Biology of Nutrition in Growing Animals

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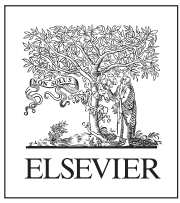

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Keynotes

Diversification of biological sciences and numbers of claims to exclusive biological function of different molecules discovered lead to unpredicted complications. Do all possible molecules, and especially their reactions, have biological function? Particular molecules can work perfectly with magnitude potency *in vitro* but their biological relevance can be limited. They can be of importance on another level of evolution. Attempts to incorporate them now are simply making noise and biological chaos.

We also need to recognize that the intellectual (regulatory) play between two molecules is much less intensive than between two tissues or two brains or two populations. There are urgent needs for descriptive studies on the functionality of different molecules; another Linneus or Mendelejev is wanted to create order in molecular biology.

A new light on this biology has been brought about by high-tech developments. A few years ago, nanotubes or superconductivity were the domain of high-tech research, but in today's biology they are very well recognized, e.g. nanotubes as brain memory storage, and, soon, superconductivity of carbon in enzymatic protein will revolutionize the understanding of enzymatic–digestive reaction in the biological world.

This series of books will attempt to select and incorporate the recent discoveries in the level of understanding of the growth and metabolism, microbial ecology, and nutrition in growing animals. The books are designed to critically evaluate the actual level of knowledge in different aspects of growing animals. In fact, the series mission was to show that gut and gut metabolism are the place of creation of "new life". "Dead" organic matter entering the gut is mysteriously, within minutes, a living part of the host metabolism.

Stefan Pierzynowski, Prof
Series Editor

Preface

This book *Biology of Nutrition in Growing Animals* is the fourth volume in the Elsevier series entitled Biology of Growing Animals. It contains a compilation of papers that have in common more of a focus on principles of the biology of nutrition rather than on quantitative aspects of nutrition and feed evaluation. A number of highly recognized active researchers from all over the world have contributed to this book.

In this book, the most recent findings relating to a new generation of feed additives and bioactive compounds are presented. A special chapter focuses on nutritional aspects in relation to the immune response and the health of the animal. Due to the ban of antibiotic growth promoters in Europe, nutrition research has become very concerned with alternatives to feed-grade antibiotics. In this context, novel functional compounds that are already in use or which have the potential to be used in the nutrition of the growing animal will be characterized and their mode of action and efficacy on nutrient and tissue metabolism will be described. Both from the consumer's and producer's perspective, safety and legal aspects in the production and the use of feed additives and bioactive compounds will be presented.

Other factors that may affect growth of the animal as a whole through effects on digestive efficiency are those compounds of raw materials that interact with digestion and metabolism, also referred to as antinutritional compounds. In particular, the role of mycotoxins in nutrition is highlighted, and strategies for detoxification are presented. Finally, special attention is drawn to the latest advances and future developments pertaining to various biotechnological, molecular and ecophysiological aspects in the nutrition of young and growing animals.

In conclusion, this book is designed to provide a comprehensive review of the state of the art, and to focus on future perspectives in the nutrition of the growing animal in this rapidly changing subject area.

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R. Mosenthin, J. Zentek and T. Żebrowska
Volume Editors

1 Intestinal fermentation: dietary and microbial interactions

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The gastrointestinal tract of growing animals represents a complex and constantly changing milieu, according to the result of complex interactions between dietary ingredients (influenced by their chemical and physical characteristics), age, production stage and immune status of the animal, environment management and microflora metabolism. The antibiotic growth promoter era is at its endpoint and new strategies to maintain high and safe production standards are needed. In this scenario, no longer bacterial inhibition, but rather bacterial modulation should be the primary target of all research efforts. Moreover, any alternative to antibiotics should be properly studied and must fit to production conditions and market requirements in order to be successful. Addition of organic acids, prebiotics and probiotics, as well as lowering the dietary buffering capacity and direct feeding of specific nutrients to sustain intestinal mucosa functions, are all strategies that require in-depth investigation. Some efforts are in progress to assess the advantages of "combo strategies" where, for example, a blend of organic acids could cumulate the effects of the different acids on animal physiology and microbial metabolism, while a symbiotic combination could maximize the efficacy of a prebiotic NDO (nondigestible oligosaccharide) by coupling it with a probiotic strain that can electively ferment it. Science in the post-antibiotic era of animal farming is facing an intriguing challenge that will give a successful return only if applicable and reliable in practical situations.

1. INTRODUCTION

The growth-promoting effects of antibiotics in animal diets have been well established for over 50 years, ever since Stokstad and Jukes (1949) demonstrated that the presence of tetracycline residues in poultry feeds increased the growth of the animals. Improved performances following the use of therapeutic antimicrobials were then described in turkeys (Stokstad and Jukes, 1950), pigs (Jukes et al., 1950), and ruminants (Jukes and Williams, 1953; Stokstad, 1954).

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The major benefits derived from the use of antibiotics in subtherapeutic doses in animal feeding involve: disease prevention, improved feed efficiency and increased performances, especially for the young stressed animals and where management and hygiene conditions are not excellent. In pig farming, feeding antibiotics is widely practiced around weaning, the time that represents the most challenging period a pig encounters during its life in terms of infection and abundance of stressors. In older pigs raised for slaughter, the use of feed antibiotics is generally regarded as unnecessary and not cost effective. Feed antibiotics have occasionally been shown to reduce the number of bacteria present in the gut (Jensen, 1988) but more often they appear to have little effect on total counts of viable bacteria.

Although the mechanism by which antibiotics promote growth is still under heated debate, the most reliable hypothesis relates to changes in the composition of the intestinal microflora. Walton (1983) identified six possible different modes of action for growth promoting agents: (1) the production of discrete lesions in the cell wall of enteric bacteria; (2) a reduction in the thickness of the intestinal mucosa; (3) an increase in intestinal alkaline phosphatase levels; (4) a reduction in amounts of bacterial toxins and toxic metabolites produced in the intestine; (5) a decrease in the level of production of intestinal ammonia; and (6) an energy-sparing effect.

The development of antimicrobial resistance over the last four decades has led to an intensification of discussions about the prudent use of antimicrobial agents, especially in veterinary medicine, animal nutrition and agriculture. One common outcome has been the conclusion that the use of antimicrobial drugs and the development of resistance in human and animals are interrelated and that systems should be established to monitor antimicrobial resistance in pathogenic and commensal bacteria of animal origin.

The magnitude of antibiotic usage in agriculture is pretty impressive. As reported by Witte (1998), in Denmark during the year 1994, a total of 24 kg of vancomycin were used for human therapy compared to 24 000 kg of the similar antibiotic, avoparcin, in the animal industry. It has to be noted that vancomycin and avoparcin have a common mode of action, which greatly increases the danger of developing cross-resistance in bacteria. As reported by the DANMAP 2002 data, after antimicrobial growth promoters (AGP) were banned in 1998, Danish usage of therapeutic antimicrobials increased (+68%) from 57 300 kg of active compound in 1998 to 96 202 kg in 2001, but total consumption of antimicrobials in food animals decreased by more than 50%.

In 1969, the Swann Committee of the United Kingdom concluded that antibiotics used in human chemotherapy or those that promote cross-resistance should not be used as growth promoters in animals, in order to reduce the risk of spreading antibiotic resistance. This recommendation led to the subdivision of antibiotics into two main categories: those for dietary use, requiring no prescription and those for medical use, requiring medical prescription.

In 1985, Sweden decided to allow the use of feeds containing antibiotics or other chemotherapeutic substances only via veterinary prescription and on a case-by-case basis.

Tylosin and virginamycin (banned in the EU since January 1, 1999) have been recently shown to induce cross-resistance to antibiotics used in human therapy (Jacobs, 1997; Witte, 1998), while other significant examples of induction of microbial resistance were reported at the WHO meeting in Berlin in 1997 (WHO, 1997).

Although the major cause of resistance to antibiotics in human pathogens is medical prescription usage of these drugs, the concerns about the spreading of antibiotic resistance culminated, as of January 1, 1999, in a ban of the use of most antibiotics utilized as growth promoters, such as bacitracin, tylosin, spiramycin, virginamycin, olaquindox and carbadox. Avoparcin had already been banned since April 1, 1997, after it was realized that enterococci isolated from the intestine of chickens and pigs fed avoparcin were resistant to vancomycin (Bager et al., 1997), an antibiotic commonly used in human therapy.

The reduced use of antibiotics would be expected to cause a progressive reduction in acquired resistance and the micro-organisms with acquired resistance should be less viable and, with reduced antibiotic-induced pressure, should be progressively eliminated by the ecosystem. However, Morrel (1997) showed that some antibiotic-resistant strains of *Escherichia coli* have evolved compensatory mutations that preclude reversion to the sensitive state, even without selective pressure.

Considering the intention of organizations and the EU to end all use of antibiotics as growth promoters by 2006, the need for novel strategies to modulate the gastrointestinal environment and microflora metabolism is of top priority.

2. STOMACH

2.1. Microflora

After birth, piglets have to rapidly adapt to significant nutritional and environmental changes throughout the postnatal and weaning periods. More precisely, this adaptation involves the gastrointestinal tract, with its digestive, fermentative, absorptive and immunological functions, as these functions will affect the health status and production performance in the subsequent periods (Pluske et al., 1997).

At birth, the intestinal tract of pigs is sterile (Sinkovics and Juhasz, 1974) and represents a good niche for rapid proliferation of environmental bacteria. Lactobacilli, streptococci, coliforms and clostridia are the main bacterial groups that can be isolated from gastric content within the first 2–3 hours of life. The major source of bacteria for the newborn pig is maternal feces. Furthermore, the piglet also acquires bacteria during birth from the sow's fecally contaminated vagina and perineum, as well as from the frequent contact with the sow's contaminated skin (Arbuckle, 1968).

The stomach is the first good site for bacterial proliferation due to the low flow rate of digesta and the nutritionally rich content present in it. Lactobacilli and streptococci can ferment milk lactose, and they increase numerically very rapidly during the first 24–48 h, and remain the dominant stomach population for the following suckling period. At this time microbial cell concentrations reach values of 10^7–10^9 per gram of gastric content (Jensen, 1998). As the main metabolic product of lactic acid bacteria is lactic acid, the pH drops consistently (3–4) inhibiting the proliferation of other bacteria (Jensen, 1998).

A number of variables such as nutrient availability, type of feed introduced, flow of digesta, pH and dry matter content, may have effects on gastrointestinal microbial diversity. At weaning, dietary shifts from a liquid to a solid feed determine a dramatic rearrangement of microbial populations. Jensen (1998) showed that at weaning time the previously dominating lactobacilli leave more space to coliforms, whose plate counts seem to be higher at day 2 and 4 postweaning. This seems to be a temporary pattern that goes back to normality (higher lactic acid bacteria and lower coliforms) one week after weaning. This kind of variation, coupled with the different stressors (regrouping, sow withdrawal, etc.), make animals more sensitive to possible microbial imbalance and susceptible to scours.

2.1.1. Microbial metabolism

Establishment of appropriate microflora at this time is of particular interest with respect to gastric pH maintenance. Cranwell et al. (1976), in their observations on gastric content and fermentation, reported that HCl secretion in suckling piglets is rather low because of mucosa

immaturity and low feed stimuli. Lactic acid, produced in an almost inverse relationship to HCl, stabilizes pH values around 3–4, which is high enough to permit lactic acid bacteria proliferation and fermentation of sow's milk lactose. The final pH reached under these conditions, together with maternal immunity are sufficient to depress growth of other potentially dangerous bacteria. A different pattern is likely to occur at weaning when market conditions force pig producers to reduce the natural weaning age (13–19 weeks) down to 3–4 weeks.

In fact, weaning pigs at 3–4 weeks exposes animals to nutritional, environmental and social stressors that usually result in a postweaning phase characterized by low weight gain, low feed intake and diarrhea (Barnett et al., 1989). Blechea et al. (1983) reported decreased cellular immunity in pigs weaned at 2–3 weeks of age, whereas cellular immunity was not altered by weaning pigs at 5 weeks. At this age the immunological status of a piglet is also low, as passive immunity acquired through maternal colostrum is dramatically decreased, and active immunity is only just beginning to develop (Gaskins and Kelly, 1995). This postweaning lag period may be related to insufficient secretions of gastric acid or pancreatic amylase, lipase and trypsin (Kidder and Manners, 1978).

Acid secretion in young pigs does not reach appreciable levels until 3–4 weeks after weaning (Cranwell and Moughan, 1989). The suckling pig uses two strategies to counteract the limitation of insufficient acid secretion and these have been discussed by Easter (1988). The primary strategy involves the conversion of lactose in sow's milk into lactic acid by the lactic acid bacteria residing in the stomach. Secondly, the nursing pig reduces the need for transitory secretion of copious amounts of acid by frequent ingestion of small meals.

Failure to maintain a low gastric pH has important implications for the digestive functions of the early-weaned pig. An elevated pH would cause a reduction in the activation of pepsinogens, which occurs rapidly at pH 2 and very slowly at pH 4 (Taylor, 1962). Pepsins have two pH optima, 2 and 3.5, and their activity declines above 3.6, with no activity at pH > 6.0 (Taylor, 1959). As a result, feed proteins may enter the small intestine almost intact. Since the end-products of pepsin digestion also stimulate the secretion of pancreatic proteolytic enzymes (Rerat, 1981) an increased gastric pH may indirectly contribute to lower pancreatic secretion with an eventual reduction in the efficiency of protein digestion.

Inefficient digestion may also provide a basis for the initiation of scours in the young pig because of the provision of abundant undigested substrates in the small intestine to support the proliferation of coliforms.

An acid gastric environment is believed to have pronounced bactericidal properties for certain micro-organisms, in particular for the Enterobacteriaceae (Sissons, 1989), whilst lactic acid bacteria can still play their beneficial role under such conditions. Viable micro-organisms entering the digestive tract via the mouth need to pass through the acidic conditions of the stomach to successfully colonize the small intestine. A rise in gastric pH would, therefore, allow increased proliferation of Enterobacteriaceae, including *Escherichia coli* (Smith and Jones, 1963), which has been associated with scours and increased mortality (White et al., 1969; Thomlinson and Lawrence, 1981). Furthermore, evidence suggests that proliferation of coliforms in the stomach may lead to a further decrease of gastric acid secretion due to the release of a bacterial polysaccharide with an inhibitory effect on acid secretion (Baume et al., 1967; Wyllie et al., 1967). Uehara et al. (1990, 1992) found that bacterial lipopolysaccharide (LPS) or endotoxin in minute doses inhibits the secretion of gastric acid and pepsin in rats. The results showed there was a dose-dependent decrease of gastric acid secretion in rats after intraperitoneal injections of LPS (10–1000 ng/rat). Subsequent histological analysis did not reveal any mucosal or parietal cell lesions, excluding a toxic mode of action of the lipopolysaccharide. Moreover, 24 h after injection, basal acid output returned to normal levels,

indicating a reversible action. Tsuji et al. (1992) observed that the effect of *Escherichia coli* lipopolysaccharide was blocked by indometacin, suggesting that LPS needs an intact prostaglandin system to exhibit its inhibitory action on gastric secretion.

2.2. Buffering capacity

During suckling, the buffering mechanisms affecting gastric pH, mainly saliva, bicarbonate and mucus secretions are not a major problem for the piglets. At weaning however, when animals begin to consume solid feed and water is drunk *ad libitum,* the buffering capacity of the diet represents a major obstacle.

In order to describe the ability of a diet to buffer HCl secretions and cause a high gastric pH, several authors have measured the acid-binding capacity (ABC) of the feed. In this case ABC is defined as the amount of acid in milliequivalents (mEq) required to lower the pH of 1 kg of feed to pH 4 (ABC-4) or pH 3 (ABC-3), respectively. As previously described, maintaining a low gastric pH may help nutrient digestion and inhibit the growth of pathogens. Several researchers reported that a reduction in the pH and/or ABC of the diet, or the addition of organic acids to the diet, improved animal performance (Partanen and Mroz, 1999; Biagi et al., 2003). A simple method to measure feed ABC (mEq) is as follows: a 2.5–5.0 g sample of feed is suspended in 50 ml of distilled deionized water and left, under continuous agitation, at 37°C for 60 minutes. This is then titrated with 0.1 N HCl or 0.1 N NaOH (depending on whether pH must be raised or lowered) until pH 3 (ABC-3) or pH 4 (ABC-4) is reached. Buffering capacity at this point is calculated as:

$$\text{ABC} = \{[(50 - ML) \times 0.1] / W\} \times 1000$$

where W is the weight of the sample and ML represents the volume of 0.1 N HCl or 0.1 N NaOH needed to reach the desired final pH.

Along with acid-binding capacity, a similar parameter that can be considered is the diet-buffering capacity calculated as follows: a feed sample (2.5–5 g) is mixed with 50 ml of HCl 0.1 N and incubated for 1 h in a shaking waterbath at 37°C. After that, the pH of the solution is brought back to 3 by using NaOH 0.1 N. The buffering capacity is then calculated as follows:

$$\text{Buffering capacity (mEq/kg)} = (50 - \text{ml NaOH}) \times 0.1 \times 1000/P$$

where P = sample weight (g).

As previously described, a low pH in the stomach of the weaning pig is ensured by the production of lactic acid and other organic acids (acetic, propionic and butyric acids are the most important) by microbial fermentation (table 1).

Defining a reliable range of values of the buffering capacity of the diet is still a matter of conjecture because of the paucity of data relative to single ingredients and their possible interactions. The mineral content and the protein fraction of the diet are the primary factors that influence ABC (Bolduan et al., 1988). Mroz et al. (2000) suggested that ABC should have a range of 530–600 mEq/kg. Low buffering-capacity diets are reported to improve feed utilization and digestibility of nutrients (Blank et al., 1999; Ange et al., 2000; Mroz et al., 2000). A low ABC diet may help to lower pH in the stomach lumen and allow a proper activation of pepsin (Taylor, 1959, 1962), leading to a higher gastric digestion of proteins to peptides and amino acids, which in turn stimulate pancreatic juice secretion (Meyer and Kelly, 1976).

Table 1

Amounts of organic acids (mmol/day) produced by microbes in the digestive tract of piglets at 6 weeks of age (source: Jensen, 1998, reproduced with permission of the Institute of Animal Physiology and Nutrition, Polish Academy of Sciences)

Organic acid	Stomach	Small intestine	Large intestine	Total
Lactic	234 ± 50	266 ± 130	0 ± 0	500 ± 162
Formic	6 ± 4	38 ± 20	11 ± 8	55 ± 23
Acetic	42 ± 18	36 ± 15	176 ± 10	254 ± 23
Propionic	4 ± 1	1 ± 1	87 ± 5	92 ± 7
Iso-butyric	0 ± 0	0 ± 0	6 ± 0	6 ± 0
Butyric	2 ± 3	2 ± 2	54 ± 3	59 ± 7
Iso-valeric	0 ± 0	0 ± 0	6 ± 1	7 ± 1
Valeric	1 ± 1	0 ± 0	9 ± 2	10 ± 2
Total	288 ± 68	343 ± 100	350 ± 23	982 ± 124

Even if these results are in agreement with Decuypere et al. (1997), the real relationship between the ABC of the diet and nutrient digestibility is still under discussion. Moreover, it is relatively difficult to standardize experimental protocols due to different feedstuff origins, as well as differences in animal genetics and rearing conditions that characterize animal production in the various countries. Nevertheless, the need for safe and natural alternatives to the use of antibiotics as growth promoters stimulates research in this field.

2.2.1. Lowering gastric pH and buffering capacity

Dietary acidification is gaining more and more interest as it reduces the buffering capacity of the ingesta, and it may support a more efficient digestion in the stomach resulting in a higher protein digestibility. Blank et al. (1999) studied the effect of fumaric acid supplemented (0, 1, 2 and 3%) to high and low buffering-capacity diets, calculated according to Bolduan et al. (1988) (56.7 and 23.5 ml of 0.1 N HCl, respectively), on ileal and fecal digestibility of amino acids in fistulated piglets. From their findings, fumaric acid exerts a beneficial activity when added to a diet with low buffering capacity, causing increased ileal digestibility of crude proteins (CP), gross energy (GE) and the majority of amino acids. On the other hand when added to a high buffering-capacity diet, fumaric acid did not significantly improve any parameter, although numerical increases in ileal digestibilities of CP, GE and amino acids were recorded. Biagi et al. (2003), in two *in vivo* studies in piglets compared six diets: (1) control diet with plasma protein and carbadox at 55 ppm (PP); (2) plant protein – high buffering-capacity diet (HB); (3) plant protein – low buffering-capacity diet (LB); (4) diet 3 plus 1% citric acid (LB+C); (5) diet 3 plus 1% fumaric acid (LB+F); (6) diet 3 plus 0.2% Tetracid® 500 (LB+T; slow-release organic acids, Vetagro, s.r.l.). Piglets fed diet 1 gained faster ($P < 0.05$) than those fed any other diet because of their supplementation with the antibiotic carbadox and plasma protein. Nevertheless, live weight, average daily gain and feed efficiency did not differ after 4 weeks. At the end of one trial, piglets fed the LB and the LB+T diets weighed, respectively, 9% and 11% more than those fed the HB diet (14.76 and 15.02 vs 13.53 kg; $P = 0.10$), and the performance of LB+T fed animals was not statistically different from those of animals on diet PP where carbadox was present. Interestingly, reducing the buffering capacity of the diet

positively influenced the composition of the intestinal microflora. Thus, there were numerical reductions of clostridia in the jejunum, and clostridia and coliforms in the cecum even if the addition of free organic acids to low buffering diets did not influence animal growth or intestinal microflora composition.

The increasing number of data suggesting a modulatory activity of various organic acids on naturally occurring microflora in the feed before and after ingestion further foster research in this field. Lærke and Jensen (2003) showed that stomach content from pigs fed a diet supplemented with lactic acid (2.2%) had a stable pH below 5 immediately after feeding, while the stomach content in pigs fed the standard diet displayed more fluctuations and a pH above 5 for up to 2.5 hours post-feeding. Similarly, Jensen et al. (2003) reported that addition of 1.8% formic acid to the diet of slaughtered pigs stabilized the pH, in the proximal GI tract, below 4 for the whole day, while a non-supplemented diet resulted in pH values of 4.7 shortly after feeding and in bactericidal (Knarreborg et al., 2002) pH levels (pH below 4) only 4 hours post-feeding.

The physical form of the feed can also play a role in the extent and efficiency of digestion. From studies on gastroesophageal ulceration in pigs (Wondra et al., 1995; Regina et al., 1999) we know that feeding pelleted or finely ground feeds result in a higher incidence of the pathology compared to coarsely ground diets. Physical aspects of feedstuffs can affect ammonia and organic acid production by the gastric microflora. Regina et al. (1999) showed that pigs fed a finely ground pelleted diet exhibit a higher concentration of ammonia, pepsin and protein in the stomach, whereas organic acids amounts, namely acetate and L-lactate, were higher in the stomach of piglets fed a coarsely ground meal. Mikkelsen and Jensen (2003) demonstrated that a coarse, non-pelleted meal stimulates production of lactic acid as well as that of acetic, propionic and butyric acids, resulting in a lower gastric pH, and reduced presence of anaerobic bacteria. The increased ammonia concentration recorded by Regina et al. (1999) could be attributed to a microbial pattern dominated by proteolytic bacteria that could metabolize the highly fermentable form of fine and pelleted feeds.

An interesting approach in lowering gastric pH comes from experiences in feeding fermented liquid feeds (FLF). When fed to piglets, FLF help piglets to overcome the stressful passage from milk to solid feed, prevent a drastic decrease of feed intake and help maintenance of low gastric pH. Piglets fed FLF have higher concentrations of lactic acid in the stomach and proximal small intestine as described by Jensen and Mikkelsen (1998) and Scholten et al. (2002). However, higher concentrations of lactic acid in the stomach do not coincide with a higher production (mmol/kg/h) of lactic acid *in vitro* (Jensen and Mikkelsen, 1998; Canibe and Jensen, 2003). This could suggest that most lactic acid in the stomach is produced from lactic acid bacteria (LAB) fermentations in the feed and not to microbial production *in situ*. Hence the higher concentration of LAB in the stomach should be attributed to a higher intake with the diet. Even if the studies of Jensen and Mikkelsen (1998) and Canibe and Jensen (2003) led to similar conclusions, the authors underline the need for new studies on a larger number of animals in order to improve the statistical power of the results. From the data discussed, the double presence of a low gastric pH and high number of LAB from dietary origin seems to be of primary importance. The presence of an already developed lactic microflora may directly exert its effect in the stomach even if LAB may not colonize that region. The acidity of FLF coupled with the *in situ* fermentation of LAB and the production of lactic acid and other weak organic acids lower the gastric pH. Thus, many enteric bacteria (*Salmonella* and *E. coli)* are killed in the stomach and do not enter the parts of the gastrointestinal tract in which they would normally proliferate.

2.2.2. Antibacterial mode of action of organic acids

The antibacterial effect of organic acids might be explained by the protons (H^+ ions) and anions ($RCOO^-$ ions) into which the acid is divided after passing the bacterial cell wall and which have a disruptive effect on bacterial protein synthesis. There is some evidence from the literature that fumaric and propionic acid, as well as formic acid, decrease intestinal microbial growth (Bolduan et al., 1988; Sutton et al., 1991; Gedek et al., 1992).

During their passage through the gastrointestinal tract, prokaryotes like *Escherichia coli, Salmonella typhimurium* or *Shigella flexneri* encounter different and stressful milieu. The most challenging situation they have to overcome is represented by low gastric pH granted by the combined actions of weak organic acids from dietary or gastric fermentations and gastric secretions of HCl. The presence of organic acids seems to be fundamental in preventing bacterial growth. Dissociation of organic acids follows the Henderson–Hasselbach equation (fig. 1), where A^- and HA are the dissociated and undissociated species, respectively:

$$pH_e = pK_a + \log[A^-]/[HA]$$

The pH value at which molecular acid and dissociated anions are in equal proportions, is defined as pK_a. As shown in fig. 2, organic acids may diffuse across membranes when in the HA form and then dissociate inside the cytoplasm (Bearson et al., 1997), because of the high internal pH (pH_i), and the anions accumulate (Russell and Diez-Gonzalez, 1998). The consequent drop in pH_i, interferes with cellular enzymatic activity, moreover bacterial cells are forced to reduce their metabolism as energy is primarily required to actively pump protons outside the cytoplasm. Bearson et al. (1997) described how cells try to raise pH_i after milieu acidification by activation of several amino acid decarboxylases that consume protons (fig. 3).

One example is lysine decarboxylase (CadA) coupled with the lysine-cadaverine antiporter (CadB) of *S. typhimurium*. The CadA decarboxylates intracellular lysine to cadaverine and consumes a proton in the process. Cadaverine is then exchanged for fresh lysine from the surrounding environment via the CadB antiporter (Park et al., 1996). Similar inducible systems, with arginine and glutamate decarboxylases, have been described for *E. coli* (Lin et al., 1995).

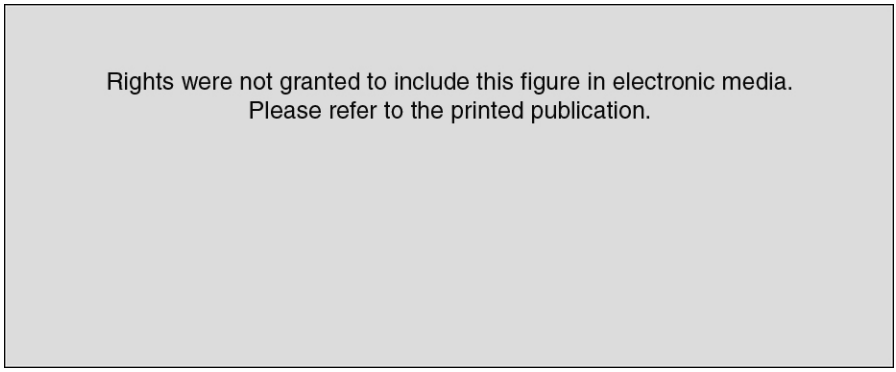

Fig. 1. Acids rate of dissociation depends on their pK_a and on the pH of the environment. As they follow Henderson–Hasselbach equation, at neutral pH, there is very little HA, but HA increases logarithmically as the pH declines. (Source: Piva, 2000.)

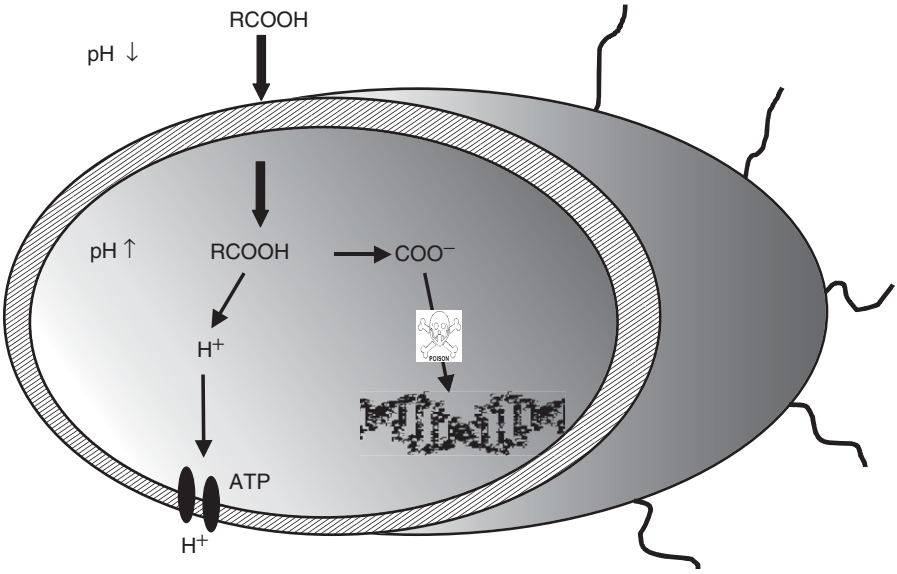

Fig. 2. Antibacterial mode of action of organic acids: the more lipophylic nondissociated form can permeate through the bacterial membrane. The higher internal pH (pH_i) allows acid to dissociate inside the cytoplasm (Bearson et al., 1997), and the anions accumulate (Russell and Diez-Gonzalez, 1998). The consequent drop in pH_i interferes with enzymatic activity and cell is forced to reduce its metabolism as energy is primarily required to actively pump protons outside the cytoplasm and subtracts energy to release protons.

In these mechanisms ΔpH between internal and external seems to be directly involved in organic acid toxicity, as suggested by Russell and Diez-Gonzalez (1998) with the equation:

$$\Delta pH = \log([A^-] + [HA])_{in}/([A^-] + [HA])_{out}$$

hence, the lower the ΔpH the higher the bacterial ability to tolerate organic acid action.

Kajikawa and Russell (1992) observed that passive potassium efflux is a mechanism for increasing membrane potential and, based on this observation, theorized a potassium-dependent

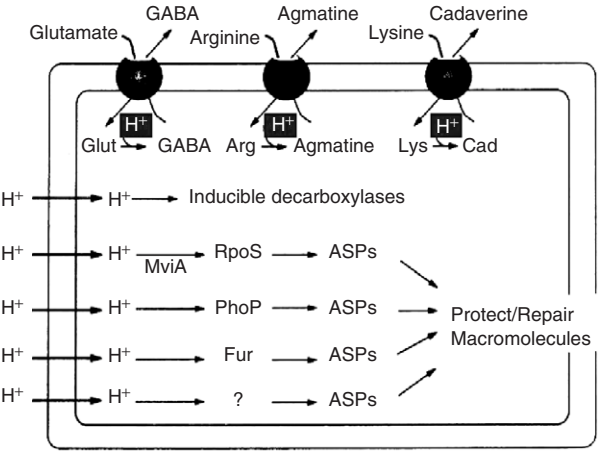

Fig. 3. Bacterial mechanisms activated to survive acid shock. Image shows both ATR systems, characterized by acid shock protein production, and AR systems, based on decarboxylases. (Source: Bearson et al., 1997, reprinted with permission from Elsevier.)

system of ΔpH and membrane potential interconversion. If a bacterium has a very high concentration of intracellular potassium, membrane potential remains high and ΔpH is low, and vice versa. This scheme is supported by a contrast between lactic acid bacteria and *E. coli*. The lactic acid bacteria, *Streptococcus bovis* and *Lactobacillus lactis,* always have very high internal potassium concentrations and never generate large ΔpH values (Cook and Russell, 1994). *E. coli,* a bacterium with lower intracellular potassium levels, is able to decrease ΔpH as the environment becomes more acidic (Kaback, 1990), while potassium addition causes an almost immediate increase in the intracellular pH of *E. coli* cells suspended in a medium at acidic pH (Kroll and Booth, 1983).

A system that could fight acid stress is the acid tolerance response (ATR) (fig. 3). After a previous exposure to mild acid conditions, the ATR is a complex stress response involving formation of acid shock proteins (ASP), that permit bacteria like *E. coli, S. typhimurium* or *S. flexneri* to resist in acid environments as low as pH 3, as well as to survive in the presence of the weak organic acids that usually predominate along the intestine (Bearson et al., 1997). Audia et al. (2001) reviewed how *S. typhimurium* ATR induced at pH 4.5–5.8, allowed the cells to survive at pH 3 for hours. Guilfoyle and Hirshfield (1996) demonstrated that *E. coli* adapted with 11.3 or 13.5 mmol/L of butyrate or propionate at pH 6.5, survive a 30-min challenge at pH 3.5, whereas Goodson and Rowbury (1989) reported survival at pH 3–3.5 after culture in nutrient broth at pH 5. Along with the previously mentioned bacteria, other harmful pathogens have also been reported to possess ATR systems, and these include: *C. perfringens* (Villarreal et al., 2000), *L. monocytogenes* (O'Driscoll et al., 1996), *C. jejuni* (Murphy et al., 2003) and *H. pylori* (Toledo et al., 2002). As such, cells undergoing acid shock in the stomach will be prepared to endure the environmental stresses in the intestine (Bearson et al., 1997).

3. SMALL INTESTINE

3.1. Morphological changes at weaning

Feeding fermented liquid feeds is also known to increase villi length and to ameliorate the villus:crypt ratio (Scholten et al., 2002). It is well known that weaning represents the most critical period in the lifespan of a pig, due to changes in nutritional and environmental conditions and the appearance of new stressors. Pluske et al. (1997) reviewed the different factors affecting structure and function of the gastrointestinal tract. Burrin and Stoll (2003) described these changes and divided weaning into an acute phase and an adaptive phase. The most important factor affecting the acute phase is the reduction of feed intake, and the consequent decrease in energy supply, due to the learning process a piglet must undergo during the change to a new feed form (from liquid to solid).

As described by Burrin and Stoll (2003), during the acute phase the intestinal wall experiences a double change: villus atrophy due to an increased cell loss, and crypt hyperplasia usually indicating an increased crypt-cell production. Hampson (1986) reported that 21 days after weaning villus height in piglets was reduced to around 75% of that in the preweaning period, i.e. from 940 μm to 694 μm. Morphological and functional changes of the intestine often lead to a reduced intestinal absorption of nutrients that can be metabolized by non-favorable intestinal bacteria, which in turn can lead to the production of noxious catabolites or to a possible overgrowth of pathogens. Intestinal changes in response to nutritional, environmental, sociological and microbiological stimuli have been well documented. As previously described, the gastrointestinal microflora is a developing "organ". At weaning piglets may easily develop diarrhea (usually within 3 days) – usually associated with hemolytic

bacteria such as *E. coli*. Nabuurs et al. (1993) postulated that the relationship between intestinal structure and scours may stem from the function of villous enterocytes and crypt cells, since shorter villi and deeper crypts have fewer absorptive and more secretory cells and this may cause decreased absorption and increased secretion (Pluske et al., 1997). Such a scenario may induce osmotic diarrhea due to over secretion, and proliferation of hemolytic *E. coli,* which may dispose of a higher amount of unabsorbed nutrients.

3.1.1. Nutritional approach

Scholten et al. (2002) tried to overcome villi shortening and crypt deepening by feeding weaning piglets with fermented wheat in liquid diets. Morphological characteristics over 4 and 8 days after weaning revealed longer villi in the first part of the small intestine of FLF piglets, as the villus/crypt ratio was higher. Moreover, the fermentation products, namely short chain fatty acids (SCFA), were more favorable for piglets fed FLF. Short chain fatty acids, produced by microbial fermentation of dietary nutrients, stimulate epithelial cell proliferation both in the small and large intestines, resulting in a larger absorptive surface (Sakata, 1988). Scheppach et al. (1992) postulated that normal colonic epithelia derive 60–70% of their energy supply from SCFA, and primarily from butyric acid. The latter induces cell differentiation and regulates the growth and proliferation of normal colonic mucosa (Treem et al., 1994) while suppressing the growth of cancer cells (Clausen et al., 1991). Piva et al. (2002a) showed *in vivo,* how such "gut nourishing" can affect piglet performances, reliably affecting small intestinal mucosa. The study was conducted using 40 weaned piglets divided into two homogeneous groups, fed a conventional nonmedicated diet without (CTR) or with sodium butyrate (SB) at 0.8 g/kg. Both diets were also supplemented with formic and lactic acid at 0.5 and 1.5 g/kg of feed, respectively. The beneficial effects of butyric acid were appreciable in the first period of the study (0–14 days) with higher average daily gain (ADG) (+20%; $P < 0.05$) and higher daily feed intake (+16%; $P < 0.05$). A higher feed intake was also recorded during the second phase (15–35 days) although it was not associated with a higher ADG. This loss of feed efficiency is most likely connected to an effective response of the intestinal architecture to SB only during the first phase (0–14 days). Conversely, in the following period SB might have stimulated feed intake without stimulating an equally effective utilization of nutrients. The improved growth performance could be associated with the beneficial effect of butyric acid on the proliferation of the intestinal epithelium. This is of greater biological value during the weaning period when the weight of the small and large intestine increases three times faster than that of the (whole) body mass growth (Sakata and Setoyama, 1997). It must be considered that the supplied amount of butyric acid (5 µmol/g DM feed) could have been of biological significance only for the small intestine where baseline values for butyric acid are about 4 µmol/g DM. Conversely, cecal concentrations of butyric acid are of about 240 µmol/g DM (Piva et al., 2002b). As such, even in the unlikely event of the entire amount of SB reaching the hindgut, the addition of SB at the tested dose would have had no influence on colonocyte metabolism. This, in turn, substantiates why the efficacy of SB is limited to the post-weaning period, when the villus structure is more negatively affected by the transition to solid feed and when it may benefit from the growth modulation effect of SB (Hodin et al., 1997). Other studies have shown positive effects of butyric acid on ileal villi and cecal crypt structure (Galfi and Bokori, 1990; Piva et al., 2002b).

As reviewed by Burrin and Stoll (2003) and from their own experiences, a large proportion of dietary nutrients are preferentially metabolized by the gut in the so-called "first-pass metabolism". Some of these, namely glutamine, glutamate and SCFA, are of particular importance

as energetic substrates for enterocytes. After ingestion, only 10% of dietary glutamate, glutamine and aspartate appear in the portal flow, indicating a large utilization by the portal drained viscera (Stoll et al., 1999). Measuring this usage it appears that a high proportion of each of these three non-essential amino acids is oxidized to CO_2 (50–70%), whilst the remainder is converted to lactate, citrulline, ornithine, arginine and alanine.

3.2. Intestinal amines

Along with glucose, other metabolic fuels for the small intestine are represented by the natural polyamines: putrescine, spermidine and spermine. These natural amines are fundamental for the proliferation and cellular evolution of living cells. Heby and Persson (1990) reported that there was an interruption of cell division in cell cultures lacking the ability to produce or absorb polyamines. From a biochemical point of view these biogenic amines are polycations, positively charged at physiological pH, that may form bridges between negative charges on the cell membrane to stabilize cell functions (Tabor and Tabor, 1984; Pegg, 1986; Osman et al., 1998). Moreover, they may act as second messengers interacting with DNA and RNA structures as well as with protein metabolism (Heby, 1981; Pignata et al., 1999; Wallace, 2000).

Polyamines in mammalian cells are mainly formed by decarboxylation of ornithine to putrescine, by the enzyme ornithine decarboxylase (ODC). Putrescine is then converted to spermidine by the enzyme spermidine synthase and consequently spermine is formed from spermidine due to the action of spermine synthase. Therefore, synthesis of these last two polyamines needs the presence of S-adenosylmethionine decarboxylase. The interconversion pathway is catalyzed by the enzyme spermidine or spermine acetyltransferase. As described by Dufour et al. (1988) and Bardocz et al. (2001) polyamines play key roles in intestinal maturation and development in the young animal. The body-pool of these polycations is used according to the needs of the different regions of the body and the amount of newly absorbed or produced compounds (White and Bradocz, 1999). The presence of these amines in enterocytes, is ensured by three sources: (1) lumenal polyamines; (2) circulating blood pool; and (3) newly synthesized inside the cell.

Luminal pool polyamines originate from the diet, defoliated cells, pancreatic secretions and bacterial metabolism. The contribution of bacterial flora is still under continuous debate and is not well understood or well described. Bardocz et al. (2001) summarized the contribution of *de novo* biosynthesis (14 μmoles/d/100 g rat), diet (16 μmoles/d/100 g rat) and intestinal microflora (3–4 μmoles/ d/100 g rat) to the body polyamine pool in the rat. Even though Bardocz et al. (1993) analyzed over 40 food ingredients and reported that high quantities (hundreds of micromoles) should enter the human gut lumen every day, the real pattern of polyamines and even biogenic amines (cadaverine, histamine, tyramine) characterizing host intestinal lumen is still nebulous. Moreover, interactions with different types of diets and feed additives and the role of bacterial polyamine production is unknown. In a recent study, Piva et al. (2002b) reported on mono-, di- and polyamine (table 2) contents in the jejunum and cecum of piglets fed a control diet with or without the addition of tributyrin and/or lactitol. As a nondigestible oligosaccharide, lactitol showed the ability to modulate intestinal microflora and reduce proteolysis (Piva et al., 1996a), whereas tributyrin is thought to be a dietary source of butyric acid for the gut. The study showed that there were no alterations of the physiological level of polyamines (Bardocz et al., 2001) within the small intestine, even though the intestinal wall was positively affected as indicated by morphometric measurements. Moreover, bacterial production of SCFA was shifted towards a significantly higher production of lactic acid, showing a positive enhancement of lactic acid bacterial activity. Interestingly, only animals

Table 2

Mono-, di- and polyamines (μmol/g DM) in the jejunum and cecum of piglets fed a control (CTR) diet with or without tributyrin (TRB) and/or lactitol (LCT) (source: Piva et al., 2002b, reproduced with permission of the American Society of Animal Science)

	Organic acid					
	Tyramine	Cadaverine	Histamine	Putrescine	Spermidine	Spermine
Jejunum[a]						
CTR	2.04 ± 1.01	1.21 ± 0.60	2.81 ± 0.39[b]	0.68 ± 0.28	0.36 ± 0.08	1.06 ± 0.12
TRB	1.18 ± 0.62	1.76 ± 1.18	2.45 ± 0.31[bc]	0.68 ± 0.33	0.38 ± 0.10	1.58 ± 0.96
LCT	1.97 ± 0.51	1.63 ± 0.36	2.90 ± 0.35[b]	0.78 ± 0.08	0.43 ± 0.12	1.33 ± 0.28
TRB+LCT	1.83 ± 1.06	1.67 ± 0.41	0.95 ± 0.32[c]	0.98 ± 0.31	0.54 ± 0.12	0.88 ± 0.21
Cecum[a]						
CTR	1.34 ± 0.20	4.32 ± 1.46	2.97 ± 0.39[b]	4.25 ± 0.71	1.37 ± 0.23	1.02 ± 0.14
TRB	0.87 ± 0.24	4.39 ± 1.97	1.66 ± 0.17[bc]	4.46 ± 1.96	1.40 ± 0.33	0.93 ± 0.16
LCT	1.54 ± 0.31	5.31 ± 1.04	2.31 ± 0.19[bc]	4.55 ± 1.02	1.52 ± 0.14	0.97 ± 0.21
TRB+LCT	0.72 ± 0.25	2.98 ± 1.24	1.51 ± 0.41[c]	3.41 ± 1.12	2.14 ± 0.12	0.91 ± 0.26

[a]Values are means ± SE, $n = 4$. [b,c]Values in the same column and in the same intestinal site with different superscripts are different ($P < 0.05$).

fed both tributyrin and lactitol showed a lower concentration of histamine in the small and large intestine. Histamine, released by mast cells, exhibits various biological effects, related to allergic enteropathy, inflammatory bowel disease (Raithel et al., 1995), and stress-related gut dysfunction (Santos et al., 1998). To counteract these noxious effects and to avoid passage of this diamine into the systemic blood circulation, the intestine usually degrades it, as well as cadaverine, by activation of diamine oxidase. This enzyme is also necessary for the oxidation of putrescine to γ-aminobutyric acid (GABA) allowing its action as a growth factor (Seild et al., 1985). These considerations are important in young developing animals, which have an immature gut and very low diamine oxidase activity, so that a high concentration of histamine or cadaverine may reduce the effective oxidation of putrescine (Bardocz et al., 2001), and consequently delay gut maturation.

The roles played by other biogenic amines, namely tyramine and cadaverine, are still unresolved. Lyons et al. (1983) indicated that cadaverine may enhance histamine toxicity by inhibiting histamine metabolism, which leads to increased uptake of nonmetabolized histamine. They did not, however, support the hypothesis that potentiation occurred via an overall increase in the absorption of histamine and its metabolites due to some disruption in the barrier function of the intestine. On the other hand, cadaverine in humans has been shown to exert beneficial effects in containing *Shigella flexnery*. It induces compartmentalization of *Shigella* species to the phagolysosome, which constitutes a protective response of the host that directly contributes to the diminished ability of polymorphonuclear leukocyte-rich inflammations to transmigrate across model intestinal epithelia (Fernandez et al., 2001). Similarly, Kohler et al. (2002) investigated whether piperidine, a cadaverine metabolite, could be used against infection with enteric pathogens. They demonstrated that piperidine treatment prevented the invasion of *S. typhimurium* into model intestinal epithelium by nearly 95%. *In vivo* studies revealed that piperidine treatment lowered the death rate in mice infected with *S. typhimurium* and reduced bacterial translocation and colonization of various organs and tissues.

Moreover, Bermudez and Firman (1998) studied biogenic amines in broilers as they were implicated in causing poor performance and intestinal lesions in chickens. They fed animals with phenylethylamine (4.8 mg/kg feed), putrescine (49 mg/kg feed), cadaverine (107 mg/kg feed), histamine (131 mg/kg feed), or a combination of all these amines. Recorded parameters at 2, 4 and 6 weeks included performance, gross lesions and histology. The authors did not observe any consistent effects on performance by any of the treatments, nor were gross lesions observed on a consistent basis and no histopathological remarks were reported.

These and other reports represent an open dilemma on the roles of these amines, their dietary origin and metabolism, their influences on intestinal microflora and whether enhancing or diminishing their concentration may have positive or negative influences on host performance and welfare.

4. LARGE BOWEL

Intestinal fermentation occurs mainly in the hindgut (Decuypere and Van der Heyde, 1972), where decarboxylation of several amino acids by bacteria can produce monoamines (tyramine and tryptamine from tyrosine and tryptophan, respectively) and polyamines (putrescine and spermidine from arginine and ornithine) (Dierick et al., 1986).

The intestinal microflora is also deeply involved in the production of ammonia. Ammonia is produced both by endogenous and bacterial enzymes within the alimentary tract. Bacterial enzymes appear to produce 75% of the alimentary tract ammonia, with urea hydrolysis being the major contributor in mammals residing in conventional, nongerm-free environments

(Visek, 1984). Energy is the limiting factor for fermentation in the hindgut (Orskov et al., 1970). As energy sources (starch and fermentable carbohydrates) are depleted, the fermentation becomes more and more proteolytic. This results in ammonia and amine production (Russell et al., 1983). Ammonia can destroy cells, alter nucleic acid synthesis, increase intestinal mucosal cell mass, increase virus infections, favor growth of cancerous cells over noncancerous cells in tissue culture (Visek, 1978), and it can reduce the villus height (Nousiainen, 1991). Furthermore, absorbed ammonia must be excreted as urea at an energy cost of approximately 7% of the total energy expenditure in monogastric as well as in ruminant animals (Eisemann and Nienaber, 1990). High plasma concentrations of ammonia may inhibit insulin release to a number of stimuli, impairing glucose metabolism and animal performances; whilst high protein diets create an environment in the reproductive tract characterized by high pH, that can reduce the vitality and motility of sperm (Visek, 1984).

At the same time, bacterial fermentation of dietery fiber may produce large quantities of SCFA, that are readily absorbed by the colonic mucosa. As previously reported, short chain fatty acids play a key role as energy sources; butyric acid being the most quickly oxidized to CO_2 among all the SCFA in the intestine (Fleming and Gill, 1997). Butyric acid has also been shown to induce cell differentiation and to regulate the growth and proliferation of normal colonic and ileal mucosa (Treem et al., 1994), whereas it can actively reduce the growth rate of colorectal cancer cells (Berry and Paraskeva, 1988).

As the origin of SCFA is mainly from nondigestible polysaccharides, modulation of these nutrients is likely to influence the distribution and concentration of SCFA in the ileal/cecal/colonic lumen. For decades, the pig industry has been utilizing the crude fiber method of Weende. Today, the more accepted method for referencing dietary fiber is the approach developed by Van Soest in 1967 in which the fibrous fraction is described by three components: neutral-detergent fiber (NDF), acid-detergent fiber (ADF) and acid-detergent lignin (ADL). These components play key roles in the digestion process by modulating the viscosity of the diet and its transit time through the gastrointestinal tract, the water-holding capacity, digestibility and cecal fermentation. For example, Berggren et al. (1993) suggested that dietary supplementation with guar gum may lead to a higher production of propionic acid whilst pectins are related to a dominant release of acetic acid (Brighenti et al., 1989). The main factor affecting fiber metabolism is fermentability and transit which together can determine the time available for bacterial fiber degradation. Henningsson et al. (2002) described how different sources of dietary fiber fed alone or in combination, may vary the SCFA pattern in rats. They investigated the fermentability and pattern of SCFA derived from rat large bowel metabolism of highly fermentable indigestible carbohydrates, i.e. guar gum (GG), pectin (Pec) and high amylose corn starch (HAS) or resistant fibers like wheat bran (WB), fed singularly or in combination. Pectin released the highest proportion of acetic acid (76 ± 2% of total SCFA) and GG of propionic (31 ± 4%; $P > 0.0005$) but they lowered butyric acid production. Interestingly, when fed in combination the butyric acid pool was doubled (9.0 ± 1.1% vs 16.6 ± 3.3%; $P > 0.05$). Incorporation of WB delayed the site of fermentation of HAS to the distal part of the hindgut. Bach Knudsen et al. (1994) also showed probable species differences in the capacity of cecal microflora to utilize different fiber sources.

Taking these suggestions into consideration, the possibility of modulating cecal fermentation through novel feed additives or diverse feed components metabolized through elective microbial pathways to release the desired SCFA takes on ever more relevance. Moreover, even if it is still highly debated, the most accredited mode of action of antibiotic growth promoters, seems to be an action on the hindgut microflora, that leads to the establishment of beneficial bacterial populations (lactic acid bacteria), i.e. that may alter intestinal metabolism to a more

beneficial pattern for the host. A desirable dietary formulation should result in low gas production, which may determine gut bloating and the so-called "abdominal pain" with consequently reduced feed-intake, reduced ammonia concentration and high levels of SCFA. On the other hand, Gaskins (2003) raised considerable doubt that antibiotics could work also against the so-called "beneficial bacteria" leaving more nutrients available for the host.

In fact, even if the positive influences of microbially produced SCFA are well known, it is still under discussion whether bacterial metabolism and nutrient transformations may be of value for the host. Over the years, two different kinds of bacterial populations have been described in the literature: the beneficial commensal bacteria (lactic acid bacteria) and the potentially harmful bacteria (coliforms, clostridia, salmonellae). Usually, commensals are described as providing nutrients such as SCFA, vitamins and amino acids, while in addition they confer some protection from pathogens by competitive exclusion. Conversely, as reviewed by Gaskins (2003) the host spends relevant energies trying to keep microbes away from the epithelial surface (pathogens and nonpathogens alike), and to quickly start-up inflammatory and immune responses against those organisms that pass the mucosal defenses. In a previous work, Anderson et al. (2000) concluded that host and microbiota are in competition for nutrients in the small intestine, whilst in the hindgut they are in symbiosis because of the final products of fermentation of indigestible feed components. Strategies directed towards ameliorating gut microbial mass, enhancing only beneficial bacteria, pose a double paradox since this increases mucosal metabolism while limiting dietary nutrient availability. The question posed by Gaskins (2003), whether energetic contributions of SCFA to whole animal metabolism are more important than their use for maintenance of a voluminous cecum-colon densely populated by fermenting bacteria, is still open and unresolved. Moreover, it seems that microbial manipulation may improve a specific bacterial population compared to others, but gastrointestinal stability is better served by a "stable diversity". Traditional culturing techniques are often limited in studying changes in the microecology of the GI tract, because of the difficulties related to growth of anaerobes, and appropriate selective media. The development of molecular techniques based on 16S rRNA genes, is now applicable to the complex intestinal environment (Vaughan et al., 2000). Favier et al. (2003) using a PCR-DGGE based method, described changes in intestinal bacteria of 60 piglets weaned at 21 days of life and sampled at 0, 2, 5, 8 and 15 days post-weaning. Their results confirmed the presence of deeply unstable microbial communities during weaning, mostly in the period between 5 and 8 days when all but one of the species detected as different gel bands seemed to disappear. Interactions between diet and bacterial changes are still not well understood due to the difficulties in approaching these subjects.

Different topics have been investigated both *in vivo* and *in vitro,* in order to evaluate non-conventional feed additives, spanning across organic acids, prebiotics, probiotics, symbiotics and botanicals. Since what was previously described relates to variations in microbial populations, trials on new strategies usually take into account and analyze indirect parameters of the activity of new additives on the gut ecosystem, such as SCFA production, total gas produced during fermentation, ammonia and amine production, etc.

4.1. *In vitro* system

An *in vitro* fermentation system was developed (Piva et al., 1996b) in order to properly investigate the relationships between diet, microbes and potential natural additives. With such an approach it is possible to study fermentation parameters over 24 – 48 h, either in the small or large intestine and, by using at least 30 fermentation vessels, a statistically correct evaluation

of dietary ingredients or additives at various inclusion rates can be made. Such a strategy is preliminary to an *in vivo* study and it narrows down the most interesting solution to be investigated *in vivo*. The method is based on two main steps to simulate ileal digestion, as described by Vervaeke et al. (1989): (1) predigestion of the basic feed diet (2 g; particle size < 1 mm), with an incubation in 40 ml of pepsin solution (2 g/L, HCl 0.075 mol/L) at 39°C for 4 h; (2) the pH is adjusted to 7.5 with NaOH (1 mmol/L), 40 ml of pancreatin solution (10 g/L in phosphate buffer pH 7.5) is added, and the mixture is incubated in a shaking water bath at 39°C for 4 h. After enzymatic digestion, the preparation is centrifuged, washed three times with distilled water and dried at 55°C overnight.

Fermentation is carried out in a batch culture system using the cecal contents from several slaughtered animals, pooled, filtered and diluted with buffer (McDougall, 1948) (ratio 1:2), before dispensing into fermentors. Samples are then taken for SCFA and ammonia analysis, while gas production is measured as described by Menke et al. (1979) using syringes with the same liquor collection procedure, the same volume of liquor and the same predigested feed concentration as the fermentation vessels. Gas production is referred to as an index of microbial metabolism, and so data are interpolated on the Gompertz bacterial growth model, assuming that substrate levels limit growth in a logarithmic relationship (Schofield et al., 1994). The Gompertz equation for gas production is as follows:

$$V = V_F \exp \{-\exp [1 + (\mu_m e/V_F) (\lambda - t)]\}$$

where symbols have the meaning assigned by Zwietering et al. (1990): V = volume of gas produced at time t, t = fermentation time, V_F = maximum volume of gas produced, μ_m = maximum rate of gas production, which occurs at the point of inflection of the gas curve and λ = the lag time, as the time-axis intercept of a tangent line at the point of inflection.

The duration of the exponential phase is calculated as the difference between the time point where the third derivative of the growth model becomes zero for the second time, and the lag time. The duration of the exponential phase can be calculated from the parameters of the modified Gompertz equation, as suggested by Zwietering et al. (1992) with the following:

$$\text{exponential phase (h)} = V_F/(\mu_m e)\{1 - \ln [(3 - \sqrt{5})/2]\}$$

4.2. Organic acids

The addition of organic acids to the diet has been already described relative to their potential ability in lowering the buffering capacity of the ration. Lactic acid bacteria are usually not influenced by their presence, whilst coliforms, salmonellae and clostridia are the more targeted bacterial strains, so that inclusion of these compounds in the diet may modulate the fermentation process in the hindgut. Even if extensively studied, real organic acid activity inside the gastrointestinal tract remains controversial. Using the above-described *in vitro* system, Biagi (2000) screened 11 different organic acids: formic, acetic, propionic, lactic, butyric, sorbic, fumaric, malic, citric, α-ketoglutaric and benzoic acid, at three different concentrations (60, 120 and 240 mmoles/L fermentation liquor). The organic acids influenced cecal fermentation and their effects varied depending on the acid and its concentration. When the acids were used at 60 mmoles/L, only sorbic acid was able to reduce the total volume of gas produced (V_F), compared to control (–34%), while citric acid and α-ketoglutaric acid increased V_F compared to control, by 92% and 32%, respectively. With acids at 120 mmoles/L, V_F was reduced by sorbic acid and benzoic acid, by –34% and –49%, respectively, whereas

lactic acid, citric acid and α-ketoglutaric acid increased V_F by 74%, 52% and 40%, respectively. When used at 240 mmoles/L, lactic acid still increased V_F by 35% compared to control.

Compared to control, ammonia concentrations at 8 h were reduced by lactic acid at 60 mmoles/L (–29%) and by sorbic acid at 240 mmoles/L (–27%). The same ammonia-lowering effect was observed at 24 h for lactic acid, fumaric acid, α-ketoglutaric acid and benzoic acid at 120 and 240 mmoles/L. On the contrary, acetic acid and malic acid at 60 mmoles/L, acetic acid, butyric acid and malic acid at 120 mmoles/L, and formic acid, acetic acid and butyric acid at 240 mmoles/L produced higher ammonia concentrations than control.

These findings suggest that organic acids can positively influence cecal fermentation in a dose-dependent manner, and that sorbic and benzoic acids are the most effective in reducing total gas and ammonia production. Benzoic acid was also reported to be effective in reducing coliforms in the stomach (Knarreborg et al., 2002). Other acids, such as citric acid, α-ketoglutaric acid and lactic acid, boost cecal fermentation, probably acting as an energy source for some cecal microflora strains, increasing total gas production or gas production rate and decreasing ammonia concentrations.

When fed to weaning piglets, organic acids have been tested extensively to achieve specific targets (e.g. pH lowering, bacterial inhibition). *In vivo* effects on microbial populations are dose dependent and usually visible at high concentrations (Jensen et al., 2003). Thus, lactic acid has a positive effect on yeast and lactic acid bacteria at doses between 0.7% to 2.8% while significantly reducing coliform counts (Maribo et al., 2000). Similar high concentrations were proposed as necessary by Tsiloyiannis et al. (2001) testing different acids in postweaning diarrhea piglets affected by ETEC strains. Because at high doses organic acids may be detrimental for operators and machinery, a coating could be applied. Moreover, the adoption of a strategy of microencapsulation can result in the slow release of coated compounds along the intestine (Piva et al., 1997a), affecting microbial metabolism throughout the intestine.

Partanen (2001) showed *in vivo* how low doses of single SCFA (< 25 g/kg) may positively affect growth performances in weaned piglets. Her meta-analysis of the published data reported significant ($P < 0.05$) improvements of average daily gain and feed to gain ration in animals fed acidified diets.

Another reliable strategy implies the use of organic acid blends, which take advantage of the synergistic effect of certain acids allowing administration of lower doses in the diet. Piva et al. (2002c) evaluated *in vitro,* at pH 6.7, the effects of adding a commercial blend of organic acids (Tetracid®500, Vetagro, Italy) providing phosphoric acid, citric acid, fumaric acid and malic acid at 1.53, 0.78, 2.59 and 1.12 mmol/L of fermentation liquor, respectively) to three diets with: 0 (low fiber, L-NDF, neutral detergent fiber; Van Soest et al., 1991), 100 (medium fiber, M-NDF), and 200 g/kg (high fiber, H-NDF) of dried sugar beet pulp.

Replacing 10% or 20% of the L-NDF diet with sugar beet pulp increased the NDF dietary level and resulted in an increased volume of gas produced (V_F) and rate of gas production (μ_m). The above information supports an increased availability of fermentable energy by increasing the NDF level of the diet, as also suggested by the shorter time required to reach the inflection point of the gas production curve. It seems that the stimulatory effect of sugar beet pulp could be accounted for by the soluble fraction (e.g. pectins) escaping NDF determination. When added to L-NDF, the acid blend resulted in an increased maximum rate of gas production. This finding could be explained by the fact that citric acid (Lutgens and Gottschalk, 1980; Marty-Teysset et al., 1996), malic acid (Renault et al., 1988; Loubiere et al., 1992) and fumaric acid (Tran et al., 1997; Tielens and Van Hellemond, 1998) may positively modulate the energy metabolism of some bacterial strains usually residing in the hindgut. Lopez et al. (1999) observed that sodium fumarate at 5 and 10 mmol/L was able to stimulate ruminal

proliferation of cellulolytic bacteria and digestion of fiber. In this study, the use of a blend of organic acids at low concentrations did not stimulate fiber digestion as indicated by the low concentration of acetic acid (Stewart and Bryant, 1988). Instead, the lower concentrations of ammonia, iso-butyric acid and iso-valeric acid in the vessels containing the organic acid blend provide an indication of effective control of the proteolytic process by the organic acids even after 24 h of fermentation. The above isoacids are formed from the deamination of valine and leucine (Van Soest, 1982) and are indicative of the extent of protein catabolism. Iso-butyric and iso-valeric acids, although in limited amounts, are extremely important as they are growth factors for many cellulolytic organisms and other species that can use them for long chain fatty acid synthesis and for amino acid synthesis through reverse reactions (Van Soest, 1982). Since fiber fermentation by cellulolytic bacteria leads generally to acetic acid production (Stewart and Bryant, 1988), the poor availability of isoacids could explain the significant reduction of acetic and *n*-butyric acids that we observed in the vessels containing organic acid blends.

4.3. Prebiotics

Another category of molecules that can play a role as microbial modulators are the prebiotics, defined as "nondigestible food ingredients" that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon (Gibson and Roberfroid, 1995). There are several categories of fermentable substrates that can act as prebiotics, including nonstarch polysaccharides (NSP; Shi and Noblet, 1993), dietary resistant starch (Jacobasch et al., 1999), nondigestible oligosaccharides (NDO; Piva et al., 1996a; Houdijk et al., 1997), and milk whey (Piva et al., 1998).

Several types of NDO are currently available: fructo-oligosaccharides (FOS), gluco-oligosaccharides (GOS), mannano-oligosaccharides (MOS), galacto-oligosaccharides (GAS), xylo-oligosaccharides (XOS). They may derive from plant origins (FOS and GAS), from enzymatic polysaccharide hydrolysis (FOS and XOS) or from *de novo* synthesis (FOS, GOS, GAS).

The use of prebiotics is aimed at enhancing beneficial bacteria (*Bifidobacterium, Lactobacillus*) inside the gut, by nourishing them with preferential substrates. The degree of selectivity of such NDO for certain types of bacteria is still under discussion. As bifidobacteria do not produce hydrogen or carbon dioxide, fermentation by bifidobacteria does not result in gastrointestinal distension and abdominal pain. Unfortunately, some prebiotics have been shown to result in gas overproduction, which may limit their usage (e.g. lactulose and α-galactosides; Levrat et al., 1991). Hartemink and Rombuts (1997) described the capability of intestinal bacteria to ferment NDO (table 3).

Even with this approach, an *in vitro* fermentation system may help to identify the best candidate for *in vivo* studies. An extremely interesting NDO tested *in vitro* and *in vivo* is lactitol. Lactitol is a disaccharide which consists of galactose and sorbitol with a β-galactoside bond. This sugar alcohol is only poorly absorbed in the small intestine (Dharmaraj et al., 1987) and reaches the hindgut where it is fermented (Nousiainen and Setälä, 1992). Jensen (1993) suggested an intriguing antiproteolytic effect of this sugar-alcohol, with reductions in deamination of amino acids and ammonia production, when lactitol is present in the lower gut.

Piva et al. (1996a) conducted a study to determine if the response of swine cecal microflora to lactitol (β-D-galactopyranosyl-(1→4)-D-sorbitol), varies when fermenting low-fiber (LF) or high-fiber (HF) predigested diets. The inoculum was collected from four sows fitted with cecal cannulas, pooled, buffered and dispensed in 27 vessels under anaerobic conditions. Lactitol (L) significantly lowered end pH and the acetic to propionic acid ratio in the first 8 hours of experiment ($P < 0.05$ and reduced ammonia by 100% and 84% in LF+L and

Table 3

Fermentation of NDO by selected intestinal bacteria (source: Hartemink and Rombouts, 1997, reproduced with permission of Wageningen University)

Bacterial group/species	FOS[a]	INU	TOS	GLL	IMO	RAF	LAT	LAC	PHGG
Bacteroides distasonis	+	+	+	+	+	+,–	+	+	–
B. fragilis	+	+	+	+	+	+,–	+	+	–
B. ovatus	+	+	+	–	+	+,–	+		+
B. thetaiotaomicron	+	+	+		+	+,–		+	–
B. vulgatus	+	+	+	+	+	+,–	+		–
Bifidobacterium spp.	+	+	+	+	+	+	+	+,–	–
Clostridium butyricum		–	–	–	–	+	+	+	+
Cl. clostridioforme	+,–	–		–	–	+,–	–	+	–
Cl. perfringens	+,–	–,+	–,+	–	+	+,–	+	+	–
Cl. ramosum	+	+		–	+	+,–	+	+	–
Escherichia coli	–,+	–	+	–	–	–	+,–	–	–
Eubacterium lentum	–	–		–	–	–	–		
Eu. limosum	–	–	–	–	–	–	–		
Fusobacterium necrophorum	–	–	–	–	–	–	–		–
Lactobacillus acidophilus group	+,–	+	+	+	+,–	–	+	+	–
Lb. casei	+,–	+	+	–	–	–	+	+	–
Megasphaera elsdenii	–	–	–	–		–	–	–	–
Mitsuokella multiacidus	+,–	+		–	+	+	+		–
Ruminococcus. productus	–			–	–	+,–	+		
Veillonella parvula	–	–	–	–	–	–	–	–	

[a]FOS: fructo-oligosaccharides; INU: inulin; TOS: trans-galactosyl-oligosaccharides; GLL: 4′-galactosyl-lactose; IMO: isomalto-oligosaccharides; RAF: raffinose; LAT: lactulose; LAC: lactitol; PHGG: partially hydrolyzed guar gum.

by 56 and 38% in HF+L diets ($P < 0.05$) at 4 and 8 h, respectively. In addition, LF+L and HF+L diets gave higher SCFA energy yields by 70% and 40% than LF and HF, respectively ($P < 0.05$). Two bacterial growth models (logistic and Gompertz) were tested to fit the gas production data and of these, the Gompertz equation provided the best fit. Lactitol reduced culture lag time by approximately 50% and increased gas production rate and maximum gas production by 60%, but only when the microflora was fermenting the LF predigested diet ($P < 0.05$). These data indicate a key role of lactitol in driving the hindgut metabolism to a better usage of nonstarch polysaccharides and eventually an increased availability of SCFA for the host. The efficacy of lactitol in containing the presence of ammonia in the LF diet and hence avoiding proteolysis would appear to confirm this hypothesis. In a subsequent study Piva et al. (1997b) confirmed these results and also showed that lactitol has the capacity to reduce indole and skatole, two L-tryptophan catabolites with detrimental effects on animal health and meat quality (Lundstrom et al., 1994; Henry, 1995).

4.4. Combo strategies

Although different approaches (organic acids, NDO) have shown beneficial influences, as alternatives to antibiotics, in modulating the fermentation process within the gastrointestinal tract when supplemented alone, evidence is growing for the efficacy of an intriguing new approach. It seems that a combination of more than one novel approach may lead to an even more favorable equilibrium of intestinal metabolism and thus animal welfare and performance. Literature concerning this strategy is still weak, even though some trials have been carried out. This approach takes into account all the different aspects of the GI tract: microbiology, nutrient metabolism and tissue requirements.

4.4.1. Pro + pre-biotic = synbiotic

The combination of a probiotic and a prebiotic can be a synergistic strategy that beneficially affects the host by improving the survival and the implantation of a direct-fed microbial in the gastrointestinal tract, and by electively stimulating the growth and/or by activating the metabolism of a limited number of health-promoting bacteria (Roberfroid, 1998). The beneficial response can be more evident when animals are challenged by pathogens or chemicals. Ziprin and DeLoach (1993) found a further reduction of intestinal colonization by *Salmonella* in chicks by administering lactose to animals that had already received probiotic cultures. Similarly, the combination of bifidobacteria and oligofructose reduced colon cancer risk in carcinogen-exposed rats (Gallaher and Khil, 1999).

Piva et al. (2005) analyzed a symbiotic effect first *in vitro* and then *in vivo* on weanling pigs. After screening to select the best combination of lactic acid bacteria and the already promising prebiotic lactitol (Piva et al., 1996a, 1997b) two synbiotics were selected: lactitol + *Lactobacillus brevis* P6 4/9 and lactitol + *Lactobacillus salivarius* 1B 4/11. The improved beneficial effects of these associations were evident by reductions of ammonia production at 8 h (10.82 and 9.81 vs 11.99 mmol/L, respectively; $P < 0.05$) and at 24 h (9.92 and 9.24 vs 12.85 mmol/L, respectively; $P < 0.05$) compared to lactitol alone, suggesting that a properly selected synbiotic can be more effective than the prebiotic component alone in controlling proteolysis. Moreover, reduced proteolysis may also be implied from the *in vivo* results. Plasma urea levels were higher in the treated groups. Rychen and Nunes (1995) described an increase of amino-nitrogen in the portal vein after feeding a probiotic to young pigs and supposed that this could be the effect of stimulating endogenous proteolytic activity, or the

consequence of an improved absorption of free amino acids in the intestinal lumen. Moreover, the synbiotic enhanced SCFA production, and hence higher energy yield, in the hindgut as observed *in vitro*. The better intestinal fermentation parameters resulted in an improved feed efficiency *in vivo* (+15%, $P < 0.05$).

4.4.2. Prebiotic + gut nutrient

As the intestine represents a complex environment, trying to promote the intestinal ecosystem may be best achieved through manipulation of nutrient availability and microbial activity. Following this concept, application of probiotic cultures, alone or in combination with prebiotic oligosaccharides, has been found to ameliorate microbial population patterns in the gastrointestinal tract and, in so doing, favorably affect the host (Howard et al., 1993; Tannock, 1999). There have also been a few reports about the development of flavorings and herbal extracts for stimulating appetite, as well as for displaying antagonism toward undesirable microbes and improving the antioxidant status of the host and, in so doing, beneficially affecting the health status in swine or poultry (Luchansky, 2000; Piva, 2000).

After *in vitro* studies on lactitol (Piva et al., 1996a, 1997b), Piva et al. (2002b) investigated tributyrin and lactitol (a prebiotic) as dietary and fermentable sources of butyrate, respectively, (US patent 6,217,915). This approach couples the needs of modulating intestinal bacteria to produce positive SCFA and at the same time supports the tissues by directly nourishing them with specific nutrients. The 28-day-old piglets in the study were fed a common commercial diet (CTR) with or without tributyrin (TRB), lactitol (LCT) alone or in combination (TRB+LCT). Compared to animals fed the control, tributyrin or lactitol diets, animals fed the TRB+LCT diet displayed the most desirable outcomes for all of the parameters measured. These animals experienced no weight loss and no mortality during the 42-day feeding period. These animals also showed an improved ADG and feed efficiency, and achieved a 34% higher total live weight at the end of the study than animals fed the control diet (237.4 vs 176.8 kg for the TRB+LCT and control groups, respectively).

Tributyrin+lactitol decreased histamine production in both the jejunum and cecum. The release of histamine by mast cells exhibits various biological effects related to allergic enteropathy, inflammatory bowel disease (Raithel et al., 1995) and stress-related gut dysfunction (Santos et al., 1998). Histamine lowers the blood pressure by dilating blood vessels and causes inflammatory reactions by promoting leukocyte chemotaxis (Mitsuoka, 1993). Histamine is also associated with increased colonic secretion (Wang et al., 1990) and ileum contraction (Bartho et al., 1987), as well as with celiac disease by inducing atrophy of villi, hyperplasia of crypts and increase of mucosal volume (Wingren et al., 1986). As such, feeding the TRB+LCT diet may be beneficial by limiting the exposure of the gut to proinflammatory conditions. Moreover, small intestinal nutrition was positively affected, as judged by villus height and crypt depth. The mucosal structure with longer villi and shorter cecal crypts observed in animals fed the lactitol or the TRB+LCT diets supports the hypothesis that nutrient absorption in the small intestine is best with the least energy-demanding configuration for the hindgut.

5. CONCLUSION

Intestinal fermentation varies dramatically due to the complex interactions between three factors: digestive tract development and nutrition, diet composition and digestibility and bacterial composition and metabolism. Such interactions evolve during the life span of the host as well as across the various sections of the gastrointestinal tract.

The change in the consumer's demand for a safe food production chain and the recent regulatory issues about the ban of antibiotic growth promoters have ensured not only a search for natural strategies to modulate gut development and health, but also a much deeper understanding of the above-described interactions.

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2 Fermentable carbohydrates: potential dietary modulators of intestinal physiology, microbiology and immunity in pigs

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Development of the gastrointestinal tract (GIT) microbiota during early life is particularly dynamic, and develops to a dense, complex and stable community. This bacterial succession involves microbe–microbe and host–microbe interactions and is dependent on host-supplied exogenous and endogenous nutrients. Research into possible alternatives for in-feed antibiotics has focused mainly on the potentially beneficial activities of the GIT microbiota. Prebiotics, or the fermentable carbohydrates, such as nondigestible oligosaccharides, are considered to have beneficial effects both on the composition and activity of the indigenous GIT microbiota, which can enhance resistance against colonization by pathogens. Additional effects of fermentable carbohydrates may also be derived from their beneficial influence on physiological aspects, including mineral absorption, reduced serum lipid levels, or reduced production of putrefactive substances. Furthermore, the short-chain fatty acids (SCFA) as end products of the fermentation process are well known for their health-promoting effects, including their trophic effects on the intestinal epithelium, and their antibacterial activities. Dietary carbohydrates may also exert immunomodulating effects mediated by changes in the intestinal microbiota, such as promotion of lactic acid bacteria which are considered to stimulate the immune response.

1. INTRODUCTION

The gastrointestinal tract (GIT) of monogastrics is colonized by a complex community of bacterial species, which are either commensal or potentially pathogenic (Berg, 1996). At weaning, the young pig faces several stresses, such as: separation from the sow, transportation, and

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usually an abrupt change in the composition of the diet, all of which may result in low voluntary feed intake, reduced performance and increased susceptibility to intestinal infections (King et al., 2003). Antibiotics are used to treat overt disease, to provide prophylaxis in situations where diseases are likely to occur, or to improve growth rates in the absence of disease (the ''growth-promoting effect'') (Hampson et al., 2001). However, in some countries, particularly in Europe, bans are being placed on the use of subtherapeutic doses of antibiotics for piglet diets. This is largely because of the rising fears regarding the risk of contamination of meat products and the possible development of antibiotic-resistant bacteria (Barton, 1999). Consequently, it is being realized that developing alternative strategies to control bacterial infections and promote growth is important.

Research into alternatives for in-feed antibiotics has focused mainly on the potentially beneficial activities of the GIT microbiota. The most promising approach until now, is the addition of specific ingredients to the diet which will enhance the ability of this indigenous microbiota to protect the host against pathogenic infections (known as ''colonization resistance'') (Van der Waaij et al., 1971; Conway, 1994). In this context, the fermentable carbohydrates are considered to have potentially beneficial effects on the composition and activity of the indigenous microbiota of the GIT (Gibson and Roberfroid, 1995; Williams et al., 2001). For human purposes, these carbohydrates must survive enzymatic digestion and reach the lower GIT intact, where they may be fermented by intestinal bacteria. Such carbohydrates include starches (resistant starch), nonstarch polysaccharides (NSP) and nondigestible oligosaccharides (NDO) (Cummings and Macfarlane, 1991).

According to Gibson and Roberfroid (1995), prebiotics are ''nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon''. More specifically in humans, prebiotics are generally considered to be food ingredients that selectively stimulate the growth and activity of bacteria such as bifidobacteria and lactobacilli, and thereby benefit health. At present, prebiotics described for human diets are carbohydrates with a degree of polymerization of sugars of between two and 60, such as several NDO. Among others, end products of the fermentation of prebiotics are the short-chain fatty acids (SCFA; mainly acetic, propionic and butyric acids) (Varel and Yen, 1997). They provide energy to the host and are well known for their health-promoting effects such as their trophic effect on the intestinal epithelium, and their stimulatory effect on sodium and water absorption from the colonic lumen (Roediger, 1980; Roediger and Moore, 1981). Short-chain fatty acids can also exert an antibacterial effect, which may prevent the establishment of pathogenic bacteria (e.g. *Salmonella* spp.) (Cummings, 1983).

Recently, there has been increased research interest in the study of the specific effects of different nutrients on the development of the GIT immune system. According to Gil and Rueda (2002), the interaction of different factors, such as the intestinal microbiota and nutrients at the local level, can influence regulation of intestinal immune function. It is generally accepted that the period soon after birth is a moment when inclusion of a specific ingredient in the diet could be especially beneficial for the development and maintenance of the immune system. The commensal bacteria may have a ''barrier'' function in times of stress such as early weaning. At this time, the host organism is not protected by a fully developed immune system. Understanding how nutrition might influence GIT physiology and immunity, holds promise in terms of controlling enteric disease without the use of antibiotics (Pluske et al., 2002). In this review, the focus is not only on pigs, but also on other species including humans, for which considerable data are available.

2. PHYSIOLOGY, MICROBIOLOGY AND IMMUNITY OF THE GASTROINTESTINAL TRACT

2.1. Physiology

The GIT extracts nutrients from the ingested diet, which are then absorbed into the blood across the intestinal mucosa. The mucosa therefore represents an interface between the metabolism of the animal and the external environment. Hydrolysis by enzymes and absorption of nutrients are the most important mechanisms for digestion in the small intestine. Furthermore, it provides a physical and immunological barrier against harmful materials such as microorganisms and dietary components. However, digestion also includes fermentation by the commensal bacteria, an aspect of digestion which is of most relevance to the large intestine of monogastric animals (Ewing and Cole, 1994; Stokes et al., 2001).

Digestion starts in the mouth, for example by comminution. Also, starch is firstly digested by α-amylase from the saliva: The α-amylase activity from the saliva continues until the HCl-containing gastric juices reduce the pH to less than 3.5, which is the lower pH limit for activity of this enzyme. The main site for the digestion of storage carbohydrates in pigs is the small intestine. A number of carbohydrases are produced in the brush border of the small intestinal mucosa, and starch hydrolysis also continues as a result of secretion of α-amylase by the pancreas. Some protein is digested by pepsin in the stomach. Upon entry into the small intestine, the increased pH renders the pepsin inactive, and the proteolytic enzymes secreted by the pancreas and intestinal brush border become active (Longland, 1991). Dietary fat digestion starts in the stomach through gastric lipase, and continues in the duodenal lumen with the synergetic action of gastric and colipase-dependent pancreatic lipases (Carey et al., 1983). The small intestine is the major site of absorption for the end products of the digestion. These digestive processes are made more efficient by the enormous epithelial surface area, which is provided by the mucosa, with the villi and microvilli increasing the surface area 10-fold and 30- to 60-fold, respectively (Caspary, 1987).

The principal physiological functions of the large intestine are considered to be the reabsorption of water and electrolytes (Ramakrishna et al., 1990), a route for the excretion of waste products of metabolism and toxic substances, and provision of an environment for the complex microbiota that concludes the digestive process by fermentation (Cummings, 1983). Another of its principal functions is the salvage of energy and nutrients through its symbiotic relationship with the GIT microbiota. Whereas the time taken for the intestinal contents to pass the length of the stomach and the small intestine is only 2–16 h in pigs, the large intestinal transit time is normally 20–80 h (Low, 1993). Consequently, there is ample time for development and activity of the microbiota. Although both digestion by endogenous enzymes and absorption from the small intestine are very effective for highly digestible feeds, there is still a constant supply of nutrients to the large intestine in the form of undigested nutrient components, host enzymes and desquamated GIT mucosal cells. The absence of glucose means that inducible enzymes such as cellulases, hemicellulases and pectinases are produced by the microbiota (Varel and Yen, 1997), and these facilitate utilization of nutrients such as resistant starch and NSP, producing SCFA and several gases (e.g. CO_2, CH_4, H_2) (Bugaut, 1987).

The colonic epithelium has the ability to actively absorb sodium against electrochemical gradients. Thus, this gives it also the capacity to dehydrate the feces. Some of the major stimuli for sodium absorption in the mammalian colon are the SCFA produced by microbial fermentation (Roediger and Moore, 1981). Large quantities of SCFA formed in the lumen are

also thought to induce HCO_3^- secretion, which acts as an important buffer for the regulation of the luminal pH. The luminal pH depends both upon the production rate of SCFA, the absorption rate of SCFA from the lumen and the luminal appearance of bicarbonate which maintain the luminal pH at 7.4 with little variation (Wrong et al., 1981).

2.2. The intestinal microbiota

There are complex consortia of microorganisms in the GIT, mainly, though not exclusively, in the large intestine. This complex microbial community develops over time, from a situation when colonization is taking place, until such a time when a stable population of bacteria becomes established in the GIT, without the need for periodic reintroduction (Gaskins, 2001). Colonization is a complex process of natural selection and ecological succession. It depends on various factors, some of which are of host origin, such as the genome and physiology of the animal, while others are of bacterial origin, such as bacterial interactions. For example, some microbial species may exert direct influences, such as the production of antimicrobial substances (bacteriocins), to exclude other microorganisms from their habitats and niches (Savage, 1977; Conway, 1996). The importance of external influences including drug administration and stress, as well as the composition of the diet for microbiota are also well known and have been well described (Ewing and Cole, 1994).

2.2.1. Development of the intestinal microbiota

After birth, the germfree GIT of neonates is rapidly colonized by anaerobic and facultative anaerobic bacteria. Microbial succession during the first few weeks of life in the alimentary tracts of humans, pigs (Moughan et al., 1992), chicks (Barrow, 1992) and calves (Smith, 1965) is remarkably similar even though, compared to human neonates, neonatal animals are exposed to greater numbers of fecal and environmental bacteria. For example, by using plasmid profiling techniques to distinguish strains of lactobacilli inhabiting the digestive tract of piglets and the feces of sows, Tannock et al. (1990) found that most of the plasmid profile types detected in the piglet GIT samples were also detected in the feces of the sows. Consequently, maternal feces seem to be a major source of lactobacilli colonizing the neonatal piglet.

Culture studies have indicated that, in general, human infants are initially colonized by enterobacteria, lactobacilli and Gram-positive cocci, which are thought to create a reduced environment favorable for the establishment of *Bacteroides, Bifidobacterium* and *Clostridium* (reviewed by Mackie et al., 1999). Favier et al. (2002) investigated the succession of bacterial communities in human neonates, by monitoring 16S ribosomal RNA (rRNA) gene diversity in fecal samples by PCR and denaturing gradient gel electrophoresis (DGGE) and by analyzing the sequences of the major ribotypes. During the first few days after birth, the profiles were simple, and it appeared that the first colonizers often belonged to *E. coli* or *Clostridium* spp. After a few days, the first signals for *Bifidobacterium* spp. appeared in the DGGE fingerprint and remained prominent during the period of breast-feeding. After weaning, the DGGE profiles became more complex and *Clostridium, Ruminococcus, Enterococcus* and *Enterobacter* spp. appeared, with microbial profiles becoming even more complex and more stable with increasing age.

In piglets, abrupt weaning results in a sudden change in the composition of the nutrients available to both the animal and to its GIT microbiota. This transition from a milk-fed to a solid-fed microbiota leads to dramatic changes in the composition of the microbial population

over a period of 7–14 days after weaning, while the animals are also adapting to a completely new environment (Hillman, 2001). According to Ewing and Cole (1994), the number of lactobacilli and other beneficial types of bacteria decreases in times of stress, as do their beneficial effects, thus allowing potential pathogens such as coliforms to increase. For example, it has been shown that within two days of weaning, the numbers of lactobacilli in the intestine decreased and *E. coli* numbers increased (Sutton and Patterson, 1996).

2.2.2. Microbiota of the adult

The GIT of the healthy, adult pig is densely colonized by a diverse population of aerobic, facultative anaerobic and strictly anaerobic bacterial species. In contrast to other omnivores such as humans, the proximal regions of the digestive tract (stomach and small intestine) of pigs are also colonized by a permanent microbiota (Savage, 1977; Jensen, 2001). This has been attributed to the fact that the young pig is coprophagic (Sansom and Gleed, 1981). The stomach wall is densely colonized, mainly by lactobacilli, but also by bifidobacteria, streptococci, clostridia and enterobacteria (Henriksson et al., 1995). The gastric microbiota, especially in the preweaned piglet, is involved in controlling the number of potential pathogens passing into the small intestine. This is achieved by the low pH, which results from lactic and acetic acid production from lactose in the sow's milk (Cranwell et al., 1976; Barrow et al., 1980). The composition of the microbiota attached to the small intestinal epithelium is comparable to that of the stomach (Conway, 1994), with the dominant cultivable bacteria species being enterobacteria, streptococci, clostridia and lactobacilli (Jensen, 2001). The total bacteria counts may increase in the distal part, while most of the bacterial species found in the ileum are also found in the large intestine (Chesson et al., 1985).

Despite the significant bacterial colonization in the small intestine, the large intestine is the major site of microbial fermentation due to the longer residence time of the digesta. The luminal contents of the colon support in excess of 400 different species, with numbers as high as 10^{10} and 10^{11} culturable bacteria/g of digesta (wet weight) (King and Kelly, 2001). The large intestine of the pig is colonized by some aerobic and facultative microorganisms, although the predominant microbes are obligate anaerobes (Varel and Yen, 1997). Major bacterial groups isolated from the pig intestine are *Streptococcus, Lactobacillus, Selenomonas, Megasphaera, Clostridium, Eubacterium, Bacteroides, Fusobacterium* and enterobacteria (Salanitro et al., 1977; Russell, 1979; Robinson et al., 1981; Moore et al., 1987).

Leser et al. (2002) investigated the phylogenetic diversity of the intestinal bacterial community in pigs by comparative 16S ribosomal RNA (rRNA) sequence analysis. Samples were collected from a total of 24 pigs, which differed according to diet, age and herd health status. In total, they identified 375 phylotypes using a 97% similarity criterion. Only 17% (66 out of 375) of the phylotypes had a sequence similarity of 97% or more to any previously cultured species for which the 16S rRNA sequence is available, i.e. represented known bacterial species. These results document that the intestinal microbial community is very complex and that the majority of the bacterial species colonizing the GIT in pigs have not yet been characterized. This is in accordance with the general assumption that bacterial diversity in natural systems extends far beyond what can currently be perceived from culture methods (Stahl, 1995).

Zoetendal et al. (2002) analyzed bacterial communities in the feces and biopsy samples from the ascending, transverse and descending colons of ten individual humans by using a 16S rRNA approach. DGGE analysis and similarity index comparisons demonstrated that the predominant mucosal-associated bacterial communities from different locations in the GIT

were both host specific and uniformly distributed along the colon, but they significantly differed from the fecal community. Therefore, it seems that the mucosal-asssociated bacteria are equally distributed along the complete colon. Differences in the structure of communities in fecal and cecal contents, observed through a dot blot hybridization and culturing approach, have also been reported by Marteau et al. (2001). Investigation of contents from different parts of the human colon (including the ascending, transverse and descending parts) of sudden death victims have revealed that lumen conditions, such as pH and concentration of fermentation products, in these parts differ considerably from one another (Macfarlane et al., 1992). This suggests that the uniform distribution of the attaching bacterial composition along the colon is very likely a result of host–bacterium interactions at the mucosal level. Several studies have already suggested that the GIT bacterial community has a strong effect on the host (Hooper et al., 2000). There has also been carried out a study in adult pigs, comparing the *in vitro* fermentation capacity of microbial inocula from the large intestine (cecum, colon and rectum) of different pigs (Bauer et al., 2004). In this *in vitro* study, it was shown that there are significant differences in microbial activity between individuals as reflected in terms of fermentation of specific substrates.

2.2.3. Colonization resistance

When animals are stressed or when dramatic alterations in environmental conditions or diet occur, the indigenous populations are disturbed and ecological niches may become temporarily accessible for colonization by invading species which can include potential pathogens. The balance between beneficial and harmful organisms may thus become disturbed, resulting in diarrhea, gastroenteritis or even death (Conway, 1994; King et al., 2003). This is best demonstrated by the changes in the intestinal microbes in relation to weaning, when piglets are more sensitive to enteropathogenic *E. coli*. The process of weaning represents a multitude of physiological shocks, which can stress the animal quite significantly. These shocks include deprivation of protective factors in the sow's milk, nutrient deprivation, since piglets often refuse feed for 1–2 days, and then a slow host response to the altered diet which results in undigested material becoming available for the potential pathogens (Conway, 1994).

In adult animals under healthy, nonstressful and constant conditions (in terms of nutrient supply and environmental conditions), a ''beneficial'' commensal microbiota colonizes the GIT surfaces in a symbiotic relationship with the host. One of the important interactions between the host and this intestinal microbiota includes their growth and thereby the chance of translocation of potentially pathogenic bacteria across the gut wall. This was termed ''colonization resistance'' (CR) by Van der Waaij et al. (1971). Colonization resistance is achieved by the action of antimicrobial metabolites (Walker and Owen, 1990), or by maintaining a lower pH, which will lead to reduced counts of pathogenic bacteria such as *E. coli* (Sutton and Patterson, 1996). The composition and flux of the indigenous microbiota along the GIT probably plays a significant role in the ability of pathogens to gain a foothold in the ecosystem. If all available niches are occupied by the indigenous microbiota, transient colonizers are unable to establish ''permanent residency'' and potential pathogens are thereby excluded (Berg, 1996). The role of this indigenous population in maintaining CR against potentially pathogenic bacteria has been shown indirectly in studies in which antibiotics were administered (e.g. Hentges et al., 1984). The stronger the suppression of ingested bacteria by the residing microbiota, i.e. the stronger the CR, the fewer potentially pathogenic bacteria were apparently able to colonize the GIT. However, there may be variations between individuals

of the same species in terms of the quality of the CR, i.e. its protective capacity (Van der Waaij and Heidt, 1990). Thus, as the intestinal ecosystem is involved in ''controlling'' ingested bacteria, it may represent a potent first line of defense in animals and man (Van der Waaij et al., 1971; Van der Waaij, 1989).

2.3. Immune function of the GIT

Apart from the protection of the GIT against pathogenic microbes by the indigenous microbiota, the host organism is also protected by a mucosal immune system. This mucosal immune system is strategically placed in areas where external pathogens and antigens may gain access to the body. The GIT immune system has to distinguish not only between self and nonself, but also between potentially dangerous foreign antigens and the common harmless foodstuffs to which it is constantly exposed (Brandtzaeg and Nilssen, 1995; Morales et al., 1996).

The mucosal immune system uses a number of mechanisms to protect the host against an aggressive immune response to luminal constituents. For example, intestinal epithelial cells are joined together apically and basally by tight junctions, which restrict the passage of even very small (2-kDa) molecules (Madara, 1998). Furthermore, bacteria and viruses can also become trapped in the mucus layer and be expelled by the peristaltic contractions of the GIT, thereby preventing potential pathogens and antigens from gaining access to the underlying epithelium. Mucins also serve as a reservoir for secretory IgA (sIgA), an antibody which is highly suited for the hostile environment of the gut.

Additional nonspecific protection is provided by the generation of antimicrobial substances, including inorganic disinfectants (e.g. hydrogen peroxide and nitric oxide) and large antimicrobial proteins (e.g. lysozyme and lactoferrin), or small antimicrobial peptides (also called defensins). Defensins are produced in several tissues by vertebrates and invertebrates to control intestinal microbiota (Lehrer and Ganz, 1999; Janeway, 2001).

2.3.1. Gut-associated lymphoid tissue (GALT)

There are several mucosal immune systems, including the gut-associated lymphoid tissue (GALT), bronchus-associated lymphoid tissue, genital tract, salivary glands, ocular tissues and mammary glands (Ogra, 1996). The GALT is responsible for the processing of antigens that interact with the intestinal mucosa and for disseminating the immune response (Gil and Rueda, 2002). This intestinal immune system is the largest immune organ in vertebrate species (Kraehenbuhl and Neutra, 1992). It has been estimated that approximately 25% of the intestinal mucosa is made up of lymphoid tissue (Kagnoff, 1987), which, in turn, constitutes approximately 50% of the total body lymphoid tissues (James, 1993). The structures of the GALT resemble lymph nodes with B cell follicles, intervening T cell areas, and antigen-presenting cells (APC), but there are no afferent lymphatics supplying antigens for immunological stimulation. Therefore, the exogenous stimuli are derived directly from the GIT lumen, probably predominantly via the M cells (Brandtzaeg et al., 1999). The GALT contains three major lymphoid compartments consisting of: (1) collections of highly organized lymphoid tissue, in the form of Peyer's patches and solitary colonic lymphoid follicles; (2) nonorganized lymphoid cells residing in the lamina propria and epithelium (lamina propria leukocytes and intraepithelial T lymphocytes, IEL, of the intestine); and (3) scattered individual or small aggregates of lymphoid follicles (Kagnoff, 1987; Langkamp-Henken et al., 1992). Although not situated within the intestinal mucosa as such, the mesenteric lymph nodes that drain the intestinal tract are also considered as part of the GALT. Mesenteric lymph nodes are

composed of immune cells leaving and entering the GIT and those that are part of the peripheral circulation (Weiner, 1997).

Peyer's patches, the inductive sites of the GALT, are aggregates of lymphoid follicles primarily found throughout the mucosa and submucosa of the distal ileum of the small intestine. Like lymph nodes, Peyer's patches contain both CD4+ and CD8+ T-cells, as well as naïve B cells, plasma cells, macrophages and dendritic cells (DCs) (Langkamp-Henken et al., 1992). The surface of the Peyer's patches is covered by a unique epithelium. This contains unique cell types, which are closely associated with lymphoid cells, and is termed the follicle-associated epithelium (FAE). The FAE is enriched with specialized antigen-sampling cells known as M cells. These exhibit thin extensions around lymphoid cells (Neutra, 1999). The brush border glycocalyx that characterizes villus enterocytes is absent from the apical surface of the M cell, and is replaced by microfolds (hence ''M'' cells). These M cells are accessible to luminal antigens. Many enteropathogenic infectious bacterial and viral agents use the M cells as portals of entry to cross the epithelial barrier, including poliovirus type 1, *Salmonella* and *Shigella* (Gebert, 1997). M-cells use transepithelial vesicular transport to carry antigens from the GIT into the Peyer's patches, where these antigens are presented by APCs (macrophages and DCs) to both immature T- and B-cells (Langkamp-Henken et al., 1992; Kraehenbuhl and Neutra, 2000). Activated T cells preferentially differentiate into CD4+ helper cells which, aided by DCs and secretion of cytokines such as transforming growth factor (TGF)-β and interleukin (IL)-10, induce the differentiation of antigen-specific B cells to predominantly IgA-committed plasma blasts (Brandtzaeg et al., 1999). The GALT-derived B-cell blasts proliferate and differentiate further on their way through the mesenteric lymph nodes and the thoracic duct into the bloodstream. After recirculating for several days, they migrate preferentially to the mucosa (mucosal effector sites: lamina propria and intraepithelial regions, but not Peyer's patches). Here they complete their terminal differentiation to IgA-producing plasma cells – a process called ''homing'' (Langkamp-Henken et al., 1992; DeWitt and Kudsk, 1999).

Apart from M cells, DCs might also be capable of transporting antigens across the epithelial barrier. According to Rescigno et al. (2001), this cell type may extend its dendritic-like processes through epithelial tight junctions and sample luminal antigens directly. However, the principal function of DCs appears to be the activation of T cells (Banchereau and Steinman, 1998). Circulating precursor DCs enter peripheral tissues where they capture microbial or viral antigens. Following antigen capture the immature DCs migrate to lymphoid organs, where, after maturation, they display antigen-derived peptides on their major histocompatability complex (MHC) molecules, which in turn select the circulating antigen-specific T cells (Palucka and Banchereau, 1999).

Apart from the Peyer's patches, the GALT also contains loosely organized mucosal effector sites, the lamina propria, and the epithelial compartment (Brandtzaeg, 2002). The lamina propria is populated by a wide range of immune cells, such as T lymphocytes, IgA-secreting plasma cells, macrophages, DCs, mast cells, eosinophils and neutrophils (Kagnoff, 1987; Gaskins and Kelley, 1995). Plasma cells (mature B-cells) are predominantly IgA+ and IgM+, but some IgG+ cells are also present (Brown and Bourne, 1976). According to Laissue and Gebbers (1992), 90% of the plasma cells in the lamina propria secrete IgA. Most of the IgA takes the form of secretory IgA (sIgA), a dimer of two monomeric IgA molecules, which is linked to the secretory component, a glycoprotein produced by epithelial cells. It is thought that the secretory component facilitates the transport of IgA through the epithelium into the GIT lumen and helps to prevent proteolytic damage to sIgA once in the lumen (Laissue and Gebbers, 1992; Mostov and Kaetzel, 1998). The main purpose of the secretory antibody

system is to perform ''immune exclusion'' (Brandtzaeg, 2003), a term coined for noninflammatory adaptive mucosal surface protection mediated by IgA in co-operation with innate nonspecific defense factors and thus referred to as the ''first line'' of microbial defense. Secretory IgA mainly functions as an inhibitor of bacterial/viral adherence and penetration of the underlying epithelium, and it can also agglutinate antigens, trapping them in the mucus layer and facilitating their removal from the host, thereby playing an important role in intestinal CR (Cunningham-Rundles, 2001; MacPherson et al., 2001; Mayer, 2003). Furthermore, it supports mucosal immunity by increasing mucus secretion (McKay and Perdue, 1993) and prevents inflammatory reactions that would cause damage to the epithelial tissues (Russell et al., 1989).

2.3.2. Development of the intestinal immune system after birth in relation to microbial colonization

In the pig, although considerable development of the systemic immune system has already taken place before and up to birth, the mucosal immune system is almost completely absent. Peyer's patches consist of primordial follicles surrounded by a small number of T-cells, and almost no immunological cells are present in the intestinal villi or between the crypts (Bailey et al., 2001). There are low numbers of intestinal MHC class II+ cells that are required for the presentation of antigen, and also low numbers of CD4+ and CD8+ T cells. Although the components of the innate immune system are present in the newborn piglet, with low numbers of macrophage and granulocyte cells evenly distributed throughout the villous and crypt regions (Vega-López et al., 1995), they may not yet be functionally mature (Stokes et al., 1992). In humans, the intestinal immune system at birth is also immature, with lymph nodes and Peyer's patches containing only primary follicles with mainly IgM+ and IgD+ cells, but very few IgA+ cells. The lamina propria contains very few immunoglobulin-containing cells, which are mainly IgM+ and almost never IgA+ (Perkkio and Savilathi, 1980; Iwase et al., 1987; Russell et al., 1990). Accordingly, no or only small amounts of sIgA are detected in fetal digesta or meconium (Rule et al., 1971; Petit et al., 1973).

Development of the mucosal immune system takes place over a period of several weeks, in a series of defined stages. It is very much dependent on microbial exposure (Vega-López et al., 1995; Pabst and Rothkötter, 1999). This has been demonstrated by studies using germfree animals, particularly rodents, which showed that little immunological development occurs in these animals (Pabst et al., 1988; Sudo et al., 1997). For example, it has been shown that the secondary lymphoid organs, i.e. the spleen, lymph nodes and Peyer's patches, are poorly developed in germfree rodents compared with conventional rodents with an indigenous GIT microflora, because of the lack of antigenic stimulation. Furthermore, immunological parameters, such as serum gamma globulins, IgA-producing lymphocytes in lamina propria, or IELs, are decreased in numbers in germfree animals (reviewed by Berg, 1996). But, when germfree mice are colonized with an intestinal microflora, there is an increase in IgA-producing cells in the ileal mucosa and Peyer's patches (Koopman et al., 1982). According to Moreau and Coste (1993), the GIT microflora is the major antigenic stimulus responsible for the migratory pathway and also for the maturation of precursor lymphoid cells present in the Peyer's patches. Consequently, it acts on the development and maturation of the IgA plasmocytes. In germfree mice, the IgA-plasmocyte number is decreased 10-fold compared with control animals.

In pigs, Pabst et al. (1988) investigated the enlargement of ileal and jejunal Peyer's patches during the postnatal period, in conventional and germfree reared animals. The authors

observed longer Peyer's patches in germfree piglets at day 39 and 59 of age, compared to normal newborn piglets. However, the Peyer's patches were shorter than in age-matched control animals. At 6 weeks of age, the vast majority of lymphocytes present in Peyer's patches of conventional pigs are B cells, whereas in germfree pigs, the T cells predominate (Rothkötter and Pabst, 1989; Pabst and Rothkötter, 1999). It seems that the enlargement and lymphocyte composition of Peyer's patches is at least partially determined by microbial influences.

3. FERMENTABLE CARBOHYDRATES: DIETARY COMPONENTS TO MODULATE INTESTINAL PHYSIOLOGY, MICROBIOLOGY AND IMMUNITY

The idea of enhancing the beneficial activity of the microbiota by addition of specific ingredients to the diet has led to the introduction of the term ''prebiotics''. The term was introduced by Gibson and Roberfroid (1995), and describes those feed/food components which fulfill several criteria (box 1).

Of all the potential food/feed ingredients, the nondigestible but fermentable carbohydrates, are considered to be very promising candidates as prebiotics. Rolfe (2000) has defined prebiotics ranging in size from small sugar alcohols and disaccharides, to oligosaccharides and large polysaccharides. Due to their chemical structure, these compounds are not absorbed in the upper GIT nor hydrolyzed by mammalian digestive enzymes. To avoid the often-used term ''dietary fiber'' which is leading to confusion since it allows no distinction between fermentable and nonfermentable carbohydrates, the term ''fermentable carbohydrates'' will be used throughout this review. This allows for the fact that the often-alleged ''health-promoting'' effects of carbohydrates are related to the fermentable rather than nonfermentable fraction. Although the latter exerts beneficial effects in terms of increasing stool bulking, it does not stimulate microbial activity.

Fermentable carbohydrates include: resistant starches, nonstarch polysaccharides (plant cell wall polysaccharides, pectins, gums), and nondigestible oligosaccharides (NDO). However, even though they can all be classified as the so-called ''colonic foods'', not all are prebiotics, since they might also stimulate the growth and activity of different bacterial species that are both potentially harmful and beneficial (Wang and Gibson, 1993). Consequently, they may lack the necessary metabolic selectivity for one or a limited number of bacteria, such as lactobacilli and bifidobacteria, which are believed to be beneficial to the health of the GIT. For example, these lactic acid bacteria suppress the growth of pathogenic bacteria by producing acetic and lactic acids, which decrease the pH and may therefore reduce the incidence of enteric disorders (Gabert et al., 1995).

Box 1

Properties of prebiotics (from Gibson and Roberfroid, 1995, reproduced with permission of the American Society for Nutritional Sciences)

A prebiotic must

1. be neither hydrolyzed nor absorbed in the proximal part of the GIT
2. be a selective substrate for one or a limited number of beneficial bacteria, which are stimulated to grow and/or be metabolically activated
3. influence the hindgut flora in favor of a healthier composition
4. induce systemic effects that are beneficial to host health

There is some evidence to show that these specific bacteria, which are generally considered to be the target of prebiotics, cannot always be detected in the GIT of the investigated host. For example, according to a recent study, no bifidobacteria could be detected in the GIT of piglets (Konstantinov et al., 2004). Therefore, it may be helpful to search for a broader definition of the term prebiotic. This would include stimulation of further indigenous bacterial strains of the GIT that might also be beneficial to the host, but which are currently less well known or understood.

3.1. Nondigestible oligosaccharides (NDO)

Currently, the most promising candidates for acting as prebiotics, in the strictest sense, are the NDO. For example, inulin is a naturally occurring storage oligomer of fructose which is found in many plants including onions, garlic, Jerusalem artichoke (*Helianthus tuberosus*) and chicory (*Cichorium intybus*). This plant fructan shows a degree of polymerization (DP) ranging from 2–60 fructose units. Inulin molecules having a degree of polymerization of <20 fructose units are generally defined as fructo-oligosaccharides (FOS). Fructo-oligosaccharides are a mixture of tri-, tetra- and penta-saccharides primarily based on fructose units, and are commonly used for their alleged prebiotic activity (Gibson and Roberfroid, 1995; Van Loo et al., 1995). Using the most widely available and accepted nomenclature, all FOS and inulins are fructans, and all FOS are inulins, but not all inulins are FOS. The term oligofructose was introduced as a synonym for FOS in 1989 (Coussement, 1999). Oligofructose has been defined by the IUB-IUPAC Joint Commission on Biochemical Nomenclature and the AOAC as ''fructose oligosaccharides containing 2–10 monosaccharide residues connected by glycosidic linkages'' (Niness, 1999).

Nondigestible oligosaccharides currently in use as prebiotic agents include: transgalacto-oligosaccharides (TOS) which are a mixture of tri-, tetra-, penta- and hexa-saccharides consisting of galactose and glucose monomers, and which are produced by transgalactosylation of lactose (Ekhart and Timmermans, 1996), e.g. by β-galactosidase from *Aspergillus oryzae* (Matsumoto et al., 1993). TOS are rarely found in common feedstuffs, though they may be present in low concentrations in yogurts (Toba et al., 1983).

Raffinose, on the other hand, is a nondigestible oligosaccharide, which is widely distributed in plants such as sugarbeet, sugar cane, cabbage, potato, grape, wheat, barley, maize and the seeds of many legumes (Rathbone, 1980). Therefore, it is ingested in a typical daily diet. Other oligomers that may have a prebiotic effect, but for which more evidence is required, include lactulose, and oligosaccharides containing xylose, mannose and galactose (Gibson, 1998). For a detailed listing and description of NDOs currently used as prebiotics see, e.g. Grizard and Barthomeuf (1999).

3.2. Nonstarch polysaccharides (NSP) and resistant starch

Apart from these ''classic'' prebiotics, other fermentable carbohydrates, such as nonstarch polysaccharides (NSP) and resistant starch (usually included as ''dietary fiber''), may also influence the activity and composition of the resident microbiota. However, they lack the required specificity for certain microbial strains.

The NSP (together with lignin) are the principal components of the cell walls, representing a group of heterogeneous compounds, which differ in their chemical composition and physical properties, both within and between plant sources. The main NSP structures commonly found in feed ingredients of plant origin are all non-α-glucan polymers such as: cellulose,

β-glucans, arabinoxylans, arabinogalactans, galactomannans, xyloglucans and rhamnogalacturonans (pectic substances) (Cummings and Englyst, 1987; De Lange, 2000). Pectins, which are usually included in the NSP fraction, are structurally based on a polymer of galacturonic acid residues with additional rhamnose and arabinose substituents. A variable proportion of the uronic carboxyl groups in pectin is esterified with methanol (Adrian, 1976). Gums, such as gum arabic and guar gum, are also known to be fermentable carbohydrates (Bauer et al., 2001). Guar gum is a galactomannan isolated from the seed of *Cyanopsis tetragonolobus* (guar). In its unmodified form, this food additive is used as a thickener in a large variety of food products. Partial enzymatic hydrolysis results in a product that can be used as a soluble dietary carbohydrate (Salyers et al., 1977). Gum arabic is an exudate from the acacia tree, which consists of a complex arabinogalactan polysaccharide associated with a glycoprotein. It has a high molecular weight and it is used as an additive in many food applications as a stabilizer and emulsifier (McLean-Ross et al., 1983).

Resistant starch consists mainly of amylose and is defined as the fraction of starch that escapes enzymatic digestion in the human small intestine (e.g. McBurney et al., 1988). The presence of resistant starch in feeds is related to many factors including the amylose:amylopectin ratio, the granule structure of the starch, the physical form of the feed, the effects of processing and the presence of NSP, amylase inhibitors, lectins and phytate (Cummings and Englyst, 1987). For example, the resistant starch content of legumes is high (Goodlad and Mathers, 1990). Starch may be resistant to hydrolysis in the small intestine for several reasons: the type RS1 includes physically inaccessible starch, which is present in grains, seeds and legumes. The second group of starches resistant to digestion is ungelatinized native starch (RS2), which is primarily found in tubers, and some peas and beans. Type RS3 is retrograded starch, an insoluble complex formed when heated starch is cooled or dried, which can result in recrystallization of amylose (Englyst et al., 1992).

3.3. Effects of fermentable carbohydrates on GIT physiology

The major physiological effects of fermentable carbohydrates originate from their interactions with colonic contents due to their fermentability by microorganisms. However, through their varying physicochemical properties, the intake of fermentable carbohydrates can also influence several metabolic processes, including the absorption of nutrients, carbohydrate, lipid and sterol metabolism or mineral balance; furthermore, production of stools is also affected by consumption of fermentable carbohydrates (Tungland and Meyer, 2002).

3.3.1. Physical properties of fermentable carbohydrates

The presence of fermentable carbohydrates in the intestine may affect the physical characteristics of the GIT contents. The water-holding capacity and nondigestibility of certain polysaccharides will directly affect the volume and bulk of the small intestinal contents. An increase in dry weight of chyme is directly related to the fact that nondigestible material is added to chyme in the small intestine. The increase in volume of the small intestinal contents, however, is related to the water-holding capacity and viscosity of certain polysaccharides (Schneeman, 1999). For example, both guar gum and glucomannan significantly increased the volume of the aqueous phase of the small intestinal contents when fed to rats, in comparison with cellulose-fed rats. The high water-holding capacity of these two polysaccharides led to the increased volume. The ability to increase viscosity is a property associated with certain polysaccharides with a high water-holding capacity. These carbohydrates are

often referred to as "soluble fibers", which typically refers to the fact that they are dispersible in water and thereby increase viscosity. Viscous polysaccharides slow the rate of gastric emptying, resulting in an overall slower rate of digestion and absorption (Lin et al., 1992, 1997). Related to its solubility, guar gum improved intestine functioning, by reducing diarrhea in enterally fed patients (Homann et al., 1994) and relieved constipation (Takahashi et al., 1994).

Some pectins also have a high water-holding capacity as well as having the ability to absorb bile salts, to form gels and to distend the intestine (Roth et al., 1995). However, not all polysaccharides that can be dispersed in water become viscous. This is important if one is to understand the variability in physiologic responses to different carbohydrate sources. The physico-chemical properties of soluble and insoluble NSP suggest that they may affect the digestion and absorption processes in the different segments of the GIT to a variable degree. According to Cummings et al. (1992), poorly fermented carbohydrates, such as cellulose, exert a stool-bulking effect in the colon. This leads to shorter fecal transit times, and a greater fecal mass, thus reducing the risk of constipation, and possibly also in the long term, of colonic cancer. Easily fermentable types of carbohydrates on the other hand, have also been reported to have a fecal-bulking effect, due to an increased bacterial mass (Macfarlane and Cummings, 1991). Bacteria contain about 80% water and have the ability to resist dehydration, thus contributing to the water-holding capacity of the fecal material. This increase in fecal water content changes the consistency and plasticity of the stools, easing excretion and increasing stool frequency (Menne et al., 2000). The higher stool weight recorded in humans consuming inulin and oligofructose (Gibson et al., 1995) was probably due to an increase in the fecal bacterial mass, as both substrates are usually completely fermented (Cherbut, 2002). Inulin and oligofructose both increase fecal water content (Gibson et al., 1995). In addition, inulin has been shown to stimulate intestine movements and to increase stool frequency, particularly in slightly constipated subjects (Kleessen et al., 1997).

3.3.2. Short-chain fatty acids (SCFA)

Microbial fermentation results in the formation of SCFA, mainly acetic, propionic and butyric acids, as well as in various gases. Short-chain fatty acids are rapidly absorbed from the hindgut and may provide up to 30% of the maintenance energy requirements for growing pigs (Yen et al., 1991), and even more for adult pigs (Varel, 1987). One of the most important properties of the SCFA is their trophic effect on the intestinal epithelium, with butyric acid considered to be the most effective, and propionic acid the least. Acetic, propionic and butyric acids are all taken up by the colonic mucosa, though butyric acid is transported preferentially and appears to be the preferred energy source for the colonocytes (Roediger, 1980). The SCFA are absorbed into the portal blood system and reach the liver and kidneys, where they can influence metabolism. This can lead to systemic effects, such as changes in glycemia, lipidemia, uremia and overall nitrogen balance (Tungland and Meyer, 2002).

Approximately 95% of the butyric acid produced by colonic bacteria is transported across the epithelium. Butyric acid can influence gene expression in the gut wall, and has anti-inflammatory effects that result, amongst others, from the reduced formation of proinflammatory cytokines (Csordas, 1996; Segain et al., 2000). Depending on its concentration, butyric acid can inhibit the growth or promote differentiation of human cells in tissue culture, and can induce apoptosis in tumor cells, while also acting as a trophic factor for cells in intact tissues (reviewed in Csordas, 1996). Additionally, SCFA have a stimulatory effect on sodium and water absorption from the colonic lumen (Argenzio, 1981; Roediger and Moore, 1981),

and are also believed to have an antibiotic effect, preventing the establishment of pathogenic bacteria, such as *Salmonella* species (Cummings, 1983).

In the case of easily fermentable carbohydrates, large quantities of SCFA are formed, the proportion varying according to substrate. Thus, for a given GIT microbial population, it may be possible to manipulate several variables such as feeding different sources and combinations of fermentable carbohydrates, in order to manipulate the specific types and amounts of SCFA (Botham et al., 1998). For example, fermentation of resistant starch using both mixed human fecal bacteria (e.g. Englyst et al., 1987) and pig large intestinal contents *in vitro* (Goodlad and Mathers, 1988), has been shown to yield high proportions of butyric acid. In contrast, fermentation of citrus pectin, resulted mainly in acetic acid (Englyst et al., 1987). Fermentation of β-glucans, raffinose and oligofructose specifically promote production of butyric acid (15–22%) (Berggren et al., 1993). The potential to modify the amounts and distribution of the SCFA and the site of their production in the colon may be important, due to their different physiological effects, thereby having varying implications for host health.

3.3.3. Effect on mineral metabolism

Another important physiological effect in terms of the function of fermentable carbohydrates is the increased bioavailability of minerals (Scholz-Ahrens et al., 2001). Mineral absorption has generally been accepted to occur by diffusion across the small intestine. However, some highly fermentable carbohydrates, such as inulin and FOS, also promote mineral absorption in the colon (Tungland and Meyer, 2002). Through their fermentation by the colonic bacteria and subsequent SCFA production, these fermentable carbohydrates stimulate the proliferation of epithelial cells in the large intestine and reduce the luminal pH (Younes et al., 1996). The SCFA and the lower pH may, in turn, increase solubility of minerals in the luminal contents, and thereby increase their diffusive absorption via the paracellular route. This may particularly be the case for calcium phosphate in the large intestine (Rémésy et al., 1993; Kashimura et al., 1996). Inulin may also stimulate calcium absorption in the large intestine, as indicated by increased concentrations of calbindin-D9k, a calcium binding protein that plays an important role in intestinal calcium transport (Ohta et al., 1998). Table 1 summarizes some effects of NDO consumption on mineral metabolism.

3.3.4. Effect on lipid metabolism

The hypotriacylglycerolemic effect of nondigestible but fermentable carbohydrates, including resistant starch or FOS, has been described in both humans (Glore et al., 1994) and animals (e.g. Tokunaga et al., 1986). This effect might result from a reduced *de novo* lipogenesis in the liver, as indicated by a reduced activity of lipogenic enzymes such as fatty acid synthase. A depression in the activity of lipogenic enzymes and fatty acid synthase mRNA suggests that FOS may modify the gene expression coding for lipogenic enzymes (Kok et al., 1996; Delzenne and Kok, 1999). Furthermore, since the transcription level of fatty acid synthase is recognized to be primarily activated by glucose and insulin (Hillgartner et al., 1995), the lower insulin level observed with the consumption of FOS could explain the metabolic effect of these fermentable carbohydrates (Kok et al., 1996). For humans, a decrease in serum triacylglycerol would be of interest, because hypertriacylglycerolemia is a known risk factor for coronary heart disease (Davignon and Cohn, 1996). Table 2 summarizes some effects of consumption of fermentable carbohydrates on lipid metabolism, which have not only been reported for FOS, but also for gums.

Table 1

Effects of NDO consumption on mineral metabolism

NDO	Subjects	Effects on mineral availability	Reference
FOS (DP 4.8, 10%) or FOS (DP 10, 10%)	Wistar rats	↑ Calcium and magnesium absorption	Delzenne et al. (1995)
FOS (50 g/100 g diet)	Rats	↑ Calcium and magnesium absorption	Ohta et al. (1995)
TOS (5 or 10 g/100 g diet)	Wistar rats	↑ Calcium and magnesium absorption and retention	Chonan and Watanuki (1995)
Chicory inulin (0, 5 and 10 g/100 g diet) diets containing 0.2, 0.5 or 1 g calcium/100 g diet	Wistar rats	↑ Whole-body bone mineral content and whole-body bone mineral density (all calcium concentrations)	Roberfroid et al. (2002)
Inulin (DP 25, 10%), FOS (DP 4, 10%) or blend of FOS and inulin	Wistar rats	↑ Magnesium absorption and retention ↑ Calcium absorption and retention (only blend of FOS and inulin)	Coudray et al. (2003)

DP: degree of polymerization (average); FOS: fructo-oligosaccharides; TOS: transgalacto-oligosaccharides.

3.3.5. Decreased formation of putrefactive substances

In the absence of sufficient energy as carbohydrates, some bacteria may use protein as a source of energy, resulting in the formation of potentially toxic substances such as NH_3, amines and amides (Cummings and Macfarlane, 1991; Macfarlane et al., 1992). On the other hand, in the presence of fermentable carbohydrates, bacteria may utilize NH_3 as a nitrogen source for their own growth (Bryant and Robinson, 1962). Accordingly, the provision of fermentable carbohydrates can increase the uptake of NH_3 by GIT bacteria. In this way, the nitrogen is then excreted as microbial protein via the feces (Mosenthin et al., 1994),

Table 2

Effects of consumption of fermentable carbohydrates on lipid metabolism

Fermentable carbohydrates	Subjects	Effects on lipid metabolism	Reference
FOS (DP 4.8, 10 g/100 g diet)	Wistar rats	↓ Serum triacylglycerol ↓ Activity of lipogenic enzymes ↓ Fatty acid synthase mRNA	Delzenne and Kok (1999)
FOS (10%, for 30 days)	Wistar rats	↓ Serum triacylglycerol ↓ Postprandial glycemia and insulinemia ↓ Activity of fatty acid synthase	Kok et al. (1996)
Gum arabic (25 g/day, for 3 weeks)	Humans	↓ Serum cholesterol	McLean-Ross et al. (1983)
Guar gum (5 g or 10 g)	Humans	↓ Postprandial glycemia	Wolever et al. (1979)

DP: degree of polymerization (average); FOS: fructo-oligosaccharides.

instead of as urea in urine, thereby saving energy to the host. Additionally, the NH_3 burden to the environment is reduced (Mosenthin et al., 1992; Canh et al., 1998). For example, the addition of NDO such as oligofructose to the diet (7.5 g per 100 g of diet) of rats, reduced blood urea and urinary nitrogen by 20–30% (Younes et al., 1995). This was also shown by Canh et al. (1997), investigating the influence of dietary NSP (sugarbeet pulp) on nitrogen partitioning of urine and feces of fattening pigs. They found that the pigs fed the sugarbeet pulp-based diet excreted 22–37% less urea in urine than the pigs fed diets with lower NSP content.

3.4. Effects of fermentable carbohydrates on the GIT microbial community

3.4.1. Studies using culture techniques

Fermentable carbohydrates may exert a powerful influence on the composition and activity of the resident GIT microbiota. This has been shown in studies using anaerobic culture methods. This effect on the microbiota is related to the availability of the carbohydrates to the bacteria as substrates, i.e. to their fermentability. For example, the microbial breakdown of specific NSP is influenced by the chemical structure of the carbohydrate polymers present (Botham et al., 1998), e.g. the degree of lignification, and other individual properties, which determine also their solubility. It is generally accepted that the more soluble carbohydrates are, the more readily available and therefore fermentable (Stephen and Cummings, 1979). However, while they are certainly readily available, they may not necessarily be readily fermentable (Bauer et al., 2001).

Using anaerobic culture techniques to investigate fecal samples from pigs, Varel et al. (1984) showed that diets high in dietary fiber increased the number of cellulolytic bacteria without changing the total number of microorganisms. Also using a culturing technique, Jonsson and Hemmingson (1991) showed that there was a correlation between the diets of piglets and the occurrence of fecal lactobacilli with an ability to degrade β-d-glucans. Gums have been shown to exert bifidogenic effects, e.g. gum arabic is completely fermented in the human colon (Ross et al., 1983). Guar gum is readily fermented by the human fecal microbiota (Salyers et al., 1977), and also showed bifidogenic effects, at least with enteral feeding (Okubo et al., 1994). Resistant starch provides a carbohydrate source for bacterial growth, which yields high levels of butyric acid (e.g. Englyst et al., 1987). In humans, the predominantly amylolytic bacteria belong to the genera *Bifidobacterium, Bacteroides, Fusobacterium* and *Butyrivibrio* (Cummings and Englyst, 1987).

In vitro fermentation using human feces, showed that inulin and FOS selectively stimulate the growth of bifidobacteria and may produce an environment (e.g. increased SCFA concentrations and/or decreased pH) that is not favorable for the growth of certain pathogenic organisms such as *E. coli* and *Clostridium perfringens* (Wang and Gibson, 1993). Generally, they can be utilized by lactobacilli, bacteroides, streptococci and enterobacteria, but cannot be utilized by *E. coli* (Hidaka et al., 1986). McDonald (2001), using weaned piglets colonized by hemolytic *E. coli* in a natural way, and Rossi et al. (2001), using an isolated jejunal loop technique, both reported decreased proliferation of *E. coli* in response to inulin in the diet.

Another carbohydrate which can influence GIT microbial composition is transgalactooligosaccharide (TOS). This generally can be utilized by bifidobacteria, lactobacilli, bacteroides, streptococci and enterobacteria (Tanaka et al., 1983). In clinical studies, Benno et al. (1987) showed that the administration of raffinose to healthy volunteers resulted in a significant increase in fecal bifidobacteria and also a decrease in bacteroides and clostridia. This result shows the potentially prebiotic effect of raffinose.

3.4.2. Studies using molecular techniques

It is becoming increasingly clear that there are serious limitations to the use of culture techniques to study complex microbial communities. Many GIT bacterial species are fastidious, require very specific growth conditions, and are not necessarily readily amenable to microbial culture in the laboratory (Tannock, 1999). The use of molecular approaches such as PCR and DGGE is now a powerful tool to gain a more complete picture of most of the species present, including those which are either difficult or impossible to culture (Tannock, 2001). For example, Satokari et al. (2001) investigated qualitative changes in fecal bifidobacterial communities in humans, by using a culture-independent approach based on genus-specific PCR and DGGE. They studied the effect of the oral administration of galacto-oligosaccharides alone or in combination (synbiotic approach) with a probiotic bacterium (*Bifidobacterium lactis* Bb-12), on the qualitative composition of the indigenous *Bifidobacterium* population. The DGGE profiles revealed that, in general, administration of 2 weeks of galacto-oligosaccharide and/or the probiotic did not affect the qualitative composition of the indigenous *Bifidobacterium* population, while *B. lactis* BB-12 colonized the GIT transiently.

Effects of fermentable carbohydrates (sugar beet pulp and FOS) on the fecal bacterial communities of weaning piglets have been reported recently by Konstantinov et al. (2003). A combination of 16S rRNA-based approaches revealed significant changes in the fecal microbiota immediately after weaning. Piglets fed a diet containing specific fermentable carbohydrates showed a higher bacterial diversity and a more rapid stabilization of the bacterial community compared to that of the animals fed the control diet. Sequence analysis of 16S rRNA genes showed that most of the DGGE bands had a low similarity to the available cultured bacteria. Amplicons related to *Ruminococcus*-like species were found in all DGGE fingerprints derived from pigs on the diet containing sugarbeet pulp and FOS, but not in pigs fed the control diet. These results indicate that these bacteria may play an important role in the fermentation of dietary carbohydrates in the GIT of newly weaned piglets.

Guo (2003) investigated the potentially prebiotic effect of different polysaccharide fractions (polysaccharides from mushrooms, *Tremella fuciformis, Lentinus edodes* and from a herb, *Astragalus membranaceus*) on the cecal bacterial community of chickens *in vitro*. Specific PCR amplification of 16S rRNA gene fragments in combination with DGGE were used to analyze the microbial community before and after *in vitro* fermentation, using chicken cecal contents as the original inoculum. The polysaccharide extracts led to significant shifts in the bacterial community when fermented *in vitro,* suggesting that these substrates had enriched the growth of certain bacterial species.

3.5. Effects on immunity

Nutritional balance is essential for the development of the immune system at the level of both organs and cells. Nutrients can influence host defense during the acute phase of the immune response, as this requires immediate changes, involving cell activation, proliferation and differentiation. Specific nutrients, e.g. nucleotides, gangliosides, polyunsaturated fatty acids (PUFA), and some minerals such as iron and zinc, appear to act as critical co-factors in the expression of the immune response (Rueda and Gil, 2000; Gil, 2002). However, according to Gil and Rueda (2002), there are only a few studies reporting the specific effects of carbohydrates on the intestinal immune system. Almost all of them mention an interaction with the intestinal microbiota as the potential mode of action. Bengmark (1998) has suggested that one should refer to this specific effect as eco-immuno-nutrition rather than immuno-nutrition.

The intestinal microbiota may modulate the GALT responses and recent findings in animal studies demonstrated that pre- and probiotics may exert beneficial effects on GIT health by enhancing GALT responses directly, or indirectly mediated by lactic acid bacteria.

According to Field et al. (1999), studies on adult dogs indicated that adding fermentable carbohydrate to the diet can modulate the type and function of cells from different regions of the GALT. The dogs used in this study were fed a diet containing 8.3 g/kg nonfermentable (cellulose) or 8.7 g/kg fermentable carbohydrate (mixture of beet pulp, oligofructose, gum arabic) for 2 weeks. The consumption of the highly fermentable carbohydrates resulted in a higher proportion of CD8+ T-cells among the intraepithelial lymphocytes (IEL), lamina propria and Peyer's patches. There was also a higher proportion of CD4+ T-cells in the mesenteric lymph nodes and peripheral blood of the small intestine (Field et al., 1999). These results agree with studies indicating proportionally more CD4+ T-cells in mesenteric lymph nodes of rats fed a diet containing 5% w/w pectin as compared to cellulose (Lim et al., 1997). Another study, also showed an increased proportion of CD8+ IEL in rats fed a diet supplemented with sugar beet fiber, compared to a fiberfree diet (Nagai et al., 2000). In the study of Lim et al. (1997), feeding pectin also resulted in increased serum IgA and IgG levels, as well as higher IgA secretion in cecal contents. Adding oat β-glucan to a diet for mice resulted in an increased level of nonspecific and antigen-specific serum IgG (Yun et al., 1997). In rats, feeding glucomannan or pectin (highly methoxylated, 5% w/w) led to higher IgA and IgG levels in spleen and mesenteric lymph nodes, as well as higher serum IgA (Yamada et al., 1999). Addition of pectin (Lim et al., 1997), or oat β-glucan (Yun et al., 1997) to a diet also resulted in altered cytokine production in the mesenteric lymph nodes of rats and mice. Such studies strongly suggest that adding fermentable carbohydrates to a diet may result in changes of immune function, though the mechanisms by which this occurs are still largely unknown.

Guo et al. (2003) reviewed several Chinese studies investigating the immuno-active properties of mushroom and herb polysaccharides. The authors concluded from these studies that different polysaccharide fractions (polysaccharides from mushrooms, *Tremella* and *Lentinus* and from a herb, *Astragalus*) may act as stimulating agents for the growth of immune organs (e.g. spleen, thymus and bursa). Further immunostimulating effects include, among others, an increase in the number and activities of B and T lymphocytes, macrophages or natural killer cells, an enhanced T cell-mediated immune response and an enhanced humoral immune response due to an increased spleen and serum antibody production. Additionally, polysaccharides derived from *Lentinus* resulted in a marked increase in the mRNA expression levels of different cytokines, including IL-1α, IL-1β, TNF-α and IFN-γ (Liu et al., 1999).

Lan (2004) investigated the effect of dietary water-soluble oligo- and polysaccharides, extracted from soybean meal, on the immune responses of broilers during the first 14 days of life. In this study, the feeding of these oligo- and polysaccharides resulted in an increase in IgA+ and IgM+ plasma cells within the cecal mucosa, suggesting their ability to promote the broilers' local immune response. Table 3 summarizes some of the immunomodulatory effects of different NDO.

3.5.1. Mechanisms for the effects of fermentable carbohydrates on the immune system

The exact mechanisms for the effect of fermentable dietary carbohydrates on immune function in the GIT have not been established, although Schley and Field (2002) recently summarized some hypotheses. As pointed out earlier, there is strong evidence indicating that for humans, the consumption of fermentable carbohydrates (prebiotics), e.g. inulin and oligofructose, increase the proportion of beneficial lactic acid bacteria in the colon

Table 3

Immunomodulatory effects of some NDO reported by several authors (adapted from Schley and Field, 2002)

NDO	Control diet	Subjects	Immune effects	Reference
Lactulose (0.5% of energy)	Infant formula	Wistar rats	↑ Phagocytic function of intraperitoneal macrophages	Nagendra and Venkat Rao (1994)
FOS (30 g/l drinking water)	Ensure® (low residue)	Mice	↑ Cecal and colonic macrophages	Gaskins et al. (1996)
FOS (from sucrose) (5.8% w/w)	Cellulose (2% w/w)	Mice	↑ Number of PP in small intestine	Pierre et al. (1997)
Lactulose (5% w/w)	Cellulose (5% w/w)	Rats	↑ κ-light chain- and IgA-positive cells in small intestine and cecal mucosa; ↓ CD4+:CD8+ ratio in spleen	Kudoh et al. (1998)
Lactulose (5% w/w)	Cellulose (5% w/w)	Rats	↑ IgA-positive cells in cecum	Kudoh et al. (1999)
FOS (10 g/d)	Whole-milk based	Veal calves	↑ Eosinophil granulocytes in blood	Kaufhold et al. (2000)
FOS (2 g/d) and MOS (2 g/d)	Poultry by-product meal/poultry fat/ brewer's rice-based	Dogs	↑ Ileal IgA	Swanson et al. (2002)
FOS (2.5% or 7.5%)	Corn starch/casein-based	Mice	↑ Fecal IgA ↑ IF-γ and IL-10 by CD4+ T cells from PP	Hosono et al. (2003)

FOS: fructo-oligosaccharides; IF: interferon; Ig: immunoglobulin; IL: interleukin; MOS: mannan-oligosaccharides; PP: Peyer's patches.

(e.g. Gibson et al., 1995). The proposed mechanisms which may explain the immunomodulating effects of dietary carbohydrates seem to be mainly related to their ability to change the GIT microbiota, and therefore, the consumption of prebiotics will behave similarly to probiotics in terms of immune function. Oral administration of probiotic bacteria increased the production of immunoglobulins, especially IgA, in GALT, and modulated both the number and activity of Peyer's patch immune cells. Therefore, one logical mechanism might be immune stimulation through direct contact of the colonic microbiota with GALT. Small numbers of bacteria can cross the intestinal epithelial barrier into the Peyer's patches inducing activation or leading to the activation of other immune cells (Berg, 1985; Schiffrin et al., 1995). This is in accordance with Yasui and Ohwaki (1991) who showed *in vitro,* that culturing murine Peyer's patch cells with *B. breve* led to an increased proliferation and antibody production by B-lymphocytes and activated macrophage-like cells. However, another possible explanation involves activation not by the bacteria themselves, but rather by microbial substances such as cell wall components that penetrate the intestinal epithelia to activate GALT (e.g. Takahashi et al., 1998).

Furthermore, the GIT microbiota may also modulate immune cells through the fermentation of fermentable carbohydrates to SCFA. It is well established that the fermentation of inulin and oligofructose increases the production of SCFA in the GIT (Gibson and Roberfroid, 1995). Immunomodulatory properties of SCFA have been shown, for example, by Pratt et al. (1996) using a rat model. They demonstrated that supplementing total parenteral nutrition with SCFA, resulted in increased natural killer cell activity. Furthermore, Segain et al. (2000) showed that butyric acid had anti-inflammatory effects that at least partly result from the reduced formation of pro-inflammatory cytokines. Finally, SCFA production, particularly butyric acid, in the colon, may reduce the requirement of epithelial cells for glutamine, which is one of the major substrates for colonocyte energy production (Zhang et al., 1998), thereby sparing it for other cells, such as those of the immune system (Jenkins et al., 1999). This hypothesis is supported by the observation that lactulose administration can increase serum levels of glutamine (Jenkins et al., 1997), which provides an essential energy source for lymphocytes (Wu et al., 1991).

A further proposed mechanism for the immunomodulatory effects of fermentable carbohydrates that change the GIT microbiota might be due to their influence on mucin production. The layer of mucus overlying the GIT mucosa prevents the adherence and subsequent translocation of some bacteria across the epithelial wall (Katayama et al., 1997). The addition of fermentable carbohydrates to a diet can increase mucin production (Satchithanandam et al., 1990). This might occur in response to the decreased pH accompanying the production of SCFA (Bustos-Fernandez et al., 1978). According to Meslin et al. (2001) and Finnie et al. (1995), SCFA production favors secretion of mucins in the colon, as shown *in vivo* in rats and man, respectively. This is in accordance with Barcelo et al. (2000), who showed by use of an *in vitro* rat colon model, that the production of acetic and butyric acids from the fermentation of pectin, gum arabic and cellulose stimulated mucin release, whereas the carbohydrates themselves did not.

3.5.2. Attachment of pathogens to host cells

Colonization of epithelial surfaces is usually the initial step in the process of infection by a pathogen. Host–pathogen interactions are often mediated by the attachment of proteins (lectins) present on the microbial surface, to oligosaccharide chains located on glycoproteins and glycolipids on the eukaryotic cell (Karlsson et al., 1992). The pathogen protein-receptor

sites have strict requirements for their oligosaccharide ligands, usually consisting of 3–5 monosaccharides. This specificity is probably one of the main factors that determines not only which host species a pathogen can colonize, but also the site of initial colonization. However, soluble oligosaccharides may prevent bacterial attachment and dislodge bacteria attached to epithelial cells (Zopf and Roth, 1996). For example, in breast milk, several oligosaccharides have been identified that protect infants from many infectious agents (Carlson, 1985). Oligosaccharides may therefore act as ''blocking factors'', by dislodging pathogens or preventing their adhesion to the attachment site on mucosal cells by steric hindrance. Prebiotics incorporating such receptor monosaccharides or oligosaccharide sequences would act as ''decoy'' molecules for potential pathogenic bacteria (Steer et al., 2000). An example is provided by type-1 fimbrial adhesions, which are common in numerous species of *E. coli* and *Salmonella,* and which are specific for mannan residues (Oyofo et al., 1989a; Spring et al., 2000). Therefore, mannans may aid in the resistance of pathogenic colonization by acting as receptor analogs for type-1 fimbriae and decrease the number of available binding sites (Oyofo et al., 1989b). Thomas and Brooks (2004) showed in a recent study, that certain polymeric saccharides are able to inhibit adhesion of *Legionella pneumophila* to a range of human respiratory cell lines, underlying the therapeutic potential of specific oligosaccharides.

4. FUTURE PERSPECTIVES

A potentially effective strategy to protect the host against colonization with pathogens could be achieved by enhancing the natural defense mechanisms of the GIT. However, development and exploitation of this approach requires a sound understanding of the composition and activity of the GIT microbiota, host health and the interactions between the microbiota and host, particularly under stressful conditions. The use of molecular methods, such as broad-range sequencing of 16S rRNA genes, will undoubtedly facilitate definition of microbial diversity in the normal GIT, and clarify how its bacterial communities respond to changes in the diet. Efforts to beneficially alter the GIT microbiota have centered on the use of prebiotics, i.e. fermentable carbohydrates that are not digested by host enzymes and which selectively stimulate beneficial GIT bacteria such as lactobacilli or bifidobacteria. Supplementation with such fermentable carbohydrates seems to affect bacterial diversity, which might help to stabilize the microbial community more rapidly in times of stress, e.g. at weaning (Konstantinov et al., 2003). Furthermore, studies using germfree animals have shown that exposure to bacterial antigens is necessary for the proper immunological development of the host (e.g. Berg, 1996). The GIT microbiota modulates the GALT responses and recent findings in animal models clearly showed that pre- and probiotics may exert beneficial effects on GIT health by enhancing GALT responses directly or indirectly by mediation of lactic acid-producing bacteria. Since young animals have an immature GIT, an undeveloped immune system and unstable bacterial communities, the benefits of protective diet components, such as prebiotics, are likely to be profound in this early stage of life (Dai and Walker, 1999).

Since candidate prebiotics vary in their molecular structure and even chain length, it is unlikely that they are fermented at equal rates nor in the same part of the intestine. Such variation could provide opportunities to generate specific metabolic and biological effects at selected sites (Priebe et al., 2002; Bauer et al., 2004). Techniques are being developed to manufacture prebiotics that might resist hydrolysis in the proximal colon, thereby allowing selective fermentation throughout the length of the hindgut. Steer et al. (2000) suggested that an increased molecular weight may enhance persistence. For example, long-chain inulin (average DP 25) may exert an extended prebiotic effect in distal colonic regions when compared with

the lower-molecular-weight FOS (average DP 4.5). This variation in composition may be reflected in a difference in fermentation rate, the longer chains being more slowly fermented than the short chains (Roberfroid et al., 1998). According to Hughes and Rowland (2001), the two supplements may therefore be metabolized at various sites in the large intestine. In fact, there is increasing evidence that some NDO are completely fermented either by the end of the terminal ileum (FOS) or within the proximal large intestine (TOS), and are therefore unavailable for microorganisms in the distal colon (Houdijk, 1998). In addition, microbial fructans such as levan have a huge molecular weight that is considerably greater than that of long-chain inulin, which has a degree of polymerization of 2–60. It is reasonable to assume that such a polysaccharide would take longer to be metabolized in the colon, thereby increasing the chances of persistence towards distal areas (Steer et al., 2000). Therefore, it might be interesting to combine rapidly fermentable carbohydrates with more slowly fermentable carbohydrates, thereby giving attention not only to manufactured prebiotics but also to more ''natural'' prebiotics, i.e. specific feedstuffs containing fermentable carbohydrates that might enhance microbial activity in a positive way and therefore improve GIT health.

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3 The quality of dietary protein digestion affects animal performance and regulates gut bacteria growth: hypotheses and facts

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The qualitative digestive processing of dietary proteins via exo- and endopeptidases in the gastrointestinal tract (GIT) leads to the appearance of amino acids and peptides in the GIT lumen. While free amino acids are easily absorbed and used as a source of substrates, both for the intestinal bacteria and the enterocytes, the peptides may already be biologically active before absorption and may also be stored in the enterocytes and blood for future protein synthesis. Thus, the regulation of protein metabolism probably starts in the GIT, and here pancreatic and stomach enzymes play an important role. The luminal digestion and enterocytic processing/metabolism is possibly the "bottleneck" for removal of protein from the intestine and from the competitive utilization by the gut bacteria. Moreover, some of the short peptides formed in the GIT due to pancreatic and stomach enzyme action in the so-called "plastein reactions", apparently protect dietary protein from microbial utilization, thus having "antibacterial" effects. These peptide antibacterial effects may play an important role in the health of humans and animals. Understanding the biological mechanisms, which are the foundation of the relationships between protein digestion and the growth/health of the host and the homeostasis of the host's gut bacterial community, is the prime scientific challenge for the modern nutritionist and integrative physiologist. Finally, proper manipulation of protein digestion with, e.g. functional food components, can reduce the therapeutic use of antibiotics in human medicine and animal production.

Biology of Nutrition in Growing Animals
R. Mosenthin, J. Zentek and T. Żebrowska (Eds.)

1. INTRODUCTION

The complicated digestive processing of dietary proteins and their amino acids leads to the appearance in the gut of an enormous amount of possibly biologically active and nonactive peptides. Thus, in comparison with the other dietary components, proteins are puzzling and complicated, but at the same time they are indispensable components of the foodstuffs. The composition and quality of the dietary proteins are probably the main factors limiting animal growth, health and productivity.

It is obvious that the influence of dietary proteins on animal performance and health is dependent on the amount and quality of dietary protein in the diets, the digestive processes in the gastrointestinal tract (GIT), primarily the small intestine, as well as the absorptive capacity for amino acids and peptides in the small intestine. Proteins/peptides/amino acids passing to the large intestine are lost for the host. Moreover, proteins, by means of their elementary components – the amino acids, and especially some of them, such as glutamine – are recognized as being the main anabolic and energy substances for enterocyte function (Pierzynowski and Sjödin, 1998).

Dietary proteins in the GIT cannot only be utilized by the host – they can also be used in any form (amino acids, peptides and whole proteins) as substrates for gut microbial growth. Thus, in the gut – especially in the small intestine – there exists a competition between the enterocytes and host bacteria for the proteins and their degradation products. These types of relationships create questions about the mechanisms in the small intestine that protect the dietary proteins and their products from degradation by the gut bacteria so they can be directed mainly for host utilization. One suspects that there are various factors affecting these interactions, and that their qualitative and quantitative effectiveness can be related to the age of the animal, while at the same time can be dependent on the intensity of the physiological processes going on in the GIT at different phases of the growth and nutritional status of the animal.

On the one hand, the composition of the gut bacterial flora in the young, suckling animal is directly dependent on the composition of the bacterial flora of the mother and on that of the bacteria colonizing the environment of the mother and offspring. On the other hand, in these early stages of development, the composition of the natural GIT microflora is closely related to the amount and quality of the receptors on the enterocyte apical membrane responsible for bacterial adherence to the intestinal wall.

The varieties of GIT microflora present can be related to the other mechanisms and status of the host metabolism, and to the sources of nitrogen available for the GIT bacteria. The neonatal state is characterized by an intensive nitrogen retention directed towards new host protein, whereas the adult state is characterized by an exchange of the nitrogen in the proteins, and not by their retention in the synthesized protein pool. In addition, other mechanisms regulating the protection of dietary protein from the gut microflora can appear at specific periods of life, e.g. during lactation or during pregnancy, and it also appears that there are species differences. In fact, only the stomach and small intestine should be considered as places where such protection is essential. In the large intestine, protection of the protein from bacterial utilization can have implications with regard to energy production and health, but no implications directly related to the competition for access to the dietary proteins. However, the *in vitro* demonstration of active absorption of small peptides through the omasal epithelium could change the axiom of protein being exclusively absorbed from the small intestine (McCollum and Webb, 1998). This would indicate another possible mechanism for the protection of proteins from bacterial utilization in the forestomach of ruminants, or perhaps it may even be present in the large intestine, since there are reports showing the existence of

peptide transporters in the rat large intestine epithelium (Ogihara et al., 1966; Shen et al., 2001).

There are unrecognized mechanisms and factors protecting dietary proteins from the endogenous gut bacteria during pathological states. This does not exclude the idea that in pathological states the protection mechanisms do not function. The limited appetite often present during sickness speaks in favor of such speculation.

2. OVERVIEW OF THE MECHANISMS PROTECTING DIETARY PROTEINS FROM THE GUT BACTERIA

2.1. Rapid digestion and absorption of the protein from the intestinal lumen

As already stressed, proteins are the most important biological components of the diet. Thus, to be of advantage to the host, they should be quickly digested and effectively absorbed from the gut. This is probably the reason why the intestinal capacity for digestion and absorption, and for the *de novo* mucosal synthesis of the protein are a few times higher than the calculated theoretical requirements. The production of the tremendous amount and variety of proteolytic digestive enzymes in the GIT supports the above statement. It is logical that this so-called ‘‘overproduction” of digestive enzymes makes it possible to produce in a minimal time the maximal variety of absorbable products of digestion. In the classical physiology handbooks, it is postulated that all proteins are digested/absorbed in the first 70 cm of the small bowel. Another aspect of this that needs to be considered, is whether or not the host metabolism is ready to accept such a bolus of the protein digestion-related nutrients for their subsequent conversion to protein rather than to energy. Thus, the massive absorption of protein digestion-related nutrients to the enterocytes must be, in one way or another, coordinated with their storage. Storage capacity is related to the quick and dynamic syntheses of the peptides and labile proteins in the enterocytes; the other possible storage location is the peptide pool in the blood plasma.

Proof of such a pattern of dietary protein metabolism has been provided by studies performed in our laboratory on pigs with ligated pancreatic ducts. The procedure eliminated pancreatic enzyme inflow to the small intestine and did not affect insulin secretion – the main factor regulating protein anabolism. In the blood of such prepared animals, which were not in a growing stage, double the amount of free amino acids in the blood was found in comparison to the amino acid levels observed in the same animals after they had received pancreatic enzymes (Creon 10,000, Solvay Pharmaceuticals) in the diet (Botermans et al., 2001). It was notable that the blood levels of the digestive enzymes did not change in these animals (Gewert et al., 2004).

There is no doubt that the intriguing question is to explain the relationships between age, health, growth of the animals, productivity and the activity of the digestive enzymes and the forms (amino acids vs peptides) in which the absorbed protein digestive products are stored in the intestine and blood. It is necessary to highlight and explain the mode of action of the potential factors and the mechanisms that determine the storage forms of proteins in the intestinal mucosa and blood during ontogeny, and especially during intensive growth. Figure 1 proposes the possible reaction chain, which can be related to the primary metabolism and storage of the products of dietary protein digestion. It is necessary to consider that in the intestinal mucosa some of the peptides and amino acids are the substrates for synthesis of labile proteins during prandial conditions. On the other hand, enterocyte labile proteins can be processed to short peptides and amino acids, and in this form can be introduced into the blood

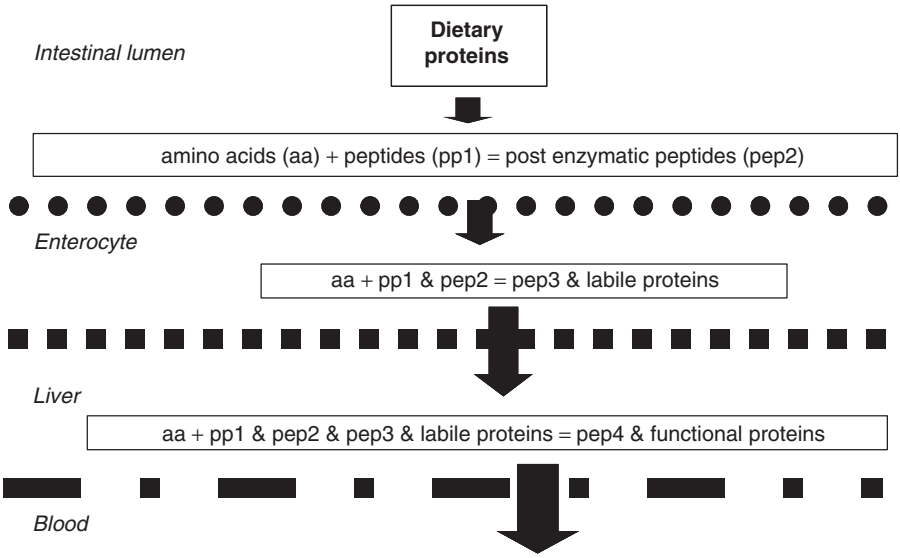

Fig. 1. Schematic concept of dietary protein digestion, absorption, primary and secondary metabolism and storage.

to meet the host's needs for synthesis of structural and functional proteins, or for the replacement of their elementary components – amino acids during interdigestive conditions, e.g. during resting or when sleeping. It is worth remembering that the rate of turnover of the membrane proteins and glycoproteins in the brush border membrane is 6–12 h, while the enterocytes exchange within 2–3 days.

It has been postulated that a large portion of the dietary amino acids reaching the portal blood is transported as peptides (Webb et al., 1992) – they are probably already partly absorbed in that form from the intestine. Their future utilization in the liver and other tissues can be directly related to their availability in the blood.

The next problem that needs to be explained is the age-dependent capability of the enterocytes for peptide absorption from the intestinal lumen. Studies performed on rat intestine show that relative expression of the small intestinal oligopeptide transporter (PEPT1) in the duodenum and jejunum is already significantly diminished at 7 days of neonatal life (Shen et al., 2001). To determine if the same relationship exists in humans and farm animals, it is necessary to estimate the conversion rates of dietary proteins to host proteins at different ages and different production periods.

It is necessary to consider studies carried out in transgenic animals in order to determine the influence of PEPT1 on nitrogen retention and utilization at different ages. Moreover, studies of peptide absorption are interesting from the physiological point of view, because it is hypothesized that the PEPT1 transporter protein appears *de novo* in the gut of pregnant females. This can be explained, since the developing fetus absorbs proteins in the form of peptides, and in some way influences the mother to return to the mechanism of protein digestion and absorption in the form of peptides, to produce peptides in the liver or placenta, since many PEPT1 transporters have been detected in the placenta (Meredith and Laynes, 1996; Botka et al., 2000).

Finally it is necessary to explain the relationship between the level of protons (one can say levels of feed acidity) in the small intestine and the degree of peptide absorption, since the mechanism of peptide absorption is proton-dependent (Fei et al., 1994).

2.2. Host antibacterial factors secreted in the GIT

There are many mechanisms and factors regulating the bacterial population in the gut, e.g. saliva lysozyme, and the antibacterial activity of the gastric and pancreatic juices (Pierzynowski et al., 1992; Kruszewska et al., 2000b,c), and bile (Kruszewska, unpublished data). Moreover, the gut-related immunological system synthesizes and secretes antibacterial factors, e.g. sIgA, defensins from neutrophils, and other cationic antibacterial peptides (Lehrer et al., 1993; for a review see Pellegrini, 2002). The proliferation of the M cells in the Peyer's patches and lymphocytes are also recognized to be factors limiting bacterial growth in the GIT. Finally, the Paneth cells in the intestinal crypts produce secretions with potent antibacterial activity (Porter et al., 2002).

2.3. Antibacterial factors related to protein digestion

Some of the products of endopeptidase protein digestion, in the form of anionic peptides, have been characterized as having antibacterial activity (Kruszewska et al., 2000a,c). Good examples of such peptides are those derived from the digestion of protein with trypsin – the main endopeptidase of the GIT (Pellegrini et al., 1997). The antibacterial activity of such digested proteins has been described (Kruszewska et al., 2000a), and the studies of scientists in Switzerland and Hungary have confirmed the existence of antibacterial activity in the digestive products of rabbit casein (Baranyi et al., 2003). This peptidic antibacterial activity is probably one of the important factors regulating bacterial homeostasis in the gut, and can directly protect dietary proteins and their other digestion products from the gut bacterial flora.

2.4. Competition between the enterocyte and gut bacteria for the ammonium ion

It has been postulated that the proper growth of enterocytes in primary culture is enhanced and protected by the ammonium ion. The *in vivo* studies of Sharma et al. (1991) demonstrated ammonium utilization in the enterocytes of rats with a porta-caval junction and reconstructed visceral venous pressure, which protects the conditions for enterocyte growth in the intestine. It is well known that for a number of bacterial strains the ammonium ion is the exclusive source of nitrogen for amino acid synthesis. Thus, it is necessary to prove the existence of competition for the ammonium ion between the enterocyte and the bacteria in specific segments of the gut due to the differing gut floral composition. Low levels of bacteria in the small intestine may be related to a low availability of ammonium for these bacteria, because of an intensive utilization by the dynamically growing enterocytes. This hypothesis needs to be tested by studies on the presence of competition between the enterocyte and the gut bacteria for utilization of ammonium. It is not excluded that the availability of ammonium is one of the important factors both for enterocyte and bacterial growth. The ammonium utilization by the enterocytes could be processed via glutamate dehydrogenase, with incorporation of the ammonium ion into alpha ketoglutarate, and also via further glutamine synthesis from glutamate and ammonium ions requiring glutamine synthetase, as seen in the perivein hepatocytes.

2.5. Intestinal electrical activity – the migrating myoelectric complex (MMC) as a factor regulating bacterial growth

Yet another mechanism which could possibly protect dietary protein from bacterial utilization, is related to the electrical activity of the intestine. *In vitro* studies have described a direct

relationship between the growth of *Escherichia coli* and the presence of electrical activity related to the MMC; the MMC, on the other hand, is directly related to the intestinal motility. Growth of *E. coli* in the electrical field generated by the MMC has been observed to be altered; an inhibition of bacterial proliferation was observed in the phase of logarithmic growth (Grzesiuk et al., 2001; Wojcik-Sikora et al., 2001). It was concluded that the electrical activity related to the MMC could regulate bacterial growth *in vitro*.

3. GUT BACTERIAL GROWTH IN RELATION TO DIETARY PROTEIN DIGESTION AND ABSORPTION

Recent studies performed by Burrin et al. (2001) and Burrin (2002), showed that a certain percentage of some dietary essential amino acids, and almost 100% of the nonessential amino acids are converted to energy in the first compartments of the small intestine.

Generally, the gut bacterial endopeptidases are nonspecific, and produce amino acids from dietary proteins, while the host endopeptidases located in the brush border glycocalyx are specific, producing specific peptides. Thus, dietary peptides produced in the glycocalyx will not be easily available for the bacteria, whereas they will be easily available for the enterocytes. The specificity of the dietary peptides and effectiveness of their peptide transporters can also limit their availability for bacteria (Payne et al., 2001). Moreover, bacterial endopeptidase digestion products are not toxic for bacteria, while the specific peptides produced by the host endopeptidases exhibit antibacterial activity (Baranyi et al., 2003).

Briefly, the following scenario can be postulated for protein digestion and the creation of the antibacterial postenzymatic peptides (Pierzynowski, 2004). During the natural processing of proteins in the alimentary tract, a vast range of defined short peptides is obtained. The particular character of the intestinal endopeptidases leads to peptides which *per se* do not possess all amino acids at the C- or N-terminal ends but only: Lys, Arg, Tyr, Trp, Thr, Cys, Glu, Asp, Met, Ala, Val, Gly, Leu or Ile. These amino acids are named “active” amino acids. In mammals and birds, and probably in all vertebrates, due to the specificity of the pancreatic endopeptidases, peptides terminating with Ser, Thr, Pro and His theoretically never appear during the natural digestion process of dietary proteins, and these amino acids are arbitrarily named “passive”. Consequently, these four amino acids do not appear in the intestines in the free form because mammalian and avian exopeptidases are also specific and do not split peptides containing these passive amino acids. This is interesting, because peptides with such terminal amino acids do not exist under these conditions. However, peptides terminated with these “passive” amino acids can appear during the nonspecific hydrolysis of proteins. This means that they can appear in the intestine after the action of gut bacteria nonspecific endopeptidases, after the action of plant enzymes, or during “technical” hydrolysis of the food proteins with, e.g. nonspecific endopeptidases. It is suspected that such peptides, terminating with “passive” amino acids, may play a role as dietary protein-related antinutritional factors. It is not excluded that the absorption of such peptides may cause metabolic and allergic disturbances.

Two amino acids, Lys and Arg, found at the C and N ends of peptides are “super-active”. This is because they appear there after the action of trypsin – the key “hormonal” enzyme of the GIT. The peptides terminating in “super-active” amino acids are easily digested by the exopeptidases, which have a high affinity for them, only interacting with these types of peptides with one exception. If proline precedes the terminal amino acid, the type of the amino acid present at the end does not matter – the intestinal enzymes carboxypeptidase A and B will interact with the peptide, leading to the release of the terminal amino acid. Thus, theoretically,

the amino acids in peptides terminating with "active" amino acids can be absorbed to a greater degree than the peptides terminating with "super-active" amino acids. The latter process probably does not take place, since there is a mechanism which causes the reintroduction of the "super-active" amino acids into peptides. It has been shown that the specific endopeptides, e.g. pepsin, trypsin, etc., in an environment not optimal for their activity, can covalently bind these "super-active" amino acids in a plastein reaction, creating a new generation of peptides with new properties – the so-called post-enzymatic peptides. Some of these can have antibacterial activity (fig. 2).

In our own studies, the tryptic/peptic digestion of soy protein in the presence of free lysine creates a hydrolysate having a high antibacterial activity (Pierzynowski, 2004). During this process, free lysine disappears from the medium and appears as peptide-bound lysine. It should be noted here that antibacterial peptides are very often rich in lysine and arginine (Lee, 2002). It is also possible that specific endopeptidases play an active role in binding metal ions into the peptides, creating "metallopeptides". This process may improve the absorption of microelements, and is known to occur during other digestive processes, where eukaryotic fermentation ensures the addition of minerals into peptides. Yeasts and fungi, as well as the higher plants, contain considerable amounts of specific endopeptidases, in contrast to the bacterial proteases, which have so far been recognized as usually being nonspecific endopeptidases.

Short, postenzymatic peptides terminating with active amino acids are absorbed via receptors into the enterocytes, blood and peripheral tissues. The intake of these peptides from the intestines depends on the pH (Winckler et al., 1999) and energy sources (Rerat et al., 1992). It has been shown that in the presence of glucose in the intestines, the proteins are absorbed into the blood in the form of short, small peptides (Rerat et al., 1992). In cases of insufficient amounts of glucose and/or glutamine and other sources of energy for the intestine, proteins are completely digested to amino acids and are absorbed as amino acids (Rerat et al., 1992). So far there is no proof that the level of intestinal energy available has an impact on the absorption of "passive" peptides.

The following aspects need to be considered in future studies in order to be able to properly understand how dietary proteins are protected from the bacteria inhabiting the small bowel (fig. 3): (1) the effectiveness of pancreatic and other gut proteolytic enzyme secretions with respect to their amount and composition; (2) the effectiveness of the absorption vs. transmission of peptides or amino acids to the enterocytes and to the blood; (3) the development of the dietary protein-related antibacterial activity and the antibacterial activity of the GIT secretions; and (4) the effectiveness of the enterocyte amino acid vs peptide metabolism/processing and secretion to the blood.

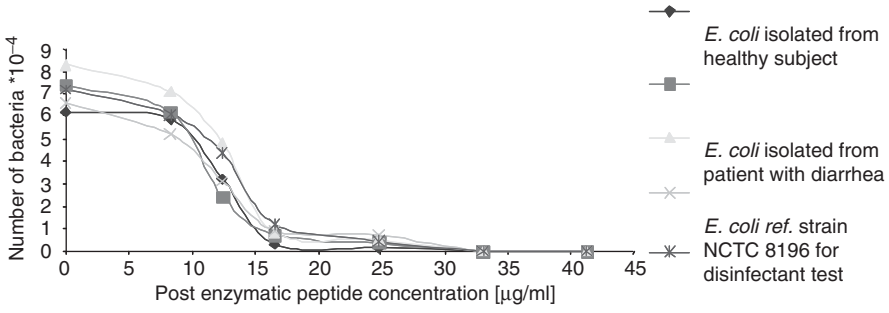

Fig. 2. Postenzymatic peptides (soy protein treated with trypsin and pepsin) inhibit growth of *E. coli* strains.

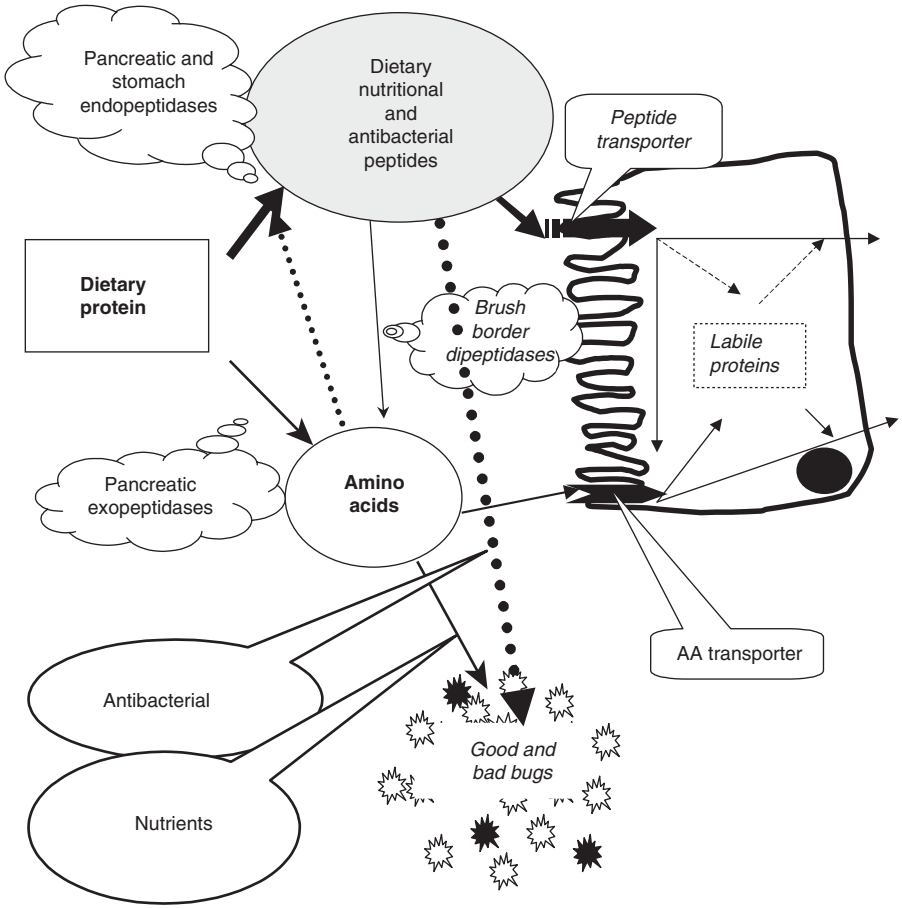

Fig. 3. Production of dietary peptides shunts proteins away from microbial utilization because of their structural specificity-dependent low availability for bacteria and exhibition of antibacterial activity.

The mechanism of the *de novo* synthesis/production of the enterocyte amino acids and their transmission to the portal blood and subsequent liver metabolism/conversion is still a mystery. This enterocyte processing/metabolism is the possible "bottleneck" for the removal of protein from the intestine away from possible bacterial utilization. The most mysterious part of this "puzzle" is how the status (fed, starved or sick) of the enterocyte can affect the intraenterocyte metabolism and disappearance from the gut of the amino acids and dietary peptides. Another question is how the blood-borne amino acid and peptide levels affect the metabolism of the dietary amino acids and peptides in the enterocyte. And finally, are there any possible dietary factors that can affect amino acids and peptide metabolism in the enterocyte, and if so, how?

New knowledge with respect to these questions is the key to maintaining the health of the enterocyte and thus the entire organism. The efficient utilization of feed protein ensures a rapid growth directly coupled to less N emission and less pollution. In order to understand and localize the mechanism of dietary protein protection from bacterial usage, we propose to concentrate the discussion to the point when dietary protein starts to be metabolized in the intestinal lumen, because proteins begin their processing already in the gut lumen. There are

strong indications that the metabolic regulation also starts there. Thus, the proposed research will exploit unique and novel concepts related to the digestion, absorption and protection of dietary protein from the gut bacteria.

Besides affecting differences in the quantity of enzymatic digestion, the composition and amount of the pancreatic secretion also affect the quality of the luminal metabolites to be absorbed (Hernell and Bläckberg, 1994). Protein digestion in pancreatectomized pigs supplemented with pancreatin produces specific postenzymatic peptides, and probably results in a pattern of digestion that shifts from the absorption of free amino acids (in the absence of pancreatic proteases) towards the absorption of short peptides to the portal bloodstream (Botermans et al., 2001). It may be that protein digestion to peptides in pancreatectomized pigs is limited because of the lack of endopeptidases. The majority of proteolytic enzymes available for protein digestion in pancreatectomized pigs are exopeptidases and dipeptidases of enterocyte origin which release amino acids from dietary protein. This may explain why pancreatectomized pigs absorb more amino acids than pigs supplemented with exogenous pancreatic enzymes. However, possible effects of pancreatin on enterocyte metabolism should also be considered. It cannot be excluded that pancreatin components can affect enterocyte metabolism resulting in altered production and excretion of amino acids and peptides into the blood. At present, two enterocyte cell wall peptide transporters have been identified and characterized in rabbits, rats and humans: PepT-1 (Fei et al., 1994; Liang et al., 1995; Saito et al., 1995) and PepT-2 (Liang et al., 1995; Liu et al., 1995). While free amino acids are easily used as an energy source (Reeds et al., 1996), peptides may be more available for protein synthesis. Pierzynowski et al. (1997) and Puchala et al. (2002) showed that peptides could directly be utilized by the productive tissues, i.e. hair growth. Thus, there are indications that the regulation of metabolism starts in the GIT, and here pancreatic exocrine secretion probably plays an important role.

In addition, the expression of peptide transport mechanisms in the intestinal brush borders (PepT-1 and PepT-2) may differ between animals according to their stage of development. In rats, the intestinal PepT-1 mRNA levels are highest in 4-day-old rats, and decrease to the adult level by 28 days after birth (Miyamoto et al., 1996).

A relatively high exocrine pancreatic secretion per kg metabolic body weight (Botermans et al., 1999) and a greater expression of peptide transporters (Miyamoto et al., 1996) may be one of the reasons for the relatively greater protein deposition and protein utilization observed in young growing animals in comparison to that of older animals, and may also help to explain the observed individual differences in growth. A more efficient protein digestion and absorption should be equivalent to more efficient nitrogen utilization.

Free amino acids are ideal nutrients for use by gut bacteria (Pedersen, 2001), while peptides probably are not. The dietary proteins ingested must be first digested by the bacterial or host peptidases to amino acids for usage by the gut flora. The intriguing question is whether bacteria actually possess peptide transporters? If not, this can be the main mechanism protecting the digestive peptides, and generally, whole dietary proteins, from bacterial utilization in the small intestine. The peptides will simply be absorbed before they become available for bacteria as short chain fatty acids – the main source of substrate for all kinds of anabolic processes in gut bacteria. Moreover, some of the short peptides formed in the GI tract after digestion apparently can protect dietary protein from microbial utilization, and thus have an antibacterial effect (Pellegrini et al., 1997).

Several bactericidal peptides have been generated by digestion from cathepsin G (Bangalore et al., 1990), lactoferrin (Bellamy et al., 1992), lysozyme (Pellegrini et al., 1997), aprotinin (Pellegrini et al., 1996) and α-lactalbumin (Pellegrini et al., 1999). It has also been

shown that efficient bactericidal peptides are produced via protein digestion (Kruszewska et al., 2000a,b). Naturally occurring antimicrobial peptides and proteins are part of the innate immunity of an organism, e.g. defensins or lysozyme. Some exert a wide spectrum of antimicrobial activity against Gram-negative and Gram-positive bacteria, fungi and viruses (Gennaro et al., 1989; Lehrer et al., 1993). These antimicrobial effects of peptides may play an important role in the health of humans and animals. Obtaining knowledge about these underlying biological mechanisms is important in order to reduce the therapeutic use of antibiotics in both human medicine and animal production (Service, 1995).

4. RELATION BETWEEN CHOLECYSTOKININ (CCK) RECEPTOR FUNCTION AND PIG GROWTH AND PERFORMANCE

CCK is released from the I-cells and neurons in the duodenum and the jejunum, in the presence of lipids and proteins from ingested food. CCK, together with impulses from the vagal-cholinergic pathway, are the main regulators of the pancreatic secretion of digestive enzymes.

Active forms of CCK arise from pro-cholecystokinin, a protein giving rise to CCK peptides of various sizes from four to 58 amino acids. CCK-33 was the first CCK-peptide isolated and was obtained from pig intestine. Later, CCK was derived in other forms as well (Morisset et al., 2003). Two receptor subtypes have been found with different binding affinities for CCK, CCK-A with high affinity and CCK-B receptors with low affinity. The CCK receptors belong to the superfamily of G-protein-coupled receptors, sharing a similar molecular structure of seven transmembrane α-helices with the N-terminal outside and the C-terminal inside the cell. When the CCK receptor is activated, the intracellular Ca^{2+} concentration increases 10–100-fold, resulting in the exocytosis of cytoplasmatic stored granules and an increased enzyme secretion (Konturek et al., 2003). Several studies (Bourassa et al., 1999; Schweiger et al., 2000; Morisset et al., 2003) have shown a varying localization of CCK receptors in the pancreas and other tissues of different species. In rats, CCK-A receptors are present on pancreatic acinar cells, while pigs and humans do not express any CCK receptors on the pancreatic acinar cells. Both CCK-A and CCK-B receptors have been located on the pancreatic islet cells (Schweiger et al., 2000; Morriset et al., 2003).

The regulation of the pancreas in pigs, as studied in our lab (Kiela et al., 1996; Evilevitch et al., 2003, 2004) appears to be highly specific and different from that of, for example, the rat. The pig pancreatic acinar cells do not possess either A or B CCK receptors (Schweiger et al., 2000; Morisset et al., 2003), and physiological doses of CCK given into the systemic circulation have failed to stimulate pancreatic secretion (Cuber et al., 1989; Evilevitch et al., 2004). In spite of this finding, it was proven that CCK is the main regulator of the exocrine pancreas in pigs. In experiments (Evilevitch et al., 2004) where CCK was administered via the duodenal arterial circulation in doses reproducing peripheral postprandial levels, a strong stimulation of the pancreatic enzyme secretion was achieved. Studies with a specific CCK-B receptor blocker showed that the stimulation was mediated via CCK-B receptors. Studies with a blockade of CCK-A receptors (Evilevitch et al., 2003, 2004) clearly showed that there were no functioning CCK-A receptors in the duodenum, and that the CCK-A receptors responsible for the exocrine pancreatic stimulation using high, pharmacological doses of CCK are located elsewhere.

Several studies have shown that pancreatic insufficiency (PI) results in a reduced weight gain in different species. In animals, pancreatic duct ligation has been used to demonstrate that a properly functioning pancreas is needed for an animal to obtain normal growth. In studies

with PI pigs (Imondi et al., 1972; Corring and Bourdon, 1977; Saloniemi et al., 1989; Gregory et al., 1999) a reduction in daily weight gain of 25–100% was found. Supplementation of pancreatic enzymes to PI pigs has been shown to stimulate their growth (Saloniemi et al., 1989). Preliminary studies performed by our group showed that pigs with a low growth rate responded weakly or not at all to CCK-A-receptor-mediated stimulation of the exocrine pancreas, while fast-growing piglets showed a strong exocrine pancreatic response after stimulation via either of the two CCK receptor subtypes (Rengman, 2004). Moreover, studies by Botermans et al. (1999) on weaned piglets and Pierzynowski et al. (2004) on suckling piglets, showed a positive correlation between growth, feed conversion, pancreatic enzyme secretion, and CCK levels in the blood. These results imply that the function and regulation of the exocrine pancreas is closely connected to an efficient feed conversion and growth rate in piglets.

Our studies suggest that the pancreatic enzyme secretion in pigs is regulated via an enteropancreatic reflex originating in the duodenum, with the CCK-B receptor being primarily involved (Evilevitch et al., 2004). However, the complete reflex arc is unknown. Direct enteropancreatic neurons have recently been identified (Kirchgessner and Liu, 2000; Lesniewska et al., 2001) and possible candidates for terminal mediators/receptors on the pancreatic acinar cells are 5-HT, acetylcholine (via M1-, M3-receptors) or adrenaline (epinephrine) (via α and β-receptors). The involvement of pancreatic ganglia with both classical and peptidergic neuromediators in this reflex is also possible. Moreover, the islet cells and their hormones are not excluded, especially since receptors for the above-mentioned classical neuromediators are present on the islet cells.

In parallel, a pancreatic, CCK-dependent reflex, which appears to originate elsewhere than in the duodenum, exists in pigs and is mediated via CCK-A receptors (Evilevitch, 2004). Although no CCK-A receptors have been found on the pancreatic acinar cells, a significant number of such receptors has been found on the pancreatic endocrine islet cells (Schweiger et al., 2000; Morisset et al., 2003). Thus, it is possible that endogenous CCK, or exogenous CCK administered in high amounts via the peripheral circulation, may activate CCK-A receptors on islet cells and stimulate the release of islet hormones, such as insulin and insulin-like growth factor (IGF) from B-cells, glucagon from A-cells or somatostatin from D-cells. Alternatively, CCK may stimulate pancreatic and visceral ganglionic neurons releasing adrenaline (epinephrine), acetylcholine or neuropeptides as mediators. High intrapancreatic levels of these hormones and mediators may then act directly on the acinar cells (Kanno et al., 1978; Williams and Goldfine, 1985; Pierzynowski et al., 1986; Ahren, 2000) since the pancreatic blood flow from the pancreatic islets and ganglions must pass the acinar cells before entering the portal circulation (Lifson et al., 1980).

In addition, these CCK-mediated reflexes appear to function in parallel but with some temporal differences. One may postulate that the CCK-B receptor-mediated entero (duodenal)-pancreatic reflex is related to the intestinal digestive phase, while the CCK-A receptor-dependent pancreatic reflex is coupled to the metabolic phase of the pancreatic regulation. After a primary function of stimulating the pancreatic enzyme secretion (in response to food stimulation), locally in the pancreas, the islet hormones also regulate the metabolism – the utilization and deposition of the absorbed dietary components being the products of this enzymatic degradation. This appears to be an ideal example of the integration of digestive function and nutrient utilization with growth (fig. 4). In fact, our preliminary studies support the possibility of this relationship since pigs characterized by slow growth do not exhibit any activity of the long pancreatic CCK-A receptor-dependent reflex and produce fewer pancreatic enzymes (Botermans et al., 1999; Rengman, 2004).

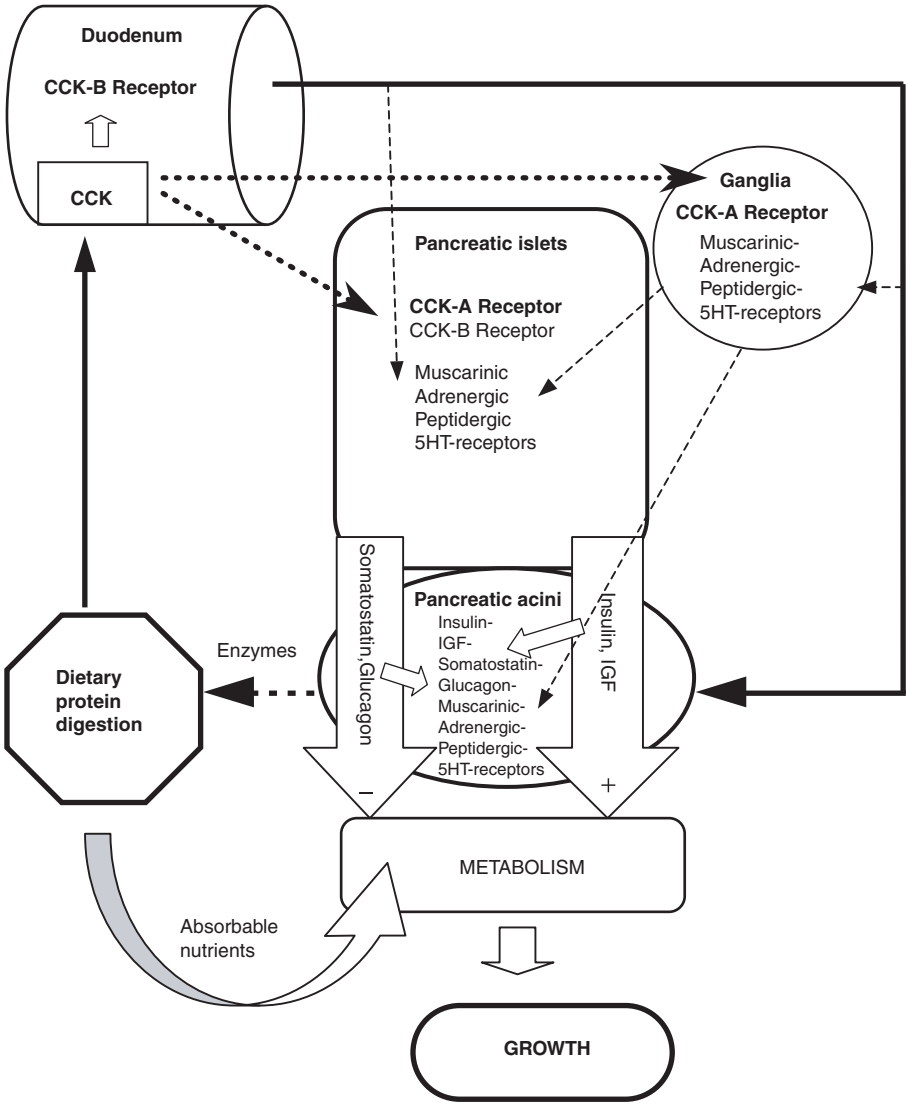

Fig. 4. Schematic drawing of the relation between CCK dependent pancreatic function, protein digestion and growth.

5. FUTURE PERSPECTIVES

The modern nutritional studies should be focused on deciphering the correlation between the growth of animals and the gut bacteria. Understanding the relations between the effectiveness of exocrine pancreatic function for host performance and gut bacterial ecology should be one of the main objectives of future dietetic and nutritional studies.

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4 Carboxylic acids as bioregulators and gut growth promoters in nonruminants

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A renewed interest in short- and medium-chain carboxylic acids as possible in-feed replacements for subtherapeutic levels of antibiotics has occurred as a result of the European Unions plan to phase out subtherapeutic levels of antibiotics from animal feed by 2006. A review of current literature relating the modes of action and effectiveness of both short and medium chain carboxylic acids relative to gut health and performance of nonruminant animals will be presented, with an emphasis on pigs.

1. INTRODUCTION

Over the last 50 years numerous studies have been addressed worldwide to evaluate four major benefits due to carboxylic acids: (1) improved health and resistance to disease; (2) faster growth; (3) increased efficiency of diet utilization; (4) better carcass quality. Secondary effects, concerning environmental pollution (less total N, volatilized ammonia, P) and/or reduced production costs have also received considerable attention.

Natural feed resources (fresh, prefermented or ensiled ingredients of plant or animal origin and additives) contain more than 100 carboxylic (fatty) acids and their derivatives. Their fate

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and effects (nutritional, biochemical, physiological or clinical) in the digestive tract and/or at the systemic level are related to physical and chemical properties such as the length of carbon chains, number of carboxylic groups (COOH), saturation degree and position of one or more double bonds, configuration (straight/branched, *cis*/*trans*), area per molecule, intermolecular distance, limiting area, melting point temperature, etc. For nutritional purposes, three major classes of carboxylic acids, i.e. short-chain fatty acids (SCFA), medium-chain fatty acids (MCFA) and long-chain fatty acids (LCFA) are usually distinguished (with 1–6, 7–12 and above 12 carbon atoms in the molecules, respectively). Despite continuous scientific efforts, our knowledge on the biological role of endogenous and intrinsic/extrinsic sources of each class of these carboxylic acids as multifunctional constituents in two principal body compartments, i.e. in the digestive tract and in the systemic/post-absorptive pool is not yet complete.

Currently, SCFA and MCFA have received renewed interest due to the phasing out of supplemental antibiotics for prophylaxis in nonruminant nutrition by 2006 in the EU. In the case of SCFA, there are some excellent reviews on their role, predominantly in the large intestine of nonruminants (Bergman, 1990; Bugaut and Bentéjac, 1993; Binder et al., 1994; Cummings et al., 1995; LeLeiko and Walsh, 1996; Topping and Clifton, 2001). In contrast to SCFA, less information is available on the role of MCFA. There is a general consensus that supplementary sources, in conjunction with intraluminally generated SCFA from fermentable substrates, can contribute up to 30% of the energy requirement for maintenance (Rerat et al., 1987). In addition, their bactericidal/bacteriostatic action in the gut leads to dynamic changes in the population of more than 400 species of both autochthonous (resident) and allochthonous (transient) microbes, as well as direct and/or indirect effects on regulating gut development, morphological structure, permeability, production of mucin and enzymes, motility, digestion, mucosal immunity, pancreas/bile secretory activity, etc. (Partanen and Mroz, 1999).

In this chapter, a special emphasis will be placed on the intraluminal and post-absorptive bioactivity of SCFA and MCFA in nonruminants, and particularly in pigs. The objective of this review is to address the following topics:

1. Some essentials on the physicochemical properties of SCFA and MCFA;
2. Intraluminal production rates and concentrations in particular sections of the gut;
3. Direct and/or indirect effects of SCFA and MCFA on gut functionality;
4. Transepithelial transport and absorptive mechanisms of SCFA;
5. Post-absorptive roles in metabolic and regulatory processes of the body.

2. SHORT-CHAIN FATTY ACIDS

2.1. Classification and physicochemical properties

Carboxylic (fatty) acids possessing from one to six carbons in the chain, and a carbon–oxygen double bond (C=O), known as the *carbonyl* group with two attached organic groups, R^1 and R^2, (R^1—C=O—R^2) are often classified as short-chain fatty acids (SCFA). Within this class are mono-, di- or tricarboxylic acids, and the monocarboxylic acids (e.g. acetic, butyric, propionic, valeric and caproic) are sometimes called volatile fatty acids (VFA) since they are derived from anaerobic breakdown of polysaccharides by intestinal microorganisms. The organic group R^1 consists either of a hydrogen (H) atom or an alkyl/aryl group, whereas the organic group R^2 always contains a hydroxide (OH) ion. The carbon–oxygen double bond consists of two types of bonds: (1) σ bond, formed by overlap of hybrid orbitals, and (2) π bond, formed by overlap of p orbitals (fig. 1).

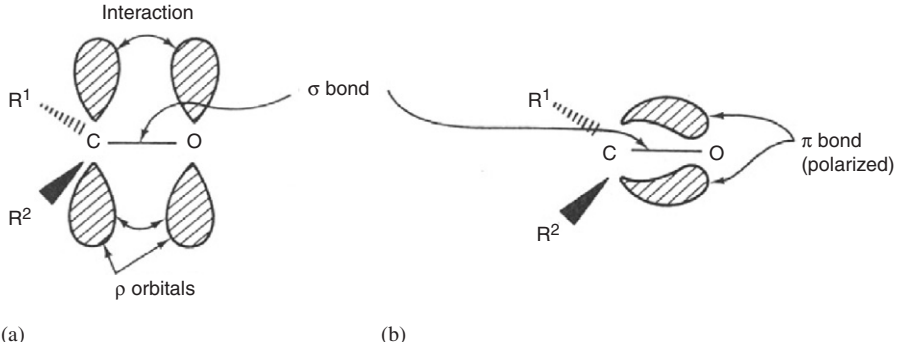

Fig. 1. Carboxylic (fatty) acids possessing from one to six carbons in the chain, and a carbon–oxygen double bond (C=O), known as the *carbonyl* group with two organic groups R^1 and R^2 attached (R^1— C=O — R^2) are often classified as short-chain fatty acids (SCFA). Within this class are mono-, di- or tricarboxylic acids, and the monocarboxylic acids are sometimes called volatile fatty acids (VFA) since they derive from anaerobic breakdown of polysaccharides by intestinal microorganisms. The organic group R^1 consists either of a hydrogen (H) atom or alkyl/aryl groups, whereas the organic group R^2 contains always a hydroxide (OH) ion. The carbon–oxygen double bonds consist of two types of bonds: (a) σ bond, formed by overlap of hybrid orbitals, and (b) π bond, formed by overlap of p orbitals.

Oxygen is considerably more electronegative than carbon, and therefore the bonds joining the two atoms are highly polarized, especially the π bond, in which the electrons are less tightly held than in the σ bond. This means that the oxygen atom effectively carries a partial negative charge and the carbon a partial positive charge. Due to this polarization, the carbonyl carbon atom is susceptible to *nucleophilic substitution* reactions (e.g. the biosynthesis of citric acid occurs by an aldol-type reaction). This tendency is often enhanced by the addition of acid (HCl), which protonates the oxygen atom and thereby increases the polarization. Water is an *amphiprotic* solvent, i.e. one capable of acting as an acid or a base, and SCFAs in aqueous solutions tend to dissociate and donate protons (H^+; fig. 2). Furthermore, the anions formed by the proton abstraction are stabilized by resonance and exhibit also acidic properties, since the negative charge is spread over the rest of the molecule (X), and particularly over the COO group (fig. 3).

Despite this stabilization resonance, most carboxylic anions are much less stable than anions such as HSO_4^-, and hence SCFAs are much less dissociated than mineral acids in aqueous solutions. For example, acetic acid (0.1 Mol) in aqueous solution is not more than 1% dissociated, whereas a comparable amount of HCl is completely dissociated. The strength of SCFA can be expressed quantitatively by means of the equilibrium constant of its

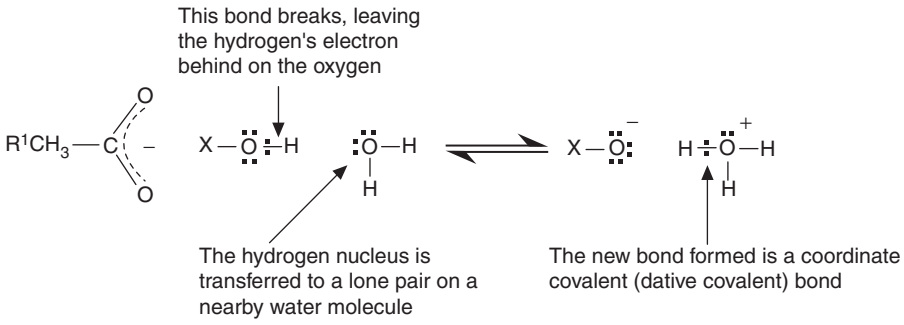

Fig. 2. Dissociation and donation of protons (H^+) by SCFA in the presence of water.

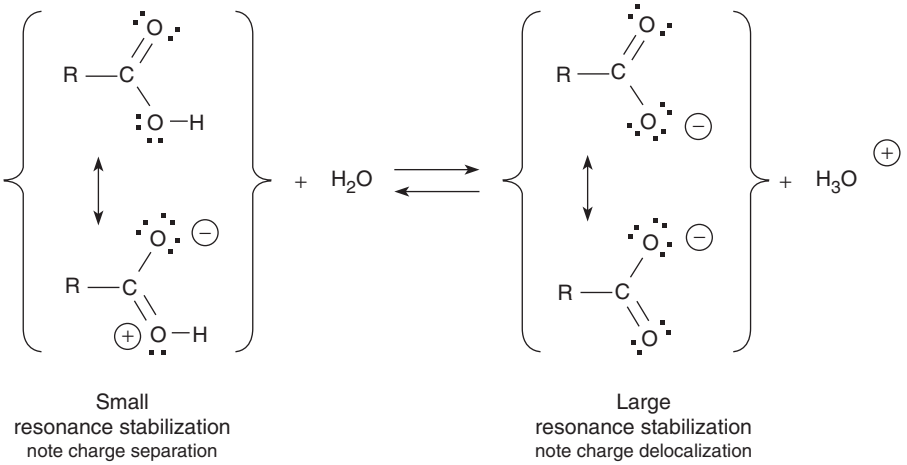

Fig. 3. Resonance stabilization of anionic substrates following proton (H^+) donation.

dissociation, known as the *dissociation constant*, K_a, and sometimes also expressed on a logarithmic scale as pK_a values.

The relationship between solution acidity (pH), dissociation constant (pK_a), and the relative concentrations of SCFA (monoprotic) is shown in fig. 4.

When the concentrations of the salt and acid are equal, the pH of the system equals the pK_a of the acid. Dicarboxylic SCFA are diprotic, i.e. possess two acid dissociation constants: K_{a1} and K_{a2}, and therefore the relationship of pH and pK_a is shown in fig. 5.

The pH of the intermediate form of a weak acid in a solution is close to midway between the two pK_a values, i.e. pH ~ $^1/_2(pK_{a1} + pK_{a2})$. Due to intramolecular interactions, the pK_a values of monocarboxylic SCFA increase up to four carbons in the chain, but not beyond.

The pK_a value represents the ionic environment of the solution where 50% of the H^+ ions are removed from the carboxyl group (COO^-) by the existing OH^- ions in the solution. Within the series of SCFA there is considerable variation in acid strength, and the smaller the pK_a value the stronger is the acid. The pK_a can be decreased by attaching an electron-accepting electronegative substitute (e.g. Cl^-), which pulls charge away from the -COO^- end (fig. 6).

Replacement of the hydrogen atoms in the alkyl group by halogen atoms (particularly at the α-positions) increases the acidity, because the carboxylate anion is stabilized by inductive electron withdrawal. For example, although ethanoic (acetic) acid is relatively weak ($pK_a = 4.76$), it may be strengthened by replacing H^+ by Cl^- in the chain to obtain monochloroethanoic acid

$$K_a = \frac{[\overset{\oplus}{H_3O}]\,[A\overset{\ominus}{:}]}{[HA]} \qquad pK_a = -\log K_a = \log\left(\frac{1}{K_a}\right)$$

(a)

$$pH = pK_a + \log\frac{[A^-]}{[HA]}$$

(b)

Fig. 4. (a) The strength of SCFA can be expressed quantitatively by means of the equilibrium constant of its dissociation, known as the *dissociation constant*, K_a, and sometimes also expressed on a logarithmic scale as pK_a values. (b) The relationship between solution acidity (pH), dissociation constant (pK_a), and the relative concentrations of SCFA (monoprotic). $[A^-]$ is the molar concentration of the dissociated salt; [HA] the concentration of the undissociated acid. When the concentrations of the salt and acid are equal, the pH of the system equals the pK_a of the acid.

$$pH = pK_{a1} + \log\left(\frac{[HA^-]}{[H_2A]}\right) \quad pH = pK_{a2} + \log\left(\frac{[A^{2-}]}{[HA^-]}\right)$$

Fig. 5. Dicarboxylic SCFA are diprotic, i.e. possess two acid dissociation constants: K_{a1} and K_{a2}, and therefore the pH is calculated as shown on the left.

($CH_2ClCOOH$; $pK_a = 2.86$) or dichloroethanoic acid ($CHCl_2COOH$; $pK_a = 1.29$). However, the effect of replacing H^+ by Cl^- along the chain of butanoic acid is not associated with a graded increase of acid strength, as the pK_a values for butanoic acid ($CH_3CH_2CH_2COOH$), 2-chlorbutanoic ($CH_3CH_2CHClCOOH$), 3-chlorbutanoic ($CH_3CHClCH_2COOH$) and 4-chlorbutanoic are 4.82, 2.84, 4.06 and 4.52, respectively.

The reactivity of SCFA depends upon several factors, including solvent, temperature and concentrations of reactants. Even under identical conditions reactions proceed at different rates, depending upon the particular reagent and the structure of the substrate and medium. Thus, under a given set of *in vivo* conditions, the rate of a single-step reaction of the type $A + B \longrightarrow C$ is given by the expression: Rate = k [A][B], where k is constant (dependent upon temperature and solvent) and [A] and [B] are the molar concentrations of A and B, respectively. These reactions can be grouped into a number of categories according to the overall result. The common categories are *addition, elimination, substitution* (displacement), and *rearrangement* (isomerization) reactions.

The reactivity of members of a particular class of carbonyl compounds depends upon the steric and electronic environment of the carbonyl group. In general, aromatic compounds are less reactive than their aliphatic counterparts because the partial positive charge on the carbonyl carbon atom can be delocalized around the benzene ring. Most of SCFA and their salts exhibit appreciable solubility in water due to the presence of the polar carboxyl group, although this solubility declines with increasing numbers of C-atoms in the molecule (table 1).

In addition to the salts listed in table 1, K-lactate, Na-sorbate, Ca-sorbate, K-sorbate, Na-tartrate, K-tartate, NaK-tartarate, NH_3-formate, Na-formate, NH_3-propionate, Na-propionate, K-acetate, Ca-acetate, Na-diacetate, Na-citrate, and K-citrate are all registered as feed acidifiers in the EU.

Liquid forms of SCFA may be volatile (up to 20%) during spraying, leading to the possibility of (if not encapsulated) corrosiveness and undesirable odor (nostril and eye irritations). In contrast to liquid SCFA, their solid salts can be relatively easily incorporated into composite diets, with less or no negative impact on the corrosiveness of equipment.

Supplemental SCFA may serve as feed preservatives, which reduce both dietary pH and buffering capacity (BC) (Blank et al., 1998; Mroz et al., 2001). The BC (also called acid-binding or cation-exchange capacity) expresses the resistance of solutes/media against declining pH,

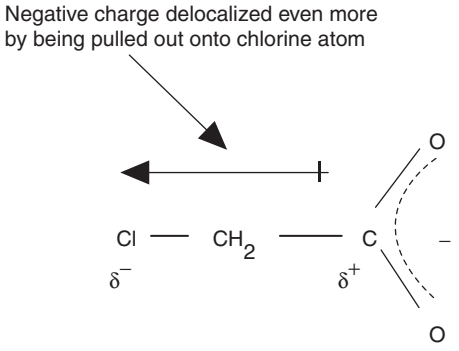

Fig. 6. There is considerable variation in acid strength of SCFA, and the smaller the pK_a value the stronger the acid is. The pK_a can be decreased by attaching an electron-accepting electronegative substitute (e.g. Cl^-), which pulls charge away from the $-COO^-$ end.

Table 1

Physical and chemical characteristics of some SCFA and their salts

Trivial name	Chemical formula	MW g/mol	Density g/cm^3	Form	pK$_a$	Solubility in water[a]	Gross energy, MJ/kg	Corrosiveness	Odor	Systemic name
Monocarboxylic										
Formic	H COOH	46.03	1.220	liquid	3.75	∞	5.54	++(+)	pungent	methanoic
Acetic	CH_3 COOH	60.05	1.049	liquid	4.76	∞	15.60	+++	vinegar	ethanoic
Propionic	CH_3CH_2 COOH	74.08	0.993	liquid	4.88	∞	20.69	++	pungent	propanoic
Butyric	CH_3 $(CH_2)_2$ COOH	88.12	0.958	liquid	4.82	∞	24.80	+	rancid butter	butanoic
Isobutyric	$(CH_3)_2$ CH COOH	88.12	0.958	liquid	4.82	*v*	24.80	+	rancid butter	2-methyl-propanoic
Valeric	CH_3 $(CH_2)_3$ COOH	102.13	0.939	liquid	4.84	∞	27.00	+	sweaty, parmesan cheese	pentanoic
Isovaleric	$(CH_3)_2$ CH CH_2 COOH	102.13	0.923	liquid	4.82	∞	27.00	+	rancid cheese	3-methyl-butanoic
Caproic	$CH_3(CH_2)_4$ COOH	116.16	0.927	liquid	4.85	*i*	30.14	+	foul, goat-like	hexanoic
Isocaproic	$(CH_3)_2$ $(CH_2)_2$ CH COOH	116.16	0.927	liquid	4.84	*i*	30.14	+	goat-like	4-methyl-pentanoic
Dicarboxylic saturated										
Oxalic	COOH COOH	90.04	1.65	crystal	1.23/ 4.19	∞	4.40	+++	odorless	ethanedioic
Malonic	COOH CH_2 COOH	104.06	1.619	crystal	2.83 5.69	∞	8.32	(+)	sugarbeet-like	propanedioic
Succinic	COOH $(CH_2)_2$ COOH	118.1	1.564	crystal	4.19 5.57	∞	14.35	+	odorless, acid taste	butanedioic
Oxalacetic	COOH C(=O) CH_2 COOH	132.04		crystalline/ powder	2.17	∞	10.68	+	odorless/ apple-like	2-oxobutanedioic
Malic	COOH CH(OH) CH_2 COOH	134.09	1.600	powder/ liquid	3.4 5.1	*v*	11.3	(+)	odorless/ apple-like	2-hydroxybutanedioic

Tartaric	COOH CH(OH) CH(OH)COOH	150.09	1.760	liquid	2.93 4.23	*v*	7.66	0	odorless	2,3-dihydroxy-butanedioic
Glutaric	COOH $(CH_2)_3$ COOH	132.1	1.400	crystal	4.31 5.41	*v*		(+)	odorless	pentanedioic
Adipic	COOH $(CH_2)_4$ COOH	146.1	1.36	crystal	4.42 5.41	*s*	19.15	(+)	nil to very faint acid taste	Hexanedioic
Glucaric	COOH $(CHOH)_4$ COOH	210.14	1.310	crystal	3.21	*s*	13.4	0	sugary, sweet	2,3,4,5-tetra-hydroxy-hexanoic
Hydroxy acids										
Carbonic	OH COOH (not possible to obtain pure, formed when carbon dioxide dissolves in water)	62.0			6.36 10.33	∞	3.58	0	weak acid	dihydrogen carbonate
Glycolic	OH CH_2 COOH	76.05	1.360	oily liquid	3.63	*v*	9.2	(+)	odorless	hydroxyethanoic
Lactic	OH CH_3 CH COOH	90.08	1.206	liquid	3.83	*v*	15.6	(+)	sour milk	2-hydroxypropanoic
Citric	COOH CH_2C(OH)(COOH) CH_2 COOH	192.14	1.665	solid	3.13 4.76 6.40	*v*	12.28	0 to ++	odorless	2-hydroxy-1,2,3-propane-tricarboxylic
Gluconic/ maltonic/ dextronic	OH CH_2 $(CHOH)_4$ COOH	196.16	1.230	syrupy liquid	3.60	∞	13.52	0	odorless/ slightly sweet	pentahydroxy-carboxylic
Keto acids										
Pyruvic	CH_3 C(=O) COOH	88.06	1.260	liquid	2.50	∞	10.8	++	grape-like pungent	2-oxo-propanoic
Diacetic	CH_3 C(=O) CH_2 COOH	102.09	1.026	powder	3.58	∞	18.72	(+)	acetone	methyl 3-oxobutanoic
Levulinic	CH_3 C(=O) $(CH_2)_2$ COOH	116.06	1.134	crystal liquid	4.59	∞	22.09	0	fruit-like	4-oxopentanoic
α-keto-glutaric	COOH $(CH_2)_2$ C(=O) COOH	146.06	1.400	crystal		∞		(+)	odorless	2-oxo-1,5-pentanedioic

Continued

Table 1

Physical and chemical characteristics of some SCFA and their salts—Cont'd

Trivial name	Chemical formula	MW g/mol	Density g/cm^3	Form	pK$_a$	Solubility in water[a]	Gross energy, MJ/kg	Corrosiveness	Odor	Systemic name
Unsaturated acids										
Fumaric	trans-COOH CH=CH COOH	116.07	1.635	crystal	2.97 4.46	*s*	13.67	0 to (+)	odorless or fruit-like	trans-butenedioic
Maleic	cis-COOH CH=CH COOH	116.07	1.635	crystal	1.88 6.23	*v*	13.67	+++	pungent	cis-butenedioic
Acrylic	CH_2=CH COOH	72.06	1.190	liquid	4.26	∞	19.93	+++	acrylic	propenoic
Methacrylic	CH_2=C (CH_3) COOH	86.1	1.015	liquid		∞	24.26		repulsive, acrid	2-methylpropenoic
Crotonic	CH_3CH=CH_2 COOH	86.1	1.020	solid	4.70	*v*	24.26	+++	acrid, croton	*trans*-2-butenoic
Isocrotonic	CH_2=$CHCH_2$ COOH	86.1		liquid		*v*	24.26	+++	oil-like	
Itaconic	COOH CH_2C(=CH_2) COOH	130.1	1.632	crystal powder	3.90 5.56	∞	18.16		croton oil-like	methylenesuccinic
Citraconic	COOH (CH_3)C=CH COOH	130.1	1.247	crystal	2.45 6.08	*s*	18.16		odorless	*cis*-methylbutenedioic
Aconitic	COOH CH=C(COOH) CH_2 COOH	174.12	1.652	crystal	2.78 4.36	∞	14.43		aromatic	1,2,3-propenetri-carboxylic

Sorbic	$CH_3CH=CHCH=CH$ COOH	112.13	1.204	crystal	4.76	*s*	27.85	++	mildly acrid	2,4-hexadienoic
Mesaconic	COOH-CH=C(CH_3) COOH	130.06		crystal	3.04 4.85	∞	18.17			*trans*-methyl-butenedioic
Propiolic	CH=C COOH	70.03	1.138	crystal		*s*	20.18	0		propynoic
Chain-branched/carbocyclic/aromatic										
Pivalic	$(CH_3)_3$ C COOH	102.13		crystal	5.03	*i*	29.49			propanoic/ neopentanoic
Benzoic	C_6H_5 COOH	122.13	1.266	crystal	4.19	*i*	26.40	+ to (++)	hot, acrid	benzene-carboxylic
Salicylic	C_6H_5 (OH) (COOH)	138.12		crystal	4.46	*i*	(30.55)[b]	++ to (+++)		2-hydroxy-benzoic
Gallic	CHCOHCOH COHCHC COOH	138.12		crystal		*i*	26.26 26.26	++ to (+++)		3,4,5-trihyroxy-benzoic
Some most common in-feed salts of SCFA										
Ca-formate		130.1		solid		*v*	11.0	0	almost odorless	
Ca-lactate		308.3		solid		*v*	30.0	0	odorless	
Ca-propionate		184.1		solid		*v*	40.0	0	faint odor of propionic acid	
K-diformate		130.0		solid		*v*	11.4	0	odorless	
Ca-butyrate		214.0		solid		*v*	48.0	0	rancid, sour milky	
Mg-citrate		214.4		solid		*v*	10.0	0	slightly orange	
Na-lactate		112.1		solid		*v*	15.0	0	odorless	

[a]∞ = soluble in all proportions; *v* = very soluble; *s* = sparingly soluble; *i* = insoluble.
[b]calculated value.

primarily due to the presence of buffering systems (bicarbonate, phosphate, and/or protein) and/or plant cell walls, in which the OH^- anions are capable of attracting protons (H^+) from the undissociated SCFA, so that the proton concentration does not change. Also, SCFA can serve as buffers in aqueous solutions, and maximal BC is achieved at the equilibrium between undissociated and dissociated forms (=pK_a). The BC of diets or SCFA in drinking water can be determined by a titration with HCl (or NaOH), and expressed as milliequivalents (mEq) of 0.1 Mol HCl needed to obtain a fixed acidity (usually pH = 5 or 4 or 3). In practical terms, the BC values at pH 5 for lactic, citric and fumaric acid are negative (–780, –738 and –1190, respectively), as their 10% solution in water has pH values lower than 5 (1.7, 1.4 and 1.8, respectively). On the other hand, salts of these acids (e.g. with Ca) in 10% solutions with water have pH values close to 5, and therefore, BC values are not far from zero. To prevent a fast dissociation of SCFA in the stomach and/or to address their bioactivity towards distal parts of the intestine, a 'micropackaging technique' (microencapsulation) may be implemented (Piva et al., 1997). Microencapsulation of blended SCFA is sometimes postulated in order to increase palatability (by masking acrid odor), to control gastric dissociation rates, and to allow for release along the whole digestive tract to prevent enteric bacterial infections (Von Felde and Rudat, 1998). Encapsulation can be chemical (coacervation, interfacial polymerization, phase separation, solvent evaporation or *in situ* polymerization) or mechanical (electrostatic methods, coextrusion: stationary/centrifugal, submerged/vibrating nozzle; spray-coating: air suspension, pan coating or atomization: spray drying, spray chilling), depending on the susceptibility of active ingredients to the different coating methods. The release period for in-feed acidifiers can be extended by coating with a rate-limiting membrane or even a totally impermeable membrane, which is perforated in selected places to allow release. Polymer systems with several layers, including a soluble core, swellable coat and semipermeable outer membrane, can be controlled by many different parameters. As more is learned about membranes and cell-signaling, the focus of new microencapsulation systems will be adjusted to allow delivery at a given location of the digestive tract to a single subset of receptors on a single subset of cells. Irrespective of the form of administration, undissociated or dissociated SCFA cannot react with freely available negatively charged Cl ions in the stomach or small intestine. Eventually, in the presence of an excess of ketonic compounds in the intraluminal environment, they may be esterified (e.g. $CH_3COOH + CH_3OH \longrightarrow CH_3COOCH_3 + H^+ + H_2O$).

2.2. Production rates and concentrations of SCFA in the digestive tract

In general, the host, environmental and microbiological factors regulate the endogenous production rates and intraluminal concentrations of SCFA along particular compartments of the digestive tract in nonruminants. The principal substrates for gut microflora are resistant starch and nonstarch polysaccharides (Topping and Clifton, 2001), and SCFA are generated together with gases (CO_2, CH_4 and H_2) and some heat. The general reaction of SCFA production and overall stoichiometry has been summarized for a hexose as follows: $59\ C_6H_{12}O_6 + 38\ H_2O \rightarrow 60\ CH_3COOH + 22\ CH_3CH_2COOH + 18\ CH_3CH_2\ CH_2COOH + 96\ CO_2 + 268\ H^+$ + heat + additional bacteria (Topping and Clifton, 2001). A simplified diagram of SCFA production in the large intestine is shown in fig. 7.

In addition to these carbohydrates, animal fats with appreciable proportions of SCFA and/or triacylglycerols (TAG) may contribute to the total intraluminal pool of SCFA. For example, Bugaut and Bentéjac (1993) indicated that bovine milk contains 10 mol% of butyric acid, 5 mol% of caproic acid and small amounts of acetic acid. In addition, three major TAG of

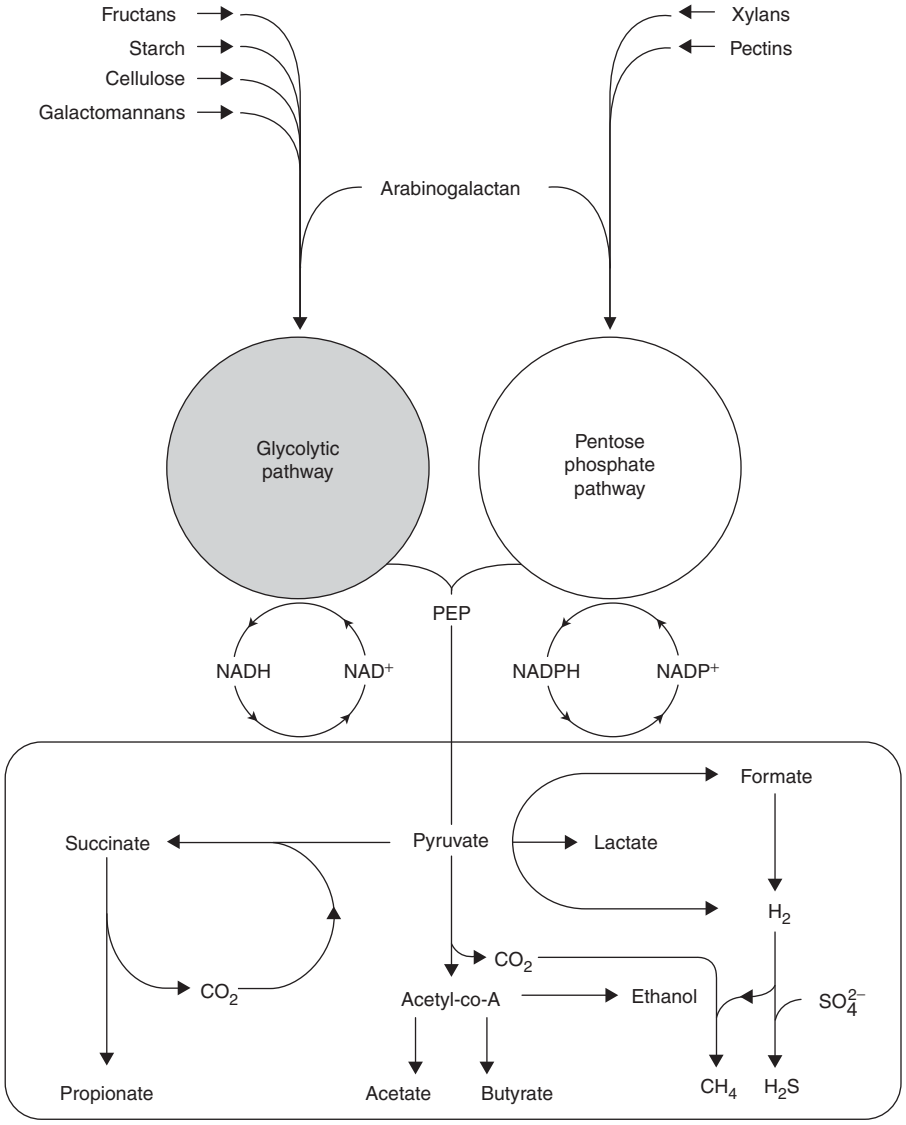

Fig. 7. A simplified diagram of polysaccharide breakdown and the main routes of carbohydrate fermentation in the large intestine. (PEP, phosphoenolpyruvate). (After Macfarlane and Macfarlane, 2003, with permission of the authors.)

bovine milk (4-16-18:1, 4-16-16 and 4-14-16, with 4.2, 3.2 and 3.1 mol% contents, respectively) bind one butyric acid per molecule, as a result of specific esterification at the sn-3 position. There is a possibility that this acid and some other VFA can be generated from dietary fats by endogenous lipoprotein lipase activity (Dierick et al., 2002a,b). Recently, Mariaca et al. (2001) found 27 optically active isomers or enantiomers of carboxylic acids in dairy products, which often have distinctly different biological properties.

The production rates and turnover of SCFA have been determined in a variety of *in vitro* and *in vivo* studies (Pouteau et al., 2003).

Table 2

***In vitro* production of SCFA (Mol%) from various sources of carbohydrates (after Topping and Clifton, 2001)**

Substrate	Incubation time, h	Degradation rate, %	SCFA produced, mMol
Pectin	10	89 (69 to 112)	62 (45 to 83)
	24	97 (83 to 117)	68 (46 to 97)
Sugarbeet fiber	10	32 (1 to 70)	26 (6 to 54)
	24	60 (14 to 98)	45 (29 to 72)
Soybean fiber	10	72 (55 to 95)	51 (29 to 70)
	24	91 (80 to 98)	64 (41 to 79)
Maize bran	10	0 (28 to 21)	5 (2 to 11)
	24	6 (21 to 27)	12 (3 to 26)
Cellulose	10	9 (9 to 38)	2 (1 to 4)
	24	7 (15 to 45)	3 (0 to 7)

2.2.1. In vitro estimates of SCFA production

In vitro studies on the production of SCFA using human fecal inoculates revealed that the total yields and proportions of acetate to propionate to butyrate vary considerably among specific substrates (tables 2 and 2a).

Few *in vitro* models for pigs have been created and/or utilized to study the modulatory effects of NSP, nondigestible oligosaccharides (NDO) or milk whey on the production rates and concentrations of SCFA. For example, Piva et al. (2002) reported that after a 24-h fermentation of cecal digesta from pigs fed various levels of polysaccharides (NSP) from sugar beet pulp, only the amounts of n-butyric acid were affected ($P < 0.05$), whereas more profound effects on cecal SCFA production were exerted by a supplementary organic acid blend (table 3).

Moreover, fermentation patterns in the cecum of pigs can be altered by different sources of N supplied to microflora inhabiting the large intestine (table 4).

Transit times in the large intestine can have profound effects on the bacterial contribution to colon SCFA pools (Macfarlane and Macfarlane, 2003). The highest concentrations of

Table 2a

***In vitro* production of SCFA (Mol%) from various sources of carbohydrates in humans (after Danone Institute of Belgium, http://www.danoneinstitute.be/communication/pdf/mono03/mono3-part4.pdf)**

Substrate	Acetate	Propionate	Butyrate	Yield[a]	n^b
Starch	62	15	23	49	7
Pectin	80	12	8	39	8
Wheat and oat bran	64	16	20	40	5
Other NSP	63	22	8	38	24
Mixed diets	63	22	8	38	24
Overall mean	67	18	11	37	57

[a]Yield = g SCFA/100 g substrate.
[b]n = number of studies.

Table 3

***In vitro* production of SCFA (mMol/L) in cecal digesta of pigs as affected by dietary graded levels of NDF (0, 10 or 20%) and adding 0.1% of organic acid (after Piva et al., 2002)**

	% in diets								
NDF:	0	10	20	0	10	20	Statistical significance[b]		
Acid[a]:				0.1	0.1	0.1	NDF	Acid	NDFxAcid
C2	17.9	17.8	18.0	16.0	16.0	16.6	ns	*	ns
C3	7.2	7.1	7.1	7.4	7.2	7.5	ns	ns	ns
C4	0.6	0.6	0.6	0.5	0.5	0.5	ns	*	ns
iC4	3.2	3.0	3.0	2.9	2.7	2.7	*	*	ns
iC5	0.5	0.5	0.5	0.4	0.4	0.4	ns	*	ns
C5	0.5	0.5	0.5	0.4	0.4	0.4	ns	*	ns
ΣVFA	29.9	29.5	29.6	27.7	27.2	28.1	ns	*	ns
C2/C3	2.5	2.5	2.5	2.2	2.2	2.2	ns	*	ns

[a]C2 = acetic; C3 = propionic; iC4 = isobutyric; C4 = *n*-butyric; iC5 = isovaleric; C5 = *n*-valeric.
[b]ns = not significant ($P > 0.05$); * = significant at $P < 0.05$.

SCFA (produced from intestinal contents incubated for 48 h with no exogenous carbon sources and under anaerobic conditions) were found in the proximal large intestine, mainly because of greater carbohydrate availability (table 5).

Other organic anions from bacterial fermentation include lactate, which is an intermediate in starch breakdown predominantly by Lactobacilli and bifidobacteria. This bacterial metabolite is found primarily in the stomach and small intestine of nonruminants, and rather seldom in the colon (Jensen, 1998). Macfarlane and Macfarlane (2003) found that lactate was not produced in substantial amounts from pectin, xylan or arabinogalactans, but predominantly starch appeared to be an important precursor of this fatty acid, which is generated primarily as the L-enantiomer.

2.2.2. In vivo estimates of SCFA production

There are numerous reports on the production rates and concentrations of SCFA in the digestive tract of nonruminants (rodents, pigs and humans), and particularly with regard to the cecal and colonic regions. Various approaches have been described, although determining the amounts of SCFA *in situ* is rather complicated, as intestinal exogenous SCFA pass in part through the splanchnic bed and reach the peripheral bloodstream, mixing with the endogenous circulating SCFA (Pouteau et al., 2003). In consequence, the whole-body turnover of SCFA is thus composed of an endogenous peripheral turnover and an exogenous production that depends on dietary intake of SCFA and/or nondigestible carbohydrates as potential substrates for intestinal microflora. Despite some limitations of each approach, it seems useful to review a few papers on this topic from studies with rats, pigs and humans.

Numerous experiments have been conducted to study SCFA production in the gut of rats in relation to host-, microflora- and dietary-related factors. In the hindgut of rats there are ~165 mMol SCFA/L of digesta (von Engelhardt et al., 1998), and feed withdrawal (starvation) leads to a decrease in SCFA concentration, particularly in the colon (table 6).

Feeding diets containing such fibrous ingredients as wheat bran, oat bran, barley bran, wheat aleurone, rice bran or oligosaccharides or resistant starch usually results in increased

Table 4

Effect of N-sources and glucose on the rate of production[1] and molar proportions of organic acids and methane in cultures of pig cecal contents (after Marounek et al., 2002, with permission of the University of Veterinary and Pharmaceutical Sciences, Brno)

Substrate	Total VFA (mMol/L)	Molar percentages						Lactate (mMol/L)	Methane (mMol/L)
		C2	C3	iC4	C4	iC5	C5		
Gluten	46.1[a]	41.2	29.6	2.3	10.9	5.4	10.6	0.05	1.11
	(5.8)	(1.9)	(4.7)	(0.9)	(2.2)	(0.8)	(0.6)	(0.05)	(0.15)
Gliadin	24.5[b]	46.5	21.5	3.0	11.1	6.5	11.4	0	0.89
	(3.1)	(0.4)	(0.1)	(0.2)	(1.8)	(0.3)	(0.7)		(0.12)
Zein	12.5[b]	50.9	18.0	2.3	14.0	9.3	5.5	0	0.83
	(3.3)	(19.8)	(10.6)	(0.7)	(6.0)	(2.5)	(1.5)		(0.48)
Mucin	114.2[c]	55.7	28.5	1.0	9.3	0.8	4.7	0	1.19
	(3.7)	(0.8)	(1.0)	(0.1)	(0.2)	(0.2)	(1.6)		(0.10)
RNA	48.6[a]	58.0	32.4	0.5	8.7	0	0.4	0	0.76
	(4.8)	(0.9)	(1.4)	(0.1)	(0.4)		(0.1)		(0.42)
Glucose	96.6[d]	48.9	41.7	0.1	8.8	0.1	0.4	0.10	1.12
	(3.1)	(0.7)	(0.4)	(0.1)	(0.4)	(0.0)	(0.1)	(0.05)	(0.15)

Means of 3 cultures. Standard deviations are given in parentheses.

C2 = acetate; C3 = propionate; iC4 = isobutyrate; C4 = butyrate; iC5 = isovalerate; C5 = valerate.

[1]Data were corrected for amounts present in incubated blanks.

[a,b,c,d]Means in columns with different superscripts differ at $P < 0.05$.

Table 5

Concentrations and rates of production of acetate, propionate, butyrate and branched chain fatty acids (BCFA) in the proximal and distal colon of humans (after Macfarlane and Macfarlane, 2003)

		Molar percentages			
Region of the colon	Total VFA	Acetate	Propionate	Butyrate	BCFA
Concentration (mMol/kg)					
Proximal	160	52	20	20	8
Distal	95	52	19	18	12
Rates of production (from intestinal contents for 48 h)					
Proximal	225	55	27	13	5
Distal	45	55	24	7	25

production rates of SCFA, although when intakes of dietary water-soluble carbohydrates exceed the fermentative capacity of the microflora, SCFA fall due to osmotic diarrhea (Topping and Clifton, 2001).

In pigs, the production rates and concentrations of SCFA in particular segments of the digestive tract have been measured *post mortem,* or by using intestinal cannulae (simple or re-entrant), and/or catheters in the vascular system (the portal vein and carotid artery) to quantify pre- and post-absorptive kinetics. With no added SCFA as acidifiers in the diet, the concentrations of SCFA in the small intestine oscillate below 40 mMol/L (Clemens et al., 1974), whereas the large intestine contains ~90 mMol/L (von Engelhardt et al., 1998). The molar ratio of acetate to propionate to butyrate is indicated to be 50:42:8 in the anterior section and 65:18:17 in the posterior section of the small intestine (Williams et al., 1997). However, there is a relatively large variation in the contents of SCFA in the colonic and ileal digesta due to differences in dietary composition and dry matter intake. For example,

Table 6

Changes in SCFA production and the proportion of acetate to propionate to butyrate in rats with starvation and refeeding with a nonpurified diet (after Topping and Clifton, 2001)

		SCFA, mM			
Time, h	Region	Acetate	Propionate	Butyrate	Total
Starvation					
0	Cecum	55	27	33	122
	Proximal colon	20	6	6	32
56	Cecum	47	10	5	59
	Proximal colon	0	0	0	0
	Distal colon	4	0	0	5
Refeeding					
5	Cecum	38	17	11	63
	Proximal colon	27	10	6	42
	Distal colon	18	6	3	21
15	Cecum	72	23	14	106
	Proximal colon	58	16	11	89
	Distal colon	39	12	4	56

Drochner (1984) found with re-entrant cannulated pigs that the influx of total VFA from the ileum into the cecum was 3.1 and 7.96 mMol/kg $BW^{0.75}$ when feeding a low fibrous diet (3.5% crude fiber) versus a high fibrous diet (6.9% crude fiber). The latter resulted in slightly greater concentrations of acetic and propionic acids (but not butyric or lactic acids) in the cecal digesta, whereas the fecal contents of total SCFA were lower than for the low fibrous diet. However, Canibe et al. (1997) was unable to detect any difference in the molar ratio of SCFA in different segments of the large intestine of pigs, despite the fact that individual sugars had vastly different rates of fermentation.

Daily production of SCFA in the hindgut is estimated to be greater than 300 mMol (Jensen, 1998), but fecal excretion is only ~10 mMol (Bugaut and Bentéjac, 1993). Jensen (2001) reported that feeding Lactobacilli prefermented diets resulted in the generation of large amounts of lactic acid in the stomach and small intestine (620 and 350 mMol/kg feed, respectively), whereas in the ceco-colonic region acetic, propionic and butyric acids (420, 370, 400 mMol/kg feed, respectively) prevailed. In the distal part, other volatile fatty acids (VFA) such as valeric, caproic, isobutyric, isovaleric and formic were also generated (table 7).

The contents of total lactic acid and its specific isomers in the ileal digesta of piglets can be affected by their age (table 8) or the access to creep feed (table 9).

Mikkelsen and Jensen (2003) demonstrated that the gastric contents of organic acids in growing pigs are also affected by the physical form of the feed (table 10).

Also, Regina et al. (1999) reported that the gastric contents of pigs fed a finely ground and pelleted diet contained 2.29 mMol of acetate and 4.04 mMol of L-lactate, whereas in those fed a coarsely ground meal diet the respective values were 13.94 and 8.65 mMol.

Substrates for bacterial fermentation (maize starch versus raw potato starch) appear to affect the contents of acetate, propionate and butyrate in the cecum, middle colon and rectum of pigs (table 11).

Jansman and Van Leeuwen (1999) found with growing-finishing pigs that the levels of acetic, propionic, butyric and lactic acids in ileal digesta varied from 1.2 to 6.9, from 0.3 to 1.6, from 0.1 to 0.6 and from 2.8 to 6.9 g/kg DM intake, respectively.

Interrelationships between supplemental SCFA and their levels in digesta from various compartments of pigs or poultry is still relatively poorly evaluated, and no consistent responses could be found. Risley et al. (1992) observed no altered profiles of VFA (acetic, propionic and butyric) or non-VFA (pyruvic, lactic, fumaric or succinic) in the stomach,

Table 7

Amounts of carboxylic acids (mMol/day ± S.D.) produced by microbes in the digestive tract of piglets at 6 weeks of age (after Jensen, 1998, with permission of Institute of Animal Physiology and Nutrition, Polish Academy of Sciences)

Organic acid	Stomach	Small intestine	Large intestine	Total
Lactic	234 ± 50	266 ± 130	0 ± 0	500 ± 162
Formic	6 ± 4	38 ± 20	11 ± 8	55 ± 23
Acetic	42 ± 18	36 ± 15	176 ± 10	254 ± 23
Propionic	4 ± 1	1 ± 1	87 ± 5	92 ± 7
Iso-butyric	0 ± 0	0 ± 0	6 ± 0	6 ± 0
Butyric	2 ± 3	2 ± 2	54 ± 3	59 ± 7
Iso-valeric	0 ± 0	0 ± 0	6 ± 1	7 ± 1
Valeric	1 ± 1	0 ± 0	9 ± 2	10 ± 2
Total	288 ± 68	343 ± 100	350 ± 23	982 ± 124

Table 8

Lactate concentrations (mMol/L) in the ileal digesta of piglets[1] as affected by their age (after Mathew et al., 1994)

Days of age	D(−) lactate	L(+) lactate	Total lactate
Preweaning			
19	38.21^{e}	34.19^{e}	73.64^{e}
22	64.01^{bcd}	54.09^{cd}	118.77^{cd}
26	46.15^{de}	48.48^{de}	97.76^{de}
29	58.89^{cd}	64.01^{cd}	126.05^{cd}
Postweaning			
33	79.67^{b}	153.14^{b}	227.64^{b}
36	69.27^{bc}	65.18^{c}	136.41^{c}
40	64.69^{bcd}	165.61^{b}	232.26^{b}
SE	7.21	7.13	14.46

[1]Data are means of two replicates of 12 piglets.
^{abcde}Values within columns without common superscripts differ ($P < 0.05$).

Table 9

Lactate concentrations (mMol/L) in ileal digesta of young piglets[1] fed without and with creep feeds (after Mathew et al., 1994)[1]

Days of age	D(−) lactate		L(+) lactate		Total lactate	
	No creep	Creep	No creep	Creep	No creep	Creep
Preweaning						
19	48.68	27.74	37.99	30.40	95.98	51.31
22	74.02	53.99	59.53	48.65	140.54	96.99
26	39.53	52.77	43.07	53.88	90.12	105.40
29	51.27	66.52	57.00	71.02	117.02	134.18
Postweaning						
33	91.25	68.09	143.20	163.08	228.13	227.15
36	77.48	61.06	77.15	53.22	161.90	110.92
40	68.33	61.54	185.89	145.32	261.50	203.02
SE	10.89		26.79		39.15	

[1]Data are means from two replicates of 6 piglets per treatment.

Table 10

Interrelationship between the physical form of diets, organic acid contents (mMol), acidity and anaerobic bacteria in the stomach of pigs (after Mikkelsen and Jensen, 2003)[1]

Organic acid	F-NP	C-NP	F-P	C-P	*P* value
Acetic	2.7^{a}	10.7^{b}	0.0^{a}	4.5^{a}	0.016
Propionic	0.0^{a}	5.7^{b}	0.0^{a}	0.2^{a}	0.036
Butyric	0.0	1.6	0.0	0.2	0.124
Lactic acid	1.6	20.3	1.9	10.9	0.089
Gastric acidity (pH) and anaerobic bacteria population (lg CFU/g)					
Acidity	3.93	3.38^{a}	3.71	4.19^{b}	0.029
Anaerobes	7.06^{a}	8.53^{b}	7.16^{a}	7.59^{a}	0.038

[1]F-NP = fine ground, nonpelleted; C-NP = coarsely ground, nonpelleted; F-P = fine ground, pelleted; C-P = coarsely ground, pelleted; CFU = colony forming units.
^{a,b}Means in rows not sharing the same superscript differ at $P < 0.05$.

Table 11

The content of SCFA (acetate, propionate, butyrate) and branched-chain short chain fatty acids (BCSCFA) in colorectal digesta of pigs (n = 6) fed diets containing 25% starch from maize or raw potato (after Martinez-Puig et al., 2003)

Item	Diet		SEM	Probability
	25% maize starch	25% raw potato starch		
Acetate				
Cecum	0.54	0.52	0.019	ns
Middle colon	0.56	0.48	0.026	ns
Rectum	0.59	0.54	0.012	$P < 0.05$
Propionate				
Cecum	0.30	0.26	0.020	ns
Middle colon	0.22	0.29	0.021	$P < 0.05$
Rectum	0.21	0.25	0.015	ns
Butyrate				
Cecum	0.13	0.18	0.016	ns
Middle colon	0.15	0.18	0.026	ns
Rectum	0.12	0.10	0.010	ns
Branched-chain short-chain fatty acids (BCSCFA)				
Cecum	0.001	0.001	0.006	ns
Middle colon	0.04	0.02	0.005	$P < 0.05$
Rectum	0.06	0.07	0.61	ns

ns = not significant ($P > 0.05$).

jejunum, cecum or lower colon of piglets weaned at 21 days when feeding a 20% CP maize–soybean meal-based diet without or with 1.5% fumaric or 1.5% citric acid. On the other hand, Mroz et al. (2001) observed a clear positive interrelationship between the dose of formate from potassium diformate on the postprandial flow of formic acid into the duodenum (table 12).

Moreover, Canibe et al. (2001) reported that K-diformate (KDF) affected the amount of lactic and SCFA in particular segments of the digestive tract in piglets (table 13).

Piva et al. (2002) studied the levels of SCFA in the jejunum and cecum of piglets weaned at 21 days of age, when feeding diets without and with tributyrin and lactitol as nutribiotics. It appeared that both agents had no impact on the VFA production, whereas lactic acid levels in the cecum were significantly affected by lactitol (table 14).

Production rates and metabolism of SCFA in the digestive tract and whole body have also been studied using stable isotopes, as the overall whole-body production of SCFA originates from an exogenous intestinal supply and from endogenous metabolism. For example, in order to show the presence of the exogenous and endogenous components of SCFA production, Pouteau et al. (2003) used intravenous infusion of ^{13}C-labeled SCFA for assessing the whole-body turnover of acetate, propionate and butyrate in rats in a fasted state, whereas the endogenous turnover of acetate and its oxidation were determined in healthy human subjects in the post-absorptive state, using intravenous infusion of [1-^{13}C] acetate. In addition, intragastric tracer infusions were performed to evaluate the splanchnic first-pass retention of acetate. Finally, an original model was developed in healthy human subjects using intravenous infusion of [1-^{13}C] acetate to determine *in vivo* the true colonic acetate production after ingestion of a nondigestible disaccharide – lactulose. The authors found that the intraluminal

Table 12

Post-prandial flow and concentrations of formic acid (FA) in fresh duodenal digesta of young pigs fed a commercial cereal–soybean meal-based diet with graded levels of K-diformate (after Mroz et al., 2001, with permission of CABI Publishing)

Post-prandial time (min)	K-diformate (%) 0.0	0.9	1.8	SED[b]	LSD	*P* value[c]
0[a]	33	1467	3001			
Flow of FA (mg)						
5	4	284	530	31.3	76.7	<.001
35	4	223	511	51.7	126.4	<.001
65	2	113	256	14.3	35.0	<.001
95	3	73	157	13.4	32.7	<.001
125	2	57	136	8.7	21.2	<.001
185	2	44	81	9.3	22.8	<.001
245	3	31	60	6.9	17.0	<.001
Concentration (mg FA/kg fresh digesta)						
5	12	727	1410	69.9	171.0	<0.001
35	16	783	1628	82.4	201.5	<0.001
65	9	453	920	32.9	80.6	<0.001
95	10	292	616	30.6	74.9	<0.001
125	9	232	518	27.9	68.1	<0.001
185	10	189	328	29.0	71.0	<0.001
245	13	129	261	24.6	60.3	<0.001

[a]Time 0 = intake of 33, 1467 and 3001 mg formate/pig in the morning meal.
[b]SED = standard error of the differences of the mean; LSD = least significant difference.
[c]*P* value = probability level.

production rate of colonic acetate after oral intake of 20 g lactulose by healthy volunteers amounted to 140.4 (± 11.7) mMol. There are some other published papers on using a [1-^{13}C] propionate tracer or a [1,2-$^{13}C_2$] butyrate in humans, but not in order to specifically evaluate their production rate in the large intestine.

2.3. Direct and/or indirect effects of SCFA in the digestive tract

There is rather a common consensus that particular SCFA in the digestive tract of nonruminants are involved in numerous beneficial (direct and/or indirect) host- and microflora-related activities such as (a) antimicrobial (bactericidal and/or bacteriostatic); (b) modulating intraluminal acid–base and buffering capacity; (c) catalyzing enzymatic processes in digestion; (d) controlling gut functionality (e.g. gastric emptying, motility and permeability); (e) modulating secretions of pancreatic and biliary juices; (f) supplying energetic precursors for epithelial cell proliferation/turnover and mucus production. On the other hand, some pathologies associated with a lack or excess of SCFA in the colonic lumen are also indicated in the literature. For example, a rapid or excessive generation of SCFA in the hindgut may lead to a lowering of luminal pH (even below 5.5) and buffering capacity, which promotes a greater accumulation of lactate (less well absorbed anion), with subsequent inhibition of SCFA-stimulated Na^+–H^+ exchange and reduction of water, Na^+ and SCFA absorption, as manifested by diarrheal fluid loss and colonocyte swelling (von Engelhardt et al., 1998; Sakata and Inagaki, 2001). Furthermore, Mariadason et al. (2000) reported that feeding diets deprived of

Table 13

Organic acids in digesta (mMol/kg wet sample) along the digestive tract of piglets (n = 9) on day 29 postweaning (after Canibe et al., 2001)

	Segments of the digestive tract							
Diet	Stomach	Small intestine proximal	Small intestine medial	Small intestine distal	Cecum	Colon proximal	Colon medial	Colon distal
Lactic acid[a]								
Control	9 ± 1.8	10 ± 1.4	23 ± 4.3	44 ± 10.9	2 ± 0.9	1 ± 0.5	1 ± 0.5	2 ± 0.5
KDF[b]	5 ± 1.8	6 ± 1.5[c]	9 ± 4.3	14 ± 10.9	2 ± 0.9	1 ± 0.5	1 ± 0.5	1 ± 0.5
Formic[a]								
Control	1 ± 5.3*	0 ± 1.7*	1 ± 1.1*	11 ± 3*	0	0	0	0
KDF	40 ± 5.3	7 ± 1.8[c]	7 ± 1.1[c]	25 ± 3	0	0	0	0
Acetic + propionic + butyric								
Control	3 ± 4.6	0	1 ± 4.6	10 ± 4.6	163 ± 4.6	149 ± 4.6	144 ± 4.6	135 ± 4.6
KDF	1 ± 4.6	0	3 ± 4.6	15 ± 4.6	167 ± 4.6	149 ± 4.6	140 ± 4.6	127 ± 4.6
Isobutyric + isovaleric								
Control	0	0	0	0	2 ± 0.5	3 ± 0.5	5 ± 0.5	6 ± 0.5
KDF	0	0	0	0	2 ± 0.5	3 ± 0.5	4 ± 0.5	5 ± 0.5

[a]Diet × segment interaction significant ($P < 0.001$).

*Differences between means for formic acid are significant (P = 0.004, 0.02, 0.009 and 0.009 for stomach, proximal, medial and distal small intestine, respectively).

[b]KDF = K-diformate.

[c]The values are least square means ± standard error of the mean; n = 8.

Table 14

Mean SCFA concentrations (µMol/g DM) in the jejunum and cecum of 21-day-old piglets fed without or with 1% tributyrin (T) and/or 0.3% lactitol (L) (after Piva et al., 2002)

	Volatile fatty acids (VFA)							
Diet	C2	C3	iC4	C4	iC5	C5	∑VFA	Lactic acid
Jejunum								
Control	29.0 ± 4.9	4.0 ± 2.3	0.4 ± 0.3	2.3 ± 1.5	0	0	37.5 ± 8.9	441.9 ± 45.4[ab]
T	27.8 ± 15.1	7.8 ± 7.7	0.8 ± 0.8	7.8 ± 3.9	0	0	44.2 ± 25.7	201.7 ± 37.9[a]
L	26.7 ± 1.1	1.6 ± 1.5	1.5 ± 1.1	0.8 ± 0.8	0	0	30.5 ± 3.1	611.2 ± 124.4[b]
T+L	14.7 ± 2.8	0	0	5.5 ± 2.5	0	0	20.2 ± 4.9	243.7 ± 83.4[a]
Cecum								
Control	1039 ± 193	701 ± 105	7.2 ± 3.2	332 ± 73	7.9 ± 5.2	98.6 ± 21.6	2186 ± 378	70.7 ± 46.4[a]
T	640 ± 117	367 ± 76	3.76 ± 2.2	170 ± 70	2.7 ± 1.8	29.2 ± 15.3	1212 ± 254	41.8 ± 24.7[a]
L	817 ± 156	500 ± 78	1.7 ± 1.7	268 ± 87	0	41.0 ± 21.6	1629 ± 324	213 ± 23[b]
T+L	671 ± 78	396 ± 58	3.2 ± 1.1	189 ± 38	0	36.2 ± 14.2	1295 ± 157	12.0 ± 12.1[a]

non starch polysaccharides (NSP) or raising animals under germfree conditions results in the absence of intestinal microflora, and therefore in the inability of dietary NSP to be fermented, as manifested by marked mucosal hypoplasia, reduced proliferative activity in the crypts, significantly reduced crypt column height and cell cycle times.

2.3.1. Antimicrobial activity: bactericidal and bacteriostatic potency

Currently, the potency of SCFA as antimicrobials has received a renewed interest in the context of phasing out supplemental antibiotics for prophylaxis in nonruminant nutrition by 2006 in the EU (Partanen and Mroz, 1999). Numerous *in vitro* and *in vivo* studies with single and blended SCFA have been addressed for assessing their antimicrobial mode of action (Mroz, 2003). In general, there is a strong body of evidence that the antimicrobial efficacy of specific SCFA *in vivo* is a resultant of such factors as: (a) the carbon chain length and inclusion level; (b) the proportion of dissociated to undissociated forms; (c) intraluminal digesta acidity and acid-binding capacity; (d) the time of retention/exposure in particular segments; (e) specific potency of pathogens for colonization and enterotoxin production; (f) intestinal status (degree of development, permeability, presence and expression of receptors at the villi).

According to an uncoupling theory, SCFA in the gastric or intestinal lumen can act as bactericidals (''bacteria killers'') or bacteriostatics (''bacterial wall permeabilizers'') in the following steps:

1. Undissociated forms can permeabilize and/or diffuse across cell membranes of pathogens, destroying their cytoplasm or inhibiting growth (inactivation of bacterial decarboxylases and catalases);
2. Intestinal dissociation liberates H^+ ions and anions serving as a pH barrier against pathogen colonization on the brush border;
3. Lowered gastric pH (complementary to suboptimal HCl production in piglets);
4. Gastric hydrolysis liberates H^+ ions activating pepsinogen and inhibiting bacterial growth (bacteriocidal/bacteriostatic effects);
5. Selective stimulation of the growth of beneficial bacteria.

As the pK_a of most SCFA ranges from 3 to 5, their nonencapsulated forms can be most effective in the stomach, as illustrated in fig. 8.

In more alkaline conditions, acids release protons, which consequently lower pH (Salmond et al., 1984). The elimination of released protons by membrane-bound ATPases is traditionally believed to result in the dissipation of the proton-motive force which is essential for ATP synthesis and substrate uptake into the cell (Cherrington et al., 1991).

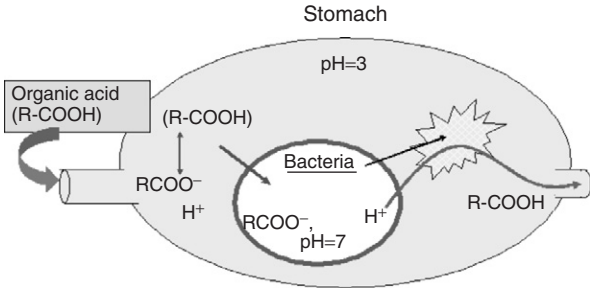

Fig. 8. Model antimicrobial action of SCFA in the stomach of nonruminants.

This uncoupling theory has been challenged by Russell (1992), who proposed that toxic effects of SCFA are caused by the accumulation of polar anions in the cell. This accumulation depends on the pH gradient across the membrane. Bacteria which try to resist internal pH change are more sensitive to organic acids than those which allow pH to decline (Russell and Diez-Gonzales, 1998). Although this aspect needs to be further elucidated, it remains relatively clear that SCFA concentrations of ~70 mMol/L in digesta of pigs are bacteriostatic, but higher levels (>100 mMol/L) are needed in order to be bactericidal (B. Miller, personal communication).

There are few *in vitro* approaches described in the literature for evaluating the antimicrobial potency of SCFA (Östling and Lindgren, 1993; Piva, 1998; Naughton and Jensen, 2001; Knarreborg et al., 2002). They rely on establishing either minimum inhibitory concentrations (MIC), gas production rates, or a growth/survival rate of coliform bacteria, *Salmonella* or *Lactobacilli* in various media (agar or in liquid broth), as well as in digesta from a specific site of the digestive tract when incubated with specific types and dosages of acidifiers under anaerobic conditions in (computer) controlled fermentation systems. Some data illustrating the MIC values for single or blended organic acids are presented in tables 15 and 15a.

Naughton and Jensen (2001) and Knarreborg et al. (2002) investigated the effects of formic, propionic, butyric, lactic, benzoic and fumaric acids, as well as potassium diformate on population changes of coliform and lactic acid bacteria in the gastric digesta at pH 3, 4 and 5, and in the content from the proximal part of the small intestine at pH 5, 6 and 7. These studies demonstrated a clear selective removal of target species, i.e. coliform bacteria (not lactic acid bacteria) from the gastric digesta at the investigated pH ranges. Their survival rate was strongly influenced by pH (a regeneration cycle lasted 70 min at pH 5, and 25 min at pH 7), irrespective of the digesta origin. The authors established the following order of killing potency of coliform bacteria: propionic < formic < butyric < lactic < fumaric < benzoic. In addition, they found that benzoic acid was superior to the other acids tested in exhibiting a bactericidal effect on coliform as well as lactic acid bacteria, irrespective of the digesta origin. In another study, Jensen et al. (2001) demonstrated that the potency of these acids against *Salmonella typhimurium* in gastric digesta at pH 4 was in the following order: acetic < formic < propionic < lactic < sorbic < benzoic. On the other hand, Roth and Kirchgessner (1998)

Table 15

An overview of the MIC values (%) measured for various SCFA or their blends (after Strauss and Hayler, 2001)[a]

Name	FA	PA	LA	Blend 1	Blend 2	Blend 3
S. typhimurium	0.1	0.15	0.3	0.15	0.15	0.25
P. aeroginosa	0.1	0.2	0.3	0.15	0.1	0.25
E. coli	0.15	0.2	0.4	0.2	0.2	0.3
S. aureus	0.15	0.25	0.4	0.15	0.2	0.3
L. monocytogenes	0.1	0.2	0.25	0.15	0.15	0.15
C. jejuni	0.1	0.2	0.3	0.15	0.1	0.25
Cl. botulinum	0.15	0.25	0.3	0.10	0.1	0.15
Cl. perfringens	0.1	0.25	0.3	0.15	0.15	0.3
MIC	0.12	0.21	0.32	0.15	0.14	0.24

[a]FA = formic acid; PA = propionic acid; LA = lactic acid; Blend 1 = 75% FA + 25% PA; Blend 2 = 50% FA + 50% PA; Blend 3 = 25% FA + 75 % PA.

Table 15a

***In vitro* effects of propionic acid, formic acid, or their combinations on the adherence of type I-fimbria-forming ETEC serotype O157:H7 (after Gedek, 1999)**

Organic acid type	Organic acid added (%)		ETEC-adherence (%)
	Calculated	Analyzed	
None	0.0	0.0	100.00
Formic	1.0	1.16	46.25
Formic + propionic (75+25%)	1.0	0.77 + 0.26	45.00
Formic + propionic (50+50%)	0.5	0.26 + 0.25	48.75
	0.75	0.39 + 0.37	52.50
	1.0	0.49 + 0.41	6.25
Formic + propionic (25+75%)	0.5	0.18 + 0.38	93.75
	0.75	0.26 + 0.56	43.75
	1.0	0.20 + 0.74	18.75

demonstrated that supplemental formic acid affected duodeno-jejunal populations of both *E. coli* and *Lactobacilli* in piglets (table 16).

There is an increasing body of evidence that certain bacteria, e.g. *Salmonella* and *E. coli* have evolved complex counteractive mechanisms, which allow them to cope with extreme acid conditions (Bearson et al., 1997). For example, Barua et al. (2002) showed that wild *E. coli* strains can grow effectively under moderately acidic organic acid-rich conditions. The authors found that the Shiga Toxin-producing *E. coli* (STEC) O157:H7 NGY9 grows more quickly than a K-12 strain in Luria-Bertani (LB)-2-morpholinoethanesulfonic acid (MES) broth supplemented with acetic acid (pH 5.4). Furthermore, the growth of some (but not all) mutants of STEC and *Salmonella* LPS were suppressed in the presence of acetic acid compared with that of the parents. These results suggest that a mutagenesis at three specific genes, i.e. fcl, wecA (rfe) and wecB (rffE), caused (1) loss of surface O-polysaccharide, (2) loss of O-polysaccharide plus enterobacterial common antigen (ECA), and (3) loss of ECA, respectively. Therefore, the authors concluded that the full expression of LPS (including O-polysaccharide) and ECA is indispensable to the resistance against acetic acid and other short-chain fatty acids in STEC O157:H7 and *Salmonella*.

Table 16

Effects of 1.2% formic acid (FA) in diets for piglets on bacterial counts (^{10}log CFU/g fresh matter) along the digestive tract of piglets (after Roth and Kirchgessner, 1998, with permission of Institute of Animal Physiology and Nutrition, Polish Academy of Sciences)

Species	Lactobacilli/ Bifidobacteria		*E. coli*		Bacteroidaceae		Enterococci	
FA, %	0 (control)	1.2	0	1.2	0	1.2	0	1.2
Duodenum	6.4	5.5	5.5^{a}	3.3^{b}	4.1^{a}	2.1^{b}	3.2^{a}	2.3^{b}
Jejunum	6.7^{a}	5.8^{b}	6.8^{a}	5.3^{b}	4.5^{a}	3.0^{b}	4.5	3.7
Ileum	7.2	6.6	7.9	6.8	5.7	4.8	5.6	5.2
Cecum	8.1	7.5	6.8	6.9	6.6	6.2	4.4	4.9
Colon	8.6	8.0	6.3	6.0	6.6	5.7	4.3	4.5

^{a,b}Means for each species within a row lacking a common superscript letter differ ($P < 0.05$).

In fact, inducible tolerance (adaptation) to acidic environments is recognized as an important feature for many prokaryotic and eukaryotic microorganisms. Pathogens, which persist in altering internal pH, are more sensitive to organic acids than those which allow pH to decline (Partanen, 2001). Moreover, Kwon and Ricke (1998) postulated that SCFA in the digestive tract of host animals or in the feed may contribute to the enhancement of the virulence of *Salmonella typhimurium* by increasing acid tolerance. Studies on acid adaptation mechanisms of *Streptococcus* mutants (Quivey et al., 2000) demonstrated that their growth at pH 5, as compared to pH 7, was associated with significant changes in the ratios of unsaturated to saturated fatty acids in the membrane. In consequence, it could affect membrane proton permeability directly by changing the base permeability of the lipid bilayer to protons or indirectly by increasing proton-excreting activity of the bacterial transport mechanism (F_1F_0-ATPase). Acid adaptation of Lactobacilli was studied by Saklani-Justforgues et al. (2000), who indicated that intragastrically inoculated acid-adapted *L. monocytogenes* (grown at pH 5.5), as compared to a non-adapted culture of *L. monocytogenes* (grown at pH 7.2), survived to a greater extent in the ceco-colonic region of mice. Specific studies on microbial adaptation to SCFA in the digestive tract of pigs are still very limited.

2.4. Transport of SCFA across the gut wall

Both *in vitro* and *in vivo* approaches have been used to study quantitative and qualitative aspects of transport (uptake/absorption) mechanisms and mucosal metabolism of SCFA using monogastric animals and humans (Fitch and Fleming, 1999; Topping and Clifton, 2001).

2.4.1. In vitro studies

In vitro approaches involve various techniques, such as Ussing chambers with isotopic enrichment (e.g. [^{14}C], [1-^{13}C], [^{2}H/^{13}C]) to measure fluxes of acetate, butyrate or propionate across colonocytes/enterocytes at short-circuit current conditions (von Engelhardt et al., 1998; Pouteau et al., 2003). In addition to the rate of SCFA absorption (% or μMol/cm^2/h), the intracellular as well as the surface pH of enterocytes within the intact epithelium using microspectrofluorometry, analysis of the lipid composition of the apical and basolateral plasma membranes of enterocytes in the different segments of the large intestine and correlation of membrane structure and permeability using the stopped-flow technique have all been measured (Bergman, 1990; Bugaut and Bentéjac, 1993; Busche et al., 2002).

In general, transport mechanisms of SCFA across species seem to be quite similar, and the large intestine of nonruminants appears to resemble the rumen epithelium in its ketogenic capability (Fitch and Fleming, 1999). It is assumed that predominantly undissociated SCFA (lipid soluble) can be absorbed through a concentration-dependent, passive diffusion process. Furthermore, their degree of absorption may be influenced by such factors as digesta transit and the surface area of the mucosa (Vogt and Wolever, 2003), as well as permeability coefficients, luminal pH and pCO_2, fluxes of water, protons, and inorganic ions (Cl^-, HCO_3^-, Na^+, and K^+) in particular intestinal compartments (Bergman, 1990). The transport of undissociated SCFA requires an equimolar disappearance of H^+ ions (von Engelhardt et al., 1998), and it generates bicarbonates in the lumen (Bergman, 1990). However, approximately 95–99% of SCFA generated in the large intestine by bacteria occur in ionized (dissociated) forms, and less than 5% of them appear in feces (Topping and Clifton, 2001). This implies that their rate of absorption is very high. For protonization of ionized SCFA entering into epithelial cells three sources are available: (1) free H^+ in digesta; (2) H^+ from dissociation of bicarbonate, and

(3) H^+ from Na^+–H^+ exchange or active K^+–H^+ transport. The absorption of SCFA increases linearly as concentration increases although saturation kinetics are seen at high concentrations (Bergman, 1990; von Engelhardt et al., 1998). From studies with Ussing chambers on isolated membrane vesicles it was concluded that a SCFA–HCO_3^- exchange mechanism exists across the apical membrane, and omission of HCO_3^- as well as the inhibition of Na^+–H^+ exchange or K^+–H^+ ATPase results in a significant reduction of SCFA transport (von Engelhardt et al., 1998). Busche et al. (2002) demonstrated, with a stopped-flow device, that there are marked segmental differences in the permeability of apical and basolateral membranes for the protonated SCFA (SCFAH) and that the apical membrane in the cecum and proximal colon of guinea pig is an effective barrier against a rapid diffusion of small lipid-soluble substances such as SCFAH. In addition, the authors found also that cholesterol and membrane proteins may contribute to this barrier property.

By using quantitative confocal microscopy, Chu and Montrose (1995) found that extracellular pH is regulated in two separate microdomains surrounding the colonic crypts: the crypt lumen and the subepithelial tissue adjacent to the crypt colonocytes. Also, apical superfusion with (1) a poorly metabolized SCFA (isobutyrate), (2) an avidly metabolized SCFA (n-butyrate), or (3) a physiologic mixture of acetate/propionate/n-butyrate produced similar results: alkalinization of the crypt lumen and acidification of subepithelial tissue. These effects were (1) dependent on the presence and orientation of a *trans*-epithelial SCFA gradient, and (2) required activation of sustained vectorial acid–base transport by SCFAs. The results suggest that the crypt lumen functions as a pH microdomain due to slow mixing with bulk superfusates and that crypts contribute significant buffering capacity to the lumen. In conclusion, physiologic SCFA gradients cause polarized extracellular pH regulation because epithelial architecture and vectorial transport synergise to establish regulated microenvironments. The addition of SCFA to isolated colonocytes acidified the intracellular pH, and the *trans*-epithelial gradients of SCFA generate pH gradients across the colonic epithelium, which are primarily regulated by mechanisms in the basolateral membrane thanks to the Na^+–H^+ exchanger and the Cl^-–HCO_3^- exchanger (Chu and Montrose, 1995; von Engelhardt et al., 1998). Nevertheless, physiological fluctuations in the intraluminal pH cannot affect SCFA absorption, as documented in humans and rats by lowering the pH from 7.4 to 5.5 in the large intestine (von Engelhardt et al., 1998).

According to Ritzhaupt et al. (1998a) butyrate is transported across the pig and human colonic luminal membrane by an electroneutral anion exchange process stimulated at pH 5.5, and it may be inhibited by several structural analogs, such as acetate, propionate, pyruvate, L-lactate or -ketobutyrate. As there are two distinct carrier-mediated anion exchange processes located in the apical and basolateral membrane (Busche et al., 2002), butyrate uptake by basolateral membrane vesicles represents both nonionic diffusion and a carrier-mediated SCFA–HCO_3^- exchange process that differs from the SCFA–HCO_3^- exchange identified in apical membrane vesicles (von Engelhardt et al., 1998).

The addition of SCFA to isolated colonocytes acidified the intracellular pH, which is mainly regulated by mechanisms in the basolateral membrane thanks to the Na^+–H^+ exchanger and the Cl^-–HCO_3^- exchanger. Also, there is some evidence that SCFA can stimulate mucus release and inorganic ion uptake (Na^+, Ca^{2+}, Mg^{2+}) and K^+ secretion in the hindgut (von Engelhardt et al., 1998). Sometimes the addition of SCFA may lead to colonocyte swelling, with subsequent Cl^- secretion (Diener et al., 1993), as manifested in diarrhea due to a decreased sodium and water absorption (von Engelhardt et al., 1998).

Hypothetical mechanisms of SCFA transport across the ceco-colonic epithelial cells, as proposed for guinea pigs are shown in fig. 9.

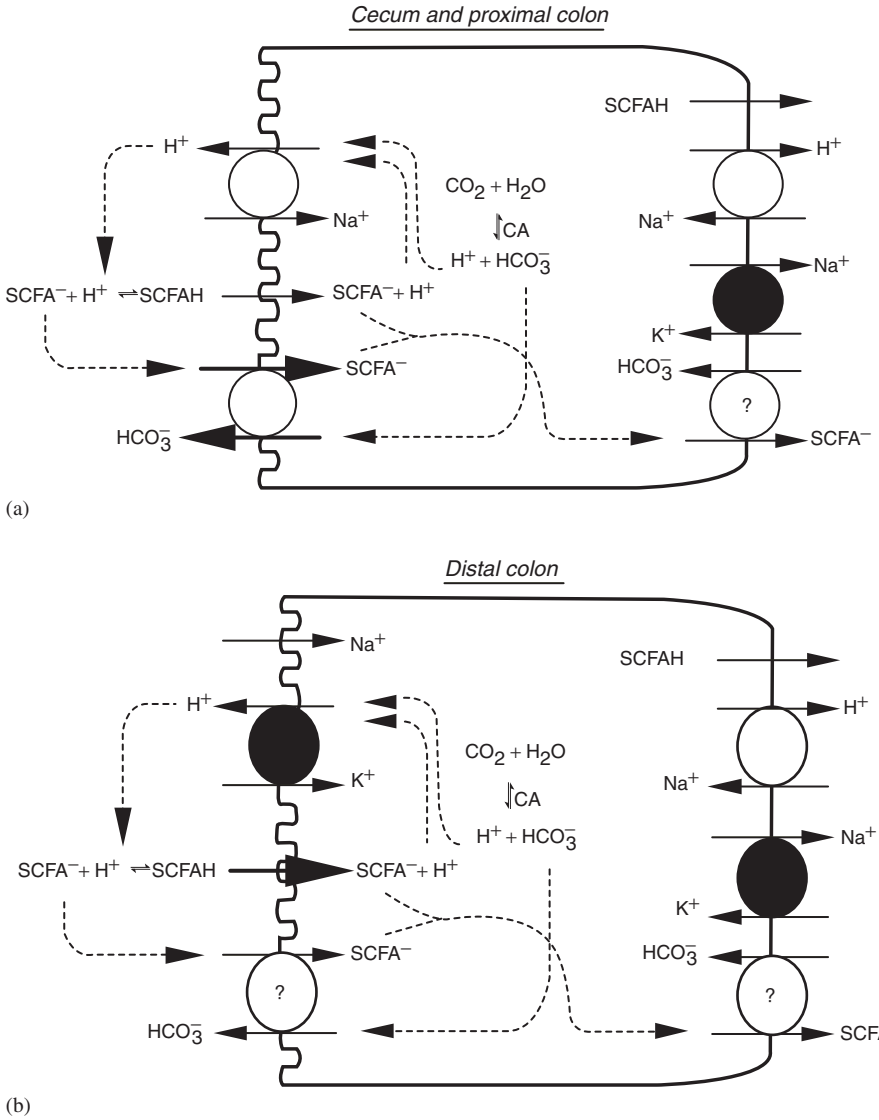

Fig. 9. Model for cellular mechanisms involved in absorption of SCFA in (a) the cecum and proximal colon or (b) distal colon. (After von Engelhardt et al., 1998, with permission of Euroscience.)

An ionized SCFA–HCO_3^- exchange mechanism across the apical membrane also exists in rats and humans (von Engelhardt et al., 1998), whereas in ruminants it is proposed that the absorption of $SCFA^-$ occurs by an anion exchange (AE) protein (fig. 10).

In this model, after passing the apical membrane of epithelial cells, undissociated/protonated and dissociated forms (HSCFA and $SCFA^-$, respectively) are partially metabolized through oxidative or anaerobic pathways. Carbon dioxide (CO_2) from oxidative breakdown can be further transformed by the activity of carbonic anhydrase (CA) to H_2CO_3, which in turn dissociates into HCO_3^- and H^+. Ketone bodies ($AcAc^-$, acetoacetate, D-3-HOB^-, D-3-hydroxybutyrate) and lactate derived from anaerobic breakdown can be extruded on the basolateral side by the proton-linked monocarboxylate transporter 1 (MCT1). Furthermore, Ritzhaupt et al. (1998b) reported

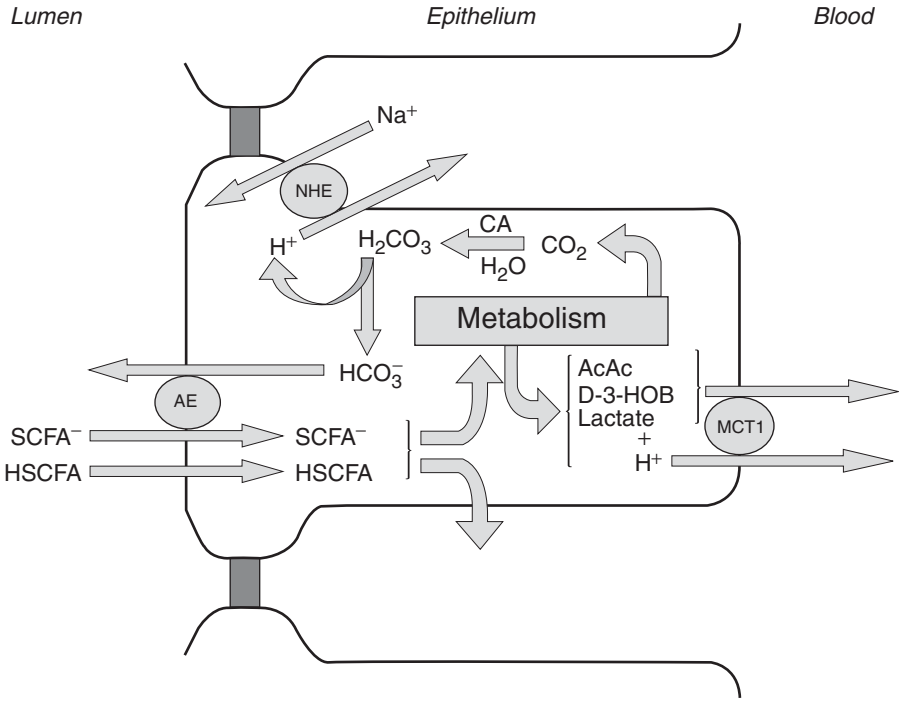

Fig. 10. Model for intracellular mechanisms involved in absorption of $SCFA^-$ by an anion exchange protein (AE) as proposed for ruminants by Gäbel and Wolffram (2004). (Reproduced with kind permission of Springer Science and Business Media.)

that a MCT1 protein is present on the luminal membrane of the pig and human colon, and is involved in the transport of both L-lactate and butyrate across the colonic luminal membrane. Moreover, the authors found that: (a) L-lactate is transported across the colonic luminal membrane by an identical mechanism to that described for butyrate transport, (b) the transport is saturable and follows Michaelis–Menten kinetics, indicative of a single transport protein, (c) L-lactate transport is inhibited by SCFA, which inhibited also colonic butyrate transport (of n-butyric acid), (d) L-lactate is both an energy substrate for colonocytes and a differentiation-inducing antineoplastic agent.

The large intestine of nonruminants appears to resemble the rumen epithelium in its ketogenic capability, since (1) instilling butyrate into the rat cecum increased acetoacetate and 3-hydroxybutyrate concentrations in the aorta blood, (2) butyrate increased net production of ketone bodies by isolated human and rat colonocytes, (3) in rat colonocytes, labeled butyrate was shown to be incorporated into ketone bodies (Fitch and Fleming, 1999). Also, SCFA stimulation coupled Na^+–Cl^- and water absorption involves an apical membrane mechanism of SCFA uptake (SCFA–HCO_3^- exchange) as well as Na^+–H^+ and Cl^-–$SCFA^-$ exchanges (Ritzhaupt et al., 1998).

2.4.2. In vivo studies

In vivo approaches to study translocation or uptake of SCFA by gut colonocytes and enterocytes have involved conscious or anesthetized rodents, dogs and pigs with various surgical modifications such as Thiry–Vella loops, intestinal T-cannulae, vascular catheters with flow

Table 17

Mean arterial and portal plasma VFA and net portal-drained viscera (PDV) flux of VFA in growing pigs (n = 4) fed diets differing in fermentable carbohydrates (after Jansman et al., 2001, and Van der Meulen et al., 1997)[1]

		VFA			
Exp.	Diet	Acetate	Propionate	Butyrate	Total
Arterial concentration, mMol/L					
Exp. 1	CS	0.17 (± 0.07)^{c}	<0.01 (± 0.01)	0.01 (± 0.01)^{c}	0.20 (± 0.07)^{c}
	CPS	0.27 (± 0.07)^{c}	0.01 (± 0.01)	0.05 (± 0.01)^{d}	0.34 (± 0.07)^{c}
	PS	0.47 (± 0.07)^{d}	0.01 (± 0.01)	0.06 (± 0.01)^{d}	0.56 (± 0.07)^{d}
Exp. 2	I	0.574 (± 0.057)	0.040 (± 0.017)	0.005 (± 0.002)	not reported
	II	0.646 (± 0.057)	0.048 (± 0.017)	0.007 (± 0.002)	not reported
Portal concentration, mMol/L					
Exp. 1	CS	0.32 (± 0.05)^{C}	0.09 (± 0.07)	0.02 (± 0.04)^{C}	0.46 (± 0.08)^{C}
	CPS	0.75 (± 0.05)^{D}	0.14 (± 0.07)	0.28 (± 0.04)^{D}	1.24 (± 0.08)^{D}
	PS	1.01 (± 0.05)^{E}	0.16 (± 0.07)	0.36 (± 0.04)^{D}	1.62 (± 0.08)^{E}
Exp. 2	I	0.931 (± 0.112)^{a}	0.202 (± 0.045)^{A}	0.046 (± 0.017)	not reported
	II	1.259 (± 0.112)^{b}	0.360 (± 0.045)^{B}	0.071 (± 0.017)	not reported
Net PDV flux, mMol/12 h					
Exp. 1	CS	137.6 (± 93.7)^{c}	71.4 (± 58.3)	15.7 (± 56.9)^{c}	240.1 (± 156.2)^{c}
	CPS	379.8 (± 93.7)^{cd}	105.4 (± 58.3)	190.0 (± 56.9)^{d}	723.9 (± 156.2)^{d}
	PS	581.0 (± 93.7)^{d}	133.4 (± 58.3)	285.1 (± 56.9)^{d}	1067.4 (± 156.2)^{e}

[1]CS = control (basal diet + 100% maize starch); CPS = basal + 50% maize starch + 50% raw potato starch); PS = basal + 100% raw potato starch; I = control (basal diet + 32.2% maize starch); II = basal diet + 8% maize starch + 15% wheat bran + 15% beet pulp.

^{abcde} Within each experiment, means (± standard error of the difference) with different superscripts differ at $P < 0.05$.

^{ABCDE} Within each experiment, means (± standard error of the difference) with different superscripts differ at $P < 0.01$.

probes or small intestine segment perfusion (Rérat, 1987, 1994; Fitch and Fleming, 1999; Mroz, 2001; Topping and Clifton, 2001; Bach Knudsen et al., 2003). In addition, tracer techniques (isotope dilution), nontracer techniques (enteral/parenteral administration of SCFA or carbohydrate substrates for luminal microflora at steady-state conditions) or postmortem examinations have been implemented.

The net portal-drained viscera (PDV) flux of VFA and glucose in pigs was also measured in relation to dietary supply of various fermentable carbohydrates (table 17; Van de Meulen et al., 1997; Jansman et al., 2001). The authors concluded that the dietary sources of fermentable carbohydrates (maize starch versus raw potato starch or versus fibrous wheat bran and beet pulp) reflect the concentrations and net PDV of acetate, propionate and butyrate in growing pigs.

In humans, SCFA absorption from the colonic lumen has been studied using an intravenous infusion of [1-^{13}C] acetate (Pouteau et al., 2003), or a rectal infusion of single/blended SCFA in the presence of polyethylene glycol (PEG) as a marker (Vogt and Wolever, 2003). The latter authors assessed the percentage of SCFA absorption as follows:

$$\% \text{ SCFA absorption} = \left[\left(\text{SCFA}_{\text{end-point}} - \text{SCFA}_{\text{baseline}}\right)/\text{SCFA}_{\text{baseline}}\right] \times 100$$

The mMol of SCFA in each sample is calculated as the SCFA/PEG ratio multiplied by the amount of PEG infused (750 mg), minus the amount of PEG withdrawn in the 20-mL baseline sample, i.e. $\text{SCFA} = \text{SCFA/PEG} \times (750 - \text{PEG}_{\text{baseline}} \times 0.2)$.

Table 18

Amounts of SCFA generated from variable amounts and sources of fermentable carbohydrates by pigs and human subjects (after Bach Knudsen et al., 2003)

	SCFA production (mMol/day)					
Item	Total	Acetate	Propionate	Butyrate	Butyrate (% total)	Reference
Catheterized pigs[a]						
Low-fiber cellulose, 6%	1184	899	213	45	3.8	Giusi-Perier et al., 1989
Low-fiber cellulose, 16%	1428	987	324	84	5.9	
Low-fiber wheat	720	317	347	46	6.4	Bach Knudsen et al., 2000
High-fiber wheat bran	738	320	335	77	10.4	
High-fiber oat bran	891	323	458	101	11.3	
Wheat bread	1563	897	558	91	5.8	Serena et al. (unpublished)
Rye bread	2064	1005	771	273	13.2	
Maize	480	275	142	31	6.5	Van der Meulen et al., 1997
Maize and raw potato	1446	760	210	380	26.3	
Raw potato	2134	1162	267	570	26.7	
Human subjects[b]						
Autopsy (UK, not fasted)	425	270	119	36	8.5	Cummings et al., 1987
Surgical (SA, not fasted)	377	197	115	65	17.2	Cummings et al., 1989
Surgical (NL, fasted)	168	114	43	11	6.6	Dankert et al., 1981
Surgical (NZ, fasted)	157	88	43	26	8.3	Peters et al., 1992

[a]The level of production of SCFA in pigs was calculated as arterio-venous concentration difference × blood flow. The latter was obtained directly (by inserting a flow probe) or indirectly via a marker para-amino-hippuric acid, PAH).

[b]The level of production of SCFA in human subjects was estimated as arterio-venous concentration difference × 1440 (assuming the blood flow of 1 L/min).

A compilation of published references on the production and absorption of SCFA as estimated *in vivo* in pigs and human subjects is presented in table 18.

In pigs, Rérat (1994) studied the chronology of VFA absorption relative to that of glucose by quantifying the net appearance of these nutrients in the portal blood using the following equation:

$$q = (C_p - C_a)\, F\, dt$$

where q is the amount absorbed during the short period (dt = 5 min) in which each factor is assumed to be constant; C_p is the portal concentration; C_a is the arterial concentration; and F is the blood flow rate in the portal vein (mL/min). The author found that VFA contribution was late as compared to that of glucose issued from the enzymatic digestion in the small intestine, but VFA partly replaced the energy supplied in the form of glucose by reducing sugars at a time when digestion was almost over. A mathematical modeling of VFA kinetics of absorption in pigs is presented in fig. 11.

2.5. Metabolism of SCFA in the gut wall and mucosal growth

Although information on the role of SCFA as intestinal bioregulators is still fragmentary and sometimes controversial, a general consensus seems to be achieved that epithelial cells of the small intestine receive energy from luminal glutamine and glucose, whereas colonic epithelial cells utilize (besides glucose and glutamine from vascular origin) SCFA from the colonic lumen (von Engelhardt et al.,1998). According to Sakata (1987), Sakata et al. (1995) and Sakata and Inagaki (2001), the presence of SCFA as metabolites of bacterial fermentation, or supplemented in diets, can stimulate mucosal growth and function in the jejunum, ileum, cecum and colon of nonruminants. It is estimated that the colonic epithelium derives 60–70% of its energy from bacterial fermentation products, and this energy serves for the gut epithelial cells, promoting villous height and mucosal DNA content (Ritzhaupt et al., 1998), and plays an important role in the mucosal protection against lesions or mucosal repair after damage (Friedel and Levine, 1992; Ramos et al., 1999). Besides, there is no doubt that SCFA are metabolized to acetyl-coenzyme A (CoA) in the following order: butyrate > acetate > propionate, and that both intraluminal and post-absorptive contribution of SCFA to the epithelial growth (enterotrophic effect) and mucosal integrity seems essential, as absorbed SCFA enter the portal vein and appear to make a significant contribution to energy through hepatic metabolism (fig. 12).

However, segmental and species differences in the extent of SCFA metabolism seem to differ substantially (Bergman, 1990). According to Cherbut (2003), SCFA affect local and remote motility of the gastrointestinal tract by mechanisms that are not completely understood. In the large intestine, where they are produced, they inhibit peristaltic activity and may stimulate tonic activity. When present in the terminal ileum, as a result of reflux of colon contents, they elicit propulsive contractions. These local motor effects could involve a neurohormonal sensory mechanism located in the mucosa of the terminal ileum and proximal colon. Finally, through a humoral pathway, which probably involves polypeptide YY release, ileal and

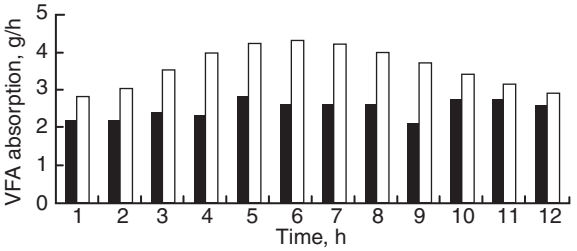

Fig. 11. Kinetics of postprandial VFA absorption in pigs as observed by Giusi (1986) [black bars] and as predicted by Bastianelli et al. (1996) [open bars].

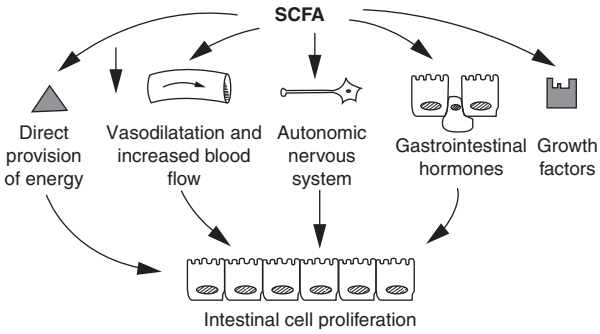

Fig. 12. Possible factors by which SCFA may mediate proliferation of the colonic/intestinal mucosa. (After von Engelhardt, 1998, with permission of Euroscience.)

colonic SCFA modify upper GI motility by inducing relaxation of the proximal stomach and lower esophageal sphincter and slowing of gastric emptying. One characteristic feature of SCFA effects is the dose-dependency of the gastrointestinal motor responses. Indeed, the effects occur only at or above a threshold of SCFA concentration in lumen contents. One putative physiological role of the motor effects of SCFA might be to maintain the physicochemical balance of the lumen environment in the terminal ileum and proximal colon. Another role might be to coregulate motility of the upper intestine. The clinical relevance of these effects is unclear. However, some recent findings suggest that excessive SCFA concentrations might induce adverse effects on gastrointestinal and colonic motility and sensitivity in certain diseases such as inflammatory bowel disease and gastroesophageal reflux disease. Also, Cuche et al. (2000) reported that ileal SCFA infusion in the intact terminal ileum of pigs decreased the amplitude of distal and terminal antral contractions (33% vs 49% of the maximal amplitude recorded before infusion) and increased their frequency (1.5/min vs 1.3/min). The authors concluded that the ileal SCFA inhibit distal gastric motility by a humoral pathway involving the release of an inhibitory factor (probably the gut hormone PYY, which also helps to restrict feed intake).

Literature data on the mucosal growth, motility, epithelial cell proliferation and apoptosis in relation to intraluminal SCFA levels and proportions in nonruminants are still relatively limited, and not always consistent. In general, Sakata (1995, 1987) indicated that daily intermittent or continuous administration of SCFA (both dissociated and undissociated) into the cecum or colon stimulates epithelial cell proliferation of the small and large intestine in a dose-dependent manner, in the following order: acetic < propionic < n-butyric. The author implied that this stimulatory effect is observed at pH 7, but not lower. On the other hand, continuous infusion of lactic acid into the cecum resulted in a greater production of crypt cells, particularly at pH 5. Also, Topping and Clifton (2001) and Williams et al. (2003) reported that intraluminal infusions of SCFA exerted a stimulatory effect on the growth of colonic mucosa in rats and human subjects. Similarly, Sakata and Inagaki (2001) indicated from studies with rats that SCFA (except for lactic acid) can increase wet tissue mass, and growth of the mucosa, submucosa and muscularis externa in distal colon. On the other hand, no SCFA-induced changes in the percentage of crypts in fission could be detected when incubating isolated pig colonic mucosa for 24 h, although crypt fission rate and crypt cell production rate remained inversely correlated. This implies that there is a systemic regulatory mechanism for integrating the total epithelial cell production rate per segment by crypt fission and cell production in the crypt. Reilly et al. (1995) found that colonic infusion of SCFA is

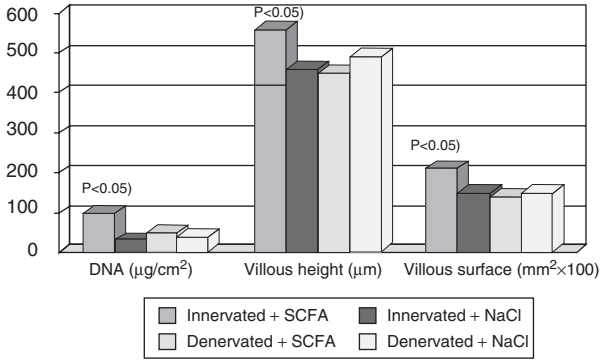

Fig. 13. Effects of the autonomic nervous system of rats on the mediation of the trophic effects of SCFA in the jejunal epithelial growth and morphology. (After Frankel et al., 1994, reprinted with permission. © 1994 American Gastroenterological Society.)

trophic to rat jejunum and is partly associated with raised jejunal gastrin concentration. Also, Bugaut and Bentéjac (1993) concluded that the trophic effects of SCFA on the intestinal mucosa cannot be explained solely by direct luminal effects, as also parenteral infusion of SCFA reduced the mucosal atrophy associated in rats. Frankel et al. (1994) demonstrated in studies with rats that both SCFA and innervation reflect on the intestinal mucosal DNA, villous height and surface (fig. 13).

According to Sakata and Inagaki (2001), lactic acid stimulates both epithelial cell proliferation and apoptosis to a similar extent, and only lactic and succinic acid, and not VFA, are major determinants of the pH in colonic digesta. Therefore, the authors concluded that for a normal fermentation in the large intestine, its content should have a pH above 6.0, and the accumulation of lactic and succinic acids should be avoided.

Some aspects of the bioregulatory roles of acetate, propionate and butyrate as principal metabolites of bacterial fermentation in mucosal growth, epithelial cell proliferation, apoptosis, regulation of transcriptional proteins, modulation of gene expression, as well as in whole body metabolism and turnover of nonruminants deserve a more detailed description.

2.5.1. Acetate

In the absence of supplemental organic acids in feeds, acetate is the main exogenous SCFA produced by bacterial fermentation of nondigestible carbohydrate in the hindgut of nonruminants, although several endogenous tissues are also capable of producing and utilizing acetate (Bergman, 1990). However, scarce and inconsistent information on the quantitative data from particular sites of its production, uptake, epithelial metabolism and whole body turnover is available. Poteau et al. (1998) indicated that acetate produced in the colon is either retained by the liver or escapes into peripheral tissues. This component can supply 6–10% of daily energy requirements for maintenance in humans (Pouteau et al., 1996, 1998) and 25% in pigs (Bergman, 1990). By comparing the relative incorporation rates of several ^{14}C-labeled substrates into lipids, acetate made a significantly larger carbon contribution to lipids than propionate, butyrate, glucose or glutamine under *in vitro* conditions (Zambell et al, 2003). Pouteau et al. (2003) proposed a model of the whole body peripheral pool of acetate in healthy adult human subjects (fig. 14).

Whole-body acetate turnover is estimated to be ~7 μmol/kg BW/min (Pouteau et al., 1998), and like glucose metabolism, acetate metabolism is sensitive to insulin control

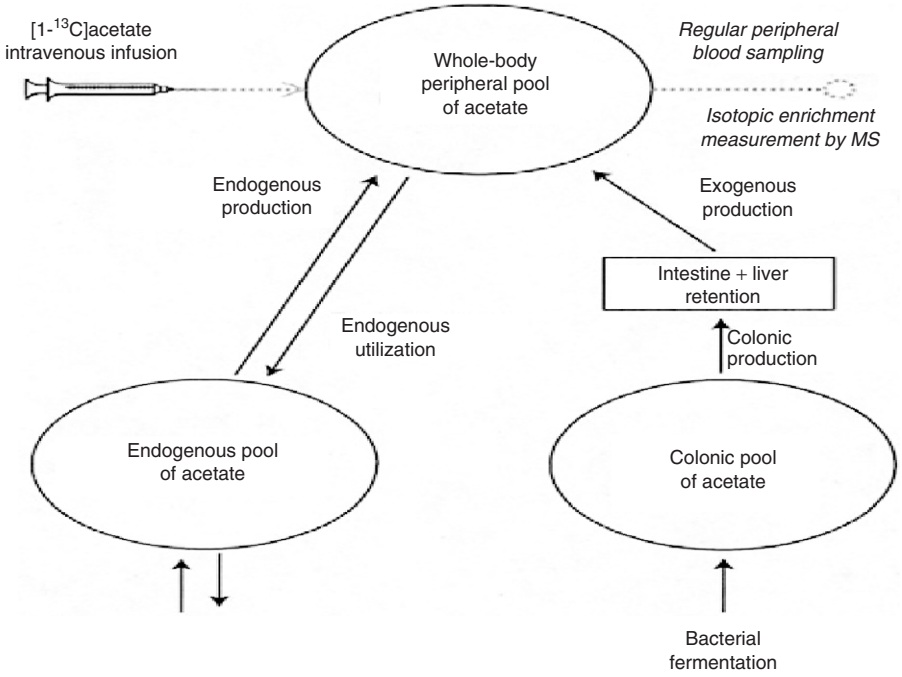

Fig. 14. Model of the whole-body peripheral pool of acetate comprising endogenous turnover and the exogenous production, as assessed from isotopic enrichment measurements on blood samples following an intravenous continuous infusion of [1-^{13}C] acetate. (After Pouteau et al., 2003, with permission of the authors.)

(Piloquet et al., 2003). Acetate produced in the colon is either retained by the liver or escapes into peripheral tissues, because it is found in measurable amounts in systemic venous blood. Exogenous acetate appearing in peripheral plasma is therefore the unique reflection of colonic fermentation of dietary carbohydrates. Acetic acid is relatively well recognized as a substrate for lipogenesis and cholesterol synthesis in the liver of humans and birds, or adipose tissues of pigs (Bergman, 1990), although few studies have considered acetate utilization and production in the intestine itself. For example, Bleiberg et al. (1992) showed in a study with dogs that infused ^{14}C-acetate can be released and taken up by the intestine in the post-absorptive state. However, because these authors did not evaluate colonic fermentation by an expired hydrogen and methane breath test, the study did not clarify whether acetate was produced by colonic fermentation or intestinal epithelial cells. An acetate-enriched solution was found to produce a trophic effect on intestinal mucosa in rats undergoing total parenteral nutrition, suggesting that acetate energy is supplied to the gut (De Michele and Karlstad, 1995). Zambell et al. (2003) concluded that acetate is the major substrate for *de novo* lipogenesis in rat colonocytes, particularly phospholipids, implying that the major role of lipogenesis is for membrane synthesis. Also, they found that colonocytes appear to synthesize lipids using a pathway distinct from that in the liver by incorporating mainly SCFA and ketone bodies into lipids, and by using citrate to a limited extent, if at all, to transport acetyl units from the mitochondria to the cytosol.

In human subjects, fecal acetate is inversely related to acetate absorption from the rectum and distal colon, although it does not seem to reflect acetate production in the colon (fig. 15).

In pigs, Breves et al. (1993) attempted to apply the tracer technique (^{13}C-labeled SCFA) to measure acetate production rates in the large intestine. However, further experiments

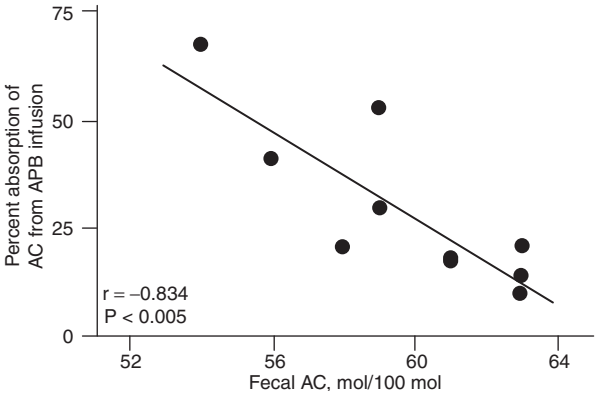

Fig. 15. Linear regression for fecal absorption (%) of acetate (AC) in healthy subjects ($n = 10$) after rectal administration of 300 mL infusate (ABP) containing acetate (60 mMol/L), propionate (20 mMol/L) and butyrate (20 mMol/L), alone or in combinations. (After Vogt and Wolever, 2003, with permission of the American Society for Nutritional Sciences.)

seem necessary to establish the dose–response curve of acetate production to validate the model.

2.5.2. Butyrate

Various nontracer and tracer techniques (*in vitro* and *in vivo*) have been used to study the role of butyrate in the digestive tract and in the post-absorptive pool of metabolites entering into the liver of nonruminants (Bergman, 1990; Bugaut and Bentéjac, 1993; Zambell et al., 2003). These studies were addressed to: (1) determine major metabolites of luminal butyrate; (2) determine how luminal butyrate concentration influences butyrate transport and metabolite formation by the proximal colon; (3) evaluate whether butyrate metabolism is influenced by the presence of other oxidative substrates; (4) compare metabolite formation from butyrate vs. acetate or propionate in a mixture of substrates; (5) compare butyrate oxidation in the proximal colon of young adult vs. aged animals; and (6) anticancer mechanisms.

Among all SCFA, butyrate was found to be the most important respiratory fuel for the epithelial cells (colonocytes, in particular), as butyrate provides energy for epithelial growth and whole body metabolism, even in the presence of glucose (Topping and Clifton, 2001). Besides, it is involved directly and/or indirectly in various mechanisms regulating cellular differentiation, growth, permeability and gene expression (e.g. PLAP, Cfos, C-myc, CEA). Moreover, Williams et al. (2003) indicated from *in vitro* studies using micro-array technology that butyrate displays a host of chemo-preventive properties including increased apoptosis, reduced proliferation, down-regulation of angiogenesis, enhanced immuno-surveillance and anti-inflammatory effects in colono-rectal cancer cell lines. Zoran et al. (1997) implied however, that high butyrate concentrations in the distal colon may not be the only mechanism through which reduced cancer formation in animal models is observed, and mitochondrial electron transport and membrane potential are critical for initiation of butyrate-mediated growth arrest and apoptosis (von Engelhardt et al., 1998). A greater understanding of the mechanisms by which butyrate reduces colon cancer risk clearly deserves further attention.

In contrast to acetic and propionic acid, butyrate seems to lower colonic blood flow (Topping and Clifton, 2001). Fitch and Fleming (1999) showed in a rat model that: (1) transport of

butyrate from the lumen into the mesenteric blood increased linearly with increasing butyrate concentrations, whereas butyrate metabolism followed saturation kinetics; (2) the major metabolites of both acetate and butyrate were CO_2, 3-hydroxybutyrate, and lactate, and the proportions of these metabolites were dependent on substrate concentration; (3) neither the transport nor metabolism of butyrate was influenced by the presence of alternative substrates, including acetate or propionate; (4) when present with other substrates, transport and metabolism were significantly lower for acetate than for butyrate; (5) the transport of butyrate and, to a lesser extent, its metabolism were lower in the proximal colon of aged, than in that of young, animals.

Although butyrate has been shown to be converted into acetyl-CoA by a fatty acid thiokinase, its further oxidation is more rapid to CO_2 than to other potential substrates such as acetate, propionate, glucose, glutamine, long-chain fatty acids, and ketone bodies (Zambell et al., 2003). The authors also compared (quantitatively) the rate of incorporation of carbon into lipids from 3-hydroxybutyrate vs. butyrate and determined whether the presence of alternative substrates would inhibit incorporation of butyrate carbon into newly synthesized lipids (table 19).

These data document that carbon provided as 3-hydroxybutyrate was incorporated into both sterols and fatty acids, although to a lesser extent than from butyrate. In this experiment, it was also shown that the incorporation of butyrate carbon into sterols and fatty acids was suppressed by a mixture of glutamine, acetate and propionate. It was previously believed that 85–90% of the butyrate produced in the gut was cleared when passing the gut epithelium, whereas more recent studies with pigs, humans (Bach Knudsen et al., 2003) and rats (Fitch and Fleming, 1999) have shown that the concentration of butyrate is strongly influenced by the production rate in the large intestine (table 20 and fig. 16).

Zambell et al. (2003) indicated that butyrate oxidation is not suppressed by the presence of other energy-providing substrates, whereas the presence of butyrate reportedly suppressed the oxidation of other SCFA including acetate. Recently, colonocytes from aged rats were reported to oxidize fatty acids at an abnormally high rate, and in both the young and aged animals butyrate was found to provide 50% of the energy for isolated colonocytes (Fitch and Fleming, 1999). These and earlier observations have caused butyrate to be recognized as the preferred energy-providing substrate for colonic epithelial cells, leading to the speculation that butyrate deprivation jeopardizes colonocyte and mucosal health. According to Blottière et al. (2003): (1) butyrate can influence cell proliferation through the release of growth factors or gastrointestinal peptides such as gastrin, or through modulation of mucosal blood flow;

Table 19

Incorporation of carbon from ketone bodies and butyrate into lipid by rat colonic epithelial cells (after Zambell et al., 2003 reproduced with permission of the American Society for Nutritional Sciences)

		nMol substrate carbon[a] (g dry weight × h)	
Substrate and tracer[b]	Other substrates	Sterols	Fatty acids
[1-^{14}C]3-Hydroxybutyrate	Mix	396 ± 110^{z}	712 ± 50^{z}
[1-^{14}C]Butyrate	Mix	799 ± 50^{y}	2050 ± 90^{y}
[1-^{14}C]Butyrate	Glucose	2130 ± 210^{x}	4630 ± 480^{x}

[a]Values are means ± SEM, $n = 3$.

[b]Tracers (20-25 kBq/μMol) were present in 10 mMol/L of the tracer substrate and in 10 mMol/L of the other substrates indicated (where mix included glucose, glutamine, acetate, propionate, butyrate and 3-hydroxybutyrate).

[x,y,z]Means in columns not sharing the same superscript differ at $P < 0.05$.

Table 20

SCFA and butyrate in the jugular vein from pigs and venous blood of human consuming a semisynthetic diet and diets based on wheat or rye breads (after Bach Knudsen et al., 2003, with permission of the authors)

Diet	Total SCFA (μMol/L)	Butyrate (μMol/L)	Butyrate % Total SCFA
Pigs			
Semisynthetic diet	136[a]	1.2[a]	0.9[a]
Wheat bread	178[b]	3.7[b]	2.0[b]
Rye bread	191[b]	7.3[c]	3.8[c]
Healthy human subjects			
Wheat bread	162	1.6	1.1
Rye bread	164	1.7	1.1

[1]$n = 6$ pigs per diet.
[2]$n = 38$ human subjects per bread type.
[a,b,c]Means in columns not sharing the same superscript differ at $P < 0.05$.

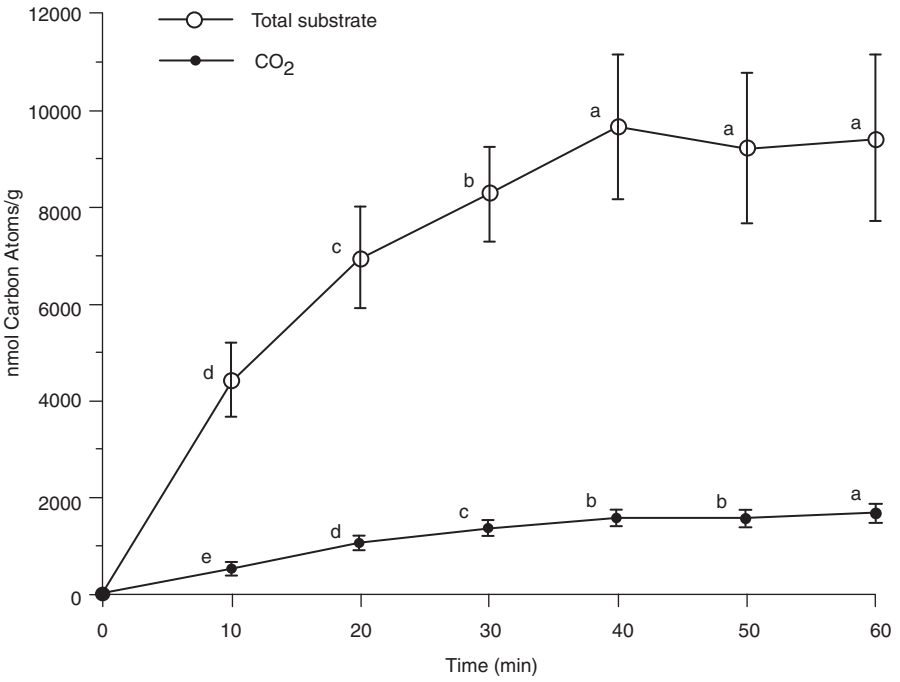

Fig. 16. Influence of intracolonic perfusion time with [1-^{14}C]butyrate on cumulative appearance in mesenteric blood of total carbon and CO_2 from butyrate in rats. Values are means ± SE; $n = 6$ for each time point. Values (○,●) with different superscripts are significantly different at $P < 0.05$. (After Fitch and Fleming, 1999, with permission of the American Physiological Society.)

(2) SCFA can act directly on genes regulating cell proliferation, and butyrate is the main SCFA to display such an effect; (3) butyrate inhibits histone deacetylase, which will allow histone hyperacetylation. (such hyperacetylation leads to transcription of several genes, and it will allow cyclin D3 hyperexpression by inhibiting its degradation); (4) the induction of cyclin-dependent kinase inhibitory proteins accounts for cell arrest in the G1 phase of the cell cycle.

In summary, butyrate has two apparently contradictory effects if served as an energy source for the normal colonic epithelium and stimulates growth of the colonic mucosa, whereas in colonic tumor lines it inhibits growth and induces differentiation and apoptosis.

2.5.3. Propionate

Propionate is a potent glucogenic compound, although in contrast to ruminants, far less quantitative data on its cellular metabolism in nonruminants are available. In general, propionate can be found in the portal blood, although some may be metabolized in the colonic epithelium, and it may stimulate the proliferation of the colonic epithelium (which may enhance the absorptive capacity of the colon), but with less power than butyrate. Topping and Clifton (2001) reported that when acetate, propionate and butyrate were infused at 75, 30 or 30 meq/L, respectively, blood flow rose by 18.1 and 3.1% for acetate and propionate, respectively, but fell by 3.4% when butyrate was infused. Besides, propionate has been shown to enhance colonic muscular contraction, an effect which contributes to the promotion of laxation and the relief of constipation (Topping, 1996). One possible effect of propionate was thought to be reduction of plasma cholesterol concentrations. It was thought that propionate, formed in the large intestine, was absorbed through the portal vein and inhibited hepatic cholesterol synthesis. This does not appear to be the case and there appears to be no major role for colonically derived propionate in the control of plasma cholesterol (Bergman, 1990; Bugaut and Bentéjac, 1993).

2.6. SCFA: Conclusions

Dietary and intraluminally generated SCFA can play a profound role as bioregulators and mucosal growth promoters via direct and/or indirect mechanisms in the gut, as well as in the whole body of nonruminants at all stages of life, from postnatal development through weaning to adulthood. Their local and systemic mode of action is orchestrated with numerous interactive factors related to diets, health status and neuro-hormonal regulatory processes. A short summary of health-related beneficial effects of SCFA is outlined in table 21.

However, SCFA metabolism in disease states of nonruminants, i.e. the development of colitis ulcerosa, diverticulosis and colorectal cancer remain to be further investigated, as factors affecting local and systemic changes, mechanisms, respective signaling cascades are still poorly understood. The morphology and function of the mucosa are malleable through most stages of life; both cell proliferation and apoptosis can be altered at all phases of the life cycle. While total SCFA availability seems to be a dominant factor in direct/indirect growth and functioning of the digestive tract, the strongest evidence is that butyric acid contributes to epithelial cell proliferation. Better understanding of nutrient interactions on these effects and of the mechanisms mediating the observations described above are still needed. Not only do these factors have importance for understanding of GI physiology but they are crucial for making practical recommendations.

Table 21

Some health-related beneficial effects of SCFA (after Topping, 1996)

SCFA	Specific effect	Benefit
Total SCFA	Lowering of pH	Diminished bioavailability of alkaline cytotoxic compounds Inhibition of growth of pH-sensitive organisms
Acetate	Possible increase in Ca and Mg absorption	Diminished fecal loss of Ca and Mg
	Relaxation of resistance vessels	Greater colonic and hepatic portal venous blood flow
Propionate	Enhanced colonic muscular contraction	Easier laxation, relief of constipation
	Relaxation of resistance vessels	Greater colonic and hepatic portal venous blood flow
	Stimulation of colonic electrolyte transport	Greater ion and fluid absorption, prevention of diarrhea
	Colonic epithelial proliferation	Possible greater absorptive capacity
Butyrate	Relaxation of resistance vessels	Greater colonic and hepatic portal venous blood flow
	Metabolism by colonocytes	Maintenance of mucosal integrity, repair of diversion and ulcerative colitis, colonocyte proliferation
	Maintenance of normal colonocyte phenotype	Diminished risk of malignancy
	Stimulation of colonic electrolyte transport	Greater ion and fluid absorption, prevention of diarrhea

3. MEDIUM-CHAIN FATTY ACIDS

In the context of searching for antibiotic alternatives for nonruminants, not only SCFA, but also medium-chain fatty acids (MCFA) seem to possess a very promising potency. To this class belong fatty acids with 7–12 carbon atoms, and their chain can be straight or branched, with odd or even lengths. For example, tomatoes contain C_{10}–C_{12} straight, medium-chain and C_{10}–C_{12} branched, medium-chain acyl acids (Kroumova et al., 1994). The most common saturated MCFA are enanthoic (C_7), caprylic (C_8), pelargonic (C_9), capric (C_{10}), undecylic (C_{11}) and lauric (C_{12}). However, in contrast to SCFA, information on the *in situ* generation of diacylglycerols, monoacylglycerols and MCFA in the stomach and intestines of monogastrics is still scarce. In fact, MCFA can be generated from short- and medium-chain triacylglycerols (TAG) due to endogenous/exogenous lipases, which can hydrolyze TAG to diacylglycerols (DAG), monoacylglycerols (MAG) and MCFA, as illustrated in fig. 17.

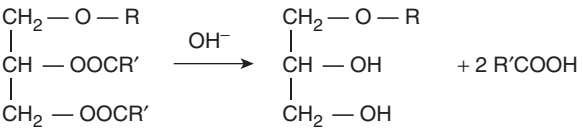

Fig. 17. Generation of medium-chain fatty acids (MCFA) from short- and medium-chain triacylglycerols (TAG) via endogenous/exogenous lipases, which hydrolyze TAG to diacylglycerols (DAG), monoacylglycerols (MAG) and MCFA.

The MCFA are recognized as potential antimicrobials in the stomach and proximal gut of monogastric animals and humans. Furthermore, it is relatively well known that lauric acid can be transformed into monolaurin, which is the antiviral, antibacterial and antiprotozoal monoglyceride used by the human or animal to destroy lipid-coated viruses, herpes, cytomegalovirus, influenza, various pathogenic bacteria including *Listeria monocytogenes* and *Heliobacter pylori*, and protozoa such as *Giardia lamblia* (Enig, 1998). Some studies have also shown some antimicrobial effects of free lauric acid.

More recent investigations have indicated that when C6:0 to C12:0 fatty acids are administered in high concentrations, they are metabolized in several tissues by carnitine-dependent mechanisms (Borum, 1992). Incomplete oxidation of C6:0 to C12:0 fatty acids may result in elevated dicarboxylic acid levels. According to Grigor and Warren (1980), C12:0 is transported mainly as the free acid via the portal vein. Unlabeled 9:0, 11:0 and 13:0 were each absorbed and transported as the next higher homolog. Optimization of the MCFA profile in the diet for nonruminants awaits an improved understanding of their fate in the gut and systemic metabolism. Their content in conventional feeds is relatively small, except for coconut fat, in which lauric acid makes up approximately 50% of the total fatty acids. Therefore, practical diets may be supplemented with commercially available MCFA and/or TAG with the aim of generating MCFA in the digestive tract by enzymatic hydrolysis of TAG. Commercial supplements of TAG for nutritional purposes are derived from lauric oils. In the process of producing MCFA, lauric oils are hydrolyzed to MCFA and glycerol. The glycerol is drawn off from the resultant mixture, and the MCFA are fractionally distilled. The MCFA fraction used commercially for nutritional purposes is finally re-esterified with glycerol, to produce TAG that are mainly glyceric esters of caproic (C_6), caprylic (C_8), capric (C_{10}) and lauric acids (C_{12}) in a ratio of approximately 2:55:42:1. Up to now, nutritional implications of dicarboxylic or chain-branched (carbocyclic/aromatic) MCFA are negligible.

Supplemental and intrinsic SMCT may consist up to 60% of total fatty acids in diets for young pigs (Dierick et al., 2002a,b). These SMCT can serve as a substrate for endogenous (gastric, pancreatic) and exogenous lipases to generate diacylglycerols, monoacylglycerols and MCFA as important energy sources and as potential antimicrobials in the stomach and duodenum of piglets (Dierick et al., 2002a,b). Physical and chemical characteristics of some MCFA are presented in table 22 and the content of some MCFA and other fatty acids in various fat sources in table 23.

Antimicrobial activity of MCFA or their derivatives (e.g. monoacylglycerols) has been known for a long time. They can serve as food-grade germicidal agents, pharmaceutical preservatives, silage additives or feed preservatives (Cherrington et al., 1990, 1991a,b; Dodge and Sagher, 1991; Russell, 1991; Boddie and Nickerson, 1992; Wang and Johnson, 1992; Oh and Marshall, 1993; Meeus, 1994; Decuypere and Meeus, 1995; Sun et al., 1998; Hsiao and Siebert, 1999). By a partial substitution of tallow in a milk replacer with tricaproin or tricaprylin, the growth rate of preruminant calves was increased by 40% (Aurousseau et al., 1984). This response resulted from the high antimicrobial efficacy of enzymatically released MCFA in the gut lumen and to their fuelling action for the enterocytes. Also, bacteria, viruses and flagellates in gastric contents of neonates were suppressed/inhibited by fatty acids or derivates generated by the action of endogenous lipases (Reiner et al., 1986; Hamosh et al., 1989; Isaacs et al., 1995; Hamosh, 1997). Preduodenal lipases are capable of generating MCFA from milk fat. Preduodenal lipase activity is high in humans, preruminant calves, young rabbits and dogs, moderate in piglets, small in carnivore avian species and absent in other birds (De Nigris et al., 1988; Moreau et al., 1988; Marounek et al., 1995).

A long time ago, supplemental TAGs were introduced in human nutrition and in clinical settings (premature infants, fat malabsorption syndromes, surgical patients, cancer patients) as

Table 22

Physical and chemical properties of MCFA

Trivial name[a]	Chemical formula	MW, g/mol	Density g/cm^3	Form	pK$_a$	Solubility in water[b]	Gross energy MJ/kg	Odor	Systemic name
Monocarboxylic									
Enanthoic	**(C7:0),** $CH_3(CH_2)_5$ COOH	130.19	0.910	oily liquid	4.85	*s*	31.78	old wine or sour sweat	heptanoic
Caprylic	**(C8:0),** CH_3 $(CH_2)_6$ COOH	144.21	0.911	oily liquid	4.89	*i*	33.61	unpleasant, irritating, goat-like	octanoic
Valproic	**(C8:0),** $(CH_3CH_2CH_2)_2$ CH COOH	144.21		oily liquid	4.80	*i*	33.61		2-propyl-pentanoic
Pelargonic	**(9:0),** $CH_3(CH_2)_7$ COOH	158.20		oily liquid	4.96	*i*	35.54	pelargonium-like	nonanoic
Capric	**(C10:0),** $CH_3(CH_2)_8$ COOH	172.30	0.905	oily liquid	4.89	*i*	36.42	goat-like	decanoic
Obtusilic	**(C10:1n-6),** $C_{10}H_{17}$ COOH	170.30				*i*	35.56		cis-4-decenoic
Caproleic	**(C10:1n-1),** $C_{10}H_{17}$ COOH	170.30				*i*	35.56		cis-9-decenoic
Undecylic	**(C11:0),** $C_{10}H_{21}$ COOH	186.30		liquid or solid	4.96	*i*	37.19	goat-like	undecanoic
Lauric	**(C12:0),** $C_{11}H_{23}$ COOH	200.40		solid	4.96	*i*	37.83	bay oil or laurel-like	dodecanoic
Lauroleic	**(C12:1n-7),** $C_{11}H_{21}$ COOH	198.40					38.22		cis-5-lauroleic
Linderic	**(C12:1n-8),** $C_{11}H_{21}$ COOH	198.40					38.22		cis-4-dodecenoic

Continued

Table 22

Physical and chemical properties of MCFA—Cont'd

Trivial name[a]	Chemical formula	MW, g/mol	Density g/cm^3	Form	pK_a	Solubility in water[b]	Gross energy MJ/kg	Odor	Systemic name
Chain-branched/carbocyclic/aromatic									
Phthalic	C_6H_5 $(COOH)_2$	166.13		solid	2.89/5.51		24.69		benzene-1,2-dicarboxylic
Mandelic	C_6H_5 CH OH COOH	152.1		powder	3.37	*v*	27.65		2-hydroxy-2-phenylacetic acid
Camphoric	$C_{10}H_{16}O_4$	200.23		crystal			28.10	camphor	1,2,2-trimethyl-1,3-cyclo-pentane-dicarboxylic
Dicarboxylic saturated MCFA[a]									
Pimelic	COOH $(CH_2)_5$ COOH	160.17		crystal	5.31	*s*	23.82		heptanedioic
Suberic	COOH $(CH_2)_6$ COOH	174.20		crystal		*s*	25.65	acid, bitter	octanedioic
Azelaic	COOH $(CH_2)_7$ COOH	188.22	1.225	solid	4.39	*v*	27.21	rancid fat-like	nonanedioic
Sebacic	COOH $(CH_2)_8$ COOH	202.25					28.54		decanedioic

[a]The trivial names in **bold** refer to those MCFA, which are the most often incorporated in practical diets for nonruminants.
[b]Solubility in water: *i* = insoluble; *v* = substantially soluble; *s* = slightly soluble.

Table 23

Contents of some MCFA and other fatty acids in various fat sources (g/100 g total fatty acids) (after Decuypere and Dierick, 2003, with permission of the authors)

Fatty acid	TAG oil	Coconut oil	Palm kernel oil	Woman milk	Horse milk	Rat milk	Cow milk	Goat milk	Sheep milk	Rabbit milk	Sow milk
C4:0							4.8	3.1	4.0	0.0	0.1
C6:0	1.0-2.0	0.5	0.3	0.1			2.2	2.5	1.7	0.0	0.1
C8:0	65.0-75.0	8.0	3.9	0.2	8.0	8.7	1.3	2.9	1.4	34.3	0.0
C10:0	25.0-35.0	6.4	4.0	1.0	17.1	23.6	2.9	10.2	2.6	21.4	0.0
C12:0	2.0	48.5	49.6	4.9	14.3	15.4	3.3	6.1	1.6	1.2	0.0
C14:0		17.6	16.0	5.6	8.7	10.6	10.8	12.5	5.0	1.0	4.0
C16:0		8.4	8.0	20.3	15.3	16.2	26.2	28.6	23.3	11.7	36.6
C16:1				3.4	4.0	1.5		2.6	1.3	1.2	10.5
C18:0		2.5	2.4	7.5	1.2	2.4	10.8	6.2	19.2	2.1	5.9
C18:1		6.5	13.7	33.6	8.3	14.5	24.1	21.6	33.5	12.6	32.3
C18:2		1.5	2.0	12.6	6.1	7.1	2.4	3.6	3.5	13.8	8.4
C18:3				1.0	4.3		1.1	0.0	2.9	0.7	1.0

medical food lipids with unique properties. Among others, these properties include their more rapid and complete hydrolysis by lingual, gastric and pancreatic lipases, their beneficial effects on intestinal mucosa, their direct transport via the portal blood to the liver (without re-esterification or chylomicron formation) and their preferential oxidation in the mitochondria to CO_2 and ketone bodies (less dependence on carnitine) providing a rapidly available energy source for the mammalian newborn (Lee and Akoh, 1998; Wilis and Marangoni, 1999; Mu and Hoy, 2000). In contrast to long-chain fatty acids, MCT do not require pancreatic enzymes or bile salts for digestion and absorption. However, an excess of MCFA (when applied as a force-fed energy booster in the form of a lipid emulsion bolus) can have very undesirable side effects in animals. In neonatal piglets, excessive amounts of MCFA can be ketogenic and narcotic (Samson et al., 1956; Lin et al., 1995). Also in man, ingestion of more than 30 g of TAG in a short period of time induces nausea and gastrointestinal discomfort (Brouns and Van der Vusse, 1998). Moreover, MCFA may induce a strong stimulus of CCK and perhaps of other intestinal hormones (Lepine et al., 1989), with a pronounced satiating activity that could interfere with gastric emptying and feed intake (Mabayo et al., 1992). However, a lower feed intake could also be the result of a strong and adverse taste of some free MCFA (Cera et al., 1989). To avoid those side effects associated with supplementing MCFA or their salts, an *in situ* generation of DAG, MAG and MCFA from TAG as substrates for exogenous lipases has been proposed (Dierick et al., 2003). Also, due to their combined usage the longer retention time in the stomach, the slower absorption rate and the stronger antimicrobial efficacy of MAG above free MCFA can be expected (Isaacs et al., 1990, 1995; Petschow et al., 1996, 1998).

Information on the effects of extrinsic TAG/MCFA at various activities of preduodenal lipase on mucosal growth, epithelial cell proliferation, digestive physiology, gut flora, feed intake, performance and carcass composition is still scarce. Zambell et al. (2003) incorporated ^3H_2O and acetate carbon into triacylglycerides (TAG), free fatty acids (FFA), and phospholipids (PL) in rat colonic epithelial cells. Cells were incubated in 15 mMol/L acetate, propionate, butyrate, glucose and glutamine with ^3H_2O (300 mBq/flask) or with [1-^{14}C] acetate (18.5 Bq/μmol). The authors found that there was net synthesis of fatty acids by rat colonocytes as indicated by incorporation of ^3H_2O into all three classes of fatty acid-containing lipid (fig. 18).

Significantly more label (61%) was incorporated into phospholipid than into TAG or FFA. Similarly, significantly more ^{14}C-acetate was incorporated into phospholipid than into FFA or TAG when the cells were incubated with ^{14}C-acetate.

In the postabsorptive phase, MCFA are preferentially available for hepatic mitochondrial β-oxidation and n-3 long-chain polyunsaturated fatty acids (n-3 LCPUFA). Combined dietary MCFA and n-3 LCPUFA without LCFA may synergistically stimulate fatty acid oxidation, resulting in blood lipid clearance and LCFA release from adipocytes. The MCFA are known to be absorbed according to a biphasic time-course mechanism, which in some respect differs from that of long-chain fatty acids (Guillot et al., 1993). Physiological studies have shown that ingestion of triglycerides containing these MCFA may result in increased energy expenditure and faster satiety. Thus, they facilitate weight control when included in the diet as a replacement for long-chain triglycerides (St-Onge and Jones, 2002).

The physiology and biochemistry of TAG are very different from those of long-chain triglycerides. MCT are rapidly absorbed from the small intestine, intact or following hydrolysis, into the portal circulation. From there, they are transported to the liver. In contrast, long-chain triglycerides are first hydrolyzed in the small intestine to long-chain fatty acids. They are in turn re-esterified in the mucosal cells of the small intestine to long-chain triglycerides, which are then carried by chylomicrons and transported via the lymphatic system to

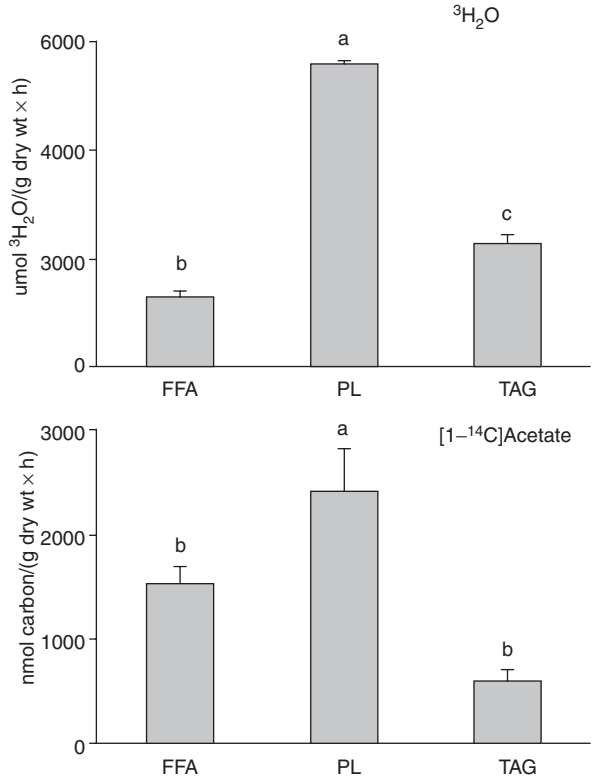

Fig. 18. Mean (± SEM) effects of incorporating ^3H_2O and [1-^{14}C] acetate into free fatty acids (FFA), phospholipids (PL) and triacylglycerides (TAG) by rat ($n = 6$) colonic epithelial cells. (After Zambell et al., 2003, with permission of the American Society for Nutritional Sciences.)

the systemic circulation. The systemic circulation in turn distributes long-chain triglycerides to various tissues of the body, including adipose tissue and the liver.

The MCFA as ketogenic compounds are taken up by hepatocytes and converted to medium-chain fatty acyl-CoA, which enter mitochondria without requiring the aid of carnitine. Besides, the metabolism of MCT in hepatocytes produces two ketone bodies, acetoacetate and beta-hydroxybutyrate. These ketone bodies are carried by the bloodstream to other tissues of the body, where they are used for energy production, as well as for other biochemical processes. It is believed that ketosis may raise the seizure threshold and reduce seizure severity. On the other hand, long-chain fatty acids (LCFA), which are also converted to their coenzyme A esters in cells, including hepatocytes, require that they are converted from coenzyme A esters to carnitine esters in order to be transported across the mitochondrial membrane. Within the hepatocyte mitochondria, medium-chain fatty acyl-CoA is converted to acetoacetate and beta-hydroxybutyrate and subsequently to carbon dioxide, water and energy. The oxidation of MCT produces 8.3 kcal of energy per gram ingested. The MCT are therefore easier to metabolize than LCFA, which could be advantageous in some carnitine deficiencies.

3.1. MCFA: Conclusions

Medium-chain fatty acids (MCFA) seem to possess a very promising potency as antimicrobials in diets for nonruminants, although in contrast to SCFA, information on the *in situ*

generation of MCFA from short- and medium-chain triacylglycerols (TAG) due to endogenous/exogenous lipases in the stomach and intestines of nonruminants is still limited.

Also, more information is needed on the effects of extrinsic TAG/MCFA at various activities of preduodenal lipase on mucosal growth, epithelial cell proliferation, digestive physiology, gut flora, feed intake, performance and carcass composition.

4. FUTURE PERSPECTIVES

The phasing out of antibiotics as growth promoters within the EU and an increasing public pressure for restricting ammonia emission from the manure of nonruminants has stimulated a worldwide scientific interest in evaluating the potency of short- and medium-chain carboxylic acids (including their salts or derivatives) as bioregulators and gut growth promoters.

In contrast to many other antibiotic alternatives, these specific acids are generally recognized as natural, safe, environmentally friendly, easy to handle in feed processing, and cost effective. It has been well established that short-chain fatty acids can provide from 7% to 80% of energy for maintenance requirements. However, these acids have a number of other important regulatory functions (via neural and humoral pathways) related to gastrointestinal functionality (mucosal development, proliferation, differentiation, maturation and apoptosis of enterocytes or colonocytes, transepithelial transport, permeability, gastric emptying, intestinal motility) and to whole body energy turnover at each phase of growth or to specific feeding strategies. Moreover, their impact on intraluminal and intramucosal acidity, cell swelling, stimulation of mucin release and mucosal blood flow has also been documented.

The contents of short-chain carboxylic acids in the stomach, duodenum, jejunum, ileum, cecum and colon (ascending, transverse and descending) during a 24-h cycle reflects the amount consumed and the rate of intraluminal production by anaerobic microorganisms from fermentable substrates. The overall whole-body production of these acids is a function of an exogenous intestinal supply and endogenous metabolism. The methods for studying carboxylic acids as substrates for gut growth and functionality have changed several times over the years, and a gradually increasing sophistication and application of modern technologies and techniques can be observed. For example, *in vivo* carboxylic acid kinetics at the level of digesta, the gut wall, organs, blood or individual tissues, can be very accurately measured using isotopically labeled specific short- or medium-chain fatty acids. The short-term and rapid nature of the technique allows for repeated measurements on individual animals and more sensitive results during periods of rapid change in gut development (postnatal growth, gestation or lactation).

Generally, butyrate seems to be the most suitable substrate for epithelial growth and minimizing apoptosis. The bioefficacy of short-chain carboxylic acids (SCFA) seems to be greater than their salts or inorganic acids, but negative nutritional interactions between SCFA and inorganic bases in the gut of the pig are not excluded, and may lead to the development of gastroduodenal erosions or ulcers, colitis ulcerosa, diverticulosis or colorectal cancer. The obscure differences between the effects of SCFA on cell proliferation, differentiation and apoptosis of enterocytes or colonocytes *in vivo* or *in vitro* indicate that the direct effects of SCFA are interrelated with systemic effects.

Cumulative data from studies on the nutritional and physiological roles of endogenous and intrinsic/extrinsic sources of each class of short- and medium-chain carboxylic acids as multifunctional constituents in two principal body compartments, i.e. in the digestive tract and in the systemic/postabsorptive pool, should be useful in increasing the precision of diet formulation and computer modeling of mucosal growth, intraluminal acid–base balance and

production rates at particular sections of the digestive tract at 24-h intervals or over the whole production cycle. Moreover, a better knowledge of various interactive effects of each class of short- and medium-chain carboxylic acid with other dietary compounds in the stomach, small intestine and large intestine on postprandial gut motility, transepithelial transport and absorption is desirable for a better 'harmonization/optimizing/steering' of intraluminal, intramucosal and postabsorptive pathways, which subsequently should improve the effectiveness of short- and medium-chain fatty acids.

Due to some bactericidal properties and limitations of individual short-chain fatty acids, there is growing interest in blending SCFA alone or in combination with some other specific compounds contributing to gastrointestinal development, maturation and functionality (e.g. glutamine, glutamate, arginine, threonine, ornithine, citrulline, polyamines, nucleotides, enzymes, electrolytes, probiotics from isolated *Lactobacilli*, prebiotics such as mannan oligosaccharides, antibodies such as IgG, bovine lactoferrins, plant- or fungal-derived biomolecules as immunostimulants, yeasts cell wall derivatives, herbs such as ginseng, garlic, oregano oil from *Origanum vulgare*, plant lectins, etc.).

A recent development is the use of prefermented ingredients in a liquid system of feeding after weaning and/or in growing-finishing phases. Despite promising preliminary results, further research should be addressed to optimize acidity, composition and dosages of each specific liquid by-product for an optimal contribution of carboxylic acids to the gut health, mucosal development, proliferation, maturation, transepithelial transport, permeability, gastric emptying, intestinal motility and whole-body turnover at each phase of growth.

Presumably, more consistent responses of nonruminants to supplementary short- or medium-chain fatty acids and their salts might be achieved by optimizing exogenous and endogenous sources and levels. Also, proper ratios of protected (microencapsulated) to non-protected forms in diets should be elaborated by using more advanced computer programming for a multifactorial approach. For this purpose, many dietary acid–base-specific characteristics (dietary electrolyte balance [Na+K-Cl], dUA [Ca+Mg+Na+K-P-Cl-S]), buffering capacity, pH, and bicarbonate concentrations could be adjusted to a desired (predicted) mode of action where physiological parameters determining intestinal health such as: gut motility, integrity, immunity and functionality (including gastric, biliary and pancreatic secretory patterns) could be related to expected levels of production (including corrosion factors, cost-effectiveness and environmental impacts).

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5 Metabolic modifiers in animal nutrition: potential benefits and risks

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Research literature describing the metabolic effects in animals of a range of substances including hormones, pharmacological agents and other biological compounds is reviewed. Somatotropin enhances the growth of animals through effects on maintenance, intake, digestibility and the efficiency of nutrient utilization, and affects these processes through direct and indirect (via IGF-1) actions. Optimal use of somatotropin requires more knowledge of nutrient requirements and details of its mechanisms of action. Estrogenic and androgenic anabolic steroids increase the rates of protein synthesis and reduce the rates of amino acid oxidation, increasing the live weight at which animals achieve the same carcass composition as well as mature size and feed efficiency as nonimplanted animals. Ghrelin and leptin are important components of the integrated pathway regulating physiological energy balance with potential to enhance animal production. Polyamines are essential modulators of cell proliferation in such tissues as the gut and immune system. Chromium picolinate supplements may enhance growth and body composition in pigs and in poultry, but in cattle, may only be beneficial during periods of stress, as in calves following transport to the feedlot. β-adrenergic agonists repartition nutrients from fat to muscle protein, which increases feed efficiency, but also reduces meat tenderness, and tissue residues are a concern. Differential expression of adrenergic receptor subtypes, as well as other hormone receptors might have implications for regulation of nutrient requirements of livestock.

1. INTRODUCTION

There has been considerable interest in health and metabolic responses of humans and livestock to a variety of biological compounds referred to as metabolic modifiers, including natural and synthetic substances which may normally be present in feeds or which may be added to feed preparations or administered as implants or injections. The greatest interest has been in those compounds which have stimulatory effects on growth, feed intake, health and other aspects of productivity. A number of previous reviews have described potential applications of metabolic modifiers (e.g. NRC, 1994; Bell et al., 1998). The effects of exogenous

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administration of somatotropin in growing ruminants have previously been summarized (Enright, 1989; Beermann and DeVol, 1991; Moseley et al., 1992), and the efficacy of various anabolic steroids is summarized in several reviews (Galbraith and Topps, 1981; Roche and Quirke, 1986; Beermann, 1989; Hancock et al., 1991).

The current chapter will focus on recent developments in knowledge concerning metabolic modifiers. We have chosen to include in our discussion, not only a consideration of feed additives, implants and pharmaceuticals, but also of endogenous factors that may modulate how feed additives, pharmaceuticals or nutrients influence biological responsiveness. For example, these might include up-regulation or down-regulation of receptors for hormones such as catecholamines, leptin or ghrelin or alteration of enzymatic processes. Some of these factors may have an environmental or genetic basis. Some of the substances considered are endogenous compounds involved in modulation of aspects of growth or cell biology in response to variations in hormone activity or nutrition. Although many of the compounds that fall into the category of metabolic modifiers show merit for improvement of growth, productivity or body composition of livestock, there are also substantial concerns regarding the health and safety aspects of using some of these products in food-producing animals.

2. SOMATOTROPIN AND IGF-1 IN GROWTH AND METABOLISM

2.1. History

Evans and Simpson (1931) first demonstrated the growth-promoting effects in rats of a crude extract from bovine pituitaries. The growth-promoting factor in the crude extract from the pituitary was called “growth hormone”. However, this growth hormone (GH) evidently did much more than just stimulate growth, since enhanced milk yield in lactating rats and other species, and reduced carcass fat in growing rats were also reported (Downs, 1930; Bierring and Nielsen, 1932; Li et al., 1945). Later, somatotropin (ST) was isolated from the anterior pituitary, which allowed extension of the results from the crude extracts to somatotropin, and it could be shown that somatotropin was the galactopoietic factor in pituitary extracts (Young, 1947). The commercial production of bovine somatotropin (bST) and porcine somatotropin (pST) became possible through the breakthroughs in recombinant DNA biotechnology in the early 1980s with the initial projects using bST being conducted by Bauman et al. (1982). Since then there has been a plethora of investigations using bST and pST to explore the biological mechanisms of somatotropin in growth and lactation biology in both basic and applied settings.

2.2. Structure

Somatotropin is a protein hormone of about 190–191 amino acids, that is synthesized and secreted by cells called somatotrophs in the anterior pituitary (Rawlings and Mason, 1989; Etherton and Bauman, 1998). There is about 90% amino acid sequence similarity between bST and pST (Bauman and Vernon, 1993; Etherton et al., 1993). On the other hand, the human somatotropin (hST) shares only about 35% of the amino acid sequence of bST and pST and that, together with the low binding affinity of bST and pST for the receptor of hST, ensures that bST and pST have no effect on human growth and metabolism (Carr and Friesen, 1976; Lesniak et al., 1977; Moore et al., 1985).

There are variant forms of somatotropin and these variant forms may differ in their potency. Thus, bST is released from the pituitary as one of four variants, with either a leucine or valine substitution at position 127 and an alanine (191-amino acid sequence) or a phenylalanine (190-amino acid sequence) at the amino (NH_2) terminus (Etherton and Bauman, 1998). Limited studies indicate that treatment with the valine-127 variant may elicit a greater increase in circulating plasma somatotropin than the leucine-127 variant (Eppard et al., 1993).

2.3. Effects of somatotropin on growth

Most of the data about the efficacy of somatotropin and its effects on growth have been collected on the pig and there is a relative paucity of data on the ruminant animal. Somatotropin acts on growth through its indirect actions as a somatogenic hormone by stimulating cell proliferation mediated by insulin-like growth factor-1 (IGF-1) (Rechler and Nissley, 1990; Burton et al., 1994). In growing animals, the effect of somatotropin on adipose tissue, skeletal muscle and bone growth is to act as a homeorrhetic control that induces marked changes in partitioning of absorbed nutrients among these target tissues (Burton et al., 1994).

2.3.1. Pigs

Porcine somatotropin has dramatic effects on growth performance, with the magnitude of the response varying according to differences in the experimental design including initial body weight, length of study, breed, sex, dose of pST, and diet composition (NRC, 1994). Examples of the dramatic effects of pST are seen in the 1.4 kg per day live weight gain in intact boars, with corresponding gains of about 273 g protein per day (Etherton and Bauman, 1998). Thus, the NRC (1994) reported that maximally effective doses of pST can increase average daily gain by as much as 10–20%, decrease the adipose tissue mass and lipid accretion rates by about 70%, and concurrently increase protein deposition by up to 50%. The partitioning effect of somatotropin is evidenced by the fact that the changes in performance indicated above are usually associated with a decrease in feed intake of approximately 10–15%, leading to improved feed to gain ratios of 15–30%. In addition, the energetic efficiency of specific processes in growing cattle and swine is not altered; however, the metabolizable energy for maintenance costs is increased by about 10–17% by pST administration in pigs, due to the fact that pST-treated animals have a greater proportion of lean tissue at a given body weight (Campbell et al., 1988; Verstegen et al., 1990; NRC, 1994).

2.3.2. Ruminants

There have been fewer studies examining the effect of ST on growth performance of ruminants relative to the number in pigs and the results of studies in which cattle and sheep were treated with ST have been collected together (Enright, 1989; Boyd et al., 1991; NRC, 1994). In growing cattle, treatment with bST has no effect on digestibilities of dry matter, carbon, nitrogen and energy (NRC, 1994). In addition, the energetic efficiency of specific processes in growing cattle is not altered; however, as noted for pigs, it is plausible that the metabolizable energy for maintenance costs are increased by bST administration in cattle, due to the fact that bST-treated animals have a greater proportion of lean tissue at a given body weight (NRC, 1994). The effects of exogenous administration of ST in growing ruminants have recently been summarized (Enright, 1989; Beermann and DeVol, 1991; Moseley et al., 1992).

Data from 21 trials with growing cattle indicate that with moderate doses of bST, average daily gain is increased by 10–15% and feed conversion efficiency is improved by 9–20%, while carcass lean (muscle) content is increased by 5–10% and carcass fat content is reduced by 10–15%. In general, it appears that the species differences in performance in response to ST may be related to the difficulty in ensuring an adequate supply of metabolizable protein to the ruminant animal and that when adequate amounts of degradable and undegradable protein are supplied the performance response in ruminants is similar to that observed in pigs (Enright, 1989; Boyd et al., 1991; NRC, 1994).

2.4. Physiological control of secretion and feedback loops

The major physiological stimuli which influence the secretion of somatostatin are growth hormone-releasing factor (GHRH) and somatostatin (SS) from the hypothalamus, which stimulate the release or inhibit the secretion of somatotropin from the pituitary gland, respectively (Tuggle and Trenkle, 1996). However, there is some evidence that ghrelin, a gastrointestinal peptide hormone, may bind to the growth hormone secretagogue receptor using a non-GHR transduction pathway to stimulate somatotropin release (Smith et al., 1997) (fig. 1). Other physiological regulators include glucagon, insulin, somatomedins and estrogen (Rawlings and Mason, 1989; Burton et al., 1994). Burton et al. (1994) reviewed the feedback control of somatotropin by GHRH and SS. These hypothalamic peptides are secreted into the hypothalamic–hypophyseal portal system, which ensures rapid transport to the pituitary to effect changes in either the secretion or inhibition of secretion of somatotropin by the pituitary. These hormones also feed back to effect a down-regulation of their own secretion by the hypothalamus. Variation in the secretion of GHRH and SS is in turn influenced by many systems, including the adrenaline (epinephrine) and opioid systems and acetylcholine and dopamine (Rawlings and Mason, 1989; Burton et al., 1994). After being secreted by the pituitary, somatotropin enters the peripheral circulation where it causes the liver to secrete IGF-1 and its associated binding proteins (Burton et al., 1994). Elevated somatotropin in blood exerts a negative feedback at the level of the hypothalamus to inhibit the release of GHRH and stimulate the release of SS. In addition, an elevated level of IGF-1 exhibits a chronic inhibitory effect on somatotropin release from the central nervous system, the hypothalamus and the anterior pituitary (Page et al., 1989; Burton et al., 1994).

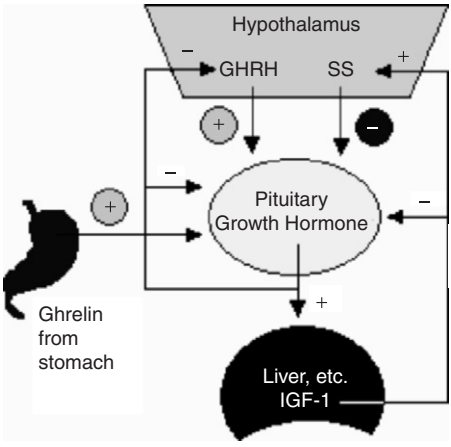

Fig. 1. Physiological control of somatotropin (growth hormone) secretion. (Adapted from colostate.edu/hbooks, 2004.)

2.4.1. Nutritional control of secretion and feedback loops

The growth hormone receptor (GHR) mediates the biological actions of growth hormone on target cells by transducing the myogenic-stimulating signal across the cell membrane and inducing the transcription of many genes, including IGF-1 (Rotwein et al., 1994; Argetsinger and Carter-Su, 1996; Ge et al., 2003). Mutations in the *GHR* gene often cause growth retardation in humans characterized as GH resistance or GH insensitivity (Rosenbloom et al., 1997; Ge et al., 2003). However, not much is known about the bST and pST receptors, although at least two types of receptors with either high affinity or low affinity have been identified (Burton et al., 1994). The low-affinity receptors have been linked to the clearance of GH and may not have any association with IGF-1 concentrations (Burton et al., 1994). The high-affinity receptors and IGF-1 response in cattle depend on the plane of nutrition, including energy and protein concentration in the diet. Thus, increases in the plane of nutrition lead to up-regulation of high-affinity ST receptors, with subsequent increases in IGF-1 and a biological response (Burton et al., 1994). On the other hand, animals in negative energy balance have little or no response in GHR high-affinity receptor numbers or in IGF-1 concentrations (Burton et al., 1994). Thus, feeding animals either a low-protein and/or low-energy diets can uncouple the GH-IGF-1 axis and lead to an ineffective biological response (Burton et al., 1994). Indeed, in the absence of ST administration and in response to low-energy diets IGF-1 secretion is normally decreased while ST increases (Elsasser et al., 1997).

2.5. Biological effects

Somatotropin has two distinct types of effects. Firstly, direct effects are the result of growth hormone directly binding to its receptor on target cells. Indeed, many of the metabolic effects are a direct action of ST. These involve a variety of tissues, and affect the metabolism of all nutrient classes – carbohydrate, lipid, protein and minerals. Thus, fat cells have growth hormone receptors that decrease lipid synthesis if the animal is in positive energy balance, increase basal lipolysis if the animal is in a negative energy balance, and increase catecholamine-stimulated lipolysis in response to ST (Etherton and Bauman, 1998) (fig. 2). Secondly, indirect effects are mediated primarily by IGF-1, a hormone that is secreted from the liver and other tissues in response to growth hormone. A majority of the growth-promoting effects of growth hormone are actually due to IGF-1 acting on its target cells.

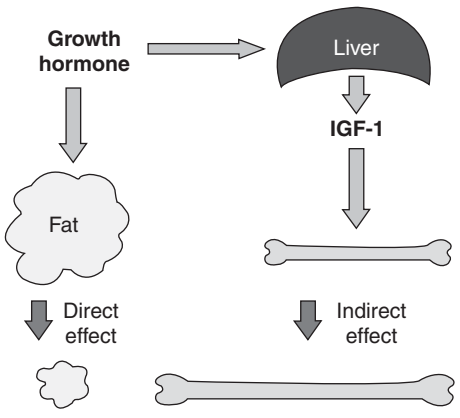

Fig. 2. Direct and indirect physiological effects of somatotropin. (Adapted from colostate.edu/hbooks, 2004.)

The dramatic biological effects on growth performance by ST are orchestrated through many diverse physiological processes, which increase lipolysis during growth and enable more nutrients to be used for lean tissue accretion. As a result, the coordinated changes in tissue metabolism alter nutrient partitioning and thus play a key role in increasing growth performance or milk yield. These somatogenic effects of ST to increase growth are mediated by IGF-1 (Rechler and Nissley, 1990). The biological effects of ST are initiated by binding to the ST receptor on the target cells. The ST receptor then mediates the biological actions of growth hormone on target cells by transducing the myogenic-stimulating signal across the cell membrane and inducing the transcription of many genes, including IGF-1 (Rotwein et al., 1994; Argetsinger and Carter-Su, 1996). The ST receptor has been found in cells such as hepatocytes, adipocytes, lymphocytes, macrophages, fibroblasts and various other cells (Burton et al., 1994). The ST receptor has been cloned for various species including cattle, sheep and swine (Adams et al., 1990; Hauser et al., 1990). Somatotropin initiates its signal transduction pathway by binding to two ST receptor extracellular domains and induces dimerization (Ultsch et al., 1991; De Vos et al., 1992).

The physiological effects of ST are both systemic and specific for various tissues. The specific actions relate to: (1) skeletal muscle (growth): increased protein accretion; increased protein synthesis; increased amino acid and glucose uptake, increased efficiency of amino acid utilization; (2) bone: increased mineral accretion; (3) adipose tissue: decreased glucose uptake and glucose oxidation; decreased lipid synthesis, if in positive energy balance; increased basal lipolysis, if in negative energy balance; decreased insulin stimulation of glucose metabolism and lipid synthesis; increased catecholamine-stimulated lipolysis; increased ability of insulin to inhibit lipolysis; decreased Glut4 translocation; decreased transcription of fatty acid synthetase; decreased adipocyte hypertrophy; (4) liver: increased glucose output; decreased ability of insulin to inhibit gluconeogenesis; (5) intestine: increased absorption of calcium and phosphorus for bone growth; increased ability of vitamin D (1,25-dihydroxycholecalciferol) to stimulate calcium binding protein; increased calcium binding protein (NRC, 1994; Etherton and Bauman, 1998). The systemic effects of ST relate to increased insulin-like growth factor (IGF)-1 and IGF-binding protein (IGFBP)-3; decreased IGFBP-2; decreased amino acid oxidation and blood urea nitrogen; decreased glucose clearance and glucose oxidation; decreased response to insulin tolerance test; increased free fatty acid oxidation, if in negative energy balance; and increased immune response (NRC, 1994; Etherton and Bauman, 1998).

2.5.1. Lipid metabolism

Biological effects of ST on lipid metabolism are not acute but rather chronic, and the extent to which they involve lipogenesis versus lipolysis is a function of energy balance. The dramatic changes in lipid metabolism play an integral role in the responses observed in ST-treated animals (fig. 3). When animals are in positive energy balance ST causes a reduction in rates of lipid synthesis, whereas effects on rates of lipolysis are minimal (Etherton and Walton, 1986; Walton and Etherton, 1986; Boyd and Bauman, 1989; Sechen et al., 1989; Houseknecht and Bauman, 1997). This represents the typical situation for growing animals treated with ST. However, when energy density is low, animals are in negative energy balance and rates of lipogenesis are low, ST treatment affects adipose tissue by increasing rates of lipid mobilization (Machlin, 1972; Eisemann et al., 1986). Thus, the effects of ST are dependent on energy status and include the mechanisms summarized below: decreased lipid synthesis, if in positive energy balance; increased basal lipolysis, if in negative energy balance; decreased

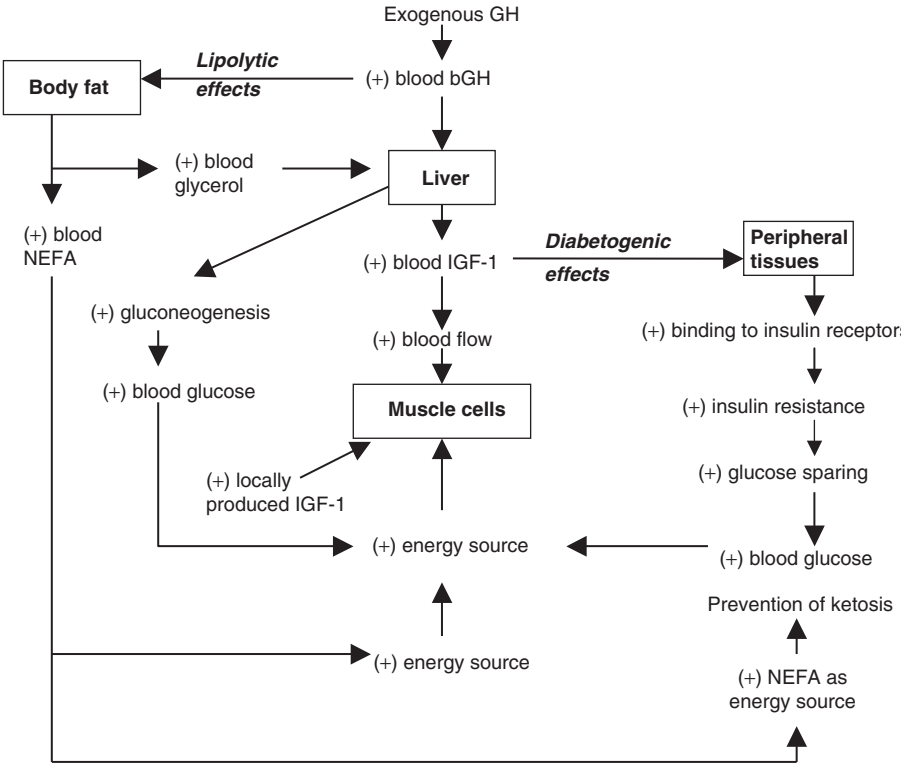

Fig. 3. Lipolytic and other nutrient portioning effects of somatotropin in growing animals (NEFA, nonesterified fatty acid; (+), positive effect). (Adapted from Burton et al., 1994.)

insulin stimulation of glucose metabolism and lipid synthesis; increased catecholamine-stimulated lipolysis; increased ability of insulin to inhibit lipolysis; decreased Glut4 translocation; decreased transcription of fatty acid synthase; decreased adipocyte hypertrophy. These actions are effected through the ability of ST to alter the response of adipose tissue to homeostatic signals. For example, there is a decrease of the stimulatory effects of insulin on glucose uptake and utilization by adipose tissue and a decrease in whole-body response when insulin tolerance tests are carried out without any change in insulin binding or insulin receptor tyrosine kinase activity in adipocytes (Walton et al., 1987; Gopinath and Etherton, 1989; Magri et al., 1990). The insulin antagonistic effect of ST, however, does not appear to universally affect all insulin-regulated genes. For example, the effects of ST on Glut 4 expression may be different than the effects on the expression of fatty acid synthase (FAS) in adipose tissue (Donkin et al., 1996). ST treatment also causes increased lipolytic responses to adrenaline (epinephrine) in growing pigs and steers (Peters, 1986; Wray-Cahen et al., 1991; Boisclair et al., 1997). Thus, ST acts to inhibit nutrient utilization in adipose tissue by suppressing the synthesis and/or activity of lipogenic enzymes such as acetyl CoA carboxylase, fatty acid synthase and glucose transporter proteins (GLUT4) either by transcriptional or post-transcriptional regulation (Mildner and Clarke, 1991; Vernon et al., 1991; Etherton and Louveau, 1992).

Overall, these processes decrease the rate of lipid accretion of meat animals and change adipocyte metabolism. The net results of these metabolic changes in the rate of lipid accretion

determine the final carcass composition in a growing animal and spare and redirect nutrients such as glucose, normally destined to be deposited as lipid, to other tissues, thereby supporting the increased lean tissue/muscle protein accretion during growth (NRC, 1994; Etherton and Bauman, 1998).

2.5.2. Carbohydrate metabolism

ST has numerous effects on carbohydrate metabolism. In the lactating cow, these effects are all geared to supply the increased demand of the mammary gland for glucose needed for lactose synthesis, together with reduced use of glucose in the hindlimb muscle (McDowell et al., 1987). In pigs (in the postabsorptive state) treated with pST, there is an increase in hepatic output of glucose (Gopinath and Etherton, 1989). In both cattle and pigs, liver responses to insulin are decreased (Gopinath and Etherton, 1989; Boisclair et al., 1994). When pigs are treated chronically with pST, plasma glucose and insulin concentrations are elevated (Gopinath and Etherton, 1989; Dunshea et al., 1992). The increase in plasma glucose is most likely related to a reduction in glucose uptake, particularly by adipose tissue. Because a significant quantity of glucose is metabolized in adipose tissue of pigs, a decrease in glucose utilization by adipose tissue redirects a considerable quantity of glucose to other tissues. For example, it has been shown that in barrows approximately 40% of whole-body glucose uptake measured in the basal state and 25–30% of that measured in the insulin-stimulated state are used by adipose tissue (Dunshea et al., 1992). In contrast, glucose utilization by adipose tissue of pST-treated pigs amounts to only about 7% of whole-body glucose turnover (Dunshea et al., 1992).

2.5.3. Protein metabolism

There has been very little work done on the effects of ST on protein metabolism of domestic animals compared to what has been achieved for either lipid or carbohydrate metabolism. Indeed, the precise mechanisms of action of ST as well as the mediation by IGF-1 remain equivocal and associative (Etherton and Bauman, 1998). However, it is now quite clear that ST treatment increases muscle protein accretion in growing animals. The increase in growth rates with ST treatment of ruminants and pigs is due to a more efficient use of metabolizable protein and absorbed amino acids, that is accompanied by increased protein synthesis and an equivocal decrease or unaltered protein degradation, with consequent reduction in urinary nitrogen loss due to a reduction in whole-body oxidation of amino acids (Pell and Bates, 1987; Eisemann et al., 1989; Boisclair et al., 1994; Tomas et al., 1996; Holzer et al., 1999, 2000; Rumsey et al., 2000; Rausch et al., 2002). Thus, ST both increases the maximal capacity for protein accretion and increases the partial efficiency by which amino acids are used for protein deposition (Etherton and Bauman, 1998; Rumsey et al., 2000; Rausch et al., 2002). In addition, growth of skeletal muscle by ST and IGF-1 is enhanced through hypertrophy of skeletal muscle fibers without a change in the number of muscle fibers (Florini et al., 1996). Indeed, ST increases nuclei proliferation and postnatal accretion of DNA, these being key steps in the regulation of muscle growth (Ono et al., 1995; Argetsinger et al., 1996).

2.5. Summary

In reviews by Boyd et al. (1991) and Etherton and Bauman (1998), the essential components of the dramatic effects of ST on animal growth were summarized in relation to its effects on

maintenance, intake, digestibility and the efficiency of nutrient utilization. The mechanism by which ST affects these processes involves coordination of many physiological processes involving both direct and indirect actions on specific targets. The future potential of ST in increasing the efficiency of production is contingent on the adoption of more dynamic methods to estimate nutrient requirement and to advance the knowledge of the mechanisms of action of ST.

3. ANABOLIC STEROIDS, GROWTH AND METABOLISM

3.1. History

Growth-promoting hormones such as estrogens and androgens have been used to improve the efficiency of growth and carcass composition of meat animals since the mid 1950s, when diethylstilbestrol (DES) was first introduced as a feed additive. Indeed, in some countries it is considered routine to implant cattle with synthetic hormones to improve growth rate and feed conversion and to reduce costs of live weight gain. Anabolic implants are categorized as being estrogenic, androgenic, or both estrogenic and androgenic in nature, and they work to increase muscle accretion and retard protein degradation (Webb et al., 2002). Anabolic steroids are not approved for use in growing swine in North America and zeranol is the only compound approved for use in lambs (Etherton and Bauman, 1998).

Steroid implants in use today include the naturally occurring hormone, estradiol, the fungal metabolite with estrogenic properties named zeranol, the hormone progesterone in combination with estradiol or estradiol benzoate, the synthetic progestin called melengestrol acetate (MGA), testosterone propionate in combination with estradiol benzoate, and a synthetic testosterone analog, trenbolone acetate (TBA). Whether the anabolic implants are categorized as being estrogenic, androgenic, or both estrogenic and androgenic in nature, they work to increase muscle accretion and retard protein degradation through their impact on somatotropin action (Duckett et al., 1996; Webb et al., 2002; Reiling and Johnson, 2003). However, increasing the meat yield without regard for effects of anabolic steroids on meat quality may be detrimental, since consumers want quality in the meat products. This literature review gives an overview of the more recent data on anabolic implants on growth parameters and the positive and negative impacts of anabolic implants on growth traits in growing beef cattle.

3.2. Structures and physiology of action

Classification of the anabolic steroids is based on their chemical structures and associated actions. Several reviews of the biosynthesis, metabolism and efficacy of both the naturally occurring estrogens and androgens as well as synthetic implants have been published (Galbraith and Topps, 1981; Roche and Quirke, 1986; Beermann, 1989; Hancock et al., 1991). In cattle, muscle accretion is regulated by response to ST whose receptors are regulated by steroids (Baldwin et al., 1991; Beerman and DeVol, 1991). Webb et al. (2002) have stated that estrogenic and estrogen-like compounds increase protein deposition by increasing the amount of ST, insulin and IGF-1 secreted and by their regulation of high-affinity estrogen receptors in bovine skeletal muscle. On the other hand, androgenic implants increase carcass protein accretion by stimulating muscle protein synthesis through an increase in the proportion of protein synthesis relative to degradation as a result of increased sensitivity to IGF-1 and fibroblast growth factor (Buttery et al., 1987; Reiling and Johnson, 2003). Furthermore, TBA and estradiol combination implants result in enhanced muscle growth through synthesis and secretion of larger amounts of ST and IGF-1, as well as by increasing the proliferation rate

and/or activation state of muscle satellite cells. These satellite cells contribute to postnatal growth by giving nuclei to the increasing fiber (Ono et al., 1995; Argetsinger et al., 1996; Smith et al., 1997). However, anabolic implants affect specific muscles and fiber types within muscles differentially (Webb et al., 2002) and, therefore, implants do not cause additional protein deposition and muscle accretion in all muscles. Indeed, the greatest muscle accretion occurs in extensor muscles associated with the long bones due to the direct effect of the hormones on increasing the rate of cell division at the bone growth plate (Baldwin et al., 1991; Elsasser et al., 1997). However, other muscles including the psoas major and semitendinosus may not be responsive to exogenous anabolic treatments, since they may be near their maximum capacity for growth, depending upon age at treatment (Pell and Bates, 1987; Baldwin et al., 1991; Elsasser et al., 1997).

3.2.1. Protein metabolism

Protein metabolism studies indicate that TBA induces a proportionally greater reduction of the fractional protein degradation rate than enhancement of the fractional protein synthesis rate, leading to an overall increased protein accretion rate in TBA-treated animals (Vernon, 1989; Elsasser et al., 1997). The reduction in protein degradation rate was associated with lower amino acid oxidation and reduced urinary 3-methyl histidine excretion. Therefore, there would be less effect on total energy retention, since reduction of protein degradation rate may have accounted for most of the improvement in efficiency of daily gain and nitrogen retention (Elsasser et al., 1997; Webb et al., 2002; Reiling and Johnson, 2003).

3.2.2. Lipid metabolism

Anabolic implants tend to reduce fat deposition, resulting in decreased marbling scores and fat thickness, as well as decreased percentages of carcasses graded as "Choice" (Duckett et al., 1996; Webb et al., 2002). However, there seems to an overwhelming lack of evidence for the effect of such implants on fat thickness and carcass quality (Duckett et al., 1996). The equivocal nature of the effects of anabolic steroids on fat metabolism relates to the relatively small body of data available regarding effects of anabolic steroid implants on lipid metabolism in growing ruminants (Duckett et al., 1996; Webb et al., 2002). Some researchers demonstrated that lipogenic enzyme activity and fatty acid synthesis *in vitro* are elevated in subcutaneous adipose tissue from bulls implanted with estradiol, while others reported that TBA implants had no effect on lipogenesis in intact heifers and only tended to reduce lipogenic enzyme activities in ovariectomized heifers treated with TBA (Duckett et al., 1996; Reiling and Johnson, 2003). Various reviews, such as the ones by Buttery and Sinnett-Smith (1984), Hayden et al. (1992), Duckett et al. (1996) and Webb et al. (2002) have reported the equivocal and inconsistent changes in ST, prolactin, insulin, or other metabolic hormones when anabolic steroids are administered to animals.

3.2.3. Growth performance response to anabolic steroids

The literature on growth responses to anabolic steroids suggests that there is much variability among experiments, ranging from no response in feedlot bulls (Etherton and Bauman, 1998; Webb et al., 2002), to other studies which indicate that implanted animals have about an 18% increase in average daily gain relative to nonimplanted animals (Duckett et al., 1996). The estrogenic compounds are generally more effective in steers and have only a variable response

in females, but androgenic steroids appear to be superior to other combinations (Etherton and Bauman, 1998). A combination of anabolics generally produces an additive response compared to use of either estrogenic or androgenic implants alone, since combination implants seem to cause the animals to synthesize and secrete larger amounts of ST and IGF-1 and also to enhance muscle growth by increasing the proliferation rate and/or activation state of muscle satellite cells. The response in bulls is generally less than that of steers, presumably because of the endogenous androgens present in the intact male, and implanted steers often achieve a similar growth performance to that observed in nonimplanted bulls (NRC, 1994).

3.2.4. Summary of effects of anabolic steroids on growth

Anabolic implants of either estrogenic, androgenic, or combinations of estrogenic and androgenic hormones have a tremendous impact on production by increasing the rates of gain and feed efficiency of cattle. This effect translates into an increase in the live weight at which carcass or empty-body fat composition equals that of nonimplanted animals, leading to an increase in their potential mature size. Livestock producers, thus, need to be aware that accelerated skeletal maturity could lead to market discounts for maturity. An increased growth rate is usually accompanied by an increase in feed intake, however, the increase in growth rate outweighs the increase in feed intake leading to an increased feed efficiency. Anabolic steroids alter protein metabolism toward increased rates of protein synthesis and reduced rates of amino acid oxidation and/or lesser rates of protein degradation. On the other hand, these agents may have little direct effect on lipid metabolism and associated fat deposition.

4. GHRELIN

One interesting aspect of the discovery of ghrelin is that its identification was an outcome of its action as a growth hormone secretagogue. In 1999, the acylated 28 amino acid peptide was identified from the rat stomach (Kojima et al., 1999) following the report of the associated receptor (GHSR-1a) (Howard et al., 1996; Smith et al., 1996). As indicated, ghrelin's first ascribable function was as a growth hormone secretagogue, which in itself dictates its importance in the regulation of metabolism, but ghrelin's metabolic influence is complemented by other activities, including orexigenic and adipogenic effects. Thus, in spite of the limited studies of ghrelin currently performed specifically in livestock, it is intuitive that all components involved in the physiological mediation of the hypothalamic–pituitary–somatotrophic axis are pertinent to whole-animal metabolism and production.

Ghrelin's release of growth hormone involves the growth hormone secretagogue receptors in the pituitary and the hypothalamus (Howard et al., 1996). Ghrelin is primarily produced by the stomach, but smaller amounts are present in the small intestine (Lee et al., 2002), entire bowel (Date et al., 2000), renal cells (Mori et al., 2000) and the pituitary itself (Korbonits et al., 2001). Plasma ghrelin levels are elevated diurnally in a similar pattern to growth hormone, but are also elevated preprandially and by fasting (Asakawa et al., 2001; Cummings et al., 2001; Sugino et al., 2002a). In a study of sheep it was further found that the preprandial ghrelin surge is influenced by different feeding regimens; it was present in Suffolk sheep fed two or four meals per day but the preprandial surge was absent in *ad libitum* fed sheep suggesting that it may be related to hunger (Sugino et al., 2002b). Furthermore, ghrelin release in sheep appears to involve autonomic regulatory pathways as indicated by a study showing inherent regulation by cholinergic neurons (Sugino et al., 2003). It is possible that preprandial release of ghrelin may serve as a preparatory mechanism for the GI tract and other tissues

regarding the ensuing nutrient exposure. Gastric ghrelin release can be observed in conditions of hypoglycemia, including those induced through exogenous administration of either leptin or insulin (Toshinai et al., 2001). Ghrelin initiates *in vitro* release of growth hormone from rat pituitary primary cells directly (Kojima et al., 1999), and *in vivo* in GH-deficient humans with GHRH receptor mutation (Gondo et al., 2001). The *in vitro* administration of either human ghrelin or rat ghrelin into Holstein anterior pituitary cells (Hashizume et al., 2003a) or porcine anterior pituitary cells (Hashizume et al., 2003b) also resulted in significant increases in GH release. In normal individuals, ghrelin`s induction of pituitary GH release appears to be primarily coordinated through GHRH pathways (Tannenbaum and Bowers, 2001). However, plasma ghrelin levels do not absolutely correspond to GH release and this is probably a reflection of effects of other GH regulators such as somatostatin (Tannenbaum and Bowers, 2001). The ghrelin-induced GH release in the cow anterior pituitary cells was additive to that induced by GHRH alone and inhibited by somatostatin, indicating similar function in bovine species as has been observed in rodents and humans (Hashizume et al., 2003a). It has further been reported that somatostatin can itself reduce gastric ghrelin release in rats (Shimada et al., 2003). These studies allude to an unsurprising integration of the agonist and antagonistic mediators of GH secretion.

Exogenous administration of ghrelin in rats results in stimulation of appetite (Nakazato et al., 2001) and a corresponding increase in adiposity (Tschop et al., 2000). Similar effects of exogenous ghrelin administration have been shown in normal human subjects and also in individuals with suppressed appetite such as cancer cachexic individuals (Wren et al., 2001). Given that the primary site of ghrelin production is the stomach and its primary site of action related to growth hormone release and perhaps the control of appetite are mediated within the CNS, it is apparent that plasma ghrelin must cross the blood–brain barrier (BBB) and it is speculated that acylation facilitates ghrelin's lipophilic properties so as to allow its transport across the BBB. Though the latter has yet to be verified it is known that the acylation of ghrelin is an essential component of its ability to serve as a ligand for the growth hormone secretagog receptor-1a (GHSR-1a) (Bednarek et al., 2000).

The lateral hypothalamus appears to be the primary site that mediates ghrelin-induced hyperphagia. It was found that lateral hypothalamic injection of ghrelin was the most effective at increasing food consumption when compared to ghrelin injections into other feeding-related brain areas, including the hypothalamic paraventricular, arcuate, and dorsomedial nuclei, amygdala, and nucleus of the solitary tract (Olszewski et al., 2003). Moreover, lateral hypothalamic nuclei are probably the domain of central circuitry that integrate the orexigenic properties of ghrelin in association of other orectic and anorectic signals.

GH-induced lipolytic activity is well characterized, but in spite of the fact that ghrelin promotes GH release, ghrelin promotes adiposity. Infusion of ghrelin, des-octanoyl ghrelin or synthetic GHS-R1a agonists into rats showed that the adipogenic effect of systemic ghrelin infusion is not mediated by the same pathway as GH release (Thompson et al., 2004). In fact, both ghrelin and des-octanoyl ghrelin promote bone marrow adipogenesis *in vivo* by a direct peripheral action, which is not mimicked by infusion with a synthetic GHSR-1a agonist (Thompson et al., 2004). Thus, it appears that the ghrelin-mediated adipogenesis may not involve the GHSR-1a receptor, at least not in rats. This is partially supported by a study in humans expressing mutant GHSR, which failed to demonstrate a significant association of these polymorphisms with incidence of obesity (Wang et al., 2004).

The administration of acylated-ghrelin directly into the third ventricle of goats, elicited a dose-dependent increase in plasma GH concentration (Hayashida et al., 2001). The same study further showed that the plasma ghrelin concentration decreased significantly 1 h postprandially in cows, followed by a subsequent recovery to prefeeding levels (Hayashida et al., 2001). This agrees with studies in both humans and rodents, which reported that ghrelin release is

decreased by acute caloric intake (Tschop et al., 2001a), especially by carbohydrates such as dextrose (Tschop et al., 2000; Toshinai et al., 2001). In a study of adult humans it was observed that ghrelin levels increased 3-fold subsequent to ingestion of an oral bolus of essential amino acids (Knerr et al., 2003).

Circulating ghrelin levels reflect acute and chronic energy balance in humans and are known to be decreased in the chronically obese (Tschop et al., 2001b). With the purpose of discerning whether ghrelin also plays a role in energy homeostasis during fetal life a comparison was made of cord blood ghrelin concentrations from small and normal-sized human infants. The results of this study indicate that cord blood ghrelin concentrations of smaller than average infants were significantly greater than those of average-sized infants (Onal et al., 2004). Furthermore, cord blood ghrelin concentrations were negatively correlated with the infants' birth weights and with body mass index values (Onal et al., 2004). This serves as preliminary evidence that cord ghrelin levels of smaller infants are greater than those of normal-sized infants, thus ghrelin expression is perhaps related to the nutritional status in the intrauterine period.

The current understanding of ghrelin indicates that it is an important component of the integrated pathway regulating physiological energy balance, that includes other regulators of orectic and satiety signaling such as insulin, leptin, NPY, AGRP, PYY and α-MSH (Holst and Schwartz, 2004). It is further possible that livestock species may have inherent differences in endogenous ghrelin release and plasma concentrations and that there is probably a range of normal individual ghrelin levels that may reflect the individuality of their respective metabolic status and efficiencies.

5. LEPTIN

It was long postulated that there must be a physiological regulator of nutrient intake and that this regulator must have the capacity to reflect the animals' nutrient and stored energy status. While working with phenotypically obese mice, the gene responsible for this obesity was identified in 1994 (Zhang et al., 1994). Furthermore, it was established that this "*ob*" gene (in mice) encoded a 167 amino acid protein called "leptin" and it was hypothesized that it would have a hypothalamic site of action (Zhang et al., 1994). As a regulator of adiposity leptin quickly became a focus for its potential involvement in the regulation of energy balance. Over the last several years it has become apparent that the leptin gene product is a critical participant in the homeostatic balance between triglyceride catabolism and storage.

Several regulatory elements have been mapped to the *ob* gene promoter, including glucocorticoid response elements (GRE), CCAAT enhancer binding protein (C/EBP) and cAMP response element binding domain (CREB, which may be a repressor in this gene). The presence of these direct regulatory mechanisms in conjunction with other identified indirect mediators of leptin expression support the idea that this gene is an important component of the regulatory pathway for metabolism. Leptin is primarily synthesized and secreted from white adipose tissue (Banks et al., 1996) and is transported in the blood to its effector organs, whose cells express a leptin-specific cell surface receptor (a product of the *db* gene) that is a member of the cytokine class of receptors.

5.1. Leptin activity and regulation

Leptin has been found to be a key endocrine peptide involved in satiety, signaling of the status of adiposity and reproduction. When exogenous leptin is delivered intravenously to ob/ob

mice it was shown that the phenotypic obesity in these mice was decreased (Halaas et al., 1995; Pelleymounter et al., 1995). These discoveries resulted in a premature conclusion that research might lead to a wonder drug that would render human obesity a remnant of the past. This failed to materialize at least partly because most obese individuals appear to have elevated serum leptin concentrations, and appear to be leptin-resistant (Considine et al., 1996). The secretion of leptin fluctuates diurnally, with a peak occurring during the night, and it has been established that plasma leptin concentrations also vary in accordance with gender and systemic adipose mass. In general, females tend to have higher levels of plasma leptin whether they are of normal body weight or obese (Considine et al., 1996). This is probably related to the effect of steroid hormones as indicated by the finding that testosterone reduces leptin mRNA levels and estrogen increases leptin mRNA (Elbers et al., 1997; Wabitsch et al., 1997). White adipose tissue (WAT), the primary secretory tissue for leptin, is well innervated sympathetically, and it has been elucidated that sympathetic activation of WAT reduces *ob* gene transcript (Trayhurn et al., 1996). In support of this relationship it has been reported that cold-exposure (known to increase sympathetic activity in adipose tissue) rapidly reduces leptin and this reverses quickly upon rewarming, indicating that it is not due to reduced adiposity (Trayhurn et al., 1995). As previously mentioned, C/EBP binding motifs are present in the promoter region of the *ob* gene and are known to induce gene transcription. A study by Hollenberg et al. (1997) found that peroxisome proliferator activated receptor-gamma (PPAR-γ) ligands (thiazoladinediones; TZDs) antagonized the C/EBP-mediated enhancement. It appears that PPARs inhibit the transcription of the leptin gene. In contrast to these studies, leptin was found to be increased by insulin (Saladin et al., 1995), glucocorticoids (De Vos et al., 1998), tumor necrosis factor-alpha (TNF-α) (Grunfeld et al., 1996), obesity and nutrient intake (Saladin et al., 1995; Considine et al., 1996).

The exogenous administration of leptin to animals results in a dose-dependent reduction in food intake, body weight and fat depots with an increase in energy metabolism (Campfield et al., 1995; Pelleymounter et al., 1995; Chen et al., 1996; Levin et al., 1996). These studies emphasize the importance of leptin in the systemic regulation of energy balance. Leptin has central effects moderated through the hypothalamus, and peripheral effects at various tissues that express the leptin receptor. The central activity of leptin is known to involve its ability to inhibit neuropeptide Y (NPY) synthesis in the hypothalamic arcuate nucleus (Stephens et al., 1995; Schwartz et al., 1996). This is not the complete story though, because animals that are NPY-deficient still become appetite-suppressed upon leptin treatment (Erickson et al., 1996). Further evaluation has determined that many other CNS peptides are also influenced by leptin, e.g. leptin inhibits agouti-related peptide (AGRP), another orectic CNS peptide. The ability of leptin to suppress appetite is accentuated by its ability to increase expression of cocaine-amphetamine-related transcript (CART, an anorectic) and proopiomelanocortin (POMC). Enzymatic cleavage of POMC results in several active peptides including α-MSH (α-melanocyte-stimulating hormone; an appetite suppressor). A key role for α-MSH is the inhibition of melanin-concentrating hormone (MCH), which is an appetite-inducing peptide. Therefore, leptin suppresses appetite by reducing central orectic signals (NPY, AGRP, MCH) while simultaneously increasing anorectic signals within the hypothalamus. The highest expression of the leptin receptor is concentrated in the arcuate nuclei and the paraventricular nuclei of the hypothalamus, but peripheral tissues have also been found to express the leptin receptor, indicating some peripheral functions for this adipostatic hormone.

Broad-spectrum screening of mouse tissue has revealed a near ubiquitous expression profile for the leptin receptor, though there are several variants of this, which will be discussed later. The reputed active long form leptin receptor (OB-Rb) is found in the lung, heart, liver,

spleen, various WAT depots, skin, bone, brain cortex, pituitary, adrenals, stomach, small intestine, colon, kidneys, bladder, testis and hypothalamus (Lollman et al., 1997). Not only is the leptin receptor expressed in peripheral tissues, leptin itself is produced by tissues other than adipose tissue. Leptin and leptin receptor are found in the stomach (Bado et al., 1998; Mix et al., 2000; Sobhani et al., 2000), skeletal muscle (Wang et al., 1998), pancreatic islet cell (Emilsson et al., 2001), fibroblasts (Glasow et al., 2001) and placenta (Reitman et al., 2001). The expression of both leptin and its receptor by tissues suggests that it may have some autocrine regulatory function related to cellular metabolism. There is good evidence to support the observation that leptin not only suppresses appetite, but further increases metabolic rate to maintain homeostatic energy balance in the face of ample nutrients. Leptin increases fatty acid oxidation in liver and muscle (Muoio et al., 1997) by a pathway involving AMP-activated protein kinase (Minokoshi et al., 2002). Additionally, leptin increases expression of uncoupling proteins; UCP-1 and UCP-3 in brown fat and thus increases thermogenesis (Scarpace et al., 1997, 1998), increases UCP-2 in WAT (Zhou et al., 1997), and increases UCP-2 and UCP-3 in skeletal muscle (Cusin et al., 1998), when administered chronically. This interplay between leptin and uncoupling proteins suggests that they are somewhat co-ordinated in their influence on systemic energy balance and play key roles in dictating whether a cell is directed towards a primarily catabolic state. Another interesting observation is that leptin-resistant mice express much less uncoupling protein in their BAT, WAT and skeletal muscle (Masaki et al., 2001), suggesting that these obese mice may be further disposed to weight gain by their inability to uncouple their mitochondria, perhaps rendering them more energetically efficient.

One study, not surprisingly, found that porcine back fat thickness is positively associated with leptin mRNA levels in this subcutaneous adipose tissue (Robert et al., 1998), supporting the observations of higher serum leptin values in obese pigs as compared to more lean animals (Ramsay et al., 1998). In a study of well-fed versus feed-restricted ewes, Dyer et al. (1997) discovered that the differential feeding regimen influenced the leptin receptor expression, and there was increased OB-receptor in both the anterior pituitary and the adipose tissue of feed-restricted sheep. A further study reported that serum leptin was substantially reduced while hypothalamic NPY expression was elevated in lactating ewes in negative energy balance (Sorensen et al., 2002). This last study establishes that hypoleptinemia in lactating ewes may activate the appetite induction pathway mediated by hypothalamic NPY, promoting hyperphagia, which suggests that it functions in the same manner as is observed in rats and humans. Houseknecht et al. (2000), found that leptin mRNA was elevated in subcutaneous adipose tissue of growth hormone-treated cattle as compared to control steers. More research is required before an effective hypothesis for this association between bovine somatotropin and leptin is established, although it is known that somatotropin has a generalized stimulatory effect on adipocyte lipolytic activity (Houseknecht et al., 1998). Another study involving cattle has reported that plasma leptin is not influenced by breed (fat Charolais vs. fat Holstein), is decreased overall by underfeeding (0.6× maintenance) and is elevated 4 h postprandially in underfed cattle (0.6× maintenance) but is decreased postprandially in well-fed cattle (1.3× maintenance) (Delavaud et al., 2002). Two more recent studies of bovine leptin have provided further insight into the potential importance of leptin towards guiding improvements in livestock production. Buchanan et al. (2002), reported an association between carcass fat content and a missense mutation in the *ob* gene of cattle, namely that a specific cytosine to thymine shift was correlated with an increased adiposity in their cattle. Geary et al. (2003) have proposed that plasma leptin itself is an accurate predictor of several carcass quality traits, and is positively correlated with marbling and fat depth measurements.

Although, these results appear to correspond to leptin research in other species, it is only recently that reliable measurements of bovine serum leptin concentrations, other than the suppressed postprandial leptin in well-fed animals, have been generated.

This brief review of leptin reinforces the idea that it is involved in the regulation of livestock metabolism. In its simplest form, if cattle were to express less leptin they might eat more, develop higher adiposity and become slightly more efficient through their reduced lipid turnover. Moreover, simple analysis of serum leptin or perhaps identification of leptin gene isoforms in livestock could be useful in directing assessment of breeding stock attributes and nutritional regimes that might positively influence livestock production systems.

6. POLYAMINES AND PRECURSOR AMINO ACIDS

Polyamines (putrescine, spermidine and spermine) are small, positively charged molecules, which are essential for cell growth. They are thought to play a role in the regulation of anabolic events such as synthesis of DNA, RNA and protein, and by complex interactions they may also be involved with cell signaling processes (Igarashi and Kashiwagi, 2000). Polyamines are essential to the proliferation, differentiation and migration of mammalian cells, including intestinal epithelial cells (Johnson, 1988). Milk contains relatively high levels of polyamines, which are expected to play an important role during intestinal development and maturation in young animals (Loser, 2000). Functional activity of the gut is induced by polyamines since, in rats, luminal polyamines increase glucose absorption in the small intestine via the rapid increase in concentration of the sodium-glucose transporter 1 (Uda et al., 2002). Early-weaned pigs experience stresses which can result in poor growth and have been shown to benefit from supplemental arginine and glutamate, which prevented intestinal villus atrophy associated with weaning (Ewtushik et al., 2000), but polyamines supplemented at a concentration of 0.39%, were not beneficial. In this case, provision of precursors to polyamines was beneficial but the polyamines were toxic, perhaps because the diet concentration was too high. On the other hand, Dorhout et al. (1997) reported that giving rat pups 3–5 μmol/d of polyamines induced precocious gut maturation. Peulen et al. (2000) in their review concluded, based on the suckling rat, that the polyamine spermidine induces all the modifications observed in the digestive tract at weaning. Greco et al. (2001) also reported that functional maturation responses were induced by 0.4 μmol/g body weight polyamines (spermine or spermidine) in the rat small intestine. Assuming that newborn farm animals respond similarly, supplemental polyamines or their precursors would be expected to benefit development and maturation of the gastrointestinal tract. Loret et al. (2000) reported that intestinal maltase activity increased in piglets by the end of the suckling period, but that orally administered spermine did not affect this activity. It was suggested, however, that polyamines contained in the pancreatic secretions, at concentrations which increased in relation to the pancreatic protein output, might have played a role in inducing intestinal epithelial maturation. Wu et al. (2000a) have demonstrated that cortisol treatment of suckling piglets induced a 13% increase in jejunal villus height and a 14% increase in intestinal weight (fig. 4). These investigators also demonstrated that cortisol enhances polyamine synthesis in piglets by inducing ornithine decarboxylase (ODC), which is a key regulatory enzyme in polyamine synthesis and which is also induced at weaning time (fig. 5). Proline and glutamine can serve as substrates for ornithine and, subsequently, polyamine synthesis in piglets, and Wu et al. (2000a) suggested that enteral provision of amino acids, including proline, glutamine, glutamate, arginine and ornithine may be important for optimal production of polyamines for promotion of intestinal development and integrity. In related studies, Wu et al. (2000b)

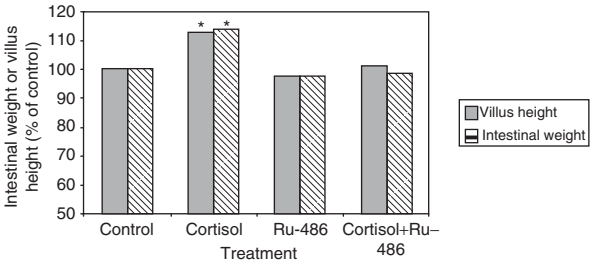

Fig. 4. Effects of cortisol, Ru-468 (inhibitor of ornithine decarboxylase) alone or in combination with cortisol in suckling pigs. (Adapted from Wu et al., 2000.) *Means differ from other treatments ($P < 0.05$), ANOVA.

described a wide array of biological functions of arginine in health and disease of animals and humans, some of which are related to its role as a precursor to polyamines. Amino acids, such as arginine, can also act to induce other anabolic hormones, which might benefit tissue growth. For example, in pigs, the maximum growth hormone level and the area under the growth hormone curve were increased in a dose-dependent manner in response to arginine treatment, but the response to a combined arginine and aspartic acid treatment was delayed compared to arginine alone and was not dose-dependent (Cochard et al., 1998). Glutamine and other amino acids are reported to have a variety of beneficial effects on metabolism, including beneficial effects on functional integrity of the bowel (Reeds and Burrin, 2001), animal production and health (Lobely et al., 2001), and immune function (Newsholme, 2001). On the other hand, there is not full agreement on the safety of dietary supplementation with glutamine and other amino acids (Sacks, 1999; Garlick, 2001). Short-term supplementation with moderate levels of glutamine and other amino acids appears to be safe, however, effects of chronic administration over long periods have not been adequately evaluated and high doses can be toxic. There may be specific groups such as the newborn or those with liver or kidney disease that are more at risk. Neurological effects have been the most frequently reported issues. Further evaluation is needed in terms of dose rate and duration of treatment.

Polyamines appear to help promote healing and recovery of the mucosa following gastrointestinal disease (Johnson and McCormack, 1999). These authors have shown, using a rat stress

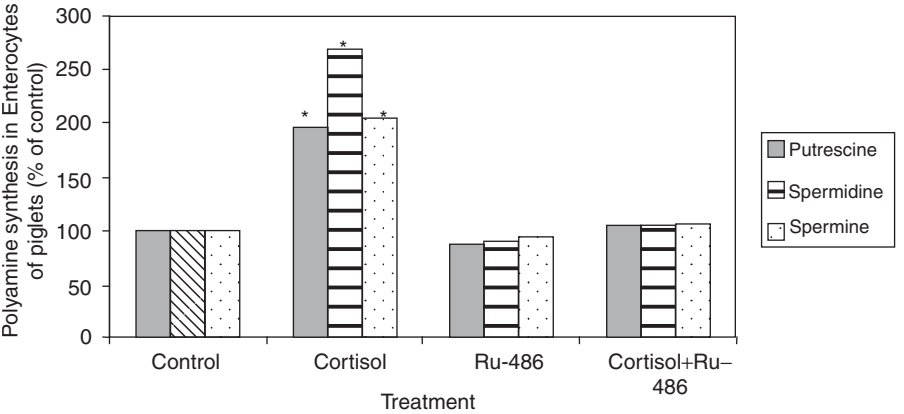

Fig. 5. Effect of cortisol, Ru-486 (inhibitor of ornithine decarboxylase) alone or in combination with cortisol. (Adapted from Wu et al., 2000.) *Means differ from other treatments ($P < 0.05$), ANOVA.

ulcer model, that polyamines are involved in two aspects of mucosal healing: cell migration and cell proliferation. They also demonstrated that supplying transforming growth factor-beta to polyamine-depleted cells, restored normal cell migration. Putrescine (1,4-diaminobutane) is the simplest of the mammalian polyamines. The feeding of 0.2% putrescine increased growth rate beyond that of controls, but higher levels of supplementation (0.8% and 1.0%) reduced growth and were toxic. It was concluded that excess tissue putrescine can be toxic to whole organisms but small, orally administered doses of this metabolite can promote growth (Smith, 1990).

The inclusion of isolated soy protein in milk-replacer diets for calves (Grant et al., 1989) and neonatal pigs (Grant et al., 1990) has been shown to inhibit development of intestinal mucosal cells, whereas simultaneous administration of putrescine partially overcomes this effect. Similarly, in chicks the feeding of supplemental putrescine largely overcame the inhibition of growth due to the feeding of raw soybeans and increased intestinal putrescine concentrations (Mogridge et al., 1996). Putrescine supplementation had no effect, however, on pancreatic and intestinal enlargement in birds fed raw soybeans and tended to depress the activity of polyamine synthetic enzymes. The beneficial effects of putrescine supplementation were confirmed in the third experiment when up to 1.0% supplemental putrescine was fed. Thus, it can be concluded that the toxicity of raw soybeans to chicks can be overcome by feeding putrescine. These effects are likely to be due to improved nutrient uptake, by overcoming the adverse effects of lectins in the intestinal tract, and are not likely to be due to alleviation of the pancreatic enlargement caused by protease inhibitors (Mogridge et al., 1996).

Experiments were conducted to evaluate the potential for dietary 1,4-diaminobutane (putrescine) to influence eggshell quality and overall laying performance in hens. Pancreatic putrescine concentrations were significantly higher ($P < 0.05$) in hens laying thick-shelled eggs compared with hens laying thin-shelled eggs, perhaps indicating that pancreatic cells synthesized more polyamines in hens laying thick-shelled eggs. This increase in polyamines might have caused an improvement in eggshell quality by increasing calcium transport. It was concluded that 0.05% supplemental putrescine improved eggshell quality; however, higher levels proved to be toxic (Chowdhury and Smith, 2001). Barnes et al. (2001) also reported the induction of gizzard erosion and proventricular lesions in broiler chicks fed diets containing histamine (0.2%) and cadaverine (0.1%). Smith et al. (1996) showed that 0.05% spermidine increased growth in chicks but that 0.4% spermidine depressed growth rate and cautioned that biogenic amine content of feed should be carefully assessed before feeding.

Polyamines are involved in embryo implantation in pigs (Rodriguez-Sallaberry et al., 2001). Spermidine/spermine N^1-acetyltransferase (SSAT) has been identified as a porcine endometrial gene whose expression is maximal at peri-implantation and which is induced by conceptus-derived factors and by progesterone. SSAT is an intracellular catabolic enzyme that acetylates the naturally occurring polyamines, spermidine and spermine, thereby facilitating their back-conversion to putrescine and/or promoting their excretion from cells, and may play a role in the maintenance of appropriate intracellular concentrations of polyamines crucial for cell growth and proliferation. Also, the observation that transgenic female mice which overexpress SSAT have underdeveloped uteri and are infertile, suggests a role for polyamines in the control of endometrial tissue growth. IGF-1 and polyamines are believed to act through distinct signaling pathways to mediate cell-type-specific growth of early-pregnancy pig uterine endometrium; it is believed that the maintenance of a threshold level of endometrial intracellular polyamines may be needed to allow the endometrium and the embryo to coordinate their development and hence initiate proper conceptus attachment. The dramatic loss of *SSAT* gene expression after day 19 of pregnancy in the pig, when conceptus attachment

is completed, lends support to this possibility (Rodriguez-Sallaberry et al., 2001). Deficiency of polyamines due to disruption of genes for their biosynthesis also results in failure of embryogenesis in mice (Alhonen et al., 2004). In agreement with this suggestion, Kwon et al. (2003) reported that the rates of polyamine synthesis in the ovine conceptus (embryo/fetus and placental membranes) are maximal during the rapid phase of embryonic growth.

In silage production, polyamines are produced by decarboxylation of certain amino acids during fermentation. Substantial amounts of the compounds are present in both silage and ruminal digesta (Eliassen, 1982). Therefore, ruminants fed silage have the potential to absorb greater levels of polyamines than other species. Polyamines are recognized as key regulators in cellular metabolism and are active when delivered orally (Smith, 1990; Forbes et al., 1994). However, little is known about the levels of polyamines in silage, their fate in the digestive tract and their effects on digestive function and intake in ruminants. Johnson and Michal (2001) described effects of diet on gut tissue ornithine decarboxylase (ODC; the rate-limiting enzyme for polyamine synthesis) and polyamines and luminal concentrations of polyamines in cattle. Diets with barley and roughage resulted in higher ruminal and duodenal tissue ODC levels than for hay diets alone, but did not influence polyamine concentrations in the tissues. Luminal polyamine levels were highest in ruminal and duodenal sites, but ruminal tissue had a lower polyamine concentration than for duodenum, jejunum or ileum. Eliassen and Sjaastad (2000) observed that ODC activity was lower in the rumen than the abomasum in kid goats, but that the activity in the rumen tissue increased in response to injury in sheep. Polyamine concentration did not parallel ODC activity in either rumen tissue or rumen liquid. Orally administered ^{14}C-labeled spermine remained mainly in the lumen during the first 2 h, whereas after intravenous injection the gastrointestinal walls became strongly labeled within 1 h, suggesting that polyamines in ruminal tissue may originate from sources other than the epithelium or ruminal lumen. The apparent slow absorption of polyamines from the rumen is in contrast to the rapid absorption from the lumen of the rat small intestine (Uda et al., 2003). Although the rate of absorption of polyamines from different parts of the small intestine in ruminants is not known, the affinity of the transporter for putrescine in the brush border membrane in chicks increases along the length of the small intestine and is highest in the ileum (Adeola et al., 2003).

In summary, polyamines are substances that are normally produced endogenously in tissues, as a response to growth and developmental signals (Hopfner et al., 2002) or to injury, and they are found in varying amounts in animal feedstuffs. They cannot be considered as the main driving force to alter growth, but nevertheless, are essential modulators at the cellular level and are required in many tissues for the proliferation responses to occur. Addition of polyamines to the diet has been shown to be beneficial in some stress situations but has also been found to be toxic in other situations, especially if high levels are given.

7. CHROMIUM PICOLINATE

There has been substantial interest in recent years in the use of organic chromium (Cr) supplements such as chromium picolinate to induce positive effects on growth and health in animals and humans (Lindemann, 1996). Chromium is involved in glucose tolerance, as a factor which enhances the action of insulin in chromium-deficient tissues (Mertz, 1969) and therefore, has beneficial effects on tissue utilization of glucose. Facilitation of the action of insulin may be a factor involved in the beneficial effects of chromium. The percentages and accretion rates of muscle tissue were increased and the percentages of fat tissue were decreased in pigs fed Cr, with chromium picolinate being more effective than chromium chloride.

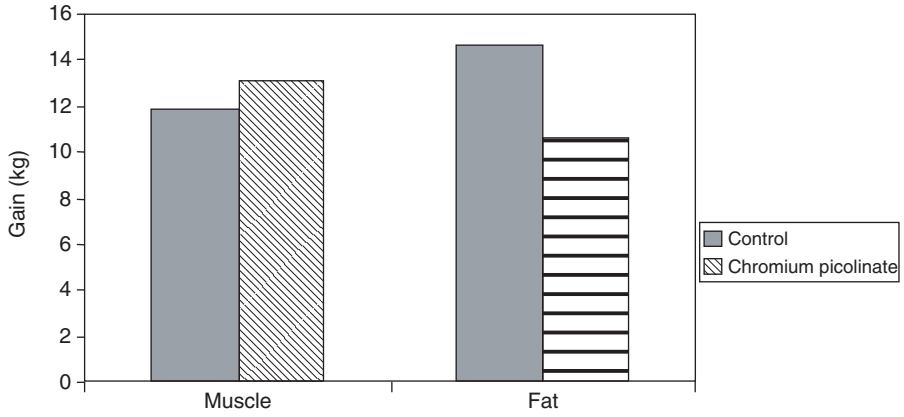

Fig. 6. Effect of feeding chromium picolinate to growing pigs. (Adapted from Mooney and Cromwell, 1997.)

Examples of changes in carcass protein and fat gain (fig. 6) and the increases in accretion rates of carcass protein and decreases in carcass lipid accretion rates in pigs fed Cr (fig. 7) are summarized from the study of Mooney and Cromwell (1997). No changes in blood metabolites were observed as a result of supplemental Cr. In other studies, pigs fed 200 ppb Cr as chromium picolinate (CrP) were shown to have larger longissimus muscle areas and improved N absorption and dry matter digestibility than controls (Kornegay et al., 1997). Furthermore, Lien et al. (2001) reported that supplementation with chromium picolinate at 200 mg/kg in the diet of pigs improved growth rate, feed consumption, reduced back fat thickness and increased loin eye area. Collectively, these results suggest that chromium picolinate is more effective than chromium chloride and that Cr must be supplemented throughout the growing-finishing period to improve the carcass composition (Mooney and Cromwell, 1997).

Pigs fed chromium picolinate (CrP) had a decreased daily fat accretion compared with pigs fed CrP. Sensory and shear force values were not affected by CrP. These results suggest that dietary supplementation of CrP in the finishing phase of pig production may increase muscle and decrease fat deposition; however, not all measures of muscling or fatness were improved by CrP (Boleman et al., 1995). Growth trials were conducted to determine the effects of Cr, as chromium picolinate, at various protein (lysine) levels on performance of growing pigs.

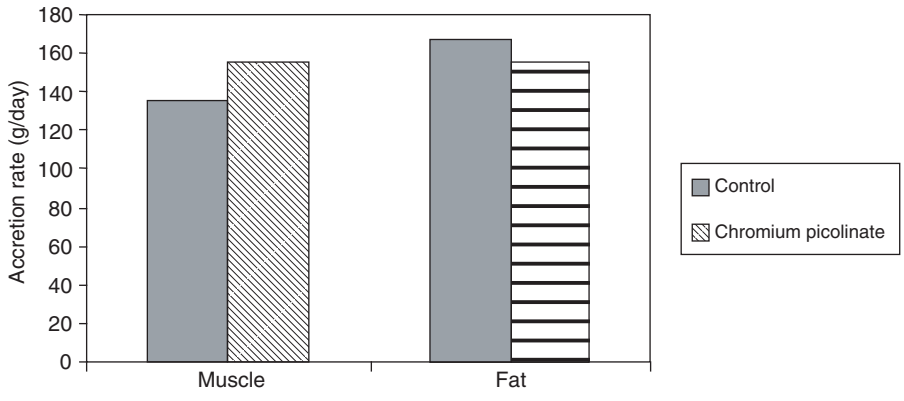

Fig. 7. Effect of feeding chromium picolinate to growing pigs. (Adapted from Mooney and Cromwell, 1997.)

A Cr × lysine interaction for gain to feed ratio demonstrated that addition of 200 ppb of Cr improved gain to feed at the level of 100% of lysine requirement but not at the 120% level. The addition of 200 ppb Cr also reduced back fat and increased longissimus muscle area regardless of lysine level in the diet (Lindemann et al., 1995). Not all experiments have demonstrated positive growth responses of pigs to chromium. In two experiments, conducted to determine the effects of dietary Cr tripicolinate or Cr propionate in pigs, average daily gain, feed intake and gain:feed ratio were not affected by treatments. Carcass length tended to be greater in pigs fed Cr tripicolinate than in pigs fed Cr propionate, but other carcass measurements were not affected. On the other hand, changes in glucose kinetics from the insulin challenge test indicated that both Cr picolinate and Cr propionate increased insulin sensitivity and that both Cr sources were bioavailable (Matthews et al., 2001).

Several studies have shown that organic chromium supplements may have beneficial effects on immunological and health responses in animals. The effect of dietary chromium picolinate (CrP) and recombinant porcine growth hormone somatotropin (rPST) administration on growth performance and cytokine production was determined in Landrace-Poland China gilts challenged with endotoxin (lipopolysaccharide, 0.2 µg/kg i.v.). The rPST treatment improved growth performance versus controls, but pigs given CrP-supplemented diets showed no differences in growth performance. The rPST and rPST+CrP treatments did not influence plasma interleukin-6 (IL-6) compared to the controls. Endotoxin challenge had no effect on either blood glucose levels or induction of TNF-α in any treatment group. On the other hand, incubated peripheral blood mononuclear cells from CrP-treated animals produced more IL-2 than peripheral blood mononuclear cells from all other groups (Myers et al., 1995), suggesting a positive effect on certain cytokines.

In Holstein cows, during late pregnancy and early lactation, Cr supplementation increased the serum glucose peak, increased the area under the response curve for serum glucose, and tended to increase IGF-1 concentrations following propionate infusion. Chromium supplementation also tended to reduce the ratio of insulin to glucagon and might have enhanced gluconeogenesis or glycogenolysis. Supplemental Cr also resulted in reduced variability of most parameters of carbohydrate metabolism during both experiments (Subiyatno et al., 1996). Supplemental Cr had no beneficial effect on health status, mastitis-related parameters or neutrophil phagocytic activity of dairy cows. However, an *in vitro* study confirmed and extended previous observations that Cr has an effect on lymphocyte proliferation, and this may have been associated with insulin or cortisol actions (Chang et al., 1996a).

Effects of supplemental chromium (Cr) from organic sources (Cr chelate and high Cr yeast) on antibody responses of feeder calves, newly arrived in the feedlot, were evaluated following vaccination with infectious bovine rhinotracheitis (IBR), para-influenza-3 (PI3), bovine respiratory syncytial virus (BRSV), bovine viral diarrhea (BVD) and *Pasteurella haemolytica* and ovalbumin (OVA). Chromium from neither source influenced the antibody responses of calves following vaccination with *P. haemolytica*. However, supplemental Cr (0.75 ppm) from Cr yeast enhanced serum antibody responses of calves to OVA during both the primary (d 14) and secondary response (d 35) following immunization. These data confirmed previous findings of this group that supplemental Cr can enhance humoral immune responses of market-transit-stressed calves, but its enhancement on vaccine efficacy was antigen-dependent and variable (Chang et al., 1996b).

In agreement with responses in stressed calves, concanavalin A-induced blastogenesis was enhanced when 1%, 10% and 20% sera, from Cr-supplemented peri-parturient cows, was added to the mononuclear cell cultures. Conversely, peripartum sera from unsupplemented cows depressed concanavalin A-induced blastogenesis. Except for a marginal rise in blood

cortisol 2–4 weeks after parturition, there were no significant effects of Cr supplementation on other hormones (insulin, GH, IGF-1 and TNF-α). These observations suggest that in Cr-supplemented cows, factors in peripheral blood serum other than these glucose-regulating hormones, modulate blastogenesis of mononuclear leukocytes (Burton et al., 1995). In feeder calves, supplemental Cr significantly reduced haptoglobin on day 7 after entering the feedlot, when morbidity was highest, an indication that chromium supplementation reduced the acute phase response in newly arrived feeder calves (Wright et al., 1995). In another study with calves, Cr supplementation increased the magnitude of the peak antibody response to the IBR, but had no effect on anti-PI-3 antibody titers. These data confirmed and extended previous observations that supplemental Cr can be immunomodulatory in cattle (Burton et al., 1994).

Chromium picolinate was approved by the U.S. FDA in 1997 for inclusion in swine diets but not in other meat-producing animals although NRC has not established recommended allowances for chromium in livestock. The circumstances in which this agent seems to have its greatest beneficial effect is during periods of stress, as in calves during transport or shortly after transport to the feedlot, and in animals receiving diets marginal in chromium. Often, no beneficial effects are observed, perhaps because basal diets contained adequate amounts of chromium to meet requirements. There are as yet no general dietary recommendations for inclusion in diets for livestock, except for swine. In human sports and athletics, there has been widespread promotion of chromium picolinate as a supplement to enhance muscle development and athletic ability. The scientific basis for this is limited, and caution is needed because of recent evidence for toxicity (oxidative damage to cells and mutagenesis) of chromium picolinate in humans (Vincent, 2003).

8. ADRENERGIC AGONISTS

8.1. β-agonists, growth and metabolism

Adrenergic receptors (AR) mediate the effects of endogenous catecholamines (epinephrine (adrenaline), norepinephrine (noradrenaline) and dopamine) released in response to stress and other metabolic challenges. Generally, the effects of catecholamines on adrenergic receptors are catabolic and lead to mobilization of body energy reserves, in association with the need for increased energy expenditure. There are two broad classes of adrenergic receptors (α and β) each of which may be further subdivided into several subtypes (Christopherson et al., 1995). Most of the emphasis has been on the effectiveness of β-agonists, although there has also been some interest in potential applications of α-receptor agonists, especially α-2 agonists. The whole body, portal drained viscera and hindquarter oxygen consumption rates increased in sheep, in response to adrenaline infusion in the presence or absence of various β-adrenergic antagonists (Miaron and Christopherson, 1997). These results illustrate that β-receptors play a major role in mediating metabolic responses to catecholamines. β-adrenergic receptors are linked to guanine nucleotide regulatory protein (G-stimulatory protein) that stimulates a variety of metabolic events including energy expenditure. Adrenergic receptors have also been shown to play major roles in body weight homeostasis of animals in a process known as diet-induced thermogenesis (Bachman et al., 2002). Adrenergic receptor density in sheep tissues has been shown to vary in response to changes in feeding level and environmental temperature (Ekpe et al., 2000a,b). Such changes might be expected to influence metabolic responses to administration of catecholamines or to endogenous release of catecholamines. However, when data from Ekpe et al. (2000a,b) were examined for evidence of possible relationships between whole-body heat production and tissue β-adrenergic

receptor density, there was a positive relationship to heat production only in the case of cardiac muscle receptor density. The relationships were negative in the case of receptor density in skeletal muscle and liver. These variable, organ-dependent relationships between receptor density and energy metabolism, however, do not rule out the possibility that administration of exogenous β-agonists could influence other aspects of metabolism, including growth and body composition.

Several synthetic analogs of catecholamines, referred to as phenethanolamine β-adrenergic agonists (e.g. clenbuterol, cimaterol, isoproterenol, ractopamine, L644.969), have been examined for growth-promoting action (Christopherson et al., 1995; Smith, 1998; Mersmann, 2002). Many of these β-agonists appear to play anabolic roles in the stimulation of growth and anabolism of muscle, at the same time as they induce catabolism in adipose tissue (Christopherson et al., 1995), which ultimately leads to the production of leaner animals (Bell et al., 1998). Whether a β-agonist in the feed is effective in growth promotion depends, in part, upon the rate of absorption from the digestive system and the half-life in the tissues and body fluids. Most β-agonists are readily absorbed from the gut (Smith, 1998). Other factors affecting efficacy include the degree of endogenous biotransformation and rate of excretion. These processes are, in turn, functions of the chemical structure, including the pattern of aromatic substitution and whether the molecule is in a halogenated or a hydroxylated form (Smith, 1998). The latter author has reported extensively on the molecular structural relationships that influence ability to bind to tissue β-2 vs. β-1 and α-receptors, and on the susceptibility of the compounds to conjugation and metabolic degradation. β-agonists with halogenated aromatic rings are metabolized more slowly and have long plasma half-lives. An example is clenbuterol, which is also absorbed effectively. On the other hand, those with hydroxylated aromatic rings (such as salbutamol) have short plasma half-lives and are eliminated more quickly (Smith, 1998). Compounds containing saligenins (salbutamol), resorcinols (terbutaline) and simple phenols (ractopamine) are resistant to degradation by catechol-*O*-methyl transferase, but are deactivated by enzymes in the liver and intestine. The rates of absorption and metabolic clearance are factors that also affect the persistence of residues in tissues from treated animals and therefore contribute to food safety (to be discussed later).

Excessive dietary energy intake may activate the sympathetic nervous system to release endogenous agonists, which stimulate β-receptors in thermogenic tissues such as brown adipocytes in many species, thus resulting in dietary-induced thermogenesis (Bachman et al., 2002). In ruminants, however, brown adipose tissue thermogenesis appears to be of significance only in very young animals (Landis et al., 2002). Many β-agonists effectively reduce lipid accumulation in adipose tissues (Christopherson et al., 1995) through activation of hormone-sensitive lipases to promote lipid mobilization (lipolysis) and deactivation of pathways leading to lipogenesis, as well as increase in the down-regulation of lipogenesis in some depots in an environment-dependent manner (Moibi et al., 2000). On the other hand, not all studies have confirmed increased lipolysis in association with reduced body fat (Mersmann, 1998). Recent studies with mice have shown that white adipose tissue cell apoptosis may be induced by β-agonist treatment, thus adding to the complexity of the process which repartitions energy from fat to lean tissues (Page et al., 2004). The apoptosis induced by clenbuterol and ractopamine was comparable in some respects to that resulting from leptin administration (Qian et al., 1998), although the mechanisms in each case are not clear. Whatever mechanisms are involved, the net result of these apparently catabolic actions is the repartitioning of energy from fat accretion towards the support of other body tissues, especially skeletal muscle, as shown by the results of Li et al. (2000) (fig. 8). There is evidence that treatment with β-agonists causes a relative decrease in the proportion of oxidative muscle fibers leading to less myoglobin pigment in the meat for cattle (Moloney et al., 1994), sheep (Warris et al., 1989) and pigs

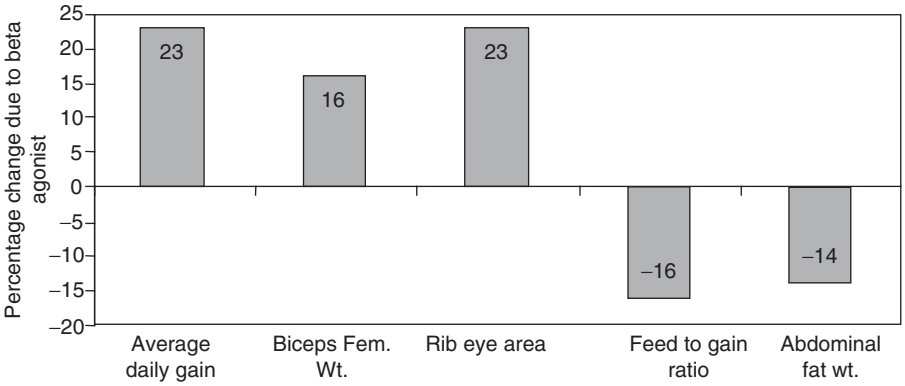

Fig. 8. Effect of the β-agonist (L-644,969) on gain, feed conversion and muscle and fat characteristics in sheep. (Adapted from Li et al., 2000.)

(Warris et al., 1990), which has a tendency to result in a lighter meat color. In circumstances where animals are stressed by preslaughter transport and other procedures, however, there is a tendency for β-agonist-treated animals to show a higher incidence of dark-cutting meat because of the enhanced glycolytic capacity of their muscles (Hanrahan et al., 1987). In addition to the repartitioning of energy from fat to muscle noted above, Nash et al. (1994) reported that β-agonist treatment also leads to repartitioning of nitrogen from wool and skin to support muscle anabolism. This, therefore, may partly explain the striking and preferential increase in skeletal muscle protein accretion by most catecholamine analogs which act on β-receptors (Beerman, 2002). Oral administration of β-adrenergic agonists usually causes an increase in daily gain accompanied, in many instances, by a slight decrease in feed intake and an improvement in efficiency of gain (Mersmann, 2002).

The repartitioning responses outlined above depend on a number of factors such as age, diet, sex, the type of agonists used, and the dose (O'Connor et al., 1991; Beerman, 2002). According to Sillence (1996) the influence of both age and sex of the animal have been variable and not consistent across different studies. On the other hand, there is compelling evidence to suggest that increases in dietary protein intake can positively influence the response to β-agonists. Effects of dietary protein on the responses of pigs to ractopamine are illustrated in figs. 9 and 10 (Dunshea et al., 1993). Similar responses have been reported by Lindsay et al. (1993), and in rats the response to clenbuterol was also dependent on the dietary protein content (Perez-Llamas et al., 1992). Mills et al. (2003) have shown that the four different ractopamine stereoisomers contained in the commercially produced ractopamine mixture, differ substantially in effectiveness for inducing growth and metabolic effects in pigs. Hence, variations in isomer ratios could conceivably contribute to variations in the response of livestock to β-agonists.

Different species of animals seem to respond to varying degrees to β-agonist treatment, although all appear to show positive responses, with ruminants responding more than swine, for example (Bell et al., 1998). The anabolic effects on muscle include fiber hypertrophy, muscle fiber frequency changes, and differential rates of muscle RNA, DNA and protein accretion. Smith et al. (1995) observed significant increases in myosin mRNA in cattle fed clenbuterol and ractopamine. Depreux et al. (2002) reported that the enhanced daily weight gain in pigs treated with ractopamine was associated with substantial changes in myosin heavy-chain isoforms in the muscles. Some studies have shown clear increases in protein

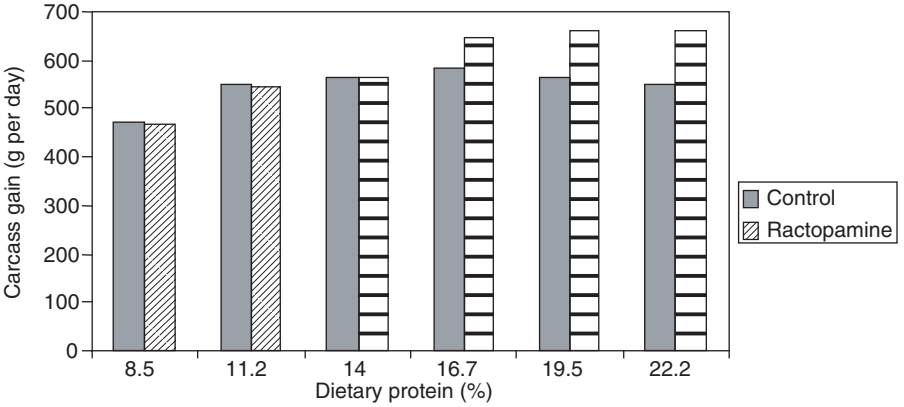

Fig. 9. The effect of dietary protein and ractopamine on carcass gain in gilts. (Adapted from Dunshea et al., 1993.)

synthesis (Bergen et al., 1989; Claeys et al., 1989) whereas others have suggested that improvements in protein accretion are due to reduced protein degradation (Bohorov et al., 1987; Dawson et al., 1991). Decreases in muscle proteolysis in association with reduced protease enzymes and increases in calpastatin concentrations (fig. 11) account, in part, for increased muscle protein accretion and reduced meat tenderness, reflected by increased shear force values (fig. 12) in response to β-agonists (Pringle et al., 1993; Shackelford et al., 1995). The increased shear force values measured in meat have also been reported by Vestergaard et al. (1994). β-adrenergic agonists have been shown to result in differential expression of certain gene fragments in cattle (McDaneld et al., 2004) with clenbuterol reducing the expression of ankyrin (protein–protein interaction) and suppressors of cytokine signaling box gene families (*ASB-15*) mRNA in skeletal muscle. Expression of this gene was not influenced by either trenbolone acetate or growth hormone treatment, but the change in response to β-agonists suggests that it may be involved in the β-agonist-induced hypertrophy of muscle.

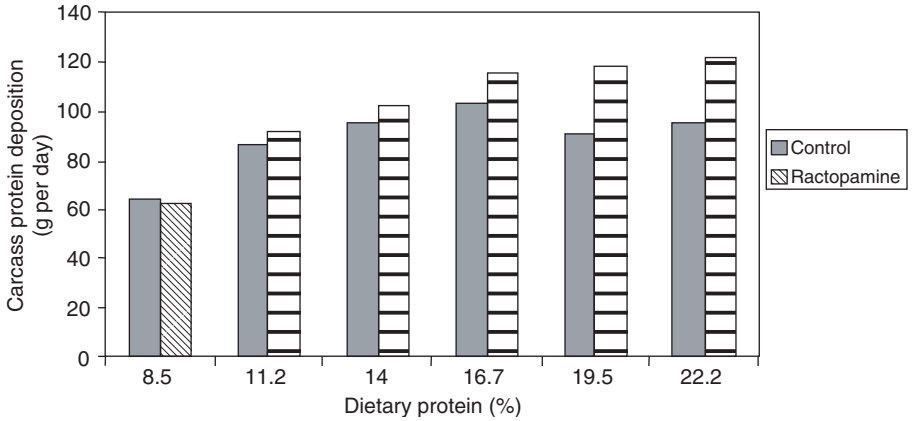

Fig. 10. The effect of dietary protein and ractopamine on carcass protein deposition in gilts. (Adapted from Dunshea et al., 1993.)

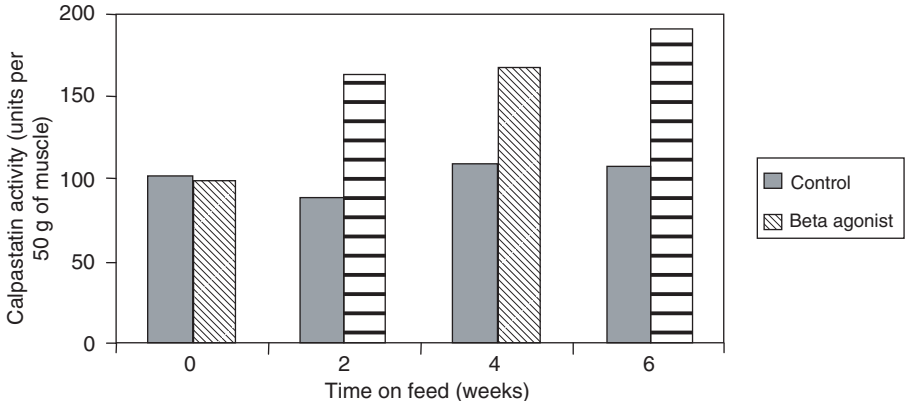

Fig. 11. The effect of the β-agonist feeding on longissimus muscle calpastatin activity in lambs. (Adapted from Pringle et al., 1993.) Treatment difference at weeks 2, 4, and 6 ($P < 0.05$).

Responses to β-agonists are often attenuated over time due to the down-regulation or desensitization of β-receptors that accompanies chronic administration of β-agonists (Sainz et al., 1993; Beermann, 2002). Mechanisms of down-regulation may include one of the following: (a) rapid uncoupling of the receptor after phosphorylation by protein kinase A and β-adrenergic receptor kinase; (b) sequestration of the receptor away from the cell surface due to a conformational change; and (c) down-regulation of receptors involving a decrease in total receptor number in the cell and possibly involving proteolytic degradation of receptors (Hadcock and Malbon, 1988; Collins et al., 1991). Therefore, receptor down-regulation may partly explain variations observed in the growth response to β-agonists in different studies, as well as the progressive decrease in the growth-promoting ability of β-agonists over time (Kim et al., 1992).

Indirect effects of β-agonists, including modulation of other endocrine influences on growth, have been suggested to partly account for the growth response. Thus, feeding

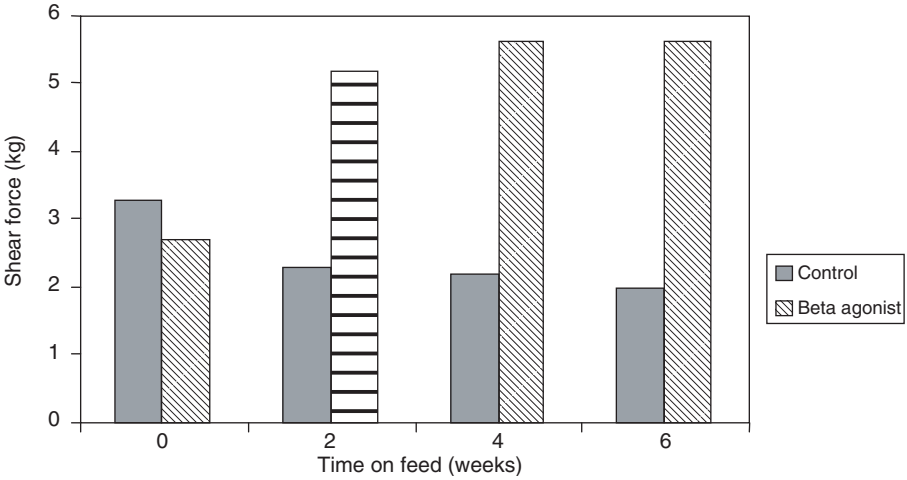

Fig. 12. The effect of feeding a β-agonist and aging on Warner-Bratzler shear force in lamb longissimus muscle. (Adapted from Pringle et al., 1993.) Treatment difference at weeks 2, 4, and 6 ($P < 0.001$).

cimaterol to growing lambs increased GH and thyroxine (T4) concentrations and decreased IGF-1 concentrations after 6 weeks, and subsequently decreased insulin concentrations by 50% (O'Connor et al., 1991). In cattle, feeding of cimaterol led to an acute decrease in GH concentration, which was then followed by chronic increases in GH and a decrease in IGF-1. However, many other studies failed to show significant differences in GH or IGF-1 concentrations in steers and lambs fed cimaterol (Beermann, 2002) and other studies, reviewed by Bell et al. (1998), also cast doubt on the suggestion that β-agonists increase growth by effects on the somatotropic axis. Meyer (2001) has discussed the various independent pathways for growth promotion by different groups of compounds. Trayhurn et al. (1998) have suggested that β-agonists could alter leptin (reducing its production) and thereby influence signals related to body energy reserves, but it is not clear how reduced leptin would lead to the repartioning of fat to lean tissue in a growing animal. This is an area that clearly needs further investigation.

A major limitation to the acceptance of β-agonists as a viable technology to improve efficiency of animal production and carcass leanness is the risk associated with drug residues in the meat products (Gonzalez et al., 1997; Smith, 1998). Tissue residues and food safety are major concerns with use of β-agonists, since acute poisonings have occurred among human consumers of livers from calves illegally treated with clenbuterol (Martinez-Navarro, 1990; Pulce et al., 1991; Salleras et al., 1995). Brambilla et al. (2000) reported an outbreak of poisoning in 15 people after consumption of meat from animals treated with clenbuterol. Europe has banned the use of β-agonists as leanness-enhancing agents and considerable resistance to any use of β-agonists in food-producing animals has developed (Witkamp and van Miert, 1992; Witkamp, 1996). Smith (1998) has argued that residue and food safety issues are less likely for certain β-agonists than for clenbuterol, which has a high oral potency and a long half-life. The latter author suggests that the risk of accidental poisoning of consumers can be avoided by proper treatment of animals with approved β-agonists, which have low oral potency and by adhering to appropriate withdrawal periods prior to slaughter (Smith, 1998). Another dimension that complicates the amount of β-agonist residues is the extent to which they may have been administered together with other modulators (such as 17-β-estradiol, dexamethasone and methylthiouracil). Groot et al. (1998) have demonstrated that some of these factors can modify excretion rates of β-agonists. The development of safe guidelines needs to be based on scientific evidence, which is currently incomplete. Indeed, recent studies indicate that modifications of β-agonists have been made resulting in new compounds that are not easily detected by conventional assays, but which have been found in feeding stuffs using special techniques and which have the potential to increase cardiovascular risk in consumers of products containing these compounds (Mazzanti et al., 2003). Unless such illegal and unethical practices can be ruled out, there will be continued resistance to the approval of the use of β-agonists in meat-producing animals. An alternative approach to feeding β-agonists is to develop site-directed antibodies, which bind to the β-2 receptor and actually activate it as well (Hill et al., 1998). This approach has been demonstrated conceptually and may be less likely to result in objectionable and hazardous tissue residues. However, studies to immunize animals with the appropriate peptide analogs of the β-2 receptor are needed to determine whether repartitioning of nutrients will be consistently accomplished by this approach.

In human medicine and biology there have been substantial efforts to study genetic polymorphisms of the β-adrenergic receptor in an attempt to relate these to health-affecting metabolic disorders, such as obesity and diabetes. Although some studies have suggested links of certain adrenergic receptor polymorphisms to health problems, many studies have not

shown clear evidence of such effects. A decrease in the expression of the β-3 adrenergic receptor gene has been reported in the adipose tissue of genetically obese rodents (Muzzin et al., 1991; Giacobino, 1995). Several lines of evidence support the concept that a defect in the β-3 receptor might be involved in the pathogenesis of obesity and diabetes. For example, in several ethnic groups an amino acid substitution in the adrenergic receptor resulted in a lower resting metabolic rate, a tendency to gain weight and insulin resistance (Strosberg, 2000). In rodent β-3 receptor knockout models there was a marked reduction in the lipolysis stimulated by β-3 agonists (Susulic et al., 1995) and a decrease in lean body mass (Revelli et al., 1997). These animals have also been observed to have impaired thermoregulatory and uncoupling protein 1 (UCP-1) mRNA responses to cold exposure (Lowell et al., 2000). There is relatively little knowledge of adrenergic receptor polymorphisms among farm animals, but the extent of variation in the β-*adrenergic receptor* of sheep has recently been studied, using a within-sire-line approach to investigate the associations between the *receptor* allele inherited from the sire and progeny performance (Forrest et al., 2003). This study describes extensive variations in the *adrenergic receptor* locus and provides evidence that this variation can be associated with important phenotypic traits such as birth weight, growth weight to weaning, carcass composition and cold survival. It is not clear whether variation at this locus would assist in the genetic selection for desirable animal production traits such as lean growth and cold tolerance, however, the evidence that certain β-adrenergic polymorphisms can be linked to growth, body composition and cold tolerance (Forrest et al., 2003) does have implications for nutrient requirements and the complexity of nutritional management in domestic animals.

8.2. Alpha-adrenergic agonists

Activation of α-adrenergic receptors can have important physiological and metabolic effects, especially concerning heat and energy conservation. Meyer and Webster (1971) demonstrated that peripheral vasoconstriction in ruminants during cold exposure is an α-adrenergic-mediated response which helps to minimize heat loss. Blocking of α-receptors prevents a normal vasoconstriction, resulting in additional heat loss in a cold environment (Webster, 1974). Hidari et al. (1991) reported that treatment of steers with the α-blocking agent, phentolamine, resulted in a 35% increase in heat production at –20°C compared to a 15% increase in control steers. This response might have been due to reduced vasoconstriction associated with α-1 receptor blockade and/or increased metabolic rate associated with α-2 receptor blockade. Most evidence suggests that α-2 adrenergic receptors, when activated by selective α-2 agonists, suppress energy metabolism in thermoneutral and cold environments (Gazzola, 1993; Miaron et al., 1995a). This suppression is either a centrally mediated suppression of sympathetic neural activity or a prejunctional inhibition of norepinephrine (noradrenaline) release by peripheral sympathetic neurons (Ruffolo and Hieble, 1994). Guanfacin, an α-2 agonist, when given to steers in a thermoneutral environment, reduced metabolic rate by up to 20% (Hunter, 1992; Hunter et al., 1993). Reduced metabolic rate has also been observed with clonidine in humans (Thompson et al., 1984) and in cattle (Lohrke et al., 1999), and with guanfacin in mice (Sillence et al., 1992). Our studies have demonstrated that the reduction in metabolic rate in steers in response to guanfacin was greatest (20%) in a cold, and smallest (<8%) in a hot environment (Miaron et al., 1995a). Metabolic rates in both fed and fasted sheep were reduced by similar amounts suggesting that guanfacin reduces basal metabolic rate rather than affecting the heat increment associated with feeding (Miaron et al., 1995b). Researchers in Australia have proposed that cattle could benefit from the energy-conserving effects of guanfacin during

dry season feeding and that this treatment could minimize weight loss during feed shortages (Hunter et al., 1993). On the other hand, studies with mice indicated that both feed intake and growth rate were suppressed by treatment with guanfacin (Sillence et al., 1992). In pigs, guanfacin was not found to have any significant effect on metabolic rate at a dose rate shown to be effective in ruminants (Cosgrove et al., 2002), suggesting that there are species differences in responsiveness to α-2 agonists. Only a few studies have been done to evaluate the potential of α-2 agonists to influence growth and body composition in livestock. Kennedy and Belluk (1987) reported that the α-2 agonist clonidine increased growth rate in Suffolk lambs but reduced growth in a Finnish-Suffolk-Shropshire strain. Clonidine reduced dressing percent and kidney fat. Longissimus dorsi muscle weight was not affected by treatment, but clonidine at a low dose increased the percent fat in the longissimus muscle. Schaefer et al. (1990) reported that clonidine fed to cattle increased plasma growth hormone and insulin concentration but was without effect on growth or carcass composition. Neither of these studies suggests that α-2 agonists have any promise as repartitioning agents in spite of their effects on growth hormone levels. Drug residues and food safety would also be an issue for α-2 agonists because of their hypotensive effects in humans.

8.3. Modifiers of thermogenesis

In relation to their modulation of metabolism, thyroid hormones (T3/T4) have the ability to dramatically influence thermogenesis through induction of cellular catabolic pathways. Webster (1975) illustrated the influence of thyroid hormones on ruminant heat production. Early studies in ruminants indicated that some changes in digestive tract function, induced through exposure to cold ambient temperatures, are associated with elevations in thyroid hormones (Christopherson et al., 1976; Kennedy et al., 1977). The increase in thermogenesis associated with thyroid hormones is a combined effect of increased catabolism, increased catecholamine sensitivity and elevated mitochondrial uncoupling protein expression (Guerra et al., 1996; Obregon et al., 1996; Jekabsons et al., 1999). It is well documented that cold-induced thermogenesis is a centrally regulated process that is controlled by the release of β-adrenergic agonists (Landsberg et al., 1984). Thyroid hormone further increases expression of β-adrenergic receptors and leptin (Fain et al., 1997), which synergistically activate lipolysis and cellular metabolism. The increase in catecholamine sensitivity associated with the elevated receptor expression promotes the catabolism and mobilization of energy supplies stored in tissue nutrient depots such as the liver, skeletal muscle, adipose tissue and especially brown adipose in some newborn and hibernating mammalian species.

Signal transduction of catecholamine ligands through β-adrenoreceptors involves the activation of adenylate cyclase and a corresponding increase in intracellular cAMP concentrations. This signal transduction cascade is usually limited by the action of phosphodiesterases that convert accumulated cAMP into 5′AMP. The inhibition of phosphodiesterases, responsible for clearing cAMP, is an effective means by which to increase thermogenesis induced by cold exposure (Wang, 1981) and extend the duration of induced catabolism. A recent study reported that acute treatment of hypothermic lambs with the phosphodiesterase inhibitor, aminophylline (a methylxanthine compound) significantly increased heat production. Fig. 13 shows the increased heat production of aminophylline-treated lambs during the period following injection. The metabolic stimulation facilitated rewarming and restoration of normal rectal temperatures (Zimmermann et al., 2003). Other methylxanthine compounds naturally produced in various plants may also be found to modify thermogenic capacity when ingested by livestock.

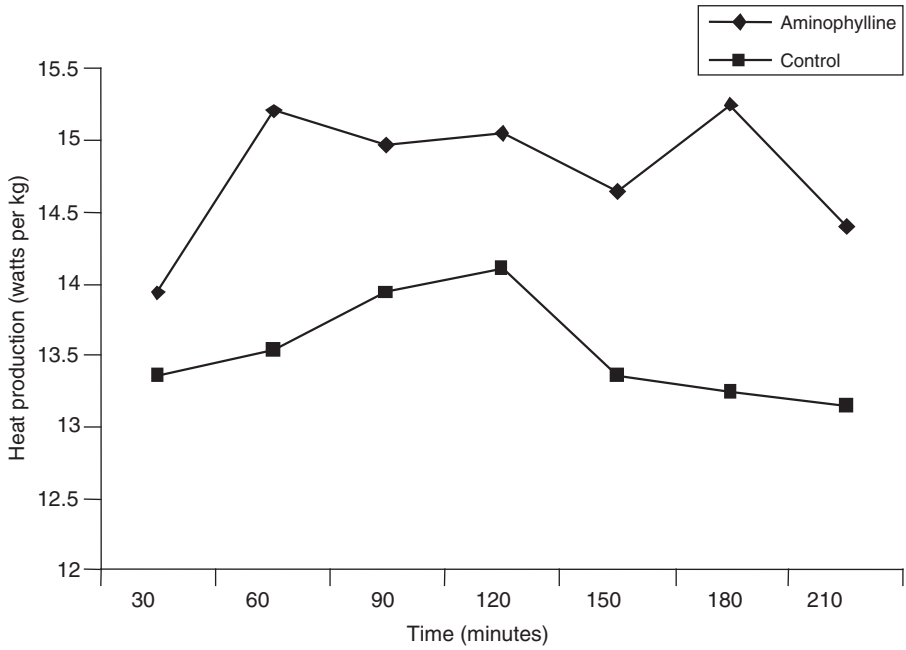

Fig. 13. Effect of aminophylline (AMP) on heat production in newborn lambs. (Adapted from Zimmermann et al., 2003.)

9. FUTURE PERSPECTIVES

There is a need for more research to determine the extent of alteration in nutrient requirements, particularly energy and amino acids, for supporting accelerated growth and altered body composition associated with higher protein accretion rates in ruminants implanted with anabolic steroids or receiving supplemental somatotropin. Better progress will be made when there is enough control over the profile and amount of amino acids and level of energy provided by the rumen which in turn contribute to adequate metabolizable protein (both in quality and quantity) and enough metabolizable energy to support protein accretion in growing animals. In monogastrics, matching nutrient supply to enhanced lean tissue growth associated with anabolic agents is also important.

Although, in the past, metabolic modifiers have been thought of as pharmaceuticals, implants or feed additives to alter growth and physiological function, in the future it may be more useful to focus attention on strategies to capitalize on inherent variability and the ability of diet and nutrients to modulate expression of genes. Given that society has concerns about residues of growth-promoting agents in animal products, livestock producers will be under increasing pressure to find alternatives to the use of anabolic agents, or to demonstrate that the ones being used are safe for use in food animals. Further research is needed in these areas. There may be opportunities to find natural substances or combinations of nutrients in feed products that will enhance endogenous expression of hormones or other gene products that exert positive influences on growth, reproduction and immune function. There is a need to identify in greater detail the genetic basis for regulation of growth, reproduction and health in livestock and to discover optimal approaches to the nutritional modulation of gene expression.

For example, the evaluation of leptin as a regulator, as well as an indicator of systemic metabolism in livestock, needs to be continued. Given that leptin expression appears variable between individuals both in levels and isoforms, can be affected by age, stress, physical body composition and components within the diet, it will nevertheless be of great value to the livestock production system to be offered information that can be utilized to improve specific breeding and feeding practices with the objective of improving efficiency. The interaction of ghrelin with the somatotrophic axis in direct association with nutrient availability and initiation of hunger emphasizes the value of continued evaluation of this endocrine peptide's involvement in livestock feed intake and growth. Improved characterization of ghrelin's physiological function may lead to useful amendments of current production practices with the objective of optimizing livestock growth, carcass composition and quality. A greater understanding of the role of different subtypes of adrenergic receptors in the regulation of growth, body composition and metabolism, may help to identify strategies for more efficient management of livestock in the future.

In consideration of the varied global environmental conditions in which livestock are raised, the improved understanding of specific adaptive strategies through which growing livestock alter their metabolic status is perhaps the most promising in terms of providing selection of optimal livestock and nutritional practices for reliable and efficient production. If we consider cold-inducible thermogenesis, it is advantageous that we retain this capacity and perhaps even facilitate the magnitude of this capacity through animal selection and feeding practices in regions with periodic or persistant cold ambient temperatures. Plant components such as the methylxanthine compounds are an example of naturally occurring substances that could potentially influence the thermogenic capacity of livestock and as such are of value as one of the metabolic modifiers that could be naturally present or supplemented within livestock feed. On the other hand, reducing maintenance requirements of livestock would appear to be possible by exploiting the capacity of animals to invoke energy-conserving responses. Examples of the latter are the reduced energy expenditure during periods of reduced feed availability, which may occur seasonally. Reduced energy expenditure may also be associated with decreasing photoperiod in livestock as well as wildlife species. A better understanding of the neuroendocrine basis of energy conservation in animals may help to identify management and nutritional strategies to optimize animal production in the future.

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6 Inorganic feed additives

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Essential trace elements (e.g. Fe, Zn, Mn, Cu, I, Se, Co, Cr, Mo, Ni) are the most important inorganic feed additives for the nutrition of laboratory animals and livestock. Their function as integral parts of diverse proteins and enzymes involved in fundamental metabolic and protective (antioxidative) processes make them indispensable. This chapter focuses on describing the physiological functions fulfilled by the trace elements. Furthermore, the current recommendations of different national committees regarding trace element supply for laboratory animals (mouse, rat, rabbit) and productive livestock (pig, beef cattle, dairy cow, goat, chicken, turkey) have been given and discussed with regard to the prevention of deficiency symptoms as well as the toxicological features of oversupply. Aspects of the bioavailability of trace elements from natural and mineral sources are discussed, including an explanation of the problems of a reduced bioavailability caused by antinutritive feed components (e.g. phytic acid). In conclusion the chapter gives both an update on the impact of trace elements on the nutrition of animals and insight into recent research possibilities using new molecular biological methods.

1. INTRODUCTION

In animal nutrition, inorganic compounds are most frequently called minerals. According to the relative amounts needed in the diet minerals can be divided into two groups, namely macrominerals and microminerals or trace elements. Another criterion by which minerals can be assigned to the macrominerals or trace elements is the amount in which they appear in the organism. By definition, macrominerals exhibit mean tissue concentrations higher than 0.05 g/kg (50 mg/kg). However, depending on the tissue, some macrominerals can reach mean concentrations up to 20 g/kg. In contrast trace elements, with the exception of iron, exhibit mean tissue concentrations lower than 0.05 g/kg (50 mg/kg).

Essential trace elements (e.g. Fe, Zn, Mn, Cu, I, Se, Co, Cr, Mo, Ni) are the most important inorganic feed additives in the nutrition of laboratory animals, livestock and companion animals. Their function as integral parts of diverse proteins, enzymes and hormones involved in fundamental metabolic and protective (antioxidative) processes make them indispensable.

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Low native dietary concentrations, as well as the existence of inorganic and organic inhibitors for some of the trace elements in natural dietary sources, make it necessary to supplement animals with trace minerals up to requirement level, especially in view of the increased performance of productive livestock in recent years.

In the following sections a description of the general physiological functions of trace elements is given. Further, the current recommendations of different committees for individual trace elements are discussed with regard to the prevention of deficiency symptoms and obtaining an optimum performance in various laboratory animals (rats, mice, rabbits, guinea pigs) and farm animals (pigs, cattle, sheep, goats, poultry). The chapter concludes with remarks on the assessment of trace element requirements and a discussion of the bioavailability of trace elements from natural and mineral sources.

2. IRON

2.1. Introduction

Iron (Fe) is the second most abundant metal (after aluminum) and the fourth most abundant element of the Earth's crust, while in the body of humans and animals iron is by far the most abundant trace element. The physiological role of iron was discovered in the late 19th century, when horse hemoglobin was shown to contain 0.34% iron. This finding was later confirmed for a range of other mammalian species. Sixty to seventy percent of body iron is present in hemoglobin in red blood cells and myoglobin in muscle, 20% is stored in labile forms in liver, spleen and other tissues and is available for hemoglobin formation. The remaining 10–20% is fixed in unavailable forms in tissues, as a component of muscle myosin and actomyosin and as an integral component of metalloenzymes (Pond et al., 1995).

For fast-growing laboratory and farm animals, especially piglets, iron supply is quite critical during the first few weeks of life when rapid expansion of red-cell mass imposes great demands upon animal and diet to deliver sufficient iron to erythropoietic tissue (Cavill, 2002). Iron can exist in various oxidation states (from –II to +VI), the principal states being +II and +III. These oxidation states are also frequently named ferrous iron (+II) and ferric iron (+III). The latter form is nearly water insoluble at a physiological pH of 7 ($K_{Sp} = 10^{-18}$ mol/l) (Römpp, 1990).

2.2. Iron in feeds

Feeds contain iron in two basically different forms: heme iron and nonheme iron (Davidsson, 2003; Oikeh et al., 2003). Table 1 summarizes typical iron concentrations in various feeds derived from plant and animal sources.

The organic form of iron, representing Fe II bound to protein structures (predominantly hemoglobin and myoglobin) is mainly called heme iron. In diets of laboratory and farm animals heme iron is derived from meat or blood components.

2.3. Metabolism and biochemical functions of iron

The specific mechanism of the absorption of heme iron in the duodenum and the proximal jejunum is shown in fig. 1.

The metal–porphyrin ring is split from globin in the lumen of the proximal small intestine, allowing the intact Fe–porphyrin to diffuse across the brush border membrane. Once in the

Table 1

Iron concentration in feeds of plant and animal origin from different locations

Feed	Location of origin and description	Fe concentration (mg/kg DM)
Pastures	Great Britain[a]	
	Pasture (legumes)	306
	Pasture (grasses)	264
	Pasture (herbs)	358
	USA (Florida)[b]	
	Pasture (hand-clipped)	155
	Pasture (ruminant selection)	153
	New Zealand[c]	
	Pasture (clover/ryegrass)	100–250
Cereals	Egypt[a]	
	Maize	10
	Barley	20
	Great Britain[a]	
	Barley	100
	Maize	100
	Oats	120
	Wheat	140
Animal proteins	Different locations[a,d]	
	Blood meal	3108
	Meat meal	439
	Fish meal	381
	Dried skimmed milk	52

[a]Underwood and Suttle, 1999; [b]Arthington, 2000; [c]Hill Laboratories Ltd., 2002; [d]Soevik et al., 1981.

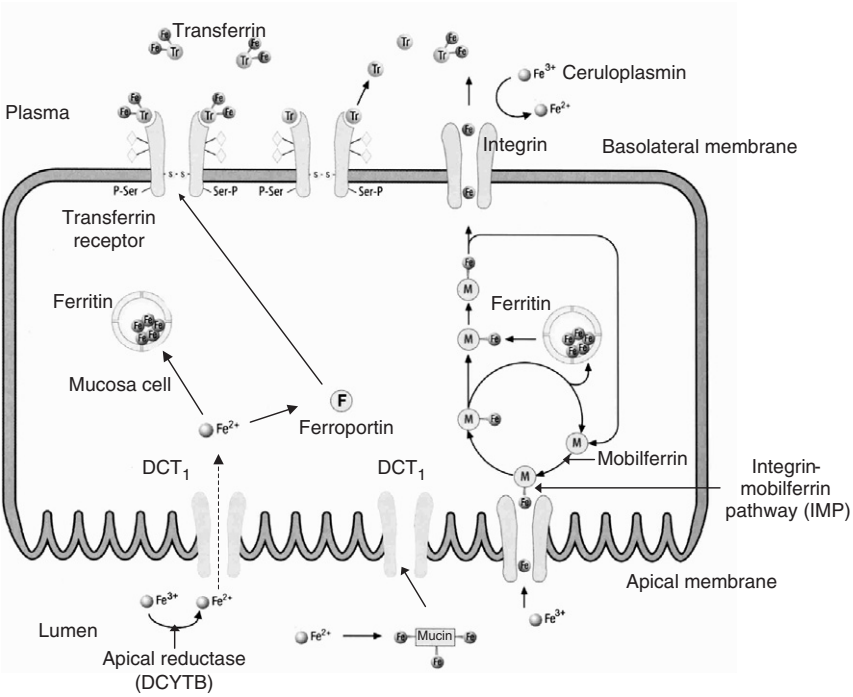

Fig. 1. Pathways of iron absorption (Löffler and Petrides, 2003 and recent findings from Smith et al., 2002 and Simovich et al., 2003).

cell, ferrous iron and bilirubin are liberated by the aid of the catalyst heme oxygenase (Wheby et al., 1970; Miret et al., 2003).

Inorganic nonheme iron, which occurs in animal diets both in the ferrous and the ferric oxidation state, must be solubilized and chelated in the acidic stomach milieu, due to the low solubility of ferric iron at pH values higher than 3. Solubilized iron in the stomach is chelated by dietary and intestinal-derived substances (e.g. mucins, certain amino acids, sugars, amines and amides), which keep iron in solution when it enters the less acidic duodenum (Wien and van Campen, 1991a, 1991b; Annibale et al., 2003).

Other nutrient compounds (e.g. phytic acid, carbonates, phosphates, oxalates and tannates) cause the precipitation of ferric iron and lead to the formation of macromolecular complexes, which prevent absorption of the iron (Benito and Miller, 1998; Miret et al., 2003).

At present two pathways are under discussion for the uptake of nontransferrin bound iron into enterocytes (Conrad and Umbreit, 2002):

1. The integrin mobilferrin pathway (IMP) (Simovich et al., 2003)
2. The divalent metal transporter 1 pathway (DMP 1) (synonyms: divalent cation transporter (DCT), natural resistance associated macrophage protein 2 (NRAMP)) (Smith et al., 2002).

The IMP pathway exclusively transports ferric iron and no other metals of nutritional importance. The formation of the 20 kDa macromolecular mobilferrin complex (consisting of calreticulin and β3 integrin) precedes the facilitated diffusion of Fe III across the apical enterocyte membrane (Simovich et al., 2003).

The DMT 1/DCT/NRAMP 2 system also enables the facilitated diffusion of other divalent cations besides iron (e.g. Mn, Zn, Co, Ni, Pb, Cd) (Smith et al., 2002). Prior to iron transport into the mucosal cell by DMT/DCT/NRAMP, ferric iron is reduced to the ferrous oxidation state by the apical reductase DCYTB (Abboud and Haile, 2000; McKie et al., 2001). In the mucosal cell iron can be stored intermediately as ferritin, from which it can be recruited again. Iron transport through the mucosal cell to the basolateral membrane is accomplished by ferroportin (synonyms: IREG and MTP1).

At the basolateral membrane iron can be bound to transferrin (Tf), a nonheme glycoprotein, which binds two ions of ferric iron per mole. This process is mediated by the apotransferrin receptor (Oates et al., 2000). For the integration of iron into transferrin the oxidation of Fe II to Fe III is necessary. This oxidation step is catalyzed by ceruloplasmin (synonym: ferroxidase), a copper-containing oxidoreductase. At this point iron metabolism is linked to copper metabolism (Harrison et al., 1994).

Iron absorption rates are low compared to the other trace metals. Indeed, mainly dependent on the chemical form, only 5–10% of the dietary iron is absorbed. In many monogastric animal species the absorption of heme iron is much more efficient than the absorption of inorganic nonheme iron (Benito and Miller, 1998; Morgan and Oates, 2002). Furthermore, an increase in nonheme iron absorption by the addition of a meat component from beef, chicken or fish to the diets could be demonstrated in several animal species, thus this effect was attributed to a so-called “meat factor” (Baech et al., 2003; Hamadaoui et al., 2004). An influence of dietary fat source and concentration on iron absorption could also be demonstrated (Shotton and Droke, 2004). A list has been drawn up to illustrate the parameters believed to influence iron absorption (table 2).

The hypothesis of a mucosal block in the regulation of iron absorption is considered to be dated. The mucosal block theory postulated that when a dose of iron was applied it saturated the mucosa, so that a subsequent dose given after a short interval would not be absorbed (Benito et al., 1998). The use of modern cellular and molecular biological approaches revealed

Table 2

Factors influencing iron absorption in animals (Wien and van Campen, 1991a, 1991b, 1994)

Intraluminal factors	Mucosal factors	Corporeal factors
Dietary iron	Anatomic factors	Body iron stores
• Quantity	• Absorptive surface	
• Chemical form	• Mucosal life span	
pH	Mucosal iron	Iron turnover
	• Quantity	Erythropoiesis
	• Chemical form	Anabolism/catabolism
Chelation and precipitation	Certain metals (Mn, Zn)	Iron regulatory-associated diseases

that the regulation of iron absorption and iron metabolism is much more complex. In rats, iron deficiency was demonstrated to affect an expansion, in length and breadth, of the duodenal microvilli. Moreover, it could be shown that mRNA expression for the apical reductase DCYTB, ferritin and the divalent cation transporter DCT are enhanced in iron deficiency (Smith et al., 2000; Tran et al., 2002). Apparently, body iron stores and further response factors regulate the above-mentioned proteins, and other proteins involved in iron metabolism, via an iron-responsive element (IRE) in the 3′ untranslated regions of their mRNA (Eisenstein and Ross, 2003; Miret et al., 2003). Transferrin delivers iron to peripheral organs where it can be taken up by mediation of the transferrin receptors 1 and 2 (TfR1 and TfR2) (Feelders et al., 1999). Storage and redistribution of iron within different tissues is mainly attributed to ferritin and transferrin (Bergamaschi et al., 1986; Feelders et al., 1999; Drysdale et al., 2002). In different cells of the organism iron is needed for the synthesis of iron-containing proteins. Examples of important heme and nonheme iron-containing proteins are listed in table 3 (Fox, 2001; Messerschmidt et al., 2001; Solomon et al., 2003).

Hemoglobin and myoglobin contain the highest portion of body iron (together 60–70%). Together, the tetrameric hemoglobin and the monomeric myoglobin iron are responsible for supplying oxygen to the tissues and cells (Acher, 1966; Braun et al., 1967; Tamura et al.,

1973; Bertini et al., 2003). By its redox function in the metalloporphyrins cytochrome oxidase, cytochrome c (Fridovich, 1966; Blomberg et al., 2003) and the metalloflavin succinic dehydrogenase (Onishi et al., 1973), iron is involved in major processes of the respiratory chain and fulfills an essential role in the energy production of cells. Thus, iron deficiency could lead to major changes in the energy metabolism of tissues (Ohira et al., 1983; Hostettler-Allen et al., 1993). Iron is also involved in the metabolism of amino acids by its function in amino acid mono- and dioxygenases (Fitzpatrick et al., 2003; Ryle et al., 2003a, 2003b). As an integral part of xanthine oxidase, iron has an essential function in the degradation of purine bases to uric acid (Furth-Walker and Amy, 1987; Ghio et al., 2002). Furthermore, iron is involved in calcium-dependent cellular signalling transduction by its function in the active center of the serine/threonine protein phosphatase calcineurin (King and Huang, 1984; Rusnak and Mertz, 2000; Namgaladze et al., 2002). The role of iron in the antioxidative balance of cells and tissues is controversial. On the one hand, an antioxidative function of iron is demonstrated by the iron porphyrin enzyme catalase, which detoxifies hydrogen peroxide (Hermel and Havemann, 1966; Andreoletti et al., 2003). On the other hand, a high iron status could cause pro-oxidative reactions. Reactive oxygen species, especially the very aggressive hydroxyl radical, could be generated by the Haber-Weiss and the Fenton reaction and effect oxidative damage to cellular proteins, cellular lipids and DNA (Minotti and Aust, 1989; Klebanoff and Waltersdorph, 1990; Toyokuni, 2002). Dietary isoflavonoids, such as quercetin derivatives, can possibly diminish iron-induced pro-oxidative reactions in the intestinal mucosa (Murota et al., 2004). There is also some evidence that fatty acids and cholesterol in transport lipoproteins are oxidized to a higher extent by an enhanced iron status (Grinshtein et al., 2003).

2.4. Iron deficiency and requirement in various animal species

Signs of iron deficiency vary among livestock. Piglets are the most susceptible to iron deficiency. Typical symptoms of iron deficiency in piglets are poor growth, listlessness, rough hair coat, anoxia, wrinkled skin, paleness of mucous membranes, hypochromic microcytic anemia, enlarged heart and ascites (Szabo and Bilkei, 2002). The iron deficiency can be confirmed by analysis of blood Hb content, which decreases to values below 9 g/100 ml (Egeli et al., 1998). The piglet is born with a total body iron reserve of about 47 mg. The iron storage will last up to 1 week given a daily requirement of 7 mg absorbed iron for normal growth and development (Steinhardt et al., 1984). Sow's milk contains only about 1.3 mg iron/kg whereas the dietary requirement for baby pigs is much higher. For the prevention of iron deficiency in pigs, it is recommended that the pigs receive an injection of at least 200 mg of iron during the first 3 days after birth (Egeli and Framstadt, 1999). Different special formulas of complexed iron added to piglet diets were also reported to be effective in protecting against iron deficiency (Egeli and Framstadt, 1998). Moreover, a sufficient iron supplementation of prestarter and starter diets should be implemented. It is recommended that iron is supplied to pigs either expressed as a daily amount which increases from 25 mg (between 3 kg and 5 kg live weight) to about 130 mg (between 50 kg and 120 kg live weight) or as an amount per kg diet (between 3 kg and 5 kg live weight: 100 mg iron/kg DM, between 50 kg and 120 kg live weight: 40–60 mg iron/kg DM) (NRC, 1998).

Iron deficiency seldom occurs in adult grazing animals. The iron reserves of the newborn calf are usually sufficient to prevent iron deficiency if calves are fed with dry feeds within a few weeks after birth (Lindt and Blum, 1994; Moser et al., 1994), but iron supplementation is needed when calves are fed exclusively whole milk (Miltenburg et al., 1992;

Blum and Hammon, 1999). Iron deficiency anemia begins at 16–20 weeks, when the hemoglobin concentration decreases below 9 g/dl (Welchman et al., 1988), and in order to prevent such an iron deficiency, as well as to optimize the performance and growth rate of calves, an iron supplementation of 100 mg/d has been recommended (Volker and Rotermund, 2000).

Iron deficiency in chickens and quails causes an anemia in which red blood cells are reduced in size and are low in hemoglobin (Washburn and Lowe, 1974; Morck and Austic, 1981). In red-feathered chickens pigmentation does not occur when the diet is deficient in iron. Taking into consideration that one egg contains about 2 mg of iron, the requirement for iron increases with the onset of laying (Manson et al., 1993). For rapidly growing broiler chickens 50–80 mg iron/kg DM are recommended, while a daily supplementation with 5.5 mg iron/animal is recommended for laying hens, assuming a feed intake of 110–120 g (NRC, 1994; GfE, 2000).

Little information is available on iron deficiency in sheep and goats, but as mentioned above for cattle, dietary iron deficiency seldom occurs in adult sheep and goats. Deficiency can develop, depending on the quality of the pasture, but in most cases the iron concentration of creep feed is sufficient. If iron deficiencies are observed and the kids remain on a milk diet, injections of iron dextran are recommended (GfE, 2003).

The iron reserves of laboratory animals (rats, mice) at birth are usually sufficient until the onset of nutrition with natural or laboratory diets. To avoid deficiency, the iron content should not be lower than 25 mg/kg diet (NRC, 1995).

In contrast to iron deficiency disorders a range of diseases exists, which are characterized by excessive iron storage. These diseases are caused exclusively by genetic defects and are listed in table 4.

3. ZINC

3.1. Introduction

The zinc concentration in the top layer of the Earth's crust is about 120 mg/kg so that zinc is the 24th most abundant element on earth. Zinc belongs to the base metals and its occurrence is linked to that of lead and cadmium. The largest zinc storages can be found in Canada, the USA, Australia, Russia and South Africa. Zinc mainly occurs in the two oxidation states 0 and +II (Römpp, 1992). Many years ago, Todd et al. (1934) investigated the essentiality of zinc in rats, and this study was followed by a large number of investigations in many other species and vertebrates. Zinc deficiency has been shown to be associated with inappetence, growth depression and an impaired reproductive performance. The identification of parakeratosis in pigs and symptoms similar to those of perosis in chickens, as indicators of severe zinc deficiency, were further important findings in the characterization of zinc as an essential trace element (Tucker and Salmon, 1955; Hoekstra, 1969). Moreover a large number of biochemical factors, which are responsive to zinc could be found, e.g. carbonic anhydrase (Keilin and Mann, 1940; Liang and Lipscomb, 1988), carboxypeptidases A and B (Vallee and Neurath, 1955; Wintersberger et al., 1965; Roth and Kirchgessner, 1974) collagenase (Starcher et al., 1980) copper–zinc superoxide dismutase (Zelco et al., 2002) and metallothionein (Bremner and Davies 1975; Maret, 2003). The transcription of about 32 000 proteins in the organism of mammals was shown to be influenced by zinc finger domains (Vallee et al., 1991) and the number of zinc-sensitive transcription processes identified increases steadily (Duran et al., 2004; Valadez-Graham et al., 2004).

Table 4

Genetic disorders affecting proteins of iron metabolism (modified from Sheth and Brittenham, 2000; reprinted, with permission, from Annual Review of Medicine, Vol. 51. © 2000 by Annual Reviews. www.annualreviews.org)

Disorder	Gene affected	Chrom. localization	Sites of increased iron deposition	Manifestation
Hereditary (HLA-Linked) hemochromatosis	HFE	6p21	Parenchymal Fe overload in liver, heart, pancreas and other organs	Liver and heart disease, diabetes, gonadal failure
Juvenile hemochromatosis	unknown	1q	Parenchymal Fe overload in liver, heart, pancreas and other organs	Liver and heart disease, diabetes, gonadal failure
Atransferrinemia	Tf gene	3q21	Fe deposition in parenchymal tissues, e.g. liver and heart. No Fe in bone marrow or spleen	Iron deficiency anaemia, growth retardation, poor survival
Aceruloplasminemia	Cp gene	3q21-24	Marked Fe accumulation in basal ganglia, liver and pancreas	Progressive neurodegeneration, diabetes
Hyperferritinemia	IRE of Ferritin gene	19q	No Fe overload or increased Fe deposition identified	Increased serum ferritin with binuclear cataracts
Friedreich's ataxia	Frataxin gene	9q13	Fe deposition in cardiomyocytes, other sites studied incompletely as yet	Spinocerebellar ataxia with cardiomyopathy
X-linked sideroplastic anemia with ataxia	ABC7	Xq13	Fe deposition in mitochondria of erythrocyte precursors and neuronal cells	Spinocerebellar ataxia with mild anemia

3.2. Zinc in feeds

Feedstuffs for animals exhibit great variation in zinc concentration. Zinc concentrations in pasture vary between 7–100 mg/kg DM, with a mean of 35 mg/kg DM. Legumes generally contain lower zinc concentrations (10–30 mg Zn/kg DM) than the main cereals barley, oats, wheat and millet (30–40 mg/kg DM) (Underwood and Suttle, 1999), while slightly lower zinc concentrations can be found in maize (Hotz et al., 2001). In grains zinc is most concentrated in the outer layer. Protein sources such as soybean, sesame seed, cotton seed and groundnut meal exhibit slightly higher zinc concentrations (50–70 mg/kg DM) than cereal grains (Edwards and Baker, 2000; Sandberg, 2002). Meat meal, fish meal and some other animal protein sources contain nearly twice as much zinc (80–120 mg/kg DM) as plant sources (Hortin et al., 1993), but cow milk (3–5 mg Zn/kg milk) on the other hand is a rather poor zinc source (Pabon and Loennerdal, 2000).

3.3. Metabolism and biochemical functions of zinc

Absorbability of zinc normally ranges between 5% and more than 90% of the intake and is regulated according to the dietary zinc concentration (Weigand and Kirchgessner, 1978; Coppen and Davies, 1987), by the expression of zinc-transporting proteins and zinc storage proteins in particular intestinal cells (Flanagan et al., 1983). A saturation of zinc absorption can be observed at very high dietary concentrations of the trace element. Comparable to the absorption of iron and manganese, gastrointestinal mucins also play an important role in zinc absorption (Crowther and Marriott, 1984; Powell et al., 1999a, 1999b). Amino acids, derived from dietary proteins, and some single amino acids (cysteine, threonine and histidine) were shown to improve both zinc absorption and zinc passage through the enterocyte. Moreover, some organic acids (citric acid, fumaric acid) were shown to enhance zinc bioavailability. Other nutritive compounds such as phytic acid, oxalates and carbonates lead to the formation of macromolecular complexes preventing zinc absorption (Turnbull et al., 1990; Rimbach and Pallauf, 1994; Pallauf and Rimbach, 1997; Loennerdal, 2000; Raboy, 2002).

Zinc absorption mainly takes place in the duodenum (58%) and the remainder of the small intestine (jejunum: 10%, ileum: 30%). The absorption process consists of two phases (Davies, 1980; Blakeborough and Slater, 1987; Hara et al., 2000):

1. Rapid uptake of zinc through the apical membrane by facilitated diffusion via the carrier ZIP1 (synonyms: zinc-regulated transporter = ZRT and IRT-like protein) and the DCT1 transporter (c.f. iron and manganese) (Cousins and McMahon, 2000);
2. Slow and stepwise release from the enterocyte into blood (Hempe and Cousins, 1992).

Absorption of zinc and homeostatic regulation of zinc metabolism in the enterocyte are shown in fig. 2.

Four major classes of proteins are involved in further zinc homeostasis in the organism:

1. The cysteine-rich intestinal protein (CRIP), a histidine- and cysteine-rich low-molecular-weight protein, which is exclusively expressed in the gastrointestinal tract, is responsible for the intermediate storage of zinc in the enterocyte and the transport of zinc from the apical enterocyte membrane to the basolateral membrane. In physiological states of low zinc intake and low zinc status, a large portion of the absorbed zinc in enterocytes is bound to the CRIP protein, whereas lower percentages are associated with metallothionein (MT) (Hempe and Cousins, 1992).
2. Metallothionein (MT), first isolated from equine kidney cortex, is a low-molecular-weight peptide, consisting of 61 amino acids and able to bind seven zinc ions or

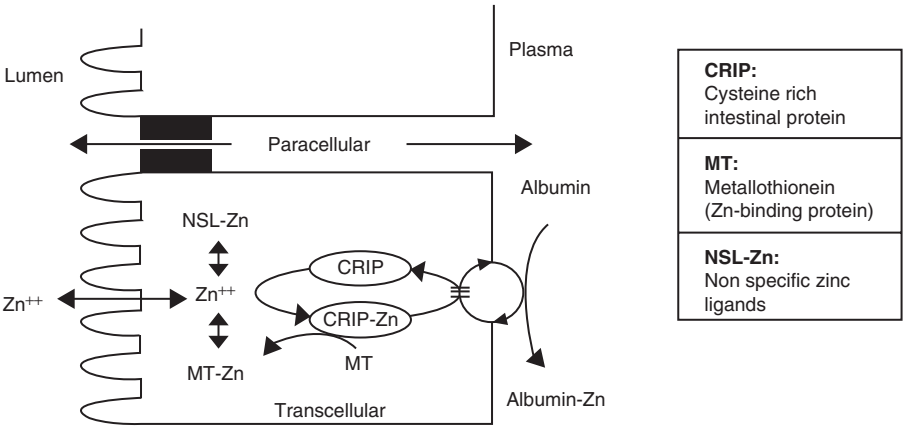

Fig. 2. Hypothetical model of zinc absorption. (Modified from Hempe and Cousins, 1992.) Zinc is transported into the enterocyte by ZIP1 (synonyms: ZRT1, IRT-like protein 1) predominantly during physiological states of low to normal zinc intake. Comparable to calcium in calbindin, zinc is associated to the cysteine-rich intestinal protein (CRIP) in the enterocyte and then stepwise released into the blood plasma where it binds to albumin.

alternatively seven cadmium ions or up to 12 copper ions (Margoshes and Vallee, 1957). Cysteine residues in the protein enable the bonding of the metal ions. The peptide forms two domains: the alpha domain holds three metal ions while the beta domain contains four metal ions. MT is expressed in a wide variety of tissues, including the gastrointestinal tract, liver, kidney and spleen. In contrast to CRIP, Zn-metallothionein concentrations are elevated by high dietary zinc intake, thus MT synthesis and association with metal ions seems to act predominantly as an intracellular buffer in order to prevent intoxication from metal ions (Maret, 2003).

3. Zinc transporters of the ZIP series (synonyms: ZRT and IRT like protein). The two main members of the ZIP series, namely ZIP1 and ZIP2, show considerable homology and possess eight transmembrane domains (Eide et al., 1996; Eng et al., 1998; Guerinot, 2000). A marked ZIP1 expression was detected in the small intestine, colon, kidney, liver and pancreas. Despite a high Km value of the transporter for zinc, sufficient amounts of zinc can be provided in the diverse tissues (Zhao and Eide, 1996; Gaither and Eide, 2001a, 2001b). Zinc absorption in the small intestine involves ZIP1, while the transporter ZIP2 is expressed in the uterus and prostate gland (Gaither and Eide, 2000).
4. Zinc transporters of the cation diffusion facilitator series (CDF, synonym: ZnT). Four transport proteins of the CDF series, namely ZnT1 to ZnT4, have so far been characterized in detail. ZnT-transporters possess six transmembrane domains and act as zinc exporters (von Heijne, 1994). ZnT1 transporters are localized in the basolateral membrane of tubulus cells and are involved in renal reabsorption of zinc. This fact explains the minor role of the kidney in zinc excretion. In the basolateral membrane of enterocytes from the duodenum, ileum and jejunum ZnT1 catalyzes the delivery of zinc ions into the blood (McMahon and Cousins, 1998a, 1998b). Expression and activity of ZnT2 transporter have been detected in different compartments of cells from the small intestine, kidney and testes, thus the function of the protein may be to distribute zinc intracellularly to different cell organelles and into vesicles (Palmiter et al., 1996a). ZnT3 fulfills functions similar to ZnT2, but its localization is restricted to the brain and

testicular cells (Palmiter et al., 1996b). ZnT4 expression is found in the mammary gland, where the protein catalyzes the export of zinc ions from mammary cells into the milk.

Recently, a model based on the application of cDNA array technology and RT-PCR was used to investigate the differential regulation of zinc transporters and zinc storage proteins in rats during an alimentary zinc deficiency. Whereas the mRNA of metallothioneine, representing an intermediary zinc storage, was found to be down-regulated in many rat tissues, the mRNA of ZnT1 and ZnT2, involved in renal Zn reabsorption and distribution of zinc within tissues was up-regulated in most of the rat tissues investigated (Pfaffl and Windisch, 2003; Pfaffl et al., 2003).

In plasma, zinc is transported to peripheral tissues by albumin (Stewart et al., 2003). In whole blood, 22% of the zinc is bound to plasma proteins, 75% derives from carbonic anhydrase of the erythrocytes and 3% is associated with alkaline phosphatase of leukocytes (Smith et al., 1979; Chesters and Will, 1981).

However, more than 99% of whole body zinc exists intracellularly in the various tissues. Relatively high zinc concentrations are present in the beta cells of the pancreas (insulin storage, as zinc-associated insulin hexamer) (Milthorpe et al., 1977; Emdin et al., 1980; Brzovic et al., 1994), in the iris and the retina of the eye (zinc function in retinal dehydrogenase) (McCormick, 1985), in the liver (main metabolic organ with many zinc-dependent enzymes), lung (high concentrations of the antioxidative enzyme copper–zinc superoxide dismutase) (Taylor and Bray, 1991; Ho, 2002), and the testes (stabilization of chromatin) (Reyes et al., 1993; Bench et al., 2000).

Zinc is involved in a large number of enzymatic processes. Indeed, up to now more than 300 enzymes are known to depend on zinc, including oxidoreductases, transferases, hydrolases, ligases, isomerases and lyases. Table 5 shows a selection of mammalian zinc metalloenzymes.

Zinc can affect the activity of enzymes in two different ways (Kimura and Kikuta, 2000; McCall et al., 2000; Löffler and Petrides, 2003).

1. Zinc is directly bound to the active center of the enzyme by histidine, cysteine, asparagic acid and glutamic acid residues and water. By cleaving the substrate and polarization of the substrate it facilitates the enzymatic reaction. This kind of zinc action can be observed in carbonic anhydrase (fig. 2) and in carboxypeptidase A and B (Silverman and Tu, 1986; Vallee and Auld, 1990; Breton, 2001). Fig. 3 illustrates the reaction mechanism of zinc in carboxypeptidase (a) and carbonic anhydrase (b) (Löffler and Petrides, 2003).
2. Zinc acts as a structural component, which stabilizes the quaternary structure of enzymes, so that the enzymes become more resistant to damaging influences such as heat and changes in pH value. Zinc functions in this manner, for example, in alcohol dehydrogenase, aspartate transcarbamylase and protein kinase C.

3.4. Zinc deficiency and requirement in various animal species

Long-term effects of zinc deficiency on the activity of a large number of enzymes result from its dual role during gene expression. The RNA polymerases II and III have been shown to depend on zinc, and thus RNA synthesis is diminished in long-term zinc deficiency (Terhune and Sandstead, 1972; Falchuk et al., 1976; Schuster et al., 1995; Schaub et al., 1997). Moreover, zinc is involved in the successful transcription of a large number of genes by its function in zinc fingers, zinc clusters and zinc twists of transcription factors.

Signs of dietary zinc deficiency in farm animals depend on the species, but there are similarities between the symptoms of a subclinical and a clinically manifested zinc deficiency. Pigs are more susceptible to a clinically manifested zinc deficiency than ruminants.

Table 5

Mammalian zinc containing enzymes (modified from Luchinat and Sola, 1994, reproduced with permission. © 1994 John Wiley & Sons Limited)

Enzyme class Name of the enzyme	Molecular weight (kDa)	Number of subunits	Zn atoms per molecule	Role of zinc within the enzyme
Oxidoreductases				
Alcohol dehydrogenase	80	2	4	C, S
Glycerol dehydrogenase	140–160	4	4	C, S
Sorbitol dehydrogenase	150	4	4	C
Superoxide dismutase	30–32	2	2	S
Transferases				
Nuclear poly A polymerase	48–60	1	<1	C
Terminal dNT transferase	33	1	1–2	C
Hydrolases				
Alkaline phosphatase	94	2	4	C?
Aminoacylase	86	2	2	S
Aminopeptidase	29–325	1–6	1–2	C, R
Aminotripeptidase	50	1	1	C
AMP deaminase	300	4	2–3	?
Angiotensin converting enzyme	130	1	1	C
Carboxypeptidase A	33–34	1	1	C
Carboxypeptidase B	34–36	1	1	C
Carboxypeptidases E, N, M	50–60	1	1	C
Collagenase	28–92	1	1	C
Dihydropyrimidine-aminohydrolase	217–226	4	4	?
Dipeptidase	85	1	1	C
Fructose-1,6-diphosphatase	140	4	12	R
Leocotriene A4-hydrolase	70	1	1	C
α-D-mannosidase	230	1	2	?
5′-nucleotidase	130–160	2	2	?
Procarboxypeptidase A	40–46	1	1	C
Procarboxypeptidase B	42–47	1	1	C
Lyases				
5-aminolevulinate dehydratase	280–300	8	8	C, S
Carbonic anhydrase	29–35	1	1	C
Glyoxalase I	46	2	2	C

C = Catalytic properties; S = Structural component; R = Regulatory function; ? = function unknown as yet.

Borderline zinc deficiency in pigs produces decreased appetite and growth in some animals, while others may show a fading or bleaching of the hair coat. A decrease in litter size and weight of piglets occurs with the sow and retarded testicular development occurs with the growing boar (Hesketh, 1982). Zinc is also critical to the immune function of pigs. One mechanism by which zinc deficiency can impair the immune system is by causing atrophy of the thymus gland (Pleau et al., 1985; Jambon et al., 1988; Mocchegiani et al., 1998). Parameters for the degree of zinc deficiency are blood zinc concentration and the activity of alkaline phosphatase. Pigs with severe zinc deficiency were reported to show the following symptoms: reduced appetite and retarded growth, skin lesions (parakeratosis) that look like mange, diarrhea and vomiting (Pond et al., 1966; Liptrap et al., 1970; Dahmer et al., 1972), but the effects

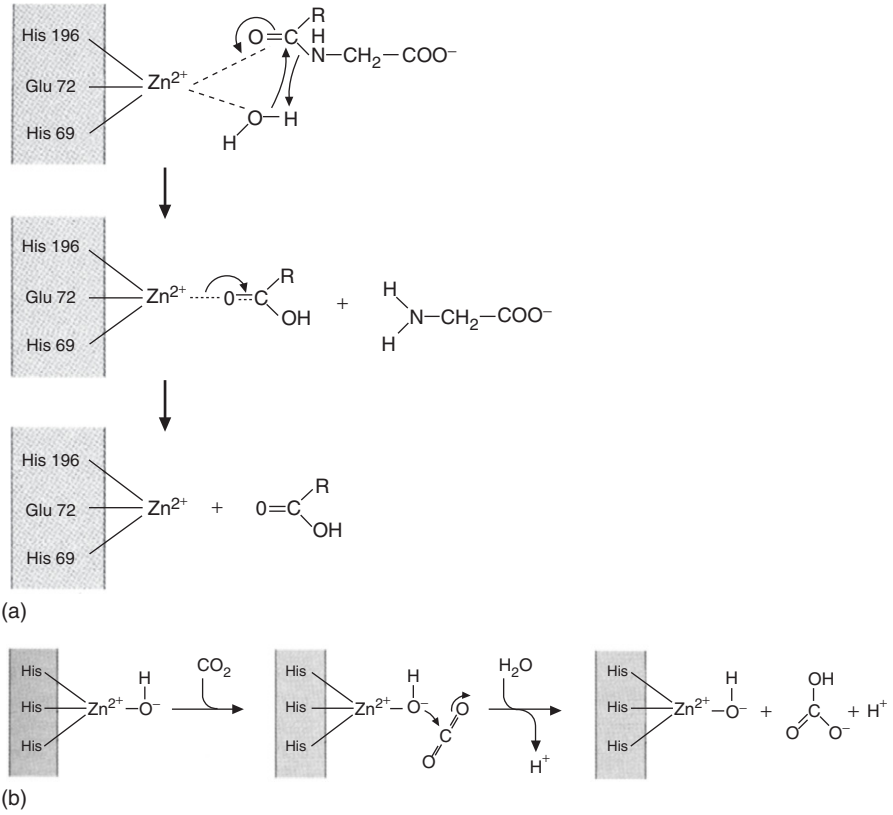

Fig. 3. Reaction mechanism of zinc in carboxypeptidase (a) and carbonic anhydrase (b).

are reversible, and zinc administration to affected animals leads to a very rapid regeneration and disappearance of the symptoms (Lansdown, 1991). The zinc requirement of growing pigs is about 50 mg/kg diet (NRC, 1998), although excessive calcium levels increase the requirement to nearly double this amount (Hoekstra et al., 1967), as is also the case in young piglets fed diets with high native phytic acid concentrations (Pallauf et al., 1994; NRC, 1998; Zhang et al., 2000). There is no danger for animal health by feeding concentrations up to 200 mg/kg, but toxic effects of zinc appear when pigs are fed levels of 2000 mg zinc/kg. Toxic signs include: growth depression, enteritis, arthritis, gastritis, and hemorrhages in the axillary spaces. Particular attention to dietary zinc deficiency must be paid in monogastric animals fed on the basis of soybean meal, cottonseed meal, sesame meal and other seeds (e.g. cereals) because of the high concentration of phytic acid, which reduces the bioavailability of the trace element. In contrast to the above mentioned recommendations, the results of a recent study have shown that the addition of 2500 mg Zn/kg to the diet of growing pigs actually improved weight gain in comparison to animals fed 200 mg and 500 mg Zn/kg diet (Davis et al., 2004). On the other hand, feeding 5000 mg zinc/kg DM to pregnant sows produced severe copper deficiency in the offspring (Hill et al., 1983).

In cattle, an early sign of zinc deficiency is excessive salivation, which may be caused by a reluctance to swallow the large amount of saliva that is normally produced. A mild zinc deficiency in cattle results in lowered weight gain (Miller et al., 1965; Mayland et al., 1980; Goetsch et al., 1991). Substantial losses of immune capacity occur, due to inadequate zinc

intake, before any other typical zinc deficiency symptoms can be observed (Spears and Kegley, 2002). Further subclinical symptoms of zinc deficiency in cattle include a reduced reproductive performance in both male and female animals and impaired wound healing (Abdullaev et al., 1979; Hidiroglou and Knipfel, 1984; Graham et al., 1994; Hostetler et al., 2003). The average zinc concentration in milk ranges between 3 mg/kg and 5 mg/kg, but as a consequence of dietary zinc deficiency these concentrations decline rapidly. Severe zinc deficiency in calves results in parakeratosis. The nose and mouth become inflamed because of submucosal hemorrhages (Miller and Miller, 1962; Engle et al., 1997). The animals also develop an unhealthy appearance, a rough hair coat and stiffness of the joints (Smart, 1985). The zinc requirement for cattle ranges between 40 mg and 50 mg zinc in the total diet (GfE, 2001), however this doubles during times of stress (Nockels et al., 1993; Campbell and Miller, 1998). The maximum tolerable dietary zinc level for cattle is 500 mg/kg and onset of toxic signs begins at zinc levels of about 1000 mg/kg diet. A lowered gain and reduced feed utilization are typical symptoms caused by high dietary zinc concentrations (Jenkins and Hidiroglou, 1991; Amici et al., 2002).

In lambs zinc deficiency results in lack of appetite, reduced growth, loss of wool, swelling around the eyes and hooves, excess salivation, general listlessness, impaired growth of the testes and cessation of spermatogenesis (Underwood and Somers, 1969; Masters et al., 1985; Droke et al., 1993; Martin et al., 1994; White et al., 1994; Kendall and Telfer, 2000). Feeding ewes low-zinc diets during pregnancy leads to a reduced survival rate of newborn lambs (Apgar et al., 1993), while reabsorption of the fetus, delivery of mummified deformed lambs and abortions have also been reported as a consequence of dietary zinc deficiency. The zinc requirement for sheep is 20–35 mg/kg total diet and the maximum tolerable level is 750 mg/kg diet, above which symptoms of excess zinc can occur, such as induction of copper deficiency (NRC, 1985). The zinc recommendation for goats is 50–80 mg/kg diet (GfE, 2003). Symptoms of zinc deficiency in goats include reduced feed intake, weight loss, parakeratosis, stiffness of joints, excessive salivation, swelling of the feet and horny overgrowth, small testes and impaired reproductive performance (Nelson et al., 1984) and show many similarities to the sheep. Zinc deficiency causes growth retardation and abnormal feather development in poultry: feather fraying occurs near the end of the feather, the severity of which varies from almost no feathers on the wings and tail to only slight defects. The hock joint may become enlarged (Hoekstra, 1969; Padanilam and Solorsh, 1996) and the long bones of the legs and wings are shortened and thickened (Litchfield et al., 1998; Sauer et al., 1998; Wang et al., 2002; Tong et al., 2003). Other symptoms of deficiency include scaling of the skin, especially on the feet, loss of appetite, reduced efficiency of feed utilization and in severe cases, mortality. Zinc deficiency in the breeder diet reduces egg production and hatchability (Stahl et al., 1986), and the embryos produced in zinc-deficient eggs show a wide variety of abnormalities (Tao et al., 2002). The recommended zinc concentration for poultry is 40–50 mg/kg diet (NRC, 1994).

In laboratory animals, e.g. rats and mice, typical signs of zinc deficiency are decreased feed intake and reduced weight gain, shaggy fur, alopecia and skin lesions. Especially in male rats an adequate zinc supplementation is needed for maintenance of fertility (Jow et al., 1993; Hamdi et al., 1997; Ishizuka et al., 2003). Characteristic changes in zinc-dependent enzymes and a disordered bone development are further typical signs of zinc deficiency in rats (Zhou et al., 1999; Zhang et al., 2003). The reduced food intake in zinc-deficient rats was found to correspond to a high dietary protein content. In this case the lack of zinc led to an impaired protein metabolism so that the generally reduced appetite was rather a reduced appetite for protein (Rains and Shay, 1995; Roth, 2003). Recent research suggests that a decreased

hypothalamic secretion of neuropeptide Y may be involved in the reduced appetite during deficiency (Levenson, 2003). In rats and mice it has been further reported that zinc deficiency can lead to severe oxidative damage to mitochondria, cellular lipids, cellular proteins and DNA (Andrews et al., 1987; Gallant and Cherian, 1987; Oteiza et al., 1996; Eck and Pallauf, 2001; Ye et al., 2001; Ho and Ames, 2002; Sakagushi et al., 2002; Klotz et al., 2003). Interestingly, recent molecular biological investigations have shown that dietary zinc deficiency modulates a large number of different genes involved in growth, bone development, antioxidative protection and immunity (Cousins et al., 2003; Moore et al., 2003; tom Dieck et al., 2003), which may explain many of the effects observed. Recommendations for zinc in laboratory animals range between 30 mg/kg and 50 mg/kg diet, depending on the dietary phytate concentration (NRC, 1995). For growing rats on a phytate-free casein diet, the zinc requirement was shown to be clearly below 20 mg/kg diet (Pallauf and Kirchgessner, 1971).

4. MANGANESE

4.1. Introduction

Manganese (Mn) is a base metal which can occur principally in 11 oxidation states (–III to +VII), more than any other element. But the aqueous chemistry and therefore the physiological chemistry of manganese is largely restricted to the oxidation states +II and +III, in which manganese exists in the body of mammals both in protein-bound and in free ionic form (Römpp, 1991). Amongst trace elements manganese is one of the least abundant, reaching total body concentrations of approximately 1% and 20% of the values for zinc and copper (Underwood and Suttle, 1999).

Over 70 years ago the essentiality of manganese was first shown in mice and rats, which exhibited retarded growth and infertility as a consequence of dietary manganese deficiency (Kemmerer et al., 1931; Orent and McCollum, 1931). Subsequently, two diseases in poultry, namely perosis and nutritional chondrodystrophy, were shown to be preventable by dietary manganese supplements (Lyons and Insko, 1937; Wilgus et al., 1937). Biochemical pathways, which are affected by manganese deficiency, remained unclear until the 1970s. By its function in the active center of pyruvate carboxylase, phosphoenolpyruvate carboxykinase and arginase, manganese fulfills fundamental functions in carbohydrate and protein metabolism (Hirsch-Kolb et al., 1971; Barns et al., 1972; Scrutton et al., 1972; Baly et al., 1984, 1985). Furthermore, as an integral constituent of the mitochondrial form of superoxide dismutase (MnSOD = SOD2) manganese exhibits antioxidative properties (Paynter, 1980; Zidenberg-Cherr et al., 1983), while activation of glycosyltransferase also makes manganese important in the synthesis of mucopolysaccharides in cartilage (Leach, 1971). Manganese ions are involved additionally in the activation of an unknown number of further enzymes, especially decarboxylases, hydrolases and kinases.

4.2. Manganese in feeds

Feeds based on pasture, cereals and other seeds vary widely in manganese concentration such that concentrations ranging from 5–200 mg manganese/kg dry matter, depending on the soil pH value, moisture level, length of the growing season and fertilization, have been reported in the literature (Underwood and Suttle, 1999). Feedstuffs derived from legumes and maize are reported to contain generally lower manganese concentrations than other seeds and cereals. Due to low manganese concentrations in animals, feedstuffs derived from animals generally contain low manganese concentrations (White et al., 1981; MAFF, 1990; Minson, 1990). On the one

hand a manganese deficiency is less likely in comparison to other trace element deficiencies but on the other hand diagnosis of manganese deficiency is more complicated (Keen et al., 1983; Clegg et al., 1986; Combs, 1987). The importance of an adequate manganese supply is most marked in poultry nutrition (Greve et al., 1987; Ochrimenko et al., 1992; Liu et al., 1994).

4.3. Metabolism and biochemical functions of manganese

Manganese absorption from diets is generally low. Indeed, true absorption rates for monogastric animals range from 2–15%, while in ruminants true absorption only reaches about 1%. Overall, there is an inverse correlation between manganese concentration in the diet and absorption rate and importantly, absorption is reduced by high dietary concentrations of calcium, phosphorus and iron (Hidiroglou, 1979; Weigand et al., 1986; Wedekind and Baker, 1990; Johnson and Korynta, 1992; Finley et al., 1997).

Manganese ions (Mn +II and Mn +III) are liberated in the acidic stomach. The absorption of manganese into enterocytes of the duodenum and of the proximal jejunum is coupled to the action of the divalent cation transporter 1 (DCT1) (synonyms: divalent cation transporter (DCT), natural resistance associated macrophage protein 2 (NRAMP)), which is also responsible for iron uptake. As in iron absorption, mucins possibly play an important role in keeping manganese in solution during absorption (Conrad and Umbreit, 2000).

After oxidation of Mn II to Mn III, manganese is bound to transferrin and distributed to the tissues (Davidsson et al., 1989). The liver seems to play a central role in manganese homeostasis because manganese is excreted to a high extent via the bile. Biliary secreted manganese passes the enterohepatic cycle. In contrast, manganese losses from urine and endogenous sources are low and seem to be even more restricted in dietary manganese deficiency (Lee and Johnson, 1988; Davis et al., 1993; Löffler and Petrides, 2003).

In a variety of tissues the highest manganese concentrations are found in the mitochondria and the nucleus. An accumulation of manganese in different tissues normally does not take place (whole body concentration in various species, depending on the tissue: 0.5–3.5 mg/kg). However, when high doses (up to 500 mg/kg diet) of the trace element are given to manganese-depleted animals, markedly increased tissue concentrations of the element can be measured, especially in the liver, kidney, pancreas, adrenal gland, spleen, muscle and bone. These increases in tissue manganese at very high intakes possibly reflect a failure of homeostasis rather than successful storage (Grace and Lee, 1990; Leach and Harris, 1997).

Depending on the species, manganese deficiency can cause a variety of disorders. Thereby, the specific symptoms develop because of the reduced synthesis and loss of activity of functional manganese proteins in which manganese ions are bound to the catalytic active center and are directly involved in enzyme catalysis. In laboratory animals and in pigs, manganese deficiency is the cause of severe damage to the mitochondrial membrane of different cells, the outer cell membrane and to DNA. The reduced activity of the manganese-dependent mitochondrial form of superoxide dismutase (MnSOD = SOD2) contributes to a large extent to the above-mentioned impairments (Zidenberg-Cherr et al., 1983; Malecki et al., 1994; Macmillan-Crow and Cruthirds, 2001). The knockout of MnSOD in mice caused the death of the animals after only 8 days. At death dilated cardiomyopathy, liver dysfunction, metabolic acidosis, mitochondrial enzymatic abnormalities and oxidative DNA damage could be shown (Lebovitz et al., 1996; Huang et al., 2001). Longevity of different animal species seems to correlate negatively with individual mitochondrial production of reactive oxygen species (ROS). For instance, with an identical metabolic rate per kilogram live weight pigeons have a five times longer life-span in comparison to rats, due to a 2–4-fold higher mitochondrial ROS production

in rats (Sohal and Weindruch, 1996). MnSOD detoxifies ROS (especially the superoxide anion radicals ($O^{2-\bullet}$) which are produced in all tissues as a normal by-product of the mitochondrial respiratory chain. During the dismutation of the superoxide anion radical, manganese in the active center of MnSOD switches between the oxidation states +II and +III (fig. 4).

In contrast to the homodimeric cytosolic copper–zinc superoxide dismutase, MnSOD is a homotetrameric enzyme (Fridovich, 1976; Roberts et al., 1991; Lamb et al., 2001) and in addition, the two enzymes originate from different gene families. The expression of MnSOD is influenced by redox-sensitive factors like NF kappa B and C/EBPNF1. The exact influence of manganese in gene expression of MnSOD and post-transcriptional processes of the enzyme synthesis is not yet clear (Xu et al., 1999; Abid et al., 2001; Murley et al., 2001; Rogers et al., 2001). In the context of MnSOD research, a synthetic superoxide anion scavenger like 5-, 10-, 15-, 20-tetrakis (4-benzoic acid) porphyrin (MnTBAP) hase been identified as a substitute for the enzyme (Patel, 2003).

Manganese also takes part in other important functions in amino acid metabolism and excretion of ammonia. In cellular metabolism, the desamination of amino acids delivers ammonia, which is cytotoxic especially for the brain. The manganese-containing glutamine synthetase fixes free ammonia by the intermediate formation of glutamine from glutamate and ammonia. Eighty percent of the manganese content in the brain is bound to glutamine synthetase. Severe neurological damage in manganese deficiency is attributed to the reduction of glutamine synthetase activity, which is additionally amplified by the sensitivity of glutamine synthetase towards oxidation (Wedler and Denman, 1984; Weber et al., 2002; Takeda, 2003).

Ammonia from amino acid desamination is excreted from the body via urea synthesis in the liver. Arginase, which liberates urea from arginine, is the final enzyme in the urea cycle. Manganese deficiency consequently reduces urea synthesis and leads to chronic poisoning of the organism via an increase in plasma ammonia concentration (Kirchgessner and Heiseke, 1978; Brock et al., 1994; Ash et al., 2000).

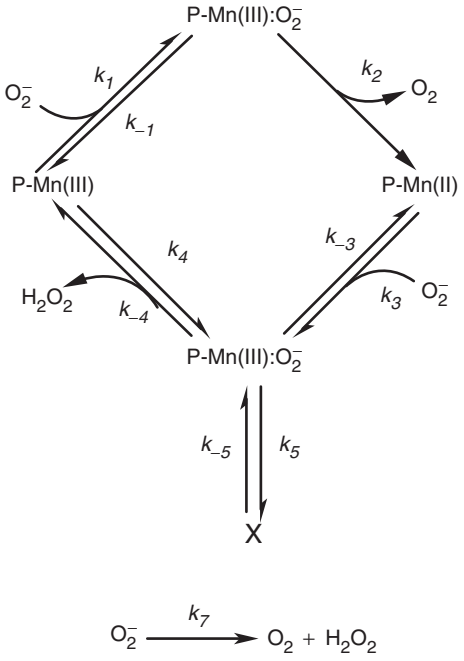

Fig. 4. The enzymatic reaction of MnSOD consists of a bimolecular reaction with a catalytic cycle involving two distinct half reactions, an oxidative reaction in which the substrate $O^{2-\bullet}$ is oxidized to dioxygen and a reductive half reaction in which $O^{2-\bullet}$ is converted to H_2O_2. (From Hsu et al., 1996, with permission of the American Society for Biochemistry & Molecular Biology.)

The function of pyruvate carboxylase (PC) and phosphoenolpyruvate carboxykinase (PEPCK), the first two marker enzymes of gluconeogenesis, also depend on manganese, and thus a long-term manganese deficiency could affect carbohydrate and lipid metabolism (Barns et al., 1972; Scrutton et al., 1972; Rognstad, 1981; Baly et al., 1985; Kai et al., 2003). Further important enzymes depending on manganese are glycosyl transferase, which is vitally important in the synthesis of mucopolysaccharides as a structural component of cartilage, and mevalonate kinase which catalyzes the conversion of mevalonic acid to squalene during cholesterol synthesis (Leach, 1971, 1988; Garcia-Martinez et al., 1982; Schulte et al., 2000).

4.4. Manganese deficiency and requirement in various animal species

Signs of manganese deficiency in livestock depend on the species. Poultry, and in particular chicks, are most susceptible to dietary manganese deficiency. Perosis is the result of a lowered proteoglycan content of the tibial growth plate, which involves a twisting and bending of the tibia, and slipping of the gastrocnemius tendon from its condyles. With increasing severity fast-growing birds are reluctant to move, squat on their hocks and subsequently die. In laying hens and breeding birds a manganese deficiency results in lowered egg production and hatchability and reduced eggshell strength (Settle et al., 1969; McNatt et al., 1976; Thomas and Lowther, 1976; Leach and Gross, 1983; Bolze et al., 1985). The recommended manganese level in diets for poultry is 50–60 mg/kg DM (GfE, 2000).

In dairy cattle symptoms of manganese deficiency include impaired growth, skeletal abnormalities, impaired reproduction and abnormalities of the newborn. Deformed calves at birth have weak legs and pasterns, enlarged joints, stiffness, twisted legs, general weakness, and reduced bone strength. Tongue rolling was also found to be associated with manganese deficiency in cattle. Deficiency symptoms in the cow are characterized by reproductive disorders. These include delayed estrus, reduced fertility and abortions. In cattle the manganese requirement for growth is lower than that required for reproduction and birth of normal calves (Anke et al., 1973a; Hidiroglou et al., 1990; Karatzias et al., 1995). The manganese recommendation for heifers and dairy cows is 50 mg manganese/kg DM (GfE, 2001).

An abnormal skeletal growth with altered fat to lean ratio, absence of or irregular estrus cycles, poor mammary development and lactation, resorption of fetuses and at birth small, weak pigs with a poor sense of balance are symptoms commonly associated with manganese deficiency in pigs. Data on the porcine requirement for manganese vary a great deal. This is probably due to the high content of calcium, phosphorus and antinutritive compounds (e.g. phytic acid) in typical diets for pigs (Neher et al., 1956; Leibholz et al., 1962; Weigand et al., 1986; Rheaume and Chavez, 1989, 1991; Pallauf et al., 1992; NRC, 1998). For growing pigs, according to NRC (1998), only 2–4 mg manganese/kg diet are required, whereby the amount decreases with increasing live weight. However, recent results with growing pigs show that 16 mg Mn/kg diet are necessary for optimal liver manganese storage and activity of manganese-depending enzymes (Kauer et al., 2005). For gestating and lactating sows the manganese requirement is met with an amount of 20 mg/kg diet (NRC, 1998).

Manganese-deficient sheep exhibit symptoms comparable to those of cattle (Anke et al., 1973a, 1973b; Hidiroglou et al., 1978). The recommendation for manganese in sheep is 20–40 mg/kg diet (NRC, 1985).

Symptoms of manganese deficiency in goats include a reluctance to walk, deformation of the forelegs and reduced reproductive efficiency. In mature goats the manganese content of the hair is considered to be a better indicator of manganese status than manganese in any other part of the body. Goats also show tarsal joint excrescences and ataxia due to manganese

deficiency (Anke et al., 1973c). For goats 60–80 mg manganese/kg DM are recommended (GfE, 2003).

In laboratory animals (e.g. mice and rats) manganese deficiency symptoms are associated with development of severe oxidative damage in different tissues, possibly due to a higher mitochondrial activity (Zidenberg-Cherr et al., 1983; Kawano et al., 1987; Lebovitz et al., 1996; Keen et al., 1999). A level of 10 mg manganese/kg diet is recommended as the requirement for rats and mice, whereas 40 mg/kg diet is recommended for guinea pigs (NRC, 1995).

5. COPPER

5.1. Introduction

Elemental copper (Cu) is a red-colored and dilatable metal. Copper belongs to the transition metals and its occurrence in the organism is restricted to the oxidation states +I (cuprous form) and +II (cupric form) (Römpp, 1990). Mammals have a total amount of about 1.0–2.0 mg copper/kg body weight. Newborn and young animals have more copper per unit of body weight and maintain these levels throughout the suckling period, followed by a steady decline until adulthood (Underwood, 1977). In animals and humans copper mainly exists in protein bound form. Copper transport proteins and oxidoreductases, such as cytochrome-c-oxidase (Lemberg, 1969; Medeiros and Jennings, 2002), superoxide dismutase (Fridovich, 1976; Zelko et al., 2002), catechol oxidase (Mason, 1976; Garcia-Borron and Solano, 2002), protein- lysine-6-oxidase (Rucker and Murray, 1978; Csiszar, 2001), ceruloplasmin (Frieden, 1969; Fuentealba and Aburto, 2003), amine oxidases (Buffoni, 1966; Dawkes and Phillips, 2001), dopamine-β-monooxygenase (Molinoff et al., 1974; Prigge et al., 2000) and peptidylglycine monooxygenase, are the most important copper-associated proteins. Free ionic forms of copper, which are generated only intermediately, are considered to be involved in the production of radical oxygen species and in the development of oxidative stress (Gaetke and Chow, 2003).

The nutritional essentiality of copper for mammals was established when rats which were anemic and stunted grew and synthesized hemoglobin rapidly after their diet based on milk protein was supplemented with 1 mg copper/kg (Hart et al., 1928). Subsequently, a number of naturally occurring diseases in different animal species were attributed to a copper deficiency. Examples of such diseases are “falling disease” in cattle (Bennets and Hall, 1939) and pica in sheep and cattle (Sjollema, 1933). Numerous cardiovascular diseases in pigs have been described as developing from copper deficiency (Shields et al., 1962; Carnes et al., 1965; Hill and Davidson, 1986). In various animal models copper-deficient diets, high in lard content, were shown to effect changes in the cardiovascular anatomy and in heart function, which could be prevented by the administration of copper as an antidote (Klevay, 1985; Stemmer et al., 1985; Vadlamudi et al., 1993; Fox et al., 2000). The discovery of copper-dependent enzymes explained the biochemical causes of many copper deficiency disorders. All enzymes known to depend on copper for activity are oxidative. Some of these enzymes are important for cardiovascular health. Crosslinking of arterial collagen and elastin requires lysyl oxidase, a copper enzyme (Rucker et al., 1977; Rucker and Dubick, 1984). Arterial proteoglycan metabolism is also disrupted by copper deficiency (Radhakrishnamurthy et al., 1989). Copper–zinc superoxide dismutase, found in various tissues (Harris, 1992; Ishida et al., 1999), is dependent on copper for activity and protects against free radicals (McCord, 1985; Southorn and Powis, 1988). It has been suggested that copper is an antioxidant nutrient important for cardiovascular health (Klevay, 2000). Malondialdehyde and thiobarbituric acid reactive substances are used as indices of peroxidation, since their concentrations are raised in the serum in coronary artery disease and angina pectoris (Mendis et al., 1995) and in the

plasma of copper-deficient rats (Saari et al., 1990). Cholesterol metabolism and copper utilization were linked when it was observed that a high ratio of zinc to copper ingested produced hypercholesterolemia in rats (Klevay, 1973). Hypercholesterolemia resulting from copper deficiency has been found in several species in at least 22 independent laboratories worldwide (Klevay, 2000). Keil and Nelson (1934) were probably the first to observe an altered physiology in copper deficiency now known as glucose intolerance. Rats depleted of copper after they reach adult size become hypertensive (Medeiros, 1987), perhaps from impaired vasodilatation in response to acetylcholine (Saari, 1992).

On the other hand, a number of diseases exist which are associated with chronic copper poisoning. Copper poisoning in sheep and cattle was first described in areas with extremely low molybdenum concentrations in the herbage and is preventable in those regions by molybdenum supplementation (Todd, 1969; MacLachlan and Johnston, 1982; Ogra et al., 1999).

5.2. Copper in feeds

Copper concentrations in animal feed exhibit great variation. Furthermore, especially for ruminant nutrition, it is important to be aware of the dietary molybdenum concentration as well as the concentrations of iron and sulfur, since these can all strongly influence copper availability and absorption. Thus, in the case of ruminants it is more important to obtain information on copper absorbability than on copper concentration (Underwood and Suttle, 1999). Copper concentrations in pasture and forages vary with the species, strain and maturity of the plant, the soil conditions and the fertilizers used (McFarlane et al., 1990). Grains contain copper concentrations ranging from 1.5–8.4 mg Cu/kg DM, with averages of 3.9 mg/kg and 4.9 mg/kg DM for oats and barley, respectively. The mean corresponding concentrations for molybdenum and copper were reported to be 0.27 mg/kg DM and 1.3 mg/kg DM, respectively (Todd, 1972; Liu et al., 2001a). Copper concentrations in legumes and oilseeds are distinctly higher than in grains and reach mean concentrations of 15–35 mg/kg DM, but the molybdenum concentrations are also found to be higher (up to 4 mg/kg). Palm-kernel cake contains high copper concentrations and was even found to cause copper toxicity in sheep (Chooi et al., 1988). With the exception of liver meal (Czarnecki and Baker, 1985) and meals from crustacea and shellfish (Lawrence et al., 1995; Turoczy et al., 2001; Correia et al., 2002), feeds of animal origin represent poor to moderate sources of copper. Meat meal and fish meal contain copper concentrations ranging from 5–15 mg/kg DM, whereas concentrations obtained in liver meal range from 80–100 mg/kg DM. Milk and milk products (normally 1 mg/kg dry matter and lower) are low in copper concentration (Underwood and Suttle, 1999).

5.3. Metabolism and biochemical functions of copper

Copper in feedstuffs for animals derives from two main sources: (1) protein-bound copper from plant and animal tissues; (2) inorganic forms of copper from mineral feed. Copper salts such as chloride, acetate, sulfate and carbonate, are highly bioavailable, but there is one exception, copper oxide, which possesses < 40% of copper chloride bioavailability in sheep and a negligible availability in chickens. Copper is primarily absorbed in the cupric +II oxidation state. The amino acid cysteine and ascorbic acid are able to reduce copper II ions to the cuprous oxidation state, so that both these substances act as inhibitors of copper absorption (van Campen and Gross, 1968; Wapnir, 1998; Eckert et al., 1999; Yost et al., 2002).

The main site of copper absorption is the small intestine, whereby all parts (duodenum, jejunum, ileum) participate equally in absorption. In contrast to other trace elements the

stomach is also considered to be involved in copper absorption. The absorption rate is inversely correlated with copper intake and can be as low as 10% with very high copper intake. In humans it has been estimated that there is a theoretical maximum absorptive capacity of about 65% (Turnlund et al., 1989), while in young milk-fed lambs absorption rates of as high as 70–85% have been reported (Suttle, 1974). The exact mechanism of copper absorption is not yet fully understood, but depending on the age and the physiological status of animals, three different mechanisms are presently under discussion as being involved in copper absorption into the enterocyte:

1. In weanling rats and in lactating rat dams copper absorption in the small intestine occurs largely by diffusion and by co-transport with water (solvent drag). Correspondingly, a lack of sodium in the lumen or inhibition of sodium transport impairs copper absorption (Wapnir, 1991; Handy et al., 2002; Schuemann et al., 2002).
2. The divalent cation transporter DCT1 (synonyms: DMP 1 and NRAMP), which is necessary for iron and manganese absorption, also seems to function in copper absorption (Wapnir, 1998).
3. Recent studies suggest that the copper transporter Ctr 1, expressed in a variety of tissues, plays an important role in intestinal copper absorption (Andrews, 2002).

Once in the enterocyte copper can be stored intermediately in a GSH-bound form and in metallothionein.

The export of copper from the enterocyte and therefore the distribution to peripheral tissues is initiated by an ATPase, which is named after the genetic defect of the protein in Menkes disease as Menkes-ATPase (Andrews, 2002). Copper entering the blood via the action of Menkes ATPase binds to serum albumin (12–17%), selected amino acids such as histidine, threonine, and glutamine (10–15%), and to transcuprein a copper transport protein with a molecular weight of 270 kDa (12–14%). Copper complexed to these carriers is quickly taken up by the liver and other tissues (Weiss and Linder, 1985). The liver is the central organ of copper metabolism. The uptake is mainly accomplished by Ctr1. Within the hepatocyte a high percentage of copper is intermediately complexed in the cytoplasm to a 10 kDa fraction similar to thionein. From this storage form copper is slowly transferred to larger molecules representing various copper enzymes.

Diverse other organs exhibit widely different copper concentrations. The heart (3.1 mg/kg FM) and kidney (3.6 mg/kg FM) are particularly copper-enriched organs. Adjustment to fluctuations in copper supply is achieved predominantly by hepatic storage and biliary copper secretion. The kidneys play only a minor part in copper excretion. Copper-containing proteins and their function in the organism are listed in table 6.

Ceruloplasmin is the major circulating form of copper, representing 60–65% of plasma copper. The protein is a single-chain β-globulin, which contains six copper atoms and possesses five identifiable functions, which are listed in table 7.

5.4. Copper deficiency and requirement in various animal species

In general, copper deficiency can occur in all mammalian species, however ruminants are perhaps the most susceptible. In pigs copper deficiency leads to poor iron mobilization and abnormal hemopoiesis. Poor keratinization and synthesis of collagen, elastin and myelin can cause sudden ruptures of major blood vessels (aneurysms). Copper deficiency also results in a broad range of cardiovascular diseases in pigs (Coulson and Carnes, 1967; Coulson and Linker, 1968; Smith et al., 1968; Wildman et al., 1996), while further diseases include microcytic and hypochromic anemia, bowing of the legs, and spontaneous fractures

Table 6

Copper containing proteins and their function in the organism of mammals (Shim and Harris, 2003)

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(Lee et al., 1968; Smith and Deans, 1969; Okonkwo et al., 1979). Copper requirement in pigs ranges from about 5 mg/kg diet in growing pigs to about 8–10 mg/kg in lactating sows. The addition of higher levels of copper (100–250 mg/kg) as $CuSO_4$ to the diet of growing pigs has been shown to enhance their performance, which is possibly a consequence of the antimicrobial actions of copper (Okonkwo et al., 1979; Stahly et al., 1980; Smith et al., 1997a; Aarestrup et al., 2002). The feed legislation of the EU allows the addition of 170 mg Cu/kg diet for pigs up to 12 weeks of age. In piglets, reduced feed intake, watery dark feces, poor growth and a rough hair coat were observed as symptoms of an intoxication with copper, when this was fed at a level of 860 mg/kg diet (Zentek et al., 1999). For growing pigs 3–6 mg copper/kg DM are recommended, whereby the amount decreases with increasing live weight. According to NRC (1998) for gestating and lactating sows the copper requirement is met with an amount of 5 mg/kg diet.

An early sign of copper deficiency in cattle is loss of hair pigmentation, particularly around the eyes. Scour is a clinical sign of copper deficiency that seems to be unique to ruminants, though the pathogenesis of this lesion is not understood (Suttle and Angus, 1976).

Table 7

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Hypochromic macrocytic anemia, as a consequence of decreased ceruloplasmin levels and activity, poor growth, fragile bones and osteoporosis and a disordered reproductive performance (delayed estrus) are further signs of copper deficiency in cattle (Thomas et al., 1981). Cardiac failure can result in "falling disease" (van der Ingh and Lenghaus, 1975). Finally, an effect of copper deficiency that is not easily observed is a reduced immune function even though the dietary copper requirement necessary for optimal immune function may exceed the amount required to prevent the more classic signs of copper deficiency (Cerone et al., 2000). The copper requirement of beef cattle and cows is estimated to be met with 10 mg copper/kg DM (GfE, 2001).

In sheep the range between copper deficiency and copper toxicity is much smaller than in other animal species. Neonatal ataxia, which is also termed swayback and lamkruis, is one of the most marked and prevalent symptoms of severe copper deficiency in lambs (O'Dell et al., 1976; Smith et al., 1976; Bourke, 1995). This symptom has also been diagnosed in goats, guinea pigs and young pigs (O'Sullivan, 1977). It is characterized by a nervous disorder and uncoordinated movement particularly of the hindquarters. The defect appears to be related to a disordered myelination of the brain and the spinal cord. It may result from low cytochrome c oxidase activity in the mitochondria, which decreases the ATP synthesis rate. This in turn leads to depressed phospholipid synthesis (Smith et al., 1976). Sheep suffering from copper deficiency also have "steely" or "stringy" wool. The characteristic physical properties of wool, including crimp, are dependent on disulfide groups that provide the cross linkages or bonding of keratin and on the alignment or orientation of the long-chain fibrillae in the fiber. Both of these are adversely affected in copper deficiency. A lack of pigmentation in black sheep (banded wool) is a further indicator of copper deficiency (Burley and de Knock, 1957; Forrest et al., 1985). Anemia, bone disorders (osteoporosis in lambs) and spontaneous bone fractures in adult sheep, and infertility of both male and female sheep have also been associated with copper deficiency in sheep (Hidiroglou et al., 1982; van Niekerk and van Niekerk, 1989a, 1989b).

The copper requirement of sheep ranges between 7–20 mg/kg diet and is largely dependent on the concentrations of molybdenum and sulfur in the diet (Lamand, 1989). Sheep tend to be much more sensitive to copper intoxication than other farm animals. For example, growing swine have often been fed copper concentrations as high as 250 mg/kg diet to improve performance (Edmonds et al., 1985; Cromwell et al., 1993), and cattle can consume diets containing 100 mg copper/kg diet without problems (Muehlenbein et al., 2001), but toxicities have occurred in sheep with concentrations as low as 10 mg/kg. Copper toxicity in sheep usually results from the accumulation of copper in the liver over a period of a few weeks and longer with no clinical signs, until there is a sudden release of copper from the liver stores, which causes the toxicity. The most common signs of copper toxicity in sheep are anorexia, excessive thirst and depression accompanied by hemoglobinemia, anemia, icterus and methemoglobinemia (Humann-Ziehank et al., 2001; Fuentabella and Aburto, 2003). Differences in breed exert a major influence on the small range between copper deficiency and copper toxicity. Whereas the Heath breed has a relatively high copper tolerance, Merino and Texel sheep tend to accumulate copper (Meyer and Coenen, 1994).

The requirement for copper in sheep is estimated to be met with 7–11 mg/kg diet, if the molybdenum content remains below 1 mg/kg diet (NRC, 1985). For goats, which are somewhat less susceptible to copper toxicity than sheep, 10–15 mg Cu/kg DM are recommended (GfE, 2003).

In poultry similar symptoms of copper deficiency occur as found in other species. Macrocytic anemia, bone deformities and lack of feather pigmentation are signs of copper

deficiency in poultry, while the occurrence of aneurysms has also been attributed to dietary copper deficiency (Rucker et al., 1999; van Veen, 1999). The recommended copper level in diets for poultry is 6–7 mg/kg DM (GfE, 2000).

In laboratory animals such as rats and mice copper deficiency leads to an increase in the number of malformed newborns (Arce and Keen, 1992). Furthermore, changes in mitochondrial metabolism have also been found in copper-deficient rats (Chen et al., 1994; Rock et al., 1995). In rabbits copper deficiency can cause myocardial disease and aneurysms. A maintenance level of 6 mg copper/kg diet has been recommended for laboratory animals (rat, mice, guinea pig), whereas 8 mg/kg diet is recommended for reproduction (NRC, 1995).

Besides the above-mentioned copper deficiency diseases in various species, there exist at least two genetic disorders, resulting from defects in the expression of copper transport proteins, namely Menkes disease and Wilson disease. In Menkes disease the expression of the above-mentioned copper export ATPase (named after the disease, Menkes ATPase = MNK), predominantly expressed in cells of the small intestine and in tubulus cells of the kidney, is disordered. As a consequence of the defect, copper is accumulated selectively in intestinal cells and in tubulus cells, while copper distribution to other tissues is largely restricted. In affected humans and animals some typical symptoms of copper deficiency occur (ataxia, mental retardation, disordered pigmentation, bone abnormalities, aneurysms). The disease leads to death at a young age because there is no treatment for the overall copper deficiency. In Wilson disease there is disordered expression of another copper export ATPase (named after the disease, Wilson ATPase = WD), mainly expressed in the liver and responsible for copper loading into ceruloplasmin. Within a short period of time copper overload in the liver and copper toxicosis occur. The disease finally leads to liver failure, movement disorders and pronounced mental disorders (Andrews, 2002). New approaches using proteomics have been carried out in order to understand the biochemical context of copper storage diseases (Simpson et al., 2004).

6. IODINE

6.1. Introduction

Elemental iodine (oxidation state 0) is a dark violet crystalline substance. Iodine belongs to the chemical group of the halides but it is more inert than the other halides. On land, iodine mainly occurs in the iodide oxidation state (–I). Iodide salts are also present in seawater and iodine concentration is high in algae, seaweed and other seafoods. In mineral iodine deposits, iodate (oxidation state +V) is the predominant chemical form of iodine (Römpp, 1990). It is believed that the Chinese, many centuries BC, knew that substances from marine products exerted a beneficial effect upon the thyroid. Burnt sponges and seaweed were added to diets according to Hippocrates (460–370 BC) to relieve enlarged thyroids. That iodine is the active substance preventing goitre was discovered by Davy in 1815 (Rosenfeld, 2000). Further milestones in the history of iodine research were the isolation of crystalline thyroxine in 1915 (Kendall, 1915) and the establishment of the empirical formula of thyroxine in 1926/27 (Harrington, 1926, 1927). Recognition of the involvement of selenium-dependent deiodinases in the conversion of the less-active tetraiodothyronine (T4 = thyroxine) form of thyroid hormone into the highly active metabolite triiodothyronine (T3) and the deactivation of thyroid hormones by reverse deiodination were further important steps in the understanding of thyroid metabolism (Larsen, 1997). Current biochemical research focuses on the molecular mechanisms of iodine uptake into the thyroid gland as well as the synthesis of thyroid hormones and their exact function in cellular metabolism (Brent, 1994; Spitzweg and Morris, 2002;

Nguyen et al., 2003; van der Putten et al., 2003). The mean iodine concentration in the body of mammals is only 0.2–0.3 mg/kg body weight with the thyroid gland containing 70–80% of the total iodine (Pond et al., 1995).

The formation of a goitre is an extrinsic sign of iodine deficiency and represents the attempt of the thyroid gland to absorb more iodine and synthesize more thyroxine in response to TSH stimulation. Thyroid hormones play a major role in controlling the rate of cellular oxidation and in the regulation of a range of processes in intermediary metabolism. Consequently, dietary iodine deficiency reduces the basal metabolic rate. Iodine deficiency in young animals causes cretinism and in adults myxoedema (NRC, 1980; Brent, 1994; Herzig and Suchy, 1996). In order to provide an adequate iodine intake in humans, modern approaches are used in animal nutrition to supply rich natural iodine sources for iodine supplementation in farm animals (Kaufmann et al., 1998; He et al., 2002).

6.2. Iodine in feeds

Iodine concentration in animal feedstuffs varies widely. Plants in particular contain iodine in highly variable concentrations due to species and strain differences, topology (proximity to the sea), climatic and seasonal conditions and the type of soil and fertilizer application. Cereals and oilseeds are poor sources of iodine, whereas fish meal from sea fish is an exceedingly rich source (Groppel et al., 1989; Underwood and Suttle, 1999). Iodine concentration from other animal protein sources depends on the iodine supplementation to the animals from which the feedstuffs were produced (Hemken, 1980; He et al., 2002). The highest iodine concentrations in feedstuffs (4–6 g/kg DM) can be found in seaweed (Tokudome et al., 2002). Table 8 gives iodine concentrations of common animal feedstuffs.

An extreme variability of iodine concentration is seen in milk from nearly all species, due to a linear increase of iodine concentration with the amount of dietary intake (Preiss et al., 1997). Special attention must be paid to the concomitance of goitrogenic substances in some feedstuffs. Examples for goitrogenic substances are the cyanogenic glycosides linamarin and lotoaustralin contained in cassava (Barret et al., 1978). Considerable amounts of glucosinolates as well as of isothiocyanates and goitrin (an oxazolidinethione), which are liberated during processing and digestion from glucosinolates, are naturally contained in brassicaceae such as rapeseed and in millet. Modern plant breeds of brassicaceae are selected for low goitrogen content (Gaitan et al., 1989; Schoene, 1993; Mawson et al., 1994a, 1994b; Underwood and Suttle, 1999; Schoene et al., 2001). Recent studies have investigated the existence of goitrogenic isoflavones in soya (Divi et al., 1997; Son et al., 2001; Doerge and Sheehan, 2002; Wade et al., 2003).

6.3. Metabolism and biochemical functions of iodine

In monogastric animals dietary iodine (iodide oxidation state, i.e. –I) is absorbed very efficiently throughout the gastrointestinal tract. Other chemical forms with a higher oxidation state (e.g. iodate, +V) are reduced prior to absorption (Burgi et al., 2001). In ruminants most iodine is absorbed in the rumen, while the abomasum is the major site of endogenous secretion (Miller et al., 1975). Iodide is almost completely absorbed over a wide concentration range (Harrington et al., 1985). Absorbed iodine is transported in the bloodstream loosely bound to plasma proteins (Thrall et al., 1992). A high percentage (normally about 90%) of the dietary iodine is taken up from plasma proteins by follicular cells of the thyroid by an active sodium–iodide symporter (NIS) (Nilsson et al., 1990; Spitzweg and Morris, 2002). Therefore, the thyroid is the organ with by far the highest iodine concentration within the body (0.5–5.0 g iodine/kg DM). Only the kidney competes with the thyroid for iodine

Table 8

Iodine concentration in feedstuffs from different countries (μg/kg DM)

Feedstuffs	Germany[a,b]	Mean conc.	Chile[c]	Mean conc.	New Zealand[d,e]	Mean conc.
Grasses, hays and straws	Hay	136	Hays and straws	100–200	Perennial ryegrass	1500
	Cereal straw	368			Cocksfoot	175
					White clover	500
Cereals	Maize	44	Cereal grains	40–90		
	Barley	95				
Leguminoses	Extr. soybean	97				
Oil seeds	Extr. rapeseed	67	Oil seed meals	100–200		
Meat meals	Fish meal	6688	Meat meals	100–200		
Fish meals			Fish meals	800–8000		
Milk products	Dried skimmed milk	376	Milk products	200–400		

[a]Groppel and Anke, 1986; [b]Groppel et al., 1989; [c]Chilean Iodine Education Bureau, 1952; [d]Johnson and Butler, 1957; [e]Barry et al., 1985.

uptake from the blood. Because of a lack of iodine storage capacity iodine is partially excreted in the urine, the level of excretion correlating well with the plasma iodine concentration. Some of the iodine can be reabsorbed by renal tubular cells, whereby the iodine reabsorption is proportional to that of chloride. Other organs have been reported to store only negligible amounts of iodine (muscle: 0.01 µg/100 g fresh matter; brain: 0.02; testes: 0.02; lymph nodes: 0.03; kidney: 0.04; lung: 0.07; ovaries: 0.07; liver: 0.20) (Oberleas et al., 1999).

The synthesis of thyroid hormones from iodine and tyrosine (fig. 5) takes place in the follicular cells of the thyroid (Cavalieri, 1997; Löffler and Petrides, 2003; Rousset, 2003). Using

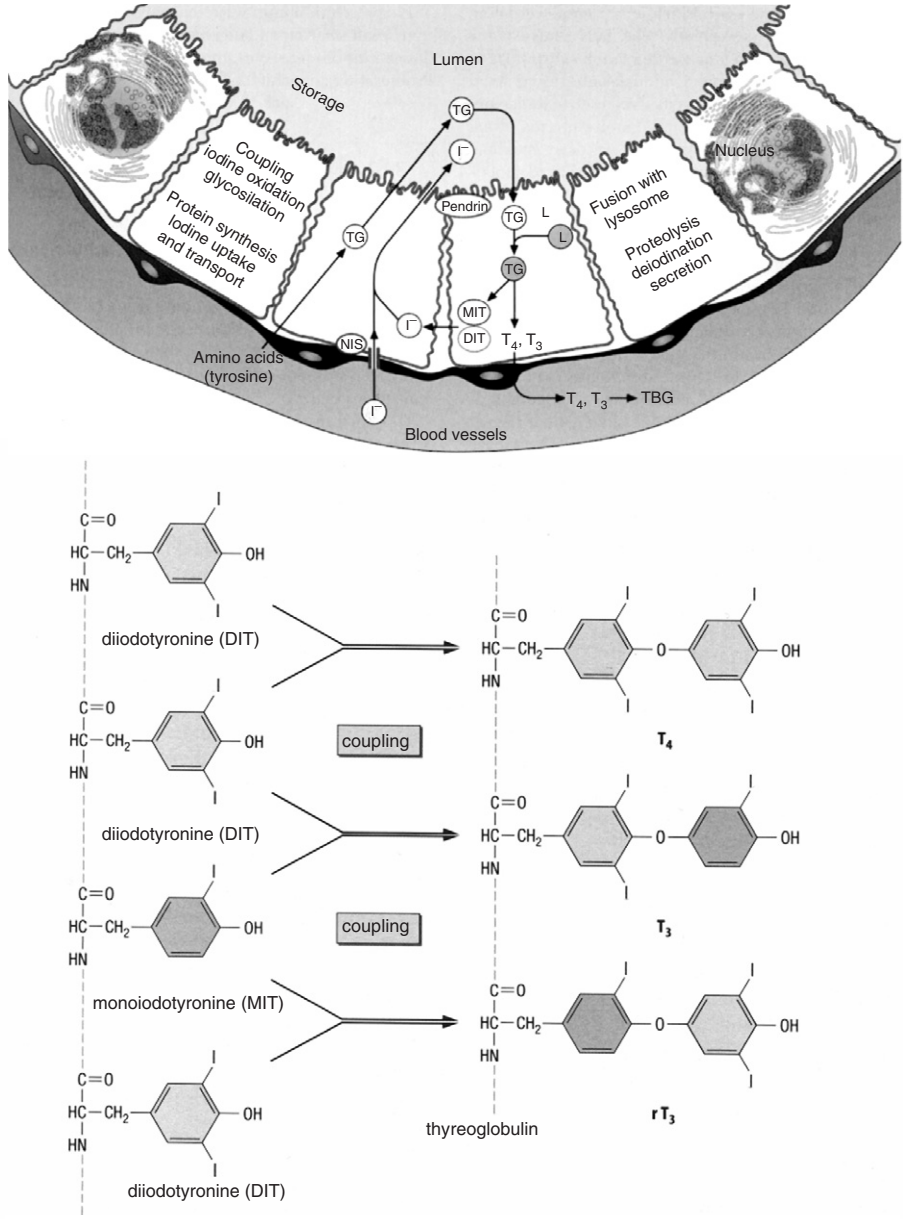

Fig. 5. Synthesis, storage and release of thyroid hormones in follicular thyroid cells. (From Löffler and Petrides, 2003, reproduced with permission of Springer-Verlag and the authors. © 2003 Springer-Verlag.)

H_2O_2 as an oxidative agent thyroperoxidase converts the imported iodide ions into iodonium ions (oxidation state +I) (Nakamura et al., 1990). At this point of thyroid hormone synthesis there occurs an interaction between selenium and iodine. Due to the high intracellular H_2O_2 levels in the follicular cells there is a considerable risk of oxidative damage to lipids of the cellular membranes and proteins in the cell. Further H_2O_2 inhibits iodine uptake into the follicular cells (Fukayama et al., 1991). Selenium-dependent glutathione peroxidases 1 and 4 therefore fulfill a crucial role in the regulation of the hydrogen peroxide tonus and in the detoxification of already-formed lipid peroxides (Beckett et al., 1991; Howie et al., 1995; Arthur and Beckett, 1999; Koehrle, 1999). The generated iodonium ions are able to iodinate tyrosine residues of thyroglobulin, a homodimeric protein with a molecular weight of 660 kDa, containing 72 tyrosine residues per molecule. The iodination reactions are carried out at the positions 3 and 5 of the aromatic ring structure of the tyrosine residues. Single mono- and diiodinated tyrosine residues are coupled with other mono- and diiodinated tyrosine residues to form thyroglobulin-bound T3 and T4 (Spiro, 1969; Rousset, 1991; Dunn and Dunn, 1999). The formation of biologically inactive rT3 is also possible by the transfer of diiodotyrosine to monoiodotyrosine. Until secretion of thyroid hormones into the plasma, iodinated forms of thyroglobulin are stored in the lumen of the follicular cells. After re-uptake of iodinated thyroglobulin the thyroid hormones T3 and T4 are liberated by proteases and released into the plasma (Rousset, 1991; Koehrle, 1996). All processes of thyroid hormone synthesis (uptake of iodine by NIS, oxidation of iodide by peroxidase and liberation of T3 and T4 by proteases) are regulated via negative feedback control (fig. 6) by two hormones, thyrotropin-releasing hormone (TRH) and thyroid-stimulating hormone (TSH) (Nilsson et al., 1990; Arthur and Beckett, 1999).

In the plasma the thyroid hormones are bound mainly to the thyroxin-binding globulin (TBG). Further transport proteins for the thyroid hormones are thyroxine-binding prealbumin (TBPA) and albumin (Schussler, 2000). In the plasma only 0.03% of T4 and 0.3% of T3 can

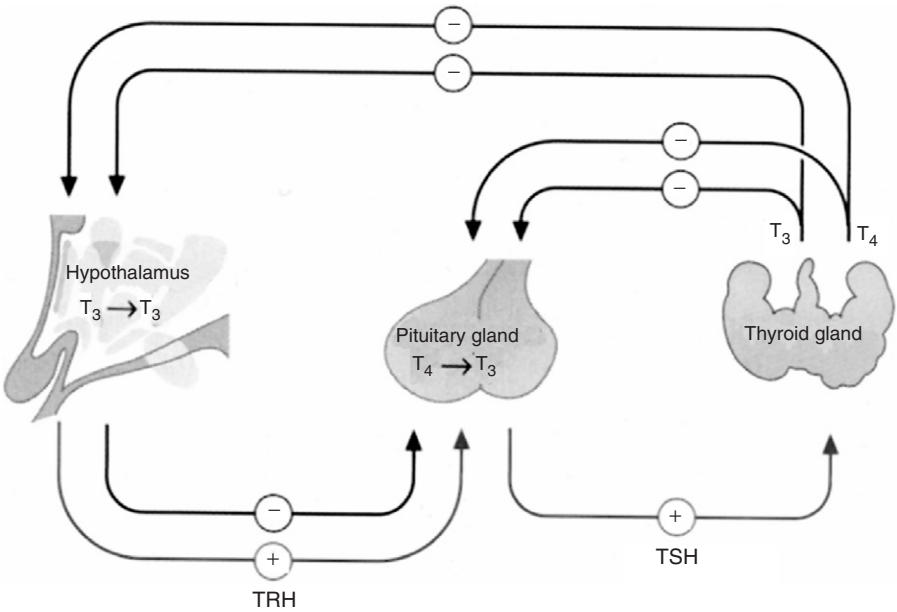

Fig. 6. Regulation of thyroid metabolism via the hypothalamic–pituitary-axis. (From Löffler and Petrides, 2003, reproduced with permission of Springer-Verlag and the authors. © 2003 Springer-Verlag.)

be found in free form. Thereby, plasma T4 concentration is 40 times higher than the T3 concentration. The more active T3 form of the thyroid hormone is mainly generated in the liver and the kidney by the 5´ monodeiodinase 1 (MDI1), which is a selenoenzyme. The plasma levels of T4, T3 and rT3 are also regulated by two further deiodinases, monodeiodinase 2 (MDI2) and monodeiodinase 3 (MDI3), which also contain selenium. MDI2 shows a quite different tissue distribution from MDI1 and is primarily found in the brain, central nervous system, brown adipose tissue and in the pituitary gland. The metabolic function of these tissues depends on T3, which is produced locally from T4. MDI3 occurs in many tissues and it functions as a regulator of the tissue-specific T3 concentration. The enzyme catalyzes the reverse deiodination of T4 and further deiodination of T3 to biologically inactive T2. The selenium status of animals therefore plays an important role for thyroid metabolism (Koehrle, 1996, 2000a, 2000b).

The thyroid hormones act via thyroid hormone receptors (T3 receptors) in the various tissues. In total, four different types of T3 receptors exist, namely T3α1, T3α2, T3β1 and T3β2, which are derived from two different gene families (Lazar, 1992; Kelly, 2000). The expression and distribution of the single receptor subtypes have genetic and developmental causes. The subtypes α1, α2 and β1 are expressed in almost all tissues, whereas expression of the β2 subtype is limited to the brain (Bradley et al., 1992; Schwartz et al., 1992; Tagami et al., 1993). T3 receptors are nuclear receptors, thus the binding of T3 leads to changes in the gene expression of metabolic enzymes and other proteins (Oppenheimer et al., 1987, 1991). Transport of T3 to its receptor-binding site is presumably carried out by fatty acid translocase (van der Putten et al., 2003).

In the cardiac muscle T3 is involved in the expression of the myosine heavy chain and thyroid hormone deficiency decreases the contractility of cardiac muscle. T3 participates further in muscle metabolism by increasing the expression of Ca-ATPases. Due to a delayed decrease in cellular calcium levels thyroid hormone deficiency leads to a prolonged diastolic relaxation of the heart and prolonged relaxation of deep tendon reflexes (Dillmann, 1998; Rohrer et al., 1991; Morkin, 1993). The thyroid hormone is an important modulator of intermediary metabolism, as exemplified by hypercholesterolemia in hypothyroidism and weight loss associated with hyperthyroidism (Thompson and Strait, 1992; Crestani et al., 1994). The thyroid hormone influences four enzymes involved in lipid metabolism, namely malic enzyme, malate dehydrogenase, glucose 6-phosphatase and fatty acid synthetase (Oppenheimer et al., 1991). Na(+),K(+)-ATPase is a very important protein that regulates cellular stability by maintenance of the intracellular sodium and potassium concentration. This protein is also the driving force for importing and exporting other ions into cells. To catalyze the ionic balance in cells Na(+),K(+)-ATPase needs energy in the form of ATP. The thyroid hormone stimulated increase in Na(+),K(+)-ATPase expression therefore contributes to an increased thermogenesis of tissues (Ismail-Beigi, 1992; Paire et al., 1998). Furthermore, thyroid hormones are involved in the expression of mitochondrial uncoupling proteins (UCPs) and growth hormone and fulfill a crucial role in fetal brain development (Hetzel, 1980; Isaacs et al., 1987; Lanni et al., 2003). Sufficient dietary iodine supply also fulfills a crucial role in bone development (Quarto et al., 1992; Ishikawa et al., 1998).

6.4. Iodine deficiency and requirement in various animal species

Symptoms of iodine deficiency in piglets born to iodine-deficient sows are thickened skin, puffy necks, hairlessness and a bloated appearance. Some of the piglets are born dead, others are alive but weak and usually die within a few hours. At necropsy the thyroid is enlarged and

hemorrhagic. Iodine deficiency can cause an approximately 6-fold enlargement of the thyroid gland (Schoene, 1999). A sufficient iodine supply for sows is important for a normal peri- and postnatal development of the piglets (Schoene et al., 2001). For growing pigs the amount of iodine required increases with increasing body weight (Schoene, 1993) such that for growing pigs the requirement is met with dietary concentrations of 0.15 mg iodine/kg diet but 0.50–0.60 mg/kg diet is required for breeding sows and boars (NRC, 1998).

Goitre in newborn calves is a sign of borderline or definite iodine deficiency, even though the cows may appear normal. Thyroid glands four to five times the normal size sometimes occur in calves, but the abnormality cannot be detected definitely until the calf is slaughtered. Depending on the severity of the iodine deficiency calves may also be born blind, hairless, weak or dead (McCoy et al., 1997). Long-term deficiencies can result in metabolic changes, depressed growth, decreased milk yield and reduced fertility in female and male animals (Morrow, 1980; Slebodzinski, 1986; Mawson et al., 1994a, 1994b; Zagrodzki et al., 1998). Iodine deficiency may occur in cattle even at recommended iodine intake levels. If one quarter of the feed used is from goitrogenic crops, iodine requirement increases to 0.5 mg/kg for growing and nonlactating cattle, and 1.0 mg/kg for gestating and lactating cows. Dairy cattle require more iodine than beef cattle because approximately 10–25% of the iodine intake is secreted into the milk (mean concentration 0.07 mg iodine/kg milk). To ensure that the iodine requirement of the high-yielding dairy cow is met, it is recommended that 0.5 mg iodine/kg DM is supplied (GfE, 2001).

In newborn lambs, the most common symptom of iodine deficiency is goitre, which the lambs may survive if the condition is not too advanced. Other symptoms which may be encountered include lambs born weak, dead or without wool (Katelige et al., 1978). Mature sheep seldom show a change in their appearance, but iodine deficiency may result in reduced yield of wool and reduced conception rate (Hidiroglou, 1980; Potter et al., 1980; Sokkar et al., 2000). Depending on the physiological status of the animal an iodine intake of 0.1–0.8 mg/kg diet is recommended for sheep (NRC, 1985). In goats, iodine-deficient kids with goitre as well as weak and dead kids have been reported at birth. The recommended iodine concentration for goats is 0.3–0.8 mg/kg DM (GfE, 2003).

Goitres also occur as a sign of iodine deficiency in poultry and the thyroid gland can grow to many times its normal size. Histological examination of the thyroid shows hyperplasia and the absence of colloid. Iodine deficiency in breeding hens results in reduced egg iodine levels, reduced egg production, decreased hatchability, prolonged hatching time and thyroid enlargement in the embryos (Akiba and Matsumoto, 1973; Decuypere et al., 1982; Carew et al., 1997). Dietary supply of 0.40–0.50 mg iodine/kg DM is recommended to meet the requirement of poultry (GfE, 1999).

In laboratory animals, e.g. rats and mice, a dietary iodine deficiency is characterized by changes in biochemical parameters of thyroid metabolism, the development of a goitre as well as the genesis of thyroid gland carcinomas and reduced growth and fertility (Kanno et al., 1992; Mitchell et al., 1996, 1998; Liu et al., 2001b). For the prevention of deficiency symptoms in all physiological states 0.15 mg iodine/kg diet is recommended for laboratory animals (NRC, 1995).

In the nutrition of all the above-mentioned animal species attention must be paid in particular to the dietary concentrations of goitrogenic glycosides and nitrate, which inhibit iodine uptake and utilization by the thyroid gland (Jahreis et al., 1987; Mawson et al., 1994a, 1994b; Szokeova et al., 2001; Doerge and Sheehan, 2002; Laurberg et al., 2002). Furthermore, consideration must be given to the physiological interaction of iodine with selenium, which is responsible for the activity of the iodothyronine deiodinases and glutathione peroxidases (Beckett et al., 1991; Koehrle, 1996, 1999, 2000a, 2000b).

7. SELENIUM

7.1. Introduction

Selenium (Se) belongs to the chalcogens and many of its chemical properties (outer valence shell electronic configuration, atomic size, bond energy, ionization potential, electronegativity) are similar to that of sulfur. Like sulfur, selenium occurs in the oxidation states –II (selenide), 0 (elemental selenium), +IV (selenite), and +VI (selenate). In its elemental oxidation state (0) selenium forms red crystals with a hexagonal ring structure (Römpp, 1993). Besides the above-mentioned similarities between selenium and sulfur there exist nevertheless some important differences between the two elements with regard to the chemistry of their oxyanions and in the acid strengths of their hydrides. Since Se compounds tend to be metabolized to more reduced states and sulfur compounds tend to be metabolized to more oxidized states, the following reaction between quadrivalent selenium and quadrivalent sulfur is the basis for many reactions in biological systems:

$$H_2SeO_3 + H_2SO_3 \rightarrow Se + 2\ H_2SO_4 + H_2O \text{ (Combs and Combs, 1986)}$$

Selenium, originally discovered by the Swedish chemist Jakob Berzelius in 1817, has been known to cause major health problems in livestock and man for more than a century. Livestock disorders, commonly referred to as alkali disease or blind stagger disease, were recognized to be endemic in areas with soil types that are rich in selenium. Selenium overexposure was identified as the underlying cause of these diseases by reproducing the symptoms (of intoxication) in experimental animals by supplying excess selenium (Hutton, 1931; Franke, 1934a, 1934b, 1934c, Franke, 1935a, 1935b). In horses, selenium intoxication is so characteristic that it could be diagnosed from historical nonscientific literature. In 1295 Marco Polo reported that, during his famous journey from Venice to China, his horses suffered from a typical necrotic hoof disease after they had eaten poisonous plants, which are now known to be selenium accumulators (Polo, 1972). Selenium exposure was even considered to cause cancer. In short, for biologists and physicians selenium remained a nuisance for several decades and it was not until the 1950s that appreciation of selenium in biological sciences began to change. In 1954 it was observed that certain bacteria grew faster in selenium-fortified media (Pinsent, 1954). In 1957 two independent research groups showed that the trace element selenium is an important nutrient for animals. Factor 3 from pig kidney prevented rats fed a torula yeast-based diet from developing liver necrosis (Schwarz and Foltz, 1957). It soon turned out that factor 3 could be replaced by various selenium compounds. In chickens it could be demonstrated that addition of selenium to their diets prevented the development of exudative diathesis (Patterson et al., 1957). During the 1960s and the early 1970s various syndromes, such as white muscle disease in cattle and mulberry heart disease as well as hepatosis dietetica in pigs, were found to be consequences of dietary selenium deficiency (Martig et al., 1972; Sharp et al., 1972; Ammerman and Miller, 1975). In rabbits the role of selenium in preventing deficiency symptoms was controversial for a long time (Draper, 1957; Wiesner et al., 1978, 1981; Turan et al., 1997), but now there is good evidence that also in this species oxidative cellular damage during dietary selenium deficiency can be prevented by selenium supplementation and that cellular glutathione peroxidase is similarly regulated as in rats (Mueller and Pallauf, 2002; Mueller et al., 2002).

A possible biochemical explanation of the beneficial effects of selenium in animals came several years after the first evidence for its essentiality, when the trace element was discovered to be an essential component of the important antioxidant enzyme cellular glutathione

peroxidase or cytosolic glutathione peroxidase = GPx1 (Flohé et al., 1973; Rotruck et al., 1973). The discovery of three further glutathione peroxidases (gastrointestinal glutathione peroxidase = GPx2, plasma glutathione peroxidase = GPx3 and phospholipid hydroperoxide glutathione peroxidase = GPx4), expressed and located in different tissues as well as of the iodothyronine deiodinases 1–3, the thioredoxin reductases 1 and 2 and of the selenoproteins P and W as functional selenoproteins, were further important steps in understanding the physiological properties of selenium (Luthman and Holmgren, 1982; Motsenbocker and Tappel, 1982; Takahashi et al., 1987; Berry et al., 1991; Maiorino et al., 1991; Vendeland et al., 1993; Ursini et al., 1995, 1999; Koehrle, 1996). Selenium deficiency leads to a decreased expression of the above-mentioned selenoproteins and a decreased activity according to a hierarchical order. Moreover, current investigations suggest that dietary selenium deficiency is not only involved in the regulation of functional selenoproteins but that a number of other genes are differentially expressed, possibly due to an altered antioxidant status of cells during selenium deficiency (Fischer et al., 2001, 2003).

7.2. Selenium in feeds

The selenium concentrations in animal feeds and forages vary widely and depend on the plant species, the part of the plant, the selenium status of the soil, the soil pH value, and on the use of selenium-containing fertilizers (Milovac et al., 1998). Selenium concentrations in pastures are generally low (<0.05 mg/kg DM), but cereal grains and other seeds show a very broad range with regard to their selenium concentration, although this mainly depends on the soil status. Selenium concentrations as low as 0.006 mg/kg DM have been reported for the most important cereals (barley, wheat, rye) from selenium-deficient areas in Sweden and New Zealand, while values up to 3.06 mg/kg DM can be found in these cereals when they are derived from certain Se-rich areas in North America (Combs and Combs, 1986; Oster and Prellwitz, 1989; Huang and Clausen, 1994; Jukola et al., 1996; Underwood and Suttle, 1999). Fish meals from sea fish are generally rich selenium sources (2.0–6.2 mg selenium/kg). Meat meal from nonmarine species can also serve as relatively good selenium sources, but the selenium concentrations reported (0.1–1.2 mg selenium/kg DM) are much more variable (Cantor and Tarino, 1982; Wen et al., 1997; Underwood and Suttle, 1999).

7.3. Metabolism and biochemical functions of selenium

In animal feeds selenium is present in two major forms. Feedstuffs derived from animal sources mainly contain selenium in the form of selenocysteine, from functional selenoproteins, while selenium in plants is present predominantly as selenomethionine. Selenomethionine- and selenocysteine-containing selenium yeasts and inorganic selenium salts (sodium selenite and sodium selenate) are further important selenium sources in animal nutrition (Wen et al., 1997). Selenium from the various dietary sources is absorbed by individual mechanisms in the jejunum and ileum of mammals. The amino acid derivatives selenomethionine and selenocysteine use the same carriers as their sulfur analogs methionine and cysteine (Wolffram et al., 1989). Selenate uses for its absorption a sodium–sulfate cotransporter, which is driven by the activity of Na(+),K(+)-ATPase at the basolateral enterocyte membrane (Wolffram et al., 1985). Prior to its absorption, one part of selenite reacts in the lumen with glutathione and other thiols to form selenotrisulfides, which are presumably taken up into the enterocytes by amino acid transporters. Another part of selenite diffuses through the apical membrane and reacts with thiols in the cytosol of the enterocytes. All the above-mentioned

selenium compounds are absorbed to a high extent (>85%) from dietary sources, but differences exist in the rate of absorption. As a result of the upstream selenotrisulfide synthesis, selenite absorption is slower than that of selenate or selenomethionine (Wolffram et al., 1985). Subsequently, the selenocompounds are liberated at the basolateral enterocyte membrane into the bloodstream and are distributed to the various peripheral tissues. The exact transport mechanism for the various selenium compounds is as yet not fully understood. Selenomethionine associates with hemoglobin, while selenate and the remaining free selenite are found to be transported with α- and γ-globulins (Beilstein and Whanger, 1986a, 1986b; Suzuki et al., 1998). The liver and the kidney are the two main organs in selenium metabolism and they exhibit the highest selenium concentrations within the mammalian body (Behne and Wolters, 1983). Table 9 summarizes typical selenium concentrations in the various tissues of mammals.

Selenomethionine is the only selenium compound which can be incorporated unspecifically into proteins instead of its sulfur analog methionine. Further cellular metabolism of all other selenium compounds requires a stepwise, glutathione-dependent reduction to the selenide oxidation state (–II). Part of the reduced selenium can be excreted from the body by the generation of dimethylselenide (excretion via exhalation through the lungs) and formation of the trimethylselenonium ion (excretion via the urine). The remaining reduced selenium is needed for selenocysteine synthesis (Foster et al., 1986; Hassoun et al., 1995).

In the active center of functional mammalian selenoproteins, selenium is conserved as selenocysteine. Remarkably, selenocysteine is encoded by the UGA-STOP-codon in the messenger RNA (mRNA) of functional selenoproteins and synthesized by a cotranslational reaction mechanism proceeding from a specific serine transfer RNA population (tRNA Ser Sec) and selenium in the reduced oxidation state –II (Sunde and Evenson, 1987; Böck et al., 1991; Park et al., 1997). The steering of these complex processes is implemented by the SECIS element (selenocysteine incorporating structure) located in the 3′ untranslated mRNA region of functional selenoproteins (Imai et al., 1995; Walczak et al., 1997). Table 10 summarizes the currently known functional selenoproteins and their individual functions.

As described earlier, the glutathione peroxidase selenoenzyme family consists of four members, GPx1, GPx2, GPx3 and GPx4. GPx1 and GPx2 are homotetrameric proteins consisting of 22 kDa subunits, while GPx4 exists as a 19 kDa monomer. GPx3 is a glycosylated tetrameric protein. All glutathione peroxidases share several common features, as they catalyze the glutathione-dependent detoxification (fig. 7) of hydrogen peroxide and other peroxides (lipid peroxides and hydroperoxides of hydrocarbons). However, each member of this family of selenoenzymes possesses several distinct characteristics (Brigelius-Flohé, 1999).

GPx1 is the glutathione peroxidase with normally the highest activity, which is expressed in all mammalian tissues, and it is assumed that GPx1 contributes to a large extent to the maintenance of cellular antioxidant balance. During dietary selenium deficiency GPx1 expression and activity decrease the most rapidly compared to the other glutathione peroxidases. GPx2, the gastrointestinal form, has been postulated as protecting the organism against foodborne hydroperoxides, though without convincing evidence. Its peculiar cellular and subcellular distribution points to more specific functions, such as regulating proliferation and apoptosis of the gastrointestinal epithelium (Brigelius-Flohé et al., 2001). Due to a lack of reduced glutathione in blood plasma, GPx3 is likewise able to detoxify peroxides using glutaredoxin and thioredoxin (Takahashi et al., 1987). GPx4 has been discovered to be an enzyme protecting biomembranes against oxidative destruction. More recent studies suggest an important role of GPx4 in immune reactions, since the enzyme is involved in the regulation of leukotriene synthesis. The suppression of interleukin-1-triggered activation of NF-κB, alleviation of the proliferative response of oxidized LDL, inhibition of apoptosis by various stimuli and involvement

Table 9

Reported selenium concentrations for different animal tissues (mg/kg)

Species	Dietary Conditions	Liver	Kidney	Heart	Skeletal muscle	Reference
Female Wistar rats, average body weight 272 g, *n*=5	Standard lab chow with a Se concentration of 0.3 mg/kg as sodium selenite	∅ 1.29	∅ 1.49	∅ 0.37	∅ 0.16	Behne and Wolters, 1983
Male Sprague Dawley rats, average body weight: 500 g, *n*=6	Torula yeast based lab chow with a Se addition of 0.5 mg/kg as sodium selenite	∅ 1.45	–	∅ 0.30	∅ 0.18	Matsuda et al., 1998
Male Sprague Dawley rats, average body weight: 300 g, *n*=10	Lab chow according to AIN-76 containing 0.1 mg Se/kg	1.60 ± 0.03	2.05 ± 0.14	–	0.19 ± 0.01	Ip and Lisk, 1994
Pigs (male castrates), average body weight: 95 kg, *n*=5	Diet based on barley and wheat with the addition of 0.1 mg Se/kg or	∅ 0.63	∅ 1.13	∅ 0.12	∅ 0.062	Mahan and Moxon, 1978
	0.4 mg Se/kg as sodium selenite	∅ 0.88	∅ 1.22	∅ 0.19	∅ 0.094	
Female sheep, slaughter weight, *n*=5	Cereal and soya based diet with a Se addition of 0.1 mg/kg as sodium selenite	∅ 0.38	∅ 1.26	–	∅ 0.09	Ullery et al., 1978
6 month old female Merino crossbred sheep, *n*=8	Corn starch and casein based diet with a Se addition of 0.2 mg/kg as sodium selenite	0.06 ± 0.02	2.1 ± 0.02	0.24 ± 0.12	0.11 ± 0.04	Hudman et al., 1988
Bulls (Holstein), average body weight: 486 kg, *n*=8	Diet based on forage, silage and cereals with a selenium addition of 0.1 mg/kg diet	∅ 0.98	∅ 1.37	–	∅ 0.13	Angelow and Yancher, 1991

Table 10

Functional selenoproteins of mammals containing selenium in a selenocysteine residuum (adapted from Gladyshev and Hatfield, 1999)

Selenoprotein	Function
1. Glutathione peroxidase 1 = GPx1 (cellular glutathione peroxidase, cytosolic glutathione peroxidase)	Glutathione-dependent hydroperoxide removal
2. Glutathione peroxidase 2 = GPx2 (gastrointestinal glutathione peroxidase, GPxGI)	Glutathione-dependent hydroperoxide removal of intracellular and foodborne hydroperoxides
3. Glutathione peroxidase 3 = GPx3 (plasma glutathione peroxidase, pGPx)	Glutathione-dependent hydroperoxide removal, antioxidant
4. Glutathione peroxidase 4 = GPx4 (phospholipid hydroperoxide glutathione peroxidase, PHGPx)	Phospholipid hydroperoxide removal
5. Thyroid hormone deiodinase 1 = D1 (Monodeiodinase 1, MDI1)	Conversion of T4 to T3, inactivation of T3 and T4
6. Thyroid hormone deiodinase 2 = D2 (Monodeiodinase 2, MDI2)	Conversion of T4 to T3
7. Thyroid hormone deiodinase 3 = D3 (Monodeiodinase 3, MDI3)	Inactivation of T3 and T4
8. Thioredoxin reductase 1 (TR1)	NADPH-dependent reduction of thioredoxin
9. Selenophosphate synthetase 2 (SPS2)	Synthesis of selenophosphate
10. Selenoprotein P (SelP)	Antioxidant, selenium storage, distribution of selenium between tissues?
11. Selenoprotein W (SelW)	Redox function, especially in muscle, selenium storage in muscle?
12. 15 kDa selenoprotein	Protein folding?

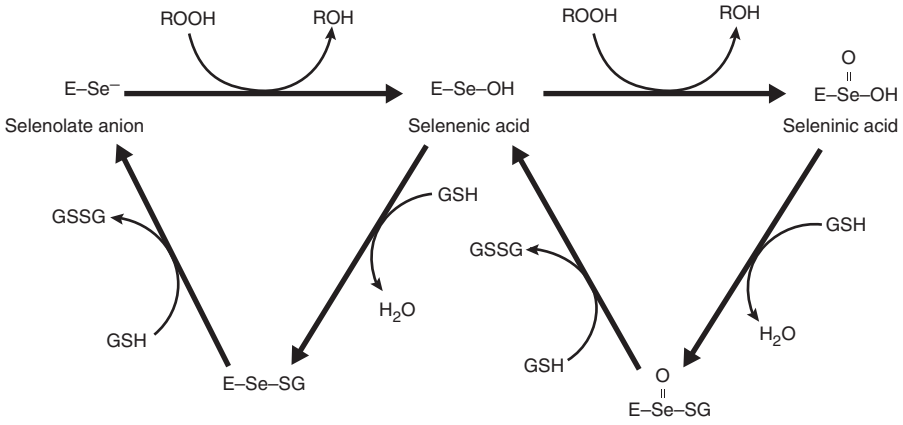

Fig. 7. Reaction mechanism of glutathione peroxidase (Ganther and Kraus, 1984). At physiological pH values selenium in glutathione peroxidase is present as a selenolate anion. The reduction of a peroxide effects the oxidation to the selenol oxidation state (selenenic acid). Starting from the selenol oxidation state, either the selenolate anion is formed via the glutathione coupled two-step regeneration (reduction), or the oxidation state of seleninic acid is reached by detoxification of a further peroxide and oxidation of the selenol.

in chromatin condensation are further properties of GPx4 (Schuckelt et al., 1991; Brigelius-Flohé, 1999). Moreover, GPx4 fulfills an interesting role with regard to male fertility. During spermatogenesis GPx4 changes from an active enzyme in spermatids into a major structural protein of the mitochondrial capsule of the mature spermatozoa (Ursini et al., 1999).

The iodothyronine deiodinases 1–3 (MDI 1–3) are a family of three oxidoreductases that regulate the activity of thyroxine by catalyzing removal of iodine from the inner (5-iodine) or outer (5´-iodine) ring of this hormone (Koehrle, 1996). MDI1 is a monomeric 29 kDa enzyme that is capable of deiodinating both the inner and the outer ring of T4, thus MDI1 can convert T4 to the active hormone T3 as well as inactivating both T4 and T3. MDI1 is located in the thyroid, liver, kidney and central nervous system and as a thyroid-responsive selenoenzyme it is mainly responsible for keeping the balance of plasma T3 and T4 concentrations (Berry et al., 1991). MDI2 is a 30.5 kDa selenoprotein that catalyzes the conversion of T4 to T3. It is found exclusively in the brain and in the pituitary gland and is responsible for the local supply of the brain and pituitary gland with receptor-bound T3 (Croteau et al., 1996). MDI3 is a 31.5 kDa selenoprotein that inactivates T3 and T4 by removal of iodine from the inner ring of either substrate. MDI3, like MDI1, is a thyroid-responsive enzyme. It mainly occurs in the placenta, the central nervous system and the skin, with the highest levels expressed in the placenta and therefore functions by impairing access of T3 and T4 to the fetus, thus playing a crucial role in fetal development (Koehrle, 1996).

Mammalian thioredoxin reductase 1 (TR1) was first characterized in the late 1970s but until 1996 it was not recognized as a selenoprotein. TR1 is a homodimeric protein consisting of two identical 55 kDa subunits, each containing a flavin adenine dinucleotide cofactor. In contrast to most other selenoproteins, the selenocysteine residue of TR1 is located in the C-terminal region of the protein (Luthman and Holmgren, 1982; Holmgren, 2000). The major function of TR1 is to catalyze the NADPH-dependent reduction of thioredoxin in the cytosol. In addition, TR1 is involved in a variety of further processes including the reduction of glutathione peroxidase, disulfide isomerase, dehydroascorbate, ascorbyl free radical, selenite, selenodiglutathione, hydroperoxides and other proteins and compounds (Bjornsted et al., 1995; May et al., 1997, 1998). TR2 is preferentially expressed in the testes, while TR3 contains a mitochondrial targeting signal and is expressed in various cell types (Rigobello et al., 1998). Selenoprotein P (SelP) is a 57 kDa glycoprotein and represents the major selenoprotein in the plasma of mammals. It contains 9–12 selenocysteine residues which are all encoded by the UGA code. SelP is rapidly expressed when selenium-depleted animals are resupplemented with selenium. More than 90% of SelP is synthesized in the kidney and released into the plasma. It has been suggested that the selenoprotein P serves as an antioxidant in plasma as well as a selenium storage protein, which distributes selenium amongst the organs (Moschos, 2000).

Selenoprotein W is an 8 kDa selenoprotein with an as yet unknown function. The protein is expressed in many tissues, with increased levels in the muscle and brain. A further selenoprotein of importance is the 50 kDa enzyme selenophosphate synthetase, which activates selenide to selenophosphate during the cotranslational synthesis of selenocysteine in all other functional selenoproteins (Vendeland et al., 1993; Burk and Hill, 1999).

7.4. Selenium deficiency and requirement in various animal species

Selenium deficiency leads to a down-regulation of the transcript levels of the functional selenoproteins, in a tissue- and selenoprotein-specific manner (Lei et al., 1995; Allan et al., 1999; Wingler et al., 1999). During dietary selenium deficiency the activities of GPx4 and GPx2 are considerably less altered than the activities of GPx1 and GPx3 (Cheng et al., 1997a, 1997b; Lei et al., 1998; Wingler et al., 1999). Recent molecular biological investigations in

rats, using the microarray technique, have shown that selenium deficiency does not only affect the expression of functional selenoproteins, but also the expression of further genes like UDP-glucuronosyl transferase 1 and bilirubin UDP-glucuronosyltransferase 2. These genes are involved in hepatic xenobiotic metabolism. Further, the expression of arachidonate 12-lipoxygenase (ALOX12), an antagonist of GPx4 in hydroperoxide metabolism, was influenced in selenium deficiency (Fischer et al., 2001, 2003).

Selenium and vitamin E exhibit some similarities with regard to their deficiency symptoms. These may derive from some overlaps in their antioxidative functions. Sudden death is a prominent feature of selenium deficiency in pigs. Gross necropsy lesions, caused by selenium deficiency, are identical to those from vitamin E deficiency (Money, 1970) and include massive hepatic necrosis and edema of the spiral colon, lungs, subcutaneous tissues and submucosa of the stomach. Bilateral paleness and dystrophy of the skeletal muscles (white muscle disease) are often found. Occasionally, mottling and dystrophy of the myocardium (mulberry heart disease) are also observed (van Vleet and Ferrans, 1977; Korpela, 1990). Mulberry heart disease in pigs is most common when cereal-based diets contain less than 0.05 mg selenium/kg. Recent research showed that adding 0.15 mg or 0.30 mg selenium/kg diet from sodium selenite from late gestation to day 14 of lactation increases milk selenium content and serum selenium concentration in nursing pigs compared to the unsupplemented controls. Organic selenium sources (selenium yeast) increase milk selenium 2.5–3.0-fold higher than sodium selenite (Mahan, 2000). Selenium supplementation of the sow reduces the incidence of mulberry heart disease in the young pig. The incidence and degree of selenium deficiency may be increased by environmental stress. Selenium recommendations for pigs vary widely. Due to the role of selenium in GPx4 an adequate selenium supplementation of boars is necessary to obtain an optimum development of Sertoli cells and sperm maturation (Marin-Guzman et al., 2000; Foresta et al., 2002). Depending on the physiological status, recommendations range from 0.15–0.30 mg selenium/kg diet (NRC, 1998). The results of a recent study in which graded selenium concentrations from 0.03–0.30 mg selenium/kg diet were fed to growing pigs suggest that a selenium supplementation of 0.20 mg selenium/kg diet for growing pigs is necessary to reach a maximum activity of the different selenoenzymes and an optimum antioxidative protection (Fischer et al., 2002; Pallauf et al., 2002).

A major symptom of selenium deficiency in cattle is white muscle disease, which usually occurs in young calves. This causes chalky white striations, degeneration, and necrosis in cardiac and skeletal muscles. Heart failure, paralysis (usually of the hind legs), a dystrophic tongue and elevated SGOT (serum glutamic oxaloacetic transaminase) values may also be evident (Hoshino et al., 1989). Selenium deficiency is also implicated in mastitis, since it has been reported that selenium injections reduced the duration, but not the incidence, of mastitis when dietary selenium was deficient (Smith et al., 1997b). Other symptoms include unthriftiness, growth depression, diarrhea, retained placenta, and lower reproductive efficiency with increased services per conception and birth of premature, weak, and dead calves (Julien et al., 1976; Bostedt and Schramel, 1990; Zust et al., 1996; Hemingway, 2003). Part of the reason why ruminants are so susceptible to selenium deficiency is that absorption is less efficient and more variable than in nonruminants. Most of the selenium that is ingested leaves the rumen attached to the cell membranes of bacteria. Part of these cell membranes pass through the small intestine unabsorbed (Serra et al., 1997). Therefore, the true selenium absorption in ruminants can be distinctly lower than in monogastric animals. The dietary recommendations are 0.15 mg Se/kg DM for growing cattle and 0.20 mg Se/kg DM for dairy cows (GfE, 2001).

Selenium deficiency in sheep has serious effects on lamb production. The manifestations are reduced growth and white muscle disease, which affects lambs at 2–8 weeks of age

(Bostedt and Schramel, 1990; Bickhardt et al., 1999). White muscle disease in lambs is preventable by specific selenium treatment of ewes (Zachara et al., 1992). However, the overall implications are probably much more severe than this, since some studies have shown that a lack of selenium causes high embryonic mortality, infertility and high lamb mortality (Bickhardt et al., 1999). The selenium requirement of sheep is given as 0.10–0.20 mg selenium/kg diet (NRC, 1985). For goats 0.1–0.2 mg/kg DM are recommended (Meshy, 2000; GfE, 2003).

The main symptom of selenium deficiency in poultry is exudative diathesis (Hassan, 1987). This disease is characterized by edema of the breast, wing and neck regions. This edema is caused by an abnormally high permeability of the capillary walls, that allows fluid to accumulate between the muscle and skin. In broilers fed low-selenium grains, chicks begin to display symptoms between 3–6 weeks of age with weight loss, leg weakness and eventual death. With severe selenium deficiency, the growth rate is reduced and mortality increased, even in the presence of adequate vitamin E (van Vleet and Ferrans, 1976; Hassan et al., 1987, 1990; Mezes et al., 1997; Jianhua et al., 2000; Avanzo et al., 2001). Pancreatic fibrosis and a reduction in pancreatic output of lipase, trypsinogen and chymotrypsinogen are also associated with selenium deficiency. Pancreatic lesions can occur as early as 6 days of age and usually return to normal within 2 weeks of selenium supplementation (Sinclair et al., 1984). In laying hens egg hatchability is the most sensitive criterion of selenium deficiency (Cantor and Scott, 1974; Latshaw et al., 1977). Encephalomalacia, membrane lipid peroxidation, erythrocyte hemolysis and muscular dystrophy are alleviated or prevented by selenium (Mezes et al., 1997). To meet the selenium requirement of poultry 0.10–0.15 mg selenium/kg DM are recommended (NRC, 1994).

Laboratory animals respond to selenium deficiency with symptoms of oxidative damage in tissues. In rats the liver is the organ most affected by dietary selenium deficiency and here increased oxidation of cellular lipids and proteins finally results in liver necrosis. Comparable symptoms have also been reported for mice (Matsuda et al., 1998; Reddy et al., 1998; Cheng et al., 1999; Hininger et al., 2002) and in addition, selenium-deficient mice are more susceptible to infections from the coxsackie virus, which leads to the development of myocarditis (Beck et al., 2003).

In rabbits the existence of symptoms of selenium deficiency, independent of vitamin E deficiency, has long been controversial (Draper, 1957; Wiesner et al., 1978, 1981; Turan et al., 1997). However, in more recent studies it could be clearly demonstrated that selenium plays an essential role in the prevention of lipid and protein oxidation also in the rabbit. Moreover, it was shown that in Se deficiency the functional selenoproteins GPx1 and GPx3 are downregulated to a comparable extent in rabbits as in rats and mice (Mueller and Pallauf, 2002; Mueller et al., 2002). The recommendation for selenium intake in rats and mice is 0.15 mg/kg diet. For an optimum reproduction in rats a higher amount of selenium (0.40 mg/kg diet) is recommended (NRC, 1995). For rabbits there is so far no recommendation on selenium requirement available, however, recent studies indicate that growing rabbits require 0.10–0.20 mg Se/kg diet (Pallauf and Blind, 2002; Pallauf, 2003).

8. FURTHER TRACE ELEMENTS: COBALT, CHROMIUM, MOLYBDENUM AND NICKEL

8.1. Cobalt

Elemental cobalt is a steel-gray, gleaming magnetic metal with a melting temperature of 1495°C. Cobalt is harder than steel and extremely ductile. At room temperature cobalt is resistant to oxygen and water, but it is soluble in diluted acids. In nature cobalt mainly occurs in the form of cobalt–nickel ores. The main oxidation states of cobalt salts are +II and +III

(Römpp, 1990). The nutritional essentiality of cobalt was first shown in sheep and cattle on account of its ability to prevent two naturally occurring diseases in these species, namely coast disease in sheep (Marston, 1935) and masting disease or enzootic marasmus in cattle (Underwood and Filmer, 1935). That the observed cobalt deficiency symptoms were actually the consequence of a vitamin B_{12} deficiency was established in 1948 when vitamin B_{12} was characterized as a cobalt-containing compound (Smith, 1948, 1952). Consequently, it was observed that the administration of vitamin B_{12} to ruminants suffering from cobalt deficiency produced the same beneficial effects as could be obtained with dietary cobalt treatment. Vitamin B_{12} synthesis in ruminants is effected by microbes in the rumen. Only 15% of dietary cobalt is converted by ruminal bacteria into the active form of vitamin B_{12} (cobalamin), which is available to the animal (Somers and Gawthorne, 1969; Raux et al., 2000). A much greater amount of dietary cobalt is wasted for the synthesis of biologically inactive vitamin B_{12} metabolites (corrinoids) (Perlmann and Toohey, 1966). With the exception of microorganisms in the large intestine, nonruminants are not able to synthesize vitamin B_{12} from cobalt and therefore a cobalt deficiency does not occur, although vitamin B_{12} deficiency may be present in these species. Cobalt concentrations in animal feeds vary widely. Forages from different locations were reported to contain 0.02–0.22 mg cobalt/kg dry matter. Cereal grains are poor cobalt sources (0.01–0.06 mg/kg DM) and can be used to formulate cobalt-deficient diets. In both ruminants and nonruminants vitamin B_{12} is absorbed from the small intestine, predominantly from the ileum. Prior to its absorption cobalamin is coupled to an intrinsic factor (IF), which derives from the abomasum of ruminants and the stomach of nonruminants (Gallagher and Foley, 1970; Roussev et al., 1975). At the serosal enterocyte membrane cobalamin is released into the plasma and is bound to transcobalamins. To date three different transcobalamins (T0, TI and TII) have been characterized in mammals (Hayem-Levy et al., 1971; Dohmann et al., 1974). The liver and kidney are the organs with the highest vitamin B_{12} concentrations. In the intermediary metabolism of ruminants and nonruminants two distinct forms of vitamin B_{12} are involved in contrasting biochemical reactions. As methylcobalamin (MeCbl) cobalt assists a number of methyltransferase enzymes by acting as a methyl group donor. Methylcobalamin is important for ruminal bacteria as well as for animals, being involved in the synthesis of acetate, methane and methionine by ruminal bacteria. In the organism methylcobalamin donates methyl groups via activated methionine to a wider range of molecules, including formate, noradrenaline (norepinephrine), myelin and phosphatidylethanolamine (Akesson et al., 1978; Marsh, 1999; Bandarian et al., 2003). As adenosylcobalamin (AdoCBl) cobalt influences energy metabolism, facilitating the formation of glucose by assisting methylmalonyl coenzyme A mutase to form succinate from propionate, mainly in the liver (Morrow and Lebowitz, 1976; Banerjee and Vlasie, 2002).

Thus, cobalt deficiency in ruminants affects both ruminal metabolism and intermediary metabolism. Clinical manifestations of cobalt deficiency in ruminants include loss of appetite, pica, weight loss succeeded by rapid muscular wasting (marasmus) and different forms of severe anemia, e.g. anemia perniciosa. The liver frequently exhibits a fatty degeneration whereas body fat in other tissues is nearly absent. Comparable symptoms in nonruminants can be attributed to a vitamin B_{12} deficiency (Robertson, 1971; Mahin and Lamand, 1982; Suttle, 1988). For fast-growing cattle and dairy cows a cobalt concentration of 0.20 mg cobalt/kg DM is recommended (Stangl et al., 2000; GfE, 2001).

8.2. Chromium

Elemental chromium is a silver-gleaming, ductile metal with a melting temperature of 1857°C. At normal temperatures elemental chromium is resistant to oxygen and water. In the

presence of oxidizing acids chromium forms a robust chromium oxide coating. On the Earth's crust chromium mainly occurs in the form of chromium–iron ores. Chromium exists in the oxidation states +II, +III, +IV, +V and +VI, the principal states being +III and +VI. Chromium salts in these oxidation states are frequently colored bright orange and green (Römpp, 1989). The essentiality of chromium for mammals was originally discovered in 1959, when it could be demonstrated that trivalent chromium improved glucose tolerance in rats (Schwarz and Mertz, 1959; Mertz, 1975). Similar results were obtained in a recent study. With new cellular biological methods it could be demonstrated that chromium supply from chromium picolinate (80 µg/kg diet) to obese rats enhanced translocation of GLUT 4 vesicles in the skeletal muscle of the animals and therefore led to ameliorated glucose tolerance. In addition, total cholesterol levels in the chromium-treated rats were half as high as in untreated rats (Cefalu et al., 2002). In quails increasing chromium supplementation (0–1200 µg chromium/kg diet) had positive effects on body weight, feed intake, feed conversion, egg production and egg shell thickness (Sahin et al., 2002). A better performance and an increased weight gain, derived from dietary chromium supplementation, were also reported for fattening bulls (Pechova et al., 2002). In livestock feeds chromium concentrations show a wide variation (0.01–4.20 mg chromium/kg DM), with cereals relatively poor and legumes relatively rich in chromium (Underwood and Suttle, 1999). Recently, palm leaves have been reported to be a rich chromium source (Pillay et al., 2003). Chromium concentrations reported for animal feeds have steadily decreased over the last 30 years due to improvements in analytical techniques and lowered chromium emission from stainless steel production. In plants chromium forms organic complexes with different molecular weights. One of the most intensely studied organic chromium complexes is the chromium-dinicotinic acid–glutathione complex. This complex was originally isolated from brewer's yeast and named the glucose tolerance factor (GTF) because of its beneficial effects on glucose metabolism in mammals (Zetic et al., 2001).

Chromium from inorganic sources is absorbed very poorly. This is one reason for the use of chromium oxide (Cr_2O_3) as an indigestible marker in experiments on the digestibility of other nutrient compounds (Walz and Pallauf, 1993; Bernabucci et al., 1999; Stein et al., 1999; Kadim et al., 2002). Currently no recommendations for chromium supply can be found in the literature for farm animals. A chromium addition of about 20 µg/kg diet is recommended for purified diets of laboratory rats and mice (NRC, 1995; Reeves, 1997).

8.3. Molybdenum

Elemental molybdenum is a silver-white heavy metal with an extremely high melting temperature of 2617°C and is very resistant to oxygen and acids. Molybdenum occurs in the oxidation states +II, +III, +IV, +V and +VI (Römpp, 1991). In nitrogen-fixing organisms molybdenum plays a crucial role in the enzyme nitrogenase, which converts elemental nitrogen to ammonia. Recent investigations have given a detailed insight into the reaction mechanism of molybdenum within nitrogenase (Leigh, 2003; Yandulov and Schrock, 2003). In sheep molybdenum was first found to influence copper metabolism and to reduce copper toxicity (Humphries et al., 1986; Barceloux, 1999; Ogra et al., 1999). With the discovery that the activity of mammalian xanthine oxidase depends on molybdenum, the essentiality of the trace element for animals could be established (de Renzo et al., 1954; Garattini et al., 2003).

The molybdenum concentration in animal feeds from plant sources varies widely and mainly depends on soil molybdenum concentration and further soil conditions. Most cereals contain about 1 mg molybdenum/kg DM, whereas pastures are reported to contain 1–60 mg

molybdenum/kg DM, depending on the location. Most animal proteins contain very low molybdenum concentrations with the exception of marine products and milk from ruminants in molybdenum-rich regions. Molybdenum from both inorganic and organic sources is highly bioavailable to animals. In monogastric species molybdenum absorption takes place in the stomach and in the small intestine and can be inhibited by sulfate (Cardin and Mason, 1976). In ruminants high dietary levels of copper and sulfur lead to the formation of insoluble thiomolybdates, thus reducing molybdenum and copper absorption drastically (Lamand, 1989; Judson and Babidge, 2002). Thiomolybdates have been found to influence trophic hormone release from the hypothalamo-adenohypophyseal system interfering with reproduction and intermediary metabolism (Haywood et al., 2004). The physiological relevance of molybdenum resides in its function as an essential cofactor of xanthine oxidase, aldehyde oxidase and mitochondrial sulfite oxidase. Molybdenum deficiency in various species was reported to lower the activities of these enzymes (Bray et al., 1982; Turner et al., 1995; Garattini et al., 2003). A further function of molybdenum is to enhance macrophage defenses (Panneerselvam and Govindasamy, 2003). Molybdenum concentrations as low as 0.025–0.1 mg/kg diet seem to be sufficient to achieve saturated molybdenum levels in various vertebrate tissues and optimum activities for the three molybdenum enzymes (Wang et al., 1992). Recently, distinct species differences were found in the molybdenum concentration of xanthine oxidase from milk (Atami et al., 2004).

8.4. Nickel

Nickel is a silver-white, easily deformable heavy metal with a melting temperature of 1453°C. Nickel is very resistant to oxygen and nonoxidizing acids. In compounds nickel most frequently occurs in the oxidation state +II (Römpp, 1991). A definite biochemical function for nickel in mammals has not yet been found. It is very difficult to induce an alimentary nickel deficiency under practical feeding conditions. For instance, in chickens signs of nickel deficiency, including depigmentation of the shank skin, thickened legs, swollen hocks, growth retardation, ultrastructural liver changes and anemia, were only observed when highly purified diets (nickel concentration: 2–15 µg/kg diet) were fed (Nielsen et al., 1974; Wilson et al., 2001). The addition of 50 µg nickel/kg diet reversed the symptoms. In growing rats and first-generation offspring, dietary nickel deficiency leads to diminished growth and fertility, anemia, and lowered activities of several enzymes of the citric acid cycle (Schnegg and Kirchgessner, 1975, 1977) as well as to altered fatty acid and cholesterol metabolism (Stangl and Kirchgessner, 1996). Furthermore, an interaction between nickel and iron metabolism has also been described (Talkvist et al., 1994). Ruminal microorganisms require nickel, which explains the changes in ruminal urease activity seen in dietary nickel deficiency. In ruminants nickel deficiency has also been associated with a reduced feed conversion, a depressed growth rate and lowered fertility (Hennig et al., 1978; Hausinger, 1986; Spears et al., 1986; Oscar and Spears, 1990).

Nickel concentrations ranging from 0.08–0.35 mg/kg have been reported for wheat, depending upon location (Underwood and Suttle, 1999). Cow milk contains 0.02–0.05 mg nickel/kg and its concentration is not influenced by feeding enhanced dietary nickel concentrations (Alanis Guzman and Castro Gongora, 1992). In mammals, nickel absorption from dietary sources ranges between 1–10%. Elemental nickel and insoluble nickel salts are absorbed very poorly, whereas soluble forms of nickel are more readily absorbed (Underwood and Suttle, 1999; Arnich et al., 2000). The nickel requirement in animal nutrition is estimated to be 50–60 µg/kg diet (Spears, 1984; Reeves, 1997).

9. ASSESSMENT OF TRACE ELEMENT REQUIREMENTS, ASPECTS OF BIOAVAILABILITY AND GENERAL CONCLUSIONS

In the first sections descriptions were given of the intestinal absorption mechanisms, biochemical functions and deficiency symptoms for trace elements in laboratory animals and farm animals. Furthermore, the current recommendations of different committees (GfE and NRC) for an adequate trace element supplementation were given. The recommendation of trace element supply in the diet consists of the scientifically derived requirement plus a certain safety margin to cover individual variations and practical eventualities.

In general, the estimation of trace element requirements can be deduced from two different approaches:

1. The optimum requirement of a trace element in an animal species can be established from dose–response studies, which should include one experimental group deficient in the trace element investigated, with all other nutrients and conditions non-limiting within the normal physiological range.
2. In the "factorial approach" the daily losses of the trace element, e.g. from feces, urine, body fluids and hair are determined for various physiological states and the requirement is calculated as the amount, necessary to compensate these losses, including a safety margin (Weigand and Kirchgessner, 1977a, 1977b, 1982; Ammermann et al., 1995; van Ryssen, 2000). Similarly, the net trace element needs for growth, reproduction and lactation have to be calculated. In order to estimate the gross requirements from the net requirements, it is crucial to know the value for the overall utilization of the trace element. This value is, however, not constant.

In the first approach the trace element requirements are a function of specific measures of response, frequently biochemical status parameters, e.g. for iron: hemoglobin concentration in whole blood, for zinc: plasma zinc concentration and the activity of alkaline phophatase in the serum, for selenium: plasma glutathione peroxidase activity, for iodine: T3 and T4 concentration in the plasma (Ammerman et al., 1995).

However in modern animal nutrition the criteria and critical values accepted as criteria for requirements are continuously changing because of the following reasons:

- The dramatic improvement in productivity of livestock deriving from the genetic improvement of farm animals and refinements in management, health control and general nutrition.
- Major advances in biochemical and molecular biological methods giving new insight into the physiological role and function of trace elements (van Ryssen, 2000).

A critical point with regard to the objective assessment of trace element requirements in various animal species arises from varying experimental conditions. On the one hand, the requirement for trace elements is strongly influenced by parameters such as age, sex and breed. On the other hand, the requirement for a trace element is considerably influenced by the dietary composition and especially by the dietary source from which the trace element is derived. Taking these aspects into consideration a somewhat controversial discussion of trace element supply with regard to "bioavailability" has taken place in recent years. Bioavailability, however, is a very complex topic rather than a clearly defined term. In the simplest case the bioavailability of nutrients and trace minerals corresponds to "absorbability" from the diet. Absorbability can be interpreted as the "apparent absorption" which calculates the difference between the intake of the trace element and its total fecal excretion, while the "true absorption" is corrected for that portion of the element, which has been absorbed and is subsequently excreted back into the gastrointestinal tract. In more comprehensive investigations

of "bioavailability", consideration is also taken of parameters such as growth response, bone development and inclusion of the trace element into bone matrix, tissue retention, synthesis and catalysis of proteins and enzymes depending on the trace element of interest, as well as further biochemical and molecular biological parameters (Ammerman et al., 1995).

When the "bioavailability" of trace elements is discussed at the level of "absorbability" two factors are important:

- The chemical form of the trace element (inorganic or organic compound).
- The presence of dietary factors, which inhibit or facilitate the absorption of the trace element.

An example of the latter is the bioavailability of zinc from natural sources, which in monogastric animals is drastically reduced in the presence of abundant phytic acid in cereals, legumes and other seeds. Feeding a phytate-rich maize–soya diet induced severe symptoms of zinc deficiency in growing pigs. But, the addition of highly available inorganic zinc, as zinc sulfate (70 mg/kg diet), to the diet abolished zinc deficiency symptoms and led to a normal development of the pigs. Zinc deficiency symptoms in growing rats and pigs could also be prevented by the addition of up to 1000 IU microbial phytase to their diets. The phytase addition caused an increase in apparent zinc absorption from under 25% to more than 45% in rats and from under 20% to more than 30% in pigs (Pallauf et al., 1992, 1994; Lei et al., 1993; Rimbach et al., 1995; Windisch and Kirchgessner, 1996). Moreover, the addition of citric acid to diets with a high molar ratio of phytic acid:zinc of 31:1, was found to enhance the availability of zinc to growing pigs and to reduce symptoms of zinc deficiency (Pallauf et al., 1990).

Besides complexing dietary components such as phytic acid, interactions of trace elements, e.g. iron, copper, zinc, manganese among each other and interactions with other minerals such as calcium can also affect the absorption and bioavailability of elements. This is because during their intestinal absorption and the absorption from tissues they compete with each other for the divalent cation transporter 1 (DCT1) (Lecoeur et al., 2002; Forbes and Gros, 2003). A further example of interactions of trace elements with each other and other dietary compounds leading to a reduced bioavailability, is the reduction of copper absorption and bioavailability with high dietary molybdenum concentrations due to the formation of thiomolybdate complexes from copper, molybdenum and sulfur in ruminants (Lamand, 1989; Underwood and Suttle, 1999; Judson and Babidge, 2002).

In some species a prolonged intestinal retention period, an optimized solubility, the more effective utilization of the known intestinal transport systems or the use of other transport mechanisms are possible reasons for a partially enhanced bioavailability of trace elements from amino acid derivatives and amino acid chelates in comparison to highly soluble inorganic compounds. Thus, it could be demonstrated that when fed to sheep and poultry, chelated zinc and zinc methionine possess a higher bioavailability than several inorganic zinc sources (Ammerman et al., 1995) (table 11).

The bioavailability of selenium is also a controversial topic. Studies on the mechanism of intestinal absorption of the inorganic selenium compounds selenite and selenate and for the organic selenium compound selenomethionine showed that each of the above-mentioned compounds possesses its own particular mechanism, by which it is almost completely absorbed (Wolffram et al., 1985, 1989).

Intraperitoneal injections of radioactive inorganic selenite and organic selenomethionine in rats led mainly to a rapid distribution of selenite to metabolic active organs, where it could be used for the synthesis of functional selenoproteins, while selenomethionine was incorporated unspecifically into peripheral proteins and therefore lowered the synthesis of biologically active functional selenoproteins (Beilstein and Whanger, 1986a, 1986b). Trials in rats and

Table 11

Relative bioavailability of zinc sources (Zn sulfate = 100) evaluated by different parameters of response (from Ammerman et al., 1995, reprinted with permission from Elsevier)

Source	Poultry	Pigs	Cattle	Sheep	Rats
Zn acetate					100
Zn chloride	100	100			
Zn sulfate	100		100	100	100
Zn carbonate	105		60		
Zn chelated				110	
Zn citrate					100
Zn elemental	100	130			
Zn lysine		100			
Zn methionine	125	100		100	
Zn oxide	100 (55)	50	100	70	
Zn piccolinate					105
Zn proteinate	100				
Zn sequestered				105	

pigs confirmed that, due to the action of glutathione peroxidase 1 and 3, there is a higher bioavailability and thus a higher tissue selenium concentration, by feeding inorganic selenocompounds than selenomethionine- or selenium-enriched plants (Mahan et al., 1999). In the case of a broader interpretation of bioavailability including constitutional aspects, it should be noted that a higher efficiency of natural organic selenium compounds could be demonstrated with regard to the prevention of colon cancer (Finley and Davies, 2001). Furthermore, in ruminants, unlike in monogastric animals, organic selenomethionine seems to possess a higher bioavailability than inorganic selenocompounds (Gunter et al., 2003). A relatively new approach, designed to optimize the selenium supply of ruminants, is the selenium fertilization of soils (van Mantgem et al., 1996; Lintschinger et al., 2000). Recent investigations into an optimized trace element nutrition of humans and animals recommend the generation of genetically modified plants in which storage proteins for the trace elements are overexpressed (Loennerdal, 2003).

In conclusion, the above-mentioned results show that in many animal species there is still a need for dose–response studies to obtain reliable data on the trace element needs. Data on dietary components which interact with individual trace elements are also an interesting field for future work. Modern biochemical and molecular biological methods can be helpful tools in searching for and evaluating new sensitive parameters which reflect the trace element supply of animals.

10. FUTURE PERSPECTIVES

This chapter has given an overview of the present knowledge of the most important trace elements and their physiological functions in laboratory animals and livestock. In the future trace element research should focus in particular on:

- aspects of bioavailability from inorganic and organic compounds
- new molecular mechanisms by which trace elements influence cellular functions and the immune system
- the prevention of environmental pollution as a possible outcome of trace element over-supply.

The bioavailability of certain divalent cations (e.g. Fe, Mn, Zn) clearly depends on the chemical form in which they are provided to the animals. In diets based on cereals these divalent cations are frequently poorly available, due to complex formation with phytic acid. Thus, in this field further research is necessary to optimize the availability of trace elements from natural sources and to find chemical compounds which are optimally available for the different species.

Taking selenium as an example, it can be seen that the different inorganic forms (selenite: oxidation state +IV and selenate: oxidation state +VI) and organic forms (selenomethionine and selenocysteine) are absorbed by fundamentally different mechanisms leading to differences in their cellular metabolism. These metabolic differences for the diverse selenium compounds can influence major cellular redox systems involved in the reduction of selenium. Moreover, redox-sensitive transcription factors for other genes can be influenced by such processes. As a result the transcription and the translation of other genes, besides genes for selenoproteins, can be influenced. Future investigations in this field should include microarray technology and proteomics to examine the differential effects of different trace element compounds on metabolic processes.

Another field of future research concerns the responsibility of animal nutrition with regard to environmental pollution. High doses of copper, for example, were used in the past to enhance the performance of growing pigs. High concentrations of copper in manure are now known to interfere with the ecosystem, e.g. Cu toxicity problems in sheep grazing on pasture fertilized with high copper manure. Feeding high concentrations of other trace elements (e.g. Zn and Mn) to animals in order to improve their immune functions and their antioxidative protection system can also lead to the accumulation of these elements in manure, which may also interfere with the ecosystem.

Future research in this area should focus on an optimal utilization of trace elements from natural sources to meet animal requirements. Natural sources such as herbs and secondary substances from plants like flavonoids, phytohormones and essential oils, may contribute in this context to an environmentally friendly, optimal nutrition of animals and in particular of productive livestock.

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7 Enzymes, bacterial direct-fed microbials and yeast: principles for use in ruminant nutrition

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This chapter provides a comprehensive review of the use of enzymes, bacterial direct-fed microbials (DFM) and yeast for ruminants. The net result of using these additives in commercial ruminant production is to maximize the utilization of feed resources and enhance production efficiency of cattle. There has been much interest in developing direct-fed enzyme products for ruminants to enhance forage digestion and animal productivity. Initial studies indicate significant potential improvements in feed digestion as a result of enzyme supplementation; however, results have been inconsistent. Thus, feed enzyme technology for ruminants is still in the developmental stage, and as such, is used on a limited basis in commercial production systems. There is a need to identify the key enzyme activities and the factors involved in ensuring positive and consistent results on-farm. Bacterial DFM are increasingly used in commercial cattle operations to advantageously alter gastrointestinal flora, thereby improving animal health and productivity. Although the mode of action of bacterial DFM is not well defined, it is generally accepted that they improve the microbial balance in the lower gastrointestinal tract. There is some evidence that bacterial DFM may also help to reduce ruminal acidosis. Yeast products based on *Saccharomyces cerevisiae* are widely used in commercial ruminant production because they tend to improve milk yield of dairy cows and liveweight gain of growing cattle. Available products vary widely in both the strain of *S. cerevisiae* used and the number and viability of yeast cells present. Not all strains of yeast are capable of stimulating digestion in the rumen possibly due to differences in metabolic activity. There is general agreement that production responses are the result of the action of the yeast within the rumen. Increased bacterial numbers within the rumen seems to

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be central to the action of yeast, driving both an increased rate of fiber degradation in the rumen and an increased flow of microbial protein from the rumen.

1. INTRODUCTION

Dairy cows and growing beef cattle raised in intensive production systems are usually fed high-energy diets to meet the demands placed on them to produce large quantities of milk or grow rapidly. However, the use of feedstuffs that are rapidly fermented in the rumen, such as grains and high-quality forages, can create conditions in the rumen that are suboptimal for the fibrolytic microorganisms, thereby impairing fiber digestion. There is presently an increased interest in using natural feed additives such as enzymes, bacterial direct-fed microbials (DFM) and yeast to help maintain optimum ruminal digestion of feed. The goal of using these additives in commercial ruminant production is to maximize the utilization of feed resources and enhance production efficiency of cattle. Enzymes and microbial additives can potentially reduce the use of antibiotics and ionophores in commercial cattle production. The existence of antibiotic resistance in bacteria is a major health concern worldwide. In an attempt to minimize agriculture's contribution to this problem, many countries have adopted policies to restrict the use of antimicrobials in animal production. In addition to the benefits that these feed additives can have on the rumen environment, bacterial DFM may play a role in disease prevention. Furthermore, public concern about pathogen contamination of meat and meat products has resulted in a recent surge of experiments evaluating the efficacy of bacterial DFM to reduce fecal shedding of harmful enteropathogens, such as *Escherichia coli* O157:H7.

This chapter discusses current information related to production responses and mode of action of enzymes, bacterial DFM and yeast additives for ruminants.

2. FEED ENZYMES

2.1. Introduction to exogenous enzymes

In recent years, there has been much interest in developing direct-fed enzyme products for ruminants to improve production efficiency, as recently reviewed by Beauchemin et al. (2001, 2002, 2003a). The main focus of enzyme research for ruminants has been directed at enhancing the digestibility of the cell wall fraction of forages. Forage dry matter (DM) typically contains 40–70% plant cell walls, and even under ideal feeding conditions, cell wall digestibility in the digestive tract is still generally less than 65% (Van Soest, 1994).

Feed enzyme products contain concentrated enzyme activities, but do not contain microbial cells. They are produced using a batch fermentation process, in which the enzymes are separated from the fermentation residues and source organism once the fermentation is complete. The types and activities of enzymes produced can vary widely depending on the microbial strain, the growth substrate and the culture conditions used (Considine and Coughlan, 1989; Gashe, 1992). Most enzyme products for ruminants are manufactured mainly for their polysaccharidase activities, but they usually also contain an array of secondary enzyme activities, such as glucosidase, protease and amylase.

While enzymes are used extensively by the poultry industry, feed enzyme technology for ruminants is still at the developmental stage, and as such, is used on a limited basis in commercial production systems. Although direct-feeding of enzymes to ruminants is limited, enzymes are added to many commercial DFM products (Kung et al., 1997; McGilliard and

Stallings, 1998; Kim et al., 2002; Pinos-Rodríguez, 2002). However, the concentration of enzymes supplied in most DFM products is usually an order of magnitude lower than levels supplied by concentrated feed enzyme products. While there have been positive effects of feeding composite DFM products to ruminants, there is little scientific evidence to demonstrate that the enzyme components of such products contribute to the response.

2.2. Polysaccharidases

Most ruminant feed enzymes contain cellulases and hemicellulases because cellulose and hemicellulose are the major structural polysaccharides in plants (Van Soest, 1994). The types of cellulases and hemicellulases can differ substantially among commercial enzyme products, and differences in the relative proportions and activities of these individual enzymes can have an impact on the efficacy of cell wall degradation by these products.

Several comprehensive reviews of cell wall chemistry and its enzymatic degradation are available elsewhere (White et al., 1993; Béguin and Aubert, 1994; Bhat and Hazlewood, 2001). In brief, the major enzymes involved in cellulose hydrolysis are: endocellulase (also referred to as endoglucanase, endo-β-1,4-glucanase, carboxymethyl cellulase or β-1,4-glucan glucanohydrolase; EC 3.2.1.4), exocellulase (also referred to as exoglucanase, exo-β-1,4-glucanase, or cellulose β-1,4-cellobiosidase; EC 3.2.1.91), and β-glucosidase (also referred to as cellobiase or glucohydrolase, EC 3.2.1.21). In general, endoglucanases hydrolyze cellulose chains at random to produce cellulose oligomers of varying degree of polymerization; exoglucanases hydrolyze the cellulose chain from the nonreducing end producing cellobiose; and β-glucosidases release glucose from cellobiose and hydrolyze short-chain cello-oligosaccharides from both reducing and nonreducing ends (Bhat and Hazlewood, 2001). A range of cellulase enzymes is necessary to hydrolyze native cellulose. The endoglucanases and exoglucanases act synergistically to hydrolyze cellulose and produce cellobiose, while β-glucosidase cleaves the cellobiose to release glucose.

The main enzymes involved in degrading the xylan core polymer to soluble sugars are xylanases (EC 3.2.1.8) and β-1,4-xylosidase (EC 3.2.1.37) (Bhat and Hazlewood, 2001). The xylanases include endoxylanases, which yield xylooligomers and β-1,4-xylosidases, which yield xylose. Other hemicellulase enzymes are involved primarily in the digestion of side chains and include β-mannosidase (EC 3.2.1.25), α-L-arabinofuranosidase (EC 3.2.1.55), α-D-glucuronidase (EC 3.2.1.139), α-D-galactosidase (EC 3.2.1.22), acetyl-xylan esterases (EC 3.1.1.72) and ferulic acid esterase (EC 3.1.1.73) (White et al., 1993; Bhat and Hazlewood, 2001).

2.3. Key activities needed to increase fiber digestibility

The key enzyme activities required in an enzyme product depend on the composition of the diet on which it is expected to act because the composition of plant cell walls is extremely variable among forages (Åman, 1993). Thus, one particular enzyme formulation is not likely to be effective for all diets (Beauchemin et al., 1995). For example, Colombatto et al. (2003) reported that the enzyme products that increased degradation of alfalfa hay were not the same ones that increased degradation of corn silage. Of the 26 enzyme candidates evaluated in that study, only one product was effective for both forages. These data clearly indicate the importance of understanding the key enzyme activities required to digest specific cell wall types, and then matching the enzyme product to the forage type.

Several *in vitro* studies have attempted to determine the key exogenous enzyme activities needed to improve forage digestion. Colombatto et al. (2003) reported a positive relationship

between added xylanase and protease activities and *in vitro* alfalfa DM degradation. In that study, enzyme activities explained about 40% of the improvements in alfalfa digestion resulting from the use of fibrolytic enzymes. Nsereko et al. (2000b) also reported a positive correlation between xylanase activity and the degradation of alfalfa neutral detergent fiber (NDF) *in vitro*. In agreement, Vanbelle and Bertin (1989) found that enzymes having high xylose- or arabinose-producing activity enhanced the *in vitro* degradation of alfalfa. Based on those studies, exogenous enzymes with high xylanase and high protease activities would be expected to enhance the digestion of alfalfa forage by ruminants.

The situation appears to be different for other forages. Wallace et al. (2001) reported that the level of cellulase activity (measured as endo-(β-1,4)-glucanase) limits the rate of fermentation of corn and grass silage in the rumen. The implication of that study is that exogenous enzymes high in endoglucanase activity would be expected to increase degradation of corn and grass silage *in vitro*. However, that relationship was not confirmed by Colombatto et al. (2003). In that study, increases in corn silage DM degradation were observed with the use of exogenous enzymes, but the increases were not related to any of the 17 enzyme activities measured, including endoglucanase activity. While the use of exogenous enzymes has been shown to increase the degradation of many different types of forages (Feng et al., 1996; Colombatto et al., 2003), the relationship between exogenous enzyme activity and digestibility has not been established with any certainty.

2.4. Forage: enzyme specificity

Ruminant diets usually contain several types of forages, which presents a major dilemma for formulating new enzyme products for ruminant feeds because of enzyme feed specificity. To achieve maximum benefit under these circumstances, a number of different enzyme sources would need to be used. One approach is to use an enzyme formulation that may not be the best for all forages, but is relatively suitable for most feeds. However, in that case, on-farm efficacy may not be high in all situations, contributing to the variability associated with enzyme technology. A more targeted approach, in which feed enzyme products are formulated for specific types of feeds, may decrease the variability associated with using enzymes in ruminant diets, thereby ensuring efficacy of feed enzyme technology on the farm. However, this targeted approach adds a degree of complexity in the marketplace.

2.5. Predicting effectiveness of enzyme products

Currently, it is not possible to predict the effectiveness of commercial feed enzyme products for ruminants based on the enzyme activities supplied by the manufacturer. This inability is mostly due to the lack of understanding of the key enzyme activities required to enhance digestion of various forages. Furthermore, there is no standardization among manufacturers in terms of the methods used to determine enzyme activities (Colombatto and Beauchemin, 2003). Enzymes are assayed based on release of hydrolysis products from standard substrates under optimal conditions for the enzyme (Wood and Bhat, 1988). However, optimal conditions for enzyme activities are usually different from those found in the rumen (Kung et al., 2002). For instance, 50°C and pH 4.8–5.3 are recommended for *Trichoderma* spp. cellulases (Ghose, 1987; Wood and Bhat, 1988), whereas the temperature in the rumen is about 39°C and the pH typically varies between 5.8 and 6.8. For ruminant applications, the pH and temperature used in the assays to measure activity should resemble the conditions found in the animal (Colombatto and Beauchemin, 2003). Furthermore, enzyme activities are measured on model

substrates that do not represent the complexity of plant cell wall material. In addition, enzyme assays are based on the initial rate of reaction with the substrate, which does not relate to overall enzyme persistency.

Conducting animal feeding studies using rapidly growing cattle or dairy cows in early lactation is the best assessment of whether an enzyme product enhances feed utilization. However, conducting experiments using cattle to determine digestibility and animal production is costly. Furthermore, using nonlactating sheep as a model for cattle is not a viable alternative (Yang et al., 2000) because of their lower intake as a percent of body weight and slower rate of passage of feed through the gastrointestinal tract. Laboratory bioassays that provide information on the rate, rather than extent, of digestion are helpful in identifying enzyme products that are likely to be effective *in vivo* (Colombatto and Beauchemin, 2003).

2.6. Animal responses

A number of studies have been published in the past decade to examine the potential role of exogenous feed enzymes in ruminant diets. Unfortunately, some studies failed to characterize the enzyme products used. Other studies provided enzyme activities of the products used, but lack of standardization of the assays used makes it impossible to make direct comparisons across studies. Furthermore, the formulation of enzyme products for ruminants used in research studies has evolved over time. It is difficult to follow these changes based on the information provided in the published literature. For example, there is no way of knowing whether an enzyme product from a particular enzyme supplier is the same from study-to-study.

Animal responses to exogenous enzymes are greatest when energy limits animal productivity, as is often the case with high-producing lactating dairy cows and rapidly growing beef cattle. Schingoethe et al. (1999) observed that dairy cows in early lactation receiving enzyme-treated forage produced 9–15% more milk and 16–23% more energy-corrected milk than did cows fed a control diet. However, milk production was not increased when cows were in mid-lactation at the start of the experiment. Similarly, others have observed greater production responses during early lactation than during later lactation (Nussio et al., 1997; Knowlton et al., 2002; Titi, 2003).

Various studies have shown that exogenous enzymes enhance the rate of *in situ* or *in vitro* digestion (Nakashima et al., 1988; Feng et al., 1996; Hristov et al., 1996; Yang et al., 1999; Bowman et al., 2002b; Colombatto et al., 2003). However, most studies have not found exogenous enzymes to improve the extent of *in situ* or *in vitro* digestion (Nakashima et al., 1988; Feng et al., 1996; Hristov et al., 1996; Iwaasa et al., 1999; Yang et al., 1999). These results suggest that enzyme additives only degrade substrates that would be naturally digested by the rumen microflora. Because these enzymes tend to increase the rate of fiber digestion, rather than the extent of fiber digestion, animal responses are expected to be greatest when the rate of passage from the rumen is relatively high and/or when the rate of digestion of feed is relatively low. These conditions are expected to cause a large discrepancy between the potential digestibility of the diet measured *in vitro* under ideal conditions and the actual digestibility of the diet obtained *in vivo*. The use of enzymes may help bridge this gap.

2.7. Practical aspects of using feed enzymes

In general, results with beef cattle and dairy cows indicate a positive response to enzymes, but the results are variable. Some of the variability has since been shown to be due to factors such

as inappropriate enzyme activities, level of enzyme supplementation, method of providing the enzyme, and the type of animal used in the experiment.

It is possible to under- as well as to over-supplement enzyme activity: *in vivo* responses to enzyme supplementation are typically nonlinear (Beauchemin et al., 1995; Kung et al., 2000). Even an enzyme product that is ideally formulated can be ineffective if provided in insufficient amounts or in excess. The optimum amount of a particular enzyme product will depend upon the diet.

Numerous methods of adding enzymes to the diet have been used across studies. Powdered enzymes have been added directly to the diet (McGilliard and Stallings, 1998), or a component of the diet (Knowlton et al., 2002; Titi, 2003), while liquid enzyme products have been applied to the total mixed ration (TMR) (Higginbotham et al., 1996; Beauchemin et al., 1999a; Yang et al., 2000; Sutton et al., 2003; Vicini et al., 2003), hay (Beauchemin et al., 1995; Lewis et al., 1996; Yang et al., 1999), ensiled forages (Beauchemin et al., 1995; Schingoethe et al., 1999; Zheng et al., 2000; Vicini et al., 2003), concentrate (Rode et al., 1999; Yang et al., 2000; Sutton et al., 2003), supplement (Bowman et al., 2002a), or premix (Bowman et al., 2002a).

Applying liquid enzymes onto feed may increase enzyme resistance to proteolytic inactivation in the rumen (Fontes et al., 1995). There is also evidence that enzymes are most effective when applied to a larger proportion of the diet rather than to a smaller component of the diet. Bowman et al. (2002a) demonstrated that adding a liquid enzyme to a premix was less effective than adding the enzymes to the concentrate. There is evidence that applying fibrolytic enzymes to feed prior to feeding alters the structure of the feed, thereby making it more amenable to degradation (Nsereko et al., 2000b). Applying enzymes to a larger portion of the diet may maximize this pre-ingestive effect. However, this explanation does not explain the increases in fiber digestion observed when enzymes are added to concentrate. We hypothesize that treating a greater portion of the diet with enzymes increases the residence time of enzymes in the rumen, because enzymes that are tightly bound onto feed will be associated with the particulate phase, rather than the liquid phase, within the rumen.

Spraying enzymes onto silage can also be an effective means of enzyme delivery, based on improvements in animal performance (Schingoethe et al., 1999; Kung et al., 2000). However, it appears that some exogenous enzymes are more effective when applied in a liquid form to dry forage as opposed to wet forage. Feng et al. (1996) observed no effect when exogenous enzymes were added to fresh or wilted forage, but when applied to dried grass, enzymes increased DM and fiber digestibility. Similarly, Yang et al. (2000) reported increased milk production and digestibility of the diet when enzymes were added to the concentrate portion of a dairy cow diet, but not when they were added directly to the TMR. The reduced efficacy of exogenous enzymes applied to ensiled feeds may be due to inhibitory compounds in fermented feeds (Nsereko et al., 2000c) or to decreased binding of the enzyme with the feed. The application of exogenous enzymes to silages can also accelerate aerobic deterioration, which could lead to a decrease of the nutritional value of the silage (Wang et al., 2002).

2.8. Effects on animal performance

Increases in animal performance due to the use of feed enzymes have been attributed to increases in feed digestion, but whether increased digestibility improves animal performance may depend on the physiological status of the cattle and the conditions of the experiment.

2.8.1. Beef cattle performance

The beef industry has been slow to adopt enzyme technology due to the relatively high cost of enzyme products compared with ionophores, antibiotics and implants. Furthermore, there are few enzyme products commercially available to the beef industry and most of these have not been widely evaluated under a range of dietary conditions.

A limited number of studies have examined the use of exogenous enzymes for growing cattle fed high-forage diets. Beauchemin et al. (1995) sprayed an enzyme mixture containing xylanase and cellulase activities (Xylanase B, Biovance Technologies Inc., Omaha, NE; and Spezyme CP®, Genencor, Rochester, NY) onto alfalfa hay or timothy hay and reported 30% and 36% improvements, respectively, in average daily gain (ADG) of steers. The increased ADG was the result of increased intake of digestible DM. However, the optimum level of enzyme product differed for the two forages, indicating the importance of the amount of enzymes added. Furthermore, the enzyme product had no effect on ADG of steers fed barley silage, demonstrating the importance of enzyme–feed specificity.

Another enzyme product was also shown to be effective for beef cattle fed high-forage diets, although improvements in animal performance were small to moderate. McAllister et al. (1999) treated whole crop barley silage (82.5% of the diet, DM basis) with a cellulase and xylanase mixture (Finnfeeds International Ltd. Marlborough, UK). In the first 2 months, feed intake, ADG and feed efficiency of growing cattle improved as a result of enzyme supplementation, but no effects were observed over the entire 17-week feeding period. Michal et al. (1996) added what may be the same enzyme mixture to a diet consisting of 85% alfalfa silage and 15% rolled barley grain (DM basis) and increased feed intake by 9%, but there were only minor effects on weight gain. Pritchard et al. (1996) also observed increased DM intake with minor effects on ADG gain when this enzyme mixture was added to a diet of alfalfa silage, chopped grass hay and barley grain (forage to concentrate ratio 70:30, DM basis). ZoBell et al. (2000) added this enzyme product to a diet containing 65% forage (DM basis; alfalfa hay, grass hay and corn silage), and found no effect on ADG or feed efficiency of growing cattle. Wang et al. (2002) added two levels of exogenous enzymes to a diet containing 65% barley silage (DM basis) and observed a 6% and 7% increase in ADG of cattle, but feed efficiency was unchanged.

Several studies have also reported positive effects of supplemental fibrolytic enzymes in high grain barley-based diets fed to growing cattle. Applying a fibrolytic enzyme that contained virtually no amylase activity (Xylanase B, Biovance Technologies Inc., Omaha, NE) to diets containing mostly barley grain improved feed efficiency by 6–12%, depending upon the level of enzyme supplementation (Beauchemin et al., 1997, 1999b; Iwaasa et al., 1997). In the study by Iwaasa et al. (1997), increased feed efficiency was due to an increase in ration digestibility. Using a similar enzyme product added to a high concentrate barley-based diet, Krause et al. (1998) reported a 28% increase in acid detergent fiber (ADF) digestibility. Using a different enzyme product (Finnfeeds International Ltd., Marlborough, UK), McAllister et al. (1999) reported that treating both the forage (ryegrass silage; 30% of the diet) and grain (barley, 70% of the diet) portions of the diet increased ADG by 10% (DM basis). However, ZoBell et al. (2000) reported no effect when what appears to be the same enzyme product was added to a high grain barley-based feedlot finishing diet containing 17% forage (DM basis).

2.8.2. Effects on milk production of dairy cows

There have been a number of studies to examine the effects of exogenous fibrolytic enzymes on milk production of dairy cows. When viewed across all studies the variability in response

is high because a range of different enzyme products and experimental conditions were used. In studies in which enzymes were effective, increases in milk production have been in the range of 4% (Yang et al., 1999) to 16% (Lewis et al., 1999). However, not all studies elicited a positive response, and therefore it is important to examine the individual studies to determine the conditions needed to ensure a positive response to enzymes.

Yang et al. (1999) added an enzyme product containing cellulases and xylanases (Promote®, Biovance Technologies Inc., Omaha, NE) to alfalfa hay cubes at the time of processing to produce cubes with a low or medium enzyme level. Another diet containing a low level of enzymes was prepared by combining treated cubes and concentrate containing enzymes. Milk yield was increased by about 1 kg/d (4%) for the low enzyme level, and by 2 kg/d (8%) for the medium enzyme level, with no effects of enzyme on feed intake. For the low level of enzyme, the response was similar whether the enzyme was added to the cubes or to both the cubes and concentrate. Thus, the effect of enzyme was dose dependent, but whether the enzyme was added to dry forage alone or dry forage and concentrate was not important. Increased milk yield was the result of increased total tract fiber digestibility, which increased mainly as a result of increased ruminal NDF digestibility.

Rode et al. (1999) applied the same enzyme product (Promote®, Biovance Technologies Inc., Omaha, NE) to the concentrate portion of a diet containing 24% corn silage and 15% alfalfa hay (DM basis). Cows in early lactation fed the enzyme-enhanced diets produced 3.6 kg/d (10%; $P = 0.11$) more milk than cows fed the control diet, although 4% fat corrected milk was not affected because milk components decreased. Feed intake was unchanged, but digestibility of nutrients in the total tract was dramatically increased by enzyme treatment. Digestibility of DM increased from 61.7% to 69.1% ($P < 0.01$) and digestibility of NDF increased from 42.5% to 51.0% ($P < 0.01$). In a follow-up study by Yang et al. (2000), a high-xylanase, low-cellulase enzyme product was added to a diet similar to that used by Rode et al. (1999). The enzyme was applied either to the concentrate or sprayed daily onto the TMR. Cows in early lactation fed the diet with enzyme applied to the concentrate produced 2 kg/d (5.9%) more milk than cows fed the control diet, without a change in percentage of milk components. However, there was no effect on milk production when the enzyme product was applied to the TMR, although total tract digestibility tended to increase. Similarly, Beauchemin et al. (1999a) reported that applying enzymes to a TMR prior to feeding did not significantly increase milk production, but increased digestibility of organic matter and fiber in the total tract. In that study, the six percentage unit increase in total tract ADF digestibility was mainly due to increased postruminal digestion.

There are various other studies in the literature that suggest products supplied by FinnFeeds Int. (Marlborough, UK) were beneficial in lactating cow diets (Nussio et al., 1997; Lewis et al., 1999; Schingoethe et al., 1999; Kung et al., 2000, 2002; Zheng et al., 2000). Lewis et al. (1999) applied an enzyme product containing cellulases and xylanases to a forage mixture consisting of alfalfa hay and alfalfa silage (1.65 ml/kg of forage DM equivalent to 0.69 ml/kg of dietary DM), fed to cows in mid-lactation. Milk production increased by 1.3 kg/d (5%). In a second study, the enzyme was applied to the forage at different levels ranging from 1–2.38 ml/kg of dietary DM. Dry matter intake increased by 7.4% and milk production increased by 6.3 kg/d (16%) at the medium level of enzyme (1.19 ml/kg of dietary DM). It is notable that higher and lower levels of the same enzyme product also increased feed intake, but increases (1.2–1.6 kg/d) in milk production were not statistically significant. Kung et al. (2000) sprayed a cellulase and xylanase mixture (enzyme EA) onto corn silage and alfalfa hay to supply 1.0 ml/kg and 2.5 ml/kg of TMR. The low level of enzyme EA increased milk production by 2.5 kg/d (7%), but the high level of enzyme was not effective. In the second

year of the same study, the same enzyme was added to forage at 1.0 ml/kg of TMR or an alternative cellulase and xylanase enzyme (EB) was added to forage to provide 0.6 ml/kg of TMR (DM basis). There was no effect of enzyme EA in this study. However, the milk yield of cows in mid-lactation receiving enzyme EB increased by 2.5 kg/d (8%), although 3.5% fat-corrected milk was not affected because milk components decreased. In another study, Kung et al. (2002) observed that an unspecified amount of the same EB enzyme resulted in 2.5 kg/d (7%) more 3.5% fat-corrected milk. Schingoethe et al. (1999) added increasing amounts of a mixture of cellulase and xylanase to corn silage and alfalfa hay to provide 0 ml/kg, 0.39 ml/kg, 0.55 ml/kg and 0.8 ml/kg of TMR (DM basis). Adding enzymes to the diet increased ($P = 0.12$) milk yield compared with the control, and the maximum response of 2.7 kg/d (11%) was observed at the highest application rate. This increase in milk production was similar to the response obtained by increasing the proportion of concentrate in the diet from 45% to 55% (DM basis). Zheng et al. (2000) added a cellulase and xylanase mixture (2:1) to the forage to supply 1.25 ml/kg of forage DM (equivalent to 0.63 ml/kg dietary DM), and observed an average response of 1.5–4.1 kg/d in milk yield, depending upon when during the lactation cycle the cows began to receive the enzymes. In contrast, Dhiman et al. (2002) observed no effect on milk yield when cows in early lactation were fed corn silage and alfalfa hay treated with a blend of cellulase and xylanase with and without ferulic acid esterase.

A comprehensive study conducted by Vicini et al. (2003) used 257 cows at four sites to validate the milk production responses reported for fibrolytic enzymes from Finnfeeds (Nussio et al., 1997; Lewis et al., 1999; Schingoethe et al., 1999; Kung et al., 2000, 2002; Zheng et al., 2000) and from Biovance Technologies (Beauchemin et al., 1999a, 1999b; Rode et al., 1999; Yang et al., 1999, 2000). These enzyme products were presumably the same formulations as those used in previous studies, although there is no way of verifying this with the information available. The diet contained corn silage and alfalfa hay and aqueous solutions of the enzymes were applied to the forage for the product from Finnfeeds (0.625 ml/kg of dietary DM) or the TMR (2 ml/kg of dietary DM) for the product from Biovance. In contrast to previous studies, there were no responses in milk production, milk composition or body weight gain for either enzyme treatment in this study. The authors speculated that the lack of response was due to the fact that the enzyme activities supplied by the products were greatly decreased at the pH and temperature conditions in the rumen. It is well established that the pH in the rumen is typically higher than the pH optima of most fungal enzymes, and ruminal temperature is lower than the optimum temperature of most fungal enzymes. However, this explanation does not explain the discrepancies between this study and the previous ones, unless the formulation of these products changed over time.

A limited number of studies have been published using other enzyme products. Beauchemin et al. (2000) used a product designed for the nonruminant market containing high β-glucanase, cellulase and xylanase activities (Natugrain, BASF Corp., Ludwigshafen, Germany). Dry matter intake and total tract digestibility increased, but no milk production response occurred. Using another product (Ruminase, Agri-Science, Liverpool, NY) containing high cellulase and xylanase activity, the milk production response was minimal (Higginbotham et al., 1996). Knowlton et al. (2002) fed a granular fibrolytic enzyme (Loveland Industries, Greeley, CO) to dairy cows and observed a 1.8 kg/d (5%) increase in 4% fat-corrected milk production of cows in early lactation, although this increase was not statistically significant because of the small number of cows used in the study. With other products, milk production increased by 1% to 15% (Stokes and Zheng,1995;

Zheng and Stokes, 1997; Jurkovich et al., 2002), but information on the products used is limited.

2.9. Mode of action

2.9.1. Pre-ingestive effects

Because enzymes are most effective when applied to feed, it is not unreasonable to assume that the mode of action is through some form of pre-ingestive attack of the enzymes upon the plant fiber, as suggested by Wallace et al. (2001). Applying exogenous enzymes onto feed causes a release of reducing sugars (Hristov et al., 1996), and in some cases, partial solubilization of NDF and ADF (Gwayumba and Christensen, 1997; Krause et al., 1998). However, the significance of a pre-ingestive release of reducing sugars is unclear. The quantity of sugars liberated represents only a minute portion of the total carbohydrate present in the diet.

There is evidence that applying fibrolytic enzymes to feed prior to feeding alters the structure of the feed, thereby making it more amenable to degradation (Nsereko et al., 2000b). While structural changes to the substrate may be an integral component of the mode of action of feed enzymes in improving digestion, this explanation does not account for improved dietary fiber digestion when exogenous enzymes are applied to the concentrate (low-fiber) portion of the diet (Rode et al., 1999; Yang et al., 2000).

Another important reason for applying enzymes to feed prior to ingestion is to enhance binding of the enzyme to the feed, thereby increasing the resistance of the enzymes to proteolysis in the rumen. Enzymes applied to feed prior to ingestion are particularly stable; the presence of substrate is known to increase enzyme resistance to proteolytic inactivation (Fontes et al., 1995).

2.9.2. Ruminal effects

Recent studies have shown that exogenous enzymes applied to feed are relatively stable in the rumen (Hristov et al., 1998a; Morgavi et al., 2000b, 2001). Thus, it is likely that exogenous enzymes survive for a considerable amount of time in the rumen where they probably maintain activity against target substrates.

It is possible that exogenous enzymes improve cell wall digestion by increasing the hydrolytic capacity within the ruminal environment. The extent to which this occurs will depend upon the amount of enzyme applied to the feed and the activity of the enzymes under ruminal conditions. For example, Vicini et al. (2003) reported that two-thirds of the maximum enzyme activity of two enzyme products was lost when enzymes were assayed at ruminal pH, and another two-thirds was lost at ruminal temperatures. In that case the amount the exogenous enzymes contributed to ruminal digestion would be relatively small, thereby indicating the importance of using exogenous enzymes with high activities under ruminal conditions (Colombatto and Beauchemin, 2003).

At the levels typically used in feeding studies, enzymic activities provided by exogenous enzymes represent only 5–15% of the enzymic activities normally present in the rumen (Wallace et al., 2001). However, this estimate disregards the synergy that occurs between exogenous enzymes and rumen microbial enzymes (Morgavi et al., 2000a). Synergy between exogenous enzymes and rumen microbial enzymes can be defined as the enhanced effect of these two entities acting cooperatively. The net effect is an increase in enzymic activity that

exceeds the additive effects of each of the individual components. Morgavi et al. (2000a) combined enzymes from *Trichoderma longibrachiatum* with ruminal enzymes extracted from cattle receiving high-fiber or high-concentrate diets. Hydrolysis of soluble cellulose and xylan increased by up to 35% and 100%, respectively. Hydrolysis of corn silage, a more complex natural plant substrate, also increased by 40%.

The significance of enhanced enzymic activity to the mode of action of exogenous enzymes in unclear, but cannot be discounted. Wallace et al. (2001) reported that the level of cellulase activity (measured as endo-(β-1,4)-glucanase) limited the rate of fermentation of corn and grass silage in the rumen. In that study, increasing the enzymic activities in ruminal fluid using exogenous enzymes increased the rate of forage digestion. Thus, it is possible that enhanced enzymic activity due to synergy of exogenous enzymes and rumen bacterial enzymes enhances fiber digestion in the rumen.

There is evidence that exogenous enzymes stimulate the attachment of ruminal microbes to plant fiber (Wang et al., 2001; Colombatto et al., 2003a; Morgavi et al., 2004). Adherence of ruminal bacteria to fiber places the enzyme system in close proximity to the substrate, and disrupts the hydrogen bonding within the cellulose matrix (White et al., 1993). There is also evidence that adding feed enzymes to the diet indirectly increases the numbers of nonfibrolytic, as well as fibrolytic, bacteria in the rumen (Wang et al., 2001; Nsereko et al., 2002a). This effect may be similar to that reported for DFM and fermentation extracts. An increase in cellulolytic microorganisms may accelerate the digestion of newly ingested feedstuffs, and may amplify the synergy between ruminal enzymes and exogenous enzymes.

2.9.3. Postruminal effects

Improved growth performance in poultry supplemented with enzymes is usually associated with a 10-fold decrease in intestinal viscosity (Bedford, 1993). The mechanism of action of feed enzymes in nonruminant diets raises the possibility that enzyme-mediated decreases in viscosity could improve nutrient absorption in the small intestine of cattle. However, intestinal viscosity in cattle is only between 1 cPoise and 2 cPoise (Mir et al., 2000) whereas intestinal viscosity in poultry may exceed 400 cPoise (Bedford, 1993). Consequently, it is difficult to comprehend how the relatively modest declines in intestinal viscosity observed in ruminants supplemented with high levels of enzymes would substantially improve nutrient absorption in the small intestine.

It must also be questioned whether supplementing ruminant diets with exogenous enzymes increases the polysaccharidase activity within the small intestine. Hristov et al. (1998b) showed that abomasal infusion of exogenous enzymes did not successfully supply cellulases and amylases to the intestine probably because of their limited resistance to low pH and pepsin proteolysis (Morgavi et al., 2001). However, xylanase activity in the duodenum increased 12–30-fold. Thus, at least some exogenous enzyme activities appear to survive in the small intestine for sufficient time to have an effect on target substrates. Hydrolysis of complex carbohydrates by exogenous enzymes in the small intestine and subsequent absorption of released sugars would offer energetic and nitrogen balance benefits to the animal that would not be accessible if these substrates remained undigested or were fermented by microbial populations residing in the large intestine. It is possible that exogenous enzymes work synergistically with the microbes even in the large intestine. Although most of the benefits of using enzyme supplements in ruminant diets are probably due to ruminal effects, the possibility of postruminal effects cannot be discounted.

3. BACTERIAL DIRECT-FED MICROBIALS

3.1. Introduction

Bacterial DFM, also referred to as probiotics, are live, naturally occurring bacterial supplements (Yoon and Stern, 1995) fed to livestock to enhance health and performance. Historical information pertaining to the use of bacterial DFM has been extensively reviewed (Stern and Storrs, 1975; Newman and Jacques, 1995; Yoon and Stern, 1995) and briefly summarized by Krehbiel et al. (2003). The original use of bacterial DFM, primarily in the form of fermented milk products, was for preventing disease caused by enteropathogens, and for restoring "normal" intestinal microorganisms following episodes of diarrhea caused by antibiotics. Since the mid 1950s, study of bacterial DFM for human and animal use has increased. However, the study of production responses by growing and lactating ruminants, and interest in the corresponding mode of action of bacterial DFM, is more recent (Yoon and Stern, 1995). The increased interest in the use of bacterial DFM in ruminant production has primarily resulted from societal concerns regarding the subtherapeutic use of antibiotics as growth promotants by the animal feed industry. Bacterial DFM might play a role in disease prevention, and thereby decrease the need for antibiotic use in ruminant production. In addition, public concern about pathogen contamination of meat and meat products has resulted in a recent surge of experiments evaluating the efficacy of bacterial DFM to reduce fecal shedding of harmful enteropathogens, such as *Escherichia coli* O157:H7.

Fuller (1989) defined probiotics as "a live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance". However, the possibility also exists that bacterial DFM improve microbial conditions in the rumen (Ghorbani et al., 2002; Beauchemin et al., 2003b). Kmet et al. (1993) defined probiotics fed to ruminants as "live cultures of microorganisms that are deliberately introduced into the rumen with the aim of improving animal health or nutrition".

3.2. Mode of action

The mode of action of bacterial DFM is not well defined for livestock species. Feeding bacterial DFM to livestock is based primarily on potential beneficial postruminal effects, however, there is some indication that certain bacterial DFM might also have beneficial effects in the rumen, in particular helping to prevent ruminal acidosis. Although responses to bacterial DFM have been positive in many experiments, enhancing our understanding of the mode of action would improve our ability to select and apply DFM to ruminant diets appropriately.

3.2.1. Ruminal effects

There is some indication that bacterial DFM might have beneficial effects for manipulating ruminal fermentation, which would aid in the prevention of ruminal acidosis (Krehbiel et al., 2003). Owens et al. (1998) suggested that acute or chronic acidosis due to the ingestion of excessive amounts of readily fermentable carbohydrate (i.e. starch) is a prominent production problem for ruminants fed high-concentrate diets. Ruminal acidosis is characterized by a decrease in ruminal pH (5.6 or below for subacute and 5.2 or below for acute) and high ruminal concentrations of total volatile fatty acids (VFAs; subacute acidosis) or lactic acid (acute acidosis). Lactate-producing bacteria (e.g. *Lactobacillus* and *Enterococcus* species) might help prevent ruminal acidosis, potentially by allowing the ruminal microorganisms to adapt to the presence of lactate in the rumen (Yoon and Stern, 1995; Ghorbani et al., 2002;

Nocek et al., 2002). In addition, inclusion of lactate-utilizing bacteria might aid in preventing the accumulation of lactate in the rumen (Nisbet and Martin, 1994; Kung and Hession, 1995). Therefore, continual inoculation with certain bacterial DFM might aid the ruminal environment in adapting to acidosis (Elam, 2003).

Results from some studies using beef and dairy cattle supplemented with *Lactobacillus* species have shown a reduction in the area under the ruminal pH curve (Huffman et al., 1992; Nocek et al., 2002), suggesting a reduced risk of subacute ruminal acidosis. Huffman et al. (1992) fed a 50% concentrate diet for 12 days to ruminally fistulated steers supplemented daily with 5×10^8 cfu (colony-forming units) of *Lactobacillus acidophilus*. On day 13, steers were dosed with a 100% concentrate diet via the ruminal cannula to induce subacute acidosis. Feeding *L. acidophilus* reduced the amount of time that ruminal pH was below 6.0 compared with control steers. Similarly, Lodge et al. (1996) induced ruminal acidosis in cannulated steers by intraruminally dosing with a 50:50 blend of fine ground corn and dry rolled wheat (1.6% of body weight; BW). Ruminal pH in steers receiving *Lactobacillus* was below 6.0 for fewer hours during a 24-hour period than control steers. Nocek et al. (2002) provided ruminally cannulated dairy cows fed a 70% concentrate diet with a mixture of *E. faecium, L. acidophilus,* and *Saccharomyces cerevisiae* (yeast) at 10^5, 10^6 or 10^7 cfu/ml of ruminal fluid daily, and measured ruminal fermentation characteristics. Compared with the control group, cows inoculated with 10^5 cfu/ml of ruminal fluid had the highest mean daily ruminal fluid pH and the lowest mean daily hours of ruminal pH < 5.5. Nocek et al. (2002) suggested that DFM that produce lactate sustain a tonic level of lactic acid in the rumen, which could potentially stimulate lactic acid-utilizing bacteria.

In contrast, Ghorbani et al. (2002) fed 10 g/steer/d of a carrier that contained 1×10^9 cfu/g of *Propionibacterium* P15 or *Propionibacterium* P15 and *E. faecium* EF212 to steers adapted to an 87% steam-rolled barley diet and found no effect on ruminal pH. Mean ruminal pH of steers fed the steam-rolled barley was 5.71. In addition, Beauchemin et al. (2003b) evaluated the effects of *E. faecium* (6×10^9 cfu/d), a lactate-producing bacterium, alone or with yeast (6×10^9 cfu/d of *Saccharomyces cerevisiae*) on the risk of ruminal and metabolic acidosis and the site and extent of digestion. Similar to the results of Ghorbani et al. (2002), there was no effect of treatment on the proportion of time or area below pH 5.8 or 5.5. In fact, the lowest daily ruminal pH was found for steers receiving *E. faecium*. The diet used in that study was typical of commercial diets fed to feedlot cattle in Western Canada. However, the authors noted that the incidence of subclinical acidosis was more severe in their experiment than in previously published studies. Steers used in their experiments spent 39% or more of the day at a ruminal pH below 5.5. In a companion study with similar treatments using continuous culture, mean fermenter pH, as well as the lowest and highest pH, was not affected by bacterial DFM (Yang et al., 2004). As noted by Beauchemin et al. (2003b), lack of an effect of bacterial DFM on ruminal pH suggests little benefit of providing DFM that either produce or utilize lactic acid when the rumen is adapted to a high-grain diet. However, in feeding situations in which lactic acid might accumulate in the rumen, providing bacterial DFM might prove beneficial (Beauchemin et al., 2003b).

Van Koevering et al. (1994) found that ruminal concentrations of D-lactate and total lactate were decreased for all steers supplemented with *L. acidophilus* BT 1389, regardless of dietary concentrate level (92% or 55% concentrate). In contrast, steers consuming an 87% steam-rolled barley diet supplemented with *Propionibacterium* P15 or *Propionibacterium* and *E. faecium* EF212 had similar ruminal concentrations of L-lactate and total VFA as control steers (Ghorbani et al., 2002). Similarly, there was no effect of *E. faecium* or *E. faecium* and *S. cerevisiae* on total VFA concentration, and lactate concentration was below detection limits

in steers fed an 87% steam-rolled barley diet (Beauchemin et al., 2003b). These results confirm that the effect of bacterial DFM supplementation on decreasing ruminal acidosis might depend on the amount of lactic acid accumulation during ruminal fermentation. Continued research with different bacterial DFM species and combinations using acidosis challenge models might be beneficial.

In the experiment by Ghorbani et al. (2002), *Propionibacterium* or the combination of *Propionibacterium* and *E. faecium* did not affect ruminal fluid concentrations of propionate, isobutyrate and isovalerate, or the actetate:propionate ratio. However, acetate concentration was greater for steers receiving *Propionibacterium* and *E. faecium* than for steers receiving *Propionibacterium* alone or no bacterial DFM. In addition, steers fed *Propionibacterium* alone had greater concentrations of ruminal butyrate (Ghorbani et al., 2002). Other researchers (Slyter et al., 1992; Kung and Hession, 1995) have reported accumulation of butyrate when *Megasphaera elsdenii* is grown in pure culture, and Lodge et al. (1996) reported a lower acetate + butyrate:propionate ratio for control steers compared with steers receiving a combined *Lactobacillus* and yeast DFM. Lodge et al. (1996) suggested that the production of propionate might be decreased during an acidosis challenge in steers supplemented with DFM. In contrast, supplementing an 87% concentrate diet with *E. faecium* increased the proportion of propionate and decreased the proportion of butyrate in ruminal fluid compared with control steers (Beauchemin et al., 2003b). As indicated by the authors, results in their study were consistent with the expectation that supplementing *E. faecium,* a lactate utilizer, would increase propionate. Similarly, Kim et al. (2000) studied the effect of increasing dosage levels (none, 10^7, 10^8, 10^9 and 10^{10} cfu) of *P. acidipropionici* on ruminal fermentation in steers fed a high-concentrate diet. When supplemented with *P. acidipropionici,* all dosage levels had numerically lower concentrations of acetate and greater concentrations of propionate, and therefore the acetate:propionate ratio decreased at all dosages except 10^8. It would appear that the *P. acidipropionici* altered ruminal metabolism toward less acetate and more propionate production. In addition, ruminal butyrate concentration decreased as the dose of *P. acidipropionici* increased, and when *P. acidipropionici* was removed, butyrate concentration returned to near pretest levels. Although reasons for discrepancies among experiments are difficult to explain, these data suggest that the energetically favorable propionate concentration might be increased in ruminants fed high-grain diets when supplemented with a lactate-utilizing DFM.

Similar to ruminal acidosis, effects of bacterial DFM on decreasing the risk of metabolic acidosis have been mixed. Ghorbani et al. (2002) reported that blood pH was not affected by bacterial DFM supplementation, but steers fed *Propionibacterium* and *E. faecium* tended to have lower concentrations of blood CO_2 and had lower concentrations of lactate dehydrogenase than control steers. The authors suggested that lower blood CO_2 and lactate dehydrogenase indicated that feeding a lactate-producing bacteria along with a lactate-utilizing bacteria might reduce the risk of metabolic acidosis. In contrast, *E. faecium* had no effect on similar blood variables measured in the study of Beauchemin et al. (2003b).

Feeding *Propionibacterium* increased protozoa (especially *Entodinium*) and decreased amylolytic bacteria in the rumen of feedlot steers (Ghorbani et al., 2002). The results of Ghorbani et al. (2002) showed amylolytic bacterial numbers were significantly decreased in steers during *Propionibacterium* supplementation compared with counts from control and *Propionibacterium*- and *E. faecium*-supplemented steers. Protozoal numbers were significantly increased in *Propionibacterium* steers over control and *Propionibacterium* and *E. faecium* steers. Although the mechanism by which bacterial DFM stimulate protozoa remains unclear, Ghorbani et al. (2002) indicated that the decrease in amylolytic species was most likely the result of the increase in protozoal numbers because protozoa are predators of

ruminal bacteria. Similar to the results of Ghorbani et al. (2002), Van Koevering et al. (1994) reported that including cultures of *L. acidophilus* BT 1389 in the diet prolonged retention of protozoa in steers fed a 92% concentrate diet. In contrast, Beauchemin et al. (2003b) found that supplementing the diet with *E. faecium* tended to decrease protozoal numbers, but had no effect on lactate-utilizing bacteria, amylolytic bacteria, or total bacterial numbers. In continuous culture, counts of total bacteria in the fermenter fluid tended to be greater when control or *Propionibacterium* P15 was included compared with *E. faecium* or *E. faecium* and yeast (Yang et al., 2004). However, lactate-utilizing bacterial numbers were greater for control or *E. faecium* than for *Propionibacterium* or *E. faecium* and yeast. As suggested by the authors, one would anticipate that the number of lactate-utilizing bacteria would be greater when *Propionibacterium* was fed.

Little information is available characterizing the effects of bacterial DFM on ruminal and total tract digestion of nutrients. Recently, Beauchemin et al. (2003b) reported that *in sacco* digestion of corn, barley and alfalfa hay were decreased by *E. faecium*. In contrast, *Propionibacterium* or *Propionibacterium* and *E. faecium* did not affect *in sacco* disappearance of DM in the study of Ghorbani et al. (2002). Supplementation with bacterial DFM had no effect on the site or extent of starch digestion (Beauchemin et al., 2003b). However, supplementing diets with *E. faecium* tended to decrease total tract digestibility of OM and intestinal digestion of NDF. The authors suggested that the lower digestion of fiber by steers supplemented with *E. faecium* might have been associated with the observed lower ruminal pH. In continuous culture, digestibilities of DM, OM, starch, NDF, ADF and nitrogen were not affected by bacterial DFM supplementation (Yang et al., 2004). However *in vivo,* feeding *E. faecium* tended to decrease the flow of microbial N from the rumen, and increased the flow of feed N (Beauchemin et al., 2003b). Decreased flow of microbial N from the rumen resulted from the numerical decrease in efficiency of microbial protein synthesis. Providing yeast (*S. cerevisiae*) with *E. faecium* negated all effects of *E. faecium* on nitrogen metabolism.

The potential for DFM to decrease the risk or severity of ruminal acidosis in cattle fed high-concentrate diets is still in question (Elam, 2003). The inconsistency of the data does not provide unequivocal evidence that DFM are efficacious for decreasing episodes of ruminal and/or metabolic acidosis. In general, it appears that bacterial DFM might be more efficacious early in the finishing period when cattle are being adapted to a high-grain diet and that once animals are adapted, bacterial DFM are less effective. It is most likely that the response also depends on the species of bacterial DFM fed, and whether or not a yeast product is included.

3.2.2. Postruminal effects

Although the mode of action of bacterial DFM is not well defined, it is generally accepted that they improve the microbial balance in the lower gastrointestinal tract (GIT). Improving gastrointestinal flora is related to the ability of bacterial DFM to decrease the concentration of harmful enteropathogens that inhabit the lower digestive tract. In a recent review, Krehbiel et al. (2003) discussed several mechanisms by which DFM might benefit inoculated animals, including competitive inhibition of pathogenic microorganisms for attachment, antibacterial effects such as hydrogen peroxide production, and immunomodulation via enhanced phagocytosis and natural killer cell activity (Fuller, 1989; Parker, 1990; Walter et al., 1992; Yoon and Stern, 1995; Salimen et al., 1996; Holzapfel et al., 1998; Reid and Burton, 2002). These mechanisms could also benefit ruminants by increasing nutrient uptake via decreased thickening of the intestinal wall as a result of inflammation. If thickening of the intestinal wall is decreased,

bacterial DFM could improve the efficiency of energy utilization by decreasing the amount of energy used for tissue turnover in the GIT (Elam, 2003).

The ability of *E. coli* O157:H7 to cause attaching/effacing lesions in bovine intestine was demonstrated by Phillips et al. (2000). In addition, Jones and Rutter (1972) indicated that for enterotoxin-producing strains of *E. coli* to cause diarrhea, attachment to the epithelial mucosa is necessary. Once attachment is established, proliferation is supported and the potential for peristaltic removal of such organisms is decreased (Yoon and Stern, 1995; Salimen et al., 1996). However, when bacterial DFM (*L. lactis*) were fed, markedly higher numbers of attached *Lactobacilli* and lower *E. coli* counts were found in the intestine of pigs compared with scouring or normal control pigs not fed the DFM (Muralidhara et al., 1977). Similarly, the fecal microbial load of lactobacilli in calves treated with *L. acidophilus* 27SC was significantly increased and that of coliforms numerically decreased compared to control animals (Abu-Tarboush et al., 1996). Therefore, the use of bacterial DFM to decrease harmful bacteria in the digestive system might prevent adherence of pathogenic species and thereby potentially improve animal performance.

Lactobacillus lactis is thought to have an antimicrobial capacity as a result of its ability to produce hydrogen peroxide via a lactoperoxidase-thiocyanate system (Reiter and Härnulv, 1984). However *in vivo,* hydrogen peroxide's involvement in the gut has been questioned due to the fact that oxygen is necessary for its formation by lactobacilli (Gilliland and Speck, 1977; Reiter et al., 1980) and many species of lactobacilli have demonstrated other inhibitory activities against enteropathogens. *Lactobacillus acidophilus* has been shown to be antagonistic toward enteropathogenic *E. coli, Salmonella typhimurium, Staphylococcus aureus* and *Clostridium perfringens* (Gilliland and Speck, 1977). The production of lactic acid might be involved in this antagonism. In support, Ratcliffe et al. (1986) showed that lactic acid decreased counts of coliforms throughout the GIT of piglets. This might result from a decrease in pH, which can prevent growth of many pathogens (Fuller, 1977; Gilliland and Speck, 1977). In a review by Walter et al. (1992), a host of antimicrobial substances produced by lactobacilli were identified, including bacteriocins, acidophilin and lactocidin, which are broad-spectrum antibiotic-like substances that have yet to be fully characterized. These antimicrobial proteins and/or bacteriocins may mediate or facilitate antagonism by *L. acidophilus,* although their role *in vivo* has been questioned due to the presence of proteolytic enzymes in the gut (Hamdan and Mikolajcik, 1974; Gilliland and Speck, 1977; Barefoot and Klaenhammer, 1983).

Attaching/effacing lesions caused by enteropathogens in the intestine of ruminants lead to inflammation (Phillips et al., 2000), resulting in a thickening of the intestinal wall. In contrast, germ-free animals have been shown to have less small-intestinal mass than conventional animals (Visek, 1978; Parker, 1990). Higher intestinal water content, a thicker lamina propria, and more reticuloendothelial elements are associated with the overall greater mass of small-intestinal tissue in conventional versus germ-free animals, and the efficiency of nutrient absorption is hypothesized to be decreased (Visek, 1978). Elam et al. (2003) fed cattle different strains of *L. acidophilus* and one strain of *Propionibacterium freudenreichii* and measured the thickness of the lamina propria. Lamina propria measurements for ileal sections taken near the cecum were 0.09 mm thinner in steers fed 1×10^9 cfu of *L. acidophilus* NP51, 1×10^6 cfu of *L. acidophilus* NP45, and 1×10^9 cfu of *P. freudenreichii* NP24, and steers fed 1×10^9 cfu of *L. acidophilus* NP51 and 1×10^9 cfu of *P. freudenreichii* NP24 daily compared with control steers. Furthermore, steers receiving some form of DFM had approximately 20% thinner lamina propria than control steers. Although not measured directly, the authors suggested that one possible mechanism by which bacterial DFM might improve feed efficiency is through lower intestinal mass. If bacterial DFM decrease inflammation in the gastrointestinal tract, then less energy would be spent repairing damaged or inflamed tissue. Less energy expended towards cellular turnover in the intestine would result in more energy available for animal growth (Elam et al., 2003).

3.2.2.1 Immune function Modulation of host immunity may represent another mechanism by which bacterial DFM might promote intestinal health and overall wellbeing of ruminants (Krehbiel et al., 2003). However, most research conducted to date has been in humans and other nonruminants species, and has recently been summarized by Krehbiel et al. (2003). Briefly, reviews by Erickson and Hubbard (2000) and Isolauri et al. (2001) have suggested that oral administration of lactobacilli generally resulted in an augmentation of innate immune responses (i.e. enhanced phagocytosis and natural killer cell activity) as well as an elevated production of immunoglobulin A (IgA) and a decreased immunoglobulin E production in both humans and animals. However, in feedlot cattle different strains of *L. acidophilus* and one strain of *P. freudenreichii* had no effect on serum IgA concentrations (Elam et al., 2003). Overall, DFM showed mixed influences on cytokine production and T and B cell responses, depending upon the strain, dose and duration of feeding DFM as well as the type of tissues and cells analyzed (Krehbiel et al., 2003).

3.2.2.2. Fecal shedding of *Escherichia coli* O157:H7 Feedlot cattle have been recognized as a host for the enteropathogen *E. coli* O157:H7, which appears to be confined to the gastrointestinal tract and is shed in feces. Lactic acid-producing *Streptococcus bovis* LCB6 and *L. gallinarum* LCB 12 isolated from adult cattle and fed to Holstein calves infected with *E. coli* O157:H7 resulted in an increase in acetate, which was correlated with a decrease in *E. coli* O157:H7 (Ohya et al., 2000). In a recent experiment (Elam et al., 2003), supplementing finishing cattle with 1×10^9 cfu of *L. acidophilus* NP51 and 1×10^9 cfu of *P. freudenreichii* NP24 daily decreased fecal shedding of *E. coli* O157 compared with control steers or steers fed 1×10^6 cfu of *L. acidophilus* NP51, 1×10^6 cfu of *L. acidophilus* NP45, and 1×10^9 cfu of *P. freudenreichii* NP24 daily. In addition, the prevalence of O157 on the hides of steers immediately before shipping was lower for cattle fed 1×10^9 cfu of *L. acidophilus* NP51, 1×10^6 cfu of *L. acidophilus* NP45, and 1×10^9 cfu of *P. freudenreichii* NP24 compared with the other bacterial DFM or control treatments. Similarly, addition of a *L. acidophilus* bacterial DFM product decreased the prevalence of *E. coli* O157:H7 in the feces of feedlot cattle in a study conducted by Folmer et al. (2003). Based on these results, supplementing cattle fed high-grain diets with certain bacterial DFM appears to decrease the incidence of fecal *E. coli* O157:H7 shedding in cattle.

3.3. Practical aspects of using bacterial direct-fed microbials

Bacterial DFM have been used to potentially replace or decrease the use of antibiotics in neonatal and stressed calves, to enhance milk production in dairy cows, and to improve daily gain and feed efficiency in beef cattle (Krehbiel et al., 2003). Throughout the late 1970s and early 1980s, bacterial DFM were primarily evaluated for efficacy in neonatal and/or stressed calves. More recently, bacterial DFM have received increased attention for finishing cattle consuming high-grain diets. However, performance results of experiments where cattle were fed bacterial DFM are generally inconsistent.

3.4. Effects on animal health and performance

3.4.1. Preruminant calves

The efficacy of bacterial DFM, such as *Lactobacillus, Enterococcus, Streptococcus* and *Bifidobacterium* species has been extensively studied in the newborn dairy calf (Newman and Jacques, 1995). In neonates, the normal population of gastrointestinal microbes that develops

upon passage through the birth canal, contact with the environment, and contact with their mother is extremely beneficial for preventing infection. In support, Fuller (1989) observed that germ-free animals are much more susceptible to disease than are inoculated animals. At birth, the gastrointestinal tract is initially colonized by coliforms and clostridial species, but colonization by lactobacilli occurs rapidly, resulting in a decrease in enteropathogenic species (Smith, 1965, 1971). However, during stress conditions, the general trend is for lactobacilli to decrease, whereas coliforms increase (Fuller, 1989). Therefore, bacterial DFM might be beneficial for young animals to establish and maintain "normal" intestinal microorganisms.

The *Lactobacillus* population decreases when animals are stressed, and stress often leads to an increased incidence of diarrhea in neonates (Tannock, 1983). Similarly, Sandine (1979) reported that fecal counts of lactobacilli normally are higher than coliforms in healthy animals and reversed in those suffering from scours. Fumiaki et al. (1995) conducted two experiments that evaluated the effects of *Bifidobacterium pseudolongum* M-602, *L. acidophilus* LAC-300, or no DFM on health and performance of young dairy calves. In one experiment, daily fecal scores (a lower score is indicative of a normal stool) averaged across 4 weeks, from 7 days to 35 days of age, were numerically lower in the DFM-treated calves, which had been supplemented with antibiotics. In a second experiment in newborn calves that received no supplemental antibiotics, cases of diarrhea were significantly lower in calves receiving DFM compared with control calves. Abu-Tarboush et al. (1996) reported similar results with 3–4-day-old calves that were given a lactobacilli DFM. Scour indices for a 9-week sampling period were significantly higher (a higher score indicated a normal stool) for calves receiving *L. acidophilus* 27SC than for calves treated with a mixed lactobacilli culture, which themselves had a significantly higher score than control calves. Given the role of *enteropathogens* as a possible cause of diarrhea, these results suggest that bacterial DFM are beneficial for establishing and maintaining a positive microbial balance in newborn calves by decreasing the prevalence of coliforms. A decreased incidence of diarrhea has generally been associated with an increased shedding of *Lactobacillus* (Ellinger et al., 1980; Gilliland et al., 1980; Jenny et al., 1991; Abu-Tarboush et al., 1996).

Performance variables may or may not be important during the first 3 weeks of the preruminant's life when enteric disease is most prevalent. Decreasing the incidence or severity of diarrhea is most likely a more important response (Krehbiel et al., 2003). Many experiments have reported no improvement in daily gain as a result of feeding lactobacilli (Morrill et al., 1977; Ellinger et al., 1980; Abu-Tarboush et al., 1996). In contrast, Beeman (1985) reported greater gains by Holstein calves treated with lactobacilli compared with control calves. By d 56 of the experiment, average BW gains were 47.3 kg and 37.8 kg for treated and control groups, respectively. These benefits were hypothesized to result from improvement of intestinal conditions because of lower fecal scores (i.e. less scouring) in calves fed DFM. Similar results were recently reported by O'Brien et al. (2003) with a novel DFM administered through a milk replacer.

3.4.2. Receiving cattle

Weaning, transport, fasting, assembly, vaccination, castration and dehorning are some of the stresses beef calves undergo upon entering the feedlot. These stresses result in decreased performance and increased morbidity and mortality, in part due to altered microorganisms in the rumen and lower gut (Williams and Mahoney, 1984). Therefore, feeding, or otherwise dosing with bacterial DFM, might reduce these changes in the microbial population.

Krehbiel et al. (2003) reported a summary of data from several research trials (Crawford et al., 1980; Hutcheson et al., 1980; Kiesling and Lofgreen, 1981; Davis, 1982; Kiesling et al., 1982; Hicks et al., 1986), which showed that feeding a DFM (combination of live cultures of *L. acidophilus, L. plantarum, L. casei* and *S. faecium*) at processing, throughout the receiving period (average of 30 days), or both resulted in a 13.2% increase in daily gain, 2.5% increase in feed consumption, and a 6.3% improvement in feed:gain. The greatest performance response to the bacterial DFM generally occurred within the first 14 days of the receiving period (Crawford et al., 1980; Hutcheson et al., 1980). Morbidity was decreased by 27.7% in cattle receiving the bacterial DFM compared with control cattle. In addition, Gill et al. (1987) conducted a 28-day receiving study in which morbidity was significantly greater in control animals than in those receiving a bacterial DFM treatment (1.4×10^9 cfu of live bacterial cells per animal daily). In contrast, Kiesling and Lofgreen (1981) needed to treat more calves from groups that were receiving some type of lactobacillus preparation than control animals. Krehbiel et al. (2001) demonstrated that calves treated for morbidity were less likely to be retreated within 96 hours if they were administered an oral gel containing 5×10^9 lactic-acid-producing bacteria (*E. faecium, L. acidophilus, Bifidobacterium thermophilum* and *B. longum*) at the time of their first antimicrobial treatment compared with those that did not receive the gel. In addition, the number of calves treated twice tended to be lower for those calves receiving the gel treatment. Daily gain did not differ among calves receiving DFM vs no DFM.

Interest in the efficacy of bacterial DFM to decrease the subtherapeutic use of antibiotics as growth promotants is increasing. Vanbelle et al. (1990) pooled data from studies where DFM (*Lactobacillus* and *Streptococcus* species) administered with or without antibiotics were compared against antibiotic-treated control or non-antibiotic-treated control cattle. Results indicated that cattle fed DFM administered with antibiotics had a 5–6% increase in ADG compared with non-antibiotic-treated control (no DFM) cattle, and a 2.5–3.5% increase in ADG compared with antibiotic-treated control (no DFM) cattle. In addition, cattle fed DFM without antibiotics had a 6–7% increase in ADG compared with non-antibiotic-treated control cattle, and a 3–4% increase compared with antibiotic-treated control cattle. Results for feed efficiency showed a similar advantage (Vanbelle et al., 1990) in feeding a bacterial DFM.

Based on these results, it seems appropriate to conclude that DFM can be beneficial for newly received feedlot cattle (Elam, 2003). A bacterial DFM used as an inoculant for stressed calves could serve as a source of beneficial microorganisms to a host animal with a compromised gastrointestinal flora. As in the neonatal calf, the response to bacterial DFM might be greater if administered to newly weaned and/or received beef calves, which are more prone to health problems. Gill et al. (1987) suggested that extremely healthy calves and extremely sick calves might be less likely to respond to bacterial DFM treatment.

3.4.3. Feedlot cattle

3.4.3.1. Growth Recently, bacterial DFM have received a great deal of attention in diets for cattle fed high-grain diets. Swinney-Floyd et al. (1999) treated feedlot calves with *Propionibacterium* P-63 alone or in combination with *L. acidophilus* LA53545, and measured performance over a 120-day feeding experiment. Cracked wheat replaced 75% of the ground corn in the diet on days 1–10 of the feeding period to increase the risk for ruminal acidosis. Although bacterial DFM did not alter DM intake, ADG (1.63 kg/d) for the animals inoculated with the combination of *Propionibacterium* and *L. acidophilus* was greater than that of calves receiving *Propionibacterium* alone (1.11 kg/d) or control calves (0.93 kg/d) from days 1–10.

Across the 120-day feeding period, calves consuming *Propionibacterium* in combination with *L. acidophilus* were more efficient, although ADG was not affected. Huck et al. (2000) reported a tendency for greater carcass-adjusted ADG (final weights were calculated as hot carcass weight/average dressing percent) in heifers that were sequentially fed a *Lactobacillus* and then a *Propionibacterium* or a *Propionibacterium* and then a *Lactobacillus* DFM between the receiving and finishing phases compared with control heifers. In contrast, Rust et al. (2000) reported no difference for carcass-adjusted ADG in control steers versus steers supplemented with *Lactobacillus* and *Propionibacterium*. However, ADG (nonadjusted) for the average of all bacterial DFM-treated steers was increased 6.2% over control steers from day 0 to finish. In addition, Rust et al. (2000) reported that feed efficiency for the entire feeding period was improved for steers receiving certain DFM treatments compared with control steers. Using similar treatments, Galyean et al. (2000) reported that DM intake for the entire feeding period was not affected by treatment, but final BW (2.1%) and ADG (4.3%) were significantly increased for the average of treated steers versus the control animals. No differences in feed efficiency were noted, but hot carcass weight was greater for steers treated with DFM than for controls. McPeake et al. (2002) compiled data from six feedlot trials conducted in several states, in which various combinations and concentrations of *Lactobacillus acidophilus* Strains 45 and 51 and *Propionibacterium freudenreichii* PF-24 were compared against controls. Steers fed a bacterial DFM had greater final weight, ADG, DM intake, hot carcass weight and carcass-adjusted ADG compared with control steers. In addition, there was also a trend for improved feed efficiency as the concentration of supplemental *L. acidophilus* increased. Most recently, Elam et al. (2003) conducted two experiments to evaluate the effects of bacterial DFM, based on different strains of *L. acidophilus* plus one strain of *Propionibacterium freudenreichii,* on performance and carcass traits in finishing beef steers. Although no differences were observed in overall DM intake, feeding DFM increased ADG by 7.5% from days 0–28, whereas overall ADG did not differ among treatments. In a second experiment, overall and carcass-adjusted ADG were greater for steers fed 1×10^9 cfu of *L. acidophilus* NP51, 1×10^6 cfu of *L. acidophilus* NP45, and 1×10^9 cfu of *P. freudenreichii* NP24 daily compared with control steers or steers fed 1×10^6 cfu of *L. acidophilus* NP51, 1×10^6 cfu of *L. acidophilus* NP45, and 1×10^9 cfu of *P. freudenreichii*.

Studies have also been conducted to determine the effects of treating high-moisture feedstuffs with bacterial DFM prior to feeding them to feedlot cattle. For example, Kreikemeier and Bolson (1995) inoculated high-moisture corn during ensiling with 100 000 cfu of lactic-acid producing bacteria and 10 000 cfu of propionate-producing bacteria/g of high-moisture corn. Over a 140-day finishing study, steers fed inoculated corn were 13 kg heavier, consumed 0.41 kg/d more feed, and gained 0.09 kg/d faster than control cattle. Feed efficiency was not influenced by the inoculant; however, cattle fed inoculated high-moisture corn produced carcasses that were 7 kg heavier than those of control cattle. This is in contrast to Weichenthal et al. (1997), who found no differences in performance over a 105-day feeding experiment when cattle were fed inoculated high-moisture corn.

3.4.3.2. Carcass In the data summarized by Ware et al. (1988b), *L. acidophilus* BT1386 did not affect yield grade, quality grade, dressing percentage, marbling score or incidence of liver abscesses. Similarly, data from six research trials (n = 1249; 184 pens) conducted in four states (CO, IA, MI and TX) were assembled to summarize the effects of varying concentrations and strains of *L. acidophilus* (LA45 and LA51) and *P. freudenreichii* (PF24) on carcass characteristics of feedlot steers (McPeake et al., 2002). Dressing percentage, quality grade and percentage of carcasses graded as Choice were not influenced by bacterial DFM. However,

hot carcass weight was generally greater when bacterial DFM were fed. Similarly, carcass characteristics were not altered by DFM treatment in the studies conducted by Swinney-Floyd et al. (1999) and Rust et al. (2000). The preplanned orthogonal contrast for control vs all DFM treatments used by Galyean et al. (2000) was significant for hot carcass weight (2.1% lower in control animals), but contrasts for other carcass characteristics were not significant. On the other hand, Huck et al. (2000) reported that the percentage of carcasses graded as Choice or Prime was greater in heifers receiving a *Propionibacterium* DFM in both the receiving and finishing phases than in control heifers. Swinney-Floyd et al. (1999) showed a decrease in liver abscesses at slaughter when feedlot steers were supplemented with a combination of *L. acidophilus* 53545 and *P. freudenreichii* P-63.

Improvements in carcass characteristics as a result of bacterial DFM are questionable, except for hot carcass weight (Elam et al., 2003). Because DFM generally improve ADG, hot carcass weight would be increased if DFM supplementation increased ADG and final BW. From the data reviewed, it seems reasonable to assume that bacterial DFM can be functional in feedlot settings, but the mechanism(s) by which they improve performance is still in question. Although the data are conflicting, the positive effects of bacterial DFM on performance might be associated with a decrease in ruminal acidosis and/or improved microbial balance in the lower gastrointestinal tract.

3.4.4. Milk yield and composition in dairy cows

Limited research has evaluated the efficacy of bacterial DFM for lactating dairy cows. Furthermore, in some of the studies the bacterial DFM were fed together with other additives making it difficult to judge the effects of the bacterial DFM themselves. In the few studies available in which DFM were fed to dairy cows, the milk yield increased by 0.75–2.0 kg/d. In general, an increased milk yield has been a consistent response, whereas changes in milk composition have been variable. For example, Jaquette et al. (1988) and Ware et al. (1988a) reported that the milk yield was 1.8 kg/d greater for cows fed a diet containing 2.0×10^9 cfu of *L. acidophilus* (BT1386) per day compared with those fed a control diet. Dry matter intake, milk fat and milk protein percentages were not affected by *L. acidophilus*. Gomez-Basauri et al. (2001) conducted a study to determine the effect of a supplement containing *L. acidophilus, L. casei, E. faecium* (total lactic acid bacteria = 1 billion cfu/g) and mannan-oligosaccharide on DM intake, milk yield, and milk component concentration. Cows fed lactic acid bacteria and mannanoligosaccharide consumed 0.42 kg/d less DM and produced 0.73 kg/d more milk. The authors reported that milk yields increased over time for lactic acid bacteria- and mannanoligosaccharide-fed cows, whereas control cows maintained constant milk yields.

In contrast to feeding bacterial DFM directly, Colenbrander et al. (1988) found that treatment of alfalfa silage with *L. acidophilus* did not improve DM intake, milk yield and milk composition in dairy cows, but efficiency (kg of fat-corrected milk/kg of feed) of milk production was improved by 7.1%. Other experiments have been conducted with combinations of fungal cultures and lactic acid bacteria (Komari et al., 1999; Block et al., 2000). Milk yields were increased by 1.08 kg/d and 0.90 kg/d, respectively when lactating cows were fed *S. cerevisiae* in combination with *L. acidophilus* or 5×10^9 cfu yeast in combination with 5×10^9 cfu *L. plantarum*/*E. faecium*. These studies suggest that bacterial DFM fed alone or in combination with fungal cultures might be efficacious for increasing milk production by lactating dairy cows. However, more research is needed before recommendations can be made.

4. YEAST

4.1. Introduction

Yeast products based on *Saccharomyces cerevisiae* are widely used in ruminant diets. The available products vary widely in both the strain of *S. cerevisiae* used and the number and viability of yeast cells present. Not all strains of yeast are capable of stimulating digestion in the rumen (Newbold and Wallace, 1992; Newbold et al., 1995; Miller-Webster et al., 2002). These differences are apparently not related to the number of viable yeast cells in the preparations (Newbold and Wallace, 1992), although their ability to stimulate rumen fermentation may be related to differences in metabolic activity.

Milk yield increased by an average of 4.5% and liveweight gain in growing adult cattle by 7.5% in response to yeast addition (Newbold, 1995). However responses were diet- and animal-dependent, with a greater response reported in early lactation and in animals fed high-concentrate diets. There is general agreement that production responses are the result of the action of the yeast within the rumen.

4.2. Mode of action

An increase in the number of total culturable bacteria that can be recovered from the rumen appears to be one of the most consistently reported responses to yeast addition and while the increases in culturable bacteria in many studies might not reach statistical significance, studies in which yeast products fail to stimulate bacterial numbers are rare. There is general agreement that the increased bacterial count seems to be central to the action of the yeast (fig. 1), driving both an increased rate of fiber degradation in the rumen and an increased flow of microbial protein from the rumen (Offer, 1990; Martin and Nisbet, 1992; Wallace and Newbold, 1992; Dawson and Girad, 1997; Kung, 2001).

What remains contentious however, is how small amounts of yeast in the diet can stimulate microbial numbers in the rumen. Low numbers of yeast (not *S. cerevisiae*) and molds occur naturally in the rumen (Lund, 1974), although growth of *S. cerevisiae* in the rumen seems unlikely (Durand-Chaucheyras et al., 1998). However, a lack of growth should not be confused with a lack of metabolic activity; Kung et al. (1997) found that *S. cerevisiae* was metabolically active in ruminal fluid for up to 48 hours. Yeast extracts that did not contain whole cells did not stimulate bacterial growth in the same way as *S. cerevisiae* (Girard and Dawson, 1995). Initial studies using rumen-simulating fermentors showed that autoclaved *S. cerevisiae* failed to stimulate bacterial numbers (Dawson et al., 1990; El Hassan et al., 1993). However, later studies indicated that *S. cerevisiae* contained both a heat-labile and a heat-stable component capable of stimulating microbial growth *in vitro* (Chaucheyras et al., 1995a, 1995b; Girard and Dawson, 1995). Koul et al. (1998) found that yeast sterilized by

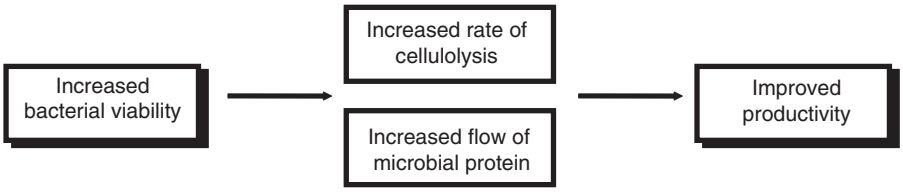

Fig. 1. The central role of an increase in bacterial numbers in the rumen in driving production responses to yeast addition.

gamma-irradiation rather than autoclaving retained 50% of its stimulatory activity, suggesting that approximately half of the activity was heat-labile. Nisbet and Martin (1990, 1993) reported that malate stimulated the growth of the prominent Gram-negative rumen bacterium *Selenomonas ruminantium* in media containing lactic acid. It has been suggested that organic acids provided by yeast might stimulate the growth of lactic acid-utilizing bacteria (Nisbet and Martin, 1991; Callaway and Martin, 1997) in the rumen, and certainly stimulation of lactic acid-utilizing bacteria by yeast has been noted *in vitro* and *in vivo* (Edwards, 1991; Dawson and Girad, 1997; Newbold et al., 1998). Reductions in rumen lactate concentrations have also been noted in animals supplemented with *S. cerevisiae* (Williams et al., 1991; Lynch and Martin, 2002).

Newbold et al. (1996) found that *S. cerevisiae* accumulated intracellular malic acid when incubated in autoclaved ruminal fluid but found no effect of adding malate to the rumen on the number of lactate-utilizing bacteria, although malate did appear to stimulate the cellulolytic bacterial population and fiber digestion. Kung et al. (1982) and Martin and Streeter (1995) found that malate, at higher concentrations than that likely to be supplied by *S. cerevisiae,* stimulated ruminal fermentation. Thus, while organic acids remain valuable tools to manipulate rumen fermentation (Martin, 1998; Lopez et al., 1999), the levels present in yeast do not appear to be sufficient to explain the action of yeast in the rumen.

It has also been suggested that yeast might supply B vitamins to the rumen (Martin and Nisbet, 1992; Callaway and Martin, 1997). Niacin and thiamine are known to affect rumen fermentation (Brent and Bartley, 1984). Chaucheyras et al. (1995b) found that the stimulation of the ruminal fungi *Neocallimastix frontalis* by *S. cerevisiae in vitro* was at least partially due to thiamine in the yeast. However, it is unlikely that *S. cerevisiae* at the levels used, could supply sufficient vitamins to stimulate fermentation *in vivo*. Rose (1987) suggested that yeast might scavenge oxygen within the rumen, thus stimulating the growth of anaerobic bacteria therein. Newbold et al. (1996) found a correlation between the ability of different yeast preparations to stimulate oxygen uptake by ruminal fluid and the ability of the yeast to stimulate the growth of rumen bacteria. The rumen is widely considered to be anaerobic; nevertheless, ruminal gas, even in nonfistulated animals, contains between 0.5% and 1.0% oxygen (McArthur and Miltimore, 1962), and dissolved oxygen is detectable *in situ* (Hillman et al., 1985). Many ruminal microorganisms are highly sensitive to the presence of oxygen (Loesche, 1969). Respiration-deficient mutants of *S. cerevisiae,* which were unable to remove oxygen from ruminal fluid, failed to stimulate bacterial numbers in rumen-simulating fermentors, in conditions in which the parent strains, capable of scavenging oxygen, did stimulate bacterial activity (Newbold et al., 1996). Recently, the possibility has been investigated that organisms other than *S. cerevisiae* might be used to stimulate oxygen uptake and microbial activity in ruminal fluid (Lee et al., 2003).

4.3. Animal responses

4.3.1. Effect of diet on the response to yeast

It is clear that the response of both lactating and growing animals to yeast addition is variable (Fiems, 1993; van Vuuren 2003) and that this variability is at least partially diet-related (Newbold, 1995). This variability is at least in part predicted by the model in fig. 1. A stimulation in the synthesis of microbial protein in ruminal fluid in response to yeast addition (Kung, 2001) will lead to an increased flow of microbial protein leaving the rumen and an enhanced supply of amino acids entering the small intestine (Erasmus et al., 1992).

However, an increase in microbial protein supply can only be expected to boost production in situations where duodenal protein supply is likely to limit productivity. Thus, Putman et al. (1997) found that milk yield increased when yeast was added to the diet of dairy cows in early lactation and fed a diet low in crude protein, but there was no response when yeast was added to a high crude protein diet. Similarly, responses to the inclusion of the yeast are often greater in early-, as opposed to mid- or late-, lactation (Harris and Lobo, 1988), again possibly reflecting the extent to which the available protein limited productivity. In addition, the stimulation in fiber digestion within the rumen (Kung, 2001) might be expected to stimulate productivity when fiber digestion is compromised and limits productivity. In support of this theory, Williams et al. (1991) observed greater responses in milk yield in response to yeast addition as the ratio of concentrate to forage in the ration increased. Similarly, yeast addition has been shown to improve dry matter intake and subsequent milk yield in prepartum cattle (Wohlt et al., 1998). As such, the model presented in fig. 1 provides a broad background to describe diets in which yeast supplementation will be beneficial (i.e. diets limited by either protein supply at the small intestine or ruminal fiber digestion).

Responses to yeast may also be modified by more subtle variations in the diet. Thus, Wallace and Newbold (1992) noted that responses in cattle fed corn silage tended to be higher than responses recorded in trials using diets based on grass silage. Furthermore, Quinonez et al. (1988) found that yeast stimulated milk yield in cows fed a diet of alfalfa hay plus wheat, but not when the wheat was replaced by corn, while Adams et al. (1981) noted that the response to yeast in dairy cows was greater on a corn silage/alfalfa hay diet than on diets of either corn silage/bermuda grass hay or corn silage alone. Clearly, the model in fig. 1 incompletely describes the interaction between diet composition and the response of ruminants to yeast supplementation, and a more detailed model, possibly reflecting changes in individual bacterial groups, needs to be developed to more completely describe the action of yeast in the rumen.

4.3.2. Variability in the response to yeast

Whether production responses or ruminal characteristics are considered, it is clear that responses to yeast addition are variable (Fiems, 1993; Newbold , 1995; van Vuuren, 2003). As discussed previously, part of the variability is diet-based, but there is also evidence that the strain of *S. cerevisiae* used in the yeast culture influences the effectiveness of the product. Not all strains of *S. cerevisiae* are capable of stimulating digestion in the rumen and care should therefore be taken in selecting yeast cultures to ensure that the strain used is capable of stimulating rumen fermentation. Similarly, yeast culture is a biological product and is adversely affected by excessive heating (El Hassan et al., 1993; Girard and Dawson, 1995; Koul et al., 1998). Commercial products have been developed which are capable of surviving pelleting but direct comparisons between strains are not within the public domain and again care should be taken to ensure that, if necessary, the product used is capable of survival during pelleting of the diet. However, the apparent variability in the response to yeast needs to be kept in context. Goodrich et al. (1984) summarized production data from a total of 228 trials using monensin and found a 7.5% improvement in the feed:gain ratio of cattle fed a variety of rations, however the response was highly variable with a standard deviation on the response of 6.5%. Despite this variation, few would doubt the value of monensin in beef cattle during the last 20 years. Recently van Vuuren (2003) presented a reanalysis of nine experiments and three field trials, involving over 1500 cattle, in which the effect of yeast on milk yield had been measured; overall the milk yield was stimulated by 4% with a 4.3% standard deviation.

5. FUTURE PERSPECTIVES

Research clearly demonstrates that feed enzymes, bacterial DFM and yeast have significant potential to improve feed efficiency of ruminants and to decrease the cost of production. Furthermore, there is strong evidence that bacterial DFM can be advantageously used to alter gastrointestinal flora, thereby stimulating the development of microbial populations that are beneficial to the host, or that decrease shedding of harmful enteropathogens such as *E. coli* O157:H7.

Yeast products are becoming more widely used commercially, particularly by the dairy sector. Yeast products are relatively inexpensive and there is increasing evidence to indicate they can be effective. However, there are conflicting hypotheses as to the mode of action. This is complicated by the paucity of data on the relative efficacy among commercial yeast products that claim functionality based on different modes of action. A better understanding of the mode of action would probably increase usage and help eliminate ineffective products from the marketplace.

Significant research is still required to understand the mode of action of bacterial DFM and enzyme products and thereby to ensure their efficacy on-farm. While the science has demonstrated the many beneficial effects of using these products, commercial availability of bacterial additives and feed enzymes will be limited by regulatory issues. In North America, new products will most likely be limited to those with source organisms from the list of approved organisms (AAFCO, 2002). Otherwise, a food additive assessment of safety and efficacy by the Center of Veterinary Medicine at the Food and Drug Administration is required (Clarkson et al., 2001), which will be cost prohibitive in most cases. In the EU, all enzymes and bacterial DFM require submission of a full dossier requiring extensive documentation to address issues of safety, functionality, labeling and manufacturing (Clarkson et al., 2001). The cost of preparing such a dossier will limit the commercial application of this technology.

Commercial use of enzymes and DFM additives is expected to increase as the use of antibiotics and ionophores in ruminant diets decreases. While the science clearly points to a bright future for these products to enhance animal productivity and health, marketing and regulatory issues may dampen some of this potential.

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8 Control of intestinal diseases by dietary supplementation with antibodies

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Due to the production conditions today about 20–40% of newborn farm animals (cattle, pigs, horses, goats, sheep) exhibit failure of passive transfer (FPT). The direct consequence of this is an increased susceptibility to diseases during the first weeks of life, which necessitates the wide use of antibiotics. As a further consequence, an increasing antibiotic-resistance of diarrheal strains of bacteria has become a fact. An alternative to using antibiotic is oral application of immunoglobulin products in the periods of greatest risk, i.e. within the first days of life and in the postweaning period. Air-dried egg yolk immunoglobulin (IgY), cow colostrum and swine serum, which can be produced on a large scale, provide the greatest chance for mass application. This work presents the most important principles of prophylactic and therapeutic, oral application of immunoglobulins on the basis of the published literature and the author's own investigations.

1. INTRODUCTION

1.1. Immunity of the neonate's gastrointestinal tract

The surface of the mucosal membranes exceeds by a hundred times that of the skin. It is at the mucosal membrane of the gastrointestinal tract that massive contact with the external environment occurs. Colonization of the host's tissue by bacteria begins by the binding of bacterial surface adhesins with appropriate ligands on the host's cells (Soto and Hultgren, 1999). Further stages involve breaking through the host's defense, proliferation and damage of the host's tissues (Kaper et al., 2004). Effective protection against potential infections via this route is a considerable challenge for the organism. The digestive tract protection system consists of natural barriers (epithelial continuity, mucus secretion, peristaltic motility, low pH of gastric juice, etc.); nonspecific humoral immunity factors (complement, lysozyme, defensins, etc.); nonspecific cellular immunity factors (macrophages, granulocytes, dendritic cells); specific humoral immunity (antibodies) and specific cellular immunity factors (T and B lymphocytes).

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The efficiency of the immune system in the healthy animal depends on the cooperation of all the mechanisms mentioned above (Mueller et al., 1983; Sheldrake and Husband, 1985; Riedel-Caspari and Schmidt, 1991; Honorio-Franca et al., 1997).

Statistics concerning the occurrence of gastrointestinal tract pathology in young animals indicate that this age group is more often prone to the failure of organ protection systems. On the day of birth no specific immune cells are found in the neonate's digestive tract. The colonization of the mucosal membrane lymphatic structures by lymphocytes takes place gradually. The first immune cells in the intestine are identified after 1–2 weeks of life (Navarro et al., 1997; Aminova et al., 2000) and their concentration comparable with that of adult animals is not achieved until after a few months of life. Until then the effectiveness of its self-active, specific, humoral and cellular immunity should be regarded as insufficient. The most important role is played by self-secretory IgA, the main mucosal immunoglobulin (Ig), of which a protective level is not achieved until after 2–4 weeks of life (Mestecky et al., 1991; Macpherson et al., 2001). However, the nonspecific immune mechanisms are efficient, although their activity may be slightly lower than that of adult animals (Mueller et al., 1983).

The observations mentioned above indicate that maintaining the gastrointestinal tract homeostasis in the first weeks of life depends to a large extent on the passive protection from maternal immunity (Stefaniak, 2000). Early drinking of colostrum provides supplemental antibodies for the gastrointestinal tract (Mellor and Murray, 1986). Due to the protection provided by maternal antibodies the microorganisms entering the neonate's digestive tract (Korhonen et al., 1995; Palmeira et al., 2001) have limited success in attacking the mucosal membrane (fig.1).

Other colostral components, such as immunomodulating and/or antimicrobial substances, including lactoferrin, lysozyme, lactoperoxidase and cytokines (Wagstrom et al., 2000; He et al., 2001; Blum and Baumrucker, 2002; Solomon, 2002) also have considerable significance for protection of the newborn. Furthermore, colostral components can also contribute to the cellular immunity, e.g. significant numbers of maternal cells may contribute to neonatal immunity, including phagocytes (neutrophils and macrophages), lymphocytes (B and T cells) and epithelial cells (Wagstrom et al., 2000). An interesting natural protective colostral mechanism is provided by the presence of soluble receptor analogs, which block the microbial adhesins and other pathogenic factors (Lindahl, 1989; Kelly and Younson, 2000). An example of this is the binding of F41 and K99 fimbrias of enterotoxigenic strains of *Escherichia coli* by glycoproteins present in cow and sow colostrum, and due to which pathogene adherence to intestinal epithelium of a neonate becomes weakened (Lindahl, 1989).

However, the most important protective role is played by specific antibodies against antigens present in the neonate's environment (Riedel-Caspari, 1993; Korhonen et al., 2000b; Le Rousic et al., 2000; Crouch et al., 2001; Pisarska et al., 2002). Maternal antibodies, obtained from the colostrum and milk, protect the neonate against infections until its own efficient specific immune protection is developed (figs. 2 and 3).

A cow should produce at least 2 L of the first colostrum, containing at least 5% of Ig (50 g/L), which equals about 100 g per calf (Kruse, 1970; Tyler et al., 1999). In the study by Kruse (1970) 12% of cows did not give 2 L of colostrum and 30% showed an IgG concentration below 50 g/L. Similar data, obtained by Levieux and Ollier (1999), point to the fact that calves originating from primiparous cows are endangered by insufficient passive protection. Immunoglobulin concentrations in secretions of the ruminant mammary gland decrease rapidly after parturition and for instance in cow milk remain below 2 g/L at the 8th milking and below 1 g/L after the 15th milking (Levieux and Ollier, 1999). Insufficient quantity of

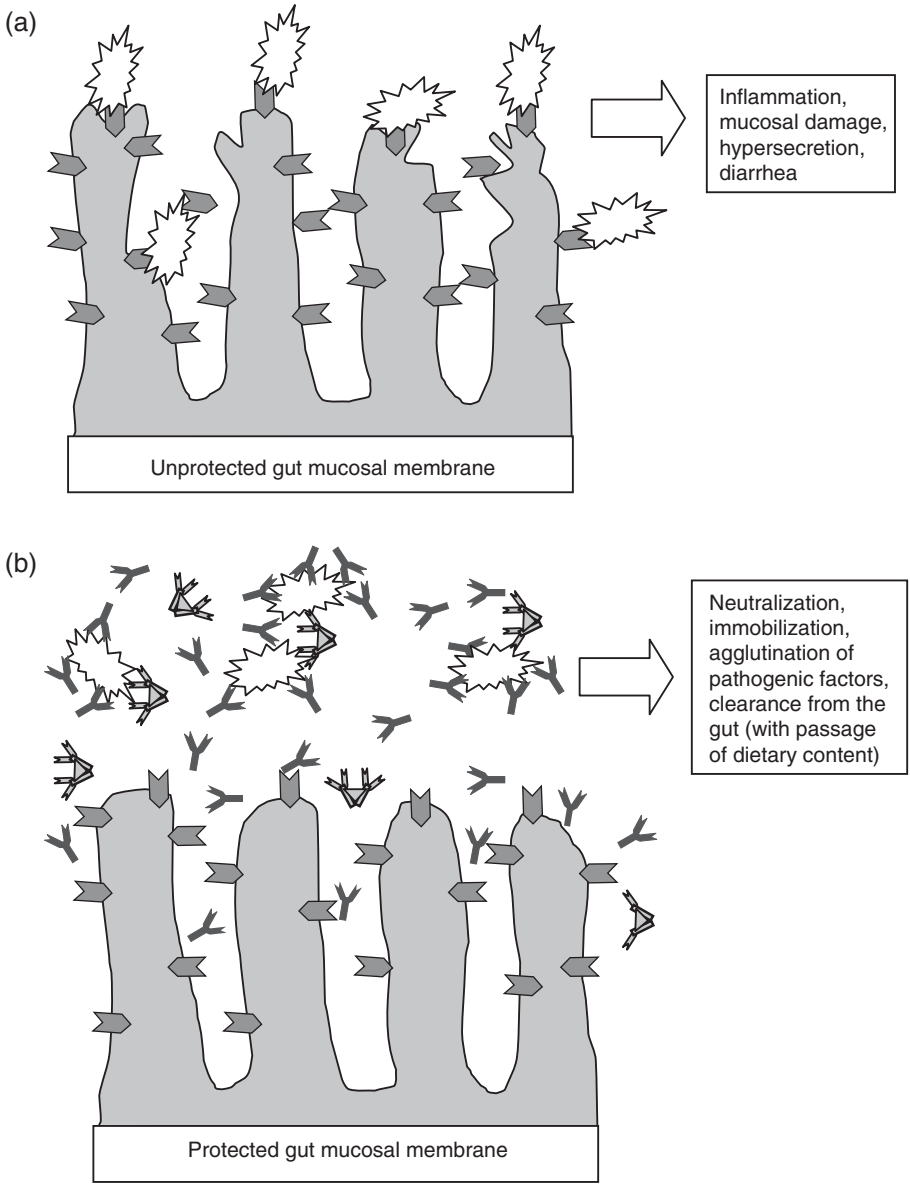

Fig. 1. Protection of the gut by specific antibodies. (a) Pathogenic factor attacking the unprotected mucosal membrane; (b) Protection due to early feeding with the immune colostrum, and/or prophylactic oral immunoglobulin supplementation. High concentration of antibodies protects the mucosal membrane. ⋋ = IgG antibodies (from colostrum or oral immunoglobulin supplementation); Ψ = SIgA antibodies (originating from colostrum or produced locally in low quantities within the gut of the young animal); ⌲ = specific receptor (to bacterial adhesins, toxins, viruses, etc.); ✲ = pathogenic factor (bacterial, viral, toxic).

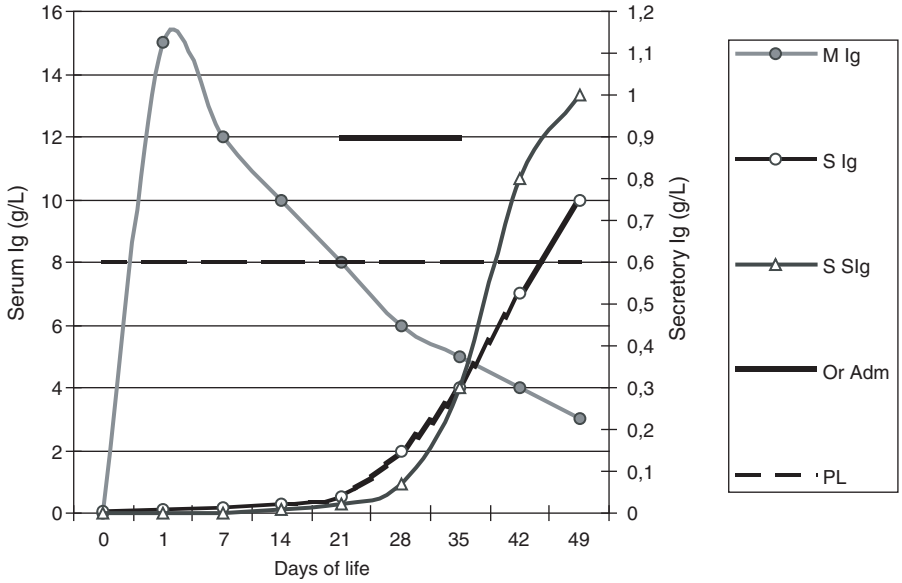

Fig. 2. Typical serum and secretory immunoglobulin levels and recommended period of oral immunoglobulin administration in healthy calves (M Ig – maternal Ig in calf serum; S Ig – self serum Ig; S SIg – self secretory Ig in the gut; Or Adm – recommended period of oral Ig administration; PL – assumed protective level).

specific antibodies, or their inadequacy towards environmental microbes, facilitate early colonization of the neonate's digestive tract as well as its susceptibility to diseases (fig. 4).

It is believed that the main role of the cellular components in mammary secretions is to interact with the development of local immunity in the newborn and to modulate active immunization of the neonatal intestine during this critical period, which is especially important to the development of the young (Le Jan, 1996; Barrington and Parish, 2001).

1.2. Failure of passive transfer (FPT) and its consequences to the neonate

In today's production practices, about 20–40% of newborns of large farm animals (cattle, swine, horses, goats, sheep) develop failure of passive transfer (FPT) (Haława and Stefaniak, 2000). FPT means the acquired humoral specific immune deficiency of the neonate due to insufficient absorption of maternal, colostral immunoglobulins (fig. 5).

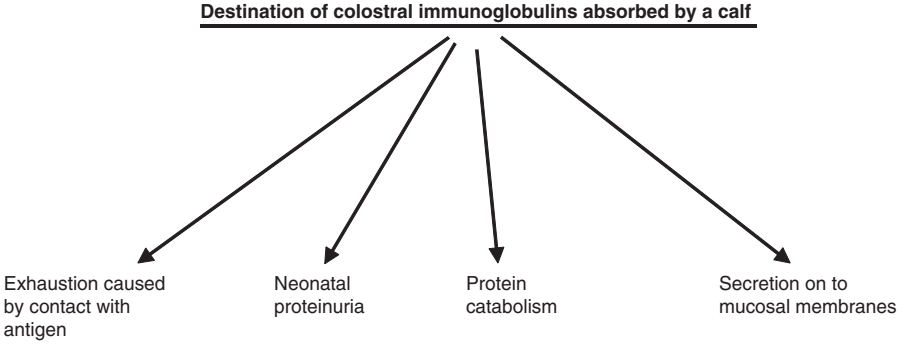

Fig. 3. Utilization of maternal immunoglobulins in neonates exemplified by a calf.

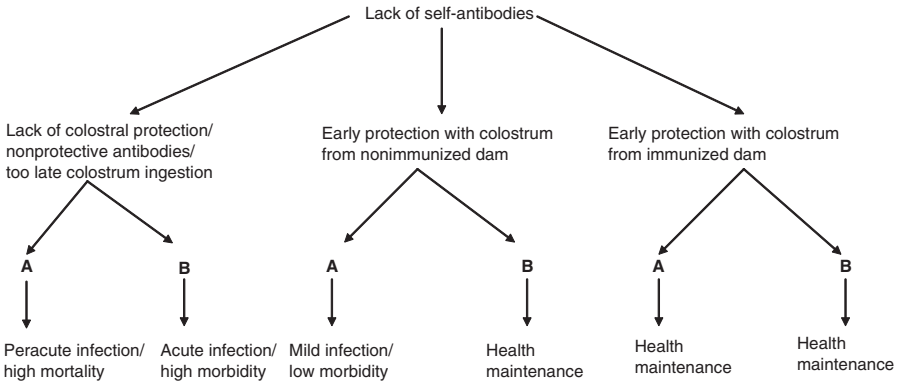

Fig. 4. Gastrointestinal tract pathology as a result of insufficient colostral protection of a neonate (A = massive infection/ high pathogenicity, B = small quantity of the infectious agent/ moderate pathogenicity).

A direct consequence of this is an increased susceptibility to diseases in the first weeks of life. Calves exhibiting inadequate IgG concentrations are at greater risk of neonatal morbidity and preweaning mortality (McGuire et al., 1976; Vermunt, 1994; Wittum and Perino, 1995; Rea et al., 1996; Tyler et al., 1999), which necessitates the wide use of antibiotics. Because of the increasing antibiotic-resistance of bacterial strains, the common use of antibiotics, to protect the young animals against diarrhea, is becoming less and less effective. This is accompanied by raised consumer awareness of safe food (antibiotic-free) and the revised definition of safe food (Nikołajczuk and Molenda, 2000). Its new understanding points to food derived from animals kept in welfare conditions, without any antibiotic treatment throughout their life. This is another reason for withdrawing antibiotics and searching for alternatives (Kelly and Younson, 2000; Mine and Kovacs-Nolan, 2002).

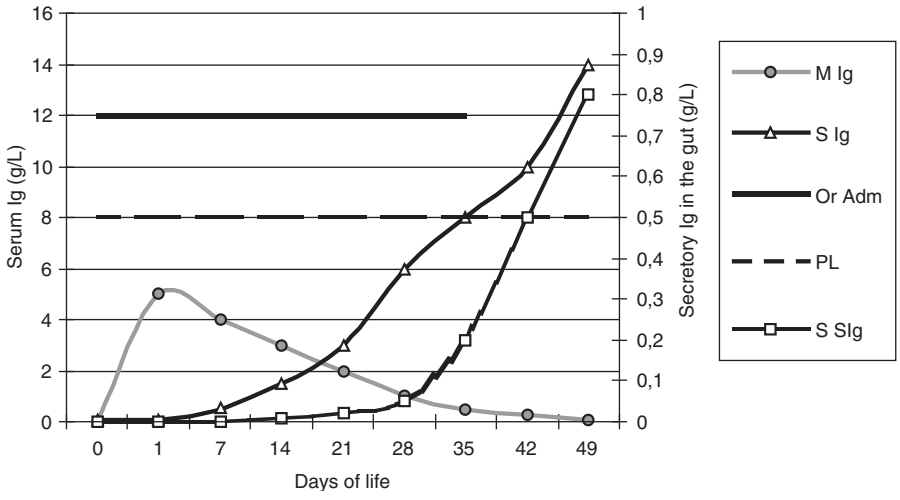

Fig. 5. Typical serum and secretory immunoglobulin levels and recommended period of oral Ig administration in calves with Failure of Passive Transfer (M Ig – maternal Ig in calf serum; S Ig – self serum Ig; Or Adm – recommended period of oral Ig administration; PL – assumed protective level; S SIg – self secretory Ig in the gut).

The binding of microbial adhesins to host receptor molecules is a critical early step in microbial infection and pathogenesis. Bacterial toxins also require at the first stage to bind with a specific ligand (Girardeau and Bertin, 1995). Exemplary well-recognized adhesins, bacterial enterotoxins and their appropriate ligands are presented in table 1.

Therefore, the possibility of blocking this interaction seems to be an attractive means of preventing infection at the early stage. Oral application of anti-adhesin antibodies is the most commonly used strategy of adhesion-blocking, but alternative aids are under investigation, especially adhesin analogues and receptor analogues (Kelly and Younson, 2000).

Orally administered specific antibodies against enteropathogenic viruses block the development of diarrhea resulting from infections with rota- and coronaviruses (Besser et al., 1988; Snodgrass et al., 1990; Heckert et al., 1999; Erhard et al., 1993; Ikemori et al., 1997).

There are two main periods of the highest risk of diarrhea (Frank and Kaneene, 1992; Vermunt, 1994; Sivula et al., 1996; Yokoyama et al., 1998), which require local immunity supplementation:

1. the neonatal period (age 0–14 days): the main risk factors are enterotoxigenic *E. coli* and rotavirus infection
2. postweaning period: associated with stress, changes in the feeding programme; the highest risk is during the first 2 weeks in the new environment.

Therefore, research into the wide application of immunoglobulins for the prophylaxis of animal gastrointestinal tract diseases ensues from ascertaining that the methods of health protection applied so far, based on massive antibiotic administration, have not been efficient enough and have caused numerous side effects. In contrast, the high efficiency of prophylactic and therapeutic oral application of antibodies is indicated in fig. 4.

2. DIETARY SUPPLEMENTATION WITH ANTIBODIES

One of the methods of treatment of acquired immune deficiency in young animals is oral application of immunoglobulin preparations containing antibodies directed against the incriminated digestive tract pathogens (Bogsted et al., 1996; Barrington et al., 2002). The most important sources of antibodies used in the protection of the alimentary tract of newborn farm animals are air-dried cow colostrum, egg yolk immunoglobulin and swine serum (Haines et al., 1990; Facon et al., 1993; LeRousic et al., 2000; Shibata et al., 2001; DeRouchey et al., 2003; Owusu-Asiedu et al., 2003a, b; Stefaniak et al., 2003). The choice of antibody source and its purification method need to be made in consideration of the circumstances of use, target animals, specificity, costs, quantity needed (table 2a and b).

2.1. The origin of orally applied antibodies

2.1.1. Allogenic (obtained from the same species, e.g. swine serum for piglets)

Allogenic antibodies cooperate with other host-specific immune mechanisms. Their main positive effect is the rapid elimination of pathogenic agents, but on the other hand, they can induce unintended tissue injury due to complement activation at the mucosal membrane (bystander effect). Bovine colostrum purified IgG was absorbed from calf neonatal intestine more effectively than native colostral immunoglobulins, air-dried colostral immunoglobulins or swine serum immunoglobulins given in similar concentrations (Arthington et al., 2000a, b). Absorbed allogenic immunoglobulins do not induce an immune response due to the intraspecies antigenic similarity. Passively acquired colostral IgG_1 anti-bovine rotavirus diffuse from blood to gut mucosal membranes up to the 10th day of calf life (Besser et al., 1988),

but in the case of intensive infections such antibodies are insufficient to protect the gastrointestinal tract against diarrhea. However, oral supplementation of calves with bovine colostral immunoglobulin concentrate resulted in higher serum immunoglobulin concentration within the first 4 weeks of life and additionally improved the weight gain and diminished the incidence of diarrhea (Nousiainen et al., 1994).

2.1.2. Xenogenic (obtained from different species, e.g. egg yolk immunoglobulin for mammals)

This kind of antibody could well merit common usage to protect the young animals. Important information about application of xenogenic antibodies is:

1. When administered during the period of Ig absorption from the gut to bloodstream (e.g. in foals before the 24th hour of life, in calves before 27–30th hour and in piglets, lambs and goat kids before 36–48th hour) xenogenic proteins appear in the blood of recipient animals – they stimulate the immune response against foreign epitopes present on the given immunoglobulins. In consequence, it leads to fast Ig elimination (removal of immune complexes) during 2–4 weeks after ingestion, although their absorption is not as effective as that of allogenic Ig (Stefaniak, 2002; Gąsowska and Stefaniak, 2003). In contrast, no absorption occurs and no such risks are observed in older animals (usually after 48 hours of life).
2. The lack of, or occurrence of less intensive interactions with the cooperating immune mechanisms, may be advantageous in the protection of a healthy gastrointestinal tract. Binding the specific antibodies to the pathogen (fig. 1) prevents interaction of the microbe with the mucosal membrane followed by immune exclusion (removal with the feces).

2.2. The most common antibody preparations administered orally

Practically, air-drying of egg yolk immunoglobulin, cow colostrum or swine serum is the only method, which allows large-scale production at low cost. Heat treatment reduces microbiological contamination of the final products, and allows their long-term storage. However, the most important disadvantage of air drying is the loss of antibody activity, which can reach over 50% (Stefaniak, 2002). The greatest denaturation and aggregation of cow immunoglobulins under the influence of temperature occur at pHs close to their isoelectric points (Lindström et al., 1994; Dominguez et al., 2001). The sensitivity of immunoglobulins to heat and their tendency to aggregation increase when there are low concentrations of salt in the solution. Although heat treatment (air-drying, pasteurization, UHT) causes a considerable decrease in the activity of specific antibodies (Korhonen et al., 2000a; Stefaniak, 2002), they still maintain restricted activity for months during their storage. Lyophilized antibodies preserve over 90% of their activity during 1 year storage (Stefaniak and Kopeć, 1997; Stefaniak, 2002, Stefaniak et al., 2004) and although they were also orally applied to young animals, relatively high costs and the low efficiency of freeze-drying has resulted in the abandonment of this method.

Egg yolk antibody from vaccinated laying hens seems to be a cheaper and good source of antibody (Jin et al., 1998). Simultaneous addition of spray-dried porcine plasma and egg yolk antibody to the fodder of early-weaned piglets appears to be an interesting idea (Owusu-Asiedu et al., 2003a).

The main immunoglobulin class in mentioned antibody sources is IgG. A comparison of the IgG of cattle, swine and hens is presented in table 3.

Table 1

Adhesins and toxins of selected diarrheagenic strains and their ligands on the enterocytes

Species/strain	Adhesin /toxin	Ligand on host enterocyte	References
Escherichia coli	fimbriae type 1 (MS)	mannose	Yuehuei and Friedman 2000
	fimbriae type P (Pap)	Gal-1,4-Gal	
	fimbriae type G	GlcNAc	
	fimbriae type S	sialic acid	
enteropathogenic	Intimin	-translocated intimin receptor, Tir	Nougayrede et al. 2003
Escherichia coli (EPEC)		-other cellular receptors (β1-integrin?)	
	bundle-forming pilus and flagella	unknown receptors	
enterotoxigenic	K88 or F4 fimbriae K88ab	pig intestine	Van den Broeck et al. 2000
Escherichia coli (ETEC)	(F4ab)	IMTGP*, GP74**	
	K88ac (F4ac)	IMTGP	Francis et al. 1999
	K88ad (F4ad)	IGLad ***	Yuehuei and Friedman 2000
		bcd receptor - collection of glycoproteins with molecular masses ranging from 45 to 70 kDa	
		bc receptor - two glycoproteins 210 and 240 kDa	
		b receptor - glycoprotein 74 kDa,	
		d receptor - glycosphingolipid with unknown molecular mass	
	K99 (F5)	ganglioside containing neuraminic acid	

Shigella dysenteriae	Vero/Shiga toxins (VT/Stx)	cause inhibition of protein synthesis in eukaryotic cells	
enterohemorrhagic *E. coli* (EHEC)	A subunit (N-glycosidase activity against 28S rRNA)	Gb3 or Gb4	Gyles 1992
vero/shiga-toxigenic *E. coli* (VTEC/STEC)	B subunit	membrane receptor	Mainil 1999
ETEC	LT enterotoxin		Gyles 1992
enterotoxic	subunit B	Glycolipids (GM1) or glycoproteins family of receptors	Kaper et al. 2004
Escherichia coli	STI (STa)- peptide of 18 or 19 amino acids.		Lucas and Corthier 1991
	STII (STb)	unknown	Nair and Takeda 1998
	STa family	no data	Nair and Takeda 1998 (rev.)
Vibrio cholerae non-O1	NAG-ST		
Hakata strains of *V. cholerae* non-O1	H-ST		
Vibrio mimicus	M-ST		
Yersinia enterocolitica	Y-ST		
Citrobacter freundi	C-ST		
Klebsiella pneumoniae	STa-like enterotoxin		
Clostridium difficile	Toxin A (enterotoxin)	trisaccharide receptor	Lyerly et al. 1988

* intestinal mucin-type sialoglycoprotein (IMTGP)
** intestinal transferrin (GP74)
*** intestinal neutral glycosphingolipid (IGLad)

Table 2

Examples of oral substitution with immunoglobulins (col. – colostrum)
(a) in the period of intestinal immunoglobulin (Ig) absorption in the newborn animals

Animal species	Source of Ig	Specificity	Dosage	References
Cattle	IgY	*Cryptosporidium* sp.	300 mg a day for 2 weeks (2 times a day 20 g of air-dried whole egg)	Erhard et al. 1997
		nonimmunized	0.5–2 g/kg of body mass	Stefaniak 2002 Gąsowska and Stefaniak 2003
			8–16 g a day for first 10 days of life	Erhard et al. 1995
		E. coli K99 and rotavirus	2–10 g of air-dried whole egg 2 times a day for 10–14 days	Heckert et al. 1999
		rotavirus/coronavirus/*E. coli* F5	10 g a day from 2–14 day of life	Erhard et al. 2000
		E. coli K99/rotavirus	11 g of air-dried whole egg twice a day for first 14 days of life	Erhard et al. 1993
		Salmonella	1 g 3 times a day	Yokoyama et al. 1998
	Cow col.	bovine rotavirus/ *E. coli* K99/F41	single additional feed 2 L within 12 h after birth	Le Rousic et al. 2000
	Bovine serum	nonimmunized	single feed 58–77 g IgG in 2.4–3.2 L	Todd et al. 1993
			10–25 g of air-dried plasma/L milk	Nollet et al. 1999
Swine	Cow col.	PED	2 weeks	Shibata et al. 2001
		nonimmunized	20% of basal liquid diet	Gomez et al. 1998
	IgY	nonimmunized	0.5–2 g/kg of fodder, from 5th day up to weaning	Stefaniak et al. 2003
		ETEC (K88)	64.5–100 mg in 5 ml solution	Yokoyama et al. 1993
		PED	2–4 ml twice a day	Kweon et al. 2000
	Whole egg IgY	ETEC (F4, F5, F6)	5% of milk replacer from 2 until 12 days of life	Hennig-Pauka et al. 2003
		ETEC (K88, K99, 987P)/ rotavirus	3 g in 5 ml once a day for 3 days	Kellner et al. 1994
Goat	Cow col.	nonimmunized		Orsel et al. 2000
Horse	Cow col.	nonimmunized		Warko et al. 1993
Sheep	Frozen cow col.	*Cryptosporidium parvum*	50 ml twice a day for seven days	Naciri et al. 1994
	Frozen ewe col.	nonimmunized	600 ml/lamb/first 30 hours	Klobasa et al. 1991
			50–125 ml/kg body weight/first day	Mellor and Murray 1986, Klobasa et al. 1994

Mouse	IgG from rabbit antiserum monoclonal antibody	*Vibrio cholerae* O1 LPS *Cryptosporidium parvum*		Mukhopadhyay et al. 2000 Arrowood et al. 1989
(b) after intestinal Ig absorption/in postweaning period				
Cattle	IgY	nonimmunized	0.5–2 g/L of milk replacer, 2 weeks after weaning	Gąsowska et al. 2001
		rotavirus	2 g three times a day, for 2 weeks	Kuroki et al. 1997
		E. coli K99+ rotavirus	2–10 g two times a day for 10–12 days	Heckert et al. 1999
		E. coli (K30, K99, F41)	0.03–0.12 g three times a day for 7 days	Ikemori et al. 1992
		nonimmunized	10–20% of spray-dried whole egg in milk replacer	Quigley 2002
	IgY or cow col.	coronavirus	twice a day for 7 days air-dried, high titer egg yolk or cow col. in 1–1.5 L of milk	Ikemori et al. 1997
Swine	cow col.	PED	2 weeks	Shibata et al. 2001
	IgY	nonimmunized	0.5–2 g/kg of fodder, 2 weeks after weaning	Stefaniak 2002
		nonimmunized	0.5–2 g/kg of fodder, from 5th day up to weaning	Stefaniak et al. 2003
		ETEC (F18)	1–5% of air-dried whole egg in fodder given 7–29 day postweaning	Zuniga et al. 1997
			1.5 g of IgY in 5 ml orally 1–3 times a day for 3 days	Marquardt et al. 1999
			ad libitum fodder containing 5% of air-dried whole egg	Erhard et al. 1996
	IgY and ADPP	ETEC (K88)	0.2–0.3% of egg yolk powder, 5–10% of ADPP	Owusu-Asiedu et al. 2003a and 2003b
		nonimmunized	5% of crude preparations to the fodder	Schmidt et al. 2003
		nonimmunized		DeRouchey et al. 2003
Mouse	cow col.	human rotavirus		Ebina et al. 1992
		E. coli O157:H7		Funatogawa et al. 2002
Geckos	cow col.	*Cryptosporidium* sp.		Graczyk et al. 1999
Snakes			1% of body weight once a week, 6 times	Graczyk et al. 1998
Guinea pig	cow col.	*Cryptosporidium parvum*		Hoskins et al. 1991
Chicken	IgY	nonimmunized	1–2 g/kg of the fodder 2–42 day of life	Stefaniak et al. 2004

2.3. Cow colostrum

Cow colostrum is the only kind of colostrum used on a large scale, due to the large volume secreted and even some "overproduction" (Klobasa et al., 1991). Cow milk and colostrum are

Table 3

Characteristics of IgG from bovine colostrum, swine plasma and egg yolk

Factor	Bovine IgG	Swine IgG	Egg yolk IgG (IgY)
molecular weight	about 150 kDa between bovine and swine		about 170 kDa
light chain	about 25 kDa	about 25 kDa	about 25 kDa
heavy chain	about 50 kDa between bovine and swine		about 68 kDa
carbohydrate content	below 3% between bovine and swine		up to 6%
number of C_H domains	3	3	4
PH-dependent destruction of secondary structure	at pH 2.0	at pH 2.0	at pH 2.0 sudden conformation changes below pH 4.0
70–72°C	about 50% lost of antibody activity on the middle		
trypsin digestion susceptibility	swine < bovine IgG_1< bovine IgG_2 < chicken on the middle		
chymotrypsin digestion susceptibility	slight for IgG_1		
pepsin digestion susceptibility	IgG_1 more susceptible than IgG_2	(20 h) limited at pH 4.5 total at pH 3.5	
protease inhibitors in the crude material	colostral trypsin inhibitor	serum protease inhibitors	ovoinhibitor ovomucoid
molecular flexibility	swine < bovine = chicken		
stepwise denaturation by guanidine-HCl	over 2.5 M solution	over 2 M solution	rapid denaturation over 2.5 M solution
protein A binding	high affinity, some differences between IgG_1, IgG_{2a} and IgG_{2b}	high affinity in 90% of serum IgG	low affinity
approximate annual yield of IgG*	160–960 g/cow	20–75 g/2 porkers	30–60 g/hen
approximate annual IgG yield/ kg of body weight*	0.27–1.6 g	0.09–0.34 g	15–30 g
aggregation in 1.5 M NaCl	slight	slight	distinct

*assumption that the source of bovine immunoglobulin is colostrum, for swine – porker's blood serum, for hens – the eggs of laying hens; the cow of 600 kg body weight supplies annually 4–8 L of first colostrum featuring Ig concentration 40–120 g/L; the porker gains its body weight of 110 kg within half a year – the blood collected at slaughter can supply 1–1.5 L blood serum/plasma of concentration 10–25 g Ig/L; the laying hen of 2 kg body weight lays about 300 eggs a year, containing 100–200 mg IgY.

(Data from: de Rham and Isliker, 1977; Brock et al., 1978; Bennell and Watson, 1980; Olsovska et al., 1982; McClead and Gregory, 1984; Shimizu et al., 1988; Linder et al., 1991; Shimizu et al., 1992; Hatta et al., 1993a,b; Shimizu et al., 1993b; Ternes et al., 1994; Quigley et al., 1995; Stefaniak, 2002.)

an attractive source of antibodies, willingly applied and easy to process. Bovine IgG_1 prevails in colostrum and milk. When applied orally it is advantageously more resistant to digestion than other classes (de Rham and Isliker, 1977); resistance of this subclass to digestion is comparable to that of IgA. Bovine colostral secretory IgA would also resist proteolysis within the digestive tract, but the amount produced is very low (McClead and Gregory, 1984). The presence of trypsin inhibitor in colostrum provides for IgG_1 protection; the inhibitor protects IgG_1 from proteolytic degradation in the intestine and moreover its concentration is positively correlated to IgG_1 concentration (Quigley et al., 1995). Orally given colostral antibodies derived from cows immunized with rotavirus or *Clostridium difficile* retain their activity after passage through human intestine (Roos et al., 1995; Kelly et al., 1997; Pacyna et al., 2001). Long-term *in vitro* digestion of colostrum whey with chymotrypsin resulted in maintenance of antibacterial IgG activity with simultaneous decline of IgM activity (Brock et al., 1978), while digestion with trypsin reduced IgG activity, but left IgM activity hardly changed. The authors determined the specific activity which required complete immunoglobulin molecules. In the digestive tract, the activity of the $(Fab)_2$ fragment alone is quite sufficient to satisfactorily restrict the possibility of contact between the organism and the mucous membrane (Reilly et al., 1997). Protection of the mucosal membrane is maintained in spite of proteolysis of IgG and IgA immunoglobulin molecules (McClead and Gregory, 1984; Hilpert et al., 1987; Molla et al., 1988).

A well-known method of preventing diarrhea in neonatal calves is twice a day administration of surplus colostrum of the first and second milking mixed with milk or milk substitute throughout the period of the greatest risk of diarrhea, usually up to 10–12 days of life (Castrucci et al., 1984; Möstl and Bürki, 1988; Gutzwiller, 2002). Cow colostrum from nonimmunized cows or cows hyperimmunized with rotavirus, coronavirus, *Escherichia coli* or *Shigella* successfully prevents diarrhea also in neonates of other species (Brunser et al., 1992; Klobasa et al., 1994; Tzipori et al., 1994; Gomez et al., 1998; Ashraf et al., 2001; Shibata et al., 2001).

There are several indications for the application of cow colostrum to foals, lambs and kids (Klobasa et al., 1991; Winter and Clarkson, 1992; Perl et al., 1995):

- lack of, or low quality of maternal colostrum
- mastitis
- twins or multiple fetuses
- control of the infectious diseases transmitted through colostrum (caprine arthritis-encephalitis, maedi-visna, mycoplasmosis).

2.4. Yolk immunoglobulin (IgY)

IgY differs from mammal IgG in having a higher molecular weight, i.e. about 170 kDa, due to the occurrence of four C_H domains in the heavy chain (Shimizu et al., 1988; Nakai et al., 1994). Egg yolk immunoglobulin displays high susceptibility to aggregation in salt solutions (1.5 M NaCl) and it is more susceptible to a decrease of pH to 2.0–4.0, as well as to digestion with proteolytic enzymes (trypsin, chymotrypsin) and is less heat-resistant than rabbit IgG (Shimizu et al., 1992; Hatta et al., 1993a, b). No considerable differences were found in the resistance to low pH, to proteolysis and to heat between IgY and cattle, goat and swine IgG (Shimizu et al., 1993b; Dominguez et al., 2001).

Egg yolk is a rich source of IgY, containing about 10–25 mg IgY/ml. From this value one can calculate that a single laying hen can produce 30–40 g IgY per year (Mine and Kovacs-Nolan, 2002). In proportion to animal body mass, the production volume of immunoglobulins

is several times higher than that from other comparable sources (such as rabbit and swine serum or cow colostrum). Noninvasive harvest of eggs, and easy IgY isolation are additional advantages (Tini et al., 2002). So far, IgY has been applied in prophylaxis and therapy in calves – against enterotoxic *E. coli* K99, *Salmonella* spp., rotaviruses, coronaviruses, *Cryptosporidium* spp.; in piglets – against enterotoxic *E. coli* K99, K88 and 987P, and against rotaviruses; in mice – against rotaviruses; in rabbits – against enterotoxic *E. coli*; also in humans – against enterotoxic and enteropathogenic *E. coli*, rotavirus (Barz et al., 1980; O'Farelly et al., 1992; Yokoyama et al., 1992; Erhard et al., 1996; Ikemori et al., 1997; Amaral et al., 2002). IgY preparations were successfully applied during the periods of the greatest risk, i.e. in neonates and in newly weaned animals. Yet, egg yolk immunoglobulin has not been used so far on a large scale in humans, except for Japan and South Korea, where it is an additive to yoghurts, sweets and baby food.

A single dose of 65 mg IgY, from hens immunized with *E. coli* K88, assured specific antibody activity was present for up to 24 h in the colon of piglets aged 1–21 days (Yokoyama et al., 1993). When broiler chickens were given 0.5–1 g of IgY/kg feed, IgY antibody activity against *E. coli* O157, *Salmonella enteritidis*, *S. typhimurium* and *Klebsiella pneumoniae* were recorded in the feces. The intensity of the ELISA reaction was proportional to the IgY dose (Stefaniak et al., 2004).

Most studies involved the use of whole yolk from hens immunized with definite antigens on an experimental scale (Barz et al., 1980; Yokoyama et al., 1992; Rzedzicki and Wernicki, 1994; Erhard et al., 1996; Ikemori et al., 1997). Air-dried immunoglobulin preparations obtained from commercial eggs displayed antibody activity against diarrheal strains of *Escherichia coli* O157, *Salmonella enteritidis*, *S. typhimurium* and *Klebsiella pneumoniae* and was successfully applied (in the dose of 1–2 g/kg feed or 1 L of a milk-substituting preparation), in the prophylaxis of diarrhea in weaned calves as well as in suckling and weaned piglets (Stefaniak, 2002; Gąsowska and Stefaniak, 2003; Stefaniak et al., 2003). High levels of addition (5–20%) of spray-dried whole eggs can restrict the calves' and piglets' weight gain because of the presence of protease inhibitors in egg white, as well as because of the differences in amino acid content in egg as compared to the needs of those mammals (Quigley, 2002; DeRouchey et al., 2003; Owusu-Asiedu et al., 2003a, b).

2.5. Sera

Serum produced from the blood of slaughtered swine allows the large-scale production of immunoglobulins. A commonly used supplement of fodder is spray-dried porcine plasma – SDPP (DeRouchey et al., 2003; Schmidt et al., 2003). It contains the whole spectrum of serum proteins, including the biologically active ones. In the commonly proposed doses (5–10% addition to the fodder) it constitutes an important source of alimentary protein (Schmidt et al., 2003). In the proposed amounts it results in a considerable increase of feeding costs. Spray-dried swine plasma is commercially available in the USA and other countries and is applied orally to suckling and early-weaned piglets. Due to the savouriness of fodder containing SDPP the piglets eat it in higher amounts and achieve higher weight gains than the control piglets fed with standard fodder.

Rabbit immune serum was successfully applied orally to rats experimentally infected with a pathogenic *E. coli* strain (Rivier and Sobotka, 1978). Immunoglobulins originating from cows immunized with *V. cholerae* were introduced with satisfactory results concerning rabbit protection (Boesman-Finkelstein et al., 1989). Swine serum possesses IgG fractions with different resistance to cleavage by trypsin or pepsin (Olsovska et al., 1982). Because of the

fact that spray-dried blood serum preparations are significantly contaminated, it is advisible for them to undergo irradiation (DeRouchey et al., 2003).

2.6. Problems associated with oral application of immunoglobulins

An important problem with oral administration is proteolysis of the immunoglobulins by digestive enzymes, while another problem is caused by inactivation of antibody activity due to low pH. Irreversible inactivation of nonprotected IgY occurs at pH below 4. There are some methods proposed to protect the applied antibodies against proteolysis by gastric juice at low pH, e.g. use of hydroxypropyl methylcellulose phthalate (Ikemori et al., 1996). In the opinion of Ikemori and coworkers (1996) spray-drying of IgY with hydroxypropyl methylcellulose phtalate resulted in the protective binding or coating of antibodies. It enabled them to withstand acidic condition and to retain the antibody activity during the passage through the small intestine. Acid stability of IgY was enhanced in the presence of 30% sorbitol (Lee et al., 2002), or by encapsulation of IgY in the liposomes (Shimizu et al., 1993a).

Bovine IgG_1 is the major immunoglobulin in cow colostrum and milk and it is more resistant to digestion than other immunoglobulin classes, which can be regarded as an advantage for oral administration (de Rham and Isliker, 1977). High doses of orally given immunoglobulins are more resistant to digestion and low pH within the gastrointestinal tract; at low concentrations their activity disappears before reaching the posterior segments of the intestine (Petschow and Talbott, 1994). On the other hand, IgG is susceptible to cleavage by bacterial proteases in the vicinity of hinge region, and indeed is more susceptible to cleavage than IgA, while IgA_1 is more susceptible than IgA_2 (Molla et al., 1988).

Orally given air-dried immunoglobulins of colostrum-supplements were only about one third as effectively absorbed by calves as immunoglobulins from fresh cow colostrum (Garry et al., 1996). Worse protection caused more frequent pathology in calves. Relatively low doses of air-dried IgY (0.5–2 g/kg body weight), supplied to newborn calves instead of or together with first colostrum, were absorbed from intestine to blood with only 4–8% efficacy (Stefaniak, 2002).

Cow colostrum given to lambs and goat kids during intestinal absorption of immunoglobulins may induce hemolytic anemia, caused by the binding of antibodies to recipient erythrocytes (Winter and Clarkson, 1992; Perl et al., 1995). Cow colostrum may also be the source of *Mycobacterium paratuberculosis* in goat kids (Orsel et al., 2000).

2.7. Hyperimmune versus normal antibodies

The products from hyperimmune animals are mostly (Ikemori et al., 1992; Kellner et al., 1994), but not always, more effective than those coming from nonimmune animals (Korhonen et al., 1995; Sarker et al., 2001). Loimaranta et al. (1998) showed that as little as 2–4 mg/ml of immune and nonimmune colostrum produced visible aggregates with *Streptococcus mutans*, which occurred in parallel to a reduction of bacterial adhesion. Lower concentrations, producing invisible aggregates, surprisingly increased bacterial adhesion. The authors believe that cow colostrum also contains other components causing *Streptococcus* aggregation.

Immunoglobulin preparations from nonimmune animals (e.g. air-dried swine serum pooled at slaughter) contain antibodies directed against a broad spectrum of antigens, which are produced during the animal's life. When applied prophylactically, they may be effective in preventing the adhesion of infectious agents to the gut mucosal membrane and in protection against gastrointestinal tract pathology (Lissner et al., 1996; Stefaniak and Kopeć, 1997).

Highly specific immunoglobulin preparations derived from hyperimmunized animals show significantly higher efficiency regarding homologous antigens, but they may not have such broad activity against other pathogens as a pool of a large number of nonimmunized slaughter animals from many herds.

2.8. Factors affecting protective efficiency of orally administered antibodies

The choice of timing and duration of oral application of antibodies, their origin, specificity and method of application must be made on the basis of good knowledge about the farm in question (fig. 6). The management and quality of the environment have to be considered. The most important pathogenic factors for the digestive tract of the young animals should be exactly identified. The essential factor to be taken into account is economic assessment, because highly purified preparations from immunized animals may be too expensive for mass prophylactic application.

The Ig additive in feed or in a milk-replacer (table 4) seems to be the most suitable for prophylaxis, whereas application in capsules or the administration of Ig concentrate is advisable in diarrhea therapy (Kelly et al., 1997; Graczyk et al., 1998, 1999; Ashraf et al., 2001; He et al., 2001; Quigley et al., 2001; Solomon, 2002; Stefaniak et al., 2003, 2004).

As presented in fig. 6, a number of factors determine whether the oral application will fulfill the expected protective function; many of these factors do not depend on people or animals (Barrington et al., 2002; Stefaniak, 2002; Stefaniak et al., 2003). Awareness of these factors should contribute to proper diagnosis and to a reduction of the risk of diarrhea on a given farm (Frank and Kaneene, 1992).

3. FUTURE PERSPECTIVES

It seems that in the future, IgY preparations will become more and more important in the protection of the digestive tract of young mammals, since their production on a large scale is undoubtedly easier and less expensive than that of other antibodies (Schade and Hlinak, 1996).

The earlier investigation carried out by our team showed that there exists a wide cross-reactivity between the most frequent Gram-negative bacteria occurring in farm animals, while

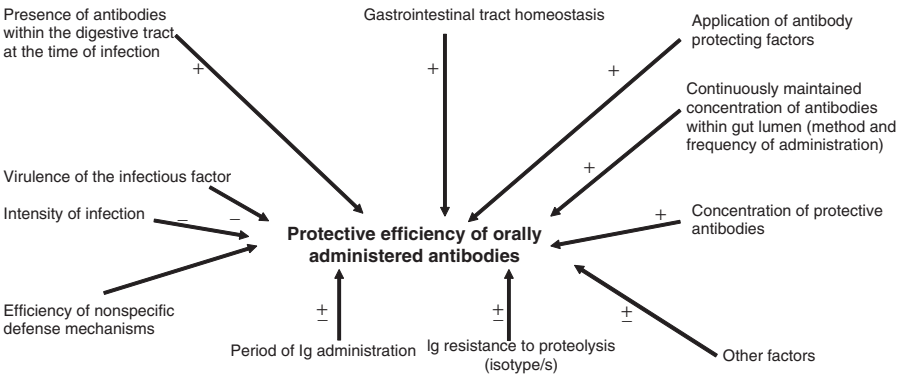

Fig. 6. Factors affecting protective efficiency of orally administered antibodies (+ positive effect; – negative effect).

Table 4

Methods of oral application of antibodies

Method	Description
Mixed with fodder	Advantages – young animals usually eat as much as they wish with voluntary access to fodder; fodder containing Ig is tastier, which helps better weight gain Disadvantages – sick animals do not eat, and will lack the protection from the antibodies
Additive to colostrum or milk-replacers	Mainly in calves, foals, lambs and goat kids Advantages – convenient and easy administration, quite precise dosage Disadvantages – preparation is usually applied twice a day, which may cause considerable fluctuations in the Ig concentration (protective effectiveness) in the digestive tract lumen
In capsules	Advantages – precise dosage, protection against low stomach pH Disadvantages – additional service is necessary
Oral application as a concentrated solution	Advantages – precise dosage Disadvantages – additional service is necessary

antibodies obtained from several animal species against *Haemophilus somnus* cross-react with antigens of *E. coli*, *Pasteurella multocida*, *P. haemolytica*, *Salmonella enteritidids*, *S. typhimurium*, *S. dublin* and *S. gallinarum-pullorum*, *Klebsiella pneumoniae* (Stefaniak et al., 1998, 1999; Wieliczko et al., 2000). Most of the cross-reacting antigens have a mass of 23–45 kDa (Stefaniak et al., 1998). Parenterally given *H. somnus* hyperimmune sera showed protective activity against infections caused by different Gram-negative bacteria (Nikołajczuk et al., 1996, Stefaniak et al., 1999).

It seems extremely attractive for field practice, to isolate the antigens responsible for induction of interspecies protection and the production of an effective subunit vaccine for immunization of laying hens, and then using egg donors as the source of IgY antibodies for the protection of young animals. Finding the least-invasive and low-cost immunization method, as well as achieving the high-titer-specific antibodies will become one of the most important tasks aimed at large-scale production (Hedlund and Hau, 2001). The decision concerning IgY doses for herds at high and low risk of infection will be a crucial step, which will contribute to the possibility of lowering the costs of protecting the young animals and obtaining the highest clinical effectiveness of the chosen preparation.

In the future, IgY is expected to be used in the prevention of epidemics of, e.g. typhus or cholera in environmental disasters, the treatment of diarrhea in risk-group patients – suffering from AIDS, in pregnant women and in the protection of the newborn with immune deficiencies or low birth weight (Kühlmann et al., 1988; Shimizu et al., 1988, 1994).

Further improvement in IgY purification is expected in the area of technological development concerning production of the preparation. It will have to take into account the relation between procedure costs and the protective effect of the preparation.

Strong expectations have been connected with experimental application of ‘‘plantibodies’’ – antibodies of required specificity and class, produced by transgenic plants (Ma et al., 1995; Larrick et al., 1998; Chadd and Chamow, 2001; Peeters et al., 2001). Transgenic plants may be suitable for diagnostic or therapeutic use, as well as for large-scale production of recombinant secretory immunoglobulin A for passive mucosal immunotherapy. The latter application may prove to be revolutionary in the field of efficient protection of young animals

against infections, because of its low costs and the large scale of immunoglobulin production (Hiatt and Ma, 1993; Sharp and Doran, 2001). Theoretically, obtaining immunoglobulins from transgenic plants considerably exceeds the amounts which can be produced from animals.

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9 Legal aspects for functional feed ingredients in the EC

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Effective livestock production is an important goal for the European economy. Adequate nutrition of animals is achieved by balanced diets and by the inclusion of compounds with specific functional effects. Feed additives have to undergo admission procedures that ensure efficacy and safety. In companion animals, trends can be observed towards foods with proven or assumed health benefits. Pet food is in this aspect similar to human food. Feedingstuffs that are brought onto the market with ”health claims” must be admitted as feedingstuffs for particular nutritional purposes.

1. INTRODUCTION

Livestock production is important for the European economy. Safe feedingstuffs ensure consumer protection, animal health and welfare and are also important for environmental protection. Feed additives have been established for the purpose of animal nutrition and have impact on the above-mentioned goals. Feed additives are regulated and subjected to admission procedures to ensure efficacy and safety. Feed materials may have biological activities that are beyond their classical nutritional value. This aspect has gained increasing attention in the food industry but also in animal nutrition and so-called nutraceuticals are offered both for food and feed applications. Reasons for this are varied. In humans, trends towards healthy eating have led to the increasing popularity of foods with proven or assumed health benefits. Similar trends can be observed in animal nutrition, especially in the area of companion animal feeding. Pet food is in many aspects much more similar to human food than to farm animal feedingstuffs. From the regulatory point of view, all complete or complementary feedingstuffs that are brought onto the market with ‘‘health claims” must be admitted as feedingstuffs for particular nutritional purposes.

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2. DEFINITIONS

2.1. Feed additives

Among the major ingredients used in diet formulation several minor compounds are used that are subjected to European Community (EC) regulations as feed additives (Regulation (EC) No. 1831/2003). These additives can have technological, sensory, nutritional or zootechnical impact. A specific group are coccidiostats and histomonostats. Depending on the claim, the intended use and their chemical and physiological properties, feed additives are categorized into the following groups: (1) chemically defined substances such as preservatives, vitamins, acidity regulators, trace elements; (2) living microorganisms; (3) spores of microorganisms; (4) enzymes.

2.2. Feedingstuffs designed for particular nutritional purposes

Feedingstuffs intended for particular nutritional purposes are regulated in the EC by the Council Directive 93/74/EEC. This directive contains a list of compound feedingstuffs that are clearly distinguished from ordinary feedingstuffs. The intention of use is to satisfy the specific nutritional needs of pets or productive livestock whose processes of assimilation, absorption or metabolism could be, or are, temporarily or irreversibly impaired.

2.3. Genetically modified feed

Genetically modified feed is covered, together with food, by the Regulation (EC) No. 1829/2003. The authorization procedure for the placing on the market of genetically modified food and feed includes feed additives consisting of, containing or produced from, genetically modified organisms (GMOs). Feed additives belonging to this category undergo an authorization procedure as laid down in Regulation (EC) No. 1829/2003 and in addition in the regulation on feed additives (Regulation (EC) No. 1831/2003).

2.4. Dietary supplements

In contrast to the situation in the United States of America, dietary supplements is not a regularly used term in the EC. Such supplements are often produced in the United States and other countries, especially for humans, but for animals as well. The definition is regulated in the Dietary Supplement Health and Education Act (DSHEA, October 25, 1994) (Hathcock, 2001). According to this Act, a supplement contains vitamins, minerals, herbs or other botanicals, amino acids or other substances. Supplements can be used to increase the total dietary intake of nutrients and are concentrates, metabolites, extracts or combinations of those. They have to be labeled and the supplier has to prove that they are safe.

2.5. Nutraceuticals

Another expression of practical importance is nutraceutical. These substances are a specific group of food or feed ingredients that are used due to a claim that is beyond the aspect of nutrition. The self-proclaimed nutraceuticals (Dzanis, 1998) are not considered in the European feed regulations and also not in the US. This term has therefore no regulatory function. Nutraceutical food or feed ingredients are designed as substances in order to treat or to prevent diseases or to affect the structure or function of the body. No claims regarding therapeutic benefits should be made and there are no exceptions from this rule for veterinary-directed nutraceuticals. The American Association of Feed Control (AAFCO) has proposed some model regulations (Dzanis, 1998).

3. FEED ADDITIVES

Feed additives are substances, microorganisms or preparations that are added to feed or water. Feed additives are used to favorably affect the characteristics of feed, the characteristics of animal-derived products, or the color of ornamental fish and birds. Feed additives are used to fulfill the nutritional needs of animals, to reduce the negative impact of animal production on the environment, to have positive effects on animal production, performance or welfare. This is particularly accomplished by effects on the gastrointestinal microflora, by enhancement of

Table 1

Groups of feed additives (Regulation EC 1831/2003)

Technological additives	• Preservatives: substances or, when applicable, microorganisms which protect feed against deterioration caused by microorganisms or their metabolites • Antioxidants: substances prolonging the storage life of feedingstuffs and feed materials by protecting them against deterioration caused by oxidation • Emulsifiers: substances that make it possible to form or maintain a homogeneous mixture of two or more immiscible phases in feeding stuffs • Stabilizers: substances which make it possible to maintain the physico-chemical state of feedingstuffs • Thickeners: substances which increase the viscosity of feedingstuffs • Gelling agents: substances which give a feedingstuff texture through the formation of a gel • Binders: substances which increase the tendency of particles of feedingstuffs to adhere • Substances for control of radionuclide contamination: substances that suppress absorption of radionuclides or promote their excretion • Anticaking agents: substances that reduce the tendency of individual particles of a feedingstuff to adhere • Acidity regulators: substances which adjust the pH of feedingstuffs • Silage additives: substances, including enzymes or microorganisms, intended to be incorporated into feed to improve the production of silage • Denaturants: substances which, when used for the manufacture of processed feedingstuffs, allow the identification of the origin of specific food or feed materials
Sensory additives	• Colorants: substances that add or restore color in feedingstuffs; substances which, when fed to animals, add colors to food of animal origin; substances which favorably affect the color of ornamental fish or birds • Flavoring compounds: substances the inclusion of which in feedingstuffs increases feed smell or palatability
Nutritional additives	• Vitamins, provitamins and chemically well-defined substances having similar effect • Compounds of trace elements • Amino acids, their salts and analogs • Urea and its derivatives
Zootechnical additives	• Digestibility enhancers: substances which, when fed to animals, increase the digestibility of the diet, through action on target feed materials • Gut flora stabilizers: microorganisms or other chemically defined substances, which, when fed to animals, have a positive effect on the gut flora • Substances which favorably affect the environment • Other zootechnical additives

the digestibility of feedingstuffs, or by a coccidiostatic or histomonostatic effect. The groups of feed additives (table 1) are defined by their physiological properties. Technological additives are substances for a technological purpose, sensory additives improve or change the organoleptic properties of feedingstuffs or the characteristics of the products obtained from animals, mainly the color of egg yolk and certain fish. Nutritional additives include zootechnical additives that affect favorably the performance of animals or affect favorably the environment. Coccidiostats and histomonostats are substances that prevent frequently occurring diseases and that are subjected to the feed regulation due to the ubiquitous character of these diseases in poultry and turkeys. Depending on the scientific development, additional feed additive categories and functional groups may be established in the future. Antibiotics as growth-promoting agents will be banned by the end of 2005. After this date, antibiotics, other than coccidiostats or histomonostats, must no longer be used as feed additives. The ban on antibiotics as feed additives has been executed to provide maximum security against antibiotic resistance, a major problem in human and veterinary medicine with multiple etiologies. This ban on antibiotics requires additional efforts to develop adequate measures to ensure optimal animal health. These future concepts will be based on the development of specific management, feeding and hygiene concepts.

3.1. Regulatory aspects of feed additives

Protecting human and animal health and the potential impact on the environment requires that feed additives undergo complex assessment procedures. Applications for an authorization have to be submitted to the European Commission. The applications can be set up by companies or an individual person. After submission of the dossier the Member States have to be informed and the application is forwarded to the European Food Safety Authority (EFSA). Directive 70/524/EEC was the regulatory framework for all admission procedures over some decades. New prerequisites have been defined in Regulation (EC) No. 1831/2003 on additives for use in animal nutrition. The application, the preparation and the presentation of an application are currently not yet redefined and, as long as other measures have not been taken, should be made in accordance with Directive 87/153/EEC. Authorizations of feed additives have to be revised regularly, therefore the authorizations are given for a limited time. All authorizations have to be renewed in 10-year periods. When authorizations are not issued to a specific person or company, the application can be submitted by the person who first places the additive on the market or by other interested parties. When the authorizations are issued to a specific holder, the holder or the successor can submit the application to the Commission. A critical point is toxicological testing in vertebrates. For obvious reasons, this testing should not be unnecessarily repeated. If parties disagree on the disclosure of information on vertebrate testing, the Commission can come to decisions to avoid new toxicological tests regarding a reasonable balance between the different parties. After the 10-year period, the scientific evaluation of the dossier or parts of it can be used for other applicants.

Certain information should not be accessible for other parties because this might have negative effects for the holder of the admission. For that reason, parts of the information can be kept confidential according to the decision of the Commission. Confidentiality is not considered for some defined parts of the scientific dossier (table 2).

The protection of the holder of an authorization is maintained for a period of 10 years from the date of authorization, during which time the information may not be used for the benefit of other parties. If the authorization of a feed additive is extended to minor species, the protection period is extended by 1 year for each minor species. Specific provisions have been

Table 2

Parts of scientific dossiers that are not subjected to confidentiality agreements

Name and composition of the feed additive
Indication of the production strain, where appropriate
Physico-chemical and biological characteristics of the feed additive
Conclusions of studies on the effects on human and animal health and on the environment
Conclusions of studies on effects of the feed additive on the characteristics of animal products and its nutritional properties
Methods for detection and identification of the feed additive
Monitoring requirements and a summary of the results of the monitoring, where applicable

made for additives in pet food. The authorization procedure is simplified for those additives which have been authorized for use in human food as laid down in the Directive 89/107/EEC.

A postmarket monitoring plan can be foreseen to monitor the impact of feed additives on human or animal health or the environment. The holder of the authorization has to perform the monitoring and the results have to be communicated to the Commission when the information has an impact on safety.

Transitional regulations are needed for additives that are currently on the market and were authorized under the Directive 70/524/EEC, as also for amino acids, their salts and analogs, urea and its derivatives that have been listed as feed additives in the Directive 82/471/EEC. Additives that are in the authorization process can remain on the market if a notification has been submitted to the Commission within 1 year after the Regulation (EC) 1831/2003 came into force (October 12, 2003). Silage agents are now also monitored by the Regulation (EC) 1831/2003.

For the application procedures, the dossiers will be evaluated by the European Food Safety Authority, and the annex of the guideline Directive 87/153/EEC will be used until new regulations are put in force. This concerns all feed additives admitted according to the Directive 70/524/EEC, and in addition to urea and derivatives, amino acids, salts of an amino acid or analogous substances (Directive 82/471/EEC).

If a feed additive is to be placed on the market this has to be notified to the Commission. Persons or companies have to be named in the notification, the feed additive has to be identified, the classification by category and by a functional group has to be included and the specifications including purity criteria have to be provided. The notification has to contain a description of the production method, the intended uses, the method of analysis that can be used for the analytical determination of residues of the additive itself or its metabolites in feeds. With all this information the product is entered in the register of feed additives. The date of expiry is referenced. The application for admission has to be submitted at the latest 1 year before the expiry date of the additive or within 7 years after the Regulation (EC) No. 1831/2003 has come into force. The submission has to be performed, even for additives that were admitted without a time limit or when the product was admitted according to the regulations of the Directive 82/471/EEC. Those additives that were not subject to the admission procedures in the given time can be withdrawn from the market. All those additives that are not authorized for a specific holder can be admitted after submission of the information by interested parties.

Coccidiostats and histomonostats can be used as feed additives until December 31 2012. A report has to be submitted (January 1, 2008) by the Commission to describe the current status of the use of both substance groups and also the alternatives. At that time the further use of these substances as feed additives will be decided.

3.2. Applications

Regulations for the preparation and the presentation of the application have not yet been established, and until the rules have been defined the annex of the Directive 87/153/EEC is valid. A guided assistance for applicants is foreseen and it will also be allowed to extrapolate studies in major species to minor species. The applications for authorization of feed additives have to contain a defined list of information (table 3). Simplified procedures will also be introduced for feed additives that have already been approved for use in food.

The basic guidelines for the assessment of feed additives are defined in the Regulation (EC) No. 1831/2003, but specific descriptions are not yet available. Therefore, the guidelines for the assessment of feed additives as laid down in the Council Directive 87/153/EEC amended by Commission Directive 2001/79/EC can still be used.

3.3. Safety aspects

Dossiers submitted to the Commission shall allow assessment of the safety of the additive based on the latest scientific knowledge. In those dossiers reports on studies have to be presented that demonstrate the safety of use of the additive regarding animals, products, humans and the environment (table 4).

All studies on feed additives have to fulfill quality standards according to good laboratory practice (GLP) as laid down in the Directive 87/18/EEC. The dossier should provide a critical appraisal of the experiments and a report including an expert report by a recognized scientist

Table 3

Information to be submitted in an application according to Regulation (EC) No. 1831/2003

Name and address of the applicant
Identification of the feed additive, a proposal for its classification by category and functional group, specifications, including, where applicable, purity criteria
A description of the method of production, manufacturing and intended uses of the feed additive, of the method of analysis of the additive in feed according to its intended use and, where appropriate, of the method of analysis for the determination of the level of residues of the feed additive, or its metabolites, in food
A copy of the studies which have been carried out and any other material which is available to demonstrate that the feed additive has no adverse effects and that the definition of a feed additive, e.g. having beneficial effects on the feedingstuff, the animal, the product or on the environment, is fulfilled
Proposed conditions for placing the feed additive on the market, including labeling requirements and, where appropriate, specific conditions for use and handling (including known incompatibilities), use levels in complementary feedingstuffs and animal species and categories for which the feed additive is intended
Written statement that three samples of the feed additive have been sent by the applicant directly to the Community reference laboratory
For certain additives with a classification by category and functional group which do not belong to either technological or sensory additives and for additives consisting of, containing or produced from GMOs, a proposal for postmarket monitoring has to be made
A summary containing the information provided in the above-mentioned points
For additives falling within the scope of Community legislation relating to the marketing of products consisting of, containing or produced from GMOs, details of any authorization granted in accordance with the applicable legislation

Table 4

Aspects of the safety of use of the feed additive that need to be filed in the application dossier

Target species
Human exposure different routes by contact while handling (additive, premixtures, feedingstuffs)
Consumer safety, residues of the additive, or its metabolites, setting of maximum residue limits (MRLs) and withdrawal periods
Selection and spread of antimicrobial resistance genes
Environment, additive itself, products of the additive, either directly and/or excreted by animals

in the relevant field. These persons should not have been directly involved in the studies themselves and the reports should cover quality, efficacy and safety of the additive. The applicant has to provide a critical evaluation of the studies and not just a summary. The dossier shall contain a summary, a proposal for the annex and it can also contain a monograph. Monographs are mandatory for certain additives that are medicinal substances and coccidiostats. The extensive tests required for additives that are to be used in feedingstuffs for food-producing animals do not necessarily apply to additives intended for pet food. Obviously, there is no need for residue studies and the tests of chronic toxicity, mutagenicity, reproductive toxicity and carcinogenicity testing may not be necessary in pets. The definition of maximum residue limits (MRLs) for additives for food-producing animals is important for consumers' protection. The level of the additive itself and its metabolites need to be determined in the target animals and also in laboratory species.

Toxicity testing is an important part of the evaluation procedure and is performed with regards to the substance as such and its metabolites. Tests are performed in laboratory animals and shall not only evaluate the toxicological effects but shall also define appropriate indicators and markers. Markers are not only used for the definition of the MRL but also for defining the withdrawal periods of the feed additive.

Data to be provided in the dossier and the sections that have to be covered are listed in table 5 according to the Directive 2001/79/EC (part I: chemically specified additives). The reader is referred to the original publication for further details.

Enzymes and microorganisms are treated as a specific group since they are in some regards different from chemically defined substances. Therefore, the dossiers have to fulfill different requirements compared to dossiers for chemically defined substances. Substances falling into this group are assumed to cause no danger to target species, humans or the environment. To fulfill this, microorganisms should produce no toxins and should also have no virulence factors. Information should be provided, that they belong to groups that are not able to induce diseases in humans or animals. The bacteria used as probiotic feed additives should not acquire resistance to antibiotics that are used in human or veterinary medicine. Because it cannot be excluded that fermentation products are present in the final form of the product it has to be assured that also those contaminants have no toxic or genotoxic effects. This has to be proven for each fermentation product that is contained in the final preparation. Because enzymes are often produced by genetically modified organisms or microorganisms are genetically modified, they have to meet the regulations of the Directives 2001/18/EC and 90/219/EEC. The requirements for dossiers on microorganisms and enzymes are described in the Commission Directive 94/40/EC, amended by Directive 95/11/EC. However, according to the new scientific knowledge the assessment of microorganisms and enzymes should be

Table 5

Overview of the contents of a dossier for the application for feed additives other than microorganisms and enzymes (Directive 2001/79/EC)

1. Section I: Summary of the data in the dossier
2. Section II: Identity, characterization and conditions of use of the additive; methods of control
   - 2.1. Identity of the additive
   - 2.2. Characterization of the active substance(s)
   - 2.3. Characterization of the additive: Physico-chemical and technological properties
   - 2.4. Conditions of use of the additive
   - 2.5. Control methods
3. Section III: Studies concerning the efficacy of the additive
   - 3.1. Studies on the effects on feedingstuffs
   - 3.2. Studies on the effects on animals
   - 3.3. Studies on the quality of animal products
   - 3.4. Studies on the effects on the characteristics of animal wastes
4. Section IV: Studies concerning the safety of use of the additive
   - 4.1. Studies on target species
   - 4.2. Studies on laboratory animals
   - 4.3. Safety evaluation for the human consumer
   - 4.4. Worker safety assessment
   - 4.5. Environmental risk assessment
5. Section V: Form of monograph
   - 5.1. Identity of the additive
   - 5.2. Specifications concerning the active substance
   - 5.3. Physico-chemical, technological and biological properties of the additive
   - 5.4. Control methods
   - 5.5. Biological properties of the additive
   - 5.6. Details of the quantitative and qualitative residues in target tissues, if any, found in animal produce following envisaged use of the additive
   - 5.7. ADI, established MRLs and the withdrawal period, if appropriate
   - 5.8. Other characteristics suitable for identification of the additive
   - 5.9. Conditions of use
   - 5.10. Dates
6. Section VI: Form of identification note
7. Section VII: Renewal of authorisation of additives submitted to a brand-specific approval
8. Section VIII: New applicant relying on the first authorization of an additive whose authorization is linked to a person responsible for putting them into circulation

provided in a dossier according to the proposal of the Scientific Committee of Animal Nutrition (table 6). The reader is referred to the original publication for further details.

Guidelines on the assessment of the efficacy of microorganisms in the animal categories of dogs, cats and horses have been published, because not all parameters for the assessment procedures usually applied to food-producing animals are appropriate and applicable for non food-producing animals. Probiotics are used for companion animals with different purposes compared to farm animals. They are primarily used to modify the composition and the metabolic profile of the intestinal microflora and thereby act positively on the digestive processes and the animal's wellbeing. Taking into consideration that the number of locations where tests can be performed and the numbers of test animals are limited, the rules laid down in the guidelines are somewhat less strict compared to those for food-producing animals. The trials have to be conducted in independent research institutes, but it is also possible to use other facilities, e.g. company-based institutions, for animal experiments. If possible, trials should be

Table 6

Overview of the contents of a dossier according to the "Guidelines for the assessment of additives in feedingstuffs. Part II: Enzymes and Micro-organisms" published by the Scientific Committee of Animal Nutrition (SCAN)

1. Section I: Summary of the data of the dossier
2. Section II: Identity, characterization and conditions of use of the additive – Methods of control
   - 2.1. Identity of the additive
     - 2.1.1. Proposed proprietary name(s)
     - 2.1.2. Type of additive according to its main function
     - 2.1.3. Qualitative and quantitative composition
     - 2.1.4. Qualitative and quantitative composition of any impurities
     - 2.1.5. Physical state of each form of the product
     - 2.1.6. Manufacturing process
   - 2.2. Characterization of the active agents(s)
     - 2.2.1. Nomenclature
     - 2.2.2. Biological origin
     - 2.2.3. Genetic modification
     - 2.2.4. Compliance with the release Directive for GMOs
     - 2.2.5. Toxins and virulence factors
     - 2.2.6. Antibiotic production and antibiotic resistance
     - 2.2.7. Other relevant properties
   - 2.3. Characterization of the additive: physico-chemical and technological properties
     - 2.3.1. Stability of the additive
     - 2.3.2. Other physico-chemical or biological properties
     - 2.3.3. Incompatibilities with other feed ingredients
   - 2.4. Conditions of use of the additive
     - 2.4.1. Technological additives
     - 2.4.2. Zootechnical additives
     - 2.4.3. Safety Data Sheet
   - 2.5. Control methods
     - 2.5.1. General methods
     - 2.5.2. Description of the qualitative and quantitative methods for routine control of the active agent in premixtures and feedingstuffs
3. Section III: Studies concerning the efficacy of the additive
   - 3.1. Technological use
   - 3.2. Studies of the effects on animals
     - 3.2.1. Evidence of efficacy
     - 3.2.2. Digestion/balance studies
     - 3.2.3. Experimental conditions
     - 3.2.4. Efficacy of multicomponent additives
   - 3.3. Studies on the quality of animal produce
   - 3.4. Studies on the effects on the characteristics of animal wastes
4. Section IV: Studies concerning the safety of use of the additive
   - 4.1. Studies on target species
     - 4.1.1. Tolerance tests on target species/animal categories
     - 4.1.2. Effects on the microflora of the digestive tract
     - 4.1.3. Metabolism and residue studies
   - 4.2. Consumer safety assessment
     - 4.2.1. Genotoxicity studies including mutagenicity
     - 4.2.2. Oral toxicity studies
   - 4.3. Worker safety assessment
     - 4.3.1. Irritancy
     - 4.3.2. Skin sensitization
     - 4.3.3. Toxic effects on the respiratory system

Continued

Table 6

Overview of the contents of a dossier according to the "Guidelines for the assessment of additives in feedingstuffs. Part II: Enzymes and Micro-organisms" published by the Scientific Committee of Animal Nutrition (SCAN)—Cont'd

4.3.4. Systemic toxicity
4.3.5. Control measures
4.4. Environmental risk assessment
4.4.1. Enzyme additives
4.4.2. Microbial additives
4.4.3. Genetically modified microorganisms
5. Section V: Form of Monograph
5.1. Identity of the additive
5.2. Specifications concerning the active agent(s)
5.3. Physico-chemical, technological and biological properties of the additive
5.4. Control methods
5.5. Biological properties of the additive
5.6. Other characteristics suitable for identification of the additive
6. Section VI: Form of identification note

performed at two different locations and experiments have to include control groups. Cross-over experiments or latin-square designs or studies with consecutive application of diets with and without microorganisms can be designed to compensate for the restricted number of animals, as long as these test systems are appropriate for the claimed parameters. *In vitro* studies or studies in other animal species can be used only as a screening method and/or as supporting data. It is stated in the guidelines that animals have to be suitable for each experiment and that specific requirements are set up if the product is for animals (dogs, cats, horses) with special performance (e.g. hunting dogs, etc.). Efficacy parameters to describe the potential effects of a microorganism have to meet accepted scientific standards of statistical and biological significance and have to be related between the added microorganisms and the observed results in target species. There is a requirement for at least two independent comparable trials at the level of $P < 10\%$ or one trial at the level of $P < 5\%$ in the target species on biologically specific parameters related to this microorganism (i.e. concerning the same parameter). However, theoretical studies or comparative models providing information that an additive will be efficacious, may also be acceptable.

3.4. Decision processes

One important goal is to keep the procedures within a reasonable time limit. The authority shall react within 6 months after the dossier has been submitted. This time can be prolonged when supplementary information is required. The authority has to make sure that the dossier is in accordance with Regulation (EC) No. 1831/2003. The information has to fulfill the conditions for authorization and has to be in line with the substantial and formal requirements. In those cases where the authority has a favorable opinion on the feed additive there are certain elements that need to be included (table 7).

The authorities have to prepare a report with a description of the assessment and a statement that describes the reasons for the conclusions. The opinion has to be made known to the Commission, the Member States and the applicant. The Commission has to prepare a regulation on the authorization. The first draft includes statements if the additive fulfills the

Table 7

Elements of the opinion of the authority on a feed additive

The name and address of the applicant
The designation of the feed additive including its categorization and allocation within functional groups; its specification, including, where applicable, purity criteria and method of analysis
Specific conditions or restrictions in relation to handling, postmarket monitoring requirements and use, including animal species and categories of animal species for which the additive is to be used
Specific additional requirements for the labeling of the feed additive necessary as a result of conditions and restrictions
A proposal for the establishment of maximum residues limits (MRLs) in the relevant foodstuffs of animal origin. Exceptions are made if MRLs are not necessary for the protection of consumers or MRLs have already been established in the Council Regulation 2377/90/EEC

requirements for a feed additive and that the product has benefits for animal health and welfare, and the products that are consumed. If the Commission disagrees with the Authority, the different opinion needs to be explained.

Specific requirements are set up for authorization of additives belonging to zootechnical additives, coccidiostats, histomonostats and for additives produced directly from or by GMOs. When residues of an additive in food, produced from animals fed with that additive, might have a detrimental effect on human health, the regulation shall include MRLs for the active substance or for its metabolites in the relevant foodstuffs of animal origin. The authorization should be valid for 10 years and is renewable. The authorized feed additives are contained in a register beginning with the date of authorization.

4. FEEDINGSTUFFS INTENDED FOR PARTICULAR NUTRITIONAL PURPOSES

4.1. Purposes

Compound feedingstuffs for particular nutritional purposes, that have to bear the word "dietetic" in the label, have an increasing role in the treatment of pet animals and are also used for farm livestock. Directive 93/74/EEC sets rules for the composition, the labeling and the intended uses of these specific feedingstuffs.

Feedingstuffs for particular nutritional purposes must have a specific composition and/or be manufactured using special methods. They are used to support animals with disturbances in body functions like assimilation, absorption or metabolism. These functions can be temporarily or irreversibly impaired. These feedingstuffs are until now mainly complete feedingstuffs, but complementary feedingstuffs are also possible.

Accurate and meaningful information should help the users to get all relevant information. Most of the dietetic feedingstuffs are sold via veterinary channels and the Directive refers to that by recommending that the opinion of a veterinarian is obtained or in some cases of a specialist that has not necessarily to be a veterinarian.

The dietetic feedingstuffs must not be marketed in a manner that could be misleading to the consumer. This is regulated by labeling provisions that go further into details compared to normal compound feed. The expression ''dietetic'' has to be used together with the description of the feedingstuff. The customer needs to be informed on the precise use and the particular nutritional purpose. This is achieved by the indication of the essential nutritional characteristics of

the feedingstuffs, the description of the particular use and the recommended length of time for use of the feedingstuff.

4.2. Positive list

The specific requirements are laid down in the Annex of the Directive as a list of intended uses. The list includes the particular nutritional purposes, the essential nutritional characteristics, the species or category of animal, the labeling declarations, the recommended length of time for use and other provisions.

The first ''positive list'' of the intended uses of animal feedingstuffs for particular nutritional purposes was published in the Annex of Directive 94/39/EC. As an example, one feedingstuff for particular nutritional purposes of the Annex is shown (Annex B of Directive 94/39/EC) (table 8). The reader is referred to the original publication for further detail.

The list of current indications is summarized in table 9. The list is not considered as closed and shall be expanded when new applications are submitted.

The list is open and might be supplemented if new applications were adopted by the Standing Committee on the Food Chain and Animal Health. As there are no specific legal authorization procedures and no specific guidelines, it is agreed upon that the applicant has to present a dossier similar to the application of feed additives.

4.3. Genetically modified feed

Genetically modified feed means feed containing, consisting of or produced from genetically modified organisms (GMOs). A genetically modified organism for feed use is a GMO that may be used itself as feed or as a source material for the production of feed. The regulations concerning genetically modified feed are laid down in the Regulation (EC) No. 1829/2003. In

Table 8

Example of the description of a feedingstuff for particular nutritional purposes as laid down in the annex of Directive 94/39/EC

Particular nutritional purposes Support of renal function in case of chronic renal insufficiency
Essential nutritional characteristics Low level of phosphorus and restricted level of protein but of high quality
Species or category of animal Dogs and cats
Labeling declarations Protein source, calcium, phosphorus, potassium, sodium, content of essential fatty acids (if added)
Recommended length of time for use Initially up to 6 months
Other provisions Indicate on the package, container or label: ''It is recommended that a veterinarian's opinion be sought before use or before extending the period of use''
Indicate in the instructions for use ''Water should be available at all times''

Table 9

Particular nutritional purposes listed in the annex of Directive 94/39/EC

Dogs and/or cats
Dissolution of struvite stones
Reduction of struvite stone recurrence
Reduction of urate stone formation
Reduction of oxalate stone formation
Reduction of cystine stone formation
Reduction of ingredient and nutrient intolerances
Reduction of acute intestinal absorptive disorders
Compensation for maldigestion
Support of heart function in case of chronic cardiac insufficiency
Regulation of glucose supply (diabetes mellitus)
Support of liver function in case of chronic liver insufficiency
Regulation of lipid metabolism in case of hyperlipidemia
Reduction of copper in the liver
Support of skin function in case of dermatosis and excessive loss of hair
Reduction of excessive body weight
Nutritional restoration, convalescence
Support of renal function in case of chronic renal insufficiency
Productive livestock
Reduction of the risk of milk fever
Reduction of the risk of ketosis
Reduction of the risk of tetany (hypomagnesaemia)
Reduction of the risk of acidosis
Stabilization of water and electrolyte balance
Reduction of the risk of urinary calculi
Reduction of stress reactions
Stabilization of physiological digestion
Reduction of the risk of constipation
Reduction of the risk of fatty liver syndrome
Compensation for malabsorption
Compensation for chronic digestive disorders of small intestine
Reduction of the risk of digestive disorders of large intestine
Reduction of stress reactions
Compensation of electrolyte loss in cases of heavy sweating
Compensation for chronic insufficiency of small intestine function
Compensation for chronic insufficiency of large intestine function
Nutritional restoration, convalescence
Support of liver function in case of chronic liver insufficiency
Support of renal function in case of chronic renal insufficiency

this directive it is established that feed that falls into the classification of ''genetically modified feed" must not have adverse effects on human health, animal health or the environment. It has to be granted that genetically modified feed must not have negative effects on the user or the consumer. The consumer may be concerned by an impairment of the characteristics of the animal products, specifically when this feed differs from normal feed to such an extent that the consumption would have different and disadvantageous features that have a negative impact on humans or animals. All genetically modified feed can only be put on the market after the product is authorized according to the procedures laid down in Regulation (EC) No. 1829/2003. To receive authorization, it has to be demonstrated that the product fulfills an extended list of requirements.

The application has to be submitted to the national competent authority of a Member State that has to deliver the application to the authorities and the Commission. The application shall contain, where applicable, a detailed description of the method of production and manufacturing and intended uses of the feed. It must contain the reports on studies that have been conducted, and, if available, independent, peer-reviewed studies. It has to be proven that the GMO feed is nutritionally not different from its conventional counterpart. Due to the natural variation of feed composition this has to be provided within the limits of the natural variations. A proposal for labeling the feed has to be presented. The feed should not give rise to ethical or religious concerns. Where appropriate, the conditions for placing the feed on the market and the specific conditions for use and handling have to be provided.

For control purposes, the methods for detection, sampling and identification of the transformation event and, where applicable, for the detection and identification of the transformation event in the feed and/or in the feed produced from or with GMOs have to be provided. The applicant has to submit control samples and information where the reference material can be accessed. When appropriate, a proposal for a postmarketing control plan has to be included. The application has to contain a summary of the dossier in a standardized form.

The authority has a time limit of 6 months after the application has been submitted to give its opinion. If supplementary data or information are required, the time limit will be extended. Specific attention is paid to the environmental aspects after the introduction of GMOs. The safety requirements as laid down in Directive 2001/18/EC have to be fulfilled. In the reference laboratory the methods submitted for the detection and identification have to be tested and validated.

Authorizations are granted for 10 years and shall be renewable. All authorized feed is entered in the register, including the date of authorization. Existing products that have been already placed on the market according to the previous regulations have to be made known to the Commission, including the date when they were first placed on the market. Applications have to be submitted within 9 years from the date when the products were first placed on the market. Authorizations are renewable after a 10-year period. The application has to be submitted to the Commission at the latest 1 year before the expiry of the authorization.

Labeling of feedingstuffs that contain, that are, or that are produced from GMOs is important for consumers (Brookes, 2002). Labeling has to be done clearly and the words ''genetically modified'' have to be included. Only feedingstuffs containing more than 0.9% of GMOs have to be labeled. Concentrations of less than 0.9% are considered as not significant or technically unavoidable. However, evidence must be provided that appropriate steps and measures have been taken to avoid the presence of such materials.

It is intended to make all information accessible to the public due to the sensitivity of the topic as such. However, confidentiality can be granted for certain parts of the dossier if the disclosure might significantly affect the competitive situation. The Commission may consult the European group on Ethics in Science and New Technologies on its own initiative when this seems indicated. A regulation for the traceability of products consisting of, or containing, GMOs is provided by Regulation (EC) No. 1830/2003. It has to be provided that the GMOs and products produced from GMOs can be traced at all stages during the production process and in the distribution chains.

5. FUTURE PERSPECTIVES

Feed safety is more and more considered as an important aspect of the safety of the total food chain. Therefore, feed additives and feedingstuffs for particular nutritional purposes will have an important role in the future, to stabilize the health and wellbeing of farm animals, to

support economic performance and to produce safe food. In the area of pet nutrition it has to be recognized that pets are more and more regarded as social partners and that the requirements of pet owners are to support the pets' wellbeing, health and longevity by adequate nutritional measures.

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10 Handling of dietary antigens: nutritional interactions with the gut immune function

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The gut mucosa is exposed to numerous exogenous factors and has differentiated regulatory mechanisms that enable selective permeability for nutrients and certain macromolecules but exclude potentially harmful dietary, environmental or bacterial antigens. Discrimination between absorption and exclusion, tolerance and reactivity result from complex regulatory processes that depend on the age of the individual, the functional and regulatory mechanisms of the immune system and the influence of exogenous factors. The interaction between luminal factors of dietary or bacterial origin and the gut wall is of particular importance. Exogenous food antigens, e.g. peptides, glycoproteins and lectins, but also microorganisms have the ability to interact with the gut wall and to induce reactions and regulatory and counter-regulatory processes. The interaction of luminal factors with the gut wall influences digestion (secretion, absorption, motility), immunological mechanisms (exclusion of antigens, regulation of the gastrointestinal immune system, on the other hand antigen-processing, sensitivity, allergy), and neuroendocrine processes and integration. Nutrition has a significant impact on the gastrointestinal tract of young animals and is of special importance for the function of the gut and the associated immune system during the early growth phase and later in adulthood.

1. INTRODUCTION

The digestive system in young animals develops according to distinct patterns of appearance, and changes in transport capacities for carbohydrates, amino acids and peptides, lipids and many other substances including chemically different macromolecules (Buddington, 2002a). Rates of qualitative and quantitative nutrient digestion and absorption match the changes in the diet, beginning with amniotic fluid, subsequently colostrum and milk and according to the different species, solid food at different times during ontogeny (Buddington, 2002b). Amino acids and glucose are the major oxidative substrates for intestinal energy generation. The oxidation

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of leucine, glutamate and glucose account for more than 80% of the total CO_2 production in piglets fed on high protein diets (Schoor et al., 2001).

The digestive tract and the gut-associated immune system are affected by important influences during early development and in adulthood; these influences include prenatal factors, such as the exposure of the developing organism to immunoglobulins E and G, amniotic fluid cytokines, allergens and maternal immune cells in the feto–maternal environment, but also the quality and quantity of maternal fat intake, e.g. the relation of n6 and n3 fatty acids (Fritsche et al., 1993; Warner and Warner, 2000; Zhou et al., 2000; Zusman et al., 2000). The intestinal epithelium and the gut-associated lymphoid tissue are the primary targets of dietary components and the incorporation of dietary fatty acids into intestinal tissues has been well studied, but the consequences of these events in relation to local immune responses have received little attention (Donnet-Hughes et al., 2001). In piglets, delivered from sows fed either lard or menhaden fish oil in the last days of gravidity and during lactation, arachidonic acid content was more than 50% lower with the fish oil diet and eicosapentaenoic acid levels matched or exceeded those of arachidonic acid. Basal release of prostaglandin E, thromboxane B and leukotriene B was lowered, but when the immune cells were stimulated with a calcium ionophore, release of leukotriene B was similar in both groups of piglets (Fritsche et al., 1993). After birth, the immune system is affected by exogenous factors, among which food or feed antigens and environmental or intestinal microorganisms are of specific importance for the development and stability of the complex digestive, immunological, endocrine and nervous processes in the gastrointestinal tract (Guy-Grand and Vassalli, 1993; Tyler et al., 1994; Pacha, 2000; Cross and Gill, 2001; Kelly et al., 2001). The exposure to dietary, bacterial and environmental antigens is necessary for the development of a stable immune system (Bailey et al., 2001), although many questions remain open at present due to the extremely complex relationship between the microflora, dietary factors and the gut. Protein intake seems to be of major importance, because it provides the major load of dietary antigens. The particular understanding of the interaction between intraluminal digestive processes and the absorption of amino acids, peptides and macromolecules and the effects of diet composition on the intestinal microflora has increased in the past years. Bacteria that colonize the intestinal mucosa can elicit a strong mucosal immune response, whereas food antigens are often only weakly immunogenic (Dahlgren et al., 1991). The explanation for this may be the physical and chemical properties of bacterial substances compared to food proteins from animals and plants, and specific stimulating properties of bacteria. The immune response of the intestinal mucosa can be reinforced by simultaneous challenge with protein and microbial antigen (Porter et al., 1987). It is worth noting, that dietary protein intake has been shown to have distinct effects on the composition and metabolic activity of the intestinal microflora (Amtsberg et al., 1989; Zentek, 1995; Steen et al., 1997), but the significance of a change in the composition of the microecology for the intestinal immune response has not yet been elucidated.

2. DEVELOPMENTAL ASPECTS OF PROTEIN DIGESTION

Proteins are of major importance as nutrients; especially in the young, providing amino acids for protein synthesis and tissue accretion as well as fuel for the gut mucosa. Digestive capacity has been investigated in different species and distinct age-related patterns have been described. The small and large intestines of piglets gain rapidly in weight and length, especially during the first 24 h after birth, the rate of growth slowing down during the next 9 days. In the jejunum the weights of both muscular and especially of mucosal tissue increases (Widdowson et al., 1976) and the total activities of lactase and acid phosphatase in the

jejunal mucosa increase during the first 24 h in suckled pigs, because of the synthesis of mucosal tissue. The total activity of digestive enzymes (chymotrypsin, trypsin, amylase and gastric proteases) increases with age, either due to higher tissue weight or to higher enzyme activity per tissue weight (Lindemann et al., 1986). The activities of sucrase, isomaltase, maltase, lactase, trehalase and pancreatic α-amylase show age-related patterns in piglets and a characteristic distribution along the intestine (Kidder and Manners, 1980), approaching the adult status by the age of 8 weeks. Glucocorticoids seem to evoke elevation of the carbohydrase enzymes necessary for initiating the hydrolysis of starch, while the normal decrease in lactase activity, regarded as a sign of gut maturation, can also be accelerated by glucocorticoid administration (Chapple et al., 1989). Furthermore, in suckling rats, corticosteroid administration increases levels of proteases in the gastric mucosa, pancreas and small intestinal contents (Britton and Koldovsky, 1988), indicating that steroids influence also protein digestive capacity during the perinatal period.

Protein digestive capacity is subjected to distinct changes during the development of the young. Prochymosin is present in the fetal gastric mucosa from about 3 weeks before birth. After birth, mean chymosin activity decreases. Prochymosin has been demonstrated in the gastric mucosa of newborn piglets (Foltmann et al., 1978), while pepsinogen is absent from the gastric mucosa for the first 5 days after birth, but increases after the first week of life (Foltmann et al., 1981). With age the total activity of chymotrypsin, trypsin and gastric proteases increases, mainly due to an increase of the organ weights (Lindemann et al., 1986). Liquid milk formulas, containing either intact bovine milk, hydrolyzed bovine milk, or isolated soybean protein as source of protein, did not affect the activities of pepsin, intestinal trypsin and chymotrypsin or pancreatic chymotrypsin, except that piglets fed the bovine milk had a lower activity of pancreatic trypsin (Moughan et al., 1990). Piglets fed on milk or a dry starter diet did not show clear differences in the intestinal enzymatic activities (Pluske et al., 1996b).

3. DEVELOPMENTAL ASPECTS OF PROTEIN ABSORPTION

The intestinal tract is capable of absorbing amino acids, di- and tripeptides and larger molecules depending on influences by age, species and physiological condition. Young piglets absorb dietary proteins very efficiently. Luminal perfusion of the jejunum and ileum with predigested and bile acid-solubilized sow's milk demonstrated increased mucosal permeability in 1-day-old piglets compared to perfusions in older animals. This increased permeability is probably not linked to a higher metabolic turnover, as the intestinal oxygen uptake remained unchanged (Crissinger and Burney, 1996). The intestinal transmission of macromolecular markers, with similar molecular weight but different susceptibility to proteolytic digestion, was investigated in newborn piglets or during the first week of life. When piglets were given a mixture of bovine serum albumin and fluorescein-isothiocyanate-labeled dextran (70 kDa) by stomach tube, a rapid decrease in the transmission of the markers was observed during the first day of life in suckled piglets and intestinal macromolecular closure was well developed after 18–36 h of life. After that time, only small amounts of the markers were transmitted to the serum. Molecules having a molecular weight greater than 3000 Daltons were excluded upon macromolecular closure, independently of whether they had protein structure or not. Smaller molecules were transmitted across the intestinal barriers unrelated to closure (Westrom et al., 1984).

Transport of porcine or human immunoglobulin G across the small intestine of the newborn piglet is mainly related to endocytosis. Preferential transport of porcine over human

immunoglobulin G was demonstrated when both were given as a single solution, but the degree of preference was small. Colostrum generally stimulates transport processes, mainly by increasing the amount of endocytosis (Burton and Smith, 1977). Trypsin inhibitors have an important role for they evidently increase the efficiency of absorption of undegraded colostral proteins (Carlsson et al., 1980). Sow milk contains a trypsin inhibitor that was suspected to be of specific importance for these effects. But, as large quantities of proteins were still absorbed even when inhibitor-free colostrum was fed, other factors must also be involved in the regulation of intestinal protein absorption. The serum concentrations of immunoglobulins G, M and A, and agglutinating antibodies for *B. bronchiseptica* were higher in piglets fed trypsin inhibitors in their diets, although, even if no trypsin inhibitors were fed, a considerable immunoglobulin absorption was still found (Jensen and Pedersen, 1982). Administration of iodinated immunoglobulin G within 3 hours of birth resulted in the appearance of the intact immunoglobulin in the piglet circulation, whereas immediate feeding of bovine colostrum followed by administration of iodinated immunoglobulin G after 3 days resulted in the appearance of fragments only. Administration of 15 g of lactose during the first 24 hours reduced absorption of immunoglobulin G by 26% compared to controls, while treatment with 54 g of lactose reduced absorption by 94%. On the other hand, when bovine colostrum was administered immediately after purified immunoglobulin G, the amount of swine immunoglobulin G absorbed was 50–70% greater than in controls. Mature milk failed to have the same influence. When the amount of purified swine immunoglobulin G administered varied from 1 g to 8 g, absorption was directly proportional to the amount administered (Werhahn et al., 1981). Piglets are obviously less selective with regard to the transport of macromolecules from gut to blood, compared to other species (rats, mice and hamsters). Enterocytes in the proximal part of the small intestine transport more proteins than those in the distal part. Bovine albumin or porcine immunoglobulin G are transported at about the same level. Mixtures of these proteins behave differently; immunoglobulin G is preferentially transported and albumin enhances immunoglobulin G transport, probably by a micropinocytotic mechanism (Leary and Lecce, 1979). In newborn calves, the absorption of colostral immunoglobulin G from the gut occurs by intracellular micropinocytosis throughout the entire small intestine, but the amounts transported increase from the duodenum to the ileum (Jochims et al., 1994). In contrast, there is no evidence for paracellular transport in the calf. However, the presence of bovine clathrin (clathrin is the major coating protein of coated specialized transport vesicles) at the microvillous membrane of the duodenal and jejunal enterocytes is evidence of an additional, selective, receptor-mediated transport of IgG during the first few hours of life.

The lymphatic system is of major importance for the transport and processing of absorbed proteins. Thus, by collection of thoracic duct lymph from nonsuckled newborn pigs it could be demonstrated, that bovine colostral proteins are absorbed from the intestine into the lymph (Kiriyama et al., 1988). Furthermore, the gut wall is not only permeable to proteins, but it may also allow cells to cross the intestinal barrier, e.g. the intestinal absorption of labeled colostral maternal lymphoid cells via the lymphatic vessels and the mesenteric lymph nodes has been demonstrated in newborn piglets. Electron microscopy revealed that absorption took place intercellularly. This route was restricted to colostral cells from the mother sow, and lymphocytes from other sows were only detected in the epithelial layer of the mucous membrane. In contrast, no absorption of lymphoid cells isolated from the sows' blood or of heat-treated colostral lymphoid cells could be detected (Tuboly et al., 1988).

The uptake and the handling of dietary antigens by the young can obviously be modified by the feeding of the dam. When breeding sows were fed ovalbumin (OvA) as a novel protein antigen, either throughout gestation and lactation or during lactation only, the uptake of OvA

into blood, colostrum and milk along with a specific immunoglobulin G response was demonstrated independently from the time of application. In piglets from sows fed the antigen from gestation, OvA and antibodies to OvA were detected in the serum after ingestion of colostrum. In a large proportion of these piglets OvA was still detected at 3 weeks of age. A significant proportion of piglets responded to OvA whilst suckling from sows fed ovalbumin from lactation. At 3 weeks of age all piglets were weaned onto an egg-based diet. A similar uptake of OvA was seen in all piglets but there was no response to OvA in the piglets from sows fed ovalbumin from gestation. In piglets from sows fed OvA only during lactation, a rapid immunoglobulin G anti-OvA response and signs of diarrhea were seen. These results have been interpreted to mean that factors of immunological importance are passed on from mother to offspring and that immunological experience of dietary antigens by the mother is important for induction of tolerance in her offspring (Telemo et al., 1991). Mice born to mothers fed with gluten-containing diets had significantly greater systemic immune responses to gliadin after parenteral immunization than mice born to mothers from a gluten-free diet. Furthermore, feeding mothers gluten-containing diets for defined periods before and during pregnancy and during lactation also resulted in priming of the specific systemic immune responses of the offspring (Troncone and Ferguson, 1988), indicating that mice can be sensitized *in utero* or shortly after birth.

4. ANTIGEN HANDLING AND THE IMMUNE RESPONSE

The normal status of the intestinal immune system is active tolerance, with the option to develop an active response when exposed to novel antigens and potential pathogens. The gastrointestinal immune system is highly developed and compartmentalized in adults, but in young animals the degree of organization is much less developed (Bailey et al., 2001). The intestine acts as a barrier preventing uncontrolled entry of dietary antigens into the gut wall and into the immune system. For this purpose, different evolutionary mechanisms have developed, including the luminal digestive processes, mainly the proteolytic activity of the stomach and of the small intestinal and pancreatic secretions, the mucus layer with different unspecific mechanisms, e.g. pH, negative charge, and nonspecific proteolytic activity, and the secretory immunoglobulin A antibodies. On the other hand, the intestine must allow the uptake of macromolecules that are important for growth and development (Sanderson, 1999; Sanderson and Walker, 2002). As long as antigens are transported across the intestine in physiological amounts, immunosurveillance is maintained and the reactivity of the gut immune system is regulated in a sense of active tolerance. Passage of small quantities of dietary antigens is an important prerequisite for the interaction of food or feed antigens with B-cells, inducing secretion of immunoglobulins and T-cells via binding of peptides to MHC molecules and interaction with T-cell receptors. When pathological transport occurs, especially when the mucosal barrier is breached, intolerance, allergy or chronic diseases may result. Development affects these mechanisms and changes in gene regulation are important for passing information from the intestinal lumen to the mucosal immune system. Some transport processes are related to specific receptors by which the molecules are shuttled through absorptive cells. Nonspecific transport mechanisms, related to the formation of vesicles and transport to the basolateral side of the cell, have also been characterized. Alternatively, antigens pass the epithelium via specialized M cells located in the area of Peyer's patches (Sanderson and Walker, 2002).

Uptake of macromolecules from the intestinal lumen by receptors, which has been characterized for immunoglobulin G, allows young animals to make some discrimination between

native and foreign immunoglobulins and a more or less selective uptake of native antibodies (Werhahn et al., 1981; Staley and Bush, 1985). In very young piglets a nonselective and massive absorption of macromolecules occurs for up to 3 days, but thereafter decreases (Mehrazar et al., 1993), although sow colostrum has an enhancing effect. A selective absorption of immunoglobulins with small quantities of ingested bovine serum albumin (0.02–0.1%) was found in 5-day-old piglets. Gut closure was delayed after feeding foreign antigens to immature rat pups. Cow milk antigens interfere with the maturational process of gut closure and jejunal permeability to macromolecules was higher in rat pups fed by gavage with cow milk on day 14 or even when cow milk was fed to the dams. Jejunal eosinophilic infiltration was found in association with increased jejunal permeability in the pups fed with cow milk and pups from dams fed with cow milk, and coupled to the increased permeability a rise in the number of antibody-secreting cells against β-lactoglobulin was also observed in peripheral blood. Mucosal barrier function can obviously be impaired due to a local hypersensitivity reaction to foreign antigens, irrespective of the protection of maternal milk or maternal antigen processing (Arvola et al., 1993).

The Fc receptor is able to cross cells and seems to be transported by membrane transport mechanisms. The neonatal Fc receptor for immunoglobulin G, an MHC class I-related molecule, functions to transport immunoglobulin G across polarized epithelial cells and to protect immunoglobulin G from degradation (Zhu et al., 2001). Transport of immunoglobulins to the newborn mammal is important for immune defense during the first weeks of life and receptors for the Fc portion of immunoglobulin G isolated from intestinal epithelial cells of suckling rats bear a resemblance to class I histocompatibility molecules (Gastinel et al., 1992). The expression of the Fc receptor gene seems to be linked to the uptake of mother milk, for a decrease in expression was described after weaning.

Additionally to these mechanisms, enterocytes can act as antigen-presenting cells and are regarded as nonprofessionals compared to the classic presenting cells like dendritic cells, B cells and macrophages. All of these cells are able to express a glycoprotein, the major histocompatibility complex II that interacts with the T cell receptor. Nonspecific transport through enterocytes can also occur in older animals after binding of macromolecules to receptors on the apical cell membrane and transport to the basolateral part of the gut cell (Sanderson and Walker, 2002). The alternative to Fc receptor-mediated transport is that macromolecules are engulfed apically in vesicles and transported through the intestinal cell. An important question is how antigens are processed in the gut epithelium. Peptide fragments could be generated during epithelial transport or antigens could be processed from the intact food or feed protein that has traversed the epithelium and reached the antigen-presenting cells. By intracellular proteolysis, the antigenicity of proteins may decrease and result in a condition where antigen-presenting cells and lymphocytes would not be able to recognize and process the ingested proteins.

Specialized cells in the gut epithelium, called M cells or microfold cells, lie directly over the lymphoid tissue in the Peyer's patches. They have a specific cell surface and the mucous layer is less compared to the adjacent gut areas. Macromolecules can enter via M cells and come into contact with immune cells located in close relation to the M cells. Macromolecules enter nonspecifically into the cells by engulfment into vesicles and can be released into the areas where lymphoid cells and antigen-presenting cells are located. M cells have a high capacity for transcytosis of a wide range of microorganisms and macromolecules, and are believed to act as an antigen-sampling system. M cells provide functional openings of the epithelial barrier, but normally, there seems to be a balance between antigen uptake and immunological response (Kucharzik et al., 2000).

5. TOLERANCE AND INTOLERANCE AGAINST DIETARY ANTIGENS

The consequence of antigen confrontation can be immune responsiveness or tolerance. The latter is not simply regarded as the absence of responses but as the result of a spectrum of responses to the ingested antigen and underlying active processes (Challacombe and Czerkinsky, 2002). Tolerance represents the most common and important response of the host to environmental antigens, including food and commensal bacterial components. Early oral exposure to antigens may affect the ability of neonatal animals to mount specific immune responses to fed proteins while leaving the response to systemic antigens largely intact (Bailey et al., 1994b).

Feeding antigens can result in a stable tolerance, but the controlling mechanisms are not completely characterized. Hyporesponsiveness relates to both humoral and cellular responses of the local and systemic immune system. The required dose of antigen for induction of tolerance is not known, but milligram quantities seem to be sufficient. Age, genetics and species as well as individual factors can be considered as the most important modifiers from the host side. Additionally, dietary antigens differ in many respects, and the type of protein, dosage, required frequency of intake, mucosal route and immunogenicity may differ considerably among the food and feed proteins. Thus, when oral administration of ovalbumin or recombinant house dust mite allergen to young dogs was compared after subcutaneous immunization with these antigens, only oral ovalbumin resulted in a nonresponsiveness to subsequent parenteral immunization (Deplazes et al., 1995). Oral tolerance is evoked by multiple low doses of antigen-induced regulatory T lymphocytes that secrete immunosuppressive cytokines, whereas feeding a single high dose of antigen induces anergy of antigen-specific Th1 lymphocytes (diminished IgG2a, interleukin (IL)-2, and interferon (IFN)-γ) with intact Th2 responses (IgG1 and IL-4) (Friedman, 1996).

Oral tolerance to fed antigen does not necessarily require organized gut lymphoid tissue. Mice lacking Peyer`s patches retain their capability to produce secretory IgA antibodies but do not develop normal oral tolerance to protein antigens (Fujihashi et al., 2001). Mice lacking Peyer's patches, B cells and the specialized antigen-handling M cells have no defect in the induction of T cell responses to dietary antigen (Alpan et al., 2001). Oral antigen intake influences the dendritic cells, such that these are conditioned to initiate the gut-oriented responses. The role of the intestinal epithelial cells that are able to present antigens to T lymphocytes is poorly understood. Antigens have been shown to be delivered to major histocompatibility complex class II antigen containing compartments, which might be one of the requirements for the induction of oral tolerance (Zimmer et al., 2000).

The underlying mechanisms of local and systemic tolerance are based on a cascade of regulatory processes. Ingestion of antigen induces secretory IgA production in the gut, preventing docking of antigen to the mucosa-associated lymphoid tissue. Uptake of small amounts of antigen via M cells induces release of down-regulatory cytokines, especially transforming growth factor β (TGF-β) from Th3 cells. Th3-type cells are a unique T-cell subset, which primarily secrete TGF-β and have suppressive properties for Th1 and other immune cells. The latter induces B cells to transform towards IgA-producing cells. High dosages of antigen induce clonal deletion of reacting T-cells, while low intake induces a switch towards a Th3- or Th2-type response with increasing TGF-β or IL-4 and IL-10 production. Oral tolerance after feeding of ovalbumin to dogs reduced OVA-specific immunoglobulin E and G production in response to subcutaneous challenge. Allergic conjunctivitis, induced by means of ocular and airway provocation, was significantly reduced in tolerized animals compared with nontolerized controls. Bronchoalveolar fluid cells had high expression of IL-10 and TGF-β

after allergen challenge (Zemann et al., 2003). Recent findings indicate that IL-18 and IL-12 can break oral tolerance when administered exogenously (Eaton et al., 2003).

Nutritional factors beside protein or peptide intake have been demonstrated to affect the development of oral tolerance. Rats fed a zinc-deficient diet had lower expressions of cytokines involved in oral tolerance, i.e. IL-4, IL-10 and TGF-β (Finamore et al., 2003). Zinc deficiency obviously suppresses oral tolerance by dysregulation of cytokine expression and lack of antigen-specific clonal deletion in rats. Long-term exposure to oral lead chloride for 10 days caused persistent down-regulation of TGF-β mRNA levels in intestinal tissue and disturbed oral tolerance induction to the dietary antigen ovalbumin in mice, associated with an increased proliferation to antigenic stimulus, increased production of IFN-γ and decreased secretion of TGF-β (Goebel et al., 2000).

6. PRACTICAL IMPLICATIONS

At weaning, dietary and often also environmental changes can stress young animals severely. In practice, diarrhea or systemic disease is a common problem in many species. Economic impact is obvious in piglet production and weaning-associated disorders are of specific importance.

The villus height in the small intestine is high in unweaned piglets and decreases subsequent to weaning. Nutrition has profound effects on intestinal morphology and function. A significant decrease in villus height was seen in piglets weaned on a dry diet on days 8 and 11 postweaning, whereas in the piglets receiving a liquid diet villus heights remained stable. Weaned pigs showed an increase in crypt depth and an increase in the complexity of villus morphology with a reduction in villus height (Hampson, 1986a). Villus height and crypt depth were maintained by feeding cows' milk after weaning (Pluske et al., 1996a). Villus height was greater in a group of piglets fed high amounts of milk than in piglets fed either a weanling diet or low amounts of milk. Apparently, villus atrophy was due more to the level of feed intake than to the composition of the diet (Beers-Schreurs et al., 1998). Villus height and crypt depth were significantly correlated with dry matter intake after weaning in milk-fed and starter-fed piglets (Pluske et al., 1996b).

A general depression in pancreatic enzymatic activities, but not in gastric proteolytic activity, was found during the first week after weaning. Subsequent increases in activity of lipase and chymotrypsin were due to the higher pancreatic weight after weaning. Amylase, trypsin and gastric protease increases were due to increased weight and increased activity per g tissue (Lindemann et al., 1986). The activities of lactase decline along the small intestine at weaning, while sucrase activity declines temporarily and then recovers. Minimum values were recorded about 4–5 days after weaning. The large loss of digestive enzyme activities at the brush border in weaned animals coincides with a reduced xylose absorption (Hampson and Kidder, 1986). The activities of pepsin, trypsin and chymotrypsin and pancreatic activities of chymotrypsin were not influenced by dietary protein source (intact bovine milk, hydrolyzed bovine milk or isolated soybean protein), but piglets receiving the bovine milk-based formula had a lower level of activity for trypsin (Moughan et al., 1990). Total absorption of galactose and glucose, adjusted for live weight and plasma volume, increased after weaning, although the galactose index (Gal:AUC for galactose ingested as lactose divided by the AUC for the same dose of galactose ingested as the monosaccharide) and fructose index (Fruc:AUC for fructose ingested as sucrose divided by the AUC for the same dose of fructose ingested as the monosaccharide), both decreased after weaning (Pluske et al., 1996a). Switching from milk to cereals increases some mucosal enzyme activities, intestinal

sodium-dependent glucose absorption, and response to secretagogs. Alkaline phosphatase- and sucrase-specific activities were higher in cereal-fed piglets than in milk-fed piglets, while dipeptidylpeptidase activity was higher in wheat-fed piglets. Sodium-dependent glucose absorption was 1.7-fold higher in cereal-fed piglets than in milk-fed piglets (Boudry et al., 2002). Aminopeptidase A activity increases after weaning compared with values at birth, while sucrase, maltase, lactase and aminopeptidase N activities change in a diet-dependent manner (Jensen et al., 2001). Spray-dried porcine plasma or casein as protein source had no effect on lactase-, sucrase- or maltase-specific activities of the small intestine (Van Dijk et al., 2002).

The weaning of piglets on diets containing soya meal is common practice. Active response to the dietary protein was demonstrated by the appearance of serum antisoya immunoglobulin G. The response of piglets to soya in the weaning diet was lower when 1 g of soya protein was given at birth. Interestingly, similar amounts of the protein itself could be detected in the serum. The response to injected soya of piglets primed orally with soya at birth was not significantly reduced, indicating that regulation of the responses to fed and systemic antigens is largely separate (Bailey et al., 1994a). Hypersensitivity responses to dietary antigen might be a predisposing factor in the etiology of postweaning diarrhea. Small amounts of feed antigens given to baby pigs before weaning significantly increased the severity and accelerated the onset of postweaning diarrhea (Miller et al., 1983). Diets based on either skimmed-milk powder, soybean-protein concentrate, soybean meal or fish meal affected postweaning feed intake, pancreatic weight, gastric pH and gastric protein breakdown, and pancreatic and jejunal trypsin and chymotrypsin activities (Makkink et al., 1994). In piglets, weaned at 28 days of age, immunoblots of serum were made to detect both the residual antigenic storage proteins of the seeds of the dietary legumes used and immunoglobulin G specific to those storage proteins. Antibodies against β-conglutin of *L. luteus*, vicilin of *V. sativa* and vicilin of *L. cicera* were detected in the sera of the piglets 28 days after feeding the diet, but no storage protein was found in the same animals. The presence of antibodies against feed proteins indicates an immune response in the weaned piglets (Seabra et al., 2001). Piglets fed protease-treated soybean meal or skimmed milk powder and fishmeal did not show significant differences between diets with respect to piglet serum anti-SBM-specific antibodies. There were no consistent changes in small intestinal enzyme-specific activities, or, following histopathological examination of the small intestine, of villus height or crypt depth (Rooke et al., 1998). Hydrolyzed casein had a protective effect on gut structure and function, probably related to the low level of antigenicity of the diet (Hampson 1986b). Macromolecule transport was shown to be affected by the dietary protein source. Newborn piglets were bottle-fed with porcine colostrum, bovine colostrum, porcine plasma, porcine milk, bovine colostrum containing porcine plasma or a milk replacer to test the potential effects of the protein source on the absorption of bovine serum albumin (BSA). The percentage of absorbed BSA just after birth was highest for piglets fed porcine colostrum (30–50%), and was reduced to 23–30% in piglets fed bovine colostrum with or without porcine plasma and to 7–20% for the piglets fed with porcine plasma or milk (Jensen et al., 2001).

7. FUTURE PERSPECTIVES

One of the major challenges in nutritional research in this area will be to develop animal models that allow study of the mechanisms of immune induction, tolerance or intolerance and allergy underdefined conditions. Species-specific reactivities have to be evaluated and adequate models for simulating the situation in humans need to be defined. Questions to be addressed include time of induction and dosage of dietary antigens, the role of maternal

nutrition and the potential impact of modifying general diet composition. The interaction of dietary factors with the intestinal microflora needs to be addressed and new technologies in food and feed production may have an impact on the development and function of the gastrointestinal immune system. Most recent work has focused on the situation in young animals, but a wide field of open questions is related to disease-induced changes in intestinal permeability and the potential impact for the regulation of the immunological network. Following the concept of a common mucosal immune system, the interactions between oral and respiratory antigen intake need to be defined. A main focus of clinical nutritionists will be the question, how reactivities of the gastrointestinal and general immune system can be influenced and modified in order to modulate immune reactions and to achieve a health-promoting or -stabilizing situation. It is already clear that the fatty acid pattern of a diet can have immunomodulating effects and it can at least be assumed that dietary carbohydrates can alter the balance between lectins, IgA and microorganisms in the gut. Thus, the potential impact of dietary factors other than proteins on antigen handling or induction of oral tolerance warrants further investigation.

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11 Immune response and nutrient intake

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The oral supply of nutrients to growing animals is essential for the establishment and maintenance of a proper balance in the immune system, between regulator and effector functions. An adequate supply of nutrients is critical when intake is the limiting factor with respect to growth requirements, and when the immune system is challenged by new antigens. This is the case at weaning, particularly when subjects from different litters are mixed.

In this light, the relevance of nutrients for innate and acquired immunity (humoral and cellular), and as vaccine adjuvants is reviewed. Some tools to assess *in vivo* and *ex vivo* the efficacy of nutrients are presented, with particular evidence for challenge models.

Some attention has been paid to the effect of supplying nutrients above the standard requirements (for example the case of zinc as a growth promoter in piglet feeding), which allows maximum growth in a clean environment. In addition, some effects of molecules that are not considered essential, but nevertheless can have the role of nutrients in special phases of growth (nucleic acids, conjugated linoleic acid, precursors of glutathione, etc.) are also discussed.

1. INTRODUCTION

The immune system is a complex of mechanisms that allow the organism to defend itself from injury by the environment in which it lives. In the fetal phase, the organism already uses some of the nutrients assigned to its body mass increase for the formation of the immune system. This situation continues after birth until maturity is reached, and subsequently some of the nutrients are assigned to maintain the ability of the organism to recognize foreign molecules from native molecules as well as noxious from harmless molecules.

According to current understanding, the immune response of animals may be influenced by several essential nutrients, which modify the functions of the immune system. Almost all nutrients in the diet play a crucial role in maintaining an optimal immune response, such that deficient as well as excessive intakes can have negative consequences on immune status and susceptibility to a variety of pathogens.

More than 50 years have passed since the first organized collections of nutrient requirements for growing domestic animals were presented (see, for example, in the case of pigs the

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NRC Report, as Hughes et al., 1950). Since then these recommendations have been more or less frequently revised and refined. Consequently, people could be tempted to believe that the nutrient requirements for maximum growth of the different species are well established and that an optimal immune status is maintained. However, there are some indications that in our main domestic species the high genetic pressure applied for growth has contributed to the development of lines in which nutrients are more directed to the growth of muscle at the expense of the immune system. Improved microenvironmental conditions favored this trend, which is particularly evident for poultry. Thus, Martin et al. (1990) observed a higher bodyweight in genotypes selected for low antibody response to sheep red blood cells (SRBC), compared to high-responsive genotypes. Lines with lower bodyweight showed greater cell-mediated (skin test against phytohaemagglutinin) and humoral (against SRBC) response, and lower lesion scores after *Escherichia coli* inoculation (Rama Rao et al., 1999).

Therefore, it is possible that the quantity of a nutrient needed to optimize a specific immune function is different to the amount of the same nutrient needed for maximal growth response. Indeed, Rama Rao et al. (2003) showed that in broilers the concentration of methionine required to maximize immunity in terms of cutaneous basophilic hypersensitivity and of antibody production against SRBC was higher than the concentration that optimized growth in most genetic lines.

Furthermore, in some cases genetic pressure reduces the feed intake of growing animals, while increasing their body mass increment, so the suggested dietary concentration of nutrients should be adjusted to maintain an optimal daily supply.

Finally, the maintenance costs for the immune system are increased by disease challenge. Klasing and Calvert (1999) calculated that the amount of lysine used for the maintenance of the immune system is around 1.2% of total intake of the young chick. However, after challenge with the bacterial molecule lipopolysaccharide (LPS), the amount of lysine used for the intense innate and adaptive response was an additional 5.5% of the total lysine intake. From the different contribution of lysine to different compartments of the immune system, it can also be estimated that the lysine cost can change with different types of challenge.

2. DEVELOPMENT, ORGANIZATION AND FUNCTION OF THE IMMUNE SYSTEM

At birth the immune system of the animal is not yet completely developed. Passive immunity, transmitted from the mother, provides protection for the first period after birth. However, in bovines, swine and horses the placenta is epitheliochorial (i.e. the uterine epithelial lining lies in apposition to the chorion). This prevents the passage of large molecules and the fetus does not receive maternal antibodies in this phase. Therefore, the newborn of these species are more undefended and, for systemic protection, need to acquire a good level of immunoglobulins (Igs) by the consumption of colostrum in the first hours of life. Afterwards, a local protection in the gut can be obtained from the Igs present in the mother´s milk.

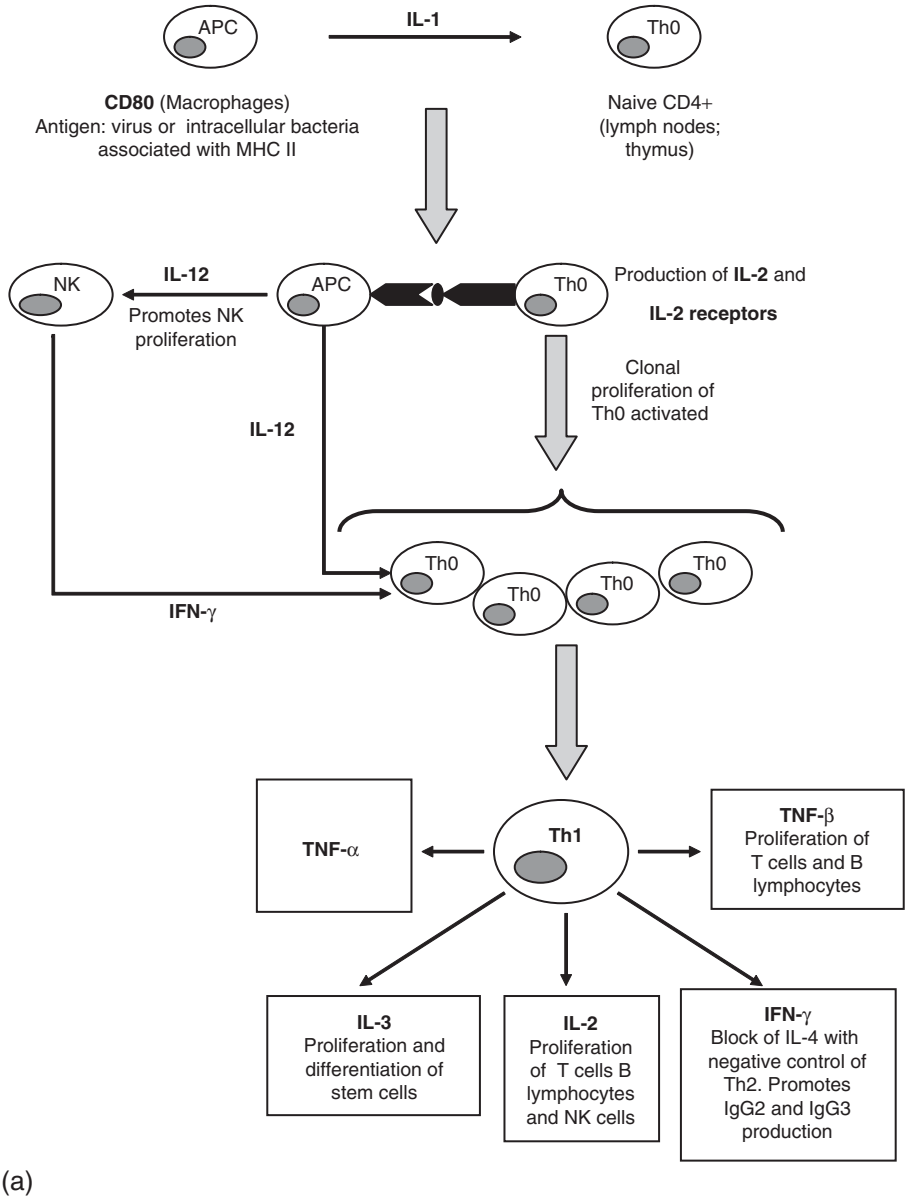
Together with the physical barrier of the mucosa, the first line of defense against infectious agents is innate immunity. This comprises mechanisms of defense already present in the organism before any encounter with the antigens, but it lacks memory of encounters with the same molecules. Innate immunity must be able to effect a rapid elimination of noxious agents and activation of this type of immunity does not require specific discrimination between various antigens. Two components of innate immunity can be recognized – a humoral and a cellular component. The humoral component consists of complement system, interferon

(IFN), C-reactive protein, lysozyme, defensins and other antimicrobial molecules. Macrophages, neutrophils and natural killer cells are the main cellular components. Many microbes have evolved strategies to resist innate immunity and if this first line of defense fails to repel the infection, the reactive immune system enters in action. This acquired immunity produces specific responses for each type of antigen.

Acquired immunity is mediated by T lymphocytes (cell-mediated immunity) and by B lymphocytes (humoral immunity). T lymphocytes organize the cell-mediated immunity, for defense against intracellular microorganisms (bacteria, virus, fungi and protozoa); two populations of these lymphocytes can be recognized: T helper lymphocytes (CD4+) and T cytotoxic lymphocytes (CD8+). These lymphocytes recognize antigens that are initially recognized, processed and exposed by other specific cells (antigen presenting cell or APC), such as dendritic cells and macrophages.

In practice, the naive T cells are activated to armed effector T cells the first time they encounter their specific antigen in the form of a peptide, exposed on the surface of an APC cell by the major histocompatibility complex (MHC) molecule. There are two classes of MHC: MHC I and MHC II. MHC I binds peptides that originate from pathogenic proteins synthesized within the host cell cytosol, and presents antigens to CD8 T cells. The peptides bound to MHC II are derived from pathogens that have been phagocytosed by macrophages or endocytosed by APC and presents antigens to CD4 T cells. These cells can differentiate into two types of effectors: T helper cells, Th1 and Th2. Pathogens that accumulate in large numbers inside macrophage and dendritic cell vesicles tend to stimulate the differentiation of Th1 cells, whereas extracellular antigens tend to stimulate the production of Th2 cells. The humoral immune response is controlled by subsets of Th2 cells, while a subset of Th1 cells controls cell-mediated immunity. Activated Th1 cells express the receptor for IL-2 and at the same time synthesize this interleukin (IL), which is necessary for its clonal expansion and also synthesize IFN-γ that activates the macrophages. Activated macrophages are thus able to kill intracellular microorganisms. Another product of Th1 cells and monocytes is tumor necrosis factor-β (TNF-β). This cytokine at low doses acts as a regulatory factor on leukocytes and other immune cells, but at high levels of secretion it is responsible for some of the systemic effects of infection, such as rise in body temperature, secretion of acute phase proteins and suppression of appetite. Th2 cells mainly synthesize IL-4, IL-5, IL-10 and IL-13. These cytokines stimulate the differentiation of B lymphocytes and thus the secretion of IgG1, IgA and IgE; Th2 lymphocytes don't stimulate the cell-mediated immunity response and in fact IL-4 and IL-10 are strong negative regulators of Th1 lymphocytes. T cytotoxic lymphocytes attack and destroy target cells directly. They recognize peptides linked to proteins coded by genes of the MHC I complex, expressed on the membrane of somatic cells. A simplified scheme of the regulation of acquired immune function by cytokines is presented in fig. 1.

B lymphocytes mature in the bursa of Fabricius in birds, or equivalent organs such as the bone marrow or ileal Peyer's patches in mammals and are specialized in the synthesis and secretion of specific antibodies in the form of Igs. IgMs are present on the cell surface and are the B cell receptors for the antigens. B lymphocytes are positioned to recognize and directly react with the antigen present on the surface of infecting agents or on any other foreign soluble substance released from the pathogenic agents. Therefore, when an antigen enters the organism, B lymphocytes make direct contact through the combination site of their antibodies. B cells must be able to generate enough different antibodies to recognize every possible antigen. After exposure to an antigen, the variable region (V) of the antibody genes acquires numerous changes. So an increase of natural polyclonal antibodies can be favorable, as a wide



(a)

Fig. 1. Simplified scheme of regulation of acquired immune function by cytokines: (a) regulation of cell-mediated immunity; *Continued*

spectrum of specific antibodies are mobilized and the basis for further specific reaction against pathogens is created.

When the antigen has been eliminated, it is necessary to arrest the activity of the immune cells, in particular the B lymphocytes, some T lymphocytes and macrophages. This function is carried out by another population of T lymphocytes (T suppressor cells). This process is

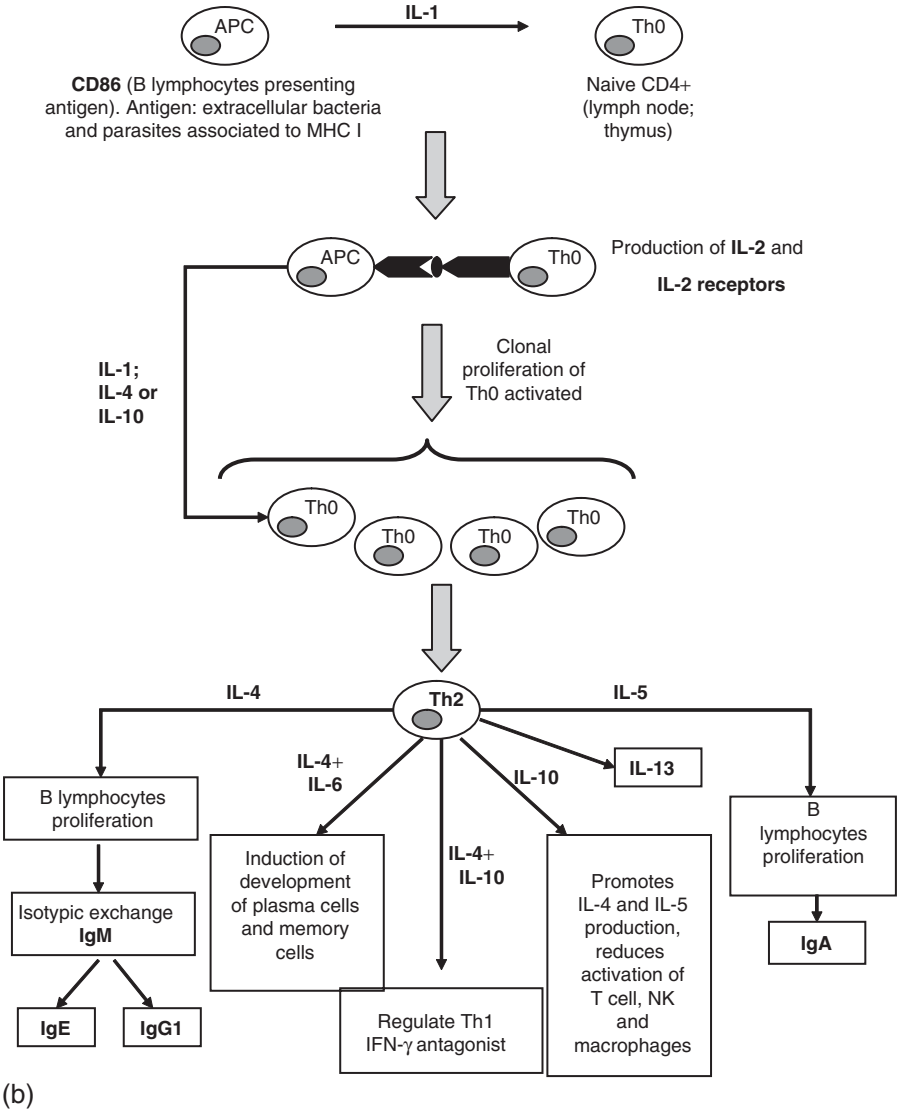


Fig. 1—Cont'd (b) regulation of humoral immunity.

important to avoid an excessive immune response; otherwise, unnecessary antibody production would cause energetic waste for the organism.

The acquired immune system includes a component of memory; this role is carried out by a population of T lymphocytes called memory cells. These cells remain quiescent until the antigen is encountered again. In this case, the response is faster and stronger than the initial response.

3. TOOLS FOR THE STUDY OF THE EFFECTS OF DIETARY SOLUTIONS ON THE IMMUNE FUNCTION OF GROWING DOMESTIC ANIMALS

A large number of parameters and tests have been used to measure the response of the immune system to contact with environmental factors and pathogens. Invasive methods and/or collection of samples after sacrifice or slaughter give important opportunities in growing domestic animals. A collection of possible parameters is presented in table 1. However, what seems most crucial is the way in which the rearing conditions of the subjects are perturbed to challenge the immune system. On experimental farms, the health of piglets is often better than on commercial farms, due to the low number of subjects and better hygienic conditions. Nevertheless, in trials on commercial farms the experimental conditions are often not well enough controlled and disturbing factors are removed with difficulty. Consequently, the effects of dietary factors have often been observed under laboratory conditions, where the immune system was stimulated by molecules such as bacterial toxins, in general parenterally injected, or by live pathogenic bacteria, viruses or parasites.

In weaning pigs, challenge with enterotoxigenic or enterotoxemic *E. coli* is frequently used. Colonization by these strains, which cause diarrhea and/or edema disease (Martineau et al., 1995), is mediated by fimbriae F18 and K88 (= F4). Predisposition to these diseases is at least partially genetically controlled and can be detected by a genetic test (F18) or by a phenotypic test (K88). The detection of individual susceptibility to such infections gives an opportunity to evaluate the effect of dietary solutions on specific immune responses, both in positive and negative subjects of a group. In fig. 3, as well as the effect of zinc supplementations on specific IgA in blood serum, we show that specific IgA are significantly increased in individual pigs susceptible to the adhesion of *Escherichia coli* K88 to intestinal villi.

In addition, other disturbing situations causing social or thermal stresses can be used. Weaning can also be considered as a disturbing factor for gut health. Jones et al. (2001) showed that a variety of stressors (mixing with non-littermates; short-term cold stress; removal from the sow) increased the IgG response in piglets after challenge with *E. coli* K88, probably as a response to increased multiplication of this pathogen in the small intestine.

4. CONTRIBUTION OF NUTRIENTS TO THE MATURATION, THE MAINTENANCE AND THE ACTIVATION OF THE IMMUNE SYSTEM

With regard to the immune system, nutrients often act at many different metabolic levels. Thus, variations of dietary supply can have different effects depending on the immune function considered. Furthermore, some nutrients, of which zinc is a typical example, are involved in the structural integrity of cells, transcription and replication of DNA and RNA and hormone synthesis. These nutrients are especially important for tissues with a high rate of replication, such as the immune system under antigenic challenge.

4.1. Primary organs of immunity

4.1.1. Bone marrow

Impaired production of lymphocytes in the marrow, causing leukopenia, is one of the signs of zinc deficiency. In mouse bone marrow B lymphocytes, it has been shown that a specific stage of maturation is affected (pre-B cells) (Osati-Ashtiani et al., 1998; King and Fraker, 2002).

Table 1

***In vivo* measures of immune function and tests with cells sampled from animals (*in italics*), valuable after normal rearing condition or challenge of animals with pathogens or toxins**

General	Innate	Humoral	Cell-mediated	Health conditions
Size and cellularity of lymphoid organ	Lysozyme	Concentration of Ig subclasses, in blood	Skin test (swelling reaction, after topical application of a previously delivered antigen)	Growth (after challenges)
Number and types of immune cells in bloodstream in tissues	Other antimicrobial peptides: histatins (salivary glands) defensins (small intestine Paneth cells) calprotectin (mouth, esophagus)	Secretory IgA, in saliva and intestinal washings	*Lymphocyte proliferation assay, in response to specific antigens or mitogens, as phytoaemagglutinin*	Febrile response Morbidity
Cytokines, in blood and tissues	Acute phase proteins, after macrophage activation (alpha1-acid glycoprotein)	Specific Ig subclasses (after challenge or after vaccination)		Diarrhea and other symptoms related to infections
Expression of cell surface molecules	*Respiratory burst (generation of oxygen-derived free radicals by phagocytic cells, after exposure to bacteria)*			Survival

This pathology is also characterized by reduced production of IgM. Conversely, zinc-deficient mice show a marked increase of granulocytes and monocytes (King and Fraker, 2002).

Specific effects of nutrients on bone marrow myelopoiesis and lymphopoiesis in domestic mammals have not been reported in official literature. Griebel et al. (1996) presented results showing that Peyer's patches B cells are developmentally distinct from other B cell populations in sheep and suggested that the bone marrow may not be a site of B lymphopoiesis in young lambs. The same research group cloned nontransformed sheep B cells that can be useful for the study of sheep B cell biology and the effects of nutrients on ruminant B cell activity (Griebel et al., 2000).

Arachidonic acid metabolites, lipoxygenase and cyclooxygenase, are found in bone marrow, and a specific requirement of this fatty acid for the development of immune cells could be expected. Most studies of this aspect have been performed in humans and Desplat et al. (1998) concluded that lipoxygenase metabolites of arachidonic acid have no important influence on the proliferation of human marrow mononuclear cells and marrow stromal cell cultures.

One important type of bone-marrow-derived immune cell is the dendritic cell. Carrasco et al. (2001) developed from bone marrow hematopoietic cells and monocytes a porcine line with the properties of dendritic cells. A comprehensive review on the early development of the immune system in the pig, with particular attention to bone marrow and thymus, has been reported by Sinkora et al. (2002).

4.1.2. Thymus

In the thymus, T lymphocytes differentiate under the effect of thymus hormones: thymocytes acquire a receptor for the antigen, MHC restriction and self-tolerance, and specialize as helper or cytotoxic cells. In this organ, marginal zinc deficiency reduces thymic development or causes thymic involution and moreover lymphocytes from zinc-deficient piglets show a reduced mitogen responsiveness. Therefore, zinc is essential for thymus function. Thymulin, a hormone produced by thymic epithelial cells and required for differentiation of immature T-cells (Saha et al., 1995), requires zinc as a cofactor in order to be biologically active (Dardenne et al., 1982). Adequate peripheral zinc levels are important in order to saturate all thymulin molecules produced (Fabris and Mocchegiani, 1995). Knowledge about maturation of thymic cells is now improving with the help of the newly available molecular techniques. Thus, using differential mRNA display to compare zinc-deficient and zinc-supplemented mice, it was identified that several genes involved in T cell development are regulated by zinc (Moore et al., 2003).

Nowadays, secondary nutrient privations are more frequent than primary in animal production. In piglets farrowed and fed from aflatoxin B_1 and G_1-exposed sows, zinc and active thymulin plasma levels were reduced, while the inactive form of the hormone was not affected (Mocchegiani et al., 1998). In plasma samples, levels of the active form of thymulin were restored by addition of zinc. Maturation of CD8+ lymphocytes takes place in the thymus. In early-weaned pigs, the substitution of a mixture of conjugated linoleic acid (CLA) for soybean oil in the diet (the mixture of CLA – predominantly *cis*-9, *trans*-11/*trans*-9, *cis*-11 and *trans*-10, *cis*-12/*cis*-10, *trans*-12 – was fed for 5 weeks) increased the presence of thymic lymphoid precursor cells (CD4–CD8–) which migrated from the bone to the marrow and affected thymus activity causing increased expression of the CD8 type lymphocyte (fig. 2) (Bassaganya-Riera et al., 2001b). This effect was seen whether or not the pigs had been vaccinated with a proteinase-digested Brachyspira hyodysenteriae bacterin.

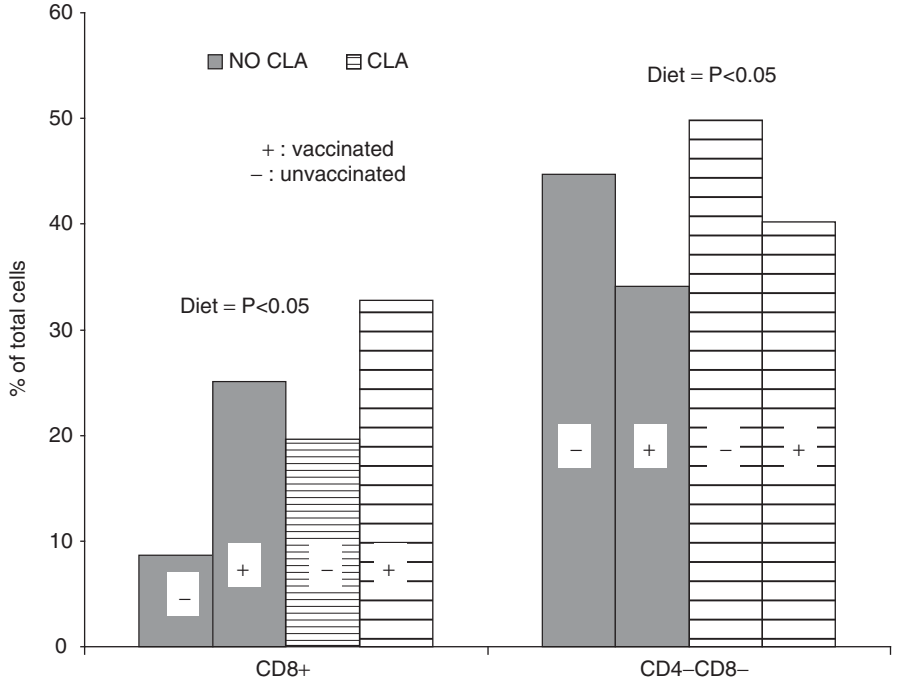


Fig. 2. Effect of conjugated linoleic acid (CLA) and vaccination with proteinase-digested *Brachyspira hyodysenteriae* bacterin on presence of phenotypes of thymocytes in pigs. (Adapted from Bassaganya-Riera et al., 2001b.)

4.2. Innate immunity

4.2.1. Soluble factors

In general, documented literature concerning relationships between the diet and soluble factors of innate immunity is very scarce.

4.2.1.1. Defensin Animals secrete many important soluble factors with antimicrobial action, particularly at different mucosal sites. However, the study of their regulation is only at the initial stages and it seems that most of these molecules play a surveillance role in maintaining the steady state of microflora on the mucosal surface. This seems, for example, the case of a porcine defensin, *p*BD-1, that is constantly expressed in the oral mucosa (Zhang et al., 2000).

4.2.1.2. Lysozyme Lysozymes hydrolyze the polysaccharide portion of bacterial cell walls (Kalfa and Brogden, 1999). In cell cultures of human middle ear epithelial cells, retinoic acid influenced the morphological phenotype and the secretory function of mucin and lysozyme (Moon et al., 2000). However, retinoic acid is a factor that influences cellular transcription and *in vivo* trials with vitamin A are necessary to assess if lysozyme secretion is affected. Interestingly, Li et al. (2001) report that daily supply of up to 150 000 IU vitamin D_3 increased serum lysozyme activity of weanling pigs. However, the number of replications per treatment was very low (= 4), and more results are required. In weaned pigs stimulated by antigenic intramuscular injection, a dietary concentration of 0.9 mg selenium/kg and various concentrations of vitamin E invariably produced serum lysozyme titers higher than 0.3 mg/kg

(Blodgett et al., 1988). In the same pig phase, the substitution of conjugated linoleic acid for 0.5% or 1.0% sunflower oil in the diet for 4 weeks increased serum lysozyme (Corino et al., 2002). In growing pigs, 150 mg α-tocopherol acetate/kg added to the feed during the first fattening period, increased lysozyme activity compared to the double dose and the negative dose (Riedel Caspari et al., 1986).

4.2.1.3. Transferrin It is well known that the iron-binding transferrin inhibits growth of a variety of bacteria and fungi *in vitro* (Weinberg, 1978). Iron is very important for the growth of microorganisms. It has been hypothesized that in the case of iron deficiency in animals, a lower iron saturation of transferrin would reduce the amount of iron available for microorganisms, thus in practice enhancing immunity. However Oppenheimer (2001) states that: (1) virulent invasive pathogens usually have efficient mechanisms to remove iron from transferrin, (2) transferrin inhibits growth of less-virulent opportunistic bacteria in plasma (such as *Escherichia coli*) over a wide range of transferrin saturations. So it does not seem probable that dietary iron can affect transferrin activity.

4.2.1.4. Cathelicidins Cathelicidins, another family of antimicrobial peptides, are expressed and primarily stored as propeptides in mature neutrophil granules in domestic animals (Wu et al., 2000). It has been shown that the expression of one protein of this family (PR-39) is stimulated in swine not only by immune stimulants such as LPS, but also by all-*trans* retinoic acid (Wu et al., 2000).

4.2.2. Cell-mediated innate functions

4.2.2.1. Zinc Monocytes respond to relatively high zinc doses (around 16 times physiological Zn concentration in humans) (Rink and Gabriel, 2000). In these cells, zinc supplementation induces the production of the cytokines IL-1, IL-6, tumor necrosis factor alpha and, in the presence of lymphocytes, IFN-γ (Rink and Gabriel, 2000). On the other hand, high doses of zinc ions inhibit IL-1 receptor-associated protein kinase and, consequently, reduce the proliferation of IL-1-dependent T cells (Wellinghausen et al., 1997).

4.2.2.2. Copper In ruminants, the first sign of copper deficiency is impaired function of neutrophils (Mills, 1987). Microbicidal activity is particularly affected (Boyne and Arthur, 1981) and neutrophil cytochrome oxidase and O_2^- generation are reduced (Mills, 1986). These effects are also found when bovines are copper depleted by administration of the antagonist molybdenum (Xin et al., 1991; Gengelbach et al., 1997).

With a copper-deficient diet (1 mg/kg), the plasma level of TNF-β in calves was lower than when the diet was supplemented to 10 mg/kg (Gengelbach et al., 1997). This result correlates well with the higher body temperature registered in the same Cu-supplemented calves, if the systemic effect of TNF-β is considered. However, there was no change in the production of TNF-β and IL-1 production by peripheral blood monocytes if calves were fed a Cu-deficient diet, or were supplemented with copper or with molybdenum (Gengelbach and Spears, 1998).

4.2.2.3. Arginine and nitric oxide Nitric oxide (NO) has an important role in the control of gene expression, particularly with respect to proteins associated with host-defense (Ehrt et al., 2001; Perez-Sala et al., 2001). NO is synthesized from L-arginine and O_2 by NO synthase (NOS) in almost all mammalian cells (Alderton et al., 2001). Three distinct isoforms of NOS – neuronal, inducible and endothelial (nNOS, iNOS, and eNOS) – have been identified. Large amounts of NO are generated by iNOS (Muscara and Wallace, 1999). This isoform

plays a pivotal role in numerous and different pathophysiological processes, particularly as a principal mediator of the microbicidal and tumoricidal actions of macrophages (Stuehr and Nathan, 1989). Inducible NOS is expressed in many cell types in response to a wide range of inflammatory cytokines including IL-1β, IL-2, interferon-γ, tumor necrosis factor-α and bacterial metabolites such as LPS (Xie and Nathan, 1994; MacMicking et al., 1997). Connelly et al. (2003) established that in macrophages eNOS also has an important role in inflammation and is stimulated by LPS.

Arginine levels in the diet could have an impact in the nutrition of young animals due to their requirement for nitric oxide synthesis. In keyhole limpet hemocyanin-immunized calves, L-arginine supplementation depressed the number of circulating lymphocytes, monocytes and neutrophils (Fligger et al, 1997). In addition, arginine supplementation reduced both specific and total IgG concentration in plasma. NO production and NOS function were not tested, however it is possible that the increased availability of arginine as substrate could have enhanced other immune functions (macrophages, acquired cellular immunity) at the expense of the tested functions. Alternatively, a dose effect could have occurred, as the action of NO is dose dependent and at high concentrations (micromolar level), it inhibits gene expression (Connelly et al., 2003).

Arginine supplementation was also tested in calves challenged with intravenous administration of the endotoxin LPS (Husier and Blum, 2002) but supplementation did not change the effects of LPS (increase of tumor necrosis-factor-α, lactate and cortisol concentrations).

NO plays also a critical role in modulating several components of mucosal defense. NO seems to exert inhibitory effects on intestinal secretion of mucus. This may depend on the concentration of NO that is produced: low amounts of NO are stimulatory, but high amounts are inhibitory (Wallace and Miller, 2000). When the mucosal defense is low, bacterial translocation is easy.

4.2.2.4. Glutamine During illness, glutamine concentrations in the blood may be low. In neutrophils, glutamine plays a role in superoxide production (Pithon-Curi et al., 2002) and delays spontaneous apoptosis (Pithon-Curi et al., 2003). In healthy humans, glutamine enhances the bactericidal function of normal neutrophils and after burns tends to restore this property to normal levels (Ogle et al., 1994).

4.3. Humoral immunity

4.3.1. Activation of a Th2 response

4.3.1.1. 1 alpha, 25-dihydroxy vitamin D_3 Activated B and T lymphocytes have high-affinity receptors for the active form of vitamin D, the hormone 1 alpha, 25-dihydroxy vitamin D_3. In activated cloned murine T cells, low doses of this hormone enhance the production of IL-4, IL-5 and IL-10 and depress the production of gamma interferon and IL-2 (Daynes et al., 1996). These data have been confirmed in normal mice topically exposed to the active form of vitamin D (Daynes et al., 1996). The observations of the authors show that adding this molecule to a vaccine preparation effectively changes a peripheral lymph node into a mucosal lymph node. Following the intramuscular vaccination of piglets with the F4 fimbriae of enterotoxigenic *E. coli,* the addition of 1 alpha, 25-dihydroxy vitamin D_3 increased the antigen-specific IgA response in serum and mucosal secretions (Van der Stede et al., 2001), seric antigen-specific IgM, F4-specific proliferation of peripheral blood mononuclear cells and initially reduced the excretion of *E. coli* F4 (Van der Stede et al., 2003).

However, production of IgG in activated normal human peripheral blood mononuclear cells was inhibited in a dose-dependent way (Lemire et al., 1984). Further research showed that this effect can be ascribed to an action on T helper subset type 1, that is responsible for IgG

subtype 2a production (Lemire et al., 1995). In pigs, this differentiation of IgG has not been reported. It is also possible that the effect of 1 alpha, 25-dihydroxy vitamin D_3 changes with the species. From a nutritional point of view, it would be very interesting to test the importance of different dietary doses of the active form of vitamin D on different immune functions in growing pigs. Furthermore, the risk of toxicity should also be taken into account. Li et al. (2001), in a very small-scale experiment, observed that addition of inadequate or excess amounts of vitamin D_3 to the diet of weanling pigs decreased the antibody response to injected bovine serum albumin.

4.3.1.2. (n-3) polyunsaturated fatty acids Programmed cell death is one way to modulate the immune response. T cells from mice fed a diet lacking in (n-3) polyunsaturated fatty acids (PUFA) are more susceptible to apoptosis when stimulated to express Th2 cytokine profile, than after stimulation to express Th1 profile (Switzer et al., 2003). This indicates that one action of (n-3) PUFA is to maintain or increase the Th2-mediated humoral immune response, at the expense of the cell-mediated response.

4.3.2. Immunoglobulin production

4.3.2.1. Vitamin E Vitamin E, via its antioxidant properties, affects expression of genes regulated by reactive oxygen species, such as nuclear factor κ B (Packer and Suzuki, 1993). In addition to effects on cytokine production by T cells, this transcription factor is also important for maturation of B cells and hence for Ig production. Thus, in steers circulating IgG for ovalbumin tended to increase dose-dependently with supplementation of up to 1140 IU/d vitamin E (Rivera et al., 2002). This trend was found 7 days after the second immunization with ovalbumin, but not before.

In contrast, doses up to 25 IU/kg in the diet of broiler chicks were sufficient to maximize the specific IgG antibody response after vaccination with killed infectious bronchitis virus and supplementation with up to 200 IU/kg did not increase the response further (Leshchinsky and Klasing, 2001). In the case of immunization with SRBC or *Brucella abortus*, the humoral response peaked at doses of 50 IU/kg vitamin E and then declined. Such moderate vitamin E levels were more effective than higher levels also for cellular and innate immunity.

On the other hand, in chickens immunized with tetanus toxoid, 250 IU/kg were necessary to increase anti-T toxoid IgA antibody titers in serum and final day intestinal scrapings, as well as the percentage of T-helper cells and IgA-positive cells in peripheral blood (Muir et al., 2002). This agrees with the positive response of anti-*E. coli* LPS antibody titers in piglets supplemented with 100 IU/kg, in comparison with values in control subjects (Ellis and Vorhies, 1976).

4.3.2.2. Glutamine, nucleotides In weaned pigs, a combination of 10 g/kg glutamine and 1 g/kg nucleotides increased serum IgG concentration (but not IgA) 2 h post LPS, and foot and mouth disease neutralizing antibody titers 3 weeks after vaccination for this disease (Yu et al., 2002). Due to the experimental design, the effects of the two different supplementations cannot be split. It may therefore be relevant to note that Cameron et al. (2001) in two different trials found no effect on total serum antibody levels or antibody level specific for injected keyhole limpet hemocyanin following addition of yeast RNA to piglet diets. For further discussion of glutamine and nucleotides, see also sections 4.4.1.2 and 4.4.1.4, respectively.

4.3.2.3. Zinc In zinc deficiency, reduced Ig secretion is mainly due to impaired maturation of B cells in bone marrow (see section 4.1.1). Nevertheless, a direct effect of zinc on human B cell activity has yet to be definitively established (Rink and Gabriel, 2001).

Indirectly, zinc values below as well as above the standard requirements can affect Ig production, due to variations in T cells functions.

In weaning pigs, research shows that dietary zinc oxide (ZnO), at pharmacological levels, improves growth and/or reduces diarrhea in weaned pigs (Hill et al., 2001). It is well known also that ZnO is effective against (mainly) Gram-positive bacteria. Consequently, knowledge of the mechanism of action of ZnO could be important to help develop more adequate dietary strategies for the general health of the animals and for the environment.

Theories to explain ZnO action are conflicting. One explanation can be found in the effects of zinc on immunity. We addressed some research to evaluate the effect of different dietary doses and sources of zinc on growth performance, health and immunity of piglets challenged *per os* with enterotoxigenic *E. coli* K88. In the first trial, the addition of 2500 mg zinc oxide/kg diet to a standard diet, did not change fecal excretion of the pathogen, and increased IgA-specific activity in the plasma of *E. coli* K88-sensible subjects (Bosi et al., 2001). In a second trial, the addition of different doses of Zn (200 mg/kg and 2500 mg/kg) from two different sources (ZnO or Zn chelated with glutamate) reduced *E. coli* K88 fecal excretion and tended to reduce IgA-specific activity in plasma (fig. 3) (Bosi et al., 2003). These results seem quite conflicting. However, in both trials the specific immune response seems to have more to do with gut health, than with metabolic effects of zinc. In fact, when no improvement was observed in gut health with zinc oxide a specific and more active immune response was stimulated, whereas when supplementary zinc induced a rapid excretion, the recruitment for a specific humoral response was lower. More knowledge on this topic could have an important impact on pig production, to find opportune dietary solutions. In fact, zinc use is restricted to 150 mg/kg feed in the European Union, mainly to reduce the transfer to the soil of zinc supplied in excess for animal requirements.

4.4. Cellular immunity

4.4.1. Activation of a Th1 response

4.4.1.1. Glutamine Rat lymphocytes use glutamine at a high rate, particularly when they are activated (O'Rourke et al., 1989). Human lymphocytes produce more IL-2, IL-10 and interferon-γ (IFN-γ) when glutamine is added to their *in vitro* culture (Yaqoob and Calder, 1998).

Yoo et al. (1997) studied the effects on immune functions of isonitrogenous substitution of 40 g glutamine/kg for the same amount of nonessential amino acids in the diet of 21-day-old weaned pigs. In subjects intraperitoneally injected with *Escherichia coli*, glutamine prevented the plasma and intramuscular glutamine depletion observed in nonglutamine-fed pigs. In the same way, the *in vitro* mitogen response in glutamine-fed, challenged pigs was as big as in the nonchallenged pigs. Again in piglets, 1–1.5% glutamine in the diet for 14 days maximized mitogen-induced proliferation of lymphocytes from peripheral blood, Peyer's patches and spleen (Lee et al., 2003), although this effect was found with only one of the mitogens used.

4.4.1.2. Conjugated linoleic acid In early-weaned pigs reared in dirty or clean environments, increasing the supplement doses of a mixture of conjugated linoleic acid (CLA) isomers (from 0% to 2% diet) for 42 days caused a linear increase in the percentage of CD8+ lymphocytes (Bassaganya-Riera et al., 2001a). This effect of CLA is confirmed also by the results of Bassaganya-Riera et al. (2001b). In this trial the effects of CLA were observed earlier in the thymus than in peripheral blood. Therefore, CLA appears to modulate primarily the functions of the thymus (see section 4.1.2), and then the secondary lymphoid pool.

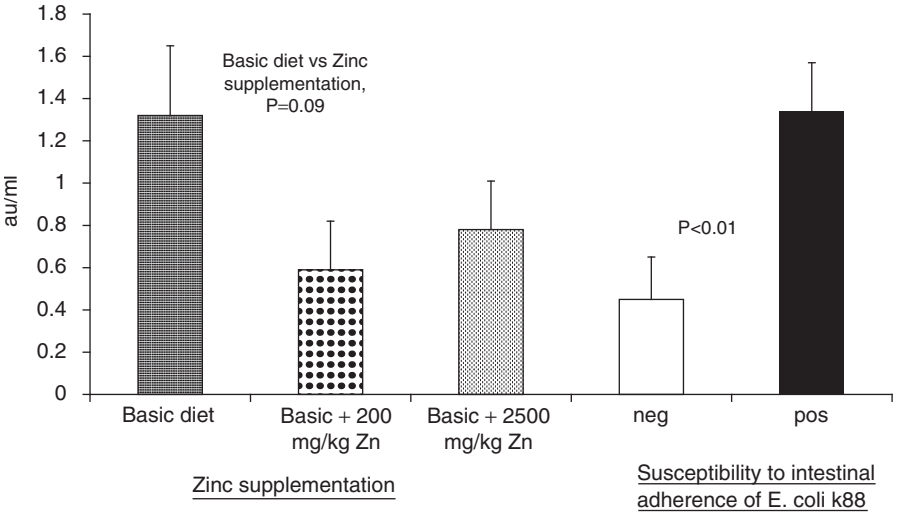


Fig. 3. Effects of Zn supplementation and of susceptibility to intestinal adherence of *Escherichia coli* K88 on specific IgA in blood serum of early-weaned pigs, 4 days after oral challenge with *E. coli* K88. Values of supplemented groups are the average of supplementation with ZnO or with Zn chelated with glutamate. Susceptibility to *E. coli* K88 intestinal adherence was determined on jejunum villi collected from each subject at sacrifice. (Adapted from Bosi et al., 2003.)

The specific effector response is also affected by CLA. In pigs fed CLA and immunized against modified-live pseudorabies virus, the proliferation of CD8+ peripheral blood mononuclear cells specific for this virus was enhanced (Bassaganya-Riera et al., 2002).

4.4.1.3. Zinc In a human model, induction of zinc deficiency caused a reduction in Th1 function, as indicated by low production of Th1 cytokines (Beck et al., 1997). T cytolytic cells were also reduced but Th2 function was maintained. This can be important, for example, in viral infections: in Zn deficiency the immune response is switched from efficient cellular antiviral action to Th2-dependent humoral immune functions.

In humans, high zinc doses (>100 mg/d) reduce delayed-type hypersensitivity (see Rink and Gabriel, 2000, for a review). *In vitro*, at three to four times the physiological level, zinc reduces the alloreactivity in mixed lymphocyte culture (Campo et al., 2001), while at higher concentrations zinc reduces T-cell proliferation *in vitro* and immunosuppressive effects have been observed *in vivo* (Rink and Gabriel, 2000).

In LPS-challenged growing pigs, zinc supplementation in the range from 10 mg/kg to 150 mg/kg does not affect mitogen-induced lymphocyte proliferation and skin thickness response to phytohemagglutinin (Roberts et al., 2002). However, zinc supplemented at 50 ppm or 150 ppm increases the febrile response in LPS-stimulated pigs.

4.4.1.4. Nucleotides In normal individuals, nucleotides are synthesized adequately *in vivo,* however, during phases of high growth and after challenge of the immune system, a dietary requirement can occur. Observations in mice showed that a nucleotide-free diet impairs antibody responses, but also that supplementation of such a diet with nucleotides induces production of IFN-γ, which is typical of the Th1 response after various antigenic stimulation (Jyonouchi et al., 2000; Sudo et al., 2000). Cytokines typical of the Th2 response are reduced and consequently a switch between antibody isotypes is found (see fig. 1).

Publications on nucleotide supplementation in animal production are very scarce. In weaned pigs, dietary supplementation with synthetic uracil and adenine (total 500 mg/kg) increases the lymphocyte blastogenesis induced by phytohemagglutinin and concanavalin A, but does not affect the *in vivo* cellular immune response (Zomborszky-Kovacs et al., 2000). Cameron et al. (2001), again in trials with piglets, confirmed the lymphocyte proliferative response *in vitro*, but *in vivo* they also observed a type of delayed hypersensitivity to intradermal challenge with keyhole limpet hemocyanin.

4.5. Reduction of drawback of immune response

4.5.1. Anti-inflammatory

4.5.1.1. n-3 PUFA Variations of quantity and quality of PUFA in the diet affect the membrane composition of immune cells. Different PUFA, in the presence of cyclooxygenase and lipoxygenase, generate different compounds and modulate the inflammatory response. In particular the isoform 2 of cyclooxygenase is important for the synthesis of prostglandins (PG) from arachidonic acid (particularly PGE2), stimulated by bacterial LPS and inflammatory cytokines. Lipoxygenase has more affinity for n-3 PUFA than for arachidonic acid and releases different leukotriene (LT) series accordingly (respectively, series 5 from the former, and series 4 from the latter). The series 5 LTs have lower chemotactic efficacy.

In chicks, experiments have been performed that were designed to test specific and inflammatory immune responses, by changing the levels and the ratio between n-3 and n-6 sources (fish oil versus corn oil) (Korver and Klasing, 1997). Only fish diets alleviated the febrile response and the growth-suppressing effect of challenge with heat-killed *S. aureus* or *S. typhimurium* LPS. Interleukin-1 release from peritoneal macrophages increased with increasing corn oil addition, but decreased with increasing fish oil addition (fig. 4).

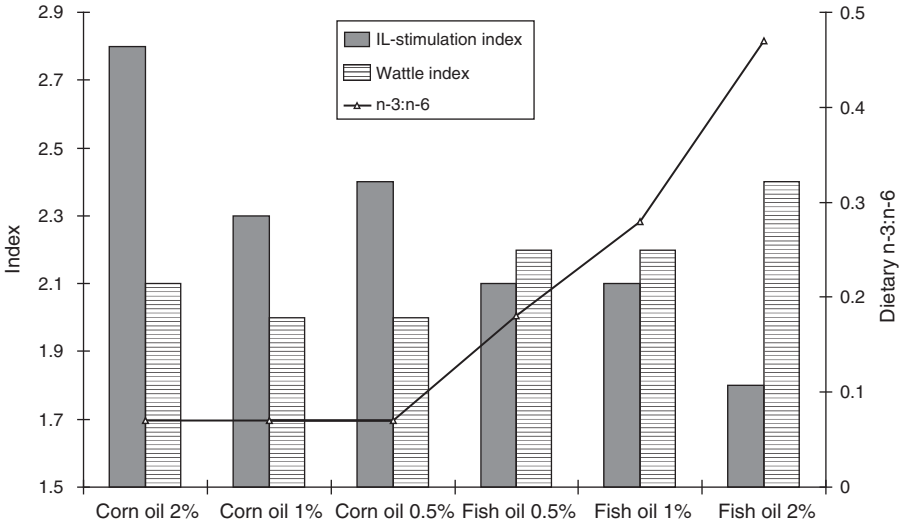


Fig. 4. Effects of the levels and the ratio between corn oil and fish oil in the diet of chicks, on indices of inflammatory response. Wattle index is the ratio of phytohemagglutinin-induced and vehicle-injected swelling. IL-1 stimulation index is the ratio of stimulation obtained in thymocytes cultured with or without surnatant containing IL-1 and produced by peritoneal macrophages cultured in the presence of *S. typhimurium* lipopolysaccharide. (Adapted from Korver and Klasing, 1997.)

Phytohemagglutinin-induced swelling increased with dietary n-3:n-6 ratio (fig. 4). Specific antibody titers after vaccination with infectious bronchitis virus were not reduced by fish oil addition. The results of a second trial, where n-3:n-6 ratio was changed by substituting linseed oil for corn oil in a mixture with fish oil, show that a moderate presence of n-3 fatty acids in the diet reduces negative effects of infection, without affecting other immune parameters. The same research group observed that Lofrin, a 5-lipoxygenase inhibitor used to reduce the synthesis of series 4 of LT, or fish oil attenuated the growth-depressing effects of *Eimeria tenella* infection (Korver et al., 1997). This confirmed that the anti-inflammatory effect of fish oil is presumable due to the competition between different PUFA series.

A dietary formula specifically designed to control ulcerative colitis, containing oligosaccharides, fish oil, gum arabic and antioxidants was compared with a control diet in growing barrows (Campbell et al., 1997). After 7 days with the anti-inflammatory diet, plasma, colonic and cecal levels of thromboxane B2 and 6-keto-prostaglandin F1 alpha were reduced, while prostaglandin E was decreased only in the colon and cecum. The observed decrease of arachidonic acid with concomitant increase in n-3 PUFA probably account for most of the anti-inflammatory effects of the experimental diet, however a contribution of the other ingredients of the formula cannot be excluded.

4.5.1.2. Conjugated linoleic acid In pigs fed CLA-supplemented diets, inflammation of the colonic mucosa induced by challenge with *Brachyspira hyodysenteriae* were lower than in pigs fed soybean oil-supplemented diet (Hontecillas et al., 2002). In the former subjects, IFN-γ and IL-10 profiles, and expression of peroxisome proliferator-activated receptor-γ (PPAR-γ) remained close to the values observed for nonchallenged pigs. PPAR-γ is a transcription factor and dietary CLA could have induced the expression of genes regulated by this molecule, such as MHC II. The action of CLA can also be related to its competition with arachidonic synthesis and with conversion of arachidonic acid into prostglandins and leukotrienes.

4.5.2. Antioxidants and precursors

4.5.2.2. Vitamin E A moderate presence of vitamin E at the site of infection should be effective to reduce the cellular damage after release of cellular free radicals, but this has yet to be directly demonstrated. Besides this putative effect, the protective action of vitamin E can favor PUFAs present in immune cells, such as macrophages. Consequently, the production of different lines of PGs and LTs can be stimulated, depending on the dietary ratio of PUFA (n-3):(n-6) (see section 4.5.1.1). Different properties of the various tocopherols need to be considered. Gamma-tocopherol (γT) is considered to have less antioxidant activity than α-tocopherol (the form generally used for dietary supplementation). However, Jiang et al. (2000) found that, in contrast to αT, γT inhibited the generation of prostaglandin E_2 activity in macrophages and epithelial cells. The anti-inflammatory action of γT has since then been confirmed *in vivo* by Jiang and Ames (2003). In mice challenged (carrageenan) to induce inflammation, γT (33 mg/kg or 100 mg/kg) reduced PGE2 synthesis at the site of inflammation, inhibited the formation of leukotriene B4, and attenuated the reduction of feed intake caused by inflammation, while in contrast αT (33 mg/kg) was ineffective.

4.5.2.3. Selenium Selenium is cofactor of antioxidative enzymes such as glutathione peroxidase and thioredoxin reductase. In humans with systemic inflammation and sepsis, plasma selenium and glutathione peroxidase are lowered, and selenium supplementation

seems to improve the outcome of the patients (Gartner et al., 2001). Furthermore, selenium supplementation should reduce the effects of induced systemic selenium deficiency in domestic animals under similar conditions.

4.5.2.4. Glutamate, cysteine, glycine for glutathione Lymphocytes, particularly of the T family, have a high demand for glutathione, both for their activity and their replication. This powerful antioxidant is endogenously produced from glutamate, cysteine and glycine. Glutamate is not an essential amino acid, however it has been shown in the piglet that dietary glutamate is the preferential source for mucosal glutathione synthesis (Reeds et al., 1997).

4.5.2.5. Tryptophan In inflammatory reactions, under the influence of IFN-γ, tryptophan is degraded and depleted by the action of indoleamine 2,3-dioxygenase (particularly in macrophages) (Chiarugi et al., 2003). This can reduce the quantity of tryptophan available for animal growth. In turn, it is possible that during infections the local availability of tryptophan limits macrophage functions.

5. FUTURE PERSPECTIVES

Basic knowledge about immune functions in humans and mice has grown very rapidly in the last 20 years, but improvements in the immunology of domestic animals encounter ever more difficulties, mainly due to increased costs and reduced research funds. Furthermore, only recently has the relevance of nutrients for the regulation of immune functions received enough research interest. This is particularly important for the interface between the growing animal, including the gut and other mucosal-associated lymphoid tissues, and the environment.

There is a need to improve knowledge about dietary factors that can regulate the response of specific immune cells, but also their interaction with nonprofessional cells. For this purpose, it is important to develop *in vitro* systems where both types of cell, derived from each domestic species are cultured. An example is the co-culture system with CaCo2 cells and lymphocytes. This system should be adapted to the different lymphocyte properties between species. In addition, it can also allow tests to be made of the response in terms of different cytokines as well as evaluations of the interactions with microorganisms.

Some of the results presented in this review were obtained in perturbed animal models. In general, the use of these models has helped to provide evidence concerning specific mechanisms of the immune system. However, the choice of the type of challenge is relevant and unfortunately only a few comparative studies of different challenge systems have been made. Models where only toxins are used can give different results compared to cases when the live organism is used. Finally, the way of contact is important: a first contact within the gut is advisable for an enteric pathogen.

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12 Dietary manipulation of infectious bowel disease

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The lumen of the gastrointestinal tract represents a critical interface between the external environment and host tissue. The tract functions in a highly ordered and integrated way in digestion and absorption of foodstuffs transiting through it, whilst necessarily accommodating a site-specific microbiota, which is large, diverse and highly metabolically active, as well as being intimately involved with digestive function. Superimposed on this complex microenvironment are pathogenic microbes that frequently enter, become established and proliferate. Depending on their specific pathogenic properties, these organisms then potentially may cause serious local or generalized disease. Young animals are particularly susceptible to such infections, since their intestinal tracts, immune system and microbiota are incompletely developed and stabilized. The structure and function of the intestinal tract, and the composition of the resident microbiota, are greatly influenced by the nature and composition of the diet consumed. This chapter explores some influences of diet on this system, and provides examples of how manipulation of the diet can help to influence colonization by certain potential enteric pathogens. Examples are drawn mainly from the problems of postweaning diarrhea in the pig, since this is an important condition that exemplifies some of the principles being explored in nutritional intervention of enteric infections.

1. INTRODUCTION

There is general agreement that diet can have an influence on intestinal disease, particularly in young animals. Surprisingly, there are relatively few examples where these influences have been studied in detail, shown to be reproducible, and the basic underlying mechanisms convincingly explained. This chapter examines some such dietary interactions, and particularly focuses on the problem of diarrhea in recently weaned pigs, since this condition has been extensively studied.

Biology of Nutrition in Growing Animals
R. Mosenthin, J. Zentek and T. Żebrowska (Eds.)

The possibility of using nutritional interventions to help control infections with specific enteric bacterial pathogens has been acknowledged for many years, but until recently the availability of cheap and effective antimicrobial drugs resulted in a general lack of investigation and application of a dietary approach to help control enteric infections. This attitude is now changing, as a result of the appearance of a reduced susceptibility to many antimicrobial drugs by a variety of commensal and pathogenic bacterial species that colonize the intestinal tract, including zoonotic bacteria such as *Salmonella* and *Campylobacter*. Fears that the excessive use of antimicrobial drugs has led to the development and spread of bacterial resistance have resulted in the reduced availability of in-feed antimicrobials. As a consequence of this action, considerable effort is now being put into finding alternative methods to maintain growth rates and protect animal health in the absence of antibiotics. This includes investigating the use of a range of different products, including vaccines and immune stimulators, whilst animal husbandry is being re-evaluated and improved to reduce management stress on production animals and exposure to potential pathogens. Targeted nutritional intervention, the subject of the current chapter, represents one of a number of such potential alternatives or supplementary means to control enteric infections. This approach considers the physical and biological properties of the whole diet, and how it might directly or indirectly interact with pathogenic bacteria in the intestinal lumen in such a way as to reduce the numbers or pathological impact of these miroorganisms. It focuses on specific diseases, rather than on ''gut-health'' in general. Finally, it does not include more specific dietary approaches to disease control, such as the use of dietary acidifiers, addition of prebiotics or chemicals such as zinc oxide to the diet, or the use of specific plant extracts or new products that have antibacterial properties.

It should be acknowledged that the use of nutritional intervention for control of enteric infections is constrained in many ways. Firstly, it is unlikely that any one dietary intervention will be universally appropriate for all enteric infections. Different animal species and different age groups are likely to respond to different diets and dietary constituents in different ways and to different extents. Even within an animal species, such as the pig, sites of infection with different pathogens vary from the stomach (e.g. *Helicobacter* species), small intestine (e.g. *Escherichia coli*) to the colon (e.g. *Brachyspira* species). Not only must the nutritional intervention be targeted at very different pathogenic bacterial species, but their activities against these microbial pathogens must be delivered in quite different macro- and microenvironments. As the ingredients of a given diet move through the tract and are digested and absorbed, their physical properties and the composition of different microbial substrates change enormously. Each of the environments inhabited by the specific pathogens is highly complex and different, and there are innumerable and often poorly defined interactions underway involving the host tissues and their products, the dietary constituents, the indigenous microbes and the specific pathogens. Even the commensal microbiota, which relies on dietary and endogenous substrate to allow it to maintain its composition and density, is itself now known to modify many aspects of cellular differentiation, gene expression and even production of antimicrobial defense molecules in the gastrointestinal tract (Hooper et al., 2002). In young animals, superimposed on this already complex situation is the changing structure, function and microbiota of the developing gastrointestinal tract, particularly in the neonatal period and in the period immediately after weaning.

There are also complications in relation to the diets; those in practical use typically contain a mixture of different raw ingredients. Not only are these different ingredients different in terms of their constituents, but even within a given macroingredient type there can be important differences in their specific microingredients. For example, different cultivars of wheat,

or the same wheat cultivar grown under different conditions, can have important differences in their composition (Kim et al., 2003). Next, the physical form of the diet resulting from processing can have important influences on disease development. Pelleting of diets has been identified as a potential risk factor for a variety of enteric conditions, including salmonellosis (Harris et al., 1997), nonspecific colitis (Connor, 1992) and development of stomach ulcers (Robertson et al., 2002). On the other hand, the use of fermented liquid diets may reduce the risk of salmonellosis (Van der Wolf et al., 1999).

Finally, in the context of production animals, any nutritional intervention to control enteric infections must be cost effective; i.e. depending on the disease in question, the specific preventative diets should not rely on ingredients or expensive dietary processing that make the intervention more costly than the disease.

Considering all these points, the most attractive targets for nutritional intervention in production animals would include those infectious bowel diseases that both cause serious production losses, and are relatively restricted in terms of the pathogens involved and sites of colonization in the tract. The best targets are probably necrotic enteritis in broiler chickens (caused by *Clostridium perfringens*), and postweaning diarrhea (PWD) in young pigs (caused by enterotoxigenic *Escherichia coli*). In addition, nutritional interventions that reduce carriage of zoonotic pathogens such as *Salmonella* and *Campylobacter* in chickens and pigs, and *E. coli* O157 in cattle, are also attractive targets because of the economic benefits that would be obtained by their control. For simplicity, the current chapter concentrates on nutritional intervention as a means of helping control PWD in the pig. The role of dietary fiber in altering susceptibility to PWD receives special attention.

2. POSTWEANING DIARRHEA

Postweaning diarrhea (PWD) is a common disease that occurs in piggeries throughout the world. Generically it can be described as a diarrheal disease that typically starts between 3 days up until about 10 days after weaning. Piglets usually develop a watery diarrhea, and show a rapid loss of condition, with most members of a litter being affected. PWD is endemic on some farms, being present in many litters, and repeatedly occurring in successive litters over many years. The disease can cause major disruptions to management, and can have a severe adverse economic impact (Cutler, 1981; Cutler and Gardner, 1988). Besides potential mortalities, and the cost of treatment, piglets fail to gain weight immediately after weaning, and this extends the total time to reach slaughter weight (Tokach et al., 1992; Williams, 2003). PWD is a complex and multifactorial disease, incorporating many aspects of management (Madec et al., 1998), but important etiological agents include *E. coli,* and sometimes rotaviruses (Lecce, 1983). Infection of the large intestine with the intestinal spirochete *Brachyspira pilosicoli* may also extend and complicate the condition (Hopwood et al., 2002).

2.1. *Escherichia coli*

The main etiological agent(s) associated with PWD is (are) specialized strains of the bacterium *Escherichia coli,* which differ from the common nonpathogenic types that occur in the intestinal tracts of healthy pigs. Unlike the nonpathogenic strains, these pathogenic strains have the ability to adhere to the luminal surface of small intestinal enterocytes or the mucus covering the villi, particularly in the anterior small intestine, preventing them from being flushed away to the more distal parts of the tract by normal peristaltic movement of the

lumenal contents. Attachment is through bacterial rod-like surface structures called fimbriae or pili. In pathogenic *E. coli* strains, adhesins K88 (also known as F4), and F18 (formerly F107) are most commonly associated with PWD, and these both exhibit several antigenic variants (Francis, 2002). At this site adjacent to the enterocyte surface they deliver powerful toxins that disrupt the normal functionality of the enterocytes. As the anterior small intestine has a critical function in both digestion and absorption, disruption of function at this site is particularly harmful.

The most common and significant pathogenic types associated with PWD are enterotoxigenic *E. coli* (ETEC). Different ETEC strains release different combinations of two toxin types, both of which provoke hypersecretory diarrhea as a result of loss of water and electrolytes into the intestinal lumen. Heat-labile toxin (LT) induces active secretion of chloride ions, sodium ions, bicarbonate ions and water into the lumen. LT binds irreversibly to the mucosal cells and activates the adenyl cyclase–cyclic AMP system in the affected villous enterocytes (Argenzio, 1992). The second toxin, heat-stable toxin (ST; variants STa and STb), interacts with the guanyl cyclase–cyclic GMP system and prevents the epithelial cell from absorbing sodium and chloride ions from the lumen (Gyles, 1993). These processes result in an excess volume of fluid and electrolyte in the gut lumen of infected pigs. This volume can only be fully reabsorbed if the colon is healthy, has a stable, well-balanced microbiota, and is not physically overloaded (Argenzio, 1992). Other virulence determinants possessed by certain less-common strains of *E. coli* involved in PWD include the genes (EAST1) encoding the enteroaggregative *E. coli* heat-stable enterotoxin 1, whose function remains uncertain (Choi et al., 2001), the gene encoding diffuse adherence (AIDA) (Ha et al., 2003), and the attaching and effacing genes (*Eae*) responsible for the production of intimins in enteropathogenic *E. coli* (Higgins et al., 1997). Intimins are outer-membrane proteins involved in attachment of the bacteria to colonic enterocytes, preceding changes in the enterocyte cytoskeleton and effacement of their microvilli (Nataro and Kaper, 1998). Some pathogenic intestinal *E. coli* strains produce a verotoxin, which is involved in the pathogenesis of edema disease, a predominantly neurological condition sometimes accompanied by diarrhea (Osek, 1999).

The *E. coli* strains that cause diarrhea after weaning usually are able to lyse the red blood cells present in the blood agar plates that are used for their isolation, and consequently these bacteria are known as β-hemolytic *E. coli*. The hemolytic activity is not considered to be a virulence factor in itself, but is a useful marker of strains that are liable to be involved in PWD. There is a small number of O-serotypes that are repeatedly observed in association with PWD, of which the most common are: O149, O138, O139, O141 and O8 (Frydendahl, 2002).

Hemolytic *E. coli* are uncommon in the intestinal tract of healthy unweaned pigs, although occasionally these strains are present in unweaned diarrheic pigs. Following weaning, these organisms frequently proliferate in the gastrointestinal tract of both healthy pigs and pigs that go on to develop diarrhea. The key difference is that the number and proportion of potentially pathogenic strains of *E. coli* in the gastrointestinal tract and feces are higher in pigs with PWD, compared to those that remain healthy (Kenworthy and Crabb, 1963; Svendsen et al., 1977; Hampson et al., 1985). Pigs with PWD have up to 10^9 colony-forming units of such hemolytic *E. coli* in the small intestine, whilst there is minimal change in other resident bacterial populations at this time (Smith and Jones, 1963). This link between excessive small intestinal multiplication of hemolytic *E. coli* and the development of diarrhea in weaner pigs was first reported by Richards and Fraser (1961). The associated disease, which has been called postweaning colibacillosis (PWC) is characterized by diarrhea, dehydration, rapid loss of weight, metabolic acidosis, poor condition and shivering (MacKinnon, 1998; Bertschinger, 1999).

Death can result if the disease is left untreated or is too advanced to treat successfully. The terms PWC and PWD tend to be used interchangeably, but PWC is a more specific term where the disease is completely or predominantly attributable to the *E. coli* infection. On the other hand, the term PWD acknowledges that the diarrhea that often occurs in piglets after weaning may have other etiologies and/or complex interactions superimposed on the *E. coli* infection (Hampson, 1994).

Consecutive infections with different strains of pathogenic *E. coli* can occur in a herd because immunity to one strain of pathogenic *E. coli* is not protective against all. This means effective vaccination is difficult to achieve, and vaccination is not generally used as a means of disease control. Many strains also show resistance to multiple antibiotics (Mateu and Martin, 2000; Amezcua et al., 2002), which limits the avenues of treatment. The duration of infection is generally between 4–14 days. Fecal–oral spread between animals is the primary means of transmission, but some aerosol and probably fomite transmission is also likely (Bertschinger, 1999).

2.2. *Brachyspira pilosicoli*

Brachyspira pilosicoli is an anaerobic spirochete that colonizes the large intestine of pigs, as well as a variety of bird species, dogs and human beings (Hampson and Trott, 1999). It is responsible for a condition called porcine intestinal spirochetosis, a mild colitis and diarrhea that can affect pigs from weaning right through the grower/finisher phases (Trott et al., 1996). A characteristic but not universal feature of the colonization is attachment of large numbers of the organism by one cell end to the colorectal epithelium. This attachment is thought to interfere with colonic absorption, and can result in a wet, cement-like diarrhea. In the same way as with hemolytic ETEC, colonization by *B. pilosicoli* is common in many pig herds, but specific disease is less commonly reported. Infection with *B. pilosicoli* can complicate cases of PWD, contributing to the extent and duration of the diarrhea that occurs.

3. DIETARY AND WEANING INFLUENCES ON THE GASTROINTESTINAL TRACT AND THE MICROBIOTA

The intestinal mucosa represents a dynamic interface where the processes of digestion, absorption, and antigen and bacterial recognition, uptake or exclusion, occur. In this regard, optimal mucosal function is vital to the integrity and protection of the gastrointestinal tract, and is determined by the orchestrated actions of specific immunological and nonimmunological defense mechanisms. The nonspecific defense mechanisms include innate immune reactivity, the indigenous microbiota, the mucin and mucous barriers, gastric acidity and bile salts, all of which represent a ‘‘front line’’ defense (Kelly and King, 2001). The indigenous microbiota plays a key role in this mechanism, and also stimulates gut function and primes the immune system. Further discussions relating to the more generalized interactions between the host and the bacteria are outside the scope of this paper, however discussion can be found in several recent reviews (e.g. Kelly and King, 2001; King et al., 2003).

The intestinal microbiota of the pig is established in the first 48 hours after birth (Conway, 1994), after which time the composite microbial populations remain stable, except at times of major dietary change such as weaning. Pigs contain more bacteria in their small intestines than do other animal species, and they have active fermentation sites in the stomach and the large intestine that are involved in the digestion of various otherwise indigestible plant materials that they may consume.

3.1. Weaning

Immediately after weaning, particularly if this is at 3 weeks of age or less, there is a short period of starvation preceding consumption of a solid diet. As a result, there is a change in specific microbial substrate throughout the tract, with the amount and type of substrate available at the different sites being influenced both by the characteristics and mass of food consumed, and by the digestive maturity of the tract. Weaning results in major changes in intestinal structure, and a transient diminished functional capacity of the small intestine, which can take several weeks to be restored (Hampson, 1986; van Beers-Schreurs, 1996; Pluske et al., 1997). These intestinal changes are also associated with changes to the mass, composition and complexity of the intestinal microbiota.

3.2. Bacterial populations in the small and large intestine at weaning

Jensen (1998) quantified changes in bacterial populations in the small and large intestine of pigs following weaning at 28 days of age. Before weaning, lactobacilli dominate the small intestinal microbiota, but in the first week after weaning, their numbers decrease whilst the total number of bacteria and the proportion of coliforms, particularly *E. coli,* increase. Upon weaning, most of the cultivable bacteria from the lumen of the large intestine are initially Gram-negative, but subsequently the microbiota restabilizes. The microbiota that evolves in the large intestine is comprised of a large and diverse selection of mostly obligate anaerobic bacteria, including *Bacteroides, Eubacterium, Bifidobacterium, Propionibacterium, Fusobacterium* and *Clostridium* species (Radecki and Yokoyama, 1991). Once established, the large intestinal microbiota is both complex and stable. It metabolically and physically provides a "colonization resistance" that inhibits colonization by other more transient, and sometimes pathogenic, bacteria (Nurmi and Rantala, 1973).

3.3. General weaning factors and PWD

Whilst all pigs show changes in the microbiota at weaning, often including a limited proliferation of hemolytic ETEC, not all go on to develop disease. A variety of dietary and other predisposing factors influence the outcome, with the risk of disease increasing when more risk factors are present (Madec et al., 1998). Risk factors include social stresses from mixing, fighting and crowding, which trigger blood cortisol release, depressing the immune response to bacterial infection. Chilling pigs also increases their susceptibility to PWD, possibly through effects on intestinal motility (Wathes et al., 1989). Besides inducing stress, moving pigs to a new pen environment causes increased exposure to microbes potentially present in fresh or dried fecal matter, including potential pathogens such as rotaviruses (Lecce, 1983; Tzipori et al., 1983).

4. DIETARY INFLUENCES ON POSTWEANING DIARRHEA

4.1. General issues

The weaning diet has a pronounced influence on postweaning intestinal structure and function, and consequently on the development of PWD (Hampson, 1987). For example, in the small intestine, diets which slow intestinal transit time and result in relative intestinal stasis immediately after weaning would allow pathogenic bacteria the opportunity to attach to the

intestinal epithelium and then to multiply. Similarly, if diets are poorly digested, and this results in undigested food particles in the lumen of the small intestine, this material may act as substrate for bacterial growth. The newly weaned pig is unable to produce sufficient pancreatic digestive enzymes to break down all the components of a typical commercial grain-based weaner diet (Cera et al., 1988). In particular, carbohydrates in the diet can present a problem to the piglet, as production of amylase, required for degradation of starch, is insufficient until around 3 weeks after weaning (Cera et al., 1988). The specific nature of the undigested food in the lumen will influence the types of bacteria that can colonize and proliferate. Careful regulation of the type and availability of microbial substrate in the intestinal lumen provides one way of using diet to modulate infectious bowel disease.

4.2. Diet form and intake

The onset of intestinal disease is influenced not only by the type of food, but also by the amount consumed. Pigs often go without food for a day or two when faced with their new unfamiliar weaning diet. This is followed by a bout of overeating, which overwhelms the functional capacity and the digestive process of the immature small intestine (Tzipori et al., 1984). This behavior has been linked to an increased incidence of PWD in individual pigs (e.g. Hampson and Smith, 1986; Rantzer et al., 1996). The detrimental effect of increased intake is thought to be related to the presence of large amounts of undigested food in the tract (Hampson, 1994), which can be alleviated by small frequent feedings. For example, in one study of PWD, feeding newly weaned pigs small meals hourly rather than three times a day reduced *E. coli* colonization and diarrhea (Lecce, 1983).

Following weaning, pigs normally show a reduced small intestinal villus height and diminished functional capacity within the small intestine (Hampson, 1987; Pluske, 1993), and these changes may inadvertently contribute to the development of PWD. Colonization of the small intestine by ETEC can result in additional small intestinal villus atrophy, and the modified villi cannot absorb water and electrolytes adequately. If a given diet influences the height of the small intestinal villi, this will modify the risk of intestinal disease (Cox et al., 1988; Nabuurs, 1998). Feeding a liquid diet can alleviate the reduction in villus height (Deprez et al., 1987; Pluske et al., 1996b) and growth rate (Lecce et al., 1979) that occur postweaning, encourage rapid adaptation to the weaning diet (Blecha et al., 1983), decrease intestinal coliform numbers (Decuypere and Van der Heyde, 1972), and prevent PWD (Armstrong and Cline, 1977; Tzipori et al., 1984). The sudden appearance of PWD after transferring from a liquid to a dry diet has been reported by several authors (Shimizu and Terashima, 1982; Miller et al., 1984a; Tzipori et al., 1984). Unfortunately, the use of a liquid diet to feed weaners is not often practical for many producers.

The feeding of fermented liquid feeds (FLF) has been shown to alter the intestinal microbiota and improve growth in young pigs. These feeds include lactic acid bacteria and yeasts in the formulation, which produce a higher lactic acid content in the feed and a more acidic pH as a result of fermentation (Jensen, 1998). Lactic acid has direct antibacterial effects on *E. coli* and *Salmonella* species (Nout et al., 1989), and lactobacilli can inhibit adhesion of *E. coli* to the intestines (Blomberg et al., 1993).

4.3. The role of the large intestine

The newly weaned pig has a relatively immature large intestine, and it is believed that this may influence the pathogenesis of nutritional and infectious diarrhea (Bolduan et al., 1988;

van Beers-Schreurs et al., 1992; Hambrecht, 1998; van Beers-Schreurs et al., 1998a). Large populations of microbes develop in the lumen and in association with the wall of the large intestine, where they break down and ferment undigested food and sloughed cells. The main fermentation products from this process are volatile fatty acids (VFA) and lactic acid. The epithelial cells lining the large intestine reabsorb these VFA together with a large amount of water and electrolytes. Even though the resorptive capabilities of the large intestine of a 3-week-old pig are considerable (Hamilton and Roe, 1977), they are somewhat reduced in the first few days to weeks after weaning. At this time, unlike in older pigs (Argenzio and Whipp, 1979; Crump et al., 1980), the absorption of VFA does not augment the absorption of water (van Beers-Schreurs et al., 1998b). Any reduction in large intestinal absorption immediately after weaning may exacerbate the effects of enterotoxins in the small intestine (Nabuurs, 1998). Consequently, it has been recommended that the formulation of weaner diets should be designed to result in higher VFA concentrations, especially butyrate, in the large intestine. This may increase the ability of the epithelial cells to absorb fluid and nutrients (van Beers-Schreurs et al., 1998b). The most logical way to increase the functional capacity of the large intestine is to include fibrous ingredients in the diet (Bolduan et al., 1988). Dietary fiber resists endogenous digestion in the small intestine, and travels into the large intestine where it is degraded and fermented by resident microbes, producing VFA which can be absorbed and then utilized by the animal.

4.4. Diet digestibility and PWD

The influence of diet digestibility on development of PWD has been somewhat controversial. Highly digestible cereal and milk-based diets have been associated with less PWD (English, 1981). On the other hand, there is evidence that less-digestible diets high in particular components of dietary fiber, namely insoluble fiber, confer some protection from PWD (Bertschinger and Eggenberger, 1978; Bolduan et al., 1988; Aumaitre et al., 1995).

4.5. Dietary protein

4.5.1. Protein source

The source of dietary protein used in weaning diets has received some attention in relation to its influence on PWD, although no clear or consistent recommendations are available. This is perhaps not surprising, given that a particular ingredient is likely to differ from any alternative ingredient in more than just its protein content. Protein sources in creep feeds have been suggested to prime postweaning intestinal hypersensitivity reactions to the weaner diet (Miller et al., 1984b), although whether or not this is a real phenomenon remains contentious. In particular, some soybean proteins are considered harmful to weaner pigs, as they have been implicated in causing intestinal mucosal damage (Li et al., 1990, 1991) and intestinal fluid accumulation (Nabuurs et al., 1996). Consistent with this, changing from animal-derived skim milk powder to plant sources of protein (soybean and maize) increased the severity of diarrhea and appearance of ETEC in 3-week-old pigs (Shimizu and Terashima, 1982). In contrast, Pouteaux et al. (1982) found no difference in diarrhea or intestinal bacterial populations when they compared diets containing buttermilk powder, soybean meal and pea protein concentrate. It has been suggested that inclusion of many protein sources in a weaner diet may increase the severity of diarrhea compared to inclusion of fewer protein sources (Okai et al., 1976; Ball and Aherne, 1982; Etheridge et al., 1984). Certain protein sources, particularly fishmeal and dried milk powders, have a high acid-binding capacity in the stomach (Bolduan et al., 1988). This activity can lead to

the stomach contents becoming less acidic, which in turn favors survival of ingested ETEC, as well as resulting in reduced pepsinogen production, and reduced proteolysis in the stomach.

4.5.2. Protein level

There is a general understanding that diets containing excessive levels of protein (> 250 g/kg) predispose to PWD, although the precise mechanism is uncertain (Prohaszka and Baron, 1980; Cutler, 1981; Bolduan et al., 1988). Excess undigested protein within the intestinal lumen becomes degraded by microbes, and can contribute to a proteolytic diarrhea independent of the presence of *E. coli,* through the production of amine by-products which irritate the mucosa and induce diarrhea (Nollet et al., 1999). On the other hand, dramatically reducing the level of dietary protein to 50 g/kg in conjunction with a high inclusion of dietary fiber (170 g/kg) into a diet with a low nutrient density was successful in reducing the severity and incidence of PWD in pigs experimentally infected with ETEC (Bertschinger and Eggenberger, 1978). Interestingly, a number of other studies investigating the level of dietary protein for weaners have found that these did not result in any significant difference in the incidence of diarrhea, or intestinal or fecal *E. coli* numbers (Smith and Jones, 1963; Palmer and Hulland, 1965; Smith and Halls, 1968; Armstrong and Cline, 1977; Pouteaux et al., 1982).

Plasma protein is used in weaner feeds in some countries, and is reported to enhance the performance and robustness of weaner pigs, primarily by stimulating feed intake (Ermer et al., 1994). In a recent study, plasma powder was added to the diet of weaner pigs at 45 g/kg and 90 g/kg, and was effective in reducing the proliferation of hemolytic *E. coli* following experimental infection (Nollett et al., 1999). The antibacterial effect was attributed to specific components of the plasma powder. However, at the higher inclusion level (90 g/kg), the pigs displayed noninfectious diarrhea, and this was attributed to the high levels of protein from the plasma powder entering the lower bowel. In the case of other protein sources that do not have such direct antibacterial actions, undigested protein may exacerbate diarrhea associated with proliferation of hemolytic *E. coli*.

4.6. Dietary fiber

Dietary fiber (DF) influences intestinal physiology and the gut microbiota in pigs (see review by Mosenthin et al., 1999). The term DF encompasses a heterogeneous class of nonstarch polysaccharides (NSP) that are resistant to hydrolysis by endogenous digestive enzymes of monogastric animals, and consequently are the main substrate for bacterial fermentation in the distal part of the gut (Trowell et al., 1976). In this chapter, the term NSP will be used rather than DF. There is an ongoing debate as to whether NSP prevents or causes the onset of PWD. The effect of NSP on progression of intestinal disease in young pigs appears to be influenced by the type and source of fiber involved, in particular whether the NSP involved is "soluble" or "insoluble". It is relevant to refer to NSP as those that are water soluble (sNSP) or insoluble (iNSP) because solubility during measurement of NSP levels *in vitro* relates to fermentability within the intestinal tract, with sNSP being more easily, rapidly and completely fermented than iNSP (Stephen, 1994).

4.6.1. Dietary fiber and PWD

The effect of different dietary fibers on the expression of PWD is variable. Protection against intestinal proliferation of ETEC has been observed in pigs fed oats, wheat and barley

(Richards and Fraser, 1961; Palmer and Hulland, 1965; Smith and Halls, 1968; Armstrong and Cline, 1976; Bertschinger and Eggenberger, 1978; Thomlinson and Lawrence, 1981), but this does not always hold true (Rivera et al., 1978; English, 1981). On closer examination of the various studies, the soluble NSP are more frequently associated with PWD than are the insoluble types. For instance, Smith and Hall (1968) found that the fibrous insoluble outer hull of barley prevented disease in weaner pigs inoculated with *E. coli,* but barley meal, which contains a higher proportion of sNSP in the form of β-glucan, increased the susceptibility of weaner pigs to development of PWD. Similarly, a diet high in crude fiber used by Bertschinger and Eggenberger (1978) was associated with reduced *E. coli* proliferation and diarrhea, whilst in another study, incorporation of the soluble fiber guar gum in the diet increased expression of PWD (McDonald et al., 1999).

As mentioned earlier, the end products of fermentation of NSP in the large intestine are predominantly VFA, which have been shown to facilitate water and electrolyte absorption from the pig colon (Argenzio and Whipp, 1979; Crump et al., 1980). This effect would help prevent dehydration and clinical diarrhea in the case of hypersecretory small intestinal disease, providing excessive fermentation did not overload the colon and induce osmotic diarrhea (Etheridge et al., 1984). Certainly a stable microbiota is more capable of controlling proliferation of ETEC than is an unstable microbiota (Hillman et al., 1994), and NSP most likely aids in the rapid establishment of a stable large intestinal microbiota (Bolduan et al., 1988; Longland et al., 1994).

4.6.2. Experiments on PWD by the authors

Over the last few years, a series of experiments conducted by the authors and colleagues have systematically explored the interaction of dietary NSP with the development and expression of PWD (McDonald et al., 2000, 2001; Hopwood, 2001; Hopwood et al., 2002, 2004). A discussion of this work follows, as it serves to illustrate the concepts and complexities involved in the manipulation of infectious diseases through dietary intervention.

To determine the contribution of dietary manipulation to the outcome of an infectious bowel disease, there must be a clear and definable distinction between the experimental diets, and a quantifiable measurement of disease expression. If there are too many interacting variables, then it may not be possible to associate an effect with a cause. Therefore, to determine how different dietary NSP might affect PWD, a diet that was particularly low in fiber was used as a starting point. Different types and amounts of NSP were added to this diet without significantly altering the basic ingredients, and with minimal variation in protein and energy levels.

The basic "control" diet used in this series of experiments contained cooked white rice (Sunwhite Calrose®, Australia) as the main ingredient. This diet had negligible amounts of dietary NSP, around 1% of the diet in total. In each experiment, the majority of the rice component of the diet was replaced by the test NSP source at an inclusion level representative of those used in piggeries. The NSP sources tested were hammer-milled wheat, extruded wheat, pearl barley, pearl barley plus exogenous enzymes, guar gum and the synthetic nonfermentable viscous compound carboxymethylcellulose (CMC) (table 1). The latter two components were incorporated as a small percentage of the diet to achieve the desired sNSP content or viscosity. This range of diets allowed comparison of NSP sources that were: (a) high in insoluble NSP content (hammer-milled and extruded wheat) and expected to undergo relatively slow and incomplete large intestinal fermentation; (b) comprised of soluble, easily fermented, moderately viscous NSP mixed with some iNSP (pearl barley diet); (c) both viscous and highly

Table 1

Percentage NSP content of the experimental weaner diets

%NSP in diet	Cooked rice	Wheat	Extruded wheat	Pearl barley	Pearl barley + enzyme	Guar gum	CMC
Soluble	0.25	0.89	0.9	2.53	2.53	9.0[a]	3.84
Insoluble	0.47	5.32	5.09	2.05	2.05	0.47[b]	0.47

[a]Estimated from guar gum inclusion of 10% in the diet and soluble NSP content of guar gum of at least 90%.
[b]Figure taken from value for cooked rice diet. There was no iNSP in the guar gum.
Analysis of NSP content was determined by the Department of Animal Science, UNE, NSW.

fermented in the large intestine (guar gum diet); (d) highly viscous but resistant to fermentation in the large intestine (CMC diet); or (e) rich in NSP but encouraged to degrade rapidly in the gut with the aid of dietary additives (pearl barley plus exogenous enzymes diet).

To determine the effect of these dietary interventions on PWD, pigs were either (a) orally infected with ETEC using 5–50 ml of culture broth containing $10^{8.5}$ ETEC/ml (serotypes O8;K88;K87 or O149;K91;K88, both with enterotoxins LT, STa, STb) in the period 48–72 hours after weaning, or (b) monitored after weaning for the development of naturally occurring PWD. The serotype isolated from natural infections was O149;K91;K88 (enterotoxins LT, STa, STb), and this strain was used for some of the experimental infection trials. All pigs were weaned at 21 days of age from the same specific pathogen-free piggery, and were split among experimental groups so that the average body weight per group was similar.

Outcomes of dietary manipulation on PWD were assessed using clinical and microbiological observations. Clinical measurements included body weight growth, and presence and liquidity of diarrhea. Cultures of fecal swabs collected daily were assigned a visual score (percentage of total bacteria cultured that were ETEC), whilst mucosal scrapings from the small and large intestine at 7–9 days after weaning were diluted and cultured to determine the count of viable numbers of ETEC at different sites along the intestinal tract.

Fecal shedding of ETEC, weight loss and watery diarrhea were evident at 3–4 days post-weaning in pigs naturally or experimentally infected with ETEC. Across five experiments, the pigs eating the control cooked white rice diet consistently had the lowest numbers of ETEC cultured from their intestines (average viable count over the experiments is presented in fig. 1), including a proportion of animals that did not have any ETEC cultured from their samples, even after inoculation with ETEC. This established that a low-fiber diet based on cooked rice was unlikely to encourage intestinal proliferation of ETEC. Addition of sNSP to the diet was associated with an increased colonization and proliferation of ETEC in the small and large intestine. The number of viable ETEC was highest in those diets containing most soluble, viscous sources of NSP (fig. 1). The highest count was in pigs fed guar gum, which is soluble, viscous and fermentable. The second highest count was in pigs fed CMC, which is soluble, viscous and nonfermentable. Interestingly, apart from the control rice-based diet, which was both low in iNSP and protective, diets with most iNSP tended to have fewest ETEC. This is consistent with many earlier observations where iNSP has been considered to confer protection against PWD (e.g. Smith and Hall, 1968). The current results could be interpreted to suggest that iNSP has a protective effect against PWD by mechanisms independent of the protection seen with the control cooked rice-based diet.

The viscosity of the intestinal contents was measured in pigs from several of the experimental groups. Of these, the diets associated with higher counts of viable intestinal ETEC

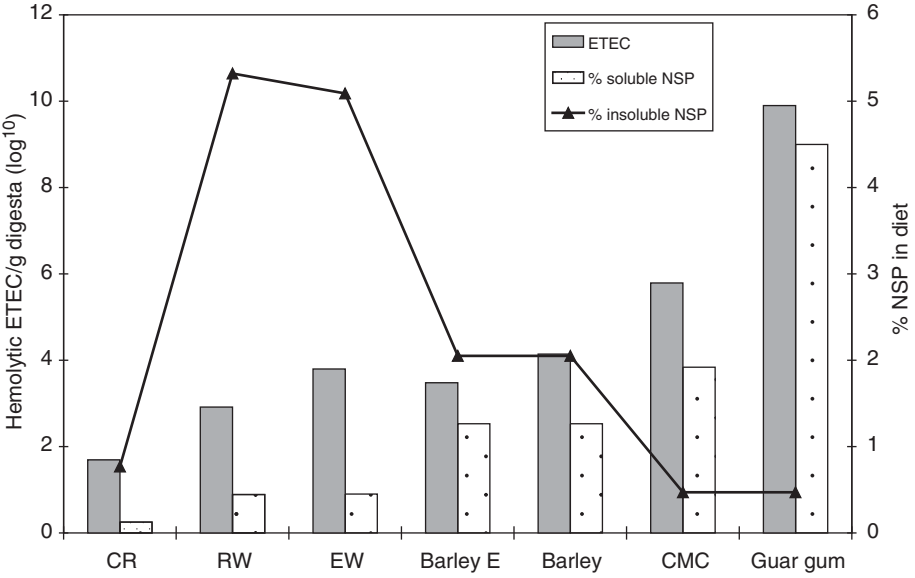

Fig. 1. Dietary NSP inclusion levels and mean viable small intestinal counts of ETEC cultured from weaner pigs with PWD fed diets containing different carbohydrates (CR, cooked rice; RW, raw wheat; EW, extruded wheat; Barley E, pearl barley plus exogenous enzymes; Barley, pearl barley; CMC, carboxymethylcellulose; Guar gum, guar gum). (Adapted from McDonald, 1996, Hopwood, 2001, and Hopwood and Hampson, 2003.)

were matched by an increased digesta viscosity in the small and large intestine, suggesting an influence of viscosity *per se* on proliferation of ETEC (fig. 2). Certainly, increasing the viscosity but not the fermentability of the diet was consistently capable of inducing a natural bout of PWD, with pigs fed CMC in their diet developing a natural infection with hemolytic ETEC in three separate sequential experiments (Hopwood, 2001; McDonald et al., 2001; Hopwood et al., 2002). The concept of increased viscosity altering the intestinal microenvironment and predisposing the animal to intestinal disease has been noted in other species. In particular, increased viscosity of the intestinal contents through consumption of sNSP has been strongly associated with enteric disease in poultry (Choct and Annison, 1992; Langhout, 1998). Furthermore, reductions in intestinal viscosity achieved through the use of exogenous enzymes has been shown to be associated with reduced colonization with *Campylobacter jejuni* in experimentally infected broilers (Fernandez et al., 2000).

The distinguishing features of the above-described work were: (a) that the control rice diet appeared to be preventative, almost protective, against the development of PWD; (b) that diets high in sNSP content were associated with greater intestinal proliferation of ETEC; and (c) that diets rich in viscous and sNSP were capable of inducing expression of PWD even when the fermentability of the diet was low. The protective effects of the rice-based diet and the way that addition of CMC predisposes to PWD have been confirmed in subsequent experiments (Montagne et al., 2004).

4.7. The protective nature of cooked rice diets

Although it is clear that a cooked white rice diet was able to reduce the intestinal proliferation of ETEC, the mechanism(s) by which it achieved this remains unknown. The success of the diet

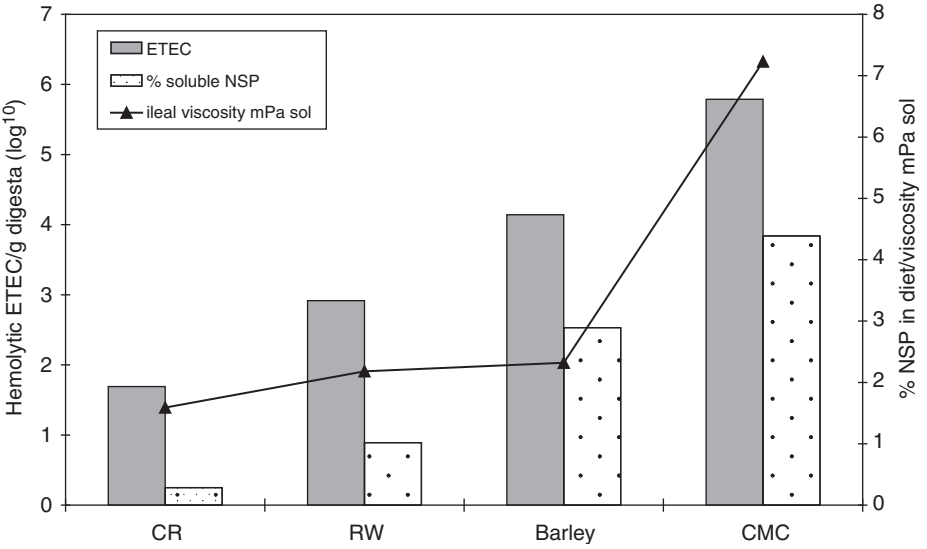

Fig. 2. Mean viscosity of the ileal contents, dietary sNSP inclusion levels and mean viable small intestinal counts of ETEC cultured from weaner pigs with PWD fed diets containing different carbohydrates (CR, cooked rice; RW, raw wheat; Barley, pearl barley; CMC, carboxymethylcellulose). (Adapted from Hopwood, 2001, and Hopwood and Hampson, 2003.)

may have related to the lack of NSP, or to the inherent properties of the rice. The cooked white rice diet was low in NSP content and viscosity, and was highly digestible. Any or all of these features could have contributed to its protective effects.

The highly digestible nature of the cooked white rice meant that it disappeared quickly in the small intestine, leaving little substrate for utilization by bacteria, pathogenic or otherwise. In the same way, white rice provides an excellent source of rapidly assimilated energy, which can aid survival in the face of diarrheic infections. Rice-based oral rehydration solutions are available for use in humans, and have proven effective with infections such as cholera and colibacillosis (Molla et al., 1982). In the authors' experiments, it was rare to observe any undigested grains of rice remaining in the large intestinal contents. This lack of substrate resulted in less hindgut fermentation, and provided little opportunity for osmotic diarrhea to occur (Etheridge et al., 1984). Such a diet may reduce the consequences of overeating by newly weaned pigs.

Special inherent properties of the cooked rice may have contributed to the protective effect of the rice diet. A substance isolated from boiled rice has been observed to inhibit the ability of bacterial enterotoxins to induce hypersecretion of fluids and electrolytes into the gut lumen of guinea pigs (Mathews et al., 1999). This possibility has not been explored in experiments with weaner pigs.

4.8. Large intestinal fermentation and PWD

Dietary NSP increases the amount of fermentation occurring within the large intestine. It is not currently clear to what extent increased fermentation affects the development of PWD. The work presented here suggests that an increased amount of fermentable substrate in the

diet (i.e. NSP) coincides with a greater intestinal proliferation of ETEC provided the substrate is easily fermentable. It appears that the addition of NSP was unable to prevent the loss of fluid resulting from ETEC-induced diarrhea. It is possible that the NSP was unable to adequately accelerate the development of the large intestine in the short time after weaning before infection occurred, or it may be that increased large intestinal function cannot compensate fully for the loss of fluid in pigs with ETEC-associated diarrhea. In all likelihood, the inability of the immature large intestine to fully degrade the NSP contributed to the onset of disease, by providing more substrate for bacterial growth, creating an osmotic force in the lumen, and drawing water into the NSP molecules by virtue of their water-holding properties. In addition, the bacteria that proliferated did not appear to provide any ''colonization resistance'' capable of offsetting the multiplication of the opportunistic pathogenic ETEC. These findings highlight the importance of designing the dietary intervention to match the specific disease and the species in question. Dietary fiber is often recommended as a treatment tool for some human diseases, yet its use in this context was not appropriate for preventing PWD in the weaner pigs described here.

Solubility and fermentability of NSP tend to reflect each other. Although iNSP are also fermentable, they ferment more slowly and incompletely, particularly in immature weaner pigs. In the work summarized in figs. 1 and 2, there was no correlation between the amount of iNSP in the diet and the occurrence of diarrhea. This again suggests that it is not fermentability *per se* that affects PWD. The structure of iNSP allows molecules and potentially bacteria to be trapped within and carried through the intestinal tract without digestion. This also may have resulted in the bacteria passing through the small intestine without contacting the epithelium. The exogenous enzymes added to the pearl barley diet helped degrade the NSP and produce VFA in the hindgut, but there was still a significant amount of digesta present in the large intestine, which may simply have provided an additional source of substrate for pathogenic bacteria in an undeveloped large intestine.

Microbial fermentation in the large intestine of pigs has been observed to increase not only with increased NSP, but also in the presence of spirochetal infection of the large intestine. In pigs experimentally infected with *Brachyspira pilosicoli,* the VFA end products of fermentation were increased in pigs colonized with *B. pilosicoli* compared to healthy control pigs (Hopwood et al., 2002). In support of this, feeding a wheat-based diet increased colonization by *B. pilosicoli* compared to feeding a highly digestible cooked white rice-based diet (Hampson et al., 2000). The relative protective effect of a rice-based diet on colonization of pigs with *B. pilosicoli* has recently been confirmed (Lindecrona et al., 2004). In some other studies, increased fermentation has also been associated with increased expression of experimental infection with *Brachyspira hyodysenteriae* (the agent of swine dysentery) (Pluske et al., 1996a, 1998; Siba et al., 1996), although this effect has not been seen in all such studies (Kirkwood et al., 2000; Lindecrona et al., 2003). These different results are difficult to reconcile, although the lack of protection from swine dysentery seen with rice-based diets in the latter experiments compared to the former may relate to the physical processing of the rice, or even to the types of rice used in the various experiments. Recent studies by Pluske et al. (unpublished data) have found marked differences between rice types (i.e. medium-grain (lower amylose) rice, long-grain (higher amylose) rice, and waxy (no amylose) rice) in the digestibility of starch when measured at the terminal ileum of pigs weaned for 14 days. Differences in starch chemical composition in rice varieties and subsequent differences in gelatinization properties may therefore affect the type and amount of bacterial substrate reaching the large intestine, and hence the nature and extent of the fermentation occurring there. This then could help explain the difference observed in disease expression.

4.9. Intestinal viscosity and PWD

There are several ways in which increased viscosity of the intestinal contents may alter the intestinal microenvironment of newly weaned pigs, and predispose them to opportunistic infection by ETEC. Greater viscosity results in a slowing of digesta as it travels along the intestines, an alteration in the types of intestinal contractions (Cherbut et al., 1990), and an increase in the thickness of the mucus layer lining the epithelium (Blackburn and Johnson, 1981; Blackburn et al., 1984). Slower digesta transit provides more time for bacterial attachment to occur, whilst altered peristaltic movements affect the flow of digesta along the gut, potentially keeping bacteria in contact with the epithelium for longer.

Mucus can provide a niche in which opportunistic bacteria can attach and multiply whilst protected from the flow of digesta, or it can physically inhibit attachment of bacteria to the epithelial cell surface. The interaction of mucus with bacterial pathogens therefore depends upon where the pathogen proliferates. In the case of *E. coli,* the bacterium has the ability to attach to mucins in mucus or to the epithelial cells, which may help explain why the pigs fed the viscous CMC compound had increased proliferation of ETEC. In addition, thick mucus delays the passage of nutrients from the lumen to the epithelium for absorption, creating a slow-moving supply of degraded nutrients suitable for supporting bacterial growth.

Increased thickness and/or altered composition of the mucus layer in the large intestine may also predispose the pig to spirochetal infection through chemotactic attraction of these bacteria to mucus (Milner and Sellwood, 1994; Witters and Duhamel, 1999). As previously stated, viscous dietary sNSP have been observed to increase the onset and amount of fecal shedding of intestinal spirochetes in an experimental situation using older weaner pigs (Siba et al., 1996; Pluske et al., 1998; Hampson et al., 2000; Hopwood et al., 2002). In one of these studies, feeding the nonfermentable viscous compound CMC to weaner pigs dually infected with *B. pilosicoli* and hemolytic *E. coli* resulted in the affected pigs shedding *B. pilosicoli* for a significantly greater number of days than dually infected pigs that were eating a cooked white rice-based diet (Hopwood et al., 2002). In this case it was not possible to determine whether the dual infection with ETEC, the increased digesta viscosity due to CMC in the diet, or both, contributed to the shedding of *B. pilosicoli*.

It is possible that increased intestinal viscosity may affect the pathogenesis of different intestinal diseases in similar basic ways. It seems likely that CMC would also exacerbate the expression of swine dysentery, and possibly a range of other enteric diseases in pigs and other species. This is consistent with what is known in poultry, where increased intestinal viscosity associated with sNSP has been identified as a precipitating factor for a number of intestinal conditions and diseases.

5. FUTURE PERSPECTIVES

Further developments concerning the ‘‘nutritional’’ control of intestinal diseases in animals are now needed in the light of increasing legislation and calls from the food service chain to deliver ‘‘clean’’ food to consumers. Disease is seen to be a welfare issue, whilst the use of antibiotics is to be avoided. In the European Union a complete ban on the use of growth-promoting antibiotics takes effect on 1st January 2006. Recently, the multinational fast-food outlet McDonalds has established a global policy on growth-promoting antibiotic use, which became effective at the end of 2004. McDonald’s ‘‘Global Policy on Antibiotics Use in Food Animals’’ creates a set of antibiotic use standards for its meat suppliers, and takes the broad position that growth-promoting antibiotics are ‘‘unsafe’’. The global nature of this policy

means that other regions besides Europe will increasingly have to acknowledge the need to find alternative nonantimicrobial strategies to ameliorate the production and welfare costs of intestinal diseases, particularly in the pig and poultry industries. Experience from Scandinavia suggests that ''alternative'' methods of control must encompass a holistic approach to each disease. Dietary interventions, such as using appropriate types and amounts of NSP in weaner diets, will form one aspect of control. From some of the studies summarized in this chapter, it can be seen that diets based on cooked rice appear to be particularly useful for newly weaned pigs, perhaps because of their low sNSP content. Unfortunately little is known of the precise mechanisms by which such diets can have a protective effect on a given enteric disease or condition. For progress to be achieved, the complex interactions that occur between the diet, the intestinal tract, the normal microbiota and potential pathogenic bacteria need to be unraveled in a systematic and coordinated way. Individual diets need to be targeted for individual animal species and specific diseases, according to need. Currently it is difficult to access funding for such projects from either governmental or commercial sources, and ultimately it may rest with the individual animal industries to provide direct financial support for this work to proceed.

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13 Feeding and disease resistance in fish

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Aquaculture is a significant and rapidly expanding seafood-producing industry worldwide, which produced approximately 40 million tonnes of seafood (shrimps, fish and shellfish) in 2001. This includes numerous carp species farmed in freshwater, anadromous salmonid species reared both in freshwater and seawater, and a minor production of pure marine species. Live production always means a risk for loss due to infectious diseases, imbalanced nutrition and environmental challenges that compromise fish welfare. These problems increase with the growth and intensity of the farming industry. On the other hand, the idea to improve performance, enhance resistance to infectious diseases, and prevent production-related diseases through dietary manipulation is fascinating, and there is an increasing body of research on the use of single nutrients and additives as health promoters and immunomodulators. The present chapter deals with the intersection between nutrition and fish diseases with emphasis on salmonids, and covers examples of nutritional deficiencies, nutritional toxicities, immunomodulation and noninfectious diseases of possible nutritional origin observed in today's aquaculture.

1. INTRODUCTION

Global aquaculture production is of increasing importance in the overall seafood production (130 million tonnes), and for some countries it is vital for the national food supply and for export revenue (Vannuccini, 2003). The dependence of production routines, including feeding regimes and feed composition for optimal growth and welfare are obvious, and are covered in the ideas of responsible aquaculture. The development of aquaculture has been made in the face of continuous challenges, which include diseases, environmental impacts, and nutrition, in addition to the basic requirements such as access to technology and financial resources for the poor. Furthermore, modern intensive aquaculture is under heavy pressure regarding improved costs, special market preferences of the products, and use of feed ingredients as replacements for limited marine resources, and consequently nutritional solutions that challenge fish physiology and resistance to diseases may be chosen during the production cycle. Moreover, under intensive farming conditions there may be a general loss of resistance towards infectious diseases relative to genetic constitution, increased fish densities and stressful conditions.

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In most countries, all parts of aquaculture production (feed, fish breed, environment and product quality) have to undergo national and international regulations, in line with other husbandry. Thus, along with the development in the aquaculture industry, there is a continuous need for research and knowledge whereby fish feed legislations can be updated. Feed legislations promote sustainability by focusing on documentation concerning fish health, environmental aspects and safety aspects for human consumption. These aspects are often integrated and have a common public interest, since, for example, unwanted substances in the aquaculture feeds may impact on fish health, cause environmental burdens, or be retained in the edible parts for human consumption. Not surprisingly, modern fish nutritional research also focuses on a sustainable production chain approach, by considering feed resources, feed production, feeding regimes, seafood quality and safety, as well as product treatment, marketing and eating preferences. In this chain of thinking, fish health has its natural place, since a stable predictable aquaculture production accepted by the market relies on healthy fish throughout the production cycle.

The feed is one of the main components determining the cost in fish farming (>50% in Atlantic salmon production), mainly through the choice of available feed ingredients, the overall feed composition and through selected feeding practices. Major changes have been made in modern intensive aquaculture, towards more energy-dense diets, improved feeding regimes and feeding efficiencies, as well as an overall shortened production cycle through manipulation of the biological processes by use of temperature and light regimes. Since the gained efficacy also introduces challenges towards fish health, it is of importance that economy is not the major driving force in fish nutrition research. The present communication discusses fields where feeding and single nutrients have been shown to impact on fish health and disease resistance, by challenging or improving immune competence and physiological systems. The focus will be on salmonid aquaculture, since this represents a developed international industry of an anadromous carnivorous species with a significant global production volume of 1.2 million tonnes in 2001 (FAO, 2003).

2. NUTRITIONAL DISEASES

2.1. Brief summary of fish immunology and defense systems

In general, it is acknowledged that fish have many of the same defense mechanisms as land-living vertebrates, such as effective outer physical and chemical barriers, defined lymphoid tissues (pronephros/anterior kidney/head kidney, thymus, spleen, liver, gut-associated lymphoid tissue), defined immune cell populations and humoral defense factors (table 1; Iwama and Nakanishi, 1996; Warr, 1997). It has been suggested, however, that fish have a relatively "simple" and less-efficient immune system, and that fish rely more on innate immunity or the nonspecific primary line of defense, than land-living vertebrates (Anderson, 1992; Warr, 1997). The development of lymphoid tissues and immune responses during ontogeny follow a sequential development, with the nonspecific immunity appearing first, and preceding the specific immunity (Tatner, 1996). Immediate and generalized nonspecific immune responses are thought to compensate for the continuous exposure to waterborne pathogens and delayed specific responses (Gannam and Schrock, 2001). The diversity among the above 20 000 teleost species is large, but considering the relatively few fish species farmed today (about 20) there seem to be less differences. However, rearing temperature plays an important role in the functional immune system, and this makes some important differences between warm-water and cold-water fish species, particularly in the specific response of the acquired immune system.

Table 1

Main protective factors in fish, which also have been used as selected markers of immunological protection and immunity in feeding experiments

	Innate immune factors	Acquired immune factors
Outer surface physical and chemical protection	Skin, mucosal membranes, mucus (containing lysozymes, complement, natural immunoglobulins (Ig)), acid, intestinal flora	Mucosal immune system with specific Ig
Humoral factors	Complement, lysozyme, lectins, transferrin, cytokines, eicosanoids	Specific Ig
Immune cells	Phagocytes, monocytes	T- and B- lymphocytes (memory cells, suppressor T-cells), natural killer cells

The significance of the acquired or specific immune system in fish is important in aquaculture, as observed from the highly effective vaccines against bacterial diseases and improving vaccines against viral diseases developed by a growing fish vaccine industry (Evelyn, 1997). The main differences in the immune systems of fish, as compared to land-living vertebrates lies in the development of antibodies after antigen stimulation, like in vaccination. Teleost fish seem to be able to generate considerable structural diversity within their tetrameric antibody molecules; in salmonids through random polymerization of the monomeric subunits, and this seems to occur later in the secretory process than in mammals (Kaattari et al., 1999). Thus, the antibodies may be of different function or potency (antigen-binding site and Fc region functionality) depending on their heterogeneously assembled structure. The practical importance of these differences is often mirrored in the difficulties in choosing reliable markers of acquired immune competence consistent with overall disease protection after vaccination, such as estimation of antibody titer (by agglutination precipitation and ELISA techniques) where structural forms are not distinguished.

Research on natural resistance and acquired protection after vaccination in fish is increasing, and it is essential to identify the mechanisms by which nutritional and environmental factors can modify the outcome (Shoemaker et al., 2001; table 2), as well as to determine the practical relevance of the findings. It is important to establish to what extent immunity may be modulated by nutrition or single nutrients, as compared to, for example, unspecific immune stimulation by β-glucans or specific stimulation after vaccination (Raa, 1996; Sakai, 1999).

Table 2

Farming or experimental conditions that impact immunity and resistance to infectious diseases (adopted from Schreck, 1996; Shoemaker et al., 2001)

	Farming or experimental condition
Environment	Temperature, photoperiod (year cycle), water quality
Stress	Suboptimal water quality, fish density, handling, transport
Pathogen	Bacteria, virus, parasites (exposure level, type, virulence)
Fish and genetics	Species, age, sexual maturation, genetic breed, inherit disease resistance
Nutrition	Quantitative and qualitative aspects, nutrient availability, antinutrients, toxicants, immunostimulants

The former would probably represent a more time-restricted modulation, varying within a certain range of response after stimulation within the frame of a natural immune regulation. Immunotoxic modulation may, however, exhaust the immunocapacity, as has been observed for the stress response after chronic toxic exposures (Wendelaar Bonga, 1996). Large individual variations in immunity and variable experimental designs and conditions (table 2) may add to the uncertainty of recorded modulation, obscuring the outcome.

During the last decade several reviews have compiled literature on how parts of the immune system and the resistance to infectious diseases of farmed fish can be modulated through nutritional means (Lall and Olivier, 1993; Waagbø, 1994; Fletcher, 1997; Sakai, 1999; Lim and Webster, 2001; Gatlin, 2002). In the present communication it is not the intention to duplicate these reviews, but to give updated examples of nutrition-related disorders and challenges in modern aquaculture. The development of *in vivo* and *in vitro* fish models, and the use of molecular techniques have made it possible to explore detailed mechanisms by which single components and cells of the immune system can be modulated in activity. One obvious direction to look has been towards the multitude of messengers within the immune system, like cytokines and eicosanoids, which allow communication between immune cells and regulate their development and functions, and to study how their synthesis and action can be modulated by nutritional means (Meydani, 1990; Secombes et al., 1999). To date, few studies on nutritional immunology and inflammation in fish include expression studies of proinflammatory cytokines, such as macrophage activating factor (MAF), tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) identified and characterized for rainbow trout (Davidson et al., 1991; Pleguezuelos et al., 2000; Laing et al., 2001). As the knowledge about fish cytokine genes increases (Secombes et al., 1999) and when recombinant cytokines are available, it will be possible to explore in detail cytokine-mediated modulation of immune responses by nutrients and toxicants.

2.2. Immunomodulation by feeding regimes and by single nutrients

Most external factors in aquaculture, including feeding regimes and feed composition, may impact fish health, the latter with respect to quality of macronutrients and contents of micronutrients, unwanted compounds (oxidation products, antinutrients and pollution), as well as immunoactive compounds (immunostimulants and probiotica). The following sections cover recent examples of these research areas.

2.2.1. Reduced immunity and disease resistance in starvation

The simplest example of nutritional impact on immunity is malnutrition, which, depending on fish size, sooner or later leads to immunodeficiency (Gatlin, 2002). Lack of nutrient supply, as in states of restricted feeding, unintended starvation (fed below maintenance level) or feeding imbalanced diets, reduces the physiological performance, and suppresses immunity and resistance to infectious diseases (Snieszko, 1972; Gatlin, 2002; Roberts, 2002). Due to lower energy and nutrient storage capacity, juvenile fish are naturally more sensitive to periods of starvation. On the other hand, a common short-term advice to the fish farmer at outbreak of infectious diseases or during blooming of algae and jellyfish is to halt feeding, probably related to reduced metabolic activity (oxygen consumption and nitrogen excretion), and access of nutrients (for example iron) for pathogens to replicate in the tissues. Thus, several studies have shown increased resistance towards infectious diseases through periods of feed deprivation of grow-out fish (reviewed by Sealy and Gatlin, 2001). However, this seems to

depend on the period of starvation. In adult Atlantic salmon, single immunological parameters, such as activity of stimulated head kidney phagocytes and plasma complement activity, showed reduced activities after 12 weeks starvation during a cold water period at 4–7°C (author's own unpublished data). That study concluded that healthy adult salmon tolerate prolonged starvation, while weakened and diseased fish (for example carriers of infectious diseases), or fish exposed to fluctuating environmental conditions should not undergo prolonged starvation or restricted feeding.

2.2.2. Classical symptoms of nutrient deficiencies

It is assumed that a functional immune system depends on optimal nutrition in fish, as in land-living animals and humans. Early manifestations of malnutrition include nonspecific symptoms, such as growth retardation, reduced feed efficiency and anorexia, and more classical symptoms of single-nutrient deficiencies, but also serious changes in immunity including susceptibility to infectious diseases, lack of resistance to stress-mediated changes and mortalities. Gross morphological signs of nutrient deficiencies and toxicities that have been reported in farmed fish are summarized in table 3 (adopted from Tacon, 1992). Interestingly, symptoms such as bone deformities, cataracts and skin damage often occur with varying incidences in modern aquaculture industry, and represent challenges to fish pathologists and nutritionists to elucidate the causative factors for such noninfectious disorders (Brown and Núñez, 1998).

Table 3

Nutritional pathology conditions and reported causes (modified according to Tacon, 1992)

Pathological condition	Reported nutrient deficiency	Reported nutrient toxicity
Bone deformities (scoliosis, lordosis, vertebra compressions, fin and jaw deformities)	Vitamin C; tryptophan; magnesium; phosphorous; vitamin K; essential fatty acids	Lead; cadmium; leucine; vitamin A; oxidized fish oil
Fin erosion	Lysine; tryptophan; zinc; riboflavin; inositol; niacin; vitamin C	Lead; vitamin A
Fin and skin hemorrhage	Riboflavin; pantothenic acid; niacin; thiamine; inositol; vitamin C; vitamin A; vitamin K	Oxidized fish oil
Gill lamellae degenerations or fusions	Essential fatty acids; vitamin E; magnesium; pantothenic acid; biotin; vitamin C	
Cataract	Methionine; tryptophan; zinc; magnesium; copper; selenium; manganese; vitamin A; riboflavin; histidine	Choline; oxidized fish oil; manganese, iron
Exophthalmia	Pantothenic acid; niacin; folate; vitamin A; vitamin E	Oxidized fish oil
Fatty liver	Choline; vitamin E, essential fatty acids	Oxidized fish oil
Anemia	Iron; selenium; vitamin C; vitamin E; vitamin D, vitamin K; all B vitamins; essential fatty acids	Lead; oxidized fish oil

The rapid growth rates of juvenile fish, as well as for fish in intensive farming, make them more sensitive to nutritional deficiencies. Without suitable biomarkers for use in establishing the requirements for essential nutrients, it is difficult to uncover states of marginal nutrient supply in adult fish. In a practical sample key to vitamin deficiencies in salmonids, based upon the type and onset of primary clinical symptoms, Hardy (2001) suggests using anemia as an initial categorization criteria. From the symptoms given in tables 3 and 4, hematological parameters are indeed among the early-appearing, nonspecific symptoms in states of nutrient and antioxidant deficiencies, as well as in toxicities.

Nutrient deficiencies in modern aquaculture may arise as a consequence of replacement of major feed ingredients (antinutrients, nutrient antagonists or nutrient imbalance), unfavorable treatment of feed and ingredients (heat destruction, oxidation, etc.), nutrient leaching during feeding of juveniles, dietary or environmental interactions that affect absorption, metabolism or excretion of nutrients, or they may occur secondarily to infectious diseases. Good progress has been achieved with respect to vitamin stability during fish feed production (pelleting and extrusion techniques) and storage (Gabaudan and Hardy, 2000), but care must still be taken to fulfill the requirements for the unstable vitamins needed to support rapid growth and optimal health. Ascorbic acid has been the most studied; however, the use of stable ascorbate phosphate derivatives has turned the focus from the instability of ascorbic acid in fish feed to its essential and (disease) prophylactic roles in preventive health care (Sandnes, 1991; Dabrowski, 2001b).

According to the compiled literature, immunity and resistance to infections in fish are compromised by deficiency of most nutrients, of which for natural reasons the antioxidant vitamins have attained highest attention (Lall and Olivier, 1993; Waagbø, 1994; Fletcher, 1997; Lim and Webster, 2001; Gatlin, 2002). Fish feed and tissue contain high levels of long chain n-3 polyunsaturated fatty acids (n-3 PUFA), which are prone to oxidation (Sargent et al., 2002), and pathological symptoms related to oxidative damages have been most commonly observed in fish (table 3). By far the most studied single nutrients are the antioxidant vitamins C and E, most probably due to the dramatic consequences during states of deficiency in the early days of aquaculture, when deficiency was observed to cause classical symptoms such as bone deformities (scoliosis and lordosis) and lipoid liver degeneration, respectively (Roberts, 2002). Compared to other micronutrients, states of deficiency of these two vitamins are easily reached due to their low natural content in most feed ingredients used, and to vitamin loss during meal and feed production.

Table 4

Examples of minimum and practical requirements for vitamin C (mg ascorbic acid/kg) in fish

Health markers	Requirement (mg/kg)	References
Optimal growth and no mortality	10–15	Sandnes et al., 1992
Vertebrae and skin collagen synthesis	10–15	Sandnes et al., 1992
Blood hemoglobin synthesis	> 10	Waagbø et al., 1993a
Measures on nonspecific immunity	1000–4000	Verlhac and Gabaudan, 1997
Measures on acquired immunity (improved T/B cell proliferation and vaccination efficacy)	500–4000	Verlhac and Gabaudan, 1997
Improved resistance to infectious diseases	2000	Wahli et al., 1998
Improved wound repair	150–1000	Wahli et al., 2003
Stress ameliorating effect	750–3000	Ishibashi et al., 1992

2.2.3. Immunomodulation by antioxidant nutrients in deficiency and excess: merely a change in antioxidant capacity?

Several classical nutritional disorders, as well as infectious diseases in fish, are related to cellular damage in tissues induced by free oxidative radical reactions, caused by lack of antioxidants or by oxidative pressure exceeding the capacity of tissue antioxidants ("oxidative stress"). Reactive oxygen species (ROS; like superoxide anions, hydrogen peroxide and singlet oxygen) may damage the genetic material, membrane-associated enzymes or biomembranes by peroxidation of the lipids, and lead to degenerative disorders, such as liver lipoid degeneration (Roberts, 2002), cardiovascular diseases (Farrell, 2002), cataracts (Waagbø et al., 2003), and increased susceptibility to infectious diseases (Obach and Laurencin, 1992). Membrane lipids and lipid stores rich in n-3 PUFA are highly susceptible to ROS-mediated oxidation. Fishes have an integrated antioxidant defense system (Winston and De Giulio, 1991) to control "wild" oxidation during normal aerobic cell respiration, consisting of endogenous antioxidants (glutathione, mercapthanes), antioxidant enzymes (superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx)), metal-chelating proteins (transferrin, ceruloplasmin, albumin) and dietary antioxidants (vitamin C (ascorbic acid), vitamin E (α-tocopherol), carotenoids (astaxanthin, cantaxanthin), vitamin A, as well as other nutrients)). They exert their antioxidant actions by radical trapping (breaking the auto-oxidation chain) and quenching, or simply by prevention of peroxidation reactions.

The status in fish of antioxidant vitamins such as ascorbic acid, α-tocopherol, retinoids and carotenoids can clearly be manipulated through the feed, in order to cover the basic requirements of single vitamins (NRC, 1993), to prevent degenerative diseases due to special rearing conditions like UV light, oxygen extremes, handling and transport (Lygren et al., 2000, 2001), to improve immunity (innate immunity or vaccination efficacy) and resistance to infectious diseases (Wahli et al., 1998), and to tailor the quality of the product through improved nutrient content and stability during storage and processing of the seafood products (Waagbø et al., 1993b; Hamre et al., 2004).

In contrast to most cells and tissues that use antioxidant defense to protect themselves, by reducing the oxidative pressure of ROS produced accidentally during oxidative metabolism, phagocytes (macrophages and leukocytes) attack pathogens via purposeful oxidative radical formation. During the process called "respiratory burst" (increased oxygen consumption) a massive, directed production of ROS and "antiseptic" radicals are produced through a series of enzymatic and chemical reactions (Lygren, 1999; Bourgeois, 2003). As observed in land-living animals, immunologically active tissues and cells in fish contain high concentrations of antioxidants (Hemre, 1995; Lygren, 1999; Gabaudan and Verlhac, 2001), probably related to the need for self-protection of immune cells against their own ROS. This is illustrated by effective quenching of the respiratory burst activity by extracellular ascorbic acid (measured as chemiluminescence), while elevated intracellular ascorbic acid, obtained by feeding the fish ascorbic acid in excess, may enhance the same response (Lygren and Waagbø, 1998; Gabaudan and Verlhac, 2001).

The requirements for the micronutrients may differ according to their biochemical and physiological roles in the body, as examplified by ascorbic acid in table 4. Ascorbic acid has been proved to be essential for most fish species, including farmed coldwater marine species (Maeland and Waagbø, 1998). Fish lack the enzyme gulonolactone oxidase, which is essential in the final reactions of ascorbic acid biosynthesis. The minimum requirement ranges between 20–50 mg/kg feed for the various species of fish examined, with the lower values representing requirements established with the use of stable and bioavailable ascorbate-phosphate

forms (Sandnes et al., 1992; Gabaudan and Verlhac, 2001; Li and Robinson, 2001; Lovell, 2001). However, it has been suggested that considerably higher practical feed supplements improve the activity of immunological parameters and wound healing, of which the outcome seems to depend on the actual concentration of ascorbic acid in the target cells or tissues (Verlhac and Gabaudan, 1997; Gabaudan and Verlhac, 2001; Waagbø et al., 1993a; Wahli et al., 2003; table 4). It has also been suggested that ascorbic acid has a role in modifying the stress response of fish. Thus, the head kidney (with integrated adrenal function), contains high levels of ascorbic acid, which is depleted after different forms of stress, as well as after administration of adrenocorticotropic hormone, ACTH (Wedemeyer, 1969; Fletcher, 1997; Gabaudan and Verlhac, 2001). Whether ascorbic acid interferes negatively with the biosynthesis of corticosteroid hormones, or ameliorates down-regulated immune responses after stress, has not been convincingly explored. Several studies have failed to show any link between ascorbic acid, corticosteroid synthesis, secondary responses to stress, and overall effects on immunity (Sandnes and Waagbø, 1991; Gouillou-Coustans and Guillaume, 1993; Thompson et al., 1993). This lack of clear results can be related to the biological importance of immune reactions in comparison with reactions to stress. Ranked according to immediate life-sustaining systems in coldwater fish, adaptive immunity can be regarded as a lesser priority compared to the response to stress. For example, rapid endocrine responses to stress (cortisol release) may temporarily suppress immune reactions (Schreck, 1996) at the expense of gaining rapid homeostatic control (Wendelaar Bonga, 1997).

Vitamin E, defined as bioactive tocopherols, is the most powerful lipid-soluble antioxidant in fish tissue, both due to its chemical properties and its abundance in cell membranes and lipid stores (Hamre, 1995). Thus, it prevents oxidation of unsaturated phospholipids in cellular and subcellular membranes. The vitamin E requirements have been shown to increase with increase in dietary PUFA, as well as at lower water temperatures (membrane PUFA also increases as part of temperature adaptation); in deficiency of other antioxidants in the integrated antioxidant defense, such as selenium (part of the antioxidant enzyme GPx) and vitamin C (Hamre et al., 1997); in relation to unfavorable farming conditions, such as oxygenation and vaccination (Lygren et al., 2000, 2001), as well as during infectious diseases. Vitamin E probably acts on the cellular immune response through protection of cellular membranes from free radicals. Vitamin E is essential for many biological activities and deficiency allows generally harmful oxidations to occur, which may compromise both immunity and resistance to infections (Lygren, 1999). Vitamin E is stored in different tissues, including lymphopoietic organs, and these stores clearly reflect the dietary vitamin E levels (Wahli et al., 1998).

Due to the protective role of vitamins C and E in immune cells, deficiencies of both vitamins seem to impact immunity and disease resistance in salmonids before any gross symptoms of deficiency can be observed (Hardie et al., 1990, 1991; Verlhac et al., 1993; Wahli et al., 1998). Thus, immunological parameters may be considered as valuable markers of states of suboptimal nutrition.

A common thought is that most nutrients show a bidirectional effect on immunity and resistance to diseases, where deficiencies lead to impaired defense systems while excess supplies, far above the requirements, improve immunity (table 5). Thus, fortification of nutrients beyond the requirements and recommended dietary levels has been considered as an interesting prophylactic and curative approach for farmed fish. Ascorbic acid has been the number one choice among feed and fish producers, probably for historical reasons and for the best scientific documentation. However, immunomodulatory levels of ascorbic acid may represent values 100 times the requirement (Verlhac and Gabaudan, 1997; table 4). The immunomodulation

Table 5

An example of a bidirectional effect on immunity and resistance to diseases. The influence of dietary vitamin C and E regimes (deficient diets, or diets supplemented adequately or in excess) on nonspecific immunity, lymphocyte proliferation and disease resistance in rainbow trout (adopted from Wahli et al., 1998, with permission of Blackwell Publishing)

Dietary vitamin C/E levels	Chemiluminescent oxidative burst (luminol)	Lymphocyte proliferation (Con A)	Mortality after artificial challenge		
			Virus	*Bacteria*	*Parasite*
0/0	17.5	9.0	a[1]	a	ab
30/30	18.7	11.2	b	b	a
2000/800	26.4	28.0	c	bc	ab

[1]The same letter within each column indicates no difference in mortality at $P \leq 0.05$.

seems to be related to the ascorbic acid status of the actual cells (Gabaudan and Verlhac, 2001), which depend not only on dietary inclusion level, but also the supplementation form of the vitamin (Sandnes et al., 1988) and the overall feeding regimes (Waagbø et al., 1993a). Several mechanisms for the immunomodulatory effects of vitamin C may be suggested, such as the role as intra- and extracellular antioxidant, cytokine-related actions, as well as interactions with other antioxidants. The modulatory effects of vitamin C are clearly different from the potential stimulatory actions of immunostimulants like β-glucans and lactoferrin (Verlhac et al., 1996; Lygren et al., 1999). The effects seem to be temporary and vary according to fish size, breed and the experimental rearing conditions, so that routine use of high levels of dietary ascorbic acid may not be advisable (Lim et al., 2001). In contrast to vitamin E deficiency, dietary vitamin E in excess increased the macrophage activity, mitogen-stimulated lymphocyte proliferation, and antibody response after vaccination (Verlhac et al., 1993). High dietary levels of both vitamin E and n-3 fatty acids may reduce activity of some acquired immune functions in salmonids, probably related to interactions in eicosanoid synthesis (Waagbø, 1994). Mechanisms for vitamin E immunomodulation may therefore be related to its antioxidant protective function, cytokine-mediated immune cell stimulation, interference with eicosanoid synthesis, or through a physicochemical stabilizing effect on cell membranes (Waagbø, 1994; Lygren, 1999).

It can be questioned whether single nutrients in excess, such as vitamins C and E, modulate immunity mainly by their antioxidant properties. This may be supported by the many inconclusive studies in fish and that the outcome of immunological markers often depends on high concentrations of the nutrients in target tissues or cells (Waagbø, 1994). On the other hand, the modulation by vitamin feeding regimes may be of a temporary nature, where the fish after long-term feeding may experience adaption to excess vitamin supply, as illustrated by Verlhac et al. (1993). Other external factors that also influence the immune system, such as water temperature, water quality, stress conditions, as well as overall feed quality (Barton, 1997; table 2) also affect the antioxidant status.

While earlier studies have considered the effects of single nutrients on immune markers, there is increasing interest to study the interaction between nutrients, and how imbalanced diets with respect to selected nutrients challenge or improve the defense system. As, an example, from a factorial vitamin C and E feeding study of rainbow trout, elevated dietary levels of one or both vitamins positively affected acquired immunity and resistance against bacteria,

virus and parasite infections, compared to levels at the minimum requirements (table 5; Wahli et al., 1998). This study indicates a synergistic role of the vitamins in unspecific strengthening of the immune defense in fish against the various classes of pathogens.

In a feeding experiment with a multivariate approach (according to a 2^{7-3} reduced factorial design) with respect to dietary pro-oxidant- (lipid level, and manganese, copper and iron supplementation) and antioxidant-nutrients (vitamin C, vitamin E and astaxanthin supplements) fed at two dietary levels, immunity and health parameters of Atlantic salmon smolt were examined over a period of 26 weeks (Lygren et al., 1999). The dietary levels were either slightly above the requirements established by NRC (1993) for cold water species, or at or below known toxic feed levels. The results showed that only dietary vitamin E influenced the markers of nonspecific immunity examined, like macrophage respiratory burst activity (Lygren et al., 1999). The dietary variation in micronutrients had minor effects on growth performance and feed conversion (Hamre et al., 2004). This study illustrates that, among the antioxidants, vitamin E was more effective in modulating the immune system than vitamin C and astaxanthin, supporting the view that these compounds may possess immunomodulating properties that are not necessarily related to pure antioxidant properties (Wu and Meydani, 1999). Thus, cellular antioxidants, like vitamin E may have additional properties, such as effects on enzyme activities and gene expressions (Özer and Azzi, 1999).

Other antioxidant compounds, like vitamin A (Thompsen et al., 1994), carotenoids (Christiansen et al., 1995; Lygren et al., 1999; Amar et al., 2001) and flavonoids have also been studied for their potential to prevent disease by boosting innate immunity or improving the efficacy of vaccination in fish. However, most of these studies failed to show improved immunocompetence at elevated levels, while low dietary levels of the nutrients often suppressed immunity and resistance to diseases.

2.3. Health aspects of vegetable feed ingredients

For farmed warm-water species, such as the American farm-raised channel catfish, *Ictalurus punctatus,* and carp species, vegetable meals make up dominant proportions of the protein and lipid contents of the feed. In farmed salmon, however, high proportions of the feed have traditionally been made up of expensive marine protein and lipid sources. A continuously rapid growth of worldwide aquaculture is expected, both in the number of farmed species and the production volumes. Limited global marine resources, as well as economical and ethical considerations related to sustainable aquaculture have put pressure on using available low-cost protein and lipid feed ingredients also for marine species. Besides differences in amino acid, fatty acid and micronutrient compositions, vegetable feed ingredients may contain variable amounts of natural (toxins of plant and microbiological origin, antinutrients etc.) and production-derived toxicants (organic chemicals like herbicides and pesticides, and additives) (Hendricks, 2002).

Growing fishes have high protein requirements and protein utilization compared to land-living production animals. Consequently, there has been a wide search for alternative protein-rich vegetable ingredients suitable as fishmeal replacement. Hertrampf and Piedad-Pascual (2000) reviewed available information on ingredients for aquaculture feeds, recommending inclusion rates of the ingredients according to the raw material production procedures, and their chemical and biological properties. In general, increased refinement of plant materials with the aim of reducing the contents of antinutrients and increasing the proportion of protein, allows higher inclusion levels in fish feed, but it also increases the feed cost. On the other hand, strategies to fulfill the requirements for the ten amino acids indispensable for optimal growth

in fish by using mixes of vegetable feed ingredients and amino acid supplements are well known (Hardy and Barrows, 2002).

While the intestine normally represents an effective barrier against pathogens, morphological changes in the intestine following the feeding of diets with plant ingredients, such as soybean meal have been reported in salmonids (van den Ingh et al., 1991; Refstie et al., 2000). Whether this represents a challenge towards increased susceptibility to feed and waterborne infectious agents remains to be sorted out. Farhangi and Carter (2001) did not find any adverse effects on markers of health and nonspecific immunity in rainbow trout fed dehulled lupin as partial, up to 50%, replacement for fishmeal.

Vegetable oils have been examined for their suitability in aquaculture feeds, especially over the last decade, since there is limited fish oil available on the world market to cover the needs for long-chain n-3 polyunsaturated fatty acids (PUFA) to the growing aquaculture industry. The major challenges with fish oil replacements lie in their different fatty acid composition, with lower proportions of long-chain n-3 PUFA and often higher concentrations of n-6 PUFA compared to fish oils, resulting in higher n-6/n-3 ratios (Sargent et al., 2002). The implications of changes in dietary fatty acid composition are related to fish health (Waagbø, 1994) and product quality aspects (Waagbø et al., 1993b). The latter problem is mainly determined by the acceptance in the market for n-6-rich seafood.

Dietary lipid composition affects nonspecific defense mechanisms, acquired immunity and resistance to bacterial diseases in fish (reviewed by Fracalossi and Lovell, 1994; Waagbø, 1994; Waagbø et al., 1995; Thompson et al., 1996; Balfry and Higgs, 2001). The effects on immunity and inflammatory processes are probably mediated through mechanisms related to immune cell membrane stability, activity of membrane-associated enzymes, receptor sites, eicosanoid production, production of cytokines, tissue lipid peroxidation or alterations in expression of selected genes following activation (fig. 1). The health impacts of dietary lipids are usually not unidirectional. The lack of consistency of the immunomodulatory effects of single or classes of fatty acids seem to relate to different mechanisms behind their modes of action. Changes in dietary lipids and tissue fatty acid composition may affect parts of the

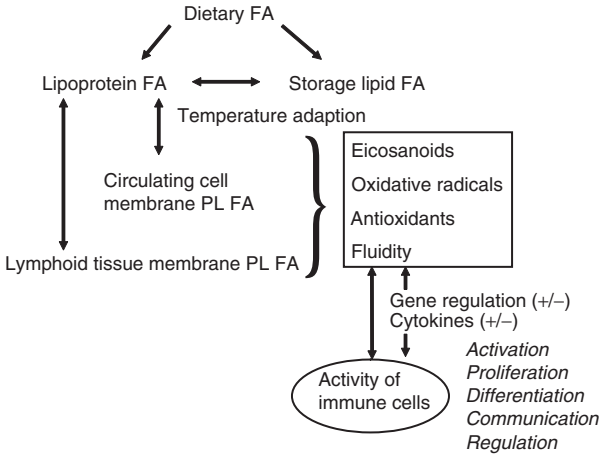

Fig. 1. A summary of possible mechanisms by which dietary fatty acids (FA) may influence immunity and disease resistance in fish by affecting the fatty acid composition of lipoproteins (LP) and the membrane phospholipids (PL) of circulating and tissue immune cells, thereby altering membrane physical strength (fluidity), enzyme activities, membrane fatty acid composition for eicosanoid production and thereby cytokine production or potency.

physiological and immunological systems differently, and the net results on fish immunity and disease resistance may vary according to the most sensitive parts of the system at the time of examination. Furthermore, as poikilotherms, fish passively adjust their body temperature according to the environment. The cell membranes respond to changes in temperature by shifting lipid class structures and fatty acid composition of the phospholipids, an acclimation process called "homeoviscous adaptation". The cell membranes increase the fractions of unsaturated fatty acids and monoenes and decrease saturated fatty acids during cold acclimatization (Hazel and Williams, 1990). Thus, both dietary lipid source and water temperature influence tissue fatty acid composition, with the main effects on storage lipid and phospholipid fatty acid compositions, respectively (Jobling and Bendiksen, 2003). This means that water temperature (or *in vitro* cell culture incubation temperature) represents a confounding aspect in lipid-immunity studies in fish. Furthermore, different subtypes of immune cells seem to respond differently to temperature changes (Bly and Clem, 1991). To illustrate this, channel catfish macrophage bactericidal activity *in vitro* was positively correlated to the level of n-3 PUFA in the diet. Vaccination enhanced this activity at normal (28°C), but not at suboptimal temperatures (19°C) (Sheldon and Blazer, 1991). On the other hand, catfish fed n-3 PUFA-rich menhaden oil diet showed reduced resistance (compared to other lipid sources) to *Edwardsiella ictaluri* infection after artificial challenge at 28°C, but not at 17°C.

Diets with a ratio of n-3/n-6 PUFA of 5.2 (obtained by adding fish oil) or a ratio of 0.3 (obtained by using sunflower oil) fed to Atlantic salmon for 4–5 months did not affect hematology or nonspecific immune parameters (Thompson et al., 1996). The high n-3 PUFA diet caused increased numbers of B-cells to respond to bacteria after vaccination and increased survival after bacterial challenges with common fish pathogens (*Vibrio anguillarum* and *Aeromonas salmonicida*). Thus, a coldwater fish species fed diets with a low ratio of n-3/n-6 PUFA may be less resistant to infections compared to fish fed diets with a high ratio (Thompson et al., 1996). Head kidney phagocytes isolated from Atlantic salmon reared at 8°C and incubated in salmon sera with defined lipid fatty acid compositions, generally increased the stimulated respiratory burst (chemiluminescent) response at 16°C compared to 8°C. Differences in chemiluminescent response among the plasma lipid groups could statistically be attributed to individual fatty acids at 16°C, but not at 8°C (Michelsen, 2000). These studies indicate that changes in membrane lipid fatty acid composition due to diet or temperature may have profound effects on measures of both innate and acquired immunity, as well as resistance to infections.

Immunomodulatory effects of lipids may be mediated by interactions between lipoproteins and circulating immune cells (fig. 1). Atlantic salmon is a hyperlipidemic species, where the lipid composition of the different lipoprotein classes clearly reflects the dietary lipids (Lie et al., 1993; Torstensen et al., 2000). Membrane phospholipid (PL) fatty acid (FA) composition in lymphoid tissue (spleen and head kidney) and circulating blood cells is influenced by the dietary lipid source, as shown in Atlantic salmon and Atlantic cod, *Gadus morhua,* fed extruded diets with soybean oil, capelin oil and sardine oil (Waagbø et al., 1993c, 1995). Tissue macrophages have a central position in pathogen killing, antigen uptake and presentation, and intercellular communication with the acquired immunity by secreting cytokines and eicosanoids upon stimulation, and this array of activities may be modulated by dietary-induced changes in membrane lipid composition (fig. 1). PUFAs in the membrane phospholipids are precursors for the synthesis of eicosanoids (prostaglandins, leukotrienes, thromboxanes, lipoxins and related compounds) by macrophages, which are potent effectors in inflammation and regulation of immune cells (Pettitt et al., 1991; Balfry and Higgs, 2001). Eicosanoid series (2-series prostaglandins (PG) and thromboxanes (TX), and 4-series

leukotrienes (LT) and lipoxins) produced from arachidonic acid (AA; 20:4 n-6) are generally more potent to modulate cells in the immune system and inflammation than those (3-series PGs and TXs, and 5-series LTs and lipoxins) derived from eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3). Thus, by incorporation into the cell membrane phospholipids, EPA can act in opposition to AA, both as a competitive substrate for eicosanoid synthesis, as well as producing eicosanoids that oppose the effects of those mediators derived from AA (Calder, 2001). In line with this, calcium ionophore stimulated head kidney macrophages, isolated from rainbow trout fed diets containing sunflower oil or fish oil, synthesized different profiles of eicosanoids, dominated by the 4-series (LTB4 and lipoxin A4) and 5-series (LTB5 and lipoxin A5), respectively (Secombes, 1994). Although scarcely explored, the changes in eicosanoids seem to cause modulation of isolated immune reactions, such as phagocyte migration, T-cell responses and serum immunoglobulins (Secombes et al., 1994; Bell et al., 1996). The role of fatty acids in inflammation and immunity in humans and animals was recently reviewed by Calder (2001). A comparison of the immune responses after intake of n-6 and n-3 PUFA suggests that the effect of fish oil cannot be regarded purely as antagonizing the n-6-derived eicosanoids, even though EPA and DHA from fish oil generally exhibit anti-inflammatory and immunosuppressive effects both in *in vitro* and animal feeding studies by inhibiting production of cytokines, such as IL-1β, TNF-α and IL-6 (Calder, 2001). In adult cod (*Gadus morhua*), the lipid fatty acid composition of circulating blood cells is more similar to plasma (lipoprotein) fatty acid composition than to spleen and head kidney tissue compositions (Waagbø et al., 1995). This study indicates that dietary long-chain monoene fatty acids from marine lipids (20:1 and 22:1) may be responsible for the outcome of the nonspecific and acquired immunological parameters studied. Since the membrane phospholipids contain very low levels of these monoenes (despite proportions >50% of the fatty acids in capelin oil), it is probable that these fatty acids affect immunity by other mechanisms than by their incorporation into biomembranes.

The discussion shows that feed lipids of different origin may affect immunity by several mechanisms, and these areas clearly needed to be explored in fish fed under different temperature regimes.

2.4. Immune suppression by feed toxicants (organic pollutants and metals)

There is an increasing concern about global and local marine pollution, since this adversely affects wild fish population stocks, as well as the marine feed ingredients based on the fisheries of these stocks. Farmed fish may be exposed to numerous unwanted chemicals and compounds from the environment, feed ingredients, and through treatments in production such as medication, anesthesia, vaccination, disinfections and treatments against algae growing, etc. The latter is normally under strict control by use of legally approved compounds and practices, and by surveillance of rest concentrations in the fish. However, in parallel to the general concern on environmental pollution, there is an increasing focus on safety with respect to pollution from the feed resources used for aquaculture feeds, both of agricultural (heavy metals, pesticides) and marine (inorganic and organic pollutants) origins. As compared to ecotoxicology, relatively few scientific contributions support the research area of feed toxicology in fish (Handy, 1996; Berntssen, 1999; Isosaari et al., 2002). There are numerous examples of industrial and agricultural loads to local fresh water and seawater environments, that have resulted in acute toxicities with compromised immunity and mortalities in fish (Arkoosh et al., 1994; Dunier, 1994; Anderson, 1996; Olsson, 1998). In line with other carnivorous wild pelagic fish species high in the marine food web, farmed salmonids

bioaccumulate dietary lipid-soluble toxicants, like dioxins and dioxin-like polychlorinated biphenyls, PCBs, in the muscle and internal organs (Isosaari et al., 2002; Haldorsen et al., 2004; Hites et al., 2004). Similarly, unwanted metals both from water and feed accumulate in organs like gills, intestine, liver, kidney and muscle of farmed fish.

Chronic exposure of fish to xenobiotics through water seems to modulate immunity, impacting both on cellular and humoral immune functions, where the rapid rate of proliferation of lymphocytes seems to be especially sensitive (Dunier, 1994). Atrophy of lymphoid organs and lymphopenia are often observed. As an example of immunotoxicity, field studies and experiments where juvenile chinook salmonids were exposed to aromatic hydrocarbon (such as PCBs) contaminated water or injections, respectively, indicate that these compounds affect splenic and anterior kidney B-cell activity (plaque-forming cell response) after stimulation with a T-cell-independent antigen (Arkoosh et al., 1994). The suppression was more pronounced for the secondary response, suggesting that the memory-generation process was more susceptible to exposure than the primary response. Most of the literature refers to administration of the pollutant through water or by injection, and not by oral exposure (Hendricks, 2002). Feeding moderate doses of the insecticide lindane or the herbicide atrazine to carp did not affect markers of the innate (such as macrophage activity delay in skin graft rejection, spleen size) and acquired immunity (vaccination efficacy), even though selected tissues reflected the dietary levels (summarized by Dunier, 1994). This indicates that chronic exposure of pollutants through the diet does not compromise fish health and measures of immunity to the same extent as water exposure (Hendricks, 2002), or it may allow the fish to compensate potential immunotoxic effects (fig. 2). On the other hand, stress reactions, with cortisol

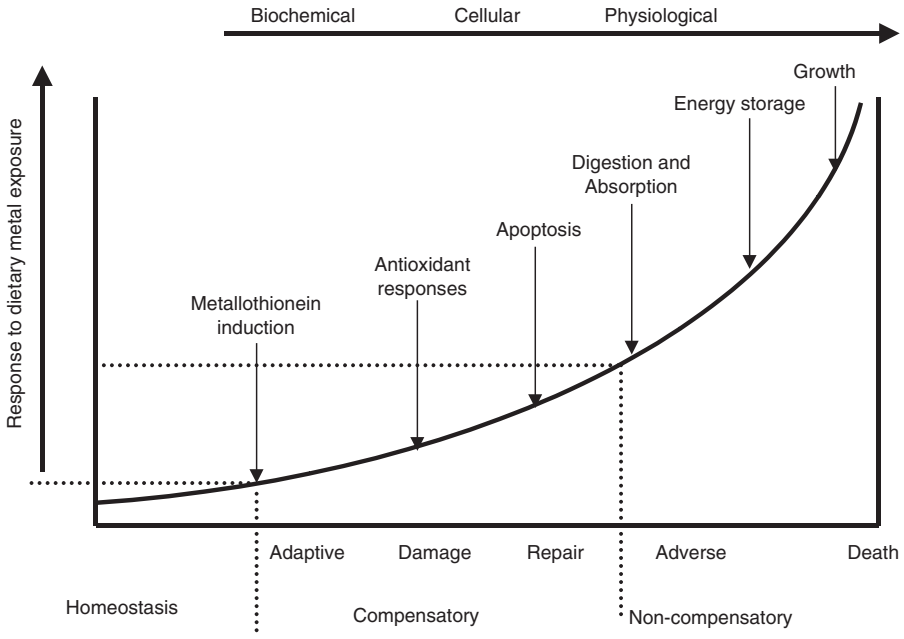

Fig. 2. Responses at biochemical, cellular and physiological levels include compensatory mechanisms to resist dietary toxic exposure. Above toxic levels noncompensatory adverse impacts occur with growth reduction and death as endpoints. (Adapted from Berntssen, 2000.) Immunological markers may be useful at all levels of exposure, from early gene expressions to failed resistance to infectious diseases.

release, in relation to exposure to environmental pollution through water may be a confounding factor to the impairment of immunity, probably mediated through suppression of interleukins and modified sensitivity of cells or components in the lymphoid system (Dunier, 1994). This makes it sometimes difficult to distinguish between immunotoxic effects and stress-related immunosuppression (Wendelaar Bonga, 1997). It cannot be excluded that long-term effects of oral exposure can lead to reduced resistance to, for example, oncogenic virus infections and development of neoplasia (Grizzle and Goodwin, 1998).

Many minerals serve essential biological functions that may affect the defensive mechanisms and immune competence in states of borderline deficiency, like magnesium (El-Mowafi et al., 1997), halides (iodine and fluorine; Lall et al., 1985) and zinc (Kiron et al., 1993). Some minerals are added to fish feed for safety purposes, since the natural contents and the availability of mineral in feed ingredients may vary. Thus, toxic concentrations of elements (heavy metals and essential minerals) in fish feed may arise from the feed ingredients (bioaccumulated, pollution or chemical part of additives), feed production equipment (storage or production containers), or unintentionally through mineral supplementation.

Unwanted metals (lead, mercury, cadmium, arsenic) and excess trace elements (zinc, iron, copper, manganese, aluminum) are most often immunosuppressive (Dunier, 1994; Anderson, 1996), affecting lymphocyte proliferation, humoral immune response and phagocytic activity. The adverse effects may be related to their pro-oxidative nature or high binding affinity to active sites of enzymes and competition to essential elements, making components and rapidly proliferating cells of the immune system susceptible to the metals. Like the organic pollutants there seem to be large differences in toxicity related to elements in water and feed, proportional to differences in rates of uptake (Handy, 1996). Therefore, studies have not demonstrated similar immunosuppression by elevated levels of dietary metals as by waterborne metals. The uptake of most metals in the intestine seems to be well regulated, even though target organs often reflect the feed burdens (Berntssen, 2000). As observed for dietary organic pollutants, toxic elements in feed may lead to more chronic or sublethal toxic effects compared to acute toxicity symptoms in fish exposed to waterborne metal pollution, even in highly contaminated feed and after prolonged feeding (Handy, 1996; Olsson, 1998). This can be related to higher bioavailability and uptake of waterborne than feedborne metals, evoking an additional or elevated stress response for waterborne metals (Wendelaar Bonga, 1997). Thus, the mechanisms of immunotoxicity seem to depend on an integrated response between the endocrine and immunological systems. Clinical signs of feed toxicity include histological changes and damage of entrance or excretion organs (intestine and kidney), changes in blood chemistry (hematology, blood metabolites and enzyme activites), changes in behavior, and rather later effects, such as reduced reproductive performance (Handy, 1996). In case of chronic exposure to toxicants in the diet it is important to develop tools to record early warning signals of known biological consequences of the dietary toxicant (fig. 2; Berntssen et al., 1999, 2004). The approach to search for markers of exposure among early molecular events, cellular reactions and gross physiological responses, represents both a strategy towards exploring detailed mechanisms behind the toxic responses of dietary nutrients and contaminants, as well as tools for nutritional toxicity testing (Berntssen, 2000).

The absorption and toxicity of heavy metals, both from water and feed, depend on the chemical forms of the elements, which are determined by external chemical factors (pH, salinity, temperature, oxygen) and complexing compounds in the water (Olsson, 1998) or intestinal fluid. As an example, large differences in absorption and retention of mercury (Hg) are seen when dietary inorganic and organic mercury (Hg) are fed to Atlantic salmon parr, where inorganic Hg accumulates mainly in the intestine, while organic methyl-Hg is absorbed

much more efficiently and is retained in the internal organs and muscle tissue (Berntssen et al., 2004).

Excess of essential minerals, such as iron, copper, manganese in the diet may influence fish immunity (Fletcher, 1997; Lygren et al., 1999). The concentration ranges to which isolated immune cells are exposed and affected by elements *in vitro* may serve, with caution, as a guidance for the critical tissue levels for oral exposure. The phagocytic response of isolated head kidney macrophages from Atlantic salmon, preincubated in plasma from salmon fed diets with different mineral content showed that elevated manganese and ascorbic acid together inhibited macrophage chemiluminescent response after PMA stimulation (Lygren and Waagbø, 1999). The range of plasma manganese was 0.04–0.19 µg/mL. Far higher *in vitro* incubation concentrations were found to be immunosuppressive for carp cells (Dunier, 1994). Feeding adult Atlantic salmon dietary copper below 10 mg/kg and above 100 mg/kg resulted in minor variations in plasma copper (1.01–1.16 µg/mL; Lygren and Waagbø, 1999), confirming the effective intestinal barrier against dietary excess of inorganic essential elements like copper (Berntssen et al., 1999) and the lack of toxic effects of elevated dietary copper on immunity (Lygren et al., 1999). The CL response of head kidney macrophages incubated in 10 µg/mL copper solution was half that seen in 0.1 µg Cu/mL and 1 µg Cu/mL incubations. The intestines of Atlantic salmon, however, showed adaptive responses of increased cell proliferation, apoptosis and metallothionein synthesis indicative of stress at 35 mg Cu/kg diet (Berntssen et al., 1999). Updated EU regulations for fish feed have recently adopted a lower dietary upper limit of copper in complete feed and feedstuffs from 35 mg/kg to 25 mg/kg, or five times the requirement (EC, 2003).

Strategies for the fish to detoxify unwanted compounds include mechanisms to reduce absorption, binding to ligands (such as metallothionein) or increased rates of excretion (Olsson, 1998). In several studies where fish have been exposed to pesticides, polychlorinated compounds and metals there have been indications of interactions between ascorbic acid status and exposure, as well as indications that excess dietary ascorbic acid may counteract immunotoxic effects, stress and mortalities (Norrgren et al., 2001). Ascorbic acid may counteract toxicity of organic compounds and metals, through its role in the metabolism and biotransformation of xenobiotics through P450-dependent enzymes, as a part of the integrated antioxidant defense, in collagen synthesis and through a potential stress-ameliorating effect.

Immunological reactions in fish should be evaluated as biomarkers of adverse outcomes after dietary exposure, to aid in the risk assessment for toxic dietary exposures (Dunier, 1994; Koller, 1993; fig 2). Recently, Calabrese and Baldwin (2003) discussed a traditional linear dose–response model of toxicity versus a hormetic dose–response model. The latter model deviates from the former in the response at low toxic concentrations, where even moderate positive responses may be observed, while adverse effects increase linearly at increasing doses following the traditional model. Thus, the hormetic model can be regarded as an expression of a compensatory response at low exposure concentrations, as illustrated in fig. 2.

National and international feed and food legislations have introduced upper limits for many contaminants, but the figures are often based upon historical data from other husbandry, and clearly need to be revised for farmed fish species. Immunological biomarkers focused on fish health and welfare may be useful in this work.

2.5. Dietary immunostimulants and probiotics

Immunostimulation through the diet involves additives that contain components that activate the immune system similarly to pathogens (Raa, 1996; Gannam and Schrock, 2001;

Sealey and Gatlin, 2001), but unlike the potential immune modulating or tuning effects by nutrients. The use of dietary immunostimulants has attained increasing interest since it gives the opportunity to work at large-scale levels and it does not involve stressors, such as with individual treatments (Sakai, 1999). Even though administration of immunostimulants by injection in vaccines is dominating the market, there is increasing evidence that oral administration of stimulants has effects on nonspecific immune markers *in vitro* and *in vivo* and improves disease protection (Raa, 1996; Sakai, 1999; Sealey and Gatlin, 2001). A promising area is the use of immunostimulants in fish larviculture to control infectious agents (Vadstein, 1997). The potential could, however, only be realized with knowledge of when the larvae are able to respond to the oral or water treatments, and the development of reliable administration procedures.

Probiotics are live organisms that beneficially affect the microbial balance of the host and thereby improve health. The research on probiotics and the potential for aquaculture purposes has also attained increasing interest (Gatesoupe, 1999; Irianto and Austin, 2002). The mode of action of probiotics is probably related to their stimulation of the immune system, production of antimicrobial substances, competition for adhesion receptors or provision of nutrients (Sealey, 2000; Sealy and Gatlin, 2001). Fish seem to have a normal gastrointestinal microbiota, modulated both by microbes from the food and the environmental water. Most bacterial cells are transient in the gut, with continuous intrusion of microbes coming from water and food. Thus, a common method of probiotic treatment in fish and shellfish larvae management is to add microbes through the water (Gatesoupe, 1999). This treatment may also have potential effects on feed utilization and fish performance.

The use of probiotics antagonistic to pathogens has the potential to reduce the use of antibiotics in disease control. Probiotics isolated for land animals have been tested in fish, but have had only variable success on fish health and performance. These experiences may be related to differences in growth and survival of these bacteria species in the aquatic environment (Gatesoupe, 1999). Selected strains of microbes from the aquatic environment (*Vibrionaceae,* pseudomonads, lactic acid bacteria, *Bacillus* spp. and yeasts) have been suggested and tried out as probiotics for fish (Gatesoupe, 1999; Sealy and Gatlin, 2001). Normally, the identified favorable bacteria are isolated from mature fish and then introduced to younger fish of the same species through the feed. For example, lactic acid bacteria isolated from Atlantic salmon (*Salmo salar*) intestines have been shown to inhibit the growth of fish pathogens such as *Vibrio anguillarum* (causing classical vibriosis) and *Aeromonas salmonicida* (causing furunculosis) *in vitro* (Gildberg et al., 1995). However, a challenge experiment in salmon fed lactic acid bacteria in the diet failed to show any effects of the dietary treatment on resistance towards *A. salmonicida* infection. On the other hand, juveniles from other fish species fed diets with lactic acid bacteria showed increased survival from artificially induced *Vibrio* infections (Gatesoupe, 1999; Sealy and Gatlin, 2001).

Experience has shown the need for controlling dosage rates and growth of the microbes within the host (Sealey, 2000). The determination of safe inclusion levels of probiotics is needed to prevent any undesired effects on fish health and performance, and safety for human consumption has to be guaranteed in probiotic-treated fish. In addition to attempts to improve vaccination, nutrition and fish management, the use of probiotics seems to have a potential in preventive disease control, including an antiviral potential. Production and economic costs of the probiotics should be weighed against positive immunological aspects, reduced labor costs related to mass treatment and possibilities to run a safety control. Thus, application of probiotics like the immunostimulants seems to be well suited to larval and juvenile fish culture (Raa, 1996; Sealey, 2000).

3. NONINFECTIOUS PRODUCTION DISORDERS

The nutrient requirements in farmed fish are not static, and may differ according to environmental challenges such as temperature, salinity, light regimes, water gas concentrations, pollution, etc. (table 4). Besides inducing changes in the intermediary metabolism and energy flow, these abiotic parameters also influence endocrinology, detoxifying systems, homeostatic mechanisms and immunity (Bly et al., 1997). In anadromous fishes like the salmonids, periods of smoltification and sexual maturation mean dramatic shifts in environment, from freshwater to seawater after smoltification, and return to freshwater for reproduction. The endocrine-mediated changes of ion homeostasis are followed by metabolic adaptations and changes in body composition (Shearer, 1994). It is believed that environmental factors and management measures can provoke nutritional imbalances, which in turn can develop into visible deficiency symptoms, especially in sensitive periods such as during the start of feeding, smoltification and reproduction (table 3).

Like other developed husbandry, modern finfish aquaculture faces problems such as bone and skeletal deformities, cataracts, as well as unspecific ulceration (table 3; Brown and Núñez, 1998). Such disorders, if related to nutrition, fast growth and environmental conditions, are most often in themselves noninfectious and not lethal, but can increase the susceptibility to secondary infectious diseases. Besides representing economical losses, and reduced fish quality, the disorders represent ethical challenges within the frame of a sustainable production. Examples of disorders linked to the intensive farming of salmonids, as well as other farmed species, are the development of cataracts (Wall, 1998) and bone deformities (Vågsholm and Djupevik, 1998; Kvellestad et al., 2000), which will be shortly discussed in relation to nutrient deficiencies or toxicities.

3.1. Cataracts

A significant occurrence of cataracts has been observed in farmed Atlantic salmon during the last decade, lately more recurrently and with varying incidence (Breck, 2004). A cataract is often defined as an opacity of the lens or its capsule, and it may occur as a reversible or permanent change (Bjerkås et al., 1996). Normally, nutrition-related cataracts are bilateral. Wall (1998) and Midtlyng et al. (1999) gave updates on the cataract situation in European salmon aquaculture, of which many forms have been related to nutrition (Hughes, 1985; Hargis, 1991; Breck, 2004).

Due to the inward growth pattern of the lens, the protein and cell structures are meant to last throughout life, despite exposure to light and other external conditions that may cause oxidative stress and increased proportions of insoluble aggregates of lens proteins (crystallins). Although sparsely explored, the natural antioxidant protection in the lens includes actors of the integrated antioxidant system, like GSH, antioxidant enzymes, and elevated concentrations of water-soluble antioxidants like ascorbic acid (Bourgeois, 2003; Waagbø et al., 2003). Besides classical nutrient deficiencies (table 3), environmental factors may cause suboptimal nutritional status that may lead to cataracts. Although not clearly connected to nutritional status, water temperature regimes under parr–smolt transformation affect growth and cataract development in Atlantic salmon (Bjerkås et al., 2001). This study showed that fluctuating temperature regimes increased both growth and cataract incidence, as compared to stable high- and low-temperature regimes. On the other hand, excess dietary nutrients may alleviate the severity of developing cataracts. In a multivariate feeding study, Waagbø et al. (2003) demonstrated that cataract development in Atlantic salmon could be influenced by

dietary means, where high levels of nutrients of pro-oxidant nature (dietary lipid, iron, copper and manganese levels) increased, while excess of antioxidant nutrients (vitamin E, vitamin C and astaxanthin) alleviated the cataract incidence. This is consistent with the hypothesis of prevention of oxidative cataracts by antioxidant nutrition in humans (Bourgeois, 2003), although lack of antioxidants was not suggested to be the primary cause of the observed salmon cataracts.

Recent research on the causative factors leading to cataractogenesis in farmed Atlantic salmon focuses on the roles of the amino acid histidine (His) and His-derivatives (N-acetyl His, anserine) in osmoregulation during smoltification and growth in the sea (Breck, 2004). Besides protein synthesis, His and imidazole compounds take part in tissue antioxidation, in tissue pH buffering, and in mechanisms of lens cell volume regulation in salmonids (Breck, 2004). Since elevated dietary His via blood meal, His-rich fishmeal, or crystalline His supplementation efficiently mitigated cataractogenesis in smolts in seawater (Breck et al., 2003), a major question has been raised as to whether dietary enrichment with His is needed to cover up for changes in homeostasis during the smoltification process and subsequent rapid growth in the sea. Normally, amino acid deficiencies are not considered to be an area of concern with respect to fish health (Hardy, 2002). This example on the cataract preventative role of histidine illustrates, however, the continuous need for re-evaluation of nutritional requirements along with the overall development of the fish farming industry.

3.2. Bone deformities

Bone deformities in juvenile and adult fish are periodically observed, with high prevalence in intensive aquaculture (Vågsholm and Djupevik, 1998), and are mostly regarded as disorders of multidisciplinary origins (Brown and Núñez, 1998; Cahu et al., 2003). Indeed, either deficiency or excess of several nutrients can cause bone disorders (table 3), and historically the largest losses have probably been caused by vitamin C deficiency (Dabrowski, 2001a). From a nutritional point of view, both development and maintenance of the bone tissue can be affected, and may include impairments in bone cell differentiation and function, matrix composition and bone tissue mineralization (Huysseune, 2000). A model modified from human research (Ziegler et al., 1995), shows that variable factors like physical activity, health status, nutrient supply and a series of endocrine factors play a complex role in the regulation of bone mass and strength (fig. 3).

Since the incidence and the appearance of deformities in modern salmon farming seem to differ from the deformities in the early stages of aquaculture, both for juvenile stages and adult fish, there is a need to characterize the bone disorders. In Atlantic salmon, the term platyspondyly has been suggested as one characterization of the vertebrate disorder (Kvellestad et al., 2000). This interpretation implies growth disturbances leading to vertebrate malformations. The processes of bone formation, remodeling and mineralization in fish are not fully described (Huysseune, 2000), but include participation of nutrients as vitamins A, D, K and C, and the bone minerals (calcium, phosphorous, magnesium, zinc, manganese) (fig. 3). Input and regulation of bone minerals influence these processes, of which phosphorus has been identified as potentially in deficit in fish diets (Shearer and Hardy, 1987), due to low water concentrations, low dietary inclusion levels (limited supplementation according to feed legislation as well as lower proportions of phosphorous-containing ingredients in high-energy diets for growers), reduced bioavailability of phosphorous from vegetable feed ingredients, and possible increased metabolic use in compensating metabolic acidosis under stressful anaerobic or hypoxic conditions.

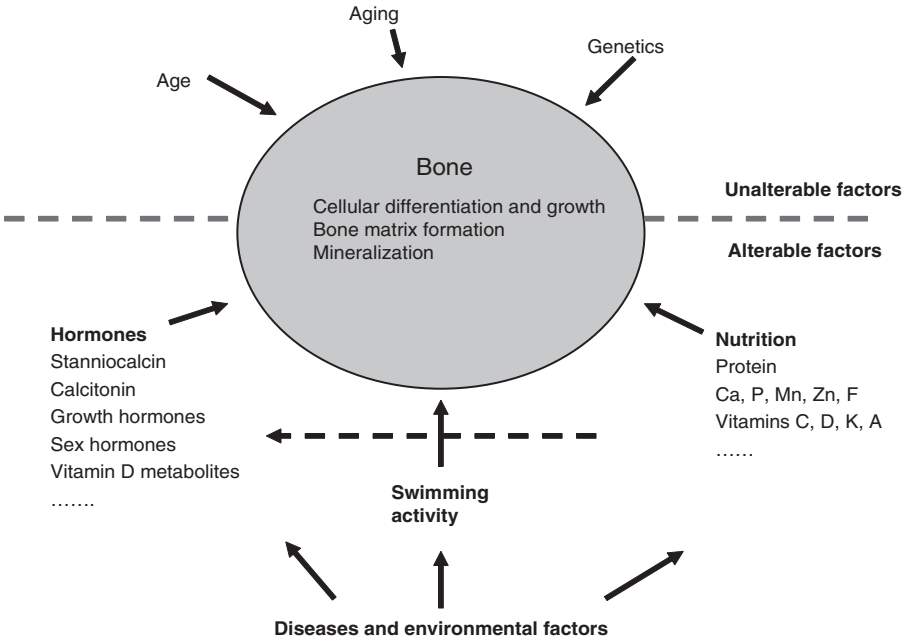

Fig. 3. Factors that influence bone health in fish include unalterable inheritable and alterable factors, which may impact cellular and matrix development and bone mineralization. (Modified from Ziegler et al., 1995, with permission of the American Society for Nutritional Sciences.)

The role of vitamin D and its active metabolites in calcium and phosphorous regulation of fishes have been discussed in relation to bone deformities, especially the potential toxic effects of excess dietary vitamin D (Graff, 2002). Fish store considerable levels of vitamin D in the liver and lipid-rich organs, and seem to tolerate very high dietary inclusion levels of vitamin D_3 compared to humans (Horvli et al., 1998; Graff et al., 2002). It has been shown that the active metabolite 1,25 dihydroxy-vitamin D_3 exerts a hypercalcemic effect in fish, and the expression of binding proteins and receptors for the metabolites indicate a similar role in Ca regulation as for humans, despite excess calcium available from water and diet (reviewed by Graff, 2002). There is also an indication that calcium regulation by different vitamin D metabolites may change in salmonids during the parr–smolt transformation, adapting the fish to calcium-rich seawater (Graff, 2002). Thus, despite tolerance for dietary vitamin D the fish seems to use its metabolites for calcium regulation, together with a series of other hormones (fig. 3; Janz, 2000). So far, excess levels of dietary vitamin D_3 supplementation have not induced deformities in fish (Graff, 2002). The focus has now turned from high levels of vitamin D to the active metabolites of vitamin D in marine feed ingredients, and their potential impact on calcium regulation in farmed fish.

In an attempt to elucidate interactions between nutritional factors and water quality on bone health, groups of Atlantic salmon parr were fed dietary regimes of vitamin D_3, vitamin K and calcium and exposed to water carbon dioxide at two levels, according to a multivariate (2^4) experimental design. Neither of the regimes provoked bone deformities or affected levels of bone minerals in freshwater, or after 11 weeks follow-up in seawater (Graff et al., 2002). Vitamin K has important functions related to bone formation and bone health, and exerts its function as a cofactor in the enzymatic carboxylation of glutamate residues of the bone and cartilage proteins osteocalcin and matrix Gla-protein, for them to be able to bind calcium

(Vermeer et al., 1996). Until now, there has been little focus on vitamin K in aquaculture nutrition, despite the supplemented forms being unstable during feed production and storage (Gabaudan and Hardy, 2000).

Selected marine feed ingredients may contain very high concentrations of vitamin A, and excess may induce bone deformities through the role of vitamin A metabolites (retinoic acid) in bone cell development and bone mineralization (Ørnsrud, 2003); e.g. retinoic acid causes developmental disorders in larval Japanese flounder (Haga et al., 2002). It has been suggested that elevated vitamin A levels might cause teratogenesis in Atlantic salmon. A study was made in which eggs of differentiated vitamin A and carotenoid contents were incubated at two temperature regimes and followed during larvae development, through the start of feeding and until adult stages at sea (Ørnsrud, 2003; Ørnsrud et al., 2004). From these studies it could not be concluded that deformities were associated with the vitamin A status of the eggs, but elevated incubation temperatures during embryogenesis and until the start of feeding affected both vitamin A metabolism and development of organ abnormalities in juveniles and adults. The exact roles of retinoids in bone health are still under investigation, and it is hoped that studies on gene expression may lead to a better understanding of bone toxicity of retinoids (Ørnsrud, 2003).

Among the heavy metals, elevated exposure to cadmium through water and feed has been associated with formation of bone deformities due to interactions with calcium metabolism. Exposure of Atlantic salmon parr to toxic dietary cadmium concentrations up to 250 mg/kg for 4 months in fresh water disturbed calcium homeostasis and decalcification of the scales, but did not induce bone deformities or affect bone calcium reservoirs (Berntssen et al., 2003). Together with the moderate levels found in commercial fish feeds (< 0.5 mg/kg), this indicates that dietary cadmium is not likely to be involved in the development of the bone disorders recently observed in salmon aquaculture.

Light regimes are among the most widely used external factors in aquaculture to manipulate the biological processes during parr–smolt transformation and sexual maturation, mediated by changes in endocrine responses (Stefansson et al., 1991). Sex hormones, for example, seem to protect bone tissue from decalcification and deformities, illustrated by the report where immature salmon showed a significantly higher incidence (22.1%) of deformities than maturing males (7.3%) and females (2.7%) (McKay and Gjerde, 1986). It is not unlikely that bone deformities or other noninfectious diseases arise from unfavorable interactions between farming conditions and nutritional regimes.

4. FUTURE PERSPECTIVES

Nutrition and fish health represents a promising field within aquaculture relative to preventive health care to sustain or improve general resistance to stress and infectious diseases. Perspectives include: (1) prevention and restoration of nutritionally related immunodepression by excess nutrient supplementation; (2) reduction of rate and severity of infectious diseases in fish of all sizes and stages of development, but especially juveniles; (3) increased immunocompetence prior to or during vaccination; (4) amelioration of stress-induced immunosuppression; and (5) counteraction of immunosuppression induced by environmental pollution and feed contaminants. Excess supplementation of selected nutrients has been used successfully to potentiate the immune system prior to stressful periods and vaccination. However, to realize this potential there is a need for information on how single nutrients or combinations of nutrients, such as dietary antioxidants, affect detailed reactions or communication in the integrated immunological system and stress response. In some fish species vaccination of single fish may

not always be practical or economically feasible. Thus, use of probiotics has a potential in preventive disease control. However considerable research efforts will be necessary to develop applications to aquaculture.

The growth in the world aquaculture industry is predicted to double the production of fish during the next two decades and the needs for feed resources to cover for the increased production exceed the sustainable catch of marine resources. Care must be taken to choose alternatives, both the types and qualities, as well as feeding strategies, to prevent nutritional challenges to immunity, such as antinutrients, contaminants and lipid fatty acid composition.

Even though the likelihood of nutritional deficiencies in modern aquaculture may be low, nutritional imbalances and diseases may occur as a result of developed husbandry measures and extreme environmental conditions. Based on scientific knowledge, feed composition and feeding regimes should be adjusted according to life cycle, health status and environmental conditions.

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14 Effects of antinutritional factors and mycotoxins on feed intake and on the morphology and function of the digestive system

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It is widely assumed that antinutritional factors and mycotoxins are limited in distribution and of minor consequence, particularly in the feeding of ruminant livestock. It is further assumed that conventional processing methods denature these compounds. However, this chapter provides a challenge to all of these issues. Thus, antinutritional factors and mycotoxins regularly occur in all the major feed raw materials including cereals, protein concentrates and forages. While rumen microbes do confer some degree of protection to herbivores, this mechanism is readily overwhelmed and in some cases exacerbated through the production of reactive metabolites. Furthermore, processing methods tend to be of limited efficacy and mostly applicable to the heat-labile compounds.

This chapter reviews the effects of selected antinutritional factors and mycotoxins on feed intake, gut morphology and function and nutrient utilization. Among the antinutritional factors, the compounds most likely to cause adverse effects include protease inhibitors, lectins, antigenic proteins, particular types of oligosaccharides and polysaccharides, saponins, glucosinolates, condensed tannins, nonprotein amino acids, gossypol and biogenic amines. The mycotoxins capable of reducing feed intake and causing abnormalities in gut morphology and nutrient absorption include deoxynivalenol and ergopeptine alkaloids. However, in view of their ubiquitous distribution there is a need to assess the effects of other mycotoxins including aflatoxins, ochratoxin A and fumonisins and also the consequences of co-contamination.

It is concluded that unless due recognition is apportioned to the adverse effects of antinutritional factors and mycotoxins, prediction models of voluntary feed intake, digestibility and nutrient utilization will continue to be of limited commercial application.

Biology of Nutrition in Growing Animals
R. Mosenthin, J. Zentek and T. Żebrowska (Eds.)

1. INTRODUCTION

The predictive efficacy of nutritional models in animal production is severely constrained by the lack of adequate quantitative data on the characterization of common feeds. Although the nutritive value of these feeds is largely determined by digestibility of the carbohydrate and protein fractions and by the bioavailability of absorbed nutrients, a significant component of quality is associated with the occurrence of antinutritional factors (ANFs) and mycotoxins in raw materials. Hitherto, little cognisance has been attached to these negative constituents due to widespread misconceptions. For example, it has been assumed that ANFs and mycotoxins are limited in distribution, being exceptions rather than the rule and the impact of these substances in nutritional modeling has, consequently, been ignored and dismissed as academic. Another misconception has been that rumen microbial metabolism confers protection to the host animal. Furthermore, it has been assumed that conventional thermal processing methods are effective for counteracting the effects of most if not all of these negative compounds. However, recent research has questioned the validity of all three assumptions. It is now clear that ANFs and mycotoxins regularly occur in all the major feed raw materials including cereals, protein concentrates and forages. Furthermore, while rumen microbes do confer some degree of protection to the host animal with respect to certain compounds, this mechanism is readily overwhelmed. More significantly, the adverse effects of other compounds may, indeed, be enhanced by microbial metabolism, with the result that ruminants become more susceptible to toxicity, compared with nonruminants. Finally, there is ample evidence now to demonstrate the inefficacy of conventional processing technologies to reduce the effects of a wide range of ANFs and mycotoxins.

The objectives of this chapter are to demonstrate the ubiquitous distribution of ANFs and mycotoxins in the important classes of feeds and forages and to review the deleterious effects of these compounds on livestock performance. Particular emphasis will be attached to the effects on morphology and function of the digestive tract in both ruminants and nonruminants. Animal factors such as age and species differences will also be considered. Morphological changes will be exemplified by an examination of histological aberrations and enterocyte architecture. Functional aspects will include changes in motility, as well as immunological, biochemical, digestive and absorptive abnormalities. The impact of ANFs and mycotoxins on rumen fermentation dynamics will form an integral part of this review. Issues relating to the acute toxicology of these compounds have been reviewed by D'Mello (2000) and will not be addressed here.

2. SEMANTICS

Over the past two to three decades, the study of ANFs and mycotoxins has led to a proliferation of new terms. In the following account, an attempt will be made to define this specialized vocabulary in the context of the various compounds to be discussed in this chapter. Although ANFs and mycotoxins are conventionally considered to represent distinct groups of compounds, by virtue of their different biological origins, there are many unifying features. Thus, amino acids are common precursors for certain compounds in both groups. Furthermore, some ANFs share common chemistry with mycotoxins while other compounds in the two groups induce similar responses in animals, precipitating adverse effects on voluntary feed intake (VFI), rumen function, reproduction and immunocompetence (D'Mello, 2000).

2.1. Antinutritional factors

Despite the voluminous data associated with this subject, a formal definition of ANFs has consistently been avoided and any definition now is unlikely to meet with universal approval.

Purists might reserve this term for any dietary component that reduces VFI of animals or impairs digestive function or partitioning and utilization of absorbed nutrients. Such a definition also implies a direct nutritional mechanism in the induction of adverse effects in animals. Depending upon assumed modes of action, compounds within this category have been referred to as ''antagonists'' or ''antimetabolites'', implying structural or biochemical inhibition, while other ANFs have been classified as ''antinutrients'' on the basis of their effects on carbohydrate, protein, vitamin and mineral uptake and utilization. However, in practice, a more inclusive definition is often employed to also cover compounds that exert their effects via indirect systemic mechanisms, for example by modulating endocrine activity, immunocompetence or organ metabolism and function. A synonym often used for this second group of compounds is ''antiphysiological'' factors. It may be argued that the distinction between ANFs with direct and systemic effects is now becoming largely academic as more evidence accumulates to show that antiphysiological compounds may also be associated with digestive dysfunction. Whatever the semantics, the term ANFs is universally applied to compounds originating from the primary and secondary metabolism of plants.

2.2. Mycotoxins

The term ''mycotoxins'' is reserved to those secondary metabolites of fungi with the capacity to induce distinct toxic effects in animals and humans. Superficially, it would appear that there is little scope for disagreement with this definition. However, it is worth noting that certain mycotoxins have been implicated in the precipitation of some plant diseases. Thus, some mycotoxins may also act like ''phytotoxins''. In addition, several mycotoxins are capable of inducing antinutritional effects in animals, particularly through induction of abnormalities in digestive morphology and function. Furthermore, a number of mycotoxins are associated with relatively low toxicity but the antiphysiological and antinutritional effects of these compounds are of much greater practical significance.

3. CLASSIFICATION

Antinutritional factors may be classified according to chemical nature or on the basis of physical properties. In addition, these compounds may be grouped in the context of the adverse effects they cause in animals. However, in the case of mycotoxins, it is more instructive to list individual compounds according to their fungal origin or biosynthetic pathway.

3.1. Antinutritional factors

The classification of the major plant compounds with antinutritional activity in animals is presented in table 1. The diversity of substances with antinutritional activity is striking. It is readily seen that the largest number of ANFs arises from the secondary metabolism of plants. However, ANFs within the protein and polysaccharide fractions provide examples of compounds with profound effects on digestive morphology and function. The inclusion of starch as one of the carbohydrate ANFs may be justified on the basis of its rapid fermentation within the rumen and its predisposition to cereal bloat in growing cattle. In any other context, starch would be considered to be an important source of carbon and energy.

An alternative system of classification is based on thermostability of ANFs. Thus, proteinase inhibitors and lectins are prime examples of heat-labile substances at standard processing temperatures, whereas the carbohydrate ANFs and most of the secondary compounds listed in table 1 are considered to be heat-stable. This is not to imply that heat is without effect on

Table 1

A classification of the important antinutritional factors (ANFs) occurring in plants

Major class	Subclass	Examples of specific ANFs
Proteins	Proteinase inhibitors	Kunitz inhibitor; Bowman-Birk inhibitor
	Lectins	Concanavalin A; kidney bean lectin
	Antigens	Glycinin; β-conglycinin
Carbohydrates	Oligosaccharides	Raffinose and stachyose
	Polysaccharides	β-Glucans
		Galactomannan gums
		Starch
		Hydrolyzable carbohydrates
Amines	Biogenic	Cadaverine
		Putrescine
		Spermidine
		Tyramine
Secondary compounds	Glycosides	Cyanogens
		Glucosinolates
		Isoflavones
		Saponins
		Phytoestrogens
		Pyrimidines
		Ptaquiloside
	Phenolic compounds	Hydrolyzable tannins
		Condensed tannins
		Gossypol
	Amino acids	Mimosine
		Lathyrogens
		Canavanine
	Alkaloids	Quinolizidine
		Glycoalkaloids
		Indolizidine
		Erythrina
	Phytic acid	Phosphoric acid derivative of myo-inositol
	Oxalic acid	Insoluble oxalate crystals

members of the second group. Indeed, in the case of gossypol in cottonseed, thermal procedures may enhance the antinutritional impact by reducing lysine availability.

3.2. Mycotoxins

The principal mycotoxins implicated in animal health conditions and syndromes may be classified according to fungal origin (table 2). It will be noted that *Aspergillus, Penicillium* and *Fusarium* fungi produce most of the mycotoxins known to impact on animal health. However, the production of mycotoxins is restricted to a small number of species within these genera. A significant feature to emerge recently is the coproduction of two or more mycotoxins by the same species of mold, offering the prospect of multiple contamination of certain feeds. The difficulty with the classification shown in table 2 is that ochratoxin A (OTA) can be considered to be an *Aspergillus* and *Penicillium* mycotoxin.

Table 2

Classification of mycotoxins

Major group	Fungal species	Mycotoxins
Aspergillus	*A. flavus; A. parasiticus*	Aflatoxins B_1, B_2 G_1 G_2 (AFB_1, AFB_2 AFG_1 AFG_2)
	A. flavus	Cyclopiazonic acid
	A. ochraceus	Ochratoxin A (OTA)
Penicillium	*P. viridicatum; P. cyclopium*	OTA
	P. citrinum; P. expansum	Citrinin
	P. expansum	Patulin
	P. citreo-viride	Citreoviridin
Fusarium	*F. culmorum; F. graminearum*	Deoxynivalenol (DON; vomitoxin); nivalenol (NIV)
	F. sporotrichioides; F. poae	T-2 toxin
	F. sporotrichioides; F. poae	Diacetoxyscirpenol (DAS)
	F. culmorum; F. graminearum; F. sporotrichioides	Zearalenone (ZEN)
	F. moniliforme	Fumonisins; moniliformin; fusaric acid
Alternaria	*A. alternata*	Tenuazonic acid; alternariol; alternariol methyl ether; altenuene
Neotyphodium	*N. coenophialum*	Ergopeptine alkaloids
	N. lolli	Lolitrem alkaloids
Claviceps	*C. purpurea*	Ergot alkaloids
Phomopsis	*P. leptostromiformis*	Phomopsins

Mycotoxins may also be categorized on the basis of their biosynthetic origin from key primary metabolites. Thus, the polyketide mycotoxins, which include for example the aflatoxins, OTA and fumonisins, are derived from acetyl coenzyme A. The terpene group including deoxynivalenol (DON), T-2 toxin and certain other *Fusarium* mycotoxins, are synthesized from mevalonic acid. Amino acids are incorporated in the formation of a third group of mycotoxins comprising cyclic polypeptides and their derivatives. The ergopeptine alkaloids fall within this third category.

It is salutary to note, however, that mycotoxin production may be strain-specific. Thus, both toxigenic and atoxigenic strains exist within the *Aspergillus flavus* species. It is conventional to subdivide toxigenic fungi into ‘‘field’’ (or plant pathogenic) and ‘‘storage’’ (or saprophytic/spoilage) organisms. *Claviceps*, *Fusarium* and *Alternaria* are classical representatives of field fungi, while *Aspergillus* and *Penicillium* exemplify storage organisms. This distinction is academic since the inoculum for postharvest spoilage of raw materials frequently originates from field sources such as soil or plant debris. Furthermore, mycotoxins from storage fungi are frequently detected on grain, nuts and fruit prior to harvest. Mycotoxigenic species may be further distinguished on the basis of geographical prevalence, reflecting specific environmental requirements for growth and secondary metabolism. Thus, *Aspergillus flavus, Aspergillus parasiticus* and *Aspergillus ochraceus* readily proliferate under warm, humid conditions whereas *Penicillium expansum* and *P. verrucosum* are essentially temperate fungi. Consequently, the *Aspergillus* mycotoxins predominate in plant products emanating from the tropics and other warm regions, while the *Penicillium* mycotoxins occur widely in temperate commodities.

4. DISTRIBUTION IN FEEDS

Both ANFs and mycotoxins are ubiquitous in cereals, oilseed meals, forages and even silage (tables 3 and 4). There appears to be no consistent taxonomic pattern in the occurrence of certain ANFs. Thus, saponins are just as likely to occur in legumes as in grasses, while condensed tannins are found in legumes as well as in sorghum grain. Antinutritional amino acids may occur as different compounds in *Brassica* forage and in certain species of legumes. On the other hand, phytoestrogens, gossypol and glucosinolates appear to be restricted to specific plants.

It will be seen that tropical forage and browse plants contain the greatest diversity of ANFs in comparison with temperate species. Nevertheless, it should be noted that the soybean (*Glycine max*) has been associated with at least six ANFs, namely proteinase inhibitors, antigenic proteins, lectins, phytoestrogens, saponins and oligosaccharides (table 3). Furthermore, in the case of *Brassica* species such as kale and oilseed rape, important ANFs exist in the form of glucosinolates and S-methylcysteine sulfoxide (SMCO). Glucosinolates may occur in both the forage and in the grain of oilseed rape.

Table 3

Distribution of antinutritional factors (ANFs) in plants and feeds

Plant type	Plant family/feed	Plant species	Major ANFs
Forage	Legumes	*Medicago* spp.	Phytoestrogens
			Saponins
		Trifolium spp.	Phytoestrogens
		Lotus spp.	Condensed tannins
	Grasses	*Brachiaria* spp.	Saponins
		Panicum spp.	Oxalates
		Panicum spp.	Saponins
		Setaria spp.	Oxalates
	Cruciferae	*Brassica* spp.	Glucosinolates
			S-methylcysteine sulfoxide
Browse	Legumes	*Acacia* spp.	Condensed tannins
			Cyanogens
		Leucaena leucocephala	Mimosine
			Condensed tannins
Grain	Legumes	*Glycine max*	Proteinase inhibitors
			Antigenic proteins
			Lectins
			Phytoestrogens
			Saponins
			Oligosaccharides
		Lupinus spp.	Quinolizidine alkaloids
			Saponins
			Oligosaccharides
	Guar	*Cyamopsis tetragonoloba*	Galactomannan gums
	Cereals	*Cyamopsis tetragonoloba*	Starch
			β-Glucans
		Sorghum spp.	Condensed tannins
	Cottonseed	*Gossypium* spp.	Gossypol
Other	Cassava	*Manihot esculenta*	Cyanogens
	Silage	Various grass species	Biogenic amines

Table 4

Mycotoxins in animal feed

Category	Type	Mycotoxins
Cereals	Wheat	Deoxynivalenol
		Zearalenone
		Citrinin
	Barley	Ochratoxin A
		Deoxynivalenol
		Zearalenone
	Maize	Aflatoxins
		Fumonisins
Oilseeds	Groundnut	Aflatoxins
	Palm kernel	Aflatoxins
Grasses (endophyte-infected)	Tall fescue	Ergopeptine alkaloids
	Perennial ryegrass	Lolitrem alkaloids
Silage	Maize	Patulin
Other	Lupin stubble	Phomopsins

Co-contamination of feed with mycotoxins has now emerged as an important issue. Thus, maize may contain both aflatoxins and fumonisins, while barley may be adulterated with OTA, DON and zearalenone (ZEN). The degree of contamination may be enhanced in complete feeds compounded from cereal grains and, for example, groundnut cake or cotton-seed cake. In the latter case it should be noted that gossypol may well act as an additional factor to limit animal performance.

5. NATURE OF COMPOUNDS

As might be gleaned from tables 1 and 2, ANFs and mycotoxins belong to diverse classes of chemical compounds. The nature of ANFs has been reviewed in detail by D'Mello (1995) and Kumar and D'Mello (1995), while details of the chemistry of mycotoxins have been presented by D'Mello (2003). Consequently, it is only intended here to summarize the principal characteristics of those ANFs and mycotoxins that impact on VFI, and on digestive morphology and function. The microbial metabolism of these compounds in the rumen is also of relevance in considering antinutritional effects in herbivores.

It is appropriate to begin with proteinase inhibitors since their occurrence in soybean represented a significant nutritional issue. Proteinase inhibitors are classical examples of heat-labile ANFs. They constitute a unique class of proteins with the ability to react in a highly specific manner with a number of proteolytic enzymes in the digestive secretions of animals. The proteinase inhibitors of soybeans are now well characterized and the complete amino acid sequences of two of these (Kunitz and Bowman-Birk inhibitors) have been elucidated. They act in a strictly competitive manner and the complex formed between inhibitor and enzyme is devoid of catalytic activity.

Lectins possess a characteristic affinity for certain sugar molecules or glycoproteins present in the membranes of different animal cells including those of the intestinal mucosa. In contrast to most feed proteins, lectins resist proteolytic degradation *in vivo* and thus substantial quantities of ingested lectins may be recovered intact from the digestive tract and feces of animals fed diets containing one of a number of legume seeds (D'Mello, 1995). The prime

example of a lectin with potent antinutritional and toxic properties is concanavalin A, a component of the jack bean (*Canavalia ensiformis*). This lectin is composed of four identical subunits each containing two metal-binding sites and an additional site for a sugar residue. Due to its particular sugar-specificity, concanavalin A binds mainly to the lower regions of the intestinal villi where membrane proteins of the less-differentiated crypt cells have predominantly polymannose-type side chains.

The antigenic proteins of soybean have been the subject of intense investigation. The bioactive agents have been identified as glycinin and conglycinin. The latter is further subdivided into α, β and γ isotypes, with the β component representing the predominating protein. Antigenic proteins are noted for their resistance to denaturation by standard thermal processing procedures and to attack by mammalian digestive enzymes.

The galactomannan component of guar has been designated with the term ''guaran''. It is a heteropolysaccharide composed of a linear chain of galactose and mannan residues in the ratio of 1:2. Processed guar meal may contain sufficient levels of the gum to induce antinutritional effects, particularly in nonruminants.

Forage and root brassica crops contain a nonprotein amino acid in the form of S-methyl cysteine sulfoxide (SMCO). It may be considered to be an antinutrient due to its structural similarity with the essential amino acid, methionine. The aromatic nonprotein amino acid, mimosine, occurs in the foliage and seeds of the tropical legume *Leucaena leucocephala*. Mimosine is widely regarded as an analog of the physiologically important amino acid tyrosine and its neurotransmitter derivatives, dopamine and norepinephrine (noradrenaline) found in the brain. Other amino acids may give rise to undesirable products. Thus, the amines, putrescine and cadaverine may occur in silage through clostridial fermentation of arginine and lysine, respectively.

Saponins are surface-active glycosides, named on the basis of their ability to form stable soap-like foams in aqueous solutions. The sugar residue generally contains glucose, galactose, glucuronic acid, xylose or rhamnose attached to an aglycone moeity which may be triterpenoid or steroidal.

Phytoestrogens are a polymorphic group of isoflavonoid compounds found primarily in legumes. Soybeans contain relatively high concentrations of the glycosides of isoflavones in the forms of daidzein, genistein and glycitein. In subterranean clover (*Trifolium subterraneum*) and in red clover (*T. pratense*) an important isoflavone is formononetin.

Tannins are complex high-molecular-weight compounds with a sufficiently large number of phenolic hydroxyl residues to form cross-linkages with proteins. Condensed tannins (CTs) are a subset of this group, being dimers or higher oligomers of variously substituted flavan-3-ols. On heating with strong acids, CTs polymerize further to yield small quantities of anthocyanidins, giving rise to the alternative term ''proanthocyanidins''.

Gossypol is a binaphthyl polyphenolic pigment occurring in different parts of the cotton plant, including the seed. It occurs in free and bound forms. The phenolic groups of gossypol are chemically reactive enabling it to bind with minerals and amino acids, particularly lysine.

The aflatoxins are a group of structurally related fluorescent heterocyclic compounds characterized by dihydrofuran or tetrahydrofuran residues fused to a substituted coumarin moeity. This group comprises aflatoxin B_1, B_2, G_1 and G_2 (designated AFB_1, AFB_2, AFG_1 and AFG_2, respectively).

Fusarium fungi are noted for the ability to synthesize the trichothecene group of mycotoxins. Four basic groups are recognized within this category, with types A and B representing the most important members. Type A trichothecenes include T-2 toxin, and diacetoxyscirpenol (DAS). Type B trichothecenes include deoxynivalenol (DON, also known as vomitoxin) and

nivalenol (NIV). All trichothecenes possess a basic tetracyclic sesquiterpene structure with a six-membered oxygen-containing ring and an epoxide group. The production of the two types of mycotoxins is characteristic for a particular *Fusarium* species. However, all toxigenic *Fusarium* species also synthesize zearalenone (ZEN), a phenolic resorcyclic lactone which, therefore, occurs as a co-contaminant with certain trichothecenes.

The fungus *Neotyphodium coenophialum* occurs in symbiotic association with perennial tall fescue. The fungus provides the grass with defensive secondary compounds while the plant serves as a source of essential nutrients for the endophyte. These secondary metabolites are also toxigenic towards animals. Ergopeptine alkaloids, mainly ergovaline, occur in *N. coenophialum*-infected tall fescue.

6. ADVERSE EFFECTS

Depending upon the individual compounds under consideration, ANFs and mycotoxins precipitate both unique and common disorders in animals. Both direct and indirect mechanisms have been implicated in the induction of adverse effects by ANFs (table 5). The direct mode includes ANFs that impact on VFI, digestive function and nutrient utilization. The indirect category includes those compounds that act in an antiphysiological role, affecting the

Table 5

Mode of action and adverse effects of antinutritional factors (ANFs)

Mechanisms	Effects	ANFs implicated
Direct	Depression of voluntary feed intake	Galactomannan gums
		Biogenic amines
		Glucosinolates
		Saponins
		Lectins
		Canavanine
		Mimosine
	Disruption of nutrient supply and utilization	Proteinase inhibitors
		Lectins
		Antigenic proteins
		Galactomannan gums
		Starch
		Saponins
		Condensed tannins
		Gossypol
		Canavanine
		Phytic acid
		Oxalates
Indirect	Organ damage	Mimosine
		S-methylcysteine sulfoxide
		Glucosinolates
		Gossypol
		Saponins
	Endocrine disruption	Glucosinolates
		Phytoestrogens
	Impaired immune function	Antigenic proteins

function of the liver, kidney, central nervous system, reproductive organs and immune system. As further research is undertaken, the distinction between direct and indirect mechanisms may become academic. It is becoming increasingly clear that several ANFs exert their effects by both mechanisms. Thus, for example, gossypol has long been associated with cardiotoxicity in pigs, lambs and calves (Risco and Chase, 1997), but there is credible evidence to indicate adverse effects on digestibility of feed components (Nagalakshmi et al., 2003).

The adverse effects of mycotoxins are commonly associated with acute toxicity and the precipitation of specific syndromes in susceptible animals (table 6). Thus, lethal doses (LD_{50}) for AFB_1 vary from 1–18 mg/kg body weight in laboratory animals; ducklings are particularly sensitive (LD_{50}, 0.5 mg/kg body weight). However, a number of mycotoxins such as DON and ZEN are much less toxic, but they exert potent effects in other respects. Thus, three mycotoxins have been implicated in specific syndromes arising from organ damage (table 6). In addition, ZEN causes hyperestrogenism in cows and infertility in sheep and pigs. It will be appreciated that the deleterious effects of some mycotoxins are mediated via diverse mechanisms not dissimilar to those for a number of ANFs (table 5). For example, both ZEN and phytoestrogens are associated with reproductive dysfunction in mammalian species. Furthermore, and of more relevance to the subject of this chapter, a number of mycotoxins are now ascribed with effects on VFI and gut morphology. In addition, the antinutritional properties of some mycotoxins and their impact on rumen function are emerging as issues worthy of further consideration (table 6).

Table 6

Adverse effects of mycotoxins

Effects	Susceptible animals	Mycotoxins
Acute toxicity	Turkeys and ducklings	Aflatoxin B_1
	Pigs	Ochratoxin A
Syndromes[a]	Turkeys (turkey X disease)	Aflatoxin B_1
	Pigs (porcine nephropathy)	Ochratoxin A
	Pigs (porcine pulmonary edema)	Fumonisins
	Equines (equine leukoencephalomalacia)	Fumonisins
Organ damage	All classes, especially poultry	Aflatoxin B_1
	Pigs	Ochratoxin A
	Pigs and cattle; equines	Fumonisins
	Cattle	Lolitrem alkaloids
	Sheep	Phomopsins
Endocrine disruption	Pigs, cattle and sheep	Zearalenone
		Ergopeptine alkaloids
		Patulin
Depression of voluntary feed intake	Pigs	Deoxynivalenol
Abnormal digestive morphology	Pigs	Deoxynivalenol; T-2 toxin
Reduced digestibility	Cattle	Ergopeptine alkaloids
Changes in nutrient partitioning	Cattle	Ergopeptide alkaloids

[a]Name of syndrome shown in parentheses.

7. DEFENSE PROTOCOLS

The primary function of the alimentary canal is to provide a site for the digestion of feed and the absorption of nutrients. In conducting these functions, the gut becomes exposed to a diverse array of exogenous antigens and other compounds, some of which escape denaturation to be absorbed and, subsequently, to evoke adverse effects in susceptible animals. However, the digestive system can also provide a first line of defense against some of these foreign substances. For example, the secretion of mucus by the goblet cells lining the intestinal tract exerts a protective function by inhibiting the attachment of feed antigens to the mucosal epithelium. The constant renewal of the mucosal lining of the intestinal tract also ensures further protection. In addition, the digestive secretions, including acid produced in the stomach, and the proteolytic activity of several enzymes may also be employed in a defensive role.

The principal defense mechanisms of the gut, however, reside in the extensive lymphoid tissue ramifying through the lamina propria of the intestine. The lymphoid tissue, particularly that located in the Peyer's patches, is responsible for the initiation and expression of the humoral immune response. The cell-mediated arm of the immune system may also become activated following absorption of antigens and other foreign compounds. This mechanism comprises the proliferation of T lymphocytes with the generation of cytotoxic T cells that attack their targets in a highly selective manner (D'Mello, 1991).

The ability of certain animal species to secrete proline-rich proteins (PRPs) in the saliva may constitute another line of defense due to their high affinity for tannins. In response to feed tannins, the deer, a browsing ruminant, produces copious quantities of salivary PRPs. In contrast, grazing ruminants such as cattle and sheep produce minimal amounts of PRPs, which may explain their susceptibility to the antinutritional effects of tannins. If the production of salivary PRPs by goats can be confirmed, then this might provide an explanation for the higher digestibilities of nutrients in goats compared to sheep when both are fed tannin-rich legume leaves, whereas no species differences emerge when low-tannin legumes are fed.

Rumen microbes may also act in a defensive role, but efficacy depends upon the nature of the ANFs or mycotoxins in question. This aspect is of sufficient merit to warrant further discussion later in this chapter.

8. VOLUNTARY FEED INTAKE

A number of ANFs and mycotoxins suppress VFI (tables 5 and 6) via physical or physiological mechanisms, but the severity of effects varies in different animal species. In growing poultry, galactomannan gums reduce VFI physically through their adhesive properties causing increased viscosity of the digesta and reduced transit times. Potato flour also limits VFI in young chicks due to the intrinsic properties of the starch granules. Particular feeds and ANFs have been associated with reduced digesta transit times. For example, high levels of lupins depress digesta transit times in pigs. Dunshea et al. (2001) observed a pronounced inverse relationship between VFI and retention time of digesta and suggested that delayed digestion might inhibit VFI in pigs fed high levels of lupins. The main ANFs considered as VFI depressants were the oligosaccharides, while the alkaloids and saponins were attributed with minor roles. Biogenic amines in some silages may inhibit rumen motility, eructation and VFI. In addition, these amines have been implicated in ketonemia and reduced nitrogen degradability in the rumen (Steidlova and Kalac, 2002).

Voluntary feed intake depression caused by CTs in browse legumes occurs as a result of complex changes in rumen dynamics and digestibility as indicated by benefits in groups receiving a drench of polyethylene glycol (Bhatta et al., 2002).

Food intake of animals is markedly reduced on feeding diets containing unprocessed forms of winged beans and jack beans. Since various gut hormones such as cholecystokinin (CCK) have been implicated in VFI regulation, it is possible that lectins present in these legume seeds may affect appetite by modulating endocrine activity. Whatever the mechanism, the effect of concanavalin A in particular on VFI of young chicks is unmistakable. Diets containing this lectin at concentrations of up to 8.5 g/kg induced a linear decline in VFI, with higher levels eliciting further, but less striking, reductions in appetite (D'Mello, 1995).

Calves given a series of liquid feeds containing heated soybean products with antigenic properties readily develop abnormalities in transit of digesta leading to diarrhea. The effect of antigenic proteins on VFI in mammalian species deserves exploration in view of recent work with rats. Nishi et al. (2003) observed that duodenal β-conglycinin peptone suppressed VFI in a CCK-dependent manner and that this effect was associated with inhibition of gastric emptying.

There is convincing evidence that VFI in pigs and poultry is affected by canavanine (D'Mello, 1995). Thus, when canavanine-rich vetch seeds were fed for 4 days, pigs reduced their VFI to less than 25% of pretreatment values. Inclusion of pure canavanine at equivalent levels precipitated similar effects in terms of the pattern and extent of VFI depression.

Initial studies with pigs indicated that DON was a potent VFI inhibitor and emetic agent. These effects were appropriately represented by the alternative term for this mycotoxin, namely ''vomitoxin''. However, recent studies have only confirmed the anorectic effects. In quantitative terms, marked effects of DON on feed intake inhibition have been observed particularly in the range 6–15 mg/kg diet. At a level of 15 mg/kg, feed intake was only 38% of control values. Even at the higher levels of DON, however, emesis was not observed. A particular feature of the appetite depression is that although the effect can be immediate, varying degrees of recovery over time have been reported, without withdrawal of DON from the feed. Thus, in one trial, partial dose-dependent adaptation to DON-contaminated diets was observed, the effects being reflected in proportionate reductions in weight gain. On the other hand, in another study, VFI depression in the initial 2 days of feeding the contaminated diet was followed by sufficient compensation to permit feed intakes and growth rates equivalent to those in control pigs. Despite these differing responses a distinct dose-related effect of dietary DON on feed intake in pigs is still unmistakable even in the long term (D'Mello et al., 1999). Pair-feeding studies with pigs indicate that at the lower dose ranges, the effects of DON on growth may be explained entirely by the effects on VFI. However, at relatively high dietary concentrations of DON (above 9 mg/kg), the deleterious effects on weight gain may not be fully explained by the suppression in VFI. It should be stressed that most of the recent studies on the effects of DON on VFI in pigs have been conducted with *Fusarium*-contaminated grain and the effects tend to be more pronounced with such diets than with those supplemented with the pure form of the mycotoxin. In at least two instances limited quantities of other *Fusarium* mycotoxins may have occurred as co-contaminants. It may be significant that other trichothecenes including NIV and T-2 toxin have been implicated in both feed refusal and anorexia in pigs. Thus, one trial showed dose-related depressions in VFI within 1 week of feeding diets contaminated with T-2 toxin. By the end of the 3-week study, pigs fed T-2 toxin at 3 mg/kg had feed intakes that were only 59% of control values. Furthermore, fusaric acid can enhance brain metabolism in pigs and a potential interaction with co-occurring DON has been proposed in feed refusal and emesis. Thus, additive or even synergistic effects on feed

intake cannot be ruled out in studies based on the feeding of *Fusarium*-contaminated grain to pigs. Furthermore, it appears that NIV and DAS reduce VFI in broiler chickens whereas DON has no effect on VFI or growth. In mink, feed choice is affected by levels of DON as low as 0.28 mg/kg diet (see D'Mello et al., 1999).

9. DIGESTIVE MORPHOLOGY

A number of ANFs and mycotoxins have the potential to precipitate profound morphological lesions in the buccal cavity and in the gastrointestinal tract. The duration of exposure of animals to these compounds and dose levels in the diet are key factors affecting the severity of these abnormalities. The nature of the compound is also important in determining the type of lesions.

The classical view of lectins is that they exert their deleterious effects via reduced nutrient absorption following extensive structural and functional disruption of the intestinal microvilli (D'Mello, 1995). Thus, concanavalin A enhances shedding of brush-border membranes and decreases villus length thereby reducing surface area for absorption in the small intestine. With other lectins, the lamina propria of the intestine may become infiltrated with eosinophils and lymphocytes.

One of the most striking effects on intestinal morphology and function occurs on feeding soybean antigens to sensitized calves and piglets (D'Mello, 1991). There is a marked degree of uniformity in these lesions, irrespective of animal species (table 7) or of the precise source of the antigenic protein. Detailed analysis of the morphological aberrations shows that while normal villi appear long and slender with tall columnar epithelial cells, those of sensitized neonates are substantially shorter and broader with some evidence of disorganized enterocyte architecture. A consistent feature accompanying these changes is the significant increase in crypt depth following antigen stimulation in both calves and piglets. Villi from sensitized animals are distinguished by prominent infiltration with lymphocytes that may penetrate into the lamina propria. It will be recalled that many of these morphological aberrations are also induced by lectins.

Table 7

Adverse effects of soyabean antigens in the calf and piglet

Adverse effects	Calf	Piglet
Intestinal lesions	Villi short and broad with extrusion of enterocytes	Atrophy of villi
	Increased crypt depth	Crypt hyperplasia
	Lymphocyte infiltration into villi and lamina propria	
Immunological reactions	Elevated serum immunoglobulin concentrations	Synthesis of anti-soybean antibodies
	Cell-mediated reactions	Cutaneous inflammation
Digestive abnormalities	Impaired xylose uptake	Transient depression of xylose uptake
	Decreased net absorption of nitrogen	Decreased digestibility of soybean protein
		Reduced lactase activity
	Diarrhea	Diarrhea

Nivalenol, T-2 toxin and DAS induce gizzard erosions and oral deformities in poultry. In the case of DAS, lesions are directly related to duration of exposure to the mycotoxin and to its concentration in the diet. Feeding a high-fat diet to broiler chicks increases the growth depression caused by DAS, suggesting that such a diet facilitates lipid micellar absorption of the mycotoxin which is then able to inhibit protein synthesis at the ribosomal level. T-2 toxin also induces lesions in pigs, specifically on the mucosa of the pars esophageal region, the incidence being dose-related. In addition, T-2 toxin can cause dermatitis of the snout, nose and buccal commissures in the pig.

10. RUMEN DYNAMICS

Rumen fermentation of plant secondary compounds provides examples of both toxigenic and protective reactions. Hitherto, much emphasis has been placed on the latter function, but there is increasing evidence of susceptibility of ruminants to the adverse effects of a wide range of ANFs.

The absence of salivary PRPs in cattle and sheep implies that these animals would be sensitive to CTs. Adverse effects may be seen in sheep when CTs, such as those in lotus (*Lotus pedunculatus*) or in browse legumes such as *Acacia* species, comprise a significant part of their diets. The evidence is now unequivocal (table 8). Primary manifestations include impaired rumen function, resulting in depressed intake, wool growth and liveweight gain. The deleterious effects on rumen function have been ascribed to complexing of CTs with microbial extracellular enzymes. However, an overall deficit of rumen-degradable N in ruminants fed high-tannin forages and browse may also occur. The inability of rumen bacteria to degrade tannin-bound proteins has been confirmed (McSweeney et al., 1999). Any undersupply of ruminal N would reduce digestibility of structural carbohydrates.

Despite the foregoing, CTs are generally attributed with beneficial properties in ruminant nutrition (D'Mello, 2000). It is consistently maintained that CTs may confer protection from degradation of leaf proteins in the rumen. Fraction 1 (F1) leaf protein predominates in forage and its digestion in the small intestine, as opposed to fermentation in the rumen, would be advantageous to ruminants since F1 is of high biological value, presumed to exceed that of

Table 8

Antinutritional effects of condensed tannins present in legumes

Legume	Animal	Antinutritional effect
Lotus corniculatus	Steer	Reduced DM digestibility in sacco, reduced VFA production *in vitro*
L. pedunculatus	Sheep	Inhibition of rumen carbohydrate digestion and reduced weight gain
Acacia aneura	Sheep	Reductions in: N digestibility, S absorption and wool yield
A. cyanophylla	Sheep	Negative N digestibility, reduced feed intake and body weight loss
A. nilotica (pods)	Sheep	Reductions in: N and NDF digestibility and growth
Calliandra calothyrsus	Goat	Reduced N digestibility in sacco
Prosopis cineraria	Sheep	Reductions in: digestibility of protein, iron absorption, feed intake and wool growth. Weight loss
Lespedeza cuneata	Sheep	Reductions in: ADF, NDF, N and cellulose digestibility. Depressed feed intake

microbial protein. It has been suggested that a complex is formed between CTs and F1 protein through reversible H-bonding which is stable at pH values between 4 and 7, but which readily dissociates on either side of this range. Consequently, it is envisaged that this complex escapes fermentation in the rumen, where the pH ranges from 5 to 7, but dissociates on exposure to gastric (pH 2.5–3.5) and pancreatic (pH 8–9) secretions. The obvious implication is that CTs protect labile plant proteins in the rumen, thereby increasing the supply of high-quality protein to the duodenum. In terms of individual amino acids, it has been shown that *Lotus pedunculatus*, a tannin-containing legume, promoted a higher degree of protection for cysteine, methionine and phenylalanine than *L. corniculatus*, a cultivar low in CTs.

Another recently proposed attribute of CTs relates to amino acid utilization in ruminants. It appears that CTs are able to markedly increase transulfuration of methionine to cystine for body synthetic reactions. This may be important, since cystine is a major component of wool protein.

Condensed tannins have also been implicated in bloat suppression in cattle. Bloat is believed to be primarily due to the formation of a stable foam in which fermentation gases are entrapped. The active foaming agent is the soluble F1 fraction of leaf protein. Bloat in grazing ruminants is often associated with low-tannin pasture legumes such as lucerne and clover, but sainfoin, lotus and tropical browse legumes are considered to confer protection by virtue of their content of CTs. Using *in vitro* methods, it has been demonstrated that CTs from a variety of legumes reduced the compressive strength of protein foams in a dose-dependent manner, irrespective of differences in the chemical structures of these tannins.

The positive attributes of certain plant secondary compounds such as tannins and saponins have come into fresh focus as possible substitutes for feed antibiotics. Plant extracts rich in secondary compounds are perceived as a natural means of modifying rumen fermentation. In this respect, Sliwinski et al. (2002) observed that tannin- and saponin-rich extracts suppressed ammonia in rumen fluid by over 20%. However, variability among batches due to origin of plant material is a limiting factor. In addition, the effects on fiber digestibility, VFI and performance need to be assessed in full-scale feeding trials.

The deleterious effects of SMCO provide a salutary, but not unique, example of how rumen microbes may promote toxigenic reactions. The toxicity of SMCO in cattle and sheep arises after its breakdown by rumen bacteria to dimethyl disulfide oxide, which is then reduced to dimethyl disulfide (D'Mello, 2000). It is the latter metabolite that precipitates the typical symptoms of hemolytic or kale anemia in ruminants. On the other hand, metabolism of mimosine by ruminants depends upon geographical differences in rumen microbial ecology. During this metabolism, 3-hydroxy-4(1H)-pyridone (3,4-DHP) is synthesized; this itself is endowed with deleterious properties, but in addition another isomer, 2,3-DHP may be produced in the rumen. Some rumen bacteria are capable of detoxifying both forms of DHP to as-yet unidentified compounds. Despite these reactions, considerable quantities of mimosine and 3,4-DHP may escape metabolism to appear in the feces. Furthermore, conjugated forms of DHP may also appear in the feces and urine, while mimosine may itself undergo decarboxylation within the tissues of the ruminant to yield mimosinamine, which is then excreted in the urine. Ruminants in Australia, the USA and Kenya are known to lack the requisite bacteria involved in the detoxification of the two DHP isomers. In other regions, where *Leucaena* is indigenous (Central America) or is naturalized (Hawaii and Indonesia), ruminants possess the full complement of bacteria required for DHP degradation, which accounts for the absence of *Leucaena* toxicity in these countries.

Phytoestrogens are actively metabolized in the rumen with the result that the activity of these substances in the rumen depends upon the extent of microbial transformations in the rumen (D'Mello, 2000). Genistein, for example, is degraded to nonestrogenic compounds.

Formononetin, however, is demethylated and reduced to the more estrogenic compound, equol. Rumen microbes may require up to 10 days to adapt to phytoestrogens, so genistein may initially evoke estrogenic effects.

The rumen has a limited capacity to deal with gossypol. The mechanism involves the binding of gossypol to soluble proteins in the rumen. Nevertheless, this mechanism is easily overwhelmed as demonstrated by consistent reports of toxicity in cattle (Risco et al., 2002). The digestibility of feed components such as organic matter may also be suppressed (Nagalakshmi et al., 2003).

Starch is a valuable source of energy and carbon skeletons for all classes of animals. Its classification as an antinutritional factor (table 1) might, therefore, appear as somewhat anomalous. Indeed, it has probably never been viewed in this perspective before. However, rapid cereal starch degradation can reduce ruminal pH and depress fiber digestion. Digestive disturbances such as acidosis, rumenitis and bloat may also occur (Ramsey et al., 2002).

The aflatoxins are actively metabolized in the rumen and in the tissues of animals, with serious implications for human health (D'Mello, 2000). In dairy animals, ruminal transformation of AFB_1 to AFM_1 results in the latter being secreted in the milk. AFM_1 has been ascribed with carcinogenic properties. Carcinogenicity of AFB_1, however, is greater, arising from the formation of a reactive epoxide which then permits covalent binding to cellular components such as DNA to yield genotoxic adducts.

The ovine metabolism of ZEN has been proposed to include the synthesis of at least five metabolites including zearalanone, α-zearalenol, β-zearalenol, α-zearalanol and β-zearalanol (D'Mello, 2000). It should be noted that α-zearalanol is used as a growth promoter with the name zeranol. High levels of some of these forms may be excreted in the urine as glucuronides by grazing sheep.

Patulin often occurs in silage and has been associated with hemorrhagic disorders in cattle. Limited evidence suggests that digestibility of organic matter and dry matter may be reduced by patulin due to effects on fermentation kinetics in the rumen. *In vitro* studies indicate that acetate production can be depressed by patulin, whereas proportions of butyrate and valerate may be increased (Tapia et al., 2002). Similarly, ergovaline in endophyte-infected tall fescue is known for its effects on thermal regulation in cattle, but there is some evidence that it may also reduce digestibility of organic matter (Aldrich et al., 1993) and fiber (Humphry et al., 2002). The rumen is also an important site for the absorption of alkaloids associated with fescue toxicosis (Hill et al., 2001).

11. INTESTINAL FUNCTION

Although the proteinase inhibitors depress proteolysis in the digestive tract, the adverse effects on growth are thought to occur via more complex mechanisms. It is envisaged that inactivation of trypsin elicits the release of CCK which stimulates pancreatic production of digestive enzymes, including trypsin and chymotrypsin. The net effect is the loss of endogenous protein rich in the sulfur-containing amino acids. It is this depletion of critical amino acids which reduces growth. A concomitant enlargement of the pancreas may also occur, particularly in broiler chickens (D'Mello, 1995). Inclusion of legume grains into diets of weaned piglets has been found to enhance ileal losses of three 25, 27 and 30 kDa proteins (Salgado et al., 2002). Their contribution to ileal losses of essential amino acids in pigs remains to be established.

The extensive morphological lesions that occur in the small intestine of animals sensitized to antigens significantly impair digestive and absorptive function. The effects arise by virtue

of markedly reduced surface area in the gut, through abnormalities in the maturation of enterocytes and through reductions in the secretion of key enzymes such as lactase (table 7). For example, xylose absorption, an indicator of absorptive competence, is substantially higher in milk-fed than in soybean-fed calves with these differences diverging with age. Similar effects are seen in piglets with soybean-hypersensitivity, except that absorptive competence is restored relatively early in the postweaning period.

Guar gum causes distinct effects on intestinal function in nonruminants, leading to reduced digestion and absorption of feed components, particularly starch. It is possible that enzyme–substrate interactions may be inhibited initially due to a physical coating of starch granules with the gum. Once starch digestion commences, however, increased digesta viscosity, caused by solubilization of the gum, may impede absorption of digestion end-products (D'Mello, 1995).

Various carbohydrate fractions of feed components have been associated with antinutritional effects and with digestive disorders. Soybean oligosaccharides are not denatured by standard processing methods and have been linked with reduced digestibility in poultry. Removal of these fractions from soybean meal can increase metabolizable energy values by up to 20% in poultry diets. However, in pigs the effects of soybean oligosaccharides on nutrient digestibility are thought to be minimal (Smiricky et al., 2002). In horses, hydrolyzable carbohydrates in grain concentrates and other feeds have been implicated in disorders such as laminitis and colic (Hoffman et al., 2001). β-Glucans are not hydrolyzed by the intestinal enzymes of poultry (Yu et al., 2002). The gel-forming characteristics promote antinutritional effects by increasing digesta viscosity and by decreasing contact between digestive enzymes and feed substrates. Nutrient absorption is thus depressed and management problems arise from the production of sticky excreta.

Saponins are associated with marked effects on the functioning of cell membranes (Francis et al., 2002). Thus, saponins cause increased permeability of mucosal cells of the intestine and inhibit active transport by these cells. Glucose transport across the brush border of the intestine can be impaired by certain saponins. Protein digestibility is also reduced, probably due to the formation of saponin–protein complexes. In addition, uptake of substances, such as allergens, may be increased.

There is growing evidence that mycotoxins may impact on digestive function. Thus, DON has long been associated with VFI depression in pigs, but it is also capable of modulating the activities of intestinal transporters. Depending upon dose level, DON can cause aqueous or inflammatory diarrhea, the latter condition arising also from the destruction of the epithelial barrier of the intestine (Maresca et al., 2002).

12. NUTRIENT UTILIZATION

The adverse effects of ANFs on nutrient utilization are best exemplified by the action of canavanine, present in the jack bean. Canavanine is structurally similar to arginine, an essential amino acid for poultry. When jack beans form a substantial proportion of a broiler diet, the resulting depression in growth of chicks can be explained on the basis of a canavanine–arginine antagonism. Such an interaction involves increased degradation of arginine and possibly synthesis of aberrant tissue proteins resulting in increased protein turnover. The overall effect is a reduction in the efficiency of utilization of absorbed arginine. In the feeding of cassava root or leaf meal, there may be an increased need for the sulfur amino acids, due to the involvement of cysteine in detoxification of HCN from these sources.

Mycotoxins may also affect nutrient partitioning and utilization, but the evidence to date is relatively sparse and might be mediated indirectly via endocrine mechanisms that modulate

nutrient metabolism. Thus, Browning et al. (2000) suggested that ergotamine is capable of altering plasma concentrations of glucagon and cortisol, important hormones in nutrient utilization.

13. CONCLUSIONS

This review has demonstrated that antinutritional factors and mycotoxins are distributed in cereals, protein feeds and forages of virtually every description. For example, barley grain contains β-glucans and may also become contaminated with deoxynivalenol and ochratoxin A. In soybeans, proteinase inhibitors, lectins and antigenic proteins are the principal antinutritional factors, whereas in cottonseed, gossypol and the aflatoxins are often the primary limiting factors. Rapeseed meal and lupins, respectively, contain glucosinolates and quinolizidine alkaloids. Brassica forages contain glucosinolates and S-methylcysteine sulfoxide, while browse and pasture legumes are renowned as sources of important antinutritional factors including condensed tannins, toxic amino acids, saponins and phytoestrogens. Depending upon species, grasses may contain saponins, oxalates and ergopeptine alkaloids, while the production of biogenic amines in certain silages has long been recognized. Although many of these antinutritional factors and mycotoxins are associated with distinct conditions and syndromes in farm animals, there is increasing evidence to indicate common effects on feed intake, gut morphology, rumen dynamics and intestinal function. The ability of rumen microbes to prevent or overcome the effects of antinutritional factors and mycotoxins is limited. As long as these deleterious compounds are ignored, reliable predictions of voluntary feed intake and livestock growth will remain an enigma.

14. FUTURE PERSPECTIVES

Although considerable advances have been made in our knowledge of the effects of antinutritional factors and mycotoxins, a number of issues remain unresolved. For example, the proteinase inhibitors of soybeans have long been recognized as the primary antinutritional factors affecting digestive function in nonruminants. However, recent studies indicate a significant role for lectins and antigenic proteins in the etiology of postweaning digestive disorders of piglets and calves. Nevertheless, there is a need to determine the practical significance of other constituents of soybeans including phytoestrogens, saponins and oligosaccharides on feed intake, digestive function and nutrient partitioning.

Another issue requiring elucidation centers on the protective role of condensed tannins in rumen fermentation of protein. It is presumed that tannins released post-ruminally do not exert any deleterious effects in the intestinal tract since pH conditions do not allow further reactions with dietary or endogenous proteins. However, it has been observed that the condensed tannins of *Prosopis cineraria* retain their capacity to precipitate pepsin at pH 2.0 and, consequently, an important gastric digestive enzyme may be inhibited. Furthermore, the benefits of protein protection may be offset, in part, by a reduction in the fractional absorption of essential as well as nonessential amino acids from the small intestine. A further question centers on the dissociation of the protein–tannin complex at pH values below 4 and above 7. Since these pH criteria also occur in nonruminants, the issue arises as to why such a dissociation of the complex does not occur in these animals or, if it does, why adverse effects are observed in ruminants but not in nonruminants.

With regard to saponins, glucosinolates, quinolizidine alkaloids and gossypol, there are still no clear indications as to their effects on gut morphology and function or on nutrient

partitioning and utilization. Due to the distribution of these compounds in important feeds, it is pertinent to ascertain the effects on rumen fermentation and dynamics.

Much work still needs to be undertaken on the effects of particular mycotoxins on feed intake, rumen function and nutrient utilization. Ochratoxin A and the fumonisins deserve further attention in this respect, as most work in the past has focused on specific syndromes associated with acute exposure. In practice, however, it is more likely that livestock are chronically exposed to much lower levels and, furthermore, to combinations of different mycotoxins. Thus, maize-based feeds may be contaminated with aflatoxins and fumonisins. In barley-based diets animals may be exposed to deoxynivalenol, ochratoxin A and zearalenone. Evaluating the effects of multiple mycotoxin contamination on gut morphology and function represents a major challenge for the future.

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15 Decontamination and detoxification of mycotoxins

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Agricultural commodities are frequently contaminated by mycotoxins worldwide. Not only the economic loss, but also the health risks for humans and animals cause the necessity to avoid or at least reduce the mycotoxin load. Fungal growth and mycotoxin formation cannot be avoided, therefore many methods were developed to decontaminate feedingstuffs, to detoxify mycotoxins or reduce the bioavailability of mycotoxins. This chapter reviews these methods and informs about the applicability in practice.

1. INTRODUCTION

Prevention of fungal growth on agricultural commodities and avoiding mycotoxin contamination are still of utmost importance. Strategies to reduce fungal growth include plant breeding for mold resistance (Snijders, 1994), considerate harvesting and careful storage practices. Nevertheless, under certain climate conditions contamination is unavoidable. Therefore, development and implementation of efficient decontamination methods become very important. Decontamination procedures should remove, destroy or inactivate mycotoxins, not lead to toxic metabolites, byproducts or substances in feedingstuffs, should retain the nutrient value and palatability of the feedingstuffs, not result in significant alterations of the product's technological properties and, if possible, destroy fungal spores (Park et al., 1988). Reduction of the concentration of the mycotoxin itself is not a sufficient criterion for the suitability of a decontamination method, because the possible formation of toxic metabolites needs also to be considered. The efficacy of decontamination should, therefore, not only be checked by chemical analysis, but also by biological tests. Finally, it is also important that the procedure is economically feasible, readily available and easily practicable. An effective way to avoid the intake of mycotoxins would be to remove or destroy the contaminated feedingstuff, which is in most cases not possible for economic reasons.

Fungal growth and the formation of mycotoxins depend upon climate conditions. The most important mycotoxins, aflatoxins, are produced by Aspergilli only under warm and humid conditions and are, therefore, restricted to specific climate regions. Other mycotoxins like

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Fusarium toxins and Ochratoxin A are typical for moderate climate areas (Leibetseder, 1995). Great efforts were undertaken to develop methods for decontamination of (primarily) aflatoxins, especially in the USA (Phillips et al., 1994), and later on for decontamination of *Fusarium* toxins and Ochratoxin A in Europe (Müller, 1982, 1983) and Canada (Charmley and Prelusky, 1994).

The decontamination methods can be categorized as physical, chemical or biological. Some of these methods have proved to be very successful, others less so, and even some of the most successful ones are difficult to implement on a commercial basis (Charmley et al., 1995a). Numerous reviews and publications have summarized the approaches already in use and being developed to minimize mycotoxin contamination (Agricultural Research Service, 1997a, 1997b; CAST, 1989, 2003; Jackson and Bullerman, 1999; Robens, 1990; Scott, 1998).

2. METHODS OF DECONTAMINATION AND DETOXIFICATION

2.1. Physical methods

A number of physical procedures like mechanical separation, cleaning, washing, dehulling, polishing and heat treatment have been used, with varying effects on decontamination or detoxification. The success depends on the initial degree of contamination and the distribution of mycotoxins throughout the grain.

2.1.1. Mechanical separation

In agricultural commodities mold infection is often not homogeneous and therefore separation of altered parts, or sorting of contaminated kernels can decrease toxin levels to a certain extent. In general, these methods are not very efficient due to incomplete removal of the contaminated parts (Phillips et al., 1994). Only peanut production made successful use of mechanical sorting, to remove the small, discolored and broken kernels with which aflatoxin contamination is correlated.

2.1.2. Sorting

Sorting is performed manually, mechanically, photoelectrically or by a combination of these methods (Dickens and Whitacker, 1975). Hand sorting is time consuming and commercially impractical, and may involve the loss of normal peanuts. Some highly contaminated kernels may appear normal, which may result in a contaminated product. The efficiency of electronic color sorting is variable resulting, on average, in the removal of 70% of the aflatoxin contaminated kernels. The efficacy of mechanical sorting in peanuts can be high (reduction of aflatoxin from 150 μg/kg to <3 μg/kg; Wogan, 1968).

2.1.3. Density segregation

In some cases moldy mycotoxin-contaminated grains show differing physical properties from normal grains and can be separated by density segregation in liquids (e.g. water, sucrose solution, sodium chloride solution, hydrogen peroxide solution) or fractionation by specific gravity. Segregation by floating, due to the different density of aflatoxin-contaminated and noncontaminated kernels, has been used successfully in cotton seed (Koltun et al., 1974), corn (Huff, 1980; Huff and Hagler, 1982) and peanuts (Henderson et al., 1989). However, in other

experiments the results were rather poor and therefore this method is not very reliable (Brekke et al., 1975a; Goldblatt and Dollear, 1977). Density segregation in hydrogen peroxide solutions can separate aflatoxin-contaminated from normal peanuts, but this treatment is not very effective unless the initial concentration of aflatoxin is <200 μg/kg and the reaction time and hydrogen peroxide concentration are strictly optimized (Clavero et al., 1993).

The concentrations of deoxynivalenol (DON) and zearalenone in sorghum (Babadoost et al., 1987) and in corn and wheat (Jackson and Bullerman, 1999) were also reduced by density segregation after removal of damaged grain. Tkachuk et al. (1991) removed tombstone kernels associated with trichothecenes, from wheat infected with *Fusarium* blight by specific gravity table. These methods led to reductions in DON, or DON and zearalenone concentrations, of 40–100%. An aerodynamic separation technique effectively decreased DON levels in contaminated wheat (de Koe, 2001). Combinations of density segregation with several other methods were reported to be effective in reducing mycotoxin concentration in grains. Rotter et al. (1995) observed a diminished toxicity to pigs from DON and zearalenone-contaminated corn after rinsing and density segregation by floating. Vasanthi and Bhat (1998) used hand picking and density segregation of moldy grains and reduced the aflatoxin and fumonisin content by up to 90%, while Malone et al. (1998) decreased fumonisins to 60% by screening and gravity separation of corn.

2.1.4. Milling

Dry milling of corn and rice contaminated with aflatoxin resulted in fractions containing different concentrations of aflatoxin. The highest concentration of aflatoxin B_1 was found in the seedlings, skin and degermer fines of corn (Brekke et al., 1975b), while in rice, 60–80% of the aflatoxin B_1 content was detected in the rice bran after milling (Schroeder et al., 1968). Similar results were obtained with zearalenone: only 20% of the original amount was found in the starch fraction whereas most was present in the skin and the fat fraction (Bennett et al., 1976). Leibetseder (1982, unpublished data) observed a similar fractionation in oats. A much higher proportion of the ochratoxin A was found in the bran and offal fractions from hard wheat than from soft wheat. Conversely, a much higher proportion of the ochratoxin A was found in the reduction flour from soft than from hard wheat (Osborne et al., 1996). After wet milling of corn about 39–42% of the total amount of aflatoxin appeared in the swelling water, 30–38% in the cellulose fraction, 15% in the gluten fraction and the rest in the seedlings. The starch fraction only contained 1% (Yahl et al., 1971). Similar results were reported by Bennett et al. (1978) with zearalenone. T-2 toxin was distributed to the fraction in the following way: about two-thirds in the swelling water, 4% in starch, the remainder in seedlings, gluten and skin (Collins and Rosen, 1981).

In general, it can be assumed that in corn, rice and oats, but probably in all kind of cereals, the concentration of mycotoxins is higher in the milling by-products than in the source material. The same problem exists with oilseeds. Enrichment of aflatoxins takes place in the by-products of oil production (Basappa and Sreenivasamurthy, 1974; Frank, 1974). This has to be considered when higher amounts of such by-products are used in animal nutrition.

2.2. Heat treatment

Pure dry aflatoxins are stable at temperatures up to the melting point (Feuell, 1966; Bösenberg, 1970). The melting points of the different aflatoxins (B_1, B_2, G_1, G_2, M_1, M_2) are between 230°C and 299°C (Milczewski et al., 1981). Aflatoxins are only destroyed to a minor

extent by heat treatment, e.g. boiling water or autoclaving (Christensen et al., 1977) or heat treatment in oil. Aflatoxin B_1 content in oil was only reduced by 20% after 0.5 h at 160°C, with no further change by continued heating (Hanssen and Hagedorn, 1969). Peers and Linsell (1975) did not find a decrease of the aflatoxin content in oil despite heating up to 250°C, which is close to the melting point. If the contaminated oil contains water, or is not refined, heat treatment can reduce the aflatoxin content to differing degrees (Dwarakanath et al., 1969). Partial decomposition of aflatoxin may be accomplished by oil roasting or dry roasting of peanuts, corn nuts and oilseed meals. Depending on the roasting conditions (temperature, duration), the water content and the initial aflatoxin content in the source material (Mann et al., 1967; Lee et al., 1968) decreases ranging from 40–50% (El-Kady and Farghaly, 1981; Waltking, 1971), 45–83% (Lee et al., 1969) and 95% (Hanssen and Hagedorn, 1969), have been reported.

For practical agriculture, the influence on the mycotoxins of drying corn with hot air (>100°C) is of great interest. A reduction of aflatoxin concentrations by two-thirds (Conway et al., 1978), and in some experiments by up to 85% (Hale and Wilson, 1979) could be achieved by this treatment.

Heat treatment of wet material reduces the aflatoxin concentration more than in dry materials. This might be explained by hydrolytic processes, which open the lactone ring, by decarboxylation and by other decomposition processes (Coomes et al., 1966). Temperature and the duration of treatment influence the extent of detoxification. There is generally no destruction of aflatoxin at temperatures below 60°C (Mann et al., 1967; El-Kady and Farghaly, 1981), although at low pH values (buffer solution of pH 1) destruction of aflatoxin B_1 and G_1 occurs already at 40°C (Pons et al., 1972). Aflatoxin in fruit juices and aqueous solutions is reduced by 8–20% after heating for 20 minutes at 120°C (Frank, 1968; Tauchmann and Leistner, 1969; Kiermeier and Rumpf, 1975), while Coomes et al. (1966) reported the complete disappearance of aflatoxin after 4 h. In highly contaminated (7 mg/kg) peanut meal with 10% moisture the aflatoxin B_1 concentration was reduced to about 5% of the initial content after 4 h autoclaving (Coomes et al., 1966), while at lower concentrations (0.76 mg B_1 and 0.7 mg G_1/kg) 60 minutes autoclaving and 90 minutes autoclaving (0.26 mg/kg and 0.18 mg/kg of B_2 and G_2), respectively, destroyed the aflatoxins completely (El-Kady and Farghaly, 1981). Heat treatment of rice in water (1:4) at 120°C for 5 minutes reduced the aflatoxin B_1 concentration (initial: 0.04–4.0 mg/kg) by 68–76% (Rehana et al., 1979).

Different results are reported about the heat treatment of aflatoxin M. Aflatoxin M concentration was not changed after 30 minutes at 60°C (Stoloff et al., 1975). Pasteurization and cylinder drying did not change the toxicity of aflatoxin M in ducks (Allcroft and Carnaghan, 1963; Allcroft and Roberts, 1968). On the other hand, Purchase et al. (1972) and Kiermeier and Mashaley (1977) observed a reduction of aflatoxin M by between 12% and 86% depending on the kind of heat treatment. Most of the studies are based on chemical analysis without toxicological evaluation. Kiermeier and Ruffer (1974) showed that compounds formed by opening of the lactone ring and decarboxylation were still toxic in broiler chickens. The effects of three extrusion variables (flour moisture, extrusion temperature and sodium metabisulfite addition) were analyzed according to a two-level factorial design (Cazzaniga et al., 2001). The process was only partially effective for the decontamination of aflatoxin B_1 (50 µg/kg) and is, therefore, of limited value for aflatoxin B_1, even if metabisulfite is added.

Among the mycotoxins known at present, trichothecens are probably the most stable compounds against heating. Trichothecens cannot be destroyed by the kind of boiling normally used for food or feed, but heat treatment for a longer period of time opens the epoxide ring, which causes a reduction of toxicity (Bamburg and Strong, 1971). But in the study of

Cazzaniga et al. (2001), with samples of corn flour experimentally contaminated with deoxynivalenol (5 mg/kg), extrusion cooking was effective for inactivation (higher than 95%) under all the conditions assessed.

Zearalenone is also relatively heat stable (Mirocha et al., 1967; Bennett et al., 1980), but the stability depends on the pH value of the substrate. Heat stability is much lower in alkaline buffer solutions (Lasztity et al., 1977; Matsuura et al., 1979) and furthermore, adding oxidants increases heat instability (Matsuura et al., 1979).

Heat treatment of ochratoxin A showed different effects. Harwig et al. (1974) and Chu et al. (1975) only found a reduction of the ochratoxin A concentration of about 10% after autoclaving or boiling whereas Trenk et al. (1971) observed a reduction of up to 89% after autoclaving. Levi et al. (1974) treated contaminated wheat and found a similar reduction of ochratoxin A. One of the factors influencing the heat stability of ochratoxin A seems to be the moisture content of the substrate. Water inhibits the decomposition of ochratoxin A by heat treatment (Osborne, 1979). The reduction of the ochratoxin A content by heat treatment does not necessarily indicate a reduced toxicity because the toxic effects of the formed compounds are not known.

Patulin and penicillic acid are also remarkably resistant against heat treatment (Scott and Somers, 1968), but this is dependent on the pH value. Heat resistance was higher at low pH values (Lovett and Peeler, 1973). On the other hand, citrinin is decomposed by heat to a relatively high extent. Autoclaving (120°C for 120 min) reduced the citrinin concentration in corn and the toxicity by about 80%, while normal drying of corn at 70°C was also effective with regard to the concentration (Jackson and Ciegler, 1978; Nelson et al., 1980), but not to the toxicity (Roberts and Mora, 1978). Rubratoxin B is also degraded quickly at temperatures above 65ºC (Engstrom and Richard, 1981).

The stability of fumonisin B_1 and fumonisin B_2 during the processing of corn flakes was investigated using three different methods for analysis of the naturally contaminated raw material (corn flour), the intermediate product (extruded, but not roasted corn flakes), and the final product (roasted corn flakes) (De Girolamo et al., 2001). About 60–70% of the initial amount of fumonisins were lost during the entire cycle of corn flake processing, with less than 30% losses occurring during the intermediate extrusion step (70–170°C for 2–5 min). Despite the fact that it was shown that some effective fumonisin decontamination occurred during corn flake processing, more work is needed to identify the thermal breakdown products of fumonisins and their relevant toxicity.

It is still unclear to what extent detoxification of mycotoxins is possible by the heat treatment that is commonly used in the processing of feed (toasting of soybean meal, pelleting, artificial drying of cereals). Mycotoxin resistance to heat differs from toxin to toxin and depends on the conditions, such as temperature, duration of treatment, moisture content, pH and concentration of the mycotoxin. Artificial drying of corn is able to reduce aflatoxin and citrinin to a certain extent, which might have some relevance in tropical and subtropical areas. In areas with moderate climatic conditions, e.g. Europe, where heat-resistant fusarium toxins are predominant, artificial drying of corn is not effective in reducing these mycotoxins.

2.3. Irradiation

Different results were obtained after irradiation of aflatoxin-containing commodities with UV light. Destruction and disappearance of mutagenicity of aflatoxin was observed by a number of researchers (Kleinwächter and Koukalova, 1979; for other references see Müller, 1982), whereas Feuell (1966) found no change in fluorescence or toxicity and Stark et al. (1990)

reported that exposure of aflatoxins to UV light activates these chemicals to mutagens. Because UV rays do not penetrate deeply, the application of UV irradiation in practice is questionable. In tropical and subtropical areas exposure to sunlight is effective to a limited extent (Okonkwo and Nwokolo, 1978; Shanta and Sreenivasamurthy, 1981). UV light decreased the concentration of aflatoxin M_1 in contaminated milk by almost 90% in the presence of 0.05% peroxide, compared to only 60% without peroxide (Yousef and Marth, 1989). However, there is concern by some that this treatment could cause peroxidation leading to more toxic products.

Microwave irradiation has also been suggested as a method for the detoxification of certain mycotoxins, especially aflatoxins (Farag et al., 1996) and T-2 toxin (Stahr et al., 1987). Irradiation with ionizing rays needs high dosages for aflatoxin destruction. Two to five Mrad were necessary to reduce aflatoxins by 90% in cereals (Frank and Grunewald, 1970). Van Dyck et al. (1982) needed 1–2 Mrad for the total inactivation of aflatoxin B_1 in the Ames test, but Feuell (1966) did not find a reduction of toxicity of aflatoxin in chicken embryos after a 2 Mrad irradiation. A certain sensitivity to light is described for ochratoxin A by Neely and West (1972) and for citrinin by Neely et al. (1972).

2.4. Extraction

The removal of mycotoxins from contaminated feed commodities by solvent extraction is well known and widely used in mycotoxin analysis. Solvent mixtures, therefore, can be efficiently used for toxin reduction in feed with minimal effects on the nutritional value of the contaminated commodity (Goldblatt and Dollear, 1977; Rayner et al., 1977). Aflatoxins can be removed by extraction with methanol (Sargeant et al., 1961; Feuell, 1966; Stahr and Obioha, 1982), ethanol (Rayner et al., 1970), acetone, chloroform (Feuell, 1966), with a mixture of acetone/water (Pons and Eaves, 1967; Gardner et al., 1968), hexane/acetone/water (Robertson et al., 1965; Vorster, 1966), isopropanol/water (Rayner and Dollear, 1968) and with water-saturated methoxymethane (Aibara and Yano, 1977). The practicability of this method of detoxification depends on the economic conditions and for that reason it is impractical for most applications (Shantha, 1987).

The extraction with aqueous solutions would be much cheaper and without nutritionally adverse effects if the reduction of the mycotoxin content is sufficiently complete. Sreenivasamurthy et al. (1965) reduced the aflatoxin content of groundnut meal by 80% using 1% $CaCl_2$ and by 100% with 1% $NaHCO_3$ aqueous solutions. Shantha and Sreenivasamurthy (1975) removed 85% of the aflatoxin in groundnut oil with 10% NaCl aqueous solution at 90°C. Aflatoxin is almost insoluble in water, therefore, the reduction by washing with aqueous solutions was probably achieved by removing contaminated particles. Fusarium toxins could also be removed from the surface of kernels by washing with water. Feed intake and body mass gain in pigs (Forsyth et al., 1976) and feed intake in rats (Uriarte et al., 1976) were improved when moldy corn was washed with water for 48 hours. The water used for washing caused feed refusal and vomiting in pigs (Curtin and Tuite, 1966).

2.5. Chemical methods

Numerous chemicals have been tested for the reduction of mycotoxin contents in agricultural commodities and products by degradation of the toxins, but only some have proved successful and very few are used in practice. Other chemicals are able to reduce the bioavailability of mycotoxins by adsorption of toxins and reduction of toxin absorption in the gastrointestinal tract.

2.5.1. Degradation

Chemicals found to be effective in degrading mycotoxins include acids, bases, aldehydes, bisulfite, oxidizing agents, other chemicals and various gases. Some of these effective chemical treatments cannot be used in practice because the chemicals themselves (Leibetseder et al., 1985) or the degradation products are potentially harmful and/or significantly alter the product quality. Every effort has been made to detoxify aflatoxins, therefore, numerous methods have been tested in this regard including ammoniation, ozonation and reaction with sodium bisulfite or chlorine gas (Feuell, 1966; Trager and Stoloff, 1967; Mann et al., 1970; Goldblat and Dollear, 1977, 1979; Moerck et al., 1980; Anderson, 1983; Altug et al., 1990; Park et al., 1988; Frayssinet and Lafarge-Frayssinet, 1990; Hagler, 1991; Hammond, 1991; Samarjeewa et al., 1991; Phillips et al., 1994). However some methods have been tested also for other mycotoxins.

2.5.2. Treatment with oxidizing agents

Aflatoxin degradation is achieved by treatment with NaOCl (Draughon and Childs, 1982), but one needs to be aware that the mutagenic and cancerogenic aflatoxin B_1-2,3-dichloride may be formed, although this can be avoided by adding acetone (Castegnaro et al., 1981). In some experiments it could be demonstrated that not only the concentration of aflatoxin was reduced, but also the toxicity was lowered in chicken embryos, mice and rats (Fischbach and Campbell, 1965; Trager and Stoloff, 1967; Yang, 1972). Aflatoxin could be removed completely when rice meal was treated with a mixture of 16.5% NaCl and 1% NaOCl for 24 hours (Okonko and Nwokolo, 1978), while hydrogen peroxide reduced alfatoxin concentrations in corn (Chakrabarti, 1981), peanut meal (Sreenivasamurthy et al., 1967) and milk (Applebaum and Marth, 1982).

The concentration and the toxicity of zearalenone were also reduced after treatment with an aqueous solution of H_2O_2 (Lasztity et al., 1977). The efficiency of H_2O_2 for destruction of zearalenone in contaminated corn was studied at different concentrations (3%, 5% and 10%) and it was found that the disappearance of zearalenone was dependent upon the concentration of H_2O_2, temperature and period of exposure. The highest degradation (83.9%) was seen with 10% H_2O_2 at 80°C for 16 hours, followed by 75% degradation at the same conditions for 8 hours, while the lowest degradation was obtained with 3% H_2O_2 at 50°C for 2 hours (Amaa, 1997). Matsuura et al. (1979) confirmed that zearalenone is destroyed by oxidation with ammonium persulfate and that raising the temperature accelerates the oxidative destruction. Some negative results may have been caused by too short treatment at low temperatures.

Another effective method of degradation is based on reaction with ozone gas. Studies have demonstrated that O_3 gas is able to degrade aflatoxins in different commodities (Dollear et al., 1968; Dwarakanath et al., 1968) and in aqueous solutions (*in vitro* studies; Maeba et al., 1988). The results in biological tests (body mass gain in ducks, protein eficiency ration in rats), however, were not very satisfying (Dollear et al., 1968). Ozone degradation has been shown to be effective also against other mycotoxins such as DON (Young, 1986; Young et al., 1986) and moniliformin (Zhang and Li, 1994). Rubratoxin B seems to be highly sensitive to oxidation (Hayes and McCain, 1975). A highly concentrated ozone produced by an electro-chemical method (Rogers et al., 1992) was able to degrade and detoxify several mycotoxins *in vitro*, including aflatoxins, cyclopiazonic acid, ochratoxin A, patulin, secalonic acid D and zearalenone (McKenzie et al., 1997). It was demonstrated that the method was effective under practical conditions to significantly degrade highly concentrated aflatoxins in corn and to

significantly protect turkeys from the deleterious effects of aflatoxins (McKenzie et al., 1998). Fumonisin B_1 was also degraded, but detoxification could not be demonstrated in bioassays. This method is applicable in practice because of the minimal cost and the minimal destruction of important nutrients.

2.5.3. Treatment with alkalines

Aflatoxins are unstable under alkaline conditions (Manabe and Matsuura, 1972; Kiermeier and Ruffer, 1974; Itoh et al., 1980), the first step of degradation being the opening of the lactone ring. Degradation of aflatoxins using ammonia has been extensively studied in laboratory experiments as well as in field trials. This kind of detoxification has also been reviewed by several authors (Goldblatt and Dollear, 1979; Anderson, 1983; Müller, 1983; Palmgren and Hayes, 1987; Park et al., 1988). Ammoniation can be performed with gaseous ammonia or ammonium hydroxide and is so successful that a reduction of the aflatoxin concentration by more than 99% could be demonstrated in some cases (Dollear et al., 1968; Masri et al., 1969; Gardner et al., 1971; Brekke et al., 1977, 1979; Park et al., 1984; Phillips et al., 1994; Weng et al., 1994). The efficacy of the process depends on the temperature, pressure, duration and the substrate. In one study it was determined that the moisture level of the product (corn) and holding temperature were the crucial factors that influenced the efficacy of aflatoxin decontamination by ammoniation (Weng et al., 1994). Because the first step of the reaction (opening of the lactone ring) is reversible, it is important to allow the reaction to proceed to completion. The procedure can be performed at high pressure and high temperature, or at atmospheric pressure and ambient temperature, which needs a longer treatment. Different results of toxicological studies with ammoniated commodities contaminated with aflatoxin may be caused by too short treatment and other factors. It is, therefore, advisable to check the suitability of the procedure for each commodity under defined conditions. The reduction of the aflatoxin B_1 content in the feed for dairy cows leads to a decrease of the aflatoxin M_1 concentration in the milk (McKinney et al., 1973; Thiesen, 1977; Lough et al., 1979; Price et al., 1982; Hoogenboom et al., 2001b). Park et al. (1988) reviewed the decontamination of aflatoxin by ammoniation and stated that the results demonstrate overwhelming support for the efficacy and safety of ammoniation as a practical solution to aflatoxin detoxification in animal feeds.

Ammoniation of aflatoxin-contaminated feedingstuffs is an accepted decontamination practice in some countries of North and South America and Africa. Detoxification by ammoniation offers advantages due to the low costs and the applicability on the farm. There are, however, some side effects regarding the nutritional value of the treated commodities. Jensen et al. (1977) and Brekke et al. (1977) observed a reduced intake of feed containing ammoniated corn in pigs and laying hens, respectively. Brekke et al. (1977) propose that one should use heat treatment after ammoniation, in order to remove ammonia residues. Some authors found a decreased protein quality of treated cottonseed and groundnut meal (Mann et al., 1971; Belebeau et al., 1974; Thiesen, 1977), while others reported a reduction in the cystine content (Waldroup et al., 1976; Viroben et al., 1978; Delort-Laval et al., 1980). In the light of all these findings, it might be advisable to restrict the use of treated commodities as ration components.

The degradation of aflatoxin in groundnut and cottonseed meal as well as in corn by sodium hydroxide and other alkaline reagents ($Ca(OH)_2$, Na_2CO_3, Na_3PO_4, methylamine, ethylendiamine, ethanolamine, urea, thiourea) has been reviewed by Müller (1983). In most cases a partial detoxification was achieved. Degradation products of aflatoxin B_1 were still toxic in chicken embryos, but to a lesser extent than aflatoxin B_1 (Park et al., 1981).

One of the most important concerns in the decontamination of aflatoxin-containing feed commodities is the safety of the products for food-producing animals and for human consumption of products derived from these animals. A new method, based on the use of florisil and C18 solid-phase extraction columns, was developed for the preparation of extracts from decontaminated peanut meal. This method allowed testing with *in vitro* genotoxicity assays without interference of the residual aflatoxin B_1 (AFB_1)(Hoogenboom et al., 2001a). Recovery of degradation products in the extracts was evaluated by the use of radiolabeled [^{14}C]-aflatoxin B_1 added to naturally contaminated peanut meal (3.5 mg/kg). The meal was treated by a small-scale version of an industrial decontamination process based on ammoniation. Following decontamination, more than 90% of the label could not be extracted from the meal. Aflatoxin B_1 accounted for about 10% of the radiolabel present in the extractable fraction, indicating a total aflatoxin B_1 reduction of more than 99%. Decontamination of the meal by a number of other small- and industrial-scale ammonia-based processes resulted in similar efficiencies. Application of the extraction procedure resulted in aflatoxin B_1-rich and aflatoxin B_1-poor fractions, the latter containing half of the extractable decontamination products, but less than 1% of the residual aflatoxin B_1. Testing in the *Salmonella*/microsome mutagenicity assay (TA 100, with S9-mix) of the original crude extracts and AFB_1-rich fractions prepared from nontreated and decontaminated meal, showed the positive results expected from the AFB_1 contents as determined by HPLC analysis. Analysis and testing of the AFB_1-poor fractions showed that the various decontamination processes not only resulted in a successful degradation of AFB_1 but also did not produce other potent mutagenic compounds. Slight positive results obtained with these extracts were similar for the untreated and treated meals and may be due to unknown compounds originally present in the meal. Results obtained with an unscheduled DNA synthesis (UDS) and Comet assay with rat hepatocytes support this conclusion. Positive results obtained with the micronucleus assay, using immortalized mouse hepatocytes (GKB), did not clearly reflect the mycotoxin levels and require further examination. It is concluded that the newly developed extraction procedure yields highly reproducible fractions and hence is very suitable for examining the possible formation of less-potent degradation products of aflatoxins in short-term genotoxicity tests.

In another study (Neal et al., 2001), differences were detected *in vivo* between samples of aflatoxin-contaminated peanut meal, following decontamination to comparable degrees by two different ammonia-based processes. In this study, a sample of peanut meal, highly contaminated with aflatoxins, was subjected to decontamination by two commercial ammonia-based processes. The original contaminated and the two decontaminated meals were fed to rats for 90 days. No lesions associated with aflatoxin-induced hepatocarcinogenesis were detected histologically following feeding with the two detoxified meals. There were, however, clear differences between the two meals in respect of growth rates of the rats. In addition, feeding one of the detoxified meals resulted in hepatic abnormalities, which were detected using novel immunohistochemical reagents. Differences between the two detoxified meals were also indicated by the results of studies using meals "spiked" with [^{14}C]-aflatoxin B_1 prior to being subjected to the detoxification processes. The meals differed in the bioavailability of the label. It was concluded that peanut meal with an initial, unacceptable level of contamination with aflatoxins, which was then reduced by two ammonia-based processes to comparable, acceptable levels, could still have different effects *in vivo* when incorporated into animal diets.

Other mycotoxins are expected to be unstable under alkaline conditions if they have a lactone ring in the molecule, like aflatoxin. This is the case for patulin, penicillic acid, mycophenolic acid, alternariol, citreoveridin, ochratoxin A, rubratoxin and zearalenone.

The instability of rubratoxin and cyclochlorotin under alkaline condition was shown by Moss (1971) and Ichikawa et al. (1970), respectively. Chelkowski et al. (1981) could demonstrate a reduction of the content of penicillic acid, citrinin, ochratoxin A, zearalenone and aflatoxin B_1 after treatment with 2% NH_3 solution, an effect that was accelerated by raising the temperature. Patulin was unstable already at pH 7,while at pH 8 it was degraded completely after 190 hours (Brackett and Marth, 1979). Toxicity was not detectable after NH_3 treatment in wheat seedlings (Ellis et al., 1980). The content of citrinin was reduced by about 30% after 24 hours at pH 11 (Müller and Widmaier, 1982).

Madsen et al. (1980) reduced the ochratoxin A content of contaminated barley by 95% using a mixture of air and 5% NH_3 for 4 days at 70ºC. Toxicity and pathological changes of kidneys in pigs were significantly reduced, but not the ochratoxin A concentration in the kidneys. At relatively low levels of contamination (up to 4 mg/kg) Chelkowski et al. (1982) concluded that the detoxification of ochratoxin A is possible by treatment for several weeks with 2% NH_3 at atmospheric pressure and ambient temperature. They could also demonstrate that ochratoxin-A-contaminated corn and wheat were not toxic after total degradation of the toxin in chicken embryos and toxicity was reduced after partial degradation in broiler chickens. Madsen et al. (1980) and Chelkowski et al. (1982) observed negative side effects of the treatment on feed intake, body weight gain and feed conversion in pigs and chicken.

Lasztity et al. (1977) treated zearalenone-contaminated corn with a NH_3 solution and dried it afterwards. The results of detoxification were satisfying in feeding trials with pigs. Bennett et al. (1980) recorded only a 20% reduction of zearalenone in contaminated corn after treatment with 10% $NaHCO_3$ or 3% NH_3 at 50°C for 16 hours, a rather weak effect that was probably caused by the short time of treatment. Trichothecenes, which contain an ester group, are transformed into the corresponding alcohol under alkaline conditions. The general toxicity of these compounds is not changed, whereas the cytotoxic and dermatotoxic effects are reduced (Ueno, 1977).

Fumonisin B_1-contaminated corn was exposed to alternative treatments, containing various combinations of $Ca(OH)_2$, $NaHCO_3$ and H_2O_2, simulating a modified nixtamalization procedure (Park et al., 1996). Treatments also included NH_4Cl alone or in combination with H_2O_2 or horseradish peroxidase. The brine shrimp assay (*Artemia* spp.) was used to monitor the toxicity of reaction products and the *Salmonella*/microsomal mutagenicity assay, using test strains TA-100 and TA-102, was used to evaluate mutagenicity. Treatments of FB_1-contaminated corn simulating modified nixtamalization, ($Ca(OH)_2$ alone or with $Na\text{-}HCO_3$ + H_2O_2), gave 100% reduction of FB_1 and reduced brine shrimp toxicity by ca. 40%. The positive mutagenic potential (without S-9) for extracts of corn naturally contaminated with FB_1, was eliminated following exposure to modified nixtamalization. Reaction products formed when pure FB_1 was treated with $Ca(OH)_2$ and H_2O_2/$NaHCO_3$ were inhibitory to *Bacillus cereus*, *B. subtilis* and *B. megaterium*. No inhibitory potential was evident for contaminated corn extracts following the chemical treatments. In another study with contaminated corn the ammonia process did reduce fumonisin levels and no mutagenic potentials were apparent in the treated corn (Norred et al., 1991).

2.5.4. Treatment with acids

Most of the known mycotoxins are resistant against acids, as reviewed by Müller (1983). However, in acid conditions aflatoxins B_1 and G_1 are transformed into aflatoxin B_{2a} and G_{2a}, respectively (Ciegler and Peterson, 1968; Dutton and Heathcote, 1968; Pohland et al., 1968; Lindenfelser and Ciegler, 1970; Pons et al., 1972; Hafez and Megalla, 1982). Nevertheless,

the acid degradation products are far less toxic than aflatoxins B_1 and G_1, as demonstrated in chicken embryos (Pohland et al., 1968), in day-old chickens (Dutton and Heathcote, 1968; Hafez and Megalla, 1982), in ducklings (Ciegler and Peterson, 1968; Lillehoj and Ciegler, 1969) and in Zebra fish larvae (Abedi and Scott, 1969) and neither compound showed mutagenic activity (Wong and Hsieh, 1976). Two other mycotoxins, citrinin (Müller and Widmaier, 1982) and PR-toxin (Scott and Kanhere, 1979) show a certain degree of acid instability.

Most investigators have found that ensiling does not change the concentration and toxicity of acid-resistant mycotoxins. Acid-degradable mycotoxins are also not detoxified to any considerable degree in silage because of the moderate acid conditions in silage, or at least degradation takes a rather long time (Lindenfelser and Ciegler, 1970; Pons et al., 1972). Only Hafez and Megalla (1982) claim that silages may result in an aflatoxin detoxification worth mentioning, derived from studies with 0.1 M lactic acid, a concentration present in silages.

2.5.5. Treatment with other chemicals

Formaldehyde is one of the most effective agents to degrade aflatoxin in oilseed meals. Indeed, after treatment of contaminated groundnut meal with 2% formaldehyde for 120 minutes at 100ºC no aflatoxins could be detected (Mann et al., 1970). Codifier et al. (1976) showed that the degradation of aflatoxins was accelerated by a combination of formaldehyde and $Ca(OH)_2$. Zearalenone was also destroyed completely by treatment with 3.7% formaldehyde solution for 16 hours at 50ºC and to 96% by formaldehyde gassing for 10 days at ambient temperature in corn, but only after grinding (Bennett et al., 1980). The toxicity after formaldehyde treatment was not tested.

Promising results have been achieved in aflatoxin B_1, G_1 and M_1 detoxification, using sodium bisulfite (Doyle and Marth 1978a, 1978b; Moerck et al., 1980; Applebaum and Marth, 1982; Hagler et al., 1982; Yagen et al., 1989; Phillips et al., 1994). Fumonisins have been shown to be very resistant to degradation and detoxification by a variety of methods however Lemke et al. (2001b) could demonstrate that fumonisin B_1 was significantly deaminated in acidic aqueous solution by the addition of $NaNO_2$.

2.5.6. Reactions of mycotoxins with feed ingredients

Ochratoxin A is obviously not stable during storage of grains. The degradation is rather fast at the beginning and slows down with time (Trenk et al., 1971; Szebiotko et al., 1980). The chemical reactions have not been clarified but Szebiotko et al. (1980) assume that there is oxidation by peroxides. Possible microbial degradation and toxicity of degradation products were not investigated. A similar degradation was observed for citrinin. The mode of action is also unclear, but the influence of light and the microbial degradation have been excluded (Harwig et al., 1977).

The concentrations of patulin (Scott and Somers, 1968; Pohland and Allen, 1970; Harwig et al., 1977) and penicillic acid (Scott and Somers, 1968) added to different grains and grain products were reduced during storage. In dried corn no degradation of patulin could be observed, whereas in moist corn degradation took place rapidly (Pohland and Allen, 1970). The accelerating effect of the water content was confirmed by Harwig et al. (1977). Patulin and penicillic acid probably react with SH-containing substances, e.g. cysteine. Both mycotoxins are able to react with thiols like cysteine and glutathione (Ciegler et al., 1972; Lieu and Bullerman, 1978). Penicillic acid may also react with some amino acids, e.g. arginine,

histidine, lysine (Ciegler et al., 1972). The reaction velocity is reduced with decreasing pH values (Hofmann et al., 1971), but at the pH values of silages remarkable degradation was observed (Scott and Somers, 1968; Lieu and Bullerman, 1978). The results of toxicity tests of the reaction products were different. In most biological tests, toxicity of patulin was significantly reduced (Hofmann et al., 1971; Lieu and Bullerman, 1978; Lindroth and von Wright, 1978), but teratogenicity was observed in chicken embryos (Ciegler et al., 1976). The toxicity of the reaction products of penicillic acid with cysteine was also reduced except in the chicken embryo test (Ciegler et al., 1972; Lieu and Bullerman, 1978).

PR-toxin reacts also with glutathione and cysteine (Nakamura et al., 1977) and with free amino acids, amines and NH_3 (Wei et al., 1973; Arnold et al., 1978; Scott and Kanhere, 1979). It is assumed therefore, that degradation in feedingstuffs containing the substances mentioned above (e.g. silage) produces reaction products which are less toxic (Moule et al., 1977; Arnold et al., 1978).

Other dietary factors may influence the toxicity of mycotoxins and especially with regard to aflatoxins such factors have been studied intensively. Methyl donors (choline, methionine) seem to be able to ameliorate the induction of preneoplastic foci in the liver. Protein deficiency was also effective in this way. The amount of fat, the fatty acid pattern, some vitamins and trace elements (vitamin A, folic acid, selenium) and antioxidants (BHT, BHA) seem to modulate the carcinogenicity of aflatoxin B_1. A number of substances in plants (phenols, garlic extracts, indole 3-carbinol, capsaicins, etc.) have been studied with regard to prevention of neoplasms (Cullen and Newberne, 1994; Eaton et al., 1994; Kensler et al., 1994; Rogers 1994; Galvano et al., 2001).

2.6. Biological methods of inactivation

Biological methods of mycotoxin inactivation are based on the use of microorganisms. Two kinds of strategy have been reported for this purpose: prevention of mycotoxin formation and product decontamination. The first method is mainly applied to prevent aflatoxin formation in peanuts and cottonseed, and attempts to decrease aflatoxin contamination by competition of nontoxigenic strains of *Aspergillus* spp. with toxigenic strains (Cole and Cotty, 1990; Bhatnager et al., 1994). The second method uses the well-known ability of ruminal and intestinal microorganisms to degrade mycotoxins such as ochratoxin A and deoxynivalenol.

Ciegler et al. (1966) were able to significantly remove aflatoxin by *Flavobacterium aurantiacum,* without producing toxic metabolites and demonstrated that certain molds could transform aflatoxin B_1 into B_{2a}, which is less toxic. The effect of the *Flavobacterium aurantiacum* was confirmed by Hao et al. (1987). Microbial detoxification of aflatoxin has been reviewed by Ciegler et al. (1966) and Marth and Doyle (1979). In a recent study (Pierides et al., 2000), lactic acid bacteria strains were tested for their ability to remove aflatoxin M_1 from liquid media. All strains, whether viable or heat-killed, could reduce the aflatoxin M_1 content of a liquid medium. The two most effective strains were also tested using contaminated skimmed and full-cream milk. The results indicate that specific lactic acid bacteria used in dairy products can offer novel means of decontaminating aflatoxin M_1 from milk. Dry contact with aflatoxins and contact with MS2 bacteriophage (surrogate of human enterovirus) in water also causes decontamination in minutes (Koper et al., 2002).

The toxicity of trichothecenes is caused by the 12,13-epoxide ring. The removal of this epoxide group leads to significant reduction of toxicity. The detoxifying ability of some ruminal and intestinal bacteria is based on the de-epoxidation reaction (transformation of DON into DOM-1) (Yoshizawa et al., 1983; He et al., 1992; Kollarczik et al., 1994). Treatment of

moldy corn, containing approximately 5 mg deoxynivalenol/kg, with microbial inoculum from the digestive tract of poultry, reduced the DON concentration by about 55% and partially alleviated its toxic effects on feed intake and body weight gain in young pigs (Ping et al., 1992; He et al., 1993). Binder et al. (2000) were the first to isolate and cultivate a new strain of *Eubacterium* spp. (BBSH 797) that is able to biotransform the epoxide group of trichothecenes. Encapsulation enhances the stability of the microorganism during storage and in the gastrointestinal tract and in this form it can be used as a feed additive for detoxification of trichothecens. It could be demonstrated, with *in vitro* studies, that bacterial counts of 3.55×10^4, 3.55×10^5 and 3.55×10^6/g transformed 31%, 83% and 100% of DON, respectively, within 24 hours, correlating with a distinct reduction of cytoxicity, when toxicity studies on DOM-1 were carried out using a lymphocyte proliferation assay. The performance of piglets and broilers fed DON-contaminated diets was significantly improved by the addition of the *Eubacterium* BBSH 797.

Poppenberger et al. (2003) reported the isolation and characterization of a gene from *Arabidopsis thaliana* encoding a UDP-glycosyltransferase that is able to detoxify deoxynivalenol. The enzyme, previously assigned the identifier UGT73C5, catalyzes the transfer of glucose from UDP-glucose to the hydroxyl group at carbon 3 of deoxynivalenol. Using a wheat germ extract-coupled transcription/translation system it was shown that this enzymatic reaction inactivates the mycotoxin. The deoxynivalenol-glucosyltransferase (DOGT1) was also found to detoxify the acetylated derivative 15-acetyl-deoxynivalenol, whereas no protective activity was observed against the structurally similar nivalenol. Expression of the glucosyltransferase is developmentally regulated and induced by deoxynivalenol as well as salicylic acid, ethylene and jasmonic acid. Constitutive overexpression in *Arabidopsis* leads to enhanced tolerance against deoxynivalenol.

Microorganisms in the rumen and in the digestive tract of pigs are able to decompose ochratoxin A into ochratoxin-α and phenylalanine (Galtier and Alvinerie, 1976; Kiessling et al., 1984); further studies have shown that ochratoxin-α is nontoxic (Yamazaki et al., 1981; Bruinik et al., 1998). Moreover, pure cultures of bacteria (Wegst and Lingens, 1983; Hwang and Draughon, 1994), aerobic bacteria cultivated from the soil (Schatzmayr et al., 2002a) and specific strains of yeast (Schatzmayr et al., 2002b) also show the same ability to degrade ochratoxin A. Enzymatic degradation of ochratoxin A by pure carboxypeptidases as well as by an undefined mixture of enzymes has also been performed (Schatzhmayr et al., 2000).

Administration of monoclonal antibodies specific for T-2 toxin neutralized the inhibitory effects of the toxin *in vitro* on protein synthesis in human lymphoblastoid cultures, caused by a net efflux of toxin from the poisoned B-lymphoblastoid cells, and restored protein synthesis to normal. These antibodies protect rats from lethal T-2 toxicosis (Feuerstein et al., 1988). Thus, administration of the monoclonal antibodies 30 minutes before an infusion of a lethal dose of T-2 toxin to rats caused sequestration of the toxin in the plasma (Hunter et al., 1990), while administration 35 minutes after dosage of toxin facilitated migration of the toxin back into the plasma from the tissues and reduced its toxic effect.

2.7. Reduction of mycotoxin bioavailability

The reduction of feedborne exposure to mycotoxins by the addition of various binding substances or sorbents to the diet has been given considerable attention. Many of these agents are purported to prevent the deleterious effects of various mycotoxins in different animal species, primarily in poultry and swine. The assumed modes of action are the diminished mycotoxin absorption and consequently the reduced distribution to the blood and the target organs.

A broad variety of agents has been tested and used in practice (table 1). The efficacy varies, depending on the mycotoxin involved. The ability of these adsorbents to react with other mycotoxins needs to be examined. There can be little doubt that if the adsorbents are free of animal/human toxicity and are easily and completely excreted from the body they could represent a most significant, easily applicable and relatively cheap means of sequestering mycotoxins in food and feed.

The first *in vitro* and *in vivo* studies on the adsorption of several mycotoxins by Antitox VANA were performed by Ehebruster in 1979. He found an absorption rate of aflatoxin B_1, B_2, G_1, G_2, zearalenone and patulin of 61%, 37%, 45%, 35%, 13% and 24%, respectively. In broilers fed an aflatoxin-contaminated diet (B_1: 0.4, G_1: 0.2 mg/kg) body weight, relative liver weight, fat content and aflatoxin concentration of the liver were different from control birds.

Table 1

Degradation of mycotoxins (except aflatoxin) by microorganisms (modified from Smith et al., 1994)

Mycotoxin	Microorganism	Concentration mg/L	Elimination %	Mechanism/ Product	Process
Patulin	*S. cerevisiae*	15.5	ca. 90	Fermentation	Cider
Patulin	*S. cerevisiae*	50	ca. 100	Non-volatile/ water soluble	*In vitro*
Ochratoxin A	*S. cerevisiae*	0.5–5	47–71	Unknown (by metabolites)	Brewing
Ochratoxin A	*S. cerevisiae*	0.02–0.1	63–82	Unknown (by metabolites	Brewing
Ochratoxin A	Rumen Organisms	0.2	ca. 95	Ochratoxin-α-phenyl-alanine	Ruminal degradation (*in vitro)*
Zearalenone	Rumen organisms	2.8	100	α-Zearalenol, β-Zearalenol	Id
T-2	Rumen organisms	20	ca. 94	Deacylated metabolites	Id
T-2 derivatives	*Butyrivibrio fibrisolvens*	10		Deacylation to RT-2, T-2 triol, neosolaniol	*In vitro*
Diacetyl-scirpenol	Rumen organisms	5	ca. 97	Deacetylation to mono-oxyscirpenol	*In vitro*
Deoxy-nivalenol	Rumen organisms	2.5	0	–	*In vitro*
Deoxy-nivalenol	Rumen and intestine organisms			DON to DOM-1	*In vitro*
Deoxy-nivalenol	Intestine organisms	5 mg/kg	55		Pigs
Deoxy-nivalenol	*Eubacterium BBSH 797*	31–100			*In vitro*
Rubratoxin A	*P. rubrum*		55	Unknown	*In vitro*
Rubratoxin B	*P. rubrum*		55	Unknown	*In vitro*

The addition of 2.5 g Antitox VANA/kg diet reduced the fat content and aflatoxin residues of the liver, but did not improve body weight gain, feed intake and feed conversion. Activated charcoal was found to increase body weight gain and feed intake in poultry fed aflatoxin-contaminated diets (Dalvi and Ademoyero, 1984; Dalvi and McGowan, 1984), but 5 g/kg in a diet containing 5 mg or 7.5 mg aflatoxin B_1/kg could not protect chickens from adverse effects (Kubena et al., 1990). Sodium bentonites improved performance and clinical indications in pigs fed diets containing aflatoxin (Lindermann et al., 1993). Adverse effects of aflatoxins were significantly reduced by 5 g HSCAS/kg diet in chickens (Phillips et al., 1988; Kubena et al., 1990, 1993), turkeys (Kubena et al., 1991), pigs (Haydon et al., 1990) and growing lambs (Harvey et al., 1991a) and the aflatoxin M_1 concentration in cow´s milk was also reduced (Harvey et al., 1991b).

Alfalfa surprisingly overcame the adverse effects of high dietary concentrations of zearalenone in rats (Smith, 1980; James and Smith, 1982; Stangroom and Smith, 1984), but had only partial or no effect on uterine enlargement in gilts fed a diet containing 10 mg and 50 mg zearalenone/kg, respectively (Smith, 1980). Cholestyramine also reduced the estrogenic effects of zearalenone in mice (Underhill et al., 1995). HSCAS at 10 g/kg diet was able to improve body weight gains in chickens fed moldy corn-based diets with low concentrations of T-2 toxin, deoxynivalenol and zearalenone (Orr, 1987) or deoxynivalenol (Patterson and Young, 1992, 1993; Charmley et al., 1995a). HSCAS was also effective in chickens given feed which was highly contaminated with fusarium and significantly increased body weight gain (Wyatt, 1987). The performance of gilts fed a diet containing 5 mg deoxynivalenonl/kg was also improved by addition of HSCAS (Trenholm et al., 1989).

Ochratoxin A absorption is reduced by activated charcoal (Rotter et al., 1989) and also cholestyramine, which reduces ochratoxin A concentration in blood, and its cumulative excretion in urine and increases fecal excretion in rats fed a contaminated diet (Madhyastha et al., 1992). On the other hand, HSCAS was ineffective against diacetoxyscirpenol (Kubena et al., 1993), T-2 toxin (Kubena et al., 1990) and fumonisin B_1 (Brown et al., 1992).

In vitro and *in vivo* studies demonstrate the ability of specific strains of lactic acid bacteria to bind a number of mycotoxins like aflatoxins, ochratoxin A and *Fusarium* toxins. This method of detoxification was recently reviewed by El-Nezami et al. (2004).

The mode of action of some of these substances includes ionic attraction between binding agent and mycotoxin, entrapment of the mycotoxin within the matrix of the binding agent and changes in the enterohepatic circulation of bile acids (Charmley et al., 1995). Besides the adsorption of mycotoxins some of these agents also bind undesirable substances present in the feedingstuffs or microorganisms in the intestinal tract, which causes additional positive effects, especially if animals suffer from diarrhea. On the other hand some essential nutrients like water-soluble vitamins and trace elements may also be bound, which leads to a reduced absorption.

3. CONCLUSION

The elimination or reduction of mycotoxins in feedstuffs is quite often of economic relevance because the limitation of the use of these feedstuffs can be avoided.

Mycotoxin contamination of crops can cause economic losses at all levels of food and feed production, including crop and animal production, and crop distribution and processing. The national economy would be affected adversely by losses incurred by crop and livestock producers and the multiplier effect this has on other industries as a result of the reduced spending power of producers. Costs of chemical analyses, quality control and regulatory programs,

research and development, extension services, law suits, and the cost of human illnesses must all be borne by the national economy. The value of the losses encountered depends on the price of grain, animals, and animal products, interest rates, degree of contamination, and other economic variables. Even during favorable seasons it is likely that millions of Euros are lost as a result of the contamination of crops with mycotoxins. Many compounds and treatments have been tested in order to reduce mycotoxin concentrations in food and feed or to alleviate their adverse effects on animals. Some of these treatments show promising prospects for commercial application, while others have already had commercial applications. However, until reliable, cost-effective, commercially applicable methods are more widely available, problems associated with mycotoxin contamination and the economic losses resulting will continue to be seen in the food and agriculture industries (Charmley et al., 1995b).

4. FUTURE PERSPECTIVES

Because of the health risks for humans and animals legal regulations for aflatoxins and rye ergot were put into force in the EU and some other countries. The EU Directive on undesirable substances in feed, which lists the substances and fixes their maximum permissible levels in feed materials, premixes, complete and complementary feedingstuffs is currently under revision. This exercise should be based on updated scientific risk assessments and should take into account the prohibition of any dilution of contaminated noncomplying material intended for animal nutrition. The terms of reference includes the identification and evaluation of undesirable substances not listed yet in the Directive. In the future a number of other mycotoxins is considered to be included in the list of the Directive. These mycotoxins are ochratoxin A, zearalenone, deoxynivalenol, T-2 toxin, fumonisins and moniliformin. For these mycotoxins gaps in the available data need to be filled in order to complete the evaluation. Because of the prohibition of any dilution of contaminated noncomplying material intended for animal nutrition the evaluation of decontamination and detoxification methods and the official approval of effective methods seem to be necessary.

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16 Minerals: functions, requirements, excessive intake and toxicity

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Optimal nutrition in young animals is crucial for their proper development and growth. To avoid disturbances of these processes, the diet needs to be well balanced to provide all the nutrients necessary for the developmental stages and for the optimal growth rate of the animal. To cover the nutritional requirements, especially during very intensive growth when the rate of metabolic processes is high, a sufficient amount of proteins, lipids, carbohydrates and vitamins must be provided. Proper whole-body functions require not only these basic nutrients, but in young animals minerals and trace elements are also indispensable, especially during the periods of very intensive growth. However, the absorption of minerals and trace elements also changes as a result of age-related changes in gastrointestinal structure and function. Similarly to proteins, lipids, carbohydrates, and vitamins, minerals must be provided in optimal concentrations and according to requirements that change during the rapid growth and development of the animal. Optimal nutrition, with adequate mineral levels, guarantees proper functions of the organism, among which the most important are structural, physiological, catalytic and regulatory functions. It is worthwhile emphasizing that deficiency of minerals and trace elements delays the growth and development of organisms by disturbing numerous physiological functions. On the other hand, their excessive supply in feed induces growth inhibition or even toxicity, especially in the case of essential elements, which are characterized by narrow margins of safety. This chapter presents the current view of the physiological importance and requirements for macro- and microelements in growing animals, and discusses also their toxicity after excessive intake.

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Biology of Nutrition in Growing Animals
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1. INTRODUCTION

Outstanding advances have been made in our understanding of the nutritional significance of minerals and the complex mechanisms by which minerals are safely transported across membranes (Bronner, 1996; Schröder et al., 2002). Although minerals and trace elements have less impact than proteins and energy on overall performance and efficiency, minerals should not be treated as less important, especially in the development of newborn animals. Over recent decades, the introduction of more sophisticated genetic selection aimed at high-productivity breeds has altered the nutritional requirements, not only in adult animals but in newborns as well. The dietary requirements for trace elements have not been adapted to account for the level of supply required to satisfy the production potential of growth in newborn animals. On the other hand, guidelines are currently lacking concerning the requirements for trace elements based on growth and production potential. As a result, it is difficult to make definitive recommendations for mineral requirements during the various stages of development in different species of animals and environmental conditions. This is further complicated by the influence of changing metabolic and nutritional factors that lead to complexes with certain trace elements, hindering or even completely blocking their availability to the animal. An example is provided by molybdenum and sulfur, which together create a thiomolybdenate complex, from which copper is unavailable to the animal. Considerable data indicate how iron, aluminum and other elements can influence the bioavailability of trace elements (Corah, 1996). Lack of knowledge of the developmental pattern of mineral requirements and how their mutual relationships influence bioavailability makes it difficult to formulate the criteria for dietary minerals and supplements required for newborn animals. In formulating supplements for newborn and developing animals, the importance of minerals is often overlooked. Indeed, evidence has recently been obtained that immune functions in developing animals may be dependent upon the levels of dietary trace elements (Chandra, 1984; Prasad, 1998, 2000). It should be underlined that the negative impact on the immune system occurs with no other clinical symptoms being present in the animals (Corah, 1996). However, an impairment of the immune system due to an insufficient supply of minerals and trace elements leads eventually to clinical symptoms (Nogowska et al., 2000; Prasad, 2000; McKenzie et al., 2002). On the other hand, excess of some elements can have a negative impact on supply of others, e.g. iron and molybdenum on utilization of copper (Kulwich et al., 1953; Corah, 1996; Suttle and Mills, 1996). An example of excessive dietary supply is very well known from uncontrolled zinc and phosphorus supplementation, especially in piglets, and soil pollution connected with manure utilization in intensive farming. Similarly, excessive dietary calcium supplementation affects skeletal growth and development, and inhibits bone remodeling (Hall et al., 1991). Although minerals and trace elements have not been accorded the range of research and importance, in practical feeding trials for formulating diets, given to energy and protein content, it is nevertheless important to introduce basic data about physiological functions, dietary requirements and excessive or even harmful influences of minerals. Because of their impact on health, growth and productivity, they should not be ignored under current standards of farming.

2. CALCIUM

Calcium (Ca) contributes about 2% of body weight in mammals and birds. Almost 99.9% of total calcium distribution is present in bone tissue and extracellular fluid (ECF). The intracellular fluid (ICF) contains only about 0.1% of total body calcium. About 1% of calcium from the bone tissue and all the calcium from the ECF is rapidly exchangeable, to fulfill the requirements of

Table 1

Limit concentration of selected elements in blood serum of animals above which toxic symptoms occur (mmol/L)

	Element					
	P^{5+}	Mg^{2+}	K^{+}	Na^{+}	Ca^{2+}	Fe^{2+}
Horse	1.9	1.15	4.7	156.5	3.12	25.1
Cattle	2.71	1.23	5.1	156.5	3.03	35.8
Sheep	2.42	1.23	5.1	160.9	3.25	39.4
Goat	4.43	1.62	4.1	157	3.05	–
Pig	3.1	1.46	5.6	156.5	3.25	–
Dog	2.87	1.19	5.4	156.5	3	21.8
Cat	2.61	1.31	5.6	156.5	3	38.5
Rabbit	2.23	2.21	6.8	155.5	3.03	–
Guinea pig	2.46	1.23	8	146	3	–
Syrian hamster	2.65	1.44	5.9	146	3	–

Adapted from: Winnicka, 2002.

the organism. Intracellular calcium concentration is about 1000 times lower than the extracellular (Kokot, 1998). The level of the maximum calcium concentration in the blood serum of breeding animals is close to 3.0–3.25 mmol/l (table 1). Calcium in plasma is present in three biologically different forms: (1) the most important form is ionic calcium (Ca^{2+}), which accounts for around 50% of the total plasma calcium concentration; (2) up to 10% of the plasma concentration occurs as calcium complexes (citrate, phosphate, sulfate); (3) the remaining pool, estimated at 35–40%, comprises protein-bound calcium, mainly with albumins and globulins (Houillier et al., 2003). Both the complexes of calcium and the protein-bound calcium exhibit no biological activity and serve as reservoirs of this element in the body (Pasternak, 2000).

Apart from its function as the major inorganic element in bone tissue, the other most important role of calcium is its function as a second messenger in muscles enabling their contraction. It is indispensable for conduction of action potentials through nerve fibers and synapses, and it converts chemical, physical and hormonal signals into biological effects (Williams, 1998; Bennett, 1999; Schröder et al., 2002). Calcium is indispensable for proper pancreatic cell signal transduction and effective excitation during pancreatic juice production (Valverde and Studziński, 1999a). It plays a crucial function in the coagulation process that guarantees proper hemostasis, but its significance is only evident when the level falls to less than half the physiological level (Kaibara, 1996). Calcium is a constituent of cell membranes and their organelles, such as mitochondria and microsomes, regulating cell permeability for various substances. Calcium ions can stimulate or inhibit many enzymes' activity (Saboury and Karbassi, 2000).

There are three different pools of this element in cells: (1) Ca bound to the cellular membrane or cellular organelle; (2) cytoplasmic-free calcium; or (3) Ca bound to ATP (adenosine triphosphate), orthophosphates and pyrophosphates. Both Na^{+}-K^{+}-ATPase and magnesium-dependent Ca^{2+}-ATPase are responsible for maintenance of the gradient of calcium between ECF and ICF (Bronner, 1996; Williams, 1998). Disturbance of intracellular calcium homeostasis may result in calcium release from mitochondrial and microsomal storage into the cytoplasm, causing membrane phospholipase activation, damage to the cytoskeleton and increased

membrane permeability. It should be emphasized that transmembrane calcium transport is coupled with that of phosphate ions and determines their intracellular transport to the intestinal epithelium and renal tubular cells as well (Kokot, 1998).

Under physiological conditions, calcium homeostasis depends on its intestinal absorption, its excretion and skeletal deposition. In the case of mammals during lactation, calcium is widely secreted in milk (table 2). Calcium absorption in monogastric animals and humans takes place mainly in the upper intestine, whereas the stomach and hindgut are less important for this process (Nordin, 1996; Schröder et al., 2002). There are three mechanisms participating in intestinal calcium absorption: transcellular, paracellular and vesicular (Kokot, 1998; Schröder et al., 2002). The first of these increases under conditions of high calcium requirements, depends on vitamin D and consists of passive entry of Ca^{2+} along its electrochemical gradient across apical brush-border membranes, transcytosis of Ca^{2+} throughout the cell body and pump-driven active secretion of Ca^{2+} across the basolateral membrane (Bronner, 1996). This mechanism takes place mainly in the duodenum. Paracellular transport is positively correlated with Ca^{2+} intestinal concentration and takes place in the lower parts of the small intestine through the tight junctions of mucosal cells. Vesicular Ca^{2+} transport is the third path of calcium absorption and proceeds according to endo- and exocytosis in enterocytes. It has been suggested that in ruminants, in contrast to monogastric animals, about half of the total calcium absorption takes place prior to entry into the small intestine, in the reticulo-rumen, omasum and abomasum. The small intestine and hindgut are responsible only for the remaining 30% and 20% of calcium absorption, respectively. Investigations of these processes in the rumen elucidated that the active calcium absorption mechanism depends on the presence of short-chain fatty acids. Moreover, there are data indicating that active calcium absorption in the rumen may be stimulated by vitamin D, similarly to the processes in the upper small intestine of monogastric species (Schröder et al., 2002).

Requirements for calcium, very high in newborn mammals – 10 g Ca per kg diet dry matter (DM) or chicks (11–13 g) – diminish gradually during their growth to half their initial requirements in finishing cattle and pigs (4–5 g) and to 7–9 g in chicks or poults finishing their growth (tables 3–5). Colostrum and milk are rich enough in this element to cover the requirements of newborn livestock mammals. Also, the majority of roughages (used in feeding ruminants or horses), are quite rich in calcium, e.g. grass contains about 6 g Ca per kg dry matter and 1 kg DM of pasture legumes contain as much as 13 g of this element. The concentrates (especially grains), however, are extremely poor in calcium, and in some cases contain only one-tenth the animals' requirements. In practical feeding of animals (especially monogastrics) a large deficit of this mineral has often been noted. Diets based on these feeds need, therefore, to be supplemented with mineral feedstuffs.

Animals tolerate a wide spectrum of this element (5–10 times), but this tolerance depends on the feed's mineral source and chemical form, which influence the element's availability for the animal. The adequacy of dietary calcium supply depends on the vitamin D status of the animal, while the level of phosphorus in the diet also has an influence on calcium availability. On the other hand, high levels of calcium in the diet negatively influence the availability of some other mineral nutrients, e.g. copper, iodine and iron but also affect availability of some toxic elements, such as lead and cadmium (Underwood and Suttle, 1999a). Calcium is absorbed according to the limits set by the absorbability of the mineral in the diet (Schneider et al., 1985). It is close to 90% for calcium in milk and usually >50% for Ca contained in solid diets (table 6).

In general, calcium is not toxic, because excess in a diet is simply excreted with the feces and the symptom of excess dietary Ca content is reduced intake. However, Ca levels also need

Table 2

Content of macroelements and microelements in 1 kg of whole milk

		Macroelements (mg)					Microelements (mg)						
	Dry matter (%)	Ca	P	Mg	Na	K	Fe	Zn	Cu	Mn	Co	I	Se
Cow[a]	12.5	1187	950	125	475	1400	0.375	1.88–4.75	0.01–0.14	0.025–0.05	0.0005–0.001	0.0125–0.025	0.0025–0.019
Mare[b]	10.5	1000	600	60	190	500		2.0	0.26				
Sow[c]	18.66	2121	1511	127	562	922	2.26	9.8	1.07	0.62	0.07		0.11
Sheep[d]	18.2	2000	1500	160	400	1500	0.6–0.7	2.0–3.0	0.05–0.15	0.06			
Goat[e]	13.5	1550	1200	150	400	2040	0.5–0.9	3.0–4.5	0.04–0.09	0.08–0.16	0.01–0.03		0.01–0.03

Adapted from: [a]NRC, 2001. Nutrient Requirements of Dairy Cattle; [b]NRC, 1989. Nutrient Requirements of Horses; [c]Migdał and Kaczmarczyk, 1995; [d]NRC, 1985. Nutrient Requirements of Sheep; [e]unpublished data.

Table 3

Requirements of growing cattle for minerals (in 1 kg of diet DM) and their content in 1 kg DM of typical feeds[a] and model diets

	Macroelements (g)							Microelements (mg)						
Item	Ca	P	Mg	Na	K	Cl	S	Fe	Mn	Zn	Cu	I	Co	Se
Calf milk replacer	10.0	7.00	0.7	4.0	6.5	2.5	2.9	100.0	40.0	40.0	10.0	0.5	0.11	0.3
Whole milk	9.5	7.6	1.0	3.8	11.2	9.2	3.2	3.0	0–0.4	15–38	0.1–1.1	0.1–0.2	0.004–0.008	0.02–0.15
Starter feed	7.0	4.5	1.0	1.5	6.5	2.0	2.0	50	40	40	10	0.25	0.10	0.30
Grower feed	6.0	4.0	1.0	1.4	6.5	2.0	2.0	50	40	40	10	0.25	0.10	0.30
Growing cattle 3–6 months (200 kg)	4.1	2.8	1.1	0.8	4.7	1.1	2.0	43	22	32	10	0.27	0.11	0.30
Growing cattle 6–12 months (300 kg)	4.1	2.3	1.1	0.8	4.8	1.2	2.0	31	20	27	10	0.30	0.11	0.30
Growing cattle 12–18 months (450 kg)	3.7	1.8	0.8	0.7	4.6	1.0	2.0	13	14	18	9	0.30	0.11	0.30

Content in 1 kg (DM basis) of typical feeds and in model summer* and winter diets[b]**														
Pasture	5.6	4.4	2.0	0.2	33.6	5.6	2.0	275	75	36	10	0.16	0.06	0.09
Maize silage	2.8	2.6	1.7	0.1	12.0	2.9	1.4	104	36	24	6	0.07	0.06	0.04
Meadow hay	6.6	2.9	2.3	0.8	21.3	9.2	2.4	194	72	25	9	0.16	0.06	0.06
SBM	3.5	7.0	2.9	0.3	24.1	1.3	3.9	206	40	58	16	0.12	0.30	0.13
Dried sugar beet pulp	9.1	0.9	2.3	3.1	9.6	1.8	3.0	642	62	22	11	0.67	0.08	0.14
Summer diets*	5.3	3.7	10.7	0.7	29	4.9	2.2	346	72	33	0		0.06	0.10
Deficiency %	Excess	Excess	Excess	50	Excess	Excess	Excess	Excess	Excess	0	22.0		45	67
Winter diets**	3.6	3.1	2.0	0.30	15	3.9	1.8	131	4	28	7	0.09	0.08	0.09
Deficiency %	12	Excess	Excess	62	Excess	Excess	10.00	Excess	Excess	12	30	67	27	70
Tolerance, multiple of max. requirements	4	2	3	10	3	5	1	10	25	12	10	100	90	6
Toxicity, in 1 kg of diet DM[c]	44	15	4	44	30	56	4	1000	1000	500	100	50	10	2

[a]Data based on NRC, 2001. Nutrient Requirements for Dairy Cattle. National Academy Press, Washington, D.C.

[b]Exemplary diets for growing cattle 3–6 months (200 kg). 1000 g daily gain, *summer: pasture + dried sugar beet pulp (80+20% DM basis), **winter: maize silage + meadow hay + SBM (70+20+10% DM basis).

[c]NRC, 1980. Mineral Tolerance of Domestic Animals. National Academy Press, Washington, D.C.

Table 4

Requirements of growing pigs for minerals in 1 kg DM and their content in typical feeds[a] and in 1 kg DM of a model diet

	Macroelements (g)						Microelements (mg)					
Body mass, kg	Ca	P total	Mg	Na	K	Cl	Fe	Zn	Cu	Mn	I	Se
3–5	10.0	7.7	0.44	2.8	3.3	2.8	110	110	6.6	4.4	0.16	0.33
5–10	8.8	7.2	0.44	2.2	3.1	2.2	110	110	6.6	4.4	0.16	0.33
10–20	7.7	6.6	0.44	1.7	2.9	1.7	88	88	5.5	3.3	0.16	0.28
20–50	6.6	5.5	0.44	1.1	2.5	0.9	66	66	4.4	2.2	0.16	0.17
50–80	5.5	5.0	0.44	1.1	2.1	0.9	55	55	3.9	2.2	0.16	0.17
80–120	5.0	4.4	0.44	1.1	1.9	0.9	44	55	3.3	2.2	0.16	0.17
Content in 1 kg of typical feeds and in a model diet: barley + corn + SBM (40 + 40 + 20%)												
1 kg of barley	0.6	3.5	1.4	0.4	4.5	1.2	78	25	7	18		0.19
1 kg of corn	0.3	2.8	1.2	0.2	3.3	0.5	29	18	3	7		0.07
1 kg of SBM	3.2	6.5	2.7	0.1	19.6	0.5	202	50	20	29		0.32
1 kg of model diet	1.1	4.21	1.74	0.29	7.75	0.86	91.5	29.9	8.8	17.4		0.19
Deficiency (%) for pig diet at 20–50 kg body mass	83	24	Excess	73	Excess	5	Excess	55	Excess	Excess		Excess
Tolerance, multiple of max requirements	2	2	5	3	6	5	20	30	50	200	50	5
Toxicity, in 1 kg of diet DM[b]	20	15	3	20–40	18–20	20–50	3000–5000	4000–5000	750	4000	800	5

[a]NRC, 1998. Nutrient Requirements of Swine. National Academy of Sciences, Washington, D.C., reproduced with permission of the National Academies Press. © 1998 National Academy of Sciences.

[b]NRC, 1980. Mineral Tolerance of Domestic Animals. National Academy Press, Washington, D.C.

Table 5

Requirements of chickens for minerals in 1 kg DM and their content in typical feeds[a] and in 1 kg DM of a model diet

		Macroelements (g)						Microelements (mg)					
Age, weeks		Ca	P, non phytate	Mg	Na	K	Cl	Fe	Zn	Cu	Mn	I	Se
Broiler chickens	0–3	11.1	5.0	0.67	2.2	3.3	2.2	89	44	8.9	67	0.39	0.17
	3–6	10.0	3.9	0.60	1.7	3.3	1.7	89	44	8.9	67	0.39	0.17
	6–8	8.9	3.3	0.67	1.3	3.3	1.3	89	44	8.9	67	0.39	0.17
Leghorn chickens	0–6	10.0	4.4	0.67	1.7	2.8	1.7	89	44	5.6	67	0.39	0.17
	6–12	8.9	3.9	0.6	1.7	2.8	1.3	67	39	4.4	33	0.39	0.11
	12–18	8.9	3.3	0.44	1.7	2.8	1.3	67	39	4.4	33	0.39	0.11
Poults	0–4	13.3	6.7	0.6	1.9	7.8	1.7	87	78	8.0	67	0.44	0.22
	4–8	11.1	5.6	0.6	1.7	6.7	1.6	67	72	8.9	67	0.44	0.22
	8–12	9.5	4.7	0.6	1.3	5.6	1.6	67	56	6.7	67	0.44	0.22
	12–16	8.3	4.2	0.6	1.3	5.6	1.3	67	44	6.7	67	0.44	0.22
	16–20	7.2	3.5	0.6	1.3	4.4	1.3	56	44	6.7	67	0.44	0.22
	20–24	6.1	3.1	0.6	1.3	4.4	1.3	56	44	6.7	67	0.44	0.22
Content in 1 kg of typical feeds and in a model diet: wheat + corn + SBM (30 + 50 + 20%)													
1 kg of wheat		0.5	3.6	1.6	0.2	4.1	0.9	64	43	7	42		0.30
1 kg of corn		0.3	2.8	1.2	0.2	3.3	0.5	29	18	3	7		0.07
1 kg of SBM		3.2	6.5	2.7	0.1	19.6	0.5	202	50	20	29		0.32
1 kg of model diet		1.03	4.16	1.78	0.20	7.48	0.69	81.1	35.1	8.4	24.1		0.21
Deficiency (%) in broiler diet by 3–6 weeks		90	Excess	Excess	88	Excess	59	10	20	6	64		Excess
Tolerance, multiple of max requirements		2	2	8	3	2	5	10	15	20	50	50	10
Toxicity, per kg of diet DM[b]		20	10	5.7–6.4	7–60	9–12	6–12	1000–4500	800–4000	250–800	3000–4500	500	5

[a]NRC, 1994. Nutrient Requirements of Poultry. National Academy Press, Washington, D.C., reproduced with permission of the National Academies Press. © 1994 National Academy of Sciences.
[b]NRC, 1980. Mineral Tolerance of Domestic Animals. National Academy Press, Washington, D.C.

Table 6

Mineral concentrations and animal apparent absorption of the most common mineral sources

Minerals	Supplement	Concentration (%)	Apparent absorption (%)
Calcium	Calcium oxide	71	30–70
	Calcium carbonate	28	50–75
	Limestone	34	45–80
	Calcium chloride (dihydrate)	28	60–95
	Dicalcium phosphate	22	65–94
	Monocalcium phosphate	17	80–95
Phosphorus	Sodium phosphate	23	40–90
	Ammonium phosphate	20	55–80
	Defluorinated phosphate	12–18	65–80
	Dicalcium phosphate	18	60–85
	Monocalcium phosphate	21	75–90
Sodium	Sodium chloride	40	85–100
	Sodium bicarbonate	27	90–95
Potassium	Potassium chloride	50	85–100
	Potassium sulfate	42	90–100
	Potassium carbonate	57	85–90
Magnesium	Magnesium oxide	55	65–80
	Magnesium sulfate	20	90–95
	Magnesium carbonate	31	35–50
	Magnesium acetate	29	80–95
Iron	Ferrous sulfate heptahydrate	20	40–80
	Ferric citrate		60–85
	Ferrous carbonate	38	5–10
Copper	Cupric sulfate	25	20–50
	Cupric chloride	37	20–75
	Cupric oxide	75	15
	Copper lysine	variable	95–100
	Copper methionine	variable	95–100
Zinc	Zinc oxide	78	15–30
	Zinc carbonate	52	10–20
	Zinc sulfate monohydrate	36	15–20
	Zinc lysine	variable	75–90
	Zinc chloride	48	15–20
Manganese	Manganese monoxide	77	20–25
	Manganese chloride	43	12–15
	Manganese carbonate	48	10–20
	Manganese sulfate monohydrate	32	10–15
	Manganese methionine	variable	90–100
Selenium	Sodium selenite	45	40–90
	Sodium selenate	21	40–85
	Selenomethionine	variable	95–100
	Selenoyeast	variable	80–100
Iodine	Sodium iodine	84	90–100
	Potassium iodine	69	85–100
	Calcium iodate	64	90–95
	Ethylenediamine dihydriodine	80	105
Cobalt	Cobaltic oxide	73	10–20
	Cobaltous oxide	70	30–55
	Cobaltous sulfate	21	70–100
	Cobaltous carbonate	46	40–90

to be considered in relation to the phosphorus levels and the maximum Ca:P ratio should not exceed 7:1 at adequate phosphorus levels. In pigs, excess calcium and phosphorus intake causes reduction of growth and development, especially when the calcium to phosphorus ratio is increased (Hall et al., 1991). Furthermore, a high level of calcium decreases phosphorus utilization and increases zinc requirements, when phytate is present in the diet of pigs (Oberleas and Harland, 1996).

3. PHOSPHORUS

The total phosphorus (P) content contributes about 1% of body weight in mammals and birds. Bone tissue and muscles contain about 85% and 6% of this element, respectively. The remaining 9% of phosphorus is distributed between all other tissues. Intracellular fluid contains 14% of phosphorus, whereas only 1% is present in extracellular fluid. Phosphates are present in serum both as inorganic ions and esters that are mainly represented by phospholipids (Kokot, 1998; Laroche, 2001). Twelve percent of the inorganic pool is bound to proteins and 43% is present as ions or salts of sodium, calcium and magnesium. Phosphates contribute to higher mechanical endurance of bones and the skeletal system thanks to their presence together with calcium as hydroxyapatites and phosphoproteins. Moreover, pyrophosphates may regulate formation and osteolysis of bone tissue and inhibit calcium absorption at the intestinal level (Laroche, 2001). Phosphorus is an essential element of nucleic acids, and the phospholipids building cellular, microsomal and mitochondrial membranes. However, its most important function in the organism is its role in energy transformation processes, where it is the indispensable constituent of high-energy molecules such as ATP, ADP and AMP (Pasternak, 2000; Laroche, 2001). Phosphates play key roles in glycolysis and glyconeogenesis and they regulate the biosynthesis of 1,25-dihydroxyvitamin D_3 (Waddell et al., 1997; Edwards, 2002). Increased serum phosphate levels inhibit vitamin D synthesis, whereas hypophosphatemia has the opposite effect. Due to protein phosphorylation processes, phosphorus participates in DNA transcription and determines the specific action of hormones in the effector cells (Kokot, 1998). Its function as a part of 2,3-DPG that regulates the oxygen affinity of hemoglobin is also noteworthy (Jänig et al., 1971). The extracellular fluid concentration of phosphorus also influences intracellular calcium transport rate. Its absorption in animals from the most common mineral sources is presented in table 6. Inorganic phosphorus absorption in monogastric mammals takes place mainly in the small intestine, especially in the duodenum and jejunum and can be stimulated by calcitriol (Wesinger and Bellorin-Font, 1998). The large intestine and stomach have no significant influence on the total phosphorus absorption. Transepithelial phosphorus absorption consists of a sodium-dependent active mechanism, that can be regulated by calcitriol, and a passive paracellular mechanism dependent upon the electrochemical gradient across the intestinal wall. Investigations in ruminants have demonstrated that about 90% of the active absorption of inorganic phosphorus occurs in the small intestine, with a major participation of the jejunum (Schröder et al., 2002).

The dietary requirement for total phosphorus in newborn calves, pigs and foals is similar and equals about 7 g/kg DM feed. This requirement is fully satisfied by the highly available phosphorus contained in colostrum and milk. The needs of growing ruminants and horses diminish gradually with age, down to about 2 g P/kg DM feed in the fully grown animals (tables 3 and 7). In pigs however, the requirement for total phosphorus remains quite high (over 4 g P/kg diet DM) (table 4). The high requirement for phosphorus in pigs, as well as in chicks utilizing solid feeds (mainly cereal grains), is primarily caused by the low availability of plant phosphorus to these animals. Most of the phosphorus (50–80%) in grain is present

Table 7

Requirements of horses for minerals in 1 kg DM and their content in typical feeds[a] and in 1 kg DM of a model diet

	Macroelements (g)						Microelements (mg)						
Age, months	Ca	P	Mg	Na	K	S	Fe	Zn	Cu	Mn	I	Se	Co
4	6.8	3.8	0.8	1.0	3.0	1.5	50	40	10	40	0.1–0.6	0.1	0.1
6 – moderate growth	5.6	3.1	0.8	1.0	3.0	1.5	50	40	10	40	0.1–0.6	0.1	0.1
6 – rapid growth	6.1	3.4	0.8	1.0	3.0	1.5	50	40	10	40	0.1–0.6	0.1	0.1
12 – moderate growth	4.3	2.4	0.8	1.0	3.0	1.5	50	40	10	40	0.1–0.6	0.1	0.1
12 – rapid growth	4.5	2.5	0.8	1.0	3.0	1.5	50	40	10	40	0.1–0.6	0.1	0.1
18 – not in training	3.4	1.9	0.8	1.0	3.0	1.5	50	40	10	40	0.1–0.6	0.1	0.1
24 – not in training	3.1	1.7	0.9	1.0	3.0	1.5	50	40	10	40	0.1–0.6	0.1	0.1
Content in 1 kg of typical feeds and in model winter* and summer** diets for 12 month horse, rapid growth													
Oats	0.9	3.8	1.6	0.6	4.5	2.3	73	39	6.7	40.5	0.13	0.24	0.06
Hay with orchard grass	2.6	3.0	1.1	0.1	26.7		84	38	20	167		0.03	0.3
Orchard grass, midbloom	2.3	1.7	3.3	2.6	20.9		68	25	50.1	136			0.1
1 kg of DM winter diet *	1.41	3.56	1.45	0.45	11.16	1.61	76.3	38.7	10.7	78.5	0.09	0.18	0.13
Deficiency (%)	67	Excess	Excess	55	Excess	Excess	Excess	3	Excess	Excess	0–83	Excess	Excess
1 kg of DM summer diet **	1.88	2.33	2.79	2.00	15.98		69.5	29.2	37.1	107		0.07	0.09
Deficiency (%)	56	4	Excess	Excess	Excess		Excess	26	Excess	Excess		30	10
Tolerance, multiple of max requirements	5	3	4	6	8	8	10	12	40	20	8	5	20
Toxicity in 1 kg of diet DM[b]	20	12	6	12	30	12.5	1000	500	800	1000	5	2	10

[a]NRC, 1989. Nutrient Requirements of Horses. National Academy Press, Washington, D.C., reproduced with permission of the National Academies Press. © 1989 National Academy of Sciences.
*Winter diet: oats + hay (70 + 30%); ** summer diet: oats + grass (30 + 70%)
[b]NRC, 1980. Mineral Tolerance of Domestic Animals. National Academy Press, Washington, D.C.

as phytate, which is well utilized by ruminants, but is poorly utilized by monogastric species. With the aim of increasing the availability of phytate P, it has been widely recommended that diets for growing pigs and chicks are supplemented with microbial phytase. Diets supplemented with this enzyme increase not only the availability of phosphorus but also that of other minerals (Ca, Zn). Because of the high proportion of poorly available phytate P in cereal grains, the phosphorus requirements for pigs and poultry are normally given not only as total P but also as available P (pigs) or nonphytate P (poultry) (National Research Council – NRC, 1994, 1998). Adequate phosphorus nutrition depends, therefore, directly on the animal species. It is also influenced by the vitamin D status of the diet and the dietary calcium concentration. A dietary Ca:P ratio between 1:1 and 2:1 is assumed to be ideal for proper growth and bone formation in all species, although most animals can radically change these ratios by homeostatic control (Underwood and Suttle, 1999b). The common diets for growing ruminants, based on roughages (grasses or legumes) and grains, supplemented with protein feed – SBM or, in some summer diets with dry sugar beet pulp, may contain adequate amounts of this mineral (table 3). However, typical pig and poultry diets, based on grains containing low levels of nonphytate phosphorus need supplementation with inorganic P sources (tables 4 and 5), the availability of that is relatively high.

Phosphorus is well tolerated by animals due to the occurrence of protective mechanisms regulating its level, both in tissues and systemic fluids. Furthermore, even though excess P in broiler's diet (5.5–8.3 g P/kg DM) causes the appearance of toxicity symptoms these can be reduced by the addition of calcium (15–17 g Ca/kg DM) (Edwards and Veltman, 1983). It was found that phosphorus toxicity might occur in horses when the dietary phosphorus content reached 12 g/kg DM (table 7).

4. MAGNESIUM

Magnesium (Mg) is the fourth most abundant cation in the body, behind sodium, potassium and calcium, and the second most prevalent intracellular cation. The total amount of magnesium in the body has been assessed at 14.5 mmol/kg BW, whereas its tissue content in land mammals oscillates between 0.1–0.47%. Most of the magnesium is stored in the bone tissue (50–60%), while the remainder is present in all the soft tissues. The majority of magnesium is distributed in an intracellular form and only 16% is exchangeable (Kokot, 1998; Wesinger and Bellorin-Font, 1998; Bober and Pasternak, 2001). Some 20–30% of serum magnesium is bound to proteins, whereas the rest is ionized and is biologically active (Paolisso and Barbagallo, 1997; Kokot, 1998; Vormann, 2003).

Among its many functions in the body concerned with processes of metabolic regulation and transmembrane transport of cations and anions, it ensures proper neuromuscular activity, thus having an opposite effect to calcium. It enables correct functioning of cardiac and vascular smooth muscles and aids muscle relaxation following physiological contractions (Paolisso and Barbagallo, 1997). It participates in resynthesis of phosphate energy sources, including ATP and creatine phosphate (Delva, 2003). Magnesium is a constituent of about 80 enzymes and it guarantees proper glycolysis, oxidation processes and nucleic acid synthesis, due to its physiological role as an enzyme activator (Paolisso and Barbagallo, 1997; Wesinger and Bellorin-Font, 1998; Nogowska et al., 2000). It also activates vitamin B_1. It provides fibrinogen and blood platelet stabilization and influences membrane permeability, catecholamine synthesis and lipid metabolism. Physiologically, magnesium is in dynamic balance with calcium and its increased intake inhibits bone growth (table 8). It is worthy of note that magnesium increases potassium absorption in the intestine and protects against

Table 8

Interaction between minerals (☺ - synergistic, ⤫ - antagonistic, ✱ - specific interaction)

Ca	P	Mg	Na	K	Cl	Fe	Zn	Cu	Mn	Co	J	Se	Cr	Pb	Hg	Cd	Si	S	
	☺	✱	✱	✱			⤫	☺	☺		⤫			⤫		⤫			Ca
				✱		⤫	⤫		⤫				⤫	⤫		⤫		☺	P
				✱		☺	☺	☺	☺	⤫				⤫				☺	Mg
				✱												☺			Na
									✱										K
																			Cl
							⤫	☺	⤫	✱	⤫			⤫		⤫			Fe
								⤫				☺	☺	⤫	⤫	⤫			Zn
									✱	☺		✱		⤫		⤫		⤫	Cu
											⤫	⤫		☺					Mn
											⤫	✱				⤫		☺	Co
												⤫			⤫				J
														✱		⤫			Se
																			Cr
																			Pb
																			Hg
																			Cd
																			Si
																			S

heart muscle injury from free radicals and toxins as well as preventing arteriosclerosis (Kokot, 1998; Robles et al., 1998; Bober and Pasternak, 2001).

Intestinal absorption of magnesium in monogastric mammals takes place mainly in the ileum and jejunum, although the colon and cecum may also contribute to magnesium uptake. In ruminants absorption of magnesium takes place in the forestomachs as well as the intestines (Kayne and Lee, 1993; Vormann, 2003). The efficiency of absorption of magnesium varies from 30–50%, under physiological conditions and normal intake, up to 80% in states of magnesium deficiency (Bober and Pasternak, 2001) (table 6). 1,25-dihydroxyvitamin D_3 and parathormone stimulate its intestinal absorption, whereas free fatty acids, inorganic phosphates, sulfur and fluorine have an inhibitory effect (Kokot, 1998; Bober and Pasternak, 2001). The half-life time of magnesium amounts to 11 days and the main excretion routes are via urine and feces. Apart from the pool of magnesium present in bone tissue, the remainder is located in the liver, heart, pancreas and brain and other organs to maintain its blood concentration at a level of 1.7 mg/100 ml. Plasma proteins bind magnesium, thus limiting the amount of this element filtered out at the kidneys.

The magnesium requirements of livestock animals are rather stable during the entire growing period and are comparatively low for monogastric (e.g. pigs–0.44 g Mg/kg diet DM) in comparison to ruminants (1 g Mg/kg diet DM) or horses (0.9 Mg/kg diet DM). The high demand for this mineral by these animals is mainly caused by the low availability of magnesium in roughages, which usually form the major dietary component for these animals (NRC, 2001). Typical diets completely cover normal magnesium requirements, however, because of its very wide spectrum of availability from particular feeds, mineral supplements of this macroelement are often used. An extremely low availability of Mg is usually noted in the young, high-nitrogen and potassium-fertilized pastures. Deficiency of available Mg can cause magnesium tetany. This is why a mineral magnesium supplement in the diet is advisable for

animals that are going to utilize this kind of pasture. On the other hand, the availability of Mg from the majority of mineral sources is high (60–95%) (table 1).

An excess of magnesium in the feed has few toxic effects in healthy animals, because they can tolerate large quantities of dietary magnesium due to limited intestinal absorption and effective kidney filtration of Mg excess. In the pig, the toxic level of magnesium is not known; however, the maximum tolerated is approximately 0.3% of diet (NRC, 1980). Moreover, 5 g/kg DM is tolerated by ruminants, whereas the amount of 14 g/kg DM is mildly toxic for sheep and steers (Chester-Jones et al., 1989, 1990). In blood serum of breeding animals, threshold concentrations of Mg above which pathological changes may occur, amount to 1.15–1.62 mmol/l (table 1). Magnesium excess of 6 g/kg DM level in a diet may cause toxic symptoms in horses as well (table 7). Excess magnesium up to a level of 36.4 mg/l in plasma remains clinically undetectable (Durlach, 1991). In ruminants, higher levels of magnesium (25–47 g/kg DM) cause acute diarrhea and somnolence (Underwood and Suttle, 1999c). In chickens, similar symptoms occur at levels above 30 g/kg DM (Lee and Britton, 1987), while levels higher than those mentioned, usually cause muscle paralysis and death.

5. SULFUR

Sulfur (S) is one of the biogenic elements that are indispensable for proper animal growth, development and organism functions. It is a constituent of the sulfur amino acids such as cysteine, cystine and methionine that build proteins in all body tissues, while lipoic acid and taurine also contain sulfur. It is especially abundant in the skin, hair and connective tissue, where it is present as a chondroitin sulfate (Murray, 1995). Sulfur participates in the processes regulating glucose metabolism as a constituent of insulin. Moreover, the sulfur-containing taurine, together with bile acids make up the taurocholic acids, which facilitate efficient intestinal lipolysis and lipid absorption. Due to its presence in glutathione, which has antioxidative functions, sulfur enables the reconstruction of protein thiolic groups after their oxygenation to sulfonic or bisulfide groups. It occurs in pantothenic acid, the starting component of coenzyme A, that participates in energy transformation of carbohydrates, lipids as well as protein metabolism processes. Vitamin B_1 as a form of thiamine pyrophosphate consists of sulfur and participates as a co-enzyme in pyruvate and α-ketoglutarate oxidative decarboxylation processes (Mayes, 1995). Sulfur is a component of biotin, which is indispensable for proper growth and skin function, and is also a constituent of the natural coagulant, heparin (Underwood and Suttle, 1999d).

Ruminants possess a rich microbial population in the forestomach, a population that is able to incorporate inorganic sulfur into microbial proteins. Sulfide, which is potentially toxic, is produced and absorbed from the rumen when excessive amounts of degradable sulfur are available to the rumen microorganisms. Nonruminants differ from ruminants and require balanced dietary sulfur amino acids as the main source of this element. The main route of sulfur excretion is through the urine in the form of sulfate; however, excretion of sulfur in the organic form occurs through the feces.

In monogastric animals the sulfur requirements can be considered in terms of their needs for sulfur-containing amino acids. Sulfur provided by these amino acids seems adequate to meet these animals' needs for synthesis of sulfur-containing compounds, such as taurine, glutathione, lapioc acid and chondroitin sulfate. In his investigation on growing-finishing pigs Miller (1975) proved that addition of inorganic sulfate to low-protein diets was not beneficial. The dietary requirement of sulfur for ruminants is primarily to provide adequate substrate to ensure maximal microbial protein synthesis. The recommended concentration of this mineral

in beef cattle diets is estimated at 1.5 g S/kg DM. Somewhat higher levels (1.8–2.6 g S/kg diet DM) are required by growing lambs (NRC, 1985) because of the approximately three times higher sulfuric acid content in wool compared to other animal proteins (Orskov, 1982). In general, the sulfur content of feedstuffs is directly related to protein concentration. The sulfur concentration found in pasture and conserved forages ranges widely from 0.5 g to >5 g S/kg DM (Underwood and Suttle, 1999d). Grasses contain ca. 2 g, lucerne ca. 3.5 g S/kg DM. Maize silage is often low in this element (0.5–1.0 g/kg DM) and generally also in protein (NRC, 2001). The protein-deficient diet, supplemented by nonprotein nitrogen must be also enriched in sulfur. Nonprotein nitrogen, such as urea, added to these diets will not be incorporated into microbial protein unless adequate sulfur is present to allow formation of methionine (NRC, 2001). The availability to ruminal microorganisms of sulfur from the following different sources has been ranked from the most to least available: as L-methionine, sodium sulfate, sodium sulfide, element sulfur, and methionine hydroxy analog (Kahlon et al., 1975). An increased sulfur level in the diet can reduce feed intake and decrease copper status. The maximum tolerable concentration of dietary sulfur for ruminants has been estimated at 4.0 g/kg diet DM (NRC, 1980).

In ruminants, there is little margin between nontoxic and toxic levels of sulfur. Some factors that increase the toxicity of sulfur derivatives include the amount of degradable organic sulfur ingested and pH value in the rumen. Excessive dietary sulfur (4.1 g/kg DM) affects the central nervous system and contributes to the development of encephalomalacia in calves and lambs receiving acidifiers, but simultaneous addition of urea can lessen the depression of appetite and digestibility caused by sulfur alone (Bird, 1974). In nonruminant animals, main toxic effect of excess S ingestion is osmotic diarrhea, although dehydration, acidosis, heart, lung and liver damage may also occur (Jeffrey et al., 1994; Cummings et al., 1995a, 1995b; Low et al., 1996).

6. SODIUM

Sodium (Na) is the major cation in the extracellular fluid compartment, which accounts for 91% of the mineral, the remaining 9% is found in the intracellular fluid. Regulation of sodium homeostasis is principally dependent on a rapidly exchangeable pool of sodium, which comprises about two-thirds of the total sodium content. The other one-third of body sodium, mainly that deposited in bone tissue, is either poorly or not at all exchangeable, and is responsible for osmotic pressure and water balance between the extracellular and intracellular spaces (Kokot, 1998; Pasternak, 2000). The sodium–potassium pump (Na^+-K^+) is an enzymatic transport process that pumps sodium ions out of the cell membrane and at the same time potassium ions from the outside to the inside of the cell. This pump is present in all cells of the body, and it is responsible for maintaining the sodium and potassium concentration gradients across the cell membrane, as well as for establishing a negative electrical potential inside the cells. It is also at the basis of nerve function to transmit signals throughout the nervous system, while an electrical potential across muscle cell membrane, determined by the Na^+-K^+ pump, is a basic requirement to transmit signals in muscle tissue as well. Finally, the Na^+-K^+ pump performs a continual surveillance role in maintaining normal cell volume, preventing swelling and bursting of cells.

The mean concentration of sodium in mammals totals 140 mmol/l in ECF and 10–20 mmol/l in ICF and is estimated at 60 mmol/kg BW. Furthermore, sodium ions participate in co-transport of glucose and amino acids, especially in the epithelial cells of the intestinal and renal tubules, to aid in the absorption of these substances into the blood. Another co-transport

mechanism is a sodium–potassium–two chloride co-transporter that allows two chloride ions to be carried into cells along with one sodium and one potassium ion. Calcium countertransport, with sodium ions moving to the interior and calcium ions to the exterior, occurs in all or almost all cell membranes as well. One very important function of Na^+ ions concerns the sodium–hydrogen countertransport that occurs in several tissues, especially in the kidneys. In the kidneys and small intestine sodium ions move from the lumen of the tubule to the interior of the tubular cells, whereas hydrogen ions are countertransported into the lumen (Guyton and Hall, 1996a). Intestinal absorption of Na^+ takes place mainly in the middle and lower part of small intestine and under physiological conditions is equal to its elimination (Kokot, 1998) (table 6).

The requirement for sodium (and also potassium and chlorine) in growing animals of all species declines as they approach their mature body weight. The American norms (NRC, 2001) recommend sodium concentrations in milk replacers of 4 g/kg DM, whereas the Na requirements of growing cattle above 3 months of age are estimated at only 0.8 g/kg dietary DM (table 3). Changes in the sodium needs of other species of growing livestock according to their age are analogous (tables 4, 5 and 7) e.g. growing broiler chicks require 2.0 g Na and 1.2 g Na in 1 kg DM in early- and late-stage growth, respectively. Common plant feedstuffs generally contain very little sodium (0.1–0.4 g/1 kg DM; tables 3, 4, 5 and 7). The diets usually cover less than a half of the animals' requirements for this mineral, but needs are usually easily met by supplementing with sodium chloride (NaCl) or alternatively sodium carbonate or sodium sulfate can be used, especially for cattle. Availability of sodium is very high – around 90%. Nevertheless, when the animals have free access to water, they can tolerate diets containing up to 20 g (pigs), or even 50 g (poultry) sodium in 1 kg DM (Underwood and Suttle, 1999e). However, application of manure from livestock fed sodium and chloride in excess of the animals' needs can increase soil salinity (Coppock, 1986).

Excessive levels of sodium and chloride disturb cell osmotic pressure. Upper threshold concentration for sodium in blood serum of breeding animals amounts to 146–160.9 mmol/l (table 1). Excessive levels of sodium occur at dietary levels of 50–80 g/kg DM (poultry), 30–60 g/kg DM (cats, sheep), 20–40 g/kg DM (swine), and 10 g/kg DM (horses) (Underwood and Suttle, 1999e). A pig is resistant to high amounts of dietary sodium chloride, but only when free access to (nonsaline) drinking water is allowed. In the event of limited access to water or of high saline concentrations in the water, toxic effects can develop. Excess of sodium ions disturbs water balance in the organism and produces severe hypertonic expansion of the extracellular fluid, usually leading to nervousness, weakness, staggering, epileptic seizures, paralysis and death. Under conditions of excessive sodium intake, arterial hypertension, vascular disorders, kidney lesions, liver dysfunction, and hypofunction of gastric glands may occur as well (Carson, 1986).

7. POTASSIUM

The mean content of potassium (K) in mammals is estimated to be 53.8 mmol/kg BW. About 10% of total body potassium is distributed in the extracellular compartment, whereas the remaining 90% reside in the intracellular space. The total amount of this element in an average 70-kg human is equal to 150 g. More than 90% of total-body potassium is exchangeable but its content in several tissues varies significantly. In adults, the highest potassium content is reported to be in the skeletal muscles, followed by bone tissue, liver and red blood cells (RBC), whereas fat tissue is relatively poor in this element. The exchangeable pool of potassium is conditioned by body fat content and decreases during aging. Under physiological

conditions there is a constant ratio of potassium to tissue nitrogen: i.e. 3 mmol to 1 g, respectively (Kokot, 1998). Potassium ions are the most important of the intracellular cations for determining osmotic pressure, the range of water intracellular space and electrical gradient across the cell membrane. Due to the activity of the Na^+-K^+ dependent ATPase, it determines membrane potential, which is especially crucial in nerve tissue and heart muscles for proper cell function (Guyton and Hall, 1996a). Furthermore, cellular potassium concentrations are important since they affect acid–base balance that is strictly connected with H^+ content in ICF. Potassium functions as an activator for numerous enzymes, shares in protein and glycogen synthesis, and participates in metabolic processes (Pasternak, 2000). The potassium absorption process is passive and takes place, mainly in the upper small intestine, in accordance with the electrochemical gradient (Kokot, 1998). Its concentration and absorption from the most common mineral sources is presented in table 6.

Due to ready availability, potassium requirements of young livestock animals do not change much with age. For example, a newborn calf requires, according to NRC (2001) norms, 6.5 g and 1-year cattle close to 5 g potassium in 1 kg diet DM; a 3–5 kg piglet and a 100 kg fattener require only 3.3 g and nearly 2 g K/kg diet DM, respectively. The roughages (green forages – fresh and preserved, roots), thanks to high-potassium fertilization, are usually rich in this mineral (10–30 g K in 1 kg DM), and often in excess of requirements (table 3). Potassium content in cereal grains (3–4 g K in 1 kg) is much lower than in roughages. However, thanks to high levels of potassium in protein additives (extracted meals), diets for monogastric animals are rarely deficient in this mineral. Ruminant diets, on the other hand, are often too rich in potassium. This can be important, since dietary K excess can decrease the availability of some other minerals, mainly magnesium and sodium, and reduced absorption of magnesium can cause tetany (NRC, 2001).

Potassium is more active than sodium, and thus is less well tolerated. Nevertheless, ruminants can tolerate relatively high potassium levels, in the range of 30–60 g/kg diet DM. Potassium toxicity symptoms become evident in swine at dietary levels of 18–20 g/kg DM, and in chickens at 9–12 g/kg DM (Underwood and Suttle, 1999f). Pigs can tolerate dietary K levels about ten times higher than the requirements if free access to drinking water is allowed. However, the toxic level of potassium has not yet been clearly established. Excessive potassium intake causes abnormalities in heart function in pigs and other animals and may also lead to edema. Excessive intake of K does not occur often and may arise accidentally in the mixing of feeds or animal treatment. Hyperkalemia can also develop from acute renal insufficiency caused by pathogenic microorganisms, such as in leptospirosis. The upper concentration limits, above which pathological states occur, are presented in table 1.

8. CHLORINE

Chloride ions (Cl^-) are the major anions in extracellular fluid and indeed, 87.6% of total body chlorine is present in the ECF, while only 12.4% occurs in the intracellular compartment. The mean chlorine content in the body is estimated at 33 mmol/kg BW and it is an important factor, along with other ions, in helping maintain the electroneutrality of the body. The mean tissue concentration of Cl^- varies between 3 mmol/l in the intracellular fluid of muscles up to 30–60 mmol/l in red blood cells. It has a concentration of 101 mmol/l in the plasma, 124 mmol/l in cerebrospinal fluid, 113 mmol/l in extracellular fluid, but only 12 mmol/l in intracellular fluid (Kokot, 1998). The highest concentration of Cl^-, up to 150 mmol/l, is found in the hydrochloric acid secreted by the stomach. Chloride ions are also present in other body fluids including saliva, bile, pancreatic juice and intestinal juice to guarantee proper digestive

processes and secretory functioning in the gastrointestinal tract. All the chloride in the organism is readily exchangeable and its metabolic activity is strictly connected with sodium ions. Intestinal absorption of chloride in monogastric organisms, which is mainly conditioned by sodium chloride supplementation, is nearly 100% of intake and takes place in the small intestine (Kokot, 1998; Underwood and Suttle, 1999e). In cattle and sheep, the effectiveness of chloride absorption is estimated at 85–91% (Agricultural Research Council, 1980).

Both requirements for absorbed chloride and dietary requirements for this mineral are very similar to those for sodium. However, the content of this mineral in most feedstuffs (especially roughages), is higher than that of sodium and some diets for growing cattle are able to cover the chloride needs without supplementation (table 3). Typical diets for pigs and poultry, however, need to be supplemented by a mineral source, usually NaCl (tables 4 and 5). If enough sodium chloride is used to meet the requirement for sodium, this will ensure that the chloride requirement is met or exceeded (Underwood and Suttle, 1999e). Like sodium, the availability of chloride is high, e.g. the absorption efficiency of this mineral in cattle and sheep fed mixed diets is estimated at 85–91% (Agricultural Research Council, 1980). Both young and grown-up mammals can tolerate dietary content of this mineral (20–80 g chloride in 1 kg diet DM) as high as that of sodium, so long as they have free access to water. However, poultry tolerate comparatively less chloride. Toxic levels of chloride were seen at dietary levels of 1–3 g/kg DM in cats and sheep, 6–12 g/kg DM in poultry and 20–49 g/kg DM in swine (Underwood and Suttle, 1999e).

9. IRON

Iron (Fe) is one of the most abundant metals in the body. The majority (60–70%) of total body iron is present in the hemoglobin of red blood cells. About 10% of body iron is a constituent of myoglobin, cytochromes and enzymes, while the remaining 20–30% of this vital element is stored as ferritins and hemosiderins in hepatocytes and reticuloendothelial macrophages (Conrad et al., 1999; Lieu et al., 2001). Iron is an essential element for several physiologic functions. It participates in metabolic processes, oxygen transport, DNA synthesis and electron transport (Bothwell et al., 1995; Andrews, 1999; Andrews et al., 1999; Lieu at al., 2001). The biological activity of iron is due to its existence in either the ferrous (Fe^{2+}) or ferric (Fe^{3+}) oxidation states, which enables it to accept or donate electrons (Wessling-Resnick, 1999; Lieu et al., 2001). The most significant iron pool, having the highest turnover, is transferrin-bound iron that contributes less than 1% of the total body pool of iron. Some 80% of transferrin-bound iron participates in hemoglobin synthesis during erythroid cell formation in the bone marrow (Conrad et al., 1999; Lieu et al., 2001). It is worth noting that the majority of iron from destroyed red blood cells is recycled and utilized for further hemoglobin synthesis. Due to its chemical properties, the iron in hemoglobin plays a crucial role in oxygen transport by RBC. Iron performs important functions in DNA, RNA and protein synthesis, it participates in electron transport, cellular respiration, cell proliferation and differentiation of monocytes and macrophages and helps to regulate gene expression by supporting transcription (Gerlach et al., 1994; Andrews et al., 1999; Boldt, 1999; Conrad et al., 1999; Wessling-Resnick, 1999; Lieu et al., 2001). There is also evidence that iron is important in myelin formation and in the development of the neuronal dendritic tree (Youdim et al., 1990, 1991; Ben-Shachar et al., 1994; Gerlach et al., 1994; Lieu et al., 2001). Among the many iron-containing enzymes, the most common are oxidases, catalases, peroxidases, cytochromes, ribonucleotide reductases, aconitases and nitric oxide synthases (Boldt, 1999; Conrad et al., 1999; Ponka, 1999; Lieu et al., 2001). Iron metabolism takes place in a variety of tissues, including muscles, testes,

brain, intestines and placenta (Youdim, 1988; Yehuda and Youdim, 1989; Andrews et al., 1999; Lieu et al., 2001).

Iron absorption takes place mainly in the small intestine via the mucosal cells lining the intestinal lumen. In the next step, iron enters capillaries, where it binds to transferrin that transports it in the blood and delivers it to the cells via the transferrin receptor (Bendich, 2001). The chemical form in which the iron is supplied and the proportion and amounts of other metals in the diet are factors that affect the absorbability of iron from the gastrointestinal tract (table 6). The absorption from the intestines depends on the iron status in the body and is diminished when iron is not depleted. Moreover, iron absorption during animal growth and development differs according to the species studied. Newborn calves are not able to meet their iron requirements from milk containing iron-saturated lactoferrin (Kume and Tanabe, 1994). At weaning, unabsorbable forms of iron may be present in the diet, which results in inefficient absorption.

The requirement for iron, in terms of dietary iron concentration, is high in the newborn animal and declines with age, since growth-needs for this element are predominantly for hemoglobin and increase in red-cell mass; this constitutes a progressively smaller component of weight gain as animals grow. Iron content in diets for growing animals is presented in table 9. Newborn pigs and calves need 110 mg Fe and 100 mg Fe/kg DM diet, respectively, whereas milk is poor in this element (sow's milk contains 18 mg Fe and cow's milk only 3 mg Fe/kg milk DM) (NRC, 1998, 2001). Shortage of this mineral in milk is responsible for the frequently noted anemia of baby animals, as the amounts of iron stored in their body are used up during the first couple of days after birth. The rapid growth rate of newborn mammals requires the supply of large amounts of Fe in the diet, e.g. the required retention of iron in piglets was estimated at 7–11 mg Fe/day, while only about 1 mg/day is obtained from milk alone. To avoid anemia, it is recommended that iron supplements are provided for the animals. Ferrous sulfate and ferric chloride are both good iron supplements for calves (Henry and Miller, 1995). Most plant feeds contain a high, though variable, iron concentration since this depends mainly on the plant species and the Fe content in the soil. Common roughages contain usually from 70–500 mg Fe/kg DM (NRC, 1996). Cereal grains are poorer in this

Table 9

Maximum microelements content in diets for growing animals

Micro-elements	Beef cattle	Sheep and goats	Pigs	Horses	Poultry
Fe	750 mg/kg diet	500 mg/kg diet	750 mg/kg diet, piglets, 1 week before weaned – 250 mg/day	750 mg/kg diet	750 mg/kg diet
Co	2 mg total	2 mg total	2 mg total	2 mg total	2 mg total
Cu	15–35 mg total	15 mg total	25 mg total piglets up to 12 weeks – 170 mg total	25 mg total	25 mg total
Mn	150 mg total	150 mg total	150 mg total	150 mg total	150 mg total
Zn	150 mg total, milk feed, 200 mg total	150 mg total	150 mg total	150 mg total	150 mg total

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element, containing 30–60 mg Fe/kg DM (Underwood and Suttle, 1999g). Iron deprivation in growing animals occurs very rarely, with the exception of newborn mammals fed only with milk. Homeostasis of iron levels in the body is maintained mainly by controlling the amount absorbed from the digestive tract. The level of absorption increases with Fe shortage in the organism and decreases when the intensity of erythropoiesis is diminished. The animals can store some iron mainly in ferritin and hemosiderin. Availability of iron from forages appears to be lower than from most supplemental iron sources (NRC, 1996). The availability of iron in natural feedstuffs, estimated by Henry and Miller (1995) in chickens, ranges from 20–60% of the availability of $FeSO_4$.

The excretion of iron excess is difficult, because there is no pathway for elimination of iron from the body. This makes the animals susceptible to iron poisoning when it is given parenterally (Kreutzer and Kirchgessner, 1991). Dietary Fe excess or injections of free iron into the blood plasma leads to bacterial growth and can promote bacterial infections and diarrhea (Klasing et al., 1980; Knight et al., 1983; Kadis et al., 1984; Dallman, 1990). Calves fed milk-replacer containing 2 g Fe/kg DM as ferrous sulfate, did not show signs of poisoning, however the iron levels in the spleen and liver were altered. Dietary iron at a level of 5 g/kg DM depressed appetite and retarded growth of calves (Jenkins and Hiridoglou, 1987). In the neonatal pig, the toxic oral dose of iron in the form of ferrous sulfate is approximately 600 mg/kg BW; however, lower doses of injectable iron (100 mg as iron dextran) are toxic to pigs from sows with vitamin E deficiency. The excess of iron accumulates in the form of hemosiderin in the liver and causes damage to this organ. Low phosphorus levels in the blood and rachitic lesions develop in piglets after a dietary iron level of 5 g/kg DM is reached, but this can be prevented by increasing the level of phosphorus in the diet. Excessive iron in the diet interferes with phosphorus utilization because it forms iron–phosphorus complexes that are not absorbed from the intestine (O'Donovan et al., 1963; Arpi and Tolerz, 1965; Patterson et al., 1969; Furugouri, 1972). Excessive iron levels in animals' diets that cause toxicity symptoms are: 3–5 g/kg DM in swine (table 4), 1 g/kg DM in horses (table 7) and 1–4.5 g/kg DM in chickens (table 5). Upper limits of Fe values in blood serum are given in table 1.

10. COPPER

Copper (Cu) is an essential trace element present in all animal tissues. The highest level of copper, reaching about 15% of total body store, is found in the liver. Brain, muscles, blood and bone tissue are also rich in copper (Peña et al., 1999; Rinaldi, 2000). Under physiological conditions plasma Cu levels oscillate from 100–130 μg/100 ml (Kabata-Pendias and Pendias, 1999a). Its importance in animals is connected with its role as an enzyme activator participating in reduction-oxidation processes. Copper and iron take part in initiating the generation of reactive oxygen species (ROS), which react with polyunsaturated fatty acid residues of cell membranes, thiol-containing proteins, and nucleic acids leading to oxidative stress and cytotoxic effects (Medici et al., 2002). On the other hand, copper is required for the activity of superoxide dismutase (SOD), which is a scavenger of reactive oxygen metabolites. As a part of cytochrome oxidase it is essential for metabolic processes and energy transformation. Tyrosinase catalyzes tyrosine's transformation to melanine, whereas monoamine oxidase has an influence on the metabolism of biogenic amines. Furthermore, galactosyltransferase participates in myelin formation processes that enable proper function of the nervous system and dopamine β-hydroxylase transforms tyrosine into norepinephrine (noradrenaline) (Turnlund, 1998; Uauy et al., 1998; Qian and Ke, 2001). Lysyl oxidase and ascorbic oxidase are both copper-dependent enzymes which play very important roles in collagen and

elastin formation, guaranteeing the proper development and function of the skeletal system and blood vessels. A total of 93% of the copper present in plasma is bound to protein and called ceruloplasmin. It is responsible for iron oxidation and mobilization in the liver, and its incorporation into transferrin. Moreover, ceruloplasmin determines copper transport and serves as its reservoir for other tissues (Wachnik, 1987). Another nonenzymatic protein containing Cu, metallothioneine (MT), is an important factor influencing metabolism of trace elements like zinc (table 8). Copper enables proper synthesis of heme and leukocyte function. Its absorption takes place mainly in the small intestine, through active transport with a carrier protein or as copper complexes with amino acids (Nogowska et al., 2000). The absorption of copper occurs in the upper gut and is enhanced by hydrochloric acid and depressed by calcium. The induction of MT is the basic mechanism of action that blocks copper absorption (Grüngreiff, 2002). There are differences in the degree of copper absorption depending on the form supplied (table 6). Copper–protein complexes are more readily absorbed than some copper salts, e.g. copper sulfate. Excretion of copper into the feces via the bile and its excretion through the urine are the main mechanisms by which Cu balance is achieved in the organism.

Copper content in diet for growing animals is presented in table 9. According to the American norms the Cu requirements of growing cattle, horses and broiler chickens is estimated at 9–10 mg Cu/kg dietary DM and they do not change with age (NRC, 1989, 1994, 2001). In ruminants however, a dramatic decrease in the availability of Cu has been noted. In newborn calves up to 70% of dietary copper is absorbed. With the development of the rumen, there comes a dramatic decrease in absorption of Cu, such that only 1–5% of dietary copper is absorbed by adult cattle (NRC, 2001). The absorption of this mineral is reduced mainly by the presence of sulfur and molybdenum in the diet, although antagonism in absorption between Cu and Fe, Zn, Ca, Cd and Pb has also been suggested (Underwood and Suttle, 1999h). Unlike ruminants, monogastric animals given digestible diets, absorb about 60% of moderate copper intakes. Common diets for fattening pigs, based on grain, are quite rich (with corn exception) in this mineral, since the protein sources usually contain enough copper (table 4). Furthermore, typical poultry diets do not always need to be supplemented by addition of mineral sources of copper. If a mineral source is needed sulfate is more effective than sulfide or copper oxide. High Cu supplementation of pig rations, causing high Cu content in the manure, can create environmental problems. In particular, copper poisoning of grazing plants highly fertilized with the manure can result. Pigs and poultry are highly tolerant of copper excess (up to 250–750 mg and 200–400 mg/kg DM, respectively). Cattle and goats are somewhat less tolerant (100–300 mg/kg DM). Among the livestock animals, sheep tolerate the least Cu in the diet (12–36 mg/kg DM) (Underwood and Suttle, 1999h).

Long-term intake of feed with elevated, even nontoxic, copper levels is associated with the risk of chronic intoxication. Young animals particularly are exposed to this. There are important variations in the susceptibility to copper toxicity in farm animals. This is related to differences in metabolic pathways and capacities of young animals. Growing calves are highly resistant, while growing sheep are highly sensitive to Cu toxicity (Eckert et al., 1999). Inadequate copper mixing in the diet can be hazardous for piglets and growing chickens. Long time intake of copper in excess of 250 mg/kg DM causes toxicity signs in the growing pig (NRC, 1980). These include low hemoglobin levels together with jaundice, as a result of copper accumulation in the liver and other organs. Copper excess in a diet induces mainly hepatic changes, followed by injury of the kidneys, brain tissue, as well as coronary vessels and myocardium. Copper shows its highest toxicity when it makes direct contact with cells, especially during their early developmental stages, and causes changes of protein structure. Copper toxicity increases when the dietary levels of zinc and iron are low or when the level

of dietary calcium is high. Low levels of zinc and iron in the diet of the baby pig may increase its sensitivity to 250 mg Cu/kg DM, because the addition of 130 mg of zinc and 150 mg Fe/kg DM prevents copper toxic effects (Suttle and Mills, 1966; Hedges and Kornegay 1973; Prince et al., 1984). Growing sheep accumulate copper in the liver more readily than other growing farm animals. When the copper content reaches a critical level or when stress conditions cause liver cell death, Cu is released into the blood causing hemolysis, hemoglobinuria, bloody nasal discharges leading to hemolytic anemia and death. The blood level of copper increases 10–20-fold and can reach levels of 5–2000 mg/dl. This precedes the clinical signs by 24–48 hours. The most common symptoms are anorexia, excessive thirst and depression. Tissue concentrations of Cu in ewes receiving various levels and different sources of this element show different patterns of distribution.

There are suggestions that the different forms of copper are utilized by the organism in different ways. In growing sheep there is an interaction between the level and source of copper and liver and kidney Cu concentrations. Copper proteinates maintain high liver Cu when fed at normal levels (10 mg/kg diet), and when fed in excess amounts (30 mg/kg diet) result in less liver Cu accumulation. Contrary to this, copper supplementation as copper sulfate ($CuSO_4$) causes increasing concentrations in the liver concomitantly with the fed dose (Stoszek et al., 1986; Eckert et al., 1999). An intermediate dose of copper (20 mg/kg diet) from Cu proteinate induces a higher kidney copper concentration compared to a 10 mg/kg or 30 mg/kg diet, while a low dose of copper (10 mg/kg diet) as Cu sulfate induces higher kidney Cu concentration than with a 20 mg/kg or 30 mg/kg diet. When liver copper is elevated (20, 30 mg Cu as $CuSO_4$/kg diet) kidney copper decreases. This inverse relationship between liver and kidney does not exist when the source of copper is copper proteinate. Copper can induce metallothionein transcription. This may be a mechanism to prevent copper toxicity; and, interestingly, the optimum concentration of the metal to induce MT production is just below a toxic concentration. Ewes fed increasing doses of copper proteinate have more copper in the small intestine tissue than those fed the same doses of copper sulfate. Thus, metallothionein synthesis in the small intestine is induced more readily with dietary copper proteinate than with copper sulfate in sheep (Eckert et al., 1999). A similar protective mechanism has been observed in rats fed high-zinc diets (180 mg/kg diet). Higher levels of zinc are bound to metallothionein in the cytosol of intestinal tissue in high-zinc compared to low-zinc (1 mg/kg diet) fed rats (Hempe and Cousins, 1991). Acute intoxication with copper salts happens very rarely. Copper sulfate ($CuSO_4$) is the most toxic form of the element. A lethal dose of copper sulfate for rabbits is 4–5 mg/kg BW after intravenous injection, and 50 mg/kg BW after oral administration. Toxic symptoms after consumption of the salt are liver, kidney and capillary vessel damage, diarrhea and pain. Death occurs after several hours with cardiac arrest at contraction, hypothermia, intensifying cyanosis and breathing paralysis. The level of dietary copper which leads to toxic effects amounts to 0.75 g/kg DM in swine (table 4), 0.8 g/kg DM in horses (table 7), and 0.25–0.8 g/kg DM in chickens (table 5).

11. ZINC

Zinc (Zn) is primarily an intracellular ion and is the most abundant trace element in the body. It is present in all tissues and body fluids. The major content of zinc in the body (about 85% of the whole body pool) is found in the muscles and bones. The skin and liver contain about 11%; other tissues contain 2–3% of the whole zinc body pool (Grüngreiff, 2002). Zinc in the bones is a relatively inert mineral, but during the periods of mobilization of calcium from the bones its enrolment is remarkable (Wastney et al., 1986). Its mean plasma concentration in

mammals is estimated at 80–90 μg/l. In leukocytes its concentration is about 20 times more than in erythrocytes (Kabata-Pendias and Pendias, 1999b).

It is a cofactor of many enzymes, among which the most common are carbonic anhydrase, carboxypeptidase, alkaline phosphatase, alcohol dehydrogenase, glutamine dehydrogenase, lactate dehydrogenase and RNA polymerase. Zinc, in coordination with cysteine and histidine residues, forms the so called "Zn-finger" domains of the DNA binding proteins (DNA-BP), thereby influencing many vital cellular processes by primary effects on gene expression (Berg, 1990; Chesters, 1992). It participates as an enzyme activator guaranteeing that metabolic processes proceed suitably (Nogowska et al., 2000). As a component of superoxide dismutase, it plays an important function in free radical elimination. Zinc stimulates protein synthesis, amino acid activation and participates in DNA and RNA synthesis. Its optimal tissue concentration determines the precise structure of skin and mucosa and guarantees physiological development and growth of the skeletal system and the whole organism. Zinc is essential for the physiological function of the immune system due to its participation in T cell formation and it regulates cytokine production by mononuclear cells (Prasad, 1996, 2000; Pasternak, 2000). One very important role of zinc is connected with insulin synthesis and its regulatory functions in the body (Tang and Shay, 2001). Moreover, it has an influence on vitamin A mobilization in the liver and its further utilization in the tissues. Zinc is indispensable for proper development of gonads, their reproductive functions and fetal organogenesis. In addition, the proper functioning of the central nervous system and sensory organs for sight, taste and smell are also conditioned by physiological zinc concentrations (Prasad, 2001).

Intracellular zinc transport is controlled primarily by metallothionein, a high-affinity, intracellular, zinc-binding ligand that is regulated directly by zinc. Changes in MT reduction-oxidation activity or intracellular levels of MT are believed to alter the concentration of labile zinc within the cell (Jacobs et al., 1998; Andrews, 2000; Kelleher and Lónnerdal, 2001). Its absorption takes places mainly in the small intestine, but small amounts are absorbed also in the stomach and large intestine (Pasternak, 2000). The digestive system plays an important role in the regulation of zinc homeostasis. The mechanisms involve adjustments in zinc absorption, accumulation and endogenous excretion into the feces (King et al., 2000). About 7% of ingested zinc is absorbed from the digestive tract. Its absorption depends on mineral source (table 6). After absorption, zinc is transported in the portal bloodstream bound to albumins and globulins and is rapidly taken up by the liver and redistributed. Most of the blood zinc (80%) is present in the erythrocytes (1 mg/10^6 cells). About 85% of this amount is present in carbonic anhydrase and 5% as copper–zinc superoxide dismutase (CuZn SOD) (Cousins, 1996). In the plasma, about two-thirds of zinc binds to α_2-globulins and one-third binds to albumins.

The albumin-bound fraction comprises the metabolically active pool of zinc, which is estimated to be 10% of the whole body zinc (Miller et al., 1994). The biological half-life of zinc ranges from 100–500 days (Elinder, 1986). The daily elimination of zinc averages about 1% of the absorbed dose. Zinc is eliminated from the organism in the feces (70–80%), and in the urine and sweat (15–25%) (Spencer et al., 1985). The liver plays an important role in the metabolism and balance of zinc in the whole organism and has the ability to induce hepatic MT synthesis, which is responsible for zinc binding and accumulation (Schröder and Cousins, 1990; Bremner, 1993). On the other hand, cytokines can influence metallothionein synthesis in the liver and the plasma level of zinc can be affected by zinc-binding proteins such as albumin, amino acids and acute phase proteins (Wellinghausen et al., 1997; Prasad, 1998; Grüngreiff, 2002).

In states of low dietary zinc intake, skin, heart and skeletal muscle concentrations of zinc are unchanged, whereas plasma, liver, pancreas, bone and testes concentrations are significantly diminished (Swinkels et al., 1996; King et al., 2000). The deficiency of zinc leads to

growth retardation, anorexia, delayed sexual maturation, iron-deficiency anemia and alterations of taste (Prasad, 1991). The metabolism of zinc is influenced by several factors. Among them glucocorticosteroids and cytokines (JL1–JL6) have the ability to reduce MT synthesis causing increased transport from plasma to the liver (Cousins, 1996). The metallothionein gene is transcriptionally regulated by zinc, thus elevations of dietary zinc increase intestinal MT synthesis (Cousins and Lee-Ambrose, 1992; Davis and Cousins, 2000). MT is also involved in systemic zinc distribution and in its cellular accumulation. The mucosal induction of the metal binding protein (MT), limits zinc absorption at high intakes and these changes can occur rapidly, within a week, depending on zinc supply (Cousins, 1996). Moreover, after epithelial cell death certain amounts of metallothionein–zinc complex are excreted, preventing excessive absorption of this mineral (Barceloux, 1999a).

Table 9 presents zinc content in diets for growing animals. They require 40–110 mg Zn/kg diet DM, e.g. newborn piglets need about 110 mg Zn/kg mixture, whereas fatteners need only half this amount. Zinc absorbed from milk covers 38–95% of calf requirements (variable content of zinc in cow's milk) and about 55% of the piglet needs. Milk, therefore, is generally deficient in this mineral. Zinc content, both in roughages and in concentrates, is usually below the animals' needs. Especially large zinc deficiencies characterize monogastric diets (tables 4 and 5). Absolute values of zinc absorbability are difficult to measure as the mammals can regulate the absorbability of this mineral quite considerably, according to their needs. Availability of zinc depends greatly on the kind of feed. It is poorly absorbed from cereal grains, where it forms unabsorbable complexes with phytates. This is a problem, especially in pig and poultry diets. Introduction of a microbial phytase supplement to the diets of these animals can increase the availability of this mineral and thus diminish the values of zinc and phosphorus needed. An elevated level of Ca in the diet has a strong negative influence on Zn absorbability. Zinc deficit, especially high in pig and poultry diets (reaching 60–80%), can be easily supplemented by zinc oxide or zinc sulfate.

The animals can tolerate rather high excesses of zinc, although this depends to some extent on the species. Weanling piglets tolerate up to 3 g Zn/kg DM (Smith et al., 1997), i.e. 27-fold of their requirement. Broilers can tolerate about 1–2 g Zn/kg DM (Oh et al., 1997). Weaned ruminants tolerate a lower zinc level in the diet (calves 0.7 g and lambs 1 g/kg DM), however, taking into consideration their lower requirement for this mineral than in nonruminants, this still represents some 25-fold of their requirement (Underwood and Suttle, 1999i).

Zinc is less toxic compared with other metals (e.g. lead, arsenic, cadmium and antimony), but adverse effects occur after oral exposure to zinc (nausea, vomiting, abdominal pain, anemia and pancreatitis) in man (Barceloux, 1999a). In growing animals the toxicity of zinc depends upon the zinc source, dietary level, the duration of feeding and the level of other minerals in the diet. Piglets fed with a diet containing 4.0–8.0 g/kg DM showed a decreased appetite and higher lethality (Brink et al., 1959). Toxicity occurred also in animals given various levels of zinc in the diet: 4.0–5.0 g/kg DM in swine (table 4), 0.5 g/kg DM in horses (table 7) and 0.8–4.0 g/kg DM in chickens (table 5). High concentrations of zinc impair lymphocyte and neutrophil functions, reduce high-density lipoprotein cholesterol and cause transient elevations in serum low-density lipoprotein cholesterol (Chandra, 1984). Excessive zinc intake interferes with copper absorption and increases copper excretion in feces. Depression, sideroblastic anemia, myelodysplasia of the bone marrow, arthritis, hemorrhage in auxillary spaces, gastritis, copper deficiency, neutropenia and death are characteristic of chronic zinc intoxication (Brown et al., 1990). In growing pigs, signs of toxicity are developed when the diet is supplemented with 2–4 g Zn/kg DM in the form of zinc carbonate, however, growing pigs fed 2–4 g/kg of zinc oxide did not reveal signs of toxicity (Cox and Hale, 1962;

Hsu et al., 1975; Hill et al., 1983). Pigs fed a diet containing 1 g/kg of zinc from zinc lactate became lame and unthrifty. Contrary to this, pigs fed a diet containing 1000 ppm of zinc as zinc sulfate did not develop toxic symptoms (Kulwich et al., 1953). Pigs from sows fed with high levels of zinc contain lower tissue concentrations of copper and easily develop anemia if the level of copper in the diet is low (Hill et al., 1983). In the growing chick during excessive zinc intake (2 g/kg DM) the greatest zinc accumulation takes place in the liver, kidney, intestine and pancreas. Feed intake of 4 g/kg DM for 3 weeks increased Zn-MT in growing chickens but rapid depletion of both zinc and Zn-MT were observed when birds returned to a normal diet. Several mechanisms may function in the young chicken to maintain Zn homeostasis, since little changes in zinc levels are observed in the kidneys. The pancreas seems to be very sensitive to Zn excess (1 g Zn/kg DM as zinc sulfate and zinc gluconate), however pancreatic exocrine dysfunction is not achieved even when Zn intake is as great as 2 g/kg DM (Sandoval et al., 1998).

12. COBALT

Cobalt (Co) is a crucial constituent of vitamin B_{12} that fulfills important biological functions in animal organisms. The absorbed cobalt is rapidly distributed to numerous tissues but the highest concentration of this element is reported in liver, kidneys, bone tissue and adrenals (Kabata-Pendias and Pendias, 1999c; Nogowska et al., 2000). Its biological role is provided by stimulation of erythropoietin synthesis and regulation of erythropoiesis at the level of the bone marrow (Barceloux, 1999b; Zaporowska, 2002). Cobalt is an important regulatory factor in protein and nucleic acid metabolism and it participates in purine and pyrimidine synthesis, while transmethylation is conditioned by cobalt as well. It is a cofactor of methylmalonyl coenzyme A mutase, methionine synthase and ribonucleotide reductase (Pasternak, 2000). Together with manganese ions, cobalt serves as an enzyme activator. It is able to replace zinc in zinc-containing enzymes and regulates thyroid gland metabolism. Cobalt is absorbed as a constituent of vitamin B_{12}, which is synthesized by microorganisms living in the gastrointestinal tract, especially in ruminants. Additional sources of this element are meat, liver, milk and yeast. Cobalt is not cumulated in the body and its requirement in humans reaches about 2 mg/year (Kabata-Pendias and Pendias, 1999c; Zaporowska, 2002). Its absorption from different mineral sources is presented in table 6. Cobalt is present in almost all feedstuffs but it is poorly absorbed from the GIT, thus feces contain approximately 80% of the ingested amount. Normally, cobalt is not stored in organisms in significant quantities. The main route of excretion of absorbed cobalt is through urine (two-thirds), and feces via the bile (one-third) (Kincaid et al., 2002).

The dietary requirement for cobalt in cattle is estimated to be 0.10 mg/kg dietary DM (NRC, 1996, 2001). Its content in diets for growing animals is presented in table 9. The common pasture plants contain cobalt in concentrations that vary according to the species and soil conditions. Grasses are poorer in this mineral than legumes. Cereal grains contain only 0.01–0.06 mg/kg DM (Field et al., 1988). Cobalt can be easily supplemented to ruminant diets, as the soluble inorganic salts, cobalt carbonate ($CoCO_3$) or sulfate ($CoSO_4$) (Underwood and Suttle, 1999k). Cobalt toxicity is not likely to occur unless an error is made in formulating a mineral supplement. Cattle can tolerate approximately 90 times the dietary requirement for cobalt (NRC, 1980).

An excess of cobalt produces polycythemia in a wide range of animals; however, growing ruminants are more resistant after microflora stabilization. In the rumen, Co is used by the bacteria for the synthesis of vitamin B_{12}. The Agricultural Research Council (1980)

summarized the available evidence, and noting that >4 mg/kg BW was toxic to sheep and >1 mg/kg BW to young cattle, recommended that dietary levels for ruminants should not exceed 30 mg Co/kg DM. High levels of 400 mg and 600 mg Co/kg DM cause anorexia, stiffness, incoordination and muscular tremor, but symptoms are alleviated by supplements of methionine or a combination of iron with manganese and zinc (Underwood and Suttle, 1999k). Most growing animals develop polycythemia when they receive 1 g/kg BW cobalt in the diet, but not growing dogs, which physiologically require more than 10 mg/kg BW. Dairy calves and lambs ingesting more than 1.5 mg of cobalt per kg feed develop toxicity signs consisting of lack of coordination, depression, rough hair and lack of body weight gain. The maximum tolerance for weanling pigs is lower than 150 mg/kg of the diet. In the young pig the level of 400 mg Co/kg DM exerts toxic effects that consist of anorexia, growth depression, stiffleggedness, humped-back, lack of coordination of movements, muscle tremor and anemia (Huck and Clawson, 1976; Kornegay et al., 1995). In day-old chicks given 125 mg, 250 mg or 500 mg Co/kg DM for 14 days, the lowest level reduced feed intake, weight gain and development, while the two higher levels caused pancreatic fibrosis, hepatic necrosis and muscle lesions (Diaz et al., 1994). Selenium, vitamin E and cysteine intake help to protect against cobalt toxicity (Van Vleet et al., 1977; Southern and Baker, 1981). On the other hand, high levels of copper may shift growth depression caused by excessive cobalt intake (Kornegay et al., 1995).

13. MANGANESE

Manganese (Mn) is an essential trace element present in all tissues but especially highly concentrated in bones, liver and kidneys. The pancreas, intestines and hair are able to cumulate Mn as well. In cells, it is bound in mitochondria, DNA and RNA, whereas in blood, it binds to β-globulin (Barceloux, 1999c; Kabata-Pendias and Pendias, 1999d) and in milk to lipids. Red blood cells contain five times higher concentrations of Mn than the plasma.

Manganese is essential for the proper development and growth of the skeletal system. It activates glycosyltransferase, so participating in glycoprotein and proteoglycan synthesis. Moreover, it influences numerous metabolic processes of carbohydrates, lipids and proteins due to activation of arginase, phosphatase, choline esterase and pyruvate carboxylase and regulates assimilation of thiamine, L-ascorbic acid and biotin (Zaporowska, 2002). It is a free radical scavenger, as a constituent of superoxide dismutase, and it participates in cellular oxidation processes and inhibits lipid oxidation (Mitrunen et al., 2001). Its physiological function as enzyme activator is not specific and it may be replaced by other metals like magnesium (table 8). Furthermore, the physiological concentration of Mn guarantees erythropoiesis and sex hormone synthesis and regulates melanin and dopamine synthesis.

Absorption of manganese takes places mainly in the small intestine (Nogowska et al., 2000). It depends on mineral source (table 6). About 3% of ingested Mn is absorbed and distributed to different compartments of the body and its turnover rate depends on the level of dietary intake. The major route of Mn excretion is in the feces via the bile, in which Mn concentration is higher than in the blood plasma.

Table 9 presents Mn content in diet for growing animals. The requirements for manganese vary with the species and strain of the animal, and in mammals the requirements increase with age while tissue content falls, as especially seen in the bones. The highest needs (67 mg/kg diet DM) are estimated for broiler chickens and poults (NRC, 1994) and these requirements do not change with age. Growing cattle have higher requirements in the first 2 months

(40 mg/kg diet DM), but these gradually diminish down to 18 mg/kg diet DM at the end of their growth (NRC, 2001). Pigs' needs, in contrast, are comparatively low (2.2–4.4 mg/kg diet DM) (NRC, 1998).

Roughages are generally moderately rich in this element (table 3), but cereal grains contain less manganese, usually between 5–40 mg Mn/kg DM, with maize having an especially low content (Underwood, 1981). Plant protein sources contain 30–50 mg Mn/kg DM. The commonly used inorganic manganese sources are $MnSO_4$ (the highest availability among inorganic sources), manganese oxide MnO and manganese carbonate. As an alternative, various organic sources can be used, e.g. manganese methionine, manganese proteinate. Compared to manganese sulfate, the relative availability of manganese from manganese methionine is approximately 120% (Henry et al., 1992).

The absorption of manganese depends mainly on the source, dietary antagonists (phytate, excess of Ca, P and Fe) and animal species (monogastric or ruminant). Absorption can also be regulated by the individual animal to maintain Mn homeostasis. It is absorbed very well (40–60%) from milk, which is rather poor in this mineral (0.4 mg in 1 kg milk DM) (Underwood and Suttle, 1999l). However, Kirchgessner and Neese (1976) proved that when milk substitute contained about 14 mg Mn/kg DM, the absorbability of manganese dropped to about 5%. The absorption of manganese from plant feeds in nonruminants is low, e.g. from a maize–soybean meal-based diet fed to chicks, it equals about 3% (Wedekind et al., 1991). Low absorbability of this mineral in grains is associated mainly with the formation of complexes with phytate and fiber (Underwood and Suttle, 1999I). Addition of phytase to phytate-rich diets improves the utilization of dietary manganese in growing nonruminants (Biehl et al., 1995). Since phytate, the main antagonist of manganese absorption, is broken down in the rumen, the absorbability of Mn in ruminants is higher and it may reach the range of 10–20%, when Mn intake is low (Underwood and Suttle, 1999I).

Manganese poisoning requires the intake of large amounts of this element. Excessive Mn intake causes elevated concentrations of the metal in the liver, alteration of copper distribution, drastic reduction in iron absorption and reduced calcium and phosphorus excretion. The toxic level of manganese is not well defined; however, most animals can tolerate 0.1% of Mn if the diet contains at least 400 mg Fe/kg DM. The maximum tolerable level of Mn, which does not depress either appetite or growth rate, has been set for calves at 1 g/kg diet DM (NRC, 1980), while for weaned lambs it was estimated by Wong-Valle et al. (1989) at between 3–4.5 g Mn/kg diet DM. Health disturbances (depressed feed intake and reduced growth rates) have been observed in growing pigs fed 4 g Mn/kg DM (Leibholz et al., 1962). Lower manganese intake (2 g/kg DM) causes lowering of hemoglobin concentration (Matrone et al., 1960), and reduced growth rate and limb stiffness (500 mg/kg DM) (Grumer et al., 1950; Khan et al., 1997). Increased dietary manganese intake (0.95 mmol Mn/kg), decreases heart magnesium and can exacerbate magnesium deficiency in the heart muscle and may be a complicating factor in the deaths of Mg-deficient piglets. The heart tissue is characterized by myodegeneration, necrosis and mineralization of the heart, moderate myocardiocyte necrosis and severe multifocal myocardial necrosis (Miller et al., 2000). It was shown in numerous animal experiments that chronic exposure to manganese causes a decrease of dopamine concentration in the basal ganglion, which may disturb catecholamine biosynthesis. Subarachnoidal administration of manganese chloride (1–2 mg/kg body weight) causes symptoms suggesting disturbances in the extrapyramidal system. In apes, manganese invokes apathy and excitation, nervousness and sudden tremor. Excessive amounts of manganese in pig diets at the level of 4.0 g/kg DM (table 4) and in poultry diets at 3.0–4.5 g/kg DM (table 5) cause the above mentioned toxic symptoms.

14. MOLYBDENUM

Molybdenum (Mo) is an essential trace element for normal growth and development of animals. The highest concentration of this element is found in the liver, kidneys and bones (Barceloux, 1999d). Physiological functions of Mo include its participation as an enzyme component in oxidative-reductive processes, it catalyzes metabolic transformations of purines and lipids as a metaloenzyme constituent. It is an indispensable enzyme activator for proper functioning of xanthine, aldehyde and sulfate oxidases (Pasternak, 2000). There is evidence that molybdenum supports the anticaries action of fluorine. Physiological actions of this element depend on its interaction with other elements, among which the most important are copper and sulfur. The optimal copper to molybdenum ratio in ruminants oscillates between 4 and 5 and is conditioned by adequate sulfur feed level. Molybdenum, sulfur, iron and zinc act as antagonists of copper metabolism. Low Mo intake can lead to Cu storage in the body, thus, growing sheep receiving a Mo-deficient diet can accumulate toxic amounts of Cu in the liver, while sulfate enhances the effects of Mo. Hepatic Mo accumulation reduces caeruloplasmin synthesis and accumulation of Cu in the tissues, thereby disrupting copper metabolism in growing heifers (Bailey et al., 2001). Growing ruminants consuming a diet high in molybdenum and sulfur can absorb one of the thiomolybdates that form trithiomolybdates, which bind to serum copper making it unavailable to the animal (Chase, 1998).

Requirements for molybdenum are not established, but are very low. In the investigations carried out by Higgins et al. (1956), chicks grew naturally on diets containing only 0.2 mg Mo/kg DM. In the experiment carried out by Anke et al. (1978) on goat kids fed a semipurified diet containing 0.06 mg Mo/kg DM, a significant growth response was noted following the addition of 1 mg Mo/kg DM. Legumes are relatively rich in molybdenum (0.5–2.5 mg Mo/kg, grasses are somewhat poorer (0.33–1.4 mg) and cereals contain only 0.16–0.92 mg Mo/kg DM. Molybdenum can be readily and rapidly absorbed by livestock, especially by cattle and sheep. Metabolism of this mineral is greatly affected by copper and sulfur, with both minerals acting antagonistically. A large variation in molybdenum toxicity between species has been noted. Cattle, which can tolerate 10 mg Mo/kg DM, are the least tolerant species. Pigs are the most tolerant of domestic livestock and can tolerate 1000 mg Mo/kg DM, while chicks tolerate levels of 200 mg Mo/kg DM (Underwood and Suttle, 1999j).

Due to its ready intestinal absorption, molybdenum deficiency is not common, but excessive feed intake of this element has a toxic influence, especially in ruminants. Excess Mo causes bone deformation, caries, protein and lipid metabolism disturbances and may induce neoplastic changes in the stomach (Xianmao, 1990; Kabata-Pendias and Pendias, 1999e). Molybdenum intake much exceeding the daily requirements may cause elevation of uric acid concentration in blood serum and sometimes the symptoms of podagra. Moreover, a high molybdenum level can act to antagonize copper, which may be the reason for anemia due to copper deficiency. The other symptoms that may appear in breeding animals fed a diet containing excessive molybdenum levels are diarrhea, anorexia, posterior weakness, delayed puberty and reproductive disturbances.

15. IODINE

Iodine (I) is one of the most important trace elements in the body and is present in all tissues. Some 80% of the total iodine pool is concentrated in the thyroid gland, where its concentration is up to 300 times higher than in other tissues (Stoś and Mojska, 1996; Nogowska et al., 2000). There are three forms of this element in the circulating blood: (1) inorganic iodine (I^-);

(2) nonhormonal, organic iodine, present in trace amounts in the form of monoiodothyronine and diiodothyronine; (3) protein-bound iodine, comprising the biologically active hormonal pool (triiodothyronine, thyroxine). Its plasma level in mammals is estimated at 35–80 µg/l (Nogowska et al., 2000).

The physiological role of iodine is strictly related to the functions of thyroid hormone in the organism. The general effect of thyroid hormone is to cause nuclear transcription of large numbers of genes and thus increase synthesis of large numbers of enzymes, proteins and other substances in all cells of the body. This results in generally enhanced functional activity throughout the body. About 90% of the thyroid hormone that binds with the receptors is triiodothyronine, and only 10% thyroxine. The high binding affinity of triiodothyronine to intracellular thyroid hormone receptors guarantees the major biological effects of iodine in the body. Metabolic activity of all, or almost all, tissues of the body is enhanced up to 60–100% above normal activity when the secretion of thyroid hormones is increased. It results in acceleration of anabolic and catabolic processes, increased growth rate and endocrine activity of most glands. The Na-K-ATPase activity is also increased by thyroid hormones (Guyton and Hall, 1996b). A very important function of iodine, performed via the thyroid hormones, is to promote the growth and development of the brain during fetal life and during the postnatal period of intensive growth (Delange et al., 2001; Dunn and Delange, 2001).

Almost all, or even all, of the iodine intake in food is readily absorbable from the gastrointestinal tract, while further sources of this element include water and air (table 6). In the intestine, iodine or iodate are oxidized to iodide, which is rapidly absorbed and distributed in the body compartments. Several organs such as the thyroid, salivary glands, skin, hair, mammary gland, placenta and ovary have the capacity to accumulate this element. The major route of iodide elimination is excretion in the urine; however large amounts can be excreted into milk in lactating animals. The metabolism of iodine may be limited by elements influencing thyroid gland function, such as arsenic, cobalt, copper, calcium and manganese, but also by selenium and fluorine (table 8) (Kabata-Pendias and Pendias, 1999e; Zaporowska, 2002).

Iodine requirements per kg diet DM have been estimated at 0.16 mg for pigs (NRC, 1998), 0.39 mg for chickens and 0.44 mg for broiler poults (NRC, 1984). The requirements do not change with the stage of growth. The National Research Council (NRC, 2001) distinguished only higher needs for this element in newborn calves, i.e. 0.5 mg I/kg DM in contrast to the concentration of 0.3 mg I/kg DM recommended for older growing dairy cattle (table 3). Iodine requirements for beef cattle are established at 0.5 mg I/kg diet DM irrespective of their age (NRC, 1996). Plants contain iodine in highly variable concentrations, due to species and strain differences, climatic and seasonal conditions and type of soil and fertilization. Pigs and poultry can tolerate comparatively high amounts (300–400 mg I/kg diet DM), while cattle and sheep tolerate up to 50 mg I/kg diet DM. Horses are the most vulnerable to iodine toxicity, tolerating only 5 mg/kg diet DM (NRC, 1980).

Iodine intoxication can develop in growing animals, but it requires large amounts of iodine to be ingested. Excessive dietary iodine intake (800 mg/kg DM) depresses growth, lowers hemoglobin levels and iron concentration in the liver of growing pigs (Newton and Clawson, 1974). Toxic symptoms are invoked by dietary iodine intake of 0.5 g/kg DM in chickens (table 5) and in pigs of 0.8 g/kg DM (table 4). Iodine toxicity is manifested as chronic intoxication, particularly in sheep, with reduced intake and growth, rough hair, weepy eyes, and as acute intoxication with excessive salivation, nasal and ocular discharge, anorexia, abortion and respiratory problems.

16. CHROMIUM

Chromium (Cr) is an essential element for proper growth and development in animals (Grela et al., 1997). All tissues contain chromium and high concentrations are reported in hair, skin, teeth, liver and kidneys. Blood proteins like albumin and transferrin bind chromium and transport it to the tissues. Moreover, amino acids cysteine and histidine bind chromium as well (Anderson, 1997; Gałuszka and Cieślak-Golonka, 1999; Stearns, 2000). There are two different oxidation states of chromium: Cr^{3+} and Cr^{6+} in animal and human tissues. The first of these dominates in most tissues except the liver, where chromium is concentrated and bound with nucleic acids. It plays a very important role in the metabolic processes regulating glucose, proteins, lipids and cholesterol. It regulates blood glucose concentrations together with insulin, supporting its biological effects on cells (Vincent, 2000). Moreover, it stimulates enzyme activity and it is also a component of trypsin and thereby participates in digestive processes. It was proven that chromium appeases symptoms of arteriosclerosis and prevents the lowering of arterial pressure (Anderson, 2000). Animals and humans may absorb this element from the air and water as well as from food, and its deficiency is not common (Kabata-Pendias and Pendias, 1999e; Zaporowska, 2002). The absorption of Cr from the intestine is poor; however some forms are better absorbed than inorganic forms (Gargas et al., 1991; Clancy et al., 1994; Ward et al., 1995). Complexing chromium to organic molecules also can influence availability. For example oxalate enhances the absorption of chromium in rats, whereas EDTA and citrate do not (Chen et al., 1973). Synthetic organic forms, such as chromium nicotinate (CrNic) and chromium picolinate (CrPic) are better absorbed than $CrCl_3$ (Olin et al., 1994). Naturally occurring chromium complexes such as chromium yeast, are known for their relatively high biologic activity (Underwood and Suttle, 1999j). The dietary requirements of livestock animals for chromium have not yet been defined, but they are increased by stress, e.g. weanling transport (NRC, 1997). According to the NRC the daily requirement for Cr in humans, to ensure normal glucose metabolism, is estimated at 0.05–0.2 mg (0.1–0.4 mg/kg diet DM). The addition of 0.5 mg Cr/kg dietary DM given in organic form (Cr yeast or Cr picolinate) considerably improves daily gain and feed conversion ratio (Wenk, 1995). Supplementing broiler diets with 0.4 mg Cr/kg DM, decreases fat content of muscles and diminishes morbidity. Inorganic forms of trivalent chromium are absorbed poorly (0.4–3.0%) and organic sources can be absorbed much more efficiently (20–30 times more) than inorganic sources (Starich and Blincoe, 1983). Chromium contents in feedstuffs range from 0.01–4.2 mg Cr/kg DM; cereals and grasses are relatively poor and legumes relatively rich in this element (Kabata-Pendias and Pendias, 1999e). More important than the amount, may be the form in which chromium is present, but this has still to be fully examined (Grela et al., 1997; Underwood and Suttle, 1999j). For livestock the maximum tolerable concentration of Cr in dietary dry matter is set at 3000 mg/kg for the oxide form and 1000 mg/kg for the chloride form of trivalent chromium. Hexavalent forms of chromium are at least five times more toxic (NRC, 1997). The maximum tolerable level of chromium oxide and chromium chloride in the diet of farm animals is set at 3 g and 1 g/kg DM, respectively (NRC, 1980). After intratracheal administration of Cr^{3+}, absorption is fast and within 10 min 69% of the administered dose can be recovered in the lungs. Chromium (Cr^{6+}) accumulates in red cells (20% of dose) and in lungs (15% of dose). Excretion of Cr occurs mainly via the urine. Some chromium (Cr^{6+}) compounds show carcinogenic effects after subcutaneous or intramuscular administration to rats, invoking tumors at the site of administration. Mutagenic properties have also been observed during *in vitro* and *in vivo* tests in prokaryotic and eukaryotic organisms. Finally, studies in hamsters provided evidence also of embryotoxic and teratogenic actions.

17. SELENIUM

Selenium (Se) is another very important trace element, which is essential for proper organism growth, development and function. The highest selenium concentration is reported for liver, pancreas, kidneys and muscles (Flohé et al., 2000; Pasternak, 2000). It is a trace element characterized by a very narrow margin between the lowest acceptable levels of intake and levels causing toxicity (Zhang et al., 2001). Selenium is a constituent of 35 enzymes and selenoproteins (Chen et al., 1999; Arteel and Sies, 2001; Behne and Kyriakopoulos, 2001). Immunostimulation, regulation of thyroid gland metabolism and protection against oxygen radicals are the most important physiological functions of this element. The antioxidant effect is mainly attributed to selenoenzymes, enzymes of the glutathione peroxidase family and thioredoxin reductase (Zhang et al., 2001). The glutathione peroxidase family detoxify a wide range of peroxidases, such as hydrogen peroxide, fatty acid hydroperoxides, phospholipid hydroperoxide and hydroperoxy groups of thymine (Ursini et al., 1985; Bao and Williamson, 2000). The thioredoxin reductase together with its substrate thioredoxin determine the redox system that have several important functions, including detoxification reactions (May et al., 1997; Jeffery, 1999; Arnér and Holmgren, 2000; Becker et al., 2000). Furthermore, selenium-dependent thyronine deiodinase catalyzes the conversion of thyroxine into triiodothyronine which, in turn, determines energy metabolic processes in the body. Cellular immunostimulation by selenium is determined by increased proliferation of T cells. Moreover, it causes B cell proliferation and enhanced antibody synthesis by plasmacytes (McKenzie et al., 1998). Other investigations performed in animal models showed a positive influence of selenium on semen quality and fertilization rate (Marin-Guzman et al., 1997). In addition, Se shows anticarcinogenic properties: it suppresses growth of tumor cells *in vivo* and *in vitro,* reduces mortality in cancer patients and decreases the incidence of many cancers including lung, colorectal and prostate cancers (Clark et al., 1996; Cho et al., 1999; Tanaka et al., 2000). Its anticarcinogenic action is believed to be due to its antioxidant properties (Schrauzer, 2000; Vinceti et al., 2000; El-Bayoumy, 2001; Raich et al., 2001). Selenium is also capable of lowering the toxicity of lead, cadmium and mercury by forming complexes with these other metals (Wesołowski and Ulewicz, 2000).

Selenium is present in almost all feedstuffs, but at different levels. Animals receive dietary selenium as organic selenoamino acids present in plants and animal proteins and in the inorganic form as dietary supplements, e.g. of sodium selenite and sodium selenate (Sembratowicz and Grela, 1997). Intestinal active absorption of selenium occurs with selenocystine and selenomethionine, and varies between 90–95%. Approximately 10% less selenium is absorbed passively from the gastrointestinal tract as selenite and selenate (Lee et al., 1996). Selenium is incorporated into selenoproteins as selenocystine residues through a cotranslational event. There are about 30–40 selenoproteins that have been characterized *in vivo* or in cell culture. Due to the large number of these compounds selenium influences a broad range of cell and organism functions. Thus, selenium exerts antioxidant functions, influences thyroid metabolism and redox status (McKenzie et al., 2002; Yu et al., 2002).

Selenoprotein expression is regulated by selenium supply, but selenium status does not seem to affect the transcription rate of the genes (Behne et al., 1988; Burk and Hill, 1993; Bermano et al., 1995; Wingler et al., 1999). Selenium can regulate intracellular signaling and transcription factor activation, therefore it may also influence the transmission of "death signals" or molecular events leading to their production, which can decide if the cell survives or initiates the apoptotic program during fetal organogenesis and early postnatal life (Burns and El- Deiry, 1999; Valverde and Studzinski, 1999b). Selenocompounds can promote cell death

via apoptosis, or cell survival against death induced by oxidative stress. The protective effect includes prevention of oxidative damage of proteins, selective regulation of gene expression, decreased oxidative damage to DNA and diminished lipid peroxidation and damage to cell membranes. Some effects are direct and others appear to be mediated by augmented expression of selenoproteins (McKenzie, 2000; Ghose et al., 2001; McKenzie et al., 2002). These effects depend on the type of cell (normal or neoplastic), the type of selenium compound (organic or inorganic) and its possible metabolites, dose of selenium compounds and the level of other antioxidants (GSH, vitamins C and E) or other metals (copper, heavy metals) (Stewart et al., 1999; Ghose et al., 2001; McKenzie et al., 2002).

Excessive selenium intake leads to decreased development of the whole body in growing animals, increased morbidity and mortality with different severity according to animal species. A significant increase in liver selenium concentration has been found in copper-loaded adult sheep. On the other hand, selenium metabolism is affected by copper, since increasing copper intake at low or normal selenium intakes in the rat decreases selenium retention. This is due to less selenium accumulation in organs other than liver, spleen, heart or kidney and enhanced urinary excretion (Yu and Beynem, 2001).

The requirements for selenium vary between different animal species. According to the Norms of the National Research Council (NRC, 1984, 1989, 2001), the selenium needs of cattle, horses and broiler chickens and poults are estimated at 0.3 mg, 0.1 mg, 0.17 mg and 0.22 mg Se/kg diet DM, respectively, and they do not change during their growth, whereas requirements of pigs decrease as they grow, from 0.33 mg to 0.17 mg Se/kg diet DM (NRC, 1998). Diets low in vitamin E may increase the amount of selenium needed to prevent certain abnormalities such as nutritional muscular dystrophy (white muscles disease) (Millar and Meads, 1988). The selenium content of forages and other feedstuffs varies greatly depending on poult species and particularly the selenium content in the soil. The Se concentration in pasture and forage is generally below 0.05 mg/Se in 1 kg DM (Underwood and Suttle, 1999m). Among cereal grains maize usually contained the lowest, and wheat the highest level of Se – about 7 mg and 30 mg per kg DM, respectively (NRC, 1994).

Selenium toxicity may occur as a result of excessive supplementation of this mineral or consumption of plants naturally high in selenium (e.g. *Actragalus* and *Stanleya*). The maximum tolerable concentration of selenium has been estimated to be 2 mg/kg diet DM (NRC, 1980). The toxicity of selenium depends greatly on the animal species and on the form of the compound supplemented as well as the level of this element in the soil and in the forage plants. Selenium salts are some of the most toxic compounds. Selenium LD_{50} for various animal species amounts to 0.4–6.4 mg/kg. Clinical symptoms of acute intoxication with selenium are as follows: atrophy of heart muscle and parenchymatous organs, anemia and sialosis. Lung inflammation and edema, hair loss, acute caries, and blindness may occur at a later stage. In the suckling calf and pig, selenium needs may be covered when Se is given to their dams in the organic form (30 mg Se/kg feed as selenium yeast), since the Se level in the milk increases after organic supplementation (17.5 μg/l cow milk and 0.188 μg/l, 0.105 μg/l for sow colostrum and milk, respectively) (Pehrson et al., 1999; Mahan, 2000). If the dam cow receives toxic levels of Se in the diet this can cause the newborn calves to be weak and lethargic because selenium can accumulate in the fetus (Puls, 1994). The level of Se supplementation is widely accepted as 0.3 mg/kg feed for pigs, however, studies on the expression of glutathione peroxidases in various tissues of weaning pigs revealed that the amount needed to meet Se physiological expression balance is about 0.2 mg/kg feed. In growing pigs fed at the level of 5 mg/kg DM as sodium selenite, sodium selenate, or selenomethionine, selenium does not cause toxic effects. Levels from 7.5–10 mg Se/kg DM cause toxic effects including

anorexia, alopecia, fatty infiltration of the liver and kidney, edema and separation of hoof and skin at the coronary band as well as neuronal necrosis (Herigstad et al., 1973; Goehring et al., 1984; Stowe and Herdt, 1992; Kim and Mahan, 2001a). The selenosis response is more severe when the diet contains a mineral rather than an organic selenium source (selenium-enriched yeast) (Kim and Mahan, 2001a, 2001b). The toxicity of selenium can be diminished by dietary arsenicals. Controlled feeding trials in growing pigs, using the same Se content in different feed sources (25 mg Se/day for up to 6 weeks), resulted in higher blood and organ Se concentrations, when pigs received seleno-DL-methionine, compared to receiving sodium selenate and *Astralagus bisulcatus* (selenium accumulating plant). These high organ Se concentrations were not correlated with the severity of pathological changes. Contrary to this, clinical signs of Se toxicity, including neurological symptoms of paralysis, occurred sooner and organ lesions were more severe in pigs receiving *A. bisulcatus* (Panter et al., 1996). Moreover, toxic selenium levels in the diet amount to 0.005 g/kg DM in pigs and chickens (tables 4 and 5).

18. FUTURE PERSPECTIVES

Further research is necessary to define more precisely the mode of action of particular elements, their modulatory influence on the immune system and the requirements for particular minerals as well. It is necessary to balance the mineral nutrition of all (but especially of young) animals, taking into consideration the mineral contents in all components of the diets (a lot of procedures of feed mixtures compile the content of minerals in their mixtures according to norms, ignoring their presence in the basic diets). From the ecological point of view, it is advisable to carefully control the utilization, especially in highly intensive production, of the highly available mineral sources (e.g. trace elements in organic forms). Particular care should be directed towards the minerals, whose toxicity limits are close to their physiological demands (magnesium, potassium, sodium, chloride, selenium). Variability in trace element contents in feeds and factors conditioning them, as well as mechanisms of their mutual relationships, need to be better documented, regarding animal production and welfare as well as environmental protection aspects.

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17 GMO in animal nutrition: potential benefits and risks

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Even a cursory look at the few papers published in peer-reviewed international journals on the potential health and metabolic effects of GM feeds/foods reveals a scarcity of published data. It is often claimed that as there are only small compositional differences between the "substantially equivalent" GM and non-GM crops, these have little biological significance. However, from the present review it has become clear that most GM and parental line crops fall short of the definition of "substantial equivalence", a concept which, in any case, has outlived its previously claimed usefulness. Thus, novel biological concepts and methods to probe into the safety of gene splicing are needed. This is made all the more urgent because the biological testing of GM feeds, as presently carried out, is rather limited in scope and mainly aimed at finding the best conditions for commercial animal production. In this review previously published animal studies have been critically examined in the light of a newly suggested testing protocol, in which the safety of GM crops is established from the effects of the GM ingredients on the physiology, pathohistology, immunology and bacterial flora of the gastrointestinal tract of young animals and the metabolic consequences of these effects. (GMO: genetically modified organisms.)

1. INTRODUCTION

1.1. Regulatory and general considerations

Assessing the production potential and value of feed components is of considerable commercial and practical importance. Indeed, in addition to safety and other nutritional considerations the effort spent by feed technologists in evaluating the feeding value of crops is considerably more extensive than that by human nutritionists to establish the nutritional value of foods for the public. Not surprisingly, this distinction is even more acute between genetically modified (GM) food or feed ingredients. Thus, except the very recently published human trial with a single dose of GM soybean-containing meal (Netherwood et al., 2004), the almost total

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absence of published data in peer-reviewed scientific literature indicates that the safety of GM foods rests more on trusting the assurances given by the biotechnology industry than on rigorous and independently verified risk assessment. A comment in Science described this in its title: "Health Risks of Genetically Modified Foods: Many Opinions but Few Data" (Domingo, 2000). Indeed, most of the attempts to establish the safety of GM food have been indirect, using animal trials with GM feed ingredients and drawing inferences from these for human health. However, even these animal studies had in most instances limited, mainly commercial, objectives, as is obvious from recent reviews (Aumaitre et al., 2002; Faust, 2002). Despite this, the main stated objective of the GM regulation is to assure the human population that GM foods are safe while animal safety is seldom discussed. The regulators, particularly in the USA, use a decision tree approach in which the authorities review the data, usually provided by the biotechnology companies, but do not carry out safety assessments of their own (Faust, 2002). Even in Europe the preferred approach is to use compositional comparisons between the GM crop and its traditional counterpart and if these results show no significant differences they are considered to be "substantially equivalent", meaning that the GM is as safe as the non-GM crop. However, even though existing legislation does not require the testing of GM crop-based feedstuffs with target animals, many new GM crops have been tested on farm animals but most of the time only to establish their effects on nutritional performance, digestibility, wholesomeness and feeding value for obvious commercial considerations (Aulrich et al., 2002; Aumaitre et al., 2002). In most of these relatively short-term and rather empirical studies the emphasis was on productivity rather than on investigating the biochemical and cell biological interactions between the GM ingredient and the digestive tract, the effect of the GM DNA and protein on the gut epithelial cellular and tissue structure, its immune and endocrine systems and bacterial ecology. This is particularly regrettable because nutritional parameters, though of great commercial interest, are rather crude measures in physiological terms of the effects of GM ingredients and may give science little guidance on what will be the likely biological consequences of long-term and heavy exposure to GM crops. Thus, as GM regulation is at present based on rather minimalistic legislative and scientific foundations, with the likely progress in the future of our understanding of the biological principles underlining the whole GM business, major efforts of clarification and updating will in time be needed.

1.2. Transgene survival in the alimentary tract and its possible consequences

In genetic modification the intended gene is incorporated into the genome of a crop, using a vector containing several other genes, including as a minimum: viral promoters, transcription terminators, antibiotic resistance or other marker genes and reporter genes. Unfortunately, the possible physiological effects on the digestive tract of these genes and their expressed proteins are seldom taken into account, even though there is some evidence that some of the other genes of the vector may have an effect on safety. This is particularly so as it is now well established that DNA does not always break down in the alimentary tract (Schubbert et al., 1994, 1997, 1998; Hohlweg and Doerfler, 2001). This opens up the possibility that the antibiotic resistance marker gene, in addition to others, may be taken up by bacteria in the digestive tract and contribute to the spreading of antibiotic resistance via human gut bacteria. In this context one potentially important observation was that a substantial proportion (6–25%) of a genetically engineered plasmid survived a 1-h exposure to human saliva (Mercer et al., 1999). Partially degraded plasmid DNA also successfully transformed *Streptococcus gordonii,* that normally lives in the human mouth. Saliva also contains factors which increase the ability of bacteria to become transformed by naked DNA. Similar results have been obtained with bacteria using artificial gut preparations (MacKenzie, 1999). Plasmid antibiotic resistance

marker gene DNA exposed to ovine saliva could transform competent *Escherichia coli* to ampicillin resistance *in vitro* (Duggan et al., 2002). Furthermore, when fed to chicks incorporated into GM maize, the plant-derived marker was shown to be present in their crop and stomach (Chambers et al., 2000). The transfer of DNA derived from GM or non-GM plant tissues to duodenal juice, lymphocytes, internal organs, etc. of animals fed on feed rations containing these is now well established (Chowdury et al., 2003; for other refs see Aumaitre et al., 2002) even though their physiological significance for humans and/or animals is unclear. However, no transgenic DNA has so far been shown to be present in milk or eggs (Phipps et al., 2003) even though it is likely that with improvements in detection techniques traces of such DNA will be found (Aumaitre et al., 2002). The only human study performed with GM soybean (Netherwood et al., 2004), to establish whether the antibiotic resistance marker gene survives in the gut and whether it is taken up by gut bacteria, confirmed the results of similar animal studies. It was shown that in the digesta of seven ileostomy patients (people whose large intestine has been surgically removed and replaced with an external pouch joined to the lower end of their small intestine) given a single meal containing GM soybean, variable but measurable amounts of the full-length transgene construct survived and that in three patients this was highly significant. Thus, transgenic DNA survives not only in mice or in artificial guts but also in humans and can be taken up by gut bacteria. Therefore, the prospect of the uptake of functional vector genes, including the antibiotic resistance gene, will have to be seriously considered. The importance of this in animal husbandry cannot be overemphasized in view of the practice of including antibiotics in animal feed in many non-European countries, although antibiotics have been banned as feed additives in the European Union from the end of 2005.

1.3. Indirect, unintended and positioning effects of genetic modification

To the generally recognized importance of the direct effects of the expression of the main transgene after its insertion into the plant genome via a gene construct, an additional concern is that this may also cause significant, indirect and unintended effects on the expression and functionality of the plant's own genes. The number of copies of the construct inserted and their location in the plant genome (positioning effect) are of particular importance. In the past, unfortunately, only scant attention has been given to their possible consequences (Ewen and Pusztai, 1999a) even though it is possible that they may cause many unexpected changes. The importance of the analysis of such unintended effects in GM foods however, has recently been recognized by including them in the *Codex Alimentarius* guidelines (Haslberger, 2003). The inadequacy of the currently used methods to detect these are frequently acknowledged (Kuiper et al., 2001, 2002). Positioning effects often occur with both conventional cross-breeding and genetic engineering and their unwanted consequences are usually eliminated by empirically selecting for the desired trait and discarding the potentially harmful ones. However, some of these changes are unpredictable. As it is only possible to compare the known properties and constituents of GM and conventional plans but not to look for, and even less analyze, unknown components, the limitations on our selection criteria are severe. Reliance based solely on chemical analysis of macro/micronutrients and known toxins is at best inadequate and, at worst, dangerous. More sophisticated analytical methods need to be devised, such as mRNA fingerprinting, proteomics, secondary metabolite profiling and other profiling techniques (Kuiper et al., 1999, 2001, 2003). However, and most importantly, there is an urgent need to develop comprehensive toxicological/physiological/nutritional methods which will equally be applicable to scientifically examine the veracity of the claimed benefits of genetic manipulation and screen for its unintended and potentially deleterious consequences

for human/animal health. The center of this effort should be the physiology of the alimentary canal, since this is the first contact point of exposure to any food/feed including those which have been genetically modified, to establish in scientific terms the short- and long-term consequences of the exposure (Ewen and Pusztai, 1999a).

This review will not deal with environmental issues or political considerations concerning GM food/feed regulation. The emphasis will be on discussing the significance of published scientific, physiological, histopathological and nutritional data of GM food/feed and the principles which ought to underpin the efforts aimed at widening our understanding. No opinions, unless supported by experimental results, will be discussed. The emphasis will be on papers published in peer-reviewed journals and only exceptionally will data from non-peer-reviewed sources be mentioned and only if they influenced the development of science-based ideas for GM food/feed safety. This is all the more important because even a cursory look at the list of references in recent major reviews on GM safety issues (Gasson and Burke, 2001; Kuiper et al., 2001; Pusztai et al., 2003) shows that most of the publications referred to were non-peer-reviewed institutional opinions or envisaged future scientific and methodological developments for safety assessments, but were short on actual published scientific papers on which a reliable database of safety could be founded.

As the compositional complexity of foods/feeds makes it difficult to use classical toxicological methods for the assessment of GM safety, a physiological/nutritional approach may be of more relevance (Pusztai, 2002). Reviewing previous work may therefore be rewarding in the framework of such an approach, particularly as most of the relevant information concerning the potential safety of GM food/feed has come from studies concentrating on gut histopathology and nutrition. This review will enlarge upon previously published reviews (Kuiper et al., 2001; Pusztai, 2001; Pryme and Lembcke, 2003; Pusztai et al., 2003).

2. SAFETY ASSESSMENT OF GM CROPS

2.1. Compositional studies

For diet formulation, a compositional analysis of the transformed and isogenic lines is essential and must always precede the feeding study. For this the parent and GM lines must be grown under identical conditions, treated and harvested the same way. One such example is given for GM potatoes in table 1 (Pusztai, 2002). Unfortunately, fulfilling this condition is the

Table 1

Compositional values for "Desiree" potato tubers and two GM lines expressing the snowdrop (*Galanthus nivalis*) bulb lectin, GNA, derived from them (from Pusztai, 2002)

exception rather than the rule, as shown in some of the examples discussed below. In addition to proteins, starch, lipids, etc., of the parent and GM lines, their contents of bioactive components should also be compared by novel methods (proteomics, fingerprinting, metabolite profiling, etc.).

2.1.1. Herbicide-resistant soybeans

Based on similarities between the macronutrient composition of parent, non-GM line and glyphosate-tolerant soybean (GTS) seeds, resulting from the transformation of conventional soybean with a gene encoding for 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* to make the soybean herbicide-resistant, it has been claimed that the GM and the non-GM lines are "substantially equivalent". This equally applies to GTS unsprayed with glyphosate (Padgette et al., 1996) or sprayed with this herbicide (Taylor et al., 1999). The results of proximate chemical analyses of the contents of crude protein, oil, ash, fiber, carbohydrates and amino acids of solvent-extracted and toasted or untoasted soybean meals of sprayed or unsprayed GTS and control soybean were also apparently substantially equivalent (Padgette et al., 1996; Taylor et al., 1999). Although several significant differences between GM and control lines, such as in ash, fat and carbohydrate contents were found (Table 2 in Padgette et al., 1996), these were not regarded to have biological significance by the authors. However, the statistical method for comparing the GM and non-GM lines was flawed. Instead of comparing sufficiently large numbers of samples of each individual GTS with its appropriate individual parent line grown side-by-side at the same location and harvested at the same time to establish whether they were compositionally "substantially equivalent", what the authors compared was a large number of different samples from different locations and harvest times. As growth conditions have a major influence on seed composition, the range of the amounts of constituents in the different samples was so great (± 10% or more) that the chances of finding statistically significant differences were unreal. This is all the more curious, because in the authors' experiment 1 in Puerto Rico the conventional and the GTS lines were grown at the same site but the results of their analyses on these soybean samples were not

Table 2

Results of lymphocyte proliferation assays in rats fed for 10 days diets containing raw GM-, control/non-GM potatoes, or control/non-GM potatoes supplemented with the gene product, GNA (from Pusztai, 2002)

included in the publication based on experiments 2 and 3 from different sites (Padgette et al., 1996). The Puerto Rico results had been deposited with the American Society for Information Science, National Auxiliary Publication Service (NAPS) as supplementary information, as referred to in Padgette et al. (1996). It could also be retrieved from the archives of the *Journal of Nutrition* and the data showed that the GM soybean contained significantly less protein and the amino acid phenylalanine, amongst many other things and therefore it could not have supported the growth of animals as well as the parent line. It is possible that from a practical point of view the variation in protein concentration of samples of the three lines between 36.8–45% would fall into the normal range of agronomic variability of soybeans and therefore may not be of major concern for agronomists. However, this comparison is not strict enough to establish whether the genetic modification introduced any unintended compositional changes. What is remarkable is that even with this approach many significant changes in macronutrient levels were found. Thus, the claim of "substantial equivalence" of GTS lines with non-GM soybean is not supported by rigorous scientific evidence. Excluding the results of the soybean samples grown in Puerto Rico, no significant differences were found in the levels of antinutrients, such as trypsin inhibitors, lectin and oligosaccharide flatulance factors between solvent-extracted, toasted or untoasted GM and non-GM soybean seeds in the study by Padgette et al. (1996). However, the comparisons were made by the same method as for the macronutrients and therefore the large range of natural variability excluded the possibility of finding significant differences. Furthermore, in single soybean meal samples of two GTS and parent lines the trypsin inhibitor (also a major allergen in soybean) content was substantially higher, by almost 30%, in one of the two GTS lines, with a smaller increase in the other. No trypsin inhibitor analyses were performed on the protein isolate or protein concentrate samples originating from the meal samples. In practically all heat-treated GM soybean samples from the Puerto Rico trial the amounts of lectin and the trypsin inhibitors were significantly higher in the GM samples than in the isogenic line. Even more curiously, heat treatment appeared to have a far lesser denaturing effect on the trypsin inhibitor content of the GM lines than on the parent line samples. Although for some unexplained reason the values were from single assays on single samples (table 3), one of the GM lines (61-67-1) appeared to have almost seven times as much trypsin inhibitor per mg sample dry weight (DW) as the parent. Indeed, the values in this GM soybean approached that found in untoasted soybean

Table 3

Relative dry organ weights of rats significantly affected by feeding for 10 days with diets containing raw or boiled GM potatoes and/or parent potatoes spiked with the gene product (GNA, *Galanthus nivalis* agglutinin; from Pusztai, 2002)

seed samples. Even the other GM line (40-3-2) contained three times as much trypsin inhibitor as the non-GM line. There were also other compositional differences in these processed soybean products. Although it is difficult to decide from single determinations what significance one can attach to them it is curious that these studies were not followed up to establish whether the differences were real or not.

GM soybean samples were consistently found to have significantly less isoflavones than the parent cultivars (Lappe et al., 1999) and the GM samples were considerably more variable in this respect than conventional soybeans. This high variability is particularly worrying in view of the known and powerful goitrogenic and estrogenic activities of soy isoflavones (Doerge, 2002).

In conclusion, even the results of the analytical work done to date, using mainly conventional methods have left many uncertainties about the chemical equivalence of the GM and non-GM soybeans, particularly as the design of some of the comparative studies was seriously flawed. Even more seriously, judging from the published literature, no attempt was apparently made to establish the equivalence of the GM to the conventional lines by more modern and high-resolving power technologies, such as proteomics, DNA microarray analysis using GM soybean RNA isolated from different tissues and plants grown under different but relevant conditions. No data could be found using NMR combined with chemometrics for the characterization of metabolite differences in the plants (Le Gall et al., 2003). Moreover, analysis of the possible different glycoforms of the 5-enolpyruvylshikimate-3-phosphate synthase and other proteins has not been attempted, although variability in glycosylation patterns can lead to different biochemical and antigenic properties. Furthermore, no comparison was made between the GM and non-GM forms in their contents of small RNA molecules that are emerging as very important and inheritable gene regulators. In view of these omissions no claims by the authors that the GM and non-GM soybeans are substantially equivalent can be accepted without carrying out further and more critical studies.

2.1.2. GM potatoes

The gene of soybean glycinin was transferred into potatoes with the aim to increase their protein content (Hashimoto et al., 1999a). However, as the expression level of glycinin in potatoes was only between 12–31 mg/g total soluble protein, the improvements in protein content or amino acid profile were minimal. In fact, the total protein content of the GM potatoes after the gene transfer became significantly less than that of the control line. Even more unfortunately, the contents of some vitamins were reduced while the amounts of both solanine and chaconine increased in the GM lines. In this light the claimed substantial equivalence of the GM and parent lines was not supported by the published results.

Furthermore, the finding of significant differences in a number of tuber components, the results of compositional analyses of some macro- and micronutrients of insect- and virus-resistant potatoes and those of untransformed lines (Rogan et al., 2000), also does not appear to support their substantial equivalence. However, in the absence of animal studies it is difficult to ascertain whether these differences could have any biological consequences for humans/animals, particularly as known antinutrients, such as lectins or enzyme inhibitors were not analyzed.

Modulating the adenylate pool by genetic manipulation of the plastidial adenylate kinase in transgenic potato plants has shown that it is possible to increase the level of starch in the tuber by 60%, the concentrations of several amino acids and, at the same time, increase tuber yield as well (Regierer et al., 2002). Unfortunately, no feeding studies have been reported on this GM potato. No modern analytical methods for establishing substantial equivalence between GM and non-GM potatoes have been used in either of these two studies.

2.1.3. GM rice

Transformed lines expressing the soybean glycinin gene have been developed (Momma et al., 1999) by a method similar to that used for GM potatoes. The glycinin expression level was between 40–50 mg glycinin/g total rice protein. The GM rice was claimed to contain 20% more protein, but its moisture content was less than that of the parent line and it was not clear whether the increased protein content was due to the decreased moisture content of the seeds, because it was not specified whether the values were expressed for air-dried or fully dried seeds.

2.1.4. GM cotton

Several lines of GM cotton plants have been developed, using the gene encoding an insecticidal protein from *Bacillus thuringiensis* subsp. *Kurstaki,* to increase the protection of the cotton plant against the lepidopteran insect pests. Cottonseed is an important source of oil for human consumption and of processed cottonseed meal for animal feed. The GM lines were claimed to be "substantially equivalent" to conventional lines (Berberich et al., 1996), because the levels of protein, fat, carbohydrate, moisture, ash, amino acids and fatty acids in the insect-protected lines appeared to be comparable to those found in commercial varieties. Moreover, the levels of antinutrients such as gossypol, cyclopropenoid fatty acids and aflatoxin were similar or less than those in conventional seeds. Unfortunately, the statistics used by the authors were identical to that used with glyphosate-resistant soybeans and therefore could be similarly criticized. In addition, no account was taken of environmental stress that could have had major and unpredictable effects on antinutrient, toxin and allergen levels (Novak and Haslberger, 2000). No attempts were made with modern methods to find whether the GM and non-GM cotton lines were compositionally equivalent or different due to possible unintended consequences of the genetic engineering.

2.1.5. GM maize

Except for a few minor differences, which the authors thought were unlikely to be of biological significance, the recently developed glyphosate-tolerant (Roundup Ready) corn line, GA21 collected from 16 field sites over two growing seasons, was shown by proximate analyses of fiber, amino acids, fatty acids and mineral contents of the grain and forage, to be comparable to the control line (Sidhu et al., 2000). The comparison however, was carried out by a statistical method similar to that for GTS soybean (Padgette et al., 1996) which is scientifically flawed.

The criticism raised above with GM soybean, that for characterization and establishing its substantial equivalence to non-GM soybean no modern analytical techniques were used, is also valid for GM maize.

2.1.6. GM wheat

A glyphosate-tolerant wheat variety called MON 71800, expressing 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. Strain CP4 (CP4 EPSPS), has been genetically engineered with reduced affinity for glyphosate in comparison with the endogenous plant EPSPS enzyme (Obert et al., 2004). Using conventional analytical techniques the authors claimed the transgenic MON 71800 wheat was substantially equivalent to the parent and other commercial wheat varieties and therefore as safe and as nutritious. While it may be understandable that a study on GM soybean carried out in 1995/6 only used rather conventional

analytical methodology for the establishment of substantial equivalence (see above), it is nevertheless clear that such a claim for GM wheat without the use of more modern and high-resolving power analytical methods is inadmissible in the present days.

2.2. Stability to degradation

It is of the utmost importance to establish the *in vivo* stability of GM products and foreign DNA, including the gene construct, promoter, antibiotic resistance marker gene, etc., to degradation in the stomach and intestines of model animals. Clearly, the safety of GM foods/feeds would be optimal if, as is often assumed, these biologically active molecules did not survive passage through the alimentary tract of animals/humans and therefore they could not interact with the gut, with possibly harmful consequences. Thus, one of the major assumptions in GM food regulation is that if the gene product protein breaks down on digestion it cannot be allergenic.

Unfortunately, at present if such studies are done at all, the stability of the gene products, DNA, etc., is only established *in vitro* by simulating gut digestion using acid and/or pepsin or other proteases in test tubes. The results of such *in vitro* digestion assays, however, can be misleading because the interactions between the digesta and the gut wall and its enzymes, which can greatly influence the stability or degradation of the components of the diet, are absent in the test tube. It is therefore questionable, for example, whether the gene product, 5-enolpyruvylshikimate-3-phosphate synthase (C4 EPSPS), that renders the soybeans glyphosate resistant (Harrison et al., 1996), truly breaks down in the gut of higher animals when in fact it has only been tested for this in a simulated digestion assay. A further complication in this study is that this *in vitro* simulated gastric/intestinal digestion assay was done with an *Escherichia coli* recombinant C4 EPSPS gene product whose stability could be different from that expressed in the soybean plant cells.

2.3. Biological, immunological and hormonal properties

To have physiologically valid results for establishing the true properties of GM plants it is essential that the gene product used for these tests must be isolated from the GM plant and that they are tested by *in vivo* assays in the rat (or other suitable animals; see Rubio et al., 1994) or in a full feeding trial. The validity of this approach can be appreciated from observations, which showed that the biological activity and stability of a gene product can be different when expressed in different life forms. Thus, it has been shown before that while the kidney bean (*Phaseolus vulgaris*) α-amylase inhibitor is fairly stable to proteolytic degradation in the rat gut (Pusztai et al., 1995, 1999), when its gene was expressed in peas (*Pisum sativum*), it was rapidly digested and inactivated in the rat stomach/small intestine *in vivo* (Pusztai et al., 1999). This may have made GM peas more safe for rats and, possibly even for other monogastric mammals.

With GM lectins, including Bt (*Bacillus thuringiensis*) toxin the presence/absence of their epithelial binding should also be demonstrated by immunohistology with the GM product isolated from the GM crop and not with a recombinant protein from *Escherichia coli,* as these two may have substantially different properties (Noteborn et al., 1995).

2.4. Nutritional testing

The genes used in present-day genetic engineering ensure that GM food is unlikely to be highly poisonous. “Toxicity” therefore is an unhelpful concept that does not easily lend itself

to quantitative assays. In contrast nutritional studies, in which GM crop-based diets are fed to young growing animals, should reveal their possible harmful effects on metabolism, organ development, immune/endocrine systems and gut flora, which together determine the safety of the GM crop and the development of the young into healthy adults. Although the validity of this approach is not always admitted, nutritional testing has often been used in the past for the safety assessment of GM foods/feeds. Indeed, historically the first such study (still unpublished) was carried out on FLAVR-SAVR™ tomato at the instigation of the FDA (Food and Drug Administration of USA). As tomatoes are of little relevance to animal nutrition and because a full review of these experiments has already been described (Pusztai et al., 2003), they will not be dealt with here.

2.4.1. Diets

For animal feeding tests iso-proteinic and iso-energetic diets need to be formulated in which most of the dietary protein is derived from the GM crop. The composition of the control diets should be the same as the GM diet, but containing the parent line with or without supplementation with the isolated gene product at the same level as expressed in the GM line. Unfortunately, although the use of the gene product-spiked control diet ought to be mandatory in these nutritional tests, they have rarely been used.

2.4.2. Feeding protocol

Groups of animals (5–6 or more animals per group) of closely similar weights, should be pair-fed in short- and long-term experiments. It is of particular importance to perform long-term nutritional experiments with GM feed components because small changes in the nutritional value of GM crops are more likely to show up with extended feeding. For example, the effect on the growth rate of rats fed GM potato-based diets was too small to be seen in the 10-day feeding experiments but a GM potato-induced reduction in rat growth was readily demonstrated in 110-day feeding trials, even when the potatoes were fully cooked (these results were published by the Rowett Research Institute (Bucksburn, Aberdeen, UK) against the authors' wishes on the institute's website (http://www.rri.sari.ac.uk)). In this experiment the GM protein in the diet was only diluted 2-fold by other dietary proteins, and the GM diet had to be supplemented with an extra 12 g lactalbumin/kg diet in order to equalize the growth rate of the rats on cooked GM potatoes to that of the control non-GM potato diets. This extra protein gives a quantitative measure of the difference in the nutritional value between GM and non-GM potatoes. Even at these similar growth rates the weights of some of the rats' vital organs, such as the gut and particularly the small intestine, the liver and kidneys were still significantly different.

In feeding experiments urine and feces should be collected for determination of net protein utilization (NPU), nitrogen balance, and feed utilization ratios. Blood samples should be taken before, during and at the end of the experiments for immune studies, such as lymphocyte proliferation assays. An example of this with GM potatoes is given in Table 2. Other assays, such as Elispot, hormone assays (insulin, CCK, etc.) and the determination of other blood constituents should also be performed. The animals should be weighed daily and any abnormalities observed. After killing the animals, the bodies are dissected, their gut rinsed and its contents saved for further studies (enzymes, GM products, DNA), gut sections taken for histology (see, for example, Pusztai et al., 2003), wet and dry weights of organs recorded and analyzed as in the example given in table 3, taken from a previous paper (Pusztai, 2002).

This recommended optimal protocol has a long history of usage for evaluating the nutritional value of conventional feedstuffs. Some of the pertinent important nutritional studies with GM crops will be fully reviewed here, to see how these previous studies were conducted and how they compare to this recommended optimal protocol and whether these methods could be incorporated into the general risk assessment procedures of GM foods/feeds.

2.5. Published nutritional/toxicological studies

2.5.1. Herbicide-resistant soybean

For the safety assessment of glyphosate-resistant soybean (GTS), the feeding value, wholesomeness (Hammond et al., 1996) and possible toxicity (Harrison et al., 1996) of two major GM lines of GTS were compared to that of the parent line. Processed GTS meal-based diets were fed to rats, broiler chickens, catfish and dairy cows for between 4–10 weeks at the same concentrations as in commercial non-GM soybean rations. According to the authors, the growth and feed conversion efficiency in rats, catfish and broilers, the fillet composition in catfish, the breast muscle and fat pad weights in broilers and milk production and composition, rumen fermentation and digestibilities in dairy cows, were similar for the GTS and parental lines. These results were therefore taken by the authors to suggest that the GTS and parental lines had similar feeding values.

2.5.1.1. Rat studies Although this study (Hammond et al., 1996) had a wider and more academic scope than many of the production-type studies (e.g. Cromwell et al., 2002; see Aumaitre et al., 2002; Faust, 2002) its design and execution could be criticized. Thus, in the published paper no primary individual data were given and no full description of the rat diet. It only reported on experiments carried out with soybean samples from experiments 2 and 3 but did not include those obtained with the equally relevant samples grown in Puerto Rico. It appears that the total protein content of the diets was adjusted to 24.7 g protein/100 g diet to be iso-nitrogenous with Purina Laboratory Rat Chow by the addition of 24.8 g of GTS and parent soybean meals, respectively (about 10% protein), to a base diet. All comparisons were made to rats fed commercial Purina Chow. The protein concentration in these diets was, however, appreciably higher than the usual 10–16% crude protein, regarded as optimal for the rat. This extra protein could have potentially masked any possible transgene product effects, particularly with the raw unprocessed soybean diets in which the GM meals were incorporated only at the level of 5% or 10% of the diet. Thus, these meals only replaced 8.5% and 17%, respectively, of the total protein of 24.7 g/100 g diet. In other words, the GM protein was diluted by other dietary proteins by 12-fold and 6-fold, respectively, producing another possible masking effect. The composition of the control Purina Chow diet in the ground raw soybean feeding study was not given and the identity of the raw control soybeans not specified.

In a 28-day feeding study four groups of singly housed rats (10 males and 10 females in each group) were fed diets containing the parental line or the GTS lines (40-3-2 or 61-67-1) for 28 days. No individual values (or their ranges) for feed intake or body weight were given. The bar diagrams of the combined bodyweights of rats were crude and uninformative. However, the Purina Chow-fed control male rats grew significantly better than most of the three experimental groups fed toasted soybeans (including the parental line). The bar diagrams also indicated that the growth of the 61-67-1 GTS line-fed rats was probably equal to that of the Purina Chow-fed control and therefore, by inference, these rats also grew significantly better than the other two experimental lines, the GM 40-3-2 GTS line and the parental line, indicating

significant differences in their nutritional value. There were no individual data for organ weights in the paper, but the kidney weights of the raw GTS line-fed (and parental control?) male rats were significantly higher than those of the controls, while the testes of the parental line-fed rats was significantly enlarged. According to the authors as these differences were not dose-related and were also shown by the parental line, they were therefore not caused by genetic modification. Unfortunately, in a major omission, no stomach or intestinal weights were recorded in the paper. No histology appears to have been done on these tissues either, apart from some qualitative microscopic observations on the pancreas that has been described as showing some minimal to mild lesions, which were claimed to be common to all groups. The absence of pancreatic hypertrophy, however, was not surprising because the unusually high dietary protein concentration, as pointed out by the authors, masked and/or diluted the biological effect of the trypsin inhibitors. This is of particular concern because the trypsin inhibitor content of GTS lines in unprocessed soybean was significantly higher than in the control line (Padgette et al., 1996). Thus, because of the major omissions in the feeding study and the lack of gut histology, more critical work is needed to decide whether the feeding value of GM and non-GM soybeans is equal or not.

2.5.1.2. Chicken study The broiler chicken feeding study's experimental design closely followed commercial practice and the results therefore at best could only be indicative of commercial feeding and production values of the various soybean lines which, according to the authors, were practically equal for both GTS and parental soybean lines.

2.5.1.3. Catfish experiment Similar to the findings with rats, one of the GTS lines, 61-67-1, was superior to the other lines (GTS 40-3-2 and the parental line) in most respects. Thus, fish ate more on GTS line 61-67-1, had better weight gain and gain/feed ratio and weighed more at the end of the 10 weeks study than the others, even though the composition of the fillets from these fish was not significantly different. Accordingly, genetic modification may not be as reproducible as claimed and the feeding value and metabolic effects of GM and parent lines are not always "substantially equivalent".

2.5.1.4. Lactating cows Milk production and composition and performance data in the lactating cow study showed some significant differences between cows fed diets containing the different lines of soybean, indicating a lack of their "substantial equivalence".

2.5.1.5. Testing the stability of the gene product In the acute gavage studies the EPSPS enzyme used was not isolated from the GTS lines but was an *Escherichia coli* recombinant product. This is a major flaw in the experimental design because, as even the authors themselves pointed out, post-translational modifications, such as amidation, acetylation and proteolytic processing of the completed polypeptide chains emerging from the ribosomes is so different in two such evolutionary distinct life forms as higher plants and prokaryotic bacteria that major differences in the conformation of the protein can be expected. As a result, these two products of the same gene may behave differently in *vivo* in the digestive system, putting a question mark to the authors' conclusion that the gene product from soybean could not have had any toxic effects because it broke down in an artificially simulated digestion test. Moreover, in gavage studies, unlike in the work described, young, rapidly growing animals must be used to establish whether the gene product has any toxic effect, affecting the growth of the animal. With older animals any effect on growth could only be shown if they were gavaged with potent toxins that by definition did not apply in this case.

The results of a separate study (Teshima et al., 2000) with toasted glyphosate-resistant GM soybean, in which rats and mice were fed with this GM soybean at 30% inclusion level in the diet for 15 weeks, could not be seriously considered because rat growth was minimal (less than 30 g over 105 days) and mice did not grow at all on either the test or control diets. This invalidates the authors' observations of finding no significant differences in nutritional performance, organ development, histopathology of the thymus, liver, spleen, mesenteric lymph nodes, Peyer's patches and small intestine and the production of IgE and IgG humoral antibodies between GM and non-GM line diets.

In an interesting paper, it was shown that the liver of mice fed on diets containing GM soybean underwent significant modifications in some nuclear features in comparison with livers from mice fed conventional soybean-based diets (Malatesta et al., 2002). Hepatocytes in GM soybean-fed mice showed irregularly shaped nuclei, indicating high metabolic rates, increased numbers of nuclear pores, suggestive of intense molecular trafficking and more irregular nucleoli with numerous small fibrillar centers, typical of increased metabolic rates. Nucleoplasmic and nucleolar splicing factors were also more abundant in GM-fed mice than in controls. Unfortunately, the design and execution of the feeding part of this study was poor which may put a question mark to the final conclusions. There is a general lack of appreciation by non-nutritionally oriented scientists that if the nutritional part of their study is not correctly designed and executed this will have an overriding influence on the results of the follow-up investigations, no matter how sophisticated these may be. Test and control diets must be iso-proteinic and iso-energetic and the starting weight of the animals must be closely similar and they must be strictly pair-fed before valid conclusions could be drawn from the studies carried out on internal organs and their ultrastructure.

An extensive production study with growing-finishing swine indicated the essential compositional and nutritional value equivalence of Roundup Ready and conventional soybeans (Cromwell et al., 2002). The results of this commercial production study are obviously useful for the industry even though their contribution to our understanding of the possible interactions between GM feedstuffs and the animal gut is limited.

Finally, as there is an inseparable link between glyphosate-resistant GM crops and the obligatory use of glyphosate with the GM plants a general warning must be given here. Although glyphosate is generally regarded as one of the most benign, wide-spectrum herbicides, there are some unconfirmed claims by Canadian scientists that spraying food crops with glyphosate may lead to elevated amounts of the toxic fungal mold, fusarium headblight, in the food crops. More serious was the published finding of French scientists (Marc et al., 2002) showing that there was a synergistic and concentration-dependent interaction between glyphosate and some of the chemicals used in herbicide formulations to delay the entry of cells into the M-phase of the cell cycle. Roundup inhibited the activation of CDK1/cyclin B *in vivo,* affecting cell cycle regulation by delaying the activation of the CDK1/cyclin B complex. These results may question the safety for human health of the widespread use of glyphosate and Roundup formulations with glyphosate-resistant GM crops.

2.5.2. GM corn

Rations containing transgenic Event 176 derived Bt corn were tested in a study involving 1280 birds (Brake and Vlachos, 1998). However, the results of this study are more relevant to commercial than to academic scientific studies. Similar conclusions could be drawn from other poultry feeding studies, such as that carried out with GA21 Roundup Ready corn-based

diets (Sidhu et al., 2000) or that with the maize line expressing the PAT protein (Flachowsky and Aulrich, 2001).

The conclusion by Kramer et al. (2000), that the GM corn developed by transferring the gene of egg white avidin to make the seed resistant to storage insect pests was safe for mice because they suffered no ill effects, can at best be regarded as premature. As the authors fed mice solely on GM or non-GM corn instead of on a balanced diet, it is not surprisingly that the mice did not grow at all with either.

The results of a study (Teshima et al., 2002) with GM corn (CBH351) expressing *Bt. thuringiensis* toxin Cry9C, in which rats and mice were fed with this GM corn at 50% inclusion level in the diet for 13 weeks in similar manner to their GM soya feeding study (Teshima et al., 2000), are open to the same criticisms as the latter study.

In a very interesting study, in which the testicular development in young mice fed on Bt-corn diets was estimated by dual parameter flow cytometry, it was shown that the GM corn had no measurable or observable effect on fetal, postnatal, pubertal or adult testicular development (Brake et al., 2004). According to the authors, if the results of this study were extrapolated to humans the consumption of Bt-corn cannot be regarded to have harmful effects on human reproductive development.

Finally, there have been some worrying reports of infertility in pigs given Bt-corn in Iowa, USA (www.organicconsumers.org/ge/pigfertility012703.cfm). However, there is no conclusive evidence whether this is linked to the genetic engineering of the corn or to the high levels of fusarium mold growth on the Bt-corn.

2.5.3. GM peas

In a 10-day feeding trial, the nutritional performance of rats fed diets containing transgenic peas expressing the transgene for insecticidal bean α-amylase inhibitor (about 3 g/kg peas!) at an inclusion level in the diet of 30% (Pusztai et al., 1999) was comparable to that of rats pair-fed iso-proteinic and iso-energetic diets containing parent-line peas and also lactalbumin diets spiked with isolated bean and pea α-amylase inhibitors, respectively. At this inclusion level, the nutritional value of diets containing transgenic or parent peas were not significantly different. Even at a 65% inclusion level the differences were small, mainly because the transgenically expressed recombinant α-amylase inhibitor in the pea was quickly (in less than 10 min) degraded in the rat digestive tract and therefore its antinutritive effect was abolished. In contrast, spiking the parental line pea diet with the stable, bean α-amylase inhibitor, as expected, reduced its nutritional value (Pusztai et al., 1995, 1999). Unfortunately, neither gut histology nor lymphocyte responsiveness assays were done and nutritional data alone are rather insensitive for finding possible differences in metabolic responses between GM and conventional food components: therefore, the results can only be regarded as preliminary. Although there were significant differences in the development of some organs of rats fed GM pea diets, mainly the enlargement of the cecum and pancreas (and a substantial but not significant increase in the size of the small intestine), most other organ weights were similar. At the end of the study cautious optimism was expressed that GM peas could be used in the diets of farm animals, particularly at the low/moderate levels recommended in commercial practice and if careful monitoring was made of the progress of the animals for the entire feeding period. However, the study cannot be taken to show that GM peas are safe for human consumption. This requires that further and more specific risk assessment will have to be designed and carried out. Moreover, only one particular line of GM peas was tested in which the endogenous antinutrient levels were selected to be similar to those of the parent peas.

In some other GM lines, however, lectin levels varied, up or down, by a factor of four and the concentration of trypsin and chymotrypsin inhibitors was usually significantly increased compared with their parent line (Pusztai, unpublished data). Therefore, to assess safety it is important that many GM lines are investigated and from the results of a single GM line no blanket approval should be given to other GM lines.

2.5.4. GM potatoes

2.5.4.1. Glycinin-expressing potatoes In a 4-week rat-feeding study both the experimental and control groups were fed the same commercial diet but they were also given by gavage 2 g/day of the respective potato lines/kg body weight. These were the parental control line and two transformed GM lines, one with the glycinin gene and another one with a designed glycinin gene (coding for four additional methionines in the gene product), respectively. Commendably, the authors (Hashimoto et al., 1999b) measured the growth, feed intake, blood cell count and blood composition and internal organ weights of the rats. However, it is unclear whether the animals were fed with raw or boiled/baked potatoes and this makes the interpretation of the results difficult.

2.5.4.2. Bt toxin potatoes A mainly histologic study was made of the ileum of mice fed with potatoes transformed with a *Bacillus thuringiensis* var. *kurstaki* CryI toxin gene compared to control mice fed potatoes treated with the toxin itself (Fares and El-Sayed, 1998). It was shown that both the δ-endotoxin and, to a lesser extent the Bt-potato, caused villus epithelial cell hypertrophy and multinucleation, disrupted microvilli, mitochondrial degeneration and increased numbers of lysosomes and autophagic vacuoles and the activation of crypt Paneth cells. Unfortunately, there were some flaws in the experimental design, such as the lack of proper description of the Bt potatoes and their gene expression level, or the uncertainty of whether the potatoes in the diet were cooked/baked or raw, or the failure to describe the amount of Bt toxin used to supplement the control potato diet. This makes it difficult to quantitatively compare the effects on the ileum of the Bt potato with the spiked control potato diets. All the same, this was an important study because once and for all it established that, in contrast to general belief, exposure of the mouse gut (ileum) to the CryI gene product caused profound hypertrophic and hyperplastic changes in the cells of the gut absorptive epithelium and these could lead to mucosal sensitization, as was later demonstrated (Vazquez Padron et al., 1999, 2000). These changes could only have occurred because, in contrast to the artificial stability shown in the *in vitro* simulated gut proteolysis tests, the Bt toxin did in fact survive, in a biologically active form, the passage through the digestive tract. Clearly, concerns about the possible biological consequences of exposure to GM food, such as those expressing the Bt toxin, should be addressed under *in vivo* conditions. As a result it was recommended that "thorough tests of these new types of genetically engineered crops must be made to avoid risks before marketing".

2.5.4.3. GNA GM potatoes Work concerning effects on the histology of the different gut compartments of feeding rats with diets based on GM potatoes expressing the snowdrop (*Galanthus nivalis*) bulb lectin (GNA) gene (Ewen and Pusztai, 1999b) revealed some major changes in gut structure and function. The significance of these results was further expanded in the authors' reply (Ewen and Pusztai, 1999a) to the invited comments by the *Lancet* (Kuiper et al., 1999) and also in a recent review (Pusztai et al., 2003). Some other selected results of the nutritional/metabolic studies published on the website of the Rowett Research Institute

(http://www.rri.sari.ac.uk), where most of the work was done (Bucksburn, Aberdeen, Scotland, UK), will only be briefly mentioned.

Young, rapidly growing rats (starting weight of 84 ± 1 g) were strictly pair-fed on isoproteinic (60 g total protein/kg diet; most of which was from potatoes) and iso-caloric diets supplemented with vitamins and minerals for 10 days. The test diets contained GM potatoes, either raw or boiled. The control diets contained the same amount of parental line potatoes (raw or boiled) alone or supplemented with GNA at the same concentration as expressed in the GM potatoes. As a system control a lactalbumin group of rats was also included. Samples of stomach, jejunum, ileum, cecum and colon were, after fixation and staining with hematoxylin and eosin, subjected to full quantitative histological evaluation. This revealed that the thickness of the stomach mucosa was increased, partly due to GNA, the gene product (Ewen and Pusztai, 1999b). However, the proliferative hyperplastic growth of the rat small intestine leading to crypt enlargement and a part of the stomach enlargement was not a GNA lectin effect. Instead this was probably due either to some other component of the gene vector used for the genetic modification and/or the disruption caused by the incorporation of the vector in the plant genome. Indeed, unlike the strongly mitotic lectins such as the kidney bean phytohemagglutinin, GNA from snowdrops is a nonmitotic lectin whose binding to, and growth-promoting activity for, the small intestinal epithelium is slight and not significant (Pusztai et al., 1990) and as measured by staining with antiGNA antibody + PAP (peroxidase–antiperoxidase), it remains unchanged after the expression of its gene in GM potatoes. Hyperplasia was also confirmed by measuring the increase in crypt cell numbers and crypt mitotic figures in the jejunum of GM potato-fed rats (Pusztai et al., 2003). However, as the solanine glycoalkaloid content of the GM potatoes was significantly less than that of the parent lines (Birch et al., 2002) the suggestion that the jejunal growth was caused by potato glycoalkaloids could be ruled out. Overall the results suggested that crypt hyperplasia and the observed epithelial T lymphocyte infiltration caused by GM potatoes might also occur with other GM plants which had been developed using the same or similar genetic vectors and method of insertion. It is therefore imperative that the effects on the gut structure and metabolism of all GM crops should be thoroughly examined as part of the regulatory process before their release into the human food chain.

2.5.4.4. Potatoes expressing cationic peptide chimeras Desiree and Russet Burbank potatoes expressing N-terminus modified cecropin-melittin cationic peptide chimeras and control line potatoes fed to mice caused severe weight loss (Osusky et al., 2000). The animals did not grow even after supplementing these potatoes with Rodent Laboratory Chow. Apparently, mice fed with tubers from transgenic potatoes were as healthy and vital (sic) as those from the control group and their fecal pellets were comparable. The severe weight loss seriously questioned the value of the results of this poorly designed feeding experiment.

2.5.5. GM tomatoes

Tomatoes are of little importance in animal nutrition, but some of the ideas in this non-peer-reviewed book chapter describing the properties of a GM tomato line developed using the *Bacillus thuringiensis* crystal protein CRYIA(b) gene may have some general relevance in GM studies. Instead of the cauliflower mosaic virus 35 s promoter (CaMV 35 s), which is used in practically all first-generation GM crops, a potentially safer plant promoter was used (Noteborn et al., 1995). However, with this promoter the expression level of the Bt toxin was only about 1/20th of that found with CaMV 35 s and therefore the validity of the results of the

feeding studies may be questionable. However, this study is still of interest because, in contrast to most other studies with GM crops, there was a commendable attempt to use immunohistology to measure the binding of the gene product to the rat gut surface *in vivo,* rather than using spurious arguments about why the gene product should not bind. Unfortunately, instead of the Bt toxin isolated from GM tomatoes, an *Escherichia coli* recombinant and potentially less stable form of the gene product was tested, which puts a serious question mark over the results. However, even with this recombinant form the *in vitro* binding of the Bt toxin to gut sections, including the cecum and colon of humans and rhesus monkeys, was demonstrated by immunocytochemistry.

More recently, a Chinese study on the safety assessment of GM tomato and GM sweet pepper expressing the coat protein (CP) gene of cucumber mosaic virus (CMV) has been published. It appears that the expression of the CMV CP gene makes these plants resistant to CMV (Chen et al., 2003). It was claimed that these two GM products showed no genotoxicity either *in vitro* or *in vivo* by the micronucleus test, sperm aberration and Ames tests. According to the authors, the 30-day animal feeding studies showed no significant differences in growth, body weight gain, food consumption, hematology, blood biochemical indices, organ weights and histopathology in rats or mice of either sex, fed with either GM sweet pepper or tomato diets compared with those on non-GM diets, and therefore it was claimed that the GM crops appeared to be as safe as their comparable non-GM counterparts. However, some of these sweeping claims are difficult to accept on the basis of the actual data in the published paper. Additionally, there is a lack of precision in defining some of the parameters measured in the work. Thus, one of the major omissions is that the coat protein expression level in the plants is not given and in the toxicity tests it is impossible to see what is measured without making comparisons with equivalent amounts of CP, particularly as no attempt has been made to isolate CP from the two GM plants. The nutrition study has not been described adequately, no starting or during-the-experiment weights of the individual animals are given. Means are no substitute, particularly when as in Figure 3 the standard deviations in the bar diagram are so big (e.g. in 3 A at 3 weeks the mean weight of the rats is about 150 ± 50 g) that makes the in-between group comparisons meaningless. No diet composition and no animal management data are described, even though without pair-feeding no valid conclusions about weight gain, organ weights, biochemical blood indices, etc., can be arrived at. The graphs and data are uninformative. The size of the most important tissues, such as the small and large intestines, pancreas, etc., has not been recorded. The methods used for histological evaluation are not detailed and therefore it is impossible to see whether the authors used appropriate methods or not. In view of these deficiencies it is difficult to accept the authors' conclusions that these GM plants are as safe as their conventional counterparts.

3. EVALUATION AND CONCLUSIONS

For the evaluation of the safety of GM food/feed the significance of differences, if any, in the parameters outlined above should be established by suitable statistical analyses (ANOVA, multiple comparisons and/or multivariate analysis). If the experiments show up differences between animals fed GM and parent-line diets this indicates that the genetic modification must have had a significant effect on the utilization and nutritional value of the GM crop and therefore this cannot be accepted as it is without further more detailed studies. However, if both the GM diet and the parent-line diet spiked with the gene product show differences, the use of this gene in GM food/feed is not acceptable. Finally, if negative effects are observed with the GM crop but not with the parent line diet containing the isolated gene product, it is

likely that the harm is caused by the use of the particular construct or by an unwanted or unforeseen effect of the gene insertion on the genome.

In this overview, peer-reviewed and published biological studies carried out with GM materials have been described and discussed in the framework of a suggested safety testing protocol (Pusztai, 2002). However, this can only be regarded as a start. There is a compelling need to further develop the concepts of biological testing, particularly for the investigation of potential long-term GM effects. Moreover, since the GM potato work with male rats showed abnormalities in the development of their sexual organs, it is imperative that similar experiments should also be done with female rats, to be followed by studies of the effects on reproductive performance of rats (or other animals) reared and maintained on GM vs non-GM diets for several generations.

4. FUTURE PERSPECTIVES

Apart from the practical considerations discussed in this review, the conceptual framework of the safety of genetically modified products also needs to move forward. In this respect it would be helpful in the interests of science to generally recognize and accept that views such as that "transgenic varieties expressing a single agronomic trait (are) not expected to (have an) altered nutrient composition" (Aumaitre et al., 2002) fly in the face of the facts and published data. The adherence of some plant molecular biologists to the almost mythical concept of "substantial equivalence" with single-trait first-generation GM crops is now a serious impediment for further progress in GM studies, as has also been recognized by the *Codex Alimentarius* guidelines. In any case, with multiple traits of the coming GM generations it will be impossible to use this as a conceptual basis for risk assessment. However, even with the present single-trait GM crops, there is a need to put some of the most important and well-established principles in the center of the science of genetic modification because, in addition to their general validity, they also apply to the recombinant gene technology work (Schubert, 2002). Thus, it needs to be recognized that the introduction of the same gene into two different cell types can produce two proteins, which can have distinct properties. Moreover, with this the overall gene expression and the phenotype of the cell may also change and it is possible that the genetically introduced enzymatic pathways could interact with endogenous pathways of the cell. Genetic engineering is therefore able to produce novel, possibly harmful, toxic, allergenic or carcinogenic molecules. As this is unpredictable, their presence can only be established from biological testing experiments, particularly from those carried out for extended time periods. Although not necessarily for the same reasons, one has to agree with the views of some biotechnologists that relatively short-term animal feeding/production experiments, particularly as they are presently carried out, do not contribute much to GM safety.

As described in this short overview, it is clear that most of our understanding of the possible consequences of GM food/feed ingestion has come from academic, basic research studies in which the digestive tract was investigated as the main target organ for the GM ingredients and then followed up by looking for the general metabolic consequences of this initial interaction between food/feed and the gut. Although, regretfully, there are only a few examples of such published studies, even these few have clearly indicated that methods based on the striking biological effects of the transgenic DNA and proteins on the gastrointestinal tract could be developed into potentially powerful tools in GM food/feed risk analysis. There is therefore a need to extend the present commercial, ad-hoc, production-oriented research of relatively crude comparisons of GM or non-GM feed rations performed with farm animals. More basic

studies are needed, in order to establish the scientific principles of the possible GM effects on the gut, including not only that of the transferred gene but also of other parts of the genetic construct, particularly the promiscuous viral promoters such as the cauliflower mosaic virus promoter, and the effect of their incorporation into, and their position in, the plant genome. It is no longer acceptable to have bland assurances, such as that CaMV 35 s promoter is specific to plants and will not work in animal cells, when indeed this is against the observed biological wisdom. It is also unacceptable to expect that the behavior and the potential hazard of the CaMV 35 s promoter incorporated into the chromosome of transgenic plants is the same as the replication and behavior of the virus whose DNA possibly never integrates into the chromosomes of the infected plant cell. These issues are open to experimentation and as such ought to be decided by *in vivo* observations.

With the development of a thorough understanding of the possible changes in the physiology, nutrition, immunology and metabolism of the animals receiving GM products, a more rational basis for further advances in animal health and production can be and indeed needs to be established. Unfortunately, considering the strikingly poor record of peer-reviewed publications of GM studies in the scientific literature, the often-publicized great potential of the genetic manipulation of our food/feed crops, is apparently based less on scientific achievements than on future promises. This is all the more curious, because some of the science and ethical considerations which appear to impede the efforts to establish risk assessment methods for human health safety with GM foods most of the time do not apply to animal work. Even a cursory look at the huge numbers of published papers on animal nutrition research with conventional feedstuffs reveals that most of the methods for investigating the interactions between novel protein-containing feeds and the gut and their metabolic consequences for the animals are well established. It is hoped, therefore, that if sufficient funding is made available, scientists working with GM will be keen to make good use of these methods, for our benefit.

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ADDENDUM

Although the release of new GM products has continued unabated in the 10 months since the main body of our review was completed, the number of papers describing the underpinning science and principles that supported their safety does not appear to have increased substantially. Nevertheless, our review could not be regarded complete without evaluating some of these.

1. SAFETY ASSESSMENT OF GM CROPS

1.1. Compositional studies

Despite great advances in analytical methodology with the introduction of proteomics, metabolite and DNA/RNA profiling, fingerprinting, microarrays, etc. into basic laboratory science, regrettably, judging from the papers published or the submissions of biotechnology companies, scientists engaged in the evaluation of the safety of GM crops have apparently been rather reluctant to rely on these novel techniques. Accordingly, rather than to aim for completeness in this review only selected examples will be given to demonstrate the rather sterile and static conventional analytical approach to safety that still appears to be the norm

and juxtapose these with the occasional examples of more novel research work attempting to unravel the actual molecular mechanisms involved in genetic modification.

1.1.1. Herbicide-resistant soybeans

With the publication of a major paper comparing the chemical composition and protein quality of soybeans from five leading soybean-producing countries (Karr-Lilienthal et al., 2004) the often-predicted quality/yield drag of genetic modification appears to have received some, albeit indirect, support by showing that the protein, some amino acid and mineral contents of the almost exclusively GM soybeans from Argentina were the lowest of the five countries. It was particularly interesting that the phenylalanine content of the Argentinian soybeans, similar to that of the Puerto Rican-produced GM soybeans (Padgette et al., 1996), was particularly low, signaling potential problems in the synthesis of some key aromatic metabolites.

1.1.2. GM potatoes

The composition of two GM lines G2 and G3 expressing *Cry V* gene from *Bacillus thuringiensis* was compared by conventional analytical methods with that of the Egyptian parent line called Spunta in a recently published paper (El Sanhoty et al., 2004). A general comment: it would have been helpful if the authors had made clear whether the potatoes used for the analyses were from the Egyptian markets as stated in the introduction or from Michigan field plots in the USA as indicated under materials and methods. According to the authors, none of the 14 main chemical components in the two GM lines and the parent potatoes was significantly different. However, a closer examination of the results in tables 2–5 of El-Sanhoty et al. (2004) showed that the contents of some components of the control and either or both of the GM lines may have differed significantly. Thus, the ascorbic acid, phosphorus and calcium contents in table 2 (El-Sanhoty et al.) appeared to be different. The contents of some amino acids in the three lines (table 3, El-Sanhoty et al.) also showed major differences, chief amongst them were methionine, cystine, tryptophan and histidine. Curiously, phenylalanine was not listed in table 3 (El-Sanhoty et al.) and each of the methionine and tryptophan contents exceeded that of aspartic acid. Both genetically modified potato lines contained significantly higher protease inhibitor activity than the parent line (table 5, El-Sanhoty et al.). The general state of the presentation of the results was rather poor and left the reader confused whether to believe the text or the tables. In fact, the results appeared to contradict the conclusions of the paper that the parent and GM potatoes were substantially equivalent.

In contrast to the traditional approach of using standard analytical techniques to establish the "substantial equivalence" of GM and non-GM lines as above, the great value of the molecular approach was demonstrated in some new research papers. These included publications in which the mechanism of the potential nutritional enhancement by genetic engineering by increasing the contents of β-carotene and other carotenoids of various crops has been investigated. A good example is the GM potato plants expressing an *Erwinia uredovora crtB* gene encoding phytoene synthase in the tuber of *Solanum tuberosum* cv. *Desiree* (Ducreux et al., 2004). Clearly, the main aim of the researchers was to understand the molecular basis of the gene transfer and the working mechanism of the genes involved in carotene biosynthesis and their regulation but not to show whether the potato tubers of higher carotenoid content were as safe as the conventional tubers. However, it would have been advantageous to use the microarray analysis not only to establish changes, up- and down-regulation in the transcription of cDNAs involved in carotenogenesis, such as that of fibrillin but also other more

nutritionally important proteins. Hopefully, together with other compositional parameters, these will come from future studies, the results of which would allow us to judge the safety of these GM potatoes, particularly if and when they will be subjected to short- and long-term animal feeding safety studies.

1.2. Published nutritional/toxicological studies

In the absence of generally agreed safety testing procedures for GM crops intended for animal/human nutrition the papers published in the last year have followed previous patterns. Whilst in some of them, particularly those associated with testing the safety of new biotechnology GM products, the methods used were similar to those established earlier for Roundup Ready soybeans (Hammond et al., 1996; Harrison et al., 1996), some others tried to find new and more functional and/or cellular methods to establish the effects on animal health of the GM crops of nutritional importance.

1.2.1. Herbicide-resistant soybean

The Malatesta group continued with their histology work aimed at establishing what effect, if any, the long-term feeding of GM-soybean-based diets had on the structure and function of some important body organs of mice. Their previous finding of reduced digestive enzyme synthesis and secretion in the pancreas (Malatesta et al., 2002b) in GM-soybean-fed mice has now been confirmed and extended by showing that in these animals the accumulation of nucleoplasmic and nucleolar splicing factors and perichromatin granules was also significantly reduced, suggesting reduced post-transcriptional hnRNA processing and/or nuclear transport (Malatesta et al., 2003) that may be the underlying mechanism of the deficiency in digestive enzyme synthesis and secretion by pancreatic acinar cells.

Due to the ever-increasing area of cultivation of glyphosate-resistant RoundupReady GM crops, the use of glyphosate has also increased. For this reason some newly published work on the possible health-damaging effects of glyphosate need to be dealt with in this review. Further findings by French scientists (Marc et al., 2005) have confirmed and extended their previous results by showing that the main ingredient of commercial Roundup formulations, glyphosate, in a millimolar concentration range, particularly when used together with the obligatory polyoxyethylene amine surfactant, significantly delayed the hatching of sea urchin embryos by inhibiting the transcription of one of the enzymes involved in hatching. As inhalation of herbicide sprays in which the active ingredient concentration exceeds by about 25 times that used in the transcription inhibition studies, health concerns associated with the use of glyphosate may have to be taken truly seriously. In another study it was shown that the oral treatment of Wistar rats to increasing concentrations of the herbicide Glyphosate-Biocarb, a formulation used in many countries such as Brazil, the number of Kupffer cells in hepatic sinusoids increased, followed by large deposition of reticulin fibers and the leakage of hepatic aspartate-aminotransferase and alanine-aminotransferase into the circulation, indicating hepatic damage in these animals (Benedetti et al., 2004). In further studies by another group of French researchers it was shown that glyphosate, particularly as used together with polyoxyethylene amine surfactant in Roundup formulations, was toxic to human placental JEG3 cells at concentrations lower than that used in agriculture. Even at subtoxic concentrations Roundup was an endocrine disruptor on aromatase activity and its mRNA level and glyphosate interacted with the active site of the purified enzyme (Richard et al., 2005). It is possible that the pregnancy problems in agricultural workers using Roundup may be traced

back to the exposure to this herbicide (Savitz et al., 2000). All these findings indicate that there is an urgent need to carry out systematic and direct studies, independent of the biotech industry, on the short- and long-term effects on animal (and human) health of exposure to glyphosate and its more effective commercial formulations. With the presently cultivated huge areas of Roundup Ready crops and the anticipated even larger future extensions of this glyphosate-dependent GM crop technology the potential danger for animal/human health needs to be dealt with in advance and not if or when it occurs.

1.2.2. GM corn

A 13-week rat-feeding safety assurance study with Roundup Ready GM corn (NK 603) was carried out to a design similar to that used previously with Roundup Ready soybean (Hammond et al., 2004). Indeed, the work could be similarly criticized. Thus, no precise composition of the test or control diets was described in the published paper. No starting weight range of the rats was given, only the mean weight of the groups with the assurance that their standard deviations were within 95% confidence limit. However, the wider the starting weight range the more difficult it is to find significant differences in growth during the feeding experiment. This would have been difficult to establish in any case because the growth and feed intake graphs were crude and uninformative. Without error bars one has to take the assurances given in the paper that there were no significant differences between the test and control groups. The low values of about 12–13% for feed conversion efficiency indicate problems with the diet. Indeed, the fact that feed intake remained static or even declined in the second part of the experiment pointed to a similar conclusion. Some of the critical hematology mean values, such as WBC (white blood cell count), NEU (leukocyte differential count) and LYC (lymphocyte count) that could indicate problems in immune responses, had huge SD values (standard deviations; almost ± 30% in some cases), so the authors' claim was not surprising that there were no significant differences between the groups. Some of the serum chemistry SD values were only little better. The two most important tissues, the small intestine and the pancreas, were not weighed. The acute mouse gavage study was conducted with the CP4 EPSPS surrogate recombinant protein and not that isolated from the GM corn although it is known that this could lead to erroneous conclusions. In view of all these flaws in the experimental design and the execution of the study it is difficult to accept the conclusion of the authors that the Roundup Ready GM corn is equivalent to its traditional counterpart. In view of the lack of experimental evidence presented in the paper, such as the use of a second control group in which the parent line is supplemented with the gene product isolated from GM corn or a third zero control group in which the parent corn was transformed with the empty vector, it is even more difficult to understand how one can accept the conclusion of the authors that their study did not detect any pleitropic effect.

The survival of transgenes and/or their products in the animal gastrointestinal tract and their uptake into body organs (e.g. Klotz et al., 2002; Chowdury et al., 2003) is a hotly debated issue with clear implications for human/animal safety. However, this issue cannot be separated from taking an account of the sensitivity of the detection methods used as shown by finding that amplifiable fragments of rabbit retrotransposon and mitochonrial DNAs were taken up from the gastrointestinal tract in human volunteers (Forsman et al., 2003). Although it is not possible to say whether one can expect any physiological effects as a result of this DNA uptake across the species barrier, it cannot be assumed either that the DNA transferred from the gut is nonfunctional. Indeed, DNA vaccines are known to have been successfully developed and oral vaccination has been achieved with an artificial promoter-driven DNA coding for an immunogenic protein (Jones et al., 1997).

Some of the possible problems in the detection of surviving gene products have been highlighted by a recent finding that the amounts of intact Cry1Ab protein in the bovine gastrointestinal tract of cows fed GM maize measured by ELISA appeared to be appreciable but only fragments of the Bt toxin could be detected by immunoblotting (Lutz et al., 2005). However, without establishing what, if any, physiological effect and/or possible toxicity for the animals of these immunoreactive Cry1Ab protein fragments can have, the assurance that GM corn is safe implied in finding no intact Bt-toxin in the bovine intestines cannot be accepted without further studies.

1.2.3. GM potatoes

GM potatoes expressing a cysteine-proteinase inhibitor, rice cystatin, are partially (or in some cases fully) resistant to nematodes without apparently harming nontarget anthropods or perturbing soil microbial communities (Urwin et al., 2003). As it occurs naturally in rice, maize and even in potatoes, cystatin is not new for the mammalian digestive system. Moreover, the expression of cystatin can be limited to the roots, further reducing the already potentially low concern of toxicity or allergenicity with these GM potatoes. According to recent claims by the Atkinson group, their nutritional studies presented *prima facie* evidence that cystatin-expressing GM potatoes do not present toxic risks to mammals (Atkinson et al., 2004). However, in reality these authors did not test the safety of the GM potatoes. Neither did they carry out a proper nutritional evaluation of them. What they actually tested was the *in vitro* stability to digestion with simulated gastric fluid at pH 1.2 and at abnormally high concentrations of pepsin, of a surrogate recombinant *E. coli* protein and not that isolated from GM potatoes or, even more importantly, not of the GM potatoes themselves. Thus, this *in vitro* simulation test was flawed both in principle and execution. Instead of a proper nutritional evaluation of the GM potatoes the authors used this recombinant surrogate protein in a LD_{50}-type oral toxicity study, the design of which was seriously flawed. Rats of unknown starting weight were kept in groups of five, and not individually, making it impossible to follow their individual feed intakes and growth and to relate therefore their body tissue weights and other measured parameters to final bodyweights. Although some body organs were weighed and some, such as the liver showed differences, the only part of the gastrointestinal tract that was measured was the cecum, but not the small intestine or the colon, the very tissues that are the most sensitive indicators of dietary effects. This omission is the more serious because the weight of the cecum, which is a relatively small part of the large intestine, was found to be significantly increased (unrelated to the final body weights!). For all these flaws the authors' conclusion that these cystatin-expressing GM potatoes do not present toxicity risks to mammals may need to be reassessed by further studies.

Raw Punta G2 and G3 GM and non-GM potatoes whose analytical composition was given above were also subjected to a 30-day feeding study with rats (El Sanhoty et al., 2004). Some of the annoying discrepancies between data in the text and the tables should have been removed during the peer-review and editing. As the potato inclusion in the diet was only 30%, less than 15% of the protein in the diet was provided by the GM potatoes. More seriously, the composition of control group 1 was substantially different from the potato-containing diets as its protein content was about 15% less. It was not made clear why all the diets were supplemented with 0.3% methionine when the potatoes were not deficient in this amino acid. Differences in rat starting weights (± 10%) were too high although they improved after prefeeding. However, despite assurances by the authors that feed efficiency values were not significantly different between the test and the control groups this cannot be accepted on the

basis of the data in table 6 of El-Sanhoty et al. (2004). Similar considerations apply to testes weights in table 7 (El-Sanhoty et al.). Apart from the specific criticisms above, the design of the feeding study was rather simplistic and static without looking at more dynamic novel features in the GM-fed rats, such as possible changes in immune, hormonal and metabolic functions, and no attempt was made to use histopathology methods to detect and/or exclude potential alterations in the cells or tissues of the rats on different diets. A particularly serious omission in the work is the absence of a critical look at the structure and function of the small intestinal epithelium.

1.2.4. GM crops expressing bioactive proteins to replace antibiotics in animal nutrition

The cultivation of a new class of GM crops expressing bioactive or pharmaceutical proteins has been on the increase in the last few years. One of the first examples of these was GM rice lines expressing lactoferrin or lysozyme designed to protect the gastrointestinal tract against bacterial infection in place of antibiotics in the feed (Humphrey et al., 2002). In a chicken-feeding study of rather limited scope and design in which some of the nutritional and physiological parameters of chickens fed on diets containing antibiotics such as bacitracin and roxarsone included in a conventional rice diet, were compared with diets containing GM rice expressing lactoferrin or lysozyme or a mixture of both. The most serious major flaw in the two experiments carried out to the same design was that in the feeding studies not all necessary control groups were included. Thus, the effects of the two GM rice lines were only compared to a conventional rice diet with or without added antibiotics but not to that of conventional rice control diets in which lactoferrin or lysozyme or both at the same concentration as they were expressed in the appropriate GM rice lines were also included. In these controls lactoferrin and/or lysozyme supplementation should have been done with the proteins isolated from the appropriate transgenic rice line. However, even the inclusion of these two proteins from conventional sources could have demonstrated whether the authors were justified in suggesting a truly "antibiotics effect" on the bacterial flora in the GM-rice-fed chickens as an explanation or whether what they measured was a direct effect of these two proteins on the gut epithelium. Although the gut histology indices were suggestive that these two proteins through their effect on the bacterial flora may have indeed been indirectly responsible for the modest nutritional improvement described in the paper but direct evidence by monitoring the gut bacterial population and counts could have given a more convincing proof for the authors' thesis. In the absence of such evidence further work is needed to substantiate the authors' suggested action mechanism. On a separate issue, the long-term human/animal safety of GM rice expressing bioactive proteins also needs to be established, particularly in view of the possibility of their cross-pollination with non-GM rice.

2. CONCLUSIONS AND PERSPECTIVES

Although there has been no great improvement in the science underpinning GM crop safety in the actual papers published in the intervening year since the completion of the main part of this review, some hopeful signs have appeared. Scientists funded by the European Union as part of the Thematic Network ENTRANSFOOD have recently reviewed the work focusing on the evaluation of current approaches to risk assessment of GM-crop-derived foods and potential needs for further improvements in safety testing methods and have come up with their recommendation of adopting a step-wise approach to safety assessment. Their recommendations have been summarized in a "Concluding Remarks" paper by Kuiper et al. (2004), together with references to the individual papers dealing with safety issues in detail.

It is hoped that this will take us a step closer to having an agreed procedure for risk assessment and safety testing of GM crops that will be based on solid scientific principles. One can but hope that in this testing procedure the recommendations of two recent influential reviews (Wilson et al., 2004; Freese and Schubert, 2003) will also be incorporated.

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18 Genetic influences on nutrient utilization in growing farm animals

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Via genetic selection, substantial improvements have been made in growth potential and carcass characteristics of farm animals. As a result the animal's growth response to nutrient intake has changed as well. Interactions between animal genotype and nutritional regime may be better understood when animal types are characterized in aspects of nutrient partitioning. The main aspects of nutrient partitioning for growth are: (1) maintenance energy and nutrient needs; (2) whole-body nutrient retention; (3) nutrient retention support costs; and (4) voluntary feed intake. In terms of nutrient retention, special consideration should be given to whole-body protein deposition and the partitioning of retained energy between whole-body protein and whole-body lipid. Whole-body protein deposition is closely associated with muscle or lean tissue growth. Whole-body protein deposition and the partitioning of retained energy between body lipid and body protein is driven by animal genotype and is influenced by environmental factors, including nutrient intake. Quantification of the main aspects of nutrient partitioning is extremely useful for estimation of nutrient requirements, for assessing the economic impact of altering feeding strategies in commercial animal production, and for the development of effective animal breeding strategies.

1. INTRODUCTION

Over the last few decades, genetic selection of farm animals has contributed substantially to improvements in growth rates, carcass characteristics and feed efficiency (e.g. Crawford, 1990; Schinckel, 1994; Bourdon, 1997; Gous, 1998). These changes in production traits have substantial implications for nutrient requirements of farm animals or responses to dietary nutrient intake (ARC, 1980, 1981; NRC, 1994, 1996, 1998). The rate of nutrient retention in the animal's body is a main determinant of dietary nutrient requirements, while the distribution of retained nutrients over the various body parts, as well as nutrient metabolism, contribute to the efficiency of converting nutrients that are supplied by the feed into high-value animal products. Moreover, when evaluating the genetic performance potential of individual animals careful consideration should be given to interactions between animal

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genotype and the animal environment. In particular, interactions between animal genotype and nutritional regime can have important consequences for the specific traits that are altered and the rate at which genetic progress is made (Falconer, 1977; Sorensen, 1985; Stern et al., 1994).

In this chapter, historical changes in productivity of growing farm animals are briefly reviewed and some examples of interactive effects between animal type and nutritional regime on animal productivity are presented. Animal growth is then described in aspects of nutrient partitioning, which will allow for understanding of the varying responses to nutrient intake in different animal types. Finally, potential implications of introducing novel traits into farm animals via modification of the animal's genome on nutrient utilization are discussed.

2. HISTORICAL CHANGES IN GROWTH PERFORMANCE POTENTIALS AND BODY COMPOSITION OF GROWING ANIMALS IN RESPONSE TO NUTRIENT INTAKE

Historically and across animal species, substantial improvements in growth rates and feed efficiency have been made (Cole, 1966; Crawford, 1990; Swatland, 1994). Whilst these historical changes in animal productivity can be attributed partly to improvement in animal management, a large proportion of these improvements can be attributed to genetic improvement in animal performance potentials (Crawford, 1990; Schinckel, 1994; Bourdon, 1997; fig. 1). In general, growth performance and carcass traits have a medium to high heritability, while genetic progress in selection schemes is determined by genetic variation and co-variation among traits that are considered in the selection scheme, selection intensity and generation interval (Bourdon, 1997). In particular, high selection intensities, short generation intervals and close control of environmental conditions have contributed to the high rate of genetic progress in poultry (Crawford, 1990). In an extreme case, 4-week bodyweight of Japanese quail was increased from 90 g to about 230 g as a result of selecting for bodyweight over 60 generations (Marks, 1985). Interestingly, greater selection progress was made during early than later generations (table 1), indicating that either genetic variability or heritability for 4-week bodyweight declined as genetic selection was continued.

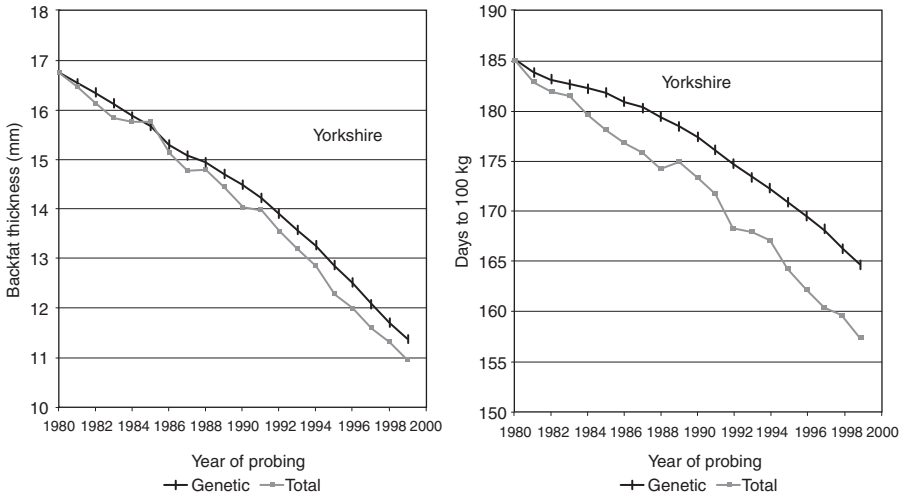

Fig. 1. Genetic and phenotypic trends in backfat thickness and days to 100 kg live bodyweight in Yorkshire pigs. (Canadian Centre for Swine Improvement, 2000, with permission.)

Table 1

Mean increases in 4-week bodyweight (g/generation) of Japanese quail during 60 generations of selection for bodyweight[a]

Generation	Selected	Control	Contrast (selected – control)
1–60	2.30	0.18	2.13
1–10	4.22	–0.12	4.29
1–20	3.06	–0.28	3.34
21–40	2.44	0.59	1.94
41–60	1.63	0.33	1.30

[a]Derived from Marks (1985); in the selected group 4-week bodyweight increased from 90 g to 228 g over 60 generation of selection.

Empirical evidence of interactive effects of nutritional regime and animal genotype on the growth performance of animals is presented in table 2 and fig. 2. The data presented in table 2 indicate that the rate of live bodyweight gain and feed efficiency in pigs of different genotype can differ by as much as 20% at similar levels of energy intake. These pig genotype effects on the partitioning of energy intake can be attributed largely to differences in fat tissue growth, and the energy required to support fat tissue growth. The lysine requirements for maximum live bodyweight gain are more than two times higher for fast-growing Hubbard broiler chickens than for slow-growing New Hampshire × Columbian crossbred chickens (fig. 2). These observed differences in lysine requirements can be attributed largely to differences in rates of whole-body protein deposition (approximately 6.6 g/d versus 3.0 g/d), which is the main determinant of dietary amino acid requirements (Hahn and Baker, 1991). Interestingly, the fast-growing Hubbard chickens ate close to two times the amount of feed consumed by the slow-growing New Hampshire × Columbian crossbred chickens, resulting in similar lysine requirements, expressed as dietary concentrations. These aspects of nutrient partitioning are addressed in detail in the next section.

Table 2

Growth performance and body composition of two types of boars (A and B) and of castrated male pigs (C) between 20 kg and 45 kg live bodyweight and at similar levels of feed intake[a]

	Pig type			
	A	B	C	SE
Feed intake, kg/d	1.50	1.50	1.51	0.01
Live bodyweight gain, g/d	809[c]	750[b]	670[b]	14
Feed/gain	1.84[c]	2.02[b]	2.25[d]	0.02
Body composition, g/kg				
Protein	166	167	164	3.4
Lipid	172[c]	205[b]	263[d]	9.6
Water	636[c]	603[b]	539[d]	6.5
Ash	25	24	24	0.5

[a]Derived from Campbell et al. (1988); all three pig types were Large White × Landrace crossbred pigs; means followed by different superscripts differ significantly.

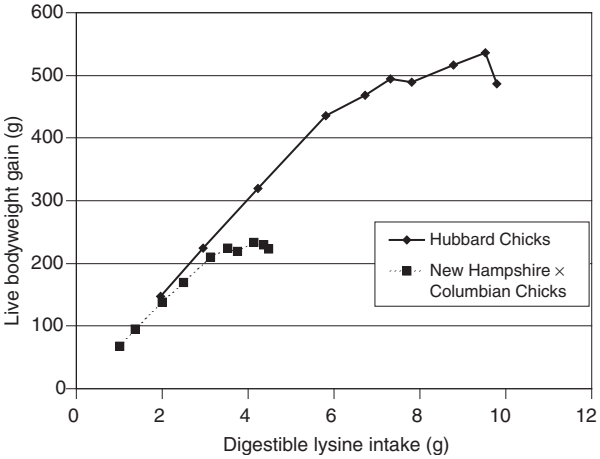

Fig. 2. Live bodyweight gain response in two types of broiler chickens to lysine intake during day 8 to 21 post hatching. (Derived from Hahn and Baker, 1991, with permission of the Poultry Science Association.)

3. ASPECTS OF NUTRIENT PARTITIONING FOR GROWTH

Growth in farm animals is generally characterized in terms of live bodyweight gain, feed efficiency and carcass composition (muscle, fat, viscera, bone, skin and hide, blood and other organs; Swatland, 1994). Alternatively, growth and animal types may be characterized in four main aspects of nutrient partitioning: (1) maintenance energy and nutrient needs; (2) whole-body nutrient retention; (3) nutrient retention support costs; and (4) voluntary feed intake. Based on these aspects of nutrient partitioning for growth, nutritional regime by animal genotype interactions may be understood and the growth response of different animal types to changes in nutritional regime may be predicted (Sorensen, 1985; Schinckel and de Lange, 1996; Gous et al., 1999). When considering the conversions of dietary nutrient into high-value animal products, such as lean meat, then the dynamic relationships between chemical and physical body composition should be considered as well (Pym, 1990; de Greef, 1995; Ferrell and Jenkins, 1998a, 1998b).

3.1. Maintenance energy and nutrient needs

Maintenance energy and nutrient requirements are traditionally defined as the animal's dietary requirements for energy and essential nutrients to support basic body functions in nonproducing animals that do not retain or mobilize body nutrient stores (ARC, 1980, 1981; NRC, 1994, 1996, 1998). Even though the relevance of the concept of maintenance for growing animals may be questioned, it provides a useful means to estimate energy and nutrient requirements of different groups of animals (e.g. van Milgen et al., 2001; Birkett and de Lange, 2001a, 2001b). Maintenance energy requirements contribute to approximately one-third of total dietary energy requirements in growing animals, while maintenance contributes only 10–15% to total amino acid requirements (ARC, 1980; 1981; NRC, 1994, 1996, 1998; Moughan, 1999). There is, however, considerable variation in maintenance energy and nutrient needs among animals within species. Variation in maintenance energy requirements within groups of animals is quite closely related to residual feed intake, i.e. the difference between actual feed intake and expected feed intake based on estimated nutrient requirements for

growth and maintenance. According to Kennedy et al. (1993) residual feed intake is a trait with a medium to high heritability.

Estimates of maintenance energy and nutrient requirements are generally expressed in dietary units, such as metabolizable energy or available amino acid intake. Since these estimates are confounded with nutritional regime and nutritional history, it is more accurate to characterize animal types in terms of basal energy and nutrient needs at the tissue level to support basic body functions (Tess et al., 1984; Birkett and de Lange, 2001a, 2001b). Energy and nutrient balances in fasting animals may be used to estimate directly basal needs, but adjustments are required for the metabolic inefficiencies of using body nutrient stores to support basic body functions and for effects of nutritional history on nutrient metabolism in fasting animals (Koong et al., 1983; Moughan 1999; van Milgen et al., 2001; Birkett and de Lange, 2001a, 2001b). Basal energy and nutrient needs may be converted to dietary requirements based on nutrient cost of ingesting and digesting dietary nutrients, metabolic inefficiencies and excretion of waste products (van Milgen et al. 2001; Birkett and de Lange 2001a, 2001b). Given the lack of solid estimates of basal energy and nutrient needs, estimates of dietary energy and essential nutrient requirements for maintenance are still routinely used in practical farm animal nutrition.

Major energy-demanding processes that contribute to maintenance requirements include those associated with blood flow, respiration, muscle tone, ion balance, tissue turnover, animal activity, ingestion of feed, excretion of waste products and utilization of dietary nutrients (Black and de Lange, 1995), while thermogenesis, increased animal activity and nutrient needs of the immune system may be included as well (Black et al., 1995). Major processes that contribute to maintenance amino acid requirements include integument losses, endogenous gut protein losses and minimum urinary losses, which are closely related to minimum amino acid catabolism rates (Moughan, 1999).

Traditionally, energy requirements for maintenance have been related to live bodyweight using allometric mathematical functions (ARC, 1980, 1981; NRC, 1994, 1996, 1998; Noblet et al., 1999). However, studies such as those conducted by Koong et al. (1983), Noblet et al. (1999) with pigs (table 3), or Gregory et al. (1994) and Ferrell and Jenkins (1998a, 1998b)

Table 3

Estimated maintenance energy requirements in different genotypes and genders of pigs[a]

Gender/genotype	Metabolizable energy requirements for maintenance (kJ/kg live bodyweight$^{0.60}$)
Males	
Synthetic line	1048
Pietrain	899
Large White	974
Females	
Large White	1004
Castrates	
Large White	1004
MS × LW	995
Meisham	874
Standard Error	56

[a]Derived from Noblet et al. (1999).

with cattle, indicate that such relationships only explain part of the variation of maintenance energy requirements that are observed between different groups of animals that are managed under similar environmental conditions. In addition to live bodyweight, the main factor that is likely to explain differences in maintenance energy requirements among different animal genotypes is animal activity (Verstegen et al., 1987; van Milgen et al., 1998). Animal activities associated with feeding and animal interactions can account for more than 20% of maintenance energy requirements (Halter et al., 1980; Verstegen et al., 1987). The distribution and energy requirements of major tissue groups (muscle, fat, visceral organs) in the animal's body should be considered as well. Because fat tissue is metabolically a relatively inactive tissue, maintenance energy requirements are lower in fat animals than in lean animals. For this reason maintenance energy requirements are better expressed relative to lean body mass or body protein mass than to live bodyweight (Whittemore, 1983). It should, however, be noted that energy requirement per kg of protein in visceral organs is much larger than that in muscle tissue (Ferrell, 1988). Recent studies suggest that there is considerable variation in the size of visceral organs between groups of pigs (Koong et al., 1983; Quiniou and Noblet, 1995; Bikker et al., 1996; van Milgen et al., 1998) and cattle (Ferrell and Jenkins, 1998a, 1998b), indicating that at least some of the variation in visceral organ size is determined by animal genotype. The latter may explain some of the variation in maintenance energy requirements among genotypes within animal types.

3.2. Whole-body nutrient retention

In terms of body nutrient retention, water, lipid and protein contribute about 95% of empty bodyweight gain. In growing animals the contribution of carbohydrate retention, primarily in the form of glycogen in the liver and muscle tissue, to daily whole-body energy retention is negligible. In growing animals, mineral retention contributes 3–6% of bodyweight gain (de Greef, 1995; Ferrell and Jenkins, 1998a, 1998b). Since water and mineral retention in the animal's body are functionally closely related to body protein deposition (PD), variation in live bodyweight gain and partitioning of retained energy can be characterized in terms of PD and body lipid deposition (LD) (Gill and Oldham, 1993; de Greef, 1995). The main focus should be on PD as it is closely associated with muscle or lean tissue growth and the main determinant of dietary amino acids requirements. Constraints on the partitioning of retained energy between LD and PD provide information on the animal's growth response to varying energy intake levels, and the amount of energy required to maximize PD in particular.

3.2.1. Body protein deposition (PD)

PD represents the balance between protein synthesis and protein degradation, while protein synthesis and protein degradation appear to be controlled by different mechanisms and are both influenced by nutritional regime (Gill and Oldham, 1993; Lobley, 2002). The rate of protein synthesis appears largely determined by the amount of ribosomal RNA in animal tissue. This is, however, not the only factor as differences have been observed in the rate of protein synthesis per unit of RNA (Millward et al., 1976; Madsen, 1983; Reeds and Fuller, 1983). In terms of nutritional influences, both protein synthesis and protein degradation increase with dietary protein intake in growing pigs, while an increase in nonprotein energy intake slightly increases protein synthesis and reduces protein degradation rates (Reeds and Fuller, 1983). Moreover, at constant protein intake, improvements in dietary protein quality enhance PD by reducing protein degradation without affecting protein synthesis (Fuller et al., 1987). Until the

mechanisms involved in controlling protein synthesis and degradation are better understood and more quantitative information on protein synthesis and protein degradation in different animal types and under varying environmental conditions becomes available, it appears reasonable to characterize protein growth in terms of PD rather than protein synthesis and degradation.

The animal's upper limit to PD (PDmax) is ultimately under genetic control. Expression of PDmax may be limited by intake of energy and essential nutrients, in particular essential amino acids, or environmental stressors. The impact of environmental stressors, such as mixing and crowding of animals or exposure to disease-causing organisms, on depression in PD are not well understood (Black et al., 1995). Since not all external factors that may influence PD are known, it is difficult to demonstrate experimentally that observed PD is determined by the animal's PDmax. For this reason, the term ''operational'' PDmax was introduced, which represents the upper limit to body protein deposition that animals can achieve under practical conditions and when fed *ad libitum* diets that are not limiting in essential nutrients (Moughan et al., 1995). Depending on environmental conditions, the operational PDmax may thus vary for a particular animal type.

The PDmax is influenced by bodyweight, or stage of maturity, and approaches zero when animals reach maturity. In young *ad libitum*-fed growing animals (less than about 20 kg bodyweight in pigs), energy intake capacity may be insufficient to allow expression of PDmax, making it difficult to establish relationships between bodyweight and PDmax (Whittemore, 1983). For practical purposes and based on some empirical evidence (Möhn and de Lange 1998; Moughan, 1999), it may be assumed that daily PDmax is constant over a considerable bodyweight range, 20–85 kg bodyweight, in growing pigs. The latter seems supported by the rather constant daily rate of body protein synthesis in growing rats between 30 g and 200 g of bodyweight, or 23 days and 130 days of age (Millward et al., 1976). This constancy of daily PDmax in young growing animals has not been verified in cattle and broiler chickens. Although changes in daily PD with increasing bodyweight have been established in cattle and broiler chickens (Robelin and Theriez, 1981; NRC 1994; Hancock et al., 1995), it has not been established whether PDmax or energy intake determines observed PD in young growing animals. In fact, the observation by Koch et al. (1979) that PD increased with feeding level in growing steers, suggests that these animals did not achieve PDmax. Moreover, cattle are generally slaughtered at a greater stage of maturity than pigs and poultry (Swatland, 1994). Therefore, as animals approach slaughter weight, PDmax tends to decrease to a greater degree in cattle than in pigs and poultry.

A variety of mathematical functions may be used to represent the relationship between bodyweight and PD (Emmans, 1981; Black et al., 1995; NRC, 1996). An elegant mathematical function that has been applied across species and that requires essentially only two parameters is the sigmoidal Gompertz function (Emmans, 1981). However, this function appears insufficiently flexible to represent accurately both the increase in PDmax in young growing animals and the decline in PDmax as animals reach maturity (Black et al., 1995). Moreover, the use of the Gompertz function often implies that PDmax rather than energy intake determines PD in young growing animals, which may not be accurate (Möhn and de Lange, 1998). A reasonable and pragmatic approach may be to represent daily PDmax as a constant that is independent of bodyweight or stage of maturity in young growing animals and to use the Gompertz function to represent the pattern of decline in PD when animals start to mature (fig. 3).

Across farm animals, PD, and possibly PDmax, may range from 80–240 g/d in pigs (Whittemore and Green 2001), 3.0–12.5 g/d in broiler chickens (Hahn and Baker, 1991, 1993; Hancock et al., 1995) and 100–200 g/d in growing cattle (Robelin and Theriez, 1981).

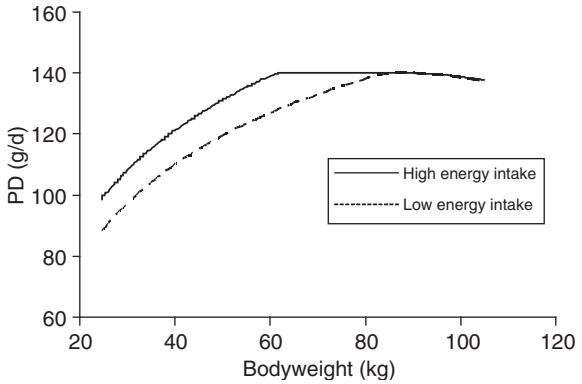

Fig. 3. Estimated whole-body protein deposition (PD) in growing pigs between 25 kg and 110 kg bodyweight (BW) and at two levels of metabolizable energy (ME) intake. High energy intake level: $47.58 \times (1 - e^{-0.0176 \times BW})$ MJ/d; low energy intake level: $42.29 \times (01 - e^{-0.0176 \times BW})$ MJ/d; maximum PD: 140 g/d, metabolizable energy (ME) requirements for maintenance: 840 kJ/kg $BW^{0.60}$/d; ME requirements for protein and lipid deposition: 43.9 and 52.8 kJ/g, respectively; minimum whole body lipid to whole body protein ratio: 0.0414 × MJ ME intake/d. (Derived from de Lange, 1995; Weis et al., 2004.)

3.2.2. Partitioning of retained energy between body lipid and protein

In growing animals retained energy in the body is partitioned mainly between PD and LD. When energy intake is insufficient to express the animal's PDmax, some of the retained energy is partitioned towards LD, while both PD and LD appear to increase linearly with increasing energy intake (fig. 2). Only when energy intake is restricted severely, approaching maintenance energy requirements, do young growing animals appear to mobilize body lipid stores to support PD, at least until some equilibrium between body lipid mass (L) and body protein mass (P) is achieved (Weis et al., 2004). On the other hand, when energy intake exceeds energy requirements for PDmax, additional energy intake is used to support additional LD (fig. 4). The energy intake level at which PDmax is just achieved (fig. 4) represents generally the level of energy intake at which feed efficiency is maximized. Carcass fatness and energy requirements per unit of live bodyweight gain increase when energy intake is increased beyond the level required to just PDmax (Black and de Lange, 1995). As mentioned earlier,

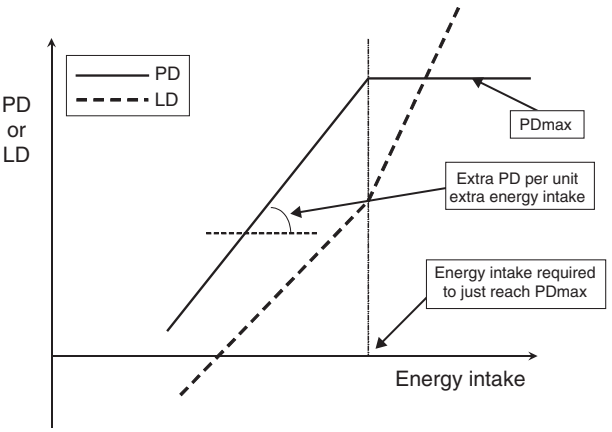

Fig. 4. Generalized relationship between energy intake and whole-body protein deposition (PD; solid line) and whole body lipid deposition (LD; dashed line) in growing animals over a fixed bodyweight range.

young growing animals have generally insufficient energy intake capacity to achieve PDmax, while finishing animals consume more energy than requirements for PDmax or maximum muscle growth (Murphy and Loersch, 1994).

When energy intake limits the expression of PDmax, the partitioning of retained energy between PD and LD may be ''controlled'' by a target bodyweight gain (marginal LD to PD ratio; minδLD/δPD; de Greef, 1992), or a target composition of the body (L to P ratios; minL/P; Moughan and Verstegen, 1988). The minδLD/δPD is the main determinant of the slope of the relationship between PD and energy intake in fig. 4 (de Greef, 1992; NRC, 1998). In various studies, effects of nutritional history on the composition of bodyweight gain have been observed, indicating that the partitioning of retained energy is determined primarily by a target body composition and not a target composition of bodyweight gain. For example, in young growing pigs with high PDmax and following a period of protein intake restriction, PD was higher and the LD to PD ratio was lower as compared to control animals that were not subjected to previous protein intake restriction (Kyriazakis et al., 1991). Following a period of catch-up growth, body composition was similar for pigs that were previously fed protein-limiting or protein-adequate diets in this study. These observations suggest that growing animals have a target body composition, which varies with energy intake level and genotype. Dynamic regulatory systems involving the endocrine system (leptin, growth hormone) likely play a role in the control of body composition (Barb et al., 1998).

The relationship between energy intake and PD changes with bodyweight (fig. 5; Black et al., 1986; de Greef, 1992; Bikker, 1994; Weis et al., 2004), reflecting an increase in minL/P with live bodyweight. However, in growing animals, energy intake level and bodyweight are usually confounded. When comparing growing pigs at similar levels of energy intake, but at different live bodyweights, the effect of live bodyweight on L to P ratios appears not significant (Weis et al., 2004). This suggests that, at least in growing pigs and when energy intake limits the expression of PDmax, minL/P increases with live bodyweight because animals eat more with increasing bodyweight; not because of increases in live bodyweight *per se*. This principle simplifies substantially the characterization of animal types in aspects of nutrient partitioning, but needs further verification, especially in species other than pigs.

In growing pigs there appears to be considerable variation in the relationship between energy intake and PD. The data presented in fig. 6 indicate that different amounts of energy intake are required just to reach PDmax in different pig genotypes and that there is no clear relationship between PDmax and the amount of energy required to reach PDmax across pig genotypes (Möhn and de Lange, 1998). Apparently, pig genotypes vary in the amount of 'essential' lipid that pigs need with increasing PD, or in minL/P at similar energy intake levels. As PDmax continues to increase, via genetic selection, energy intake capacity is more likely

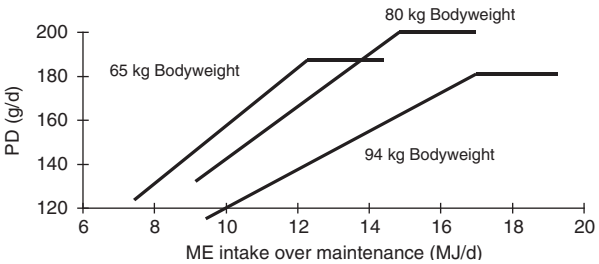

Fig. 5. Relationship between metabolizable energy (ME) intake and whole-body protein deposition (PD) at various bodyweights in a defined population of pigs. (Derived from Quiniou et al., 1995, with permission of the American Society of Animal Science.)

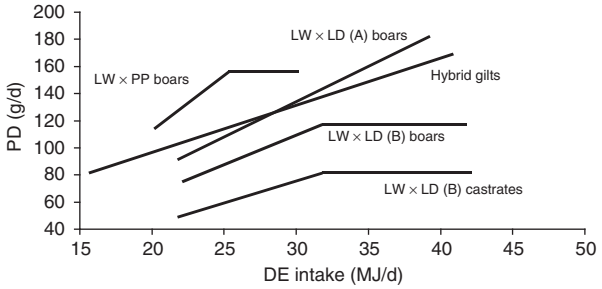

Fig. 6. Relationship between digestible energy (DE) intake and whole-body protein deposition (PD) in various pig genotypes; derived from Bikker (1994; hybrid gilts), Campbell and Taverner (1988; three types of Large White × Landrace crosses; LW × LD), Quiniou et al. (1995; Large White × Pietrain cross; LW × PP).

to limit the expression of PDmax. This increases the need to better understand and characterize the relationship between energy intake and PD in different species and genotypes (Black and de Lange, 1995). Knowledge of whether PD in growing animals is determined by energy intake or PDmax is critical to understand the animal's response to changes in feeding levels and for the development of effective cross-breeding strategies. For example, based on simple additivity of PDmax and minL/P values, crossing two lines of animals that have similar PD at the same level of energy intake, but that differ in PDmax and minL/P, will result in cross-bred animals that will outperform both of the parent lines at the same level of energy intake (fig. 7; feeding level 90% of NRC). This "heterosis" in PD can be attributed to complementarity between the two underlying traits PDmax and minL/P.

3.3. Nutrient retention support costs

Nutrient retention support costs represent the marginal inefficiency of using nutrient intake over and above basal nutrient needs for PD and LD. Part of this inefficiency can be attributed

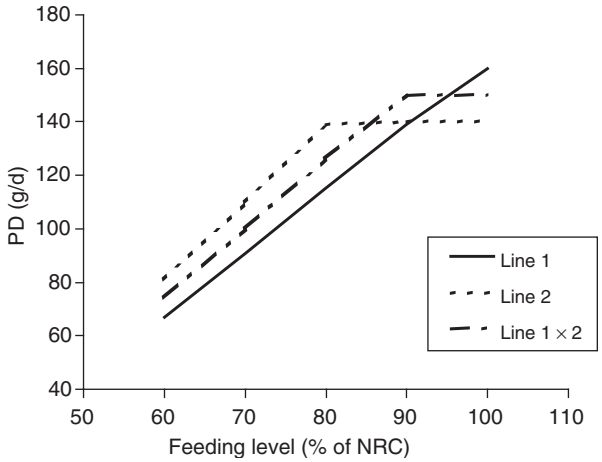

Fig. 7. Generalized relationship between energy intake, expressed as a percentage of voluntary feed according to NRC (1998)(% of NRC), and whole-body protein deposition (PD) in two lines of growing animals and cross-bred animals derived by crossing the two pure lines.

to incomplete digestion of ingested nutrients, while the main contributors to the postabsorptive nutrient utilization efficiency are inevitable catabolism of dietary essential nutrients and energy needs at the tissue level, in the form of ATP, to support LD and PD (Schultz, 1978; Gill et al., 1984; Moughan, 1999). Since the different energy-yielding nutrients yield ATP with differing energetic efficiency, diet nutrient composition has a direct effect on nutrient retention support costs.

Nutrient retention support costs have been discussed in detail elsewhere (Schultz, 1978; Gill et al., 1984; Moughan, 1999; Birkett and de Lange, 2001a, 2001b; Lobley and Lapierre, 2003) and have been estimated with reasonable accuracy (ARC, 1980, 1981; NRC, 1994, 1996, 1998). However, effects of animal genotype within species on these efficiencies have not been explored extensively. It is generally assumed that nutrient retention support costs are not influenced by animal genotype (Pym, 1990). This is supported by the similar growth response in fast- and slow-growing broiler chickens fed lysine-limiting diets (fig. 2; Hahn and Baker, 1991). Moreover, Marks (1985) could not establish changes in animal physiology and biology in Japanese quail that were selected for live bodyweight over 60 generations. This implies that differences in nutrient needs across animal genotypes are attributed primarily to differences in basal nutrient needs and nutrient retention rates.

Within this context, an interesting observation is that the marginal efficiency of amino acid utilization for PD is enhanced in growing pigs that are treated with porcine somatotrophin (PST), which effectively enhances PDmax (Krick et al., 1993). This observation appears consistent with the observed reduction in inevitable amino acid catabolism when growing pigs are fed diets that are substantially limiting in either lysine or threonine (Möhn et al., 1999; de Lange et al., 2001). Apparently, inevitable amino acid catabolism is a passive process that is driven largely by plasma amino acid levels (Lobley and Lapierre, 2003). Exogenous PST appears to widen the gap between achieved PD and PDmax, increasing amino acid uptake by peripheral tissue for PD and effectively reducing plasma amino acid levels and the extent of inevitable amino acid catabolism. These observations suggest that animals with improved PDmax may have lower minimal plus inevitable amino acid catabolism rates and thus reduced amino acid support costs for PD. This needs further exploring.

3.4. Voluntary feed intake

Within animal species there is considerable variation in voluntary feed intake between groups of animals. This variation may be attributed to the genetic make-up of the animals, diet characteristics or environmental conditions (NRC, 1987; Forbes et al., 1989). As mentioned in the previous section, energy or feed intake is more likely to limit the expression of PDmax when PDmax or lean growth potentials in growing animals continue to increase. It will thus become increasingly important to maintain high levels of energy (feed) intake, both in breeding programs and under commercial conditions.

Given the medium to high heritability of feed intake and the genetic correlation with other traits such as live bodyweight gain, feed efficiency and carcass fat content, genetic selection has had substantial impact on voluntary feed intake (Forbes et al., 1989). In selection strategies voluntary feed intake may be either increased or decreased without direct selection for feed intake, given the positive genetic correlation between voluntary feed intake and live bodyweight gain or the negative correlation between voluntary feed intake and feed efficiency or carcass leanness.

The initiation and cessation of feeding and voluntary feed intake is ultimately under hormonal and neural control. In particular, the satiety and hunger centers in the hypothalamus

play an important role in the regulation of feed intake (e.g. Forbes et al., 1989). This is illustrated by recent experiments where leptin was injected into the brain, resulting in reduced feed intake and increased secretion of growth hormone (Barb et al., 1998). However, the physiological control mechanisms involved in the short- and long-term regulation of feed intake are still poorly understood (e.g. Forbes et al., 1989; Kyriazakis and Emmans, 1999). A pragmatic approach to understanding *ad libitum* feed intake in different groups of animals is to recognize that voluntary feed intake is driven by the animal's requirements for nutrients, and that feed intake is reduced because of various constraints imposed on the animal (Black et al., 1986; Kyriazakis and Emmans, 1999; fig. 8). These constraints relate to diet characteristics (bulk density, nature and rate of digestion of fiber, water-holding capacity, nutrient and antinutrient contents), environment (thermal, social, physical, presence of disease-causing organisms) and the animal's physical capacity to ingest feed. Important first steps in characterizing voluntary feed intake in different groups of animals are to determine PD and LD in a "non-limiting" environment (e.g. Ferguson and Gous, 1993), and the physical capacity to ingest feed (e.g. Black, 1995) at the various bodyweight ranges and in various genotypes. Based on this approach, either energy or the first limiting essential nutrient may drive the animal's desired nutrient intake, while the animal's physical feed intake capacity, or gut fill, may prevent the animal from achieving its desired nutrient intake.

According to Black (1995), the pig's physical capacity to ingest feed (gut fill) is likely to limit performance in growing pigs up to approximately 40 kg bodyweight. In these pigs, an increase in the nutrient density of the diet will thus not affect feed intake but it will increase the daily nutrient intake. At body weights higher than approximately 40 kg bodyweight, the daily nutrient intake is more likely to determine feed intake; in this situation pigs tend to compensate for changes in diet nutrient content with changes in feed intake in such a manner that the daily nutrient intake is constant.

In growing pigs, gut fill and physical feed intake capacity have been expressed in units of feed dry matter intake (Black et al., 1986) or units of indigestible feed dry matter intake (Whittemore, 1983). Even though these approaches yield similar estimates of feed intake, both approaches fail to recognize the impact of the dietary fat (Revell and Williams, 1993) and

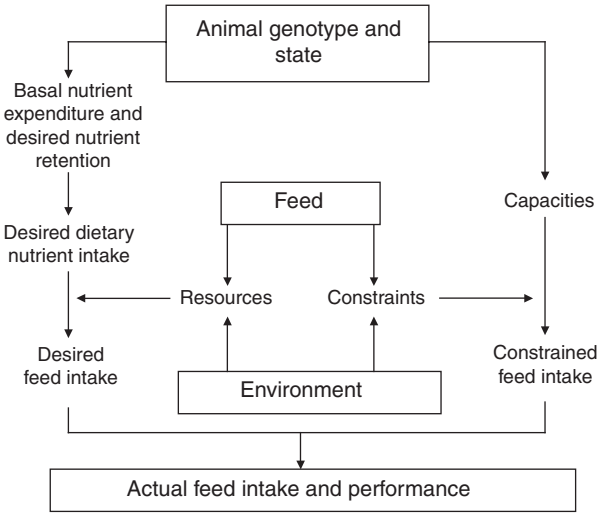

Fig. 8. Schematic representation of voluntary feed intake control in growing animals.

dietary fiber (Eastwood et al., 1983; Kyriazakis and Emmans, 1995) on voluntary feed intake. In cattle, dietary neutral detergent fiber content appears a reasonable descriptor of gut fill and physical feed intake capacity (Mertens, 1987). However in growing pigs, for a series of diets with extreme ingredient compositions, diet water-holding capacity appeared a better predictor of voluntary feed intake than dry matter, crude fiber or neutral detergent fiber content (Kyriazakis and Emmans, 1995). Moreover, the addition of a combination of xylanase, glucanase and cellulase to wheat-based diets largely eliminated the substantial effect of wheat variety on feed intake in young pigs (Choct et al., 1999). Clearly, more effort is required to characterize the dietary constituent that contributes to gut fill and physical feed intake capacity and to determine animal genotype and environmental effects on physical feed intake capacity in individual groups of animals.

4. GENETIC MODIFICATION OF FARM ANIMALS AND NUTRIENT UTILIZATION

Modifying the animal's genome provides a powerful means to alter the nutrient requirements and response to nutrient intake of farm animals. Targeted genetic alterations may relate to improved digestive capacity, metabolism of specific nutrients and antinutrients, endogenous synthesis of specific essential nutrients, or improved nutrient retention rates. An example of altered digestive capacity in farm animals is Enviropig™ (Golovan et al., 2001). A gene that controls the production of a phytase enzyme and that has been isolated from bacteria was combined with a salivary protein promoter gene and inserted into pig embryos. This gene is expressed in salivary glands of Enviropig™ and allows these pigs to efficiently use phytic acid-bound phosphors in plant feed ingredients (table 4). As a result, the total dietary phosphorus requirements of these pigs fed plant material-based diets are reduced substantially, while excretion of phosphorus with swine manure into the environment is reduced by up to 75%. In a similar manner, the capacity of monogastric animals to digest dietary fiber, particularly cellulose, or to inactivate antinutritional factors in feed ingredients may be enhanced. The latter is likely to enhance the utilization of nutrients other than dietary fiber as well, since fiber and antinutritional factors often reduce the digestive utilization of dietary protein, fat, starch and even selected vitamins and minerals.

Farm animals cannot metabolize some of the nutrients that are absorbed from the digestive tract. Examples include the pentose monomer sugars: xylose and arabinose. These sugars are components of arabinoxylans in cereal grains, such as wheat and corn, and can be released in the digestive tract of monogastric animals when exogenous xylanases are added to the diet (Choct et al., 1999). Once absorbed xylose and arabinose are mostly excreted in the urine, and

Table 4

True phosphorus digestibility (mean ± standard error) in conventional pigs and transgenic Enviropigs™ fed soybean meal as the only phosphorus source[a]

Pigs	Conventional	Transgenic
Weanling	48.5 ± 5.4	87.9 ± 3.4
Growing-finishing	51.9 ± 10.3	98.8 ± 3.4

[a]Derived from Golovan et al. (2001); digestibility was determined by a regression analysis technique.

yield no metabolizable energy to pigs and poultry (Schutte, 1991). Genes controlling the production of xylose and arabinose metabolizing enzymes may thus be isolated from rumen microbes and incorporated into DNA of farm animals.

Farm animals often lack only one or few enzymes that are required for the synthesis of nutrients that are considered dietary essential. Examples are the lack of the ω3 and ω6 fatty acid desaturase enzymes, which are required for the synthesis of ω3 and ω6 families of polyunsaturated fatty acids (Mayes, 1993). For the synthesis of nutritionally essential amino acids from appropriate intermediates between six and 13 enzymes are required, while only 1–3 enzymes are required to synthesize nutritionally nonessential amino acids (Rodwell, 1993). These enzymes exist in plants, fish or microbes and genes from these organisms could be transferred to farm animals and thus eliminate the need to include some of the essential nutrients in animal diets.

Obviously, the public acceptance of products derived from genetically modified farm animals requires careful consideration and the risk to consumers of consuming these foods needs to be assessed scientifically.

5. FUTURE PERSPECTIVES

Based on quantitative and marker assist genetic selection, improvements will continue to be made in growth potential and carcass characteristics of farm animals. As a result the animals' growth response to nutrient intake will continue to change as well. The latter can be attributed to between animal variation in: (1) maintenance energy and nutrient needs; (2) whole-body nutrient retention; (3) nutrient retention support costs; and (4) voluntary feed intake. In terms of nutrient retention, special consideration should be given to whole-body protein deposition, the proportion of whole-body protein present in edible muscle, or lean tissue, and the partitioning of retained energy between whole-body protein and whole-body lipid. Animal, feed and environmental factors all influence these aspects of nutrient partitioning. Quantification of the main aspects of nutrient partitioning for growth in different animal types will become increasingly important for estimation of nutrient requirements, for assessing the economic impact of altering feeding strategies in commercial animal production, and for the development of effective animal breeding strategies. Targeted alterations of the animal's genome will provide a means to alter the nutrient requirements and response to nutrient intake of farm animals as well.

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19 Manipulation and characterization of the rumen ecosystem through biotechnology

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Many of the biotechnological approaches originally employed in the rumen focused on direct genetic modification of microbes, in an effort to enhance processes such as cell wall digestion, toxin degradation and the balance of nutrients delivered to the ruminant small intestine. Although some progress was made using these approaches, the failure to establish a regulatory policy anchored in science has precluded their release into commercial livestock systems. To date, the most significant contribution of biotechnology has been to define the diversity, dynamics and evolution of the ruminal ecosystem in a manner that was previously impossible using traditional culture techniques. Cloning techniques have identified myriad genes encoding a variety of unique enzyme activities, many of which may have potential applications in livestock production and industry. Novel bacteriocins and other antimicrobial agents have also been identified, and the rumen may prove to be a rich source of novel antibiotics. Undoubtedly, gene discovery will continue to accelerate as genome sequencing of cultivated rumen bacteria continues and uncultivated genomes become available with the production of metagenome libraries. Such approaches may provide detailed knowledge of ruminal enzyme systems, the absence of which has hampered rumen scientists in solving age-old challenges such as improving the digestion of low-quality forages and the efficiency of ruminal nitrogen utilization.

1. INTRODUCTION: MEMBERS OF THE RUMINAL ECOSYSTEM

The microflora of the rumen is exceedingly diverse and contains representatives of all three domains, Eucarya, Archaea and Bacteria. Bacteria are responsible for the majority of feed digestion in the rumen and are by far the most numerous of these organisms, at 10^{10}–10^{11} cells/g

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of digesta. While over 200 species of bacteria have been isolated from the rumen, cloning of 16S genes from the rumen has revealed that these represent less than 15% of the diversity of the rumen bacterial population. Of these cultivated species, only about 20 occur in numbers exceeding 10^7 per gram of digesta. Other cultivated species form a small part of the total population, but the relative importance of a microbial species cannot be defined on population density alone, as complex microbial consortia are required for efficient feed digestion (McAllister et al., 1994).

Protozoa are present in the rumen at 10^3–10^6 cells/ml of ruminal fluid, with over 100 species identified that represent over 25 genera. Protozoa are thought to be responsible for one-quarter to one-third of the fiber digestion in the rumen (Williams and Coleman, 1988). Although protozoa are often associated with ruminal fluid, large numbers may also attach to the surface of feed particles (fig. 1). As a result of predation by protozoa on ruminal bacteria, the number of bacteria in the rumen fluctuates inversely with the number of protozoa (Teather et al., 1984). Ruminants can survive, and in fact prosper, without any protozoa in the rumen (Lindsay and Hogan, 1972), and protozoa may reduce the availability of dietary and microbial protein to the animal (Leng and Nolan, 1984; Ushida et al., 1986). Protozoa are thought to have a symbiotic relationship with ruminal methanogens, as the hydrogenosomes of the protozoa produce hydrogen that is used by methanogens to reduce carbon dioxide to methane (Finlay et al., 1994). Considerable effort has been devoted to the development of technologies to eliminate or alter the species composition of the rumen protozoal population, with the goals of improving ruminal protein metabolism and reducing methane production. Although several compounds show promise as selective defaunation agents (Hristov et al., 2003), none are presently routinely employed at a production level.

Ruminal fungi exhibit the most complex life cycle of all ruminal microorganisms, alternating between a motile zoospore stage and a sessile vegetative stage (fig. 2). Zoospores occur in relatively low numbers (10^3–10^4/ml of ruminal fluid), but when one considers the vegetative stage,

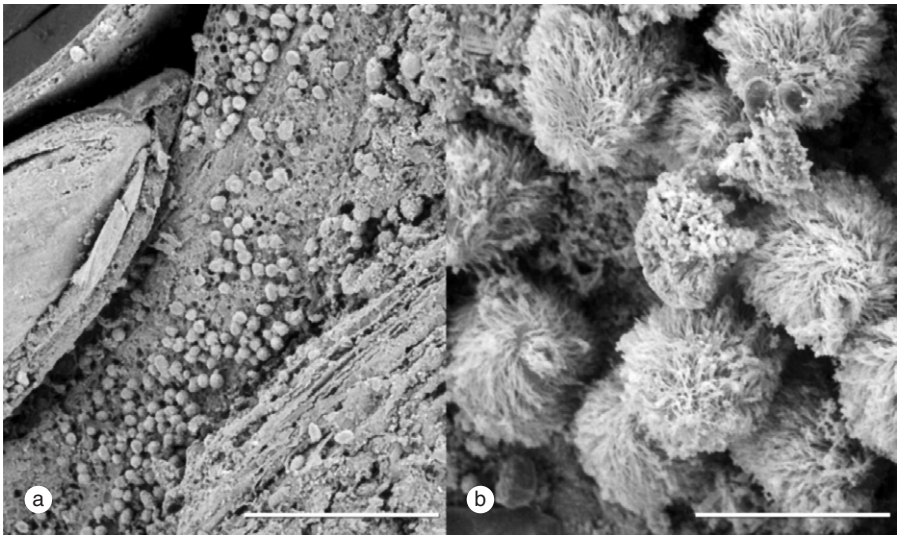

Fig. 1. Scanning electron micrographs of ground corn incubated for 30 min in the rumen of a cow fed an early lactation diet. (a) Colonization of the endosperm by numerous protozoa is evident. (b) Higher magnification reveals that the protozoa are heavily ciliated. Bar in (a) = 600 μm; (b) = 50 μm. (McAllister and Wang, unpublished data.)

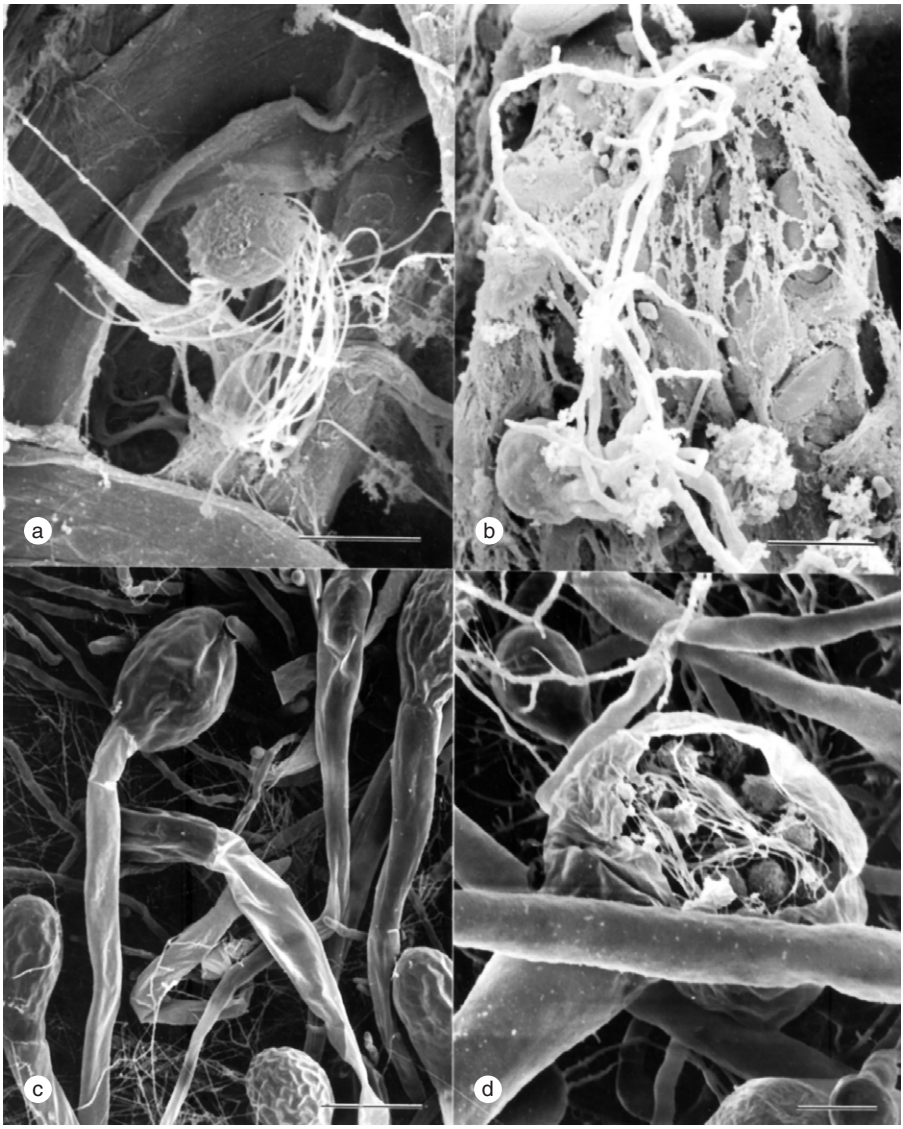

Fig. 2. Scanning electron micrographs illustrating the various stages of the life cycle of anaerobic ruminal fungi. (a) Multi-flagellated *Neocallimastix patriciarum* zoospore on the surface of cellulose filter paper. (b) Early developmental stage of *Piromyces communis* attached to an endosperm cell in barley. Numerous rhizoids originate from the initial site of encystment. (c) Extensive rhizomycelium formed by *P. communis* on the surface of filter paper. Numerous zoosporangia are visible in this preparation, each of which would have developed from a single zoospore in this monocentric species. (d) Initial dissolution of the wall of a mature zoosporangium from *Neocallimastix frontalis.* Numerous flagellated zoospores are visible within the zoosporangium. Bars in (a), (b), and (d) = 10 μm; bar in (c) = 50 μm. (McAllister, unpublished data.)

fungi may contribute up to 8% of the total rumen microbial biomass (Orpin, 1983). Fungi are thought to be involved in the digestion of the most recalcitrant components of plant cell walls.

Methanogens occur in the rumen at approximately 10^9 cells/g of digesta. All ruminal methanogens utilize H_2 and CO_2 and play a key role in the microbial ecosystem as scavengers of the hydrogen produced during fermentation. This prevents the accumulation of reducing equivalents, allowing digestion to proceed efficiently. In the rumen, methanogens are either free-living (Sharp et al., 1998), attached to protozoa (Vogels et al., 1980), or reside as endosymbionts inside protozoa (Finlay et al., 1994). Methanogens in general are difficult to isolate because of their fastidious growth requirements. As a result, only five species of ruminal methanogens have been successfully cultured. Early research on ruminal methanogens was aimed at recovering the metabolizable energy lost from ruminants as methane but now, because of increasing concern with global warming, they are under study with a view to controlling methane emissions from ruminants (van Nevel and Demeyer, 1995; Joblin, 1996; Mathison et al., 1998).

Although mycoplasmas were first identified over 35 years ago (Hungate, 1966), they are perhaps the least studied of the ruminal microorganisms. There are at least five distinct groups of mycoplasmas within the rumen, with numbers ranging from 10^5–10^7 cells/g of digesta. Several species of *Anaeroplasma* are bacteriolytic and specifically degrade the cell walls of Gram-negative bacteria (Joblin and Naylor, 2002). Further characterization of this fraction of mycoplasmas may provide a means of limiting the establishment of undesirable Gram-negative bacteria such as *Escherichia coli* O157:H7 within the digestive tract. Because of their resistance to antibiotics, ruminal mycoplasmas are commonly co-isolated with protozoa, fungi and methanogens (Joblin and Naylor, 2002). Ruminal mycoplasmas have also been shown to inhibit cellulolysis by fungi and by bacteria (Joblin and Naylor, 1999), but the nature of their interactions with other ruminal microorganisms remains largely unknown.

Both lytic and temperate bacteriophage have been identified within the rumen (Lockington et al., 1988; Kleive et al., 1989), but their biology and population dynamics are poorly understood. Using pulse-field gel electrophoresis (PFGE) coupled with laser densitometry, Klieve and Swain (1993) estimated that there are 10^7–10^9 bacteriophage particles/ml of ruminal fluid, but concentrations vary significantly with time of sampling and diet (Swain et al., 1996; Klieve et al., 1998). Interest in the study of rumen bacteriophage has been growing in response to recognition of phage therapy as a potential means of favorably altering ruminal ecology (Klieve and Hegarty, 1999; Klieve et al., 1999; Ambrozic et al., 2001). Tarakanov (1994) demonstrated that the *Streptococcus bovis* population in the rumen was reduced following the addition of *S. bovis*-specific bacteriophage to the diet.

Members of the microbial community within the rumen have adapted to compete under conditions of anaerobiosis, high protease activity, high turnover rates, high population density, and great diversity, and they are capable of efficiently digesting plant polymers (e.g. cellulose, hemicellulose, phytate) embedded in a biochemically and structurally complex matrix. Consequently, the ruminal ecosystem represents a unique source of genes coding for hydrolytic enzymes (i.e., cellulases, hemicellulases, proteases, phytases) and antimicrobial agents (i.e., bacteriophage, bacteriocins).

2. DEFINING MICROBIAL DIVERSITY USING MOLECULAR TECHNIQUES

Traditional methods for estimating microbial diversity and changes in rumen microbial populations have relied almost exclusively on the ability to culture microorganisms in the laboratory (Mackie et al., 1998). Current representations of ruminal ecology are based on

those microorganisms that allow culture-based enumeration and characterization (e.g. substrate utilization, fermentation products, etc.). However, it has commonly been found that only 10–15% of the bacteria observed by direct microscopic examination of ruminal fluid can be grown in the laboratory using traditional anaerobic plating techniques.

Unlike traditional, culture-based identification methods, molecular characterization techniques enable differentiation of strains on the basis of genotype rather than phenotype, and can also be applied to organisms that cannot be grown in the laboratory. Many species of rumen bacteria that were isolated and classified as a single bacterial species on a phenotypic basis have been determined subsequently to be genetically diverse when assessed using molecular tools. For instance, it has been recognized that many cultivated strains identified as *Butyrivibrio* are not closely related to the type strain of this genus (Forster et al., 1996; Kopecny et al., 2002). These problems and emerging techniques have resulted in many researchers developing alternative ways of describing microbial diversity in the rumen.

A variety of nucleic acid-based approaches to studying ruminal ecology are presently employed, but one of the first was based on using a 1.9-kb labeled fragment of genomic DNA to monitor the fate of a laboratory strain of *Prevotella (Bacteroides) bryantii* introduced into the rumen (Attwood et al., 1988). The strain had a half-life of only 30 min in the rumen, but the work did demonstrate the ability of this technique to track the fate of a single bacterial species within the complexity of the ruminal ecosystem. Subsequent to these early studies (Attwood et al., 1988; Stahl et al., 1988), DNA hybridization techniques were used to assess the diversity of several cultivated species of ruminal bacteria, including *Butyrivibrio* and *Lachnospira* (Mannarelli et al., 1990), *Prevotella* (Hudman and Gregg, 1989), *Fibrobacter* (Flint et al., 1990), *Selenomonas ruminantium* (Ning et al., 1991), *Streptococcus bovis* (Whitehead and Cotta, 1993), and *Ruminococcus, Fibrobacter,* eukaryotes, Gram-positive bacteria, the *Bacteroides-Porphromonas-Prevotella* group and the anaerobic ruminal fungi (Krause et al., 2000).

In more recent years, 16S rRNA gene sequence information has been used to characterize the diversity of microorganisms within the ruminal ecosystem. Unlike methods based on organism-specific gene sequences, rRNA-based methods have been developed on the basis of bacterial phylogeny. Consequently, their specificity is more appropriate for the evaluation of taxonomic diversity.

The determination of 16S rRNA gene sequence and subsequent phylogenetic analysis is becoming a common tool in many laboratories for identifying isolates to the genus, species and under some circumstances, even the strain-specific level (Stahl and Amann, 1991; Chen and Weimer, 2001). More significantly, methods based on the direct amplification of 16S rRNA genes from ruminal samples using the polymerase chain reaction (PCR) have been applied in evaluating diversity and structure in the rumen microbial community. The most commonly applied technique is to isolate DNA from ruminal contents, amplify the 16S rRNA gene using primers based on highly conserved regions of the 16S rRNA gene, clone the amplification products and sequence the inserts. The sequences are then subjected to multiple sequence alignment and phylogenetic analysis. The advantage of this approach is that it is cultivation-independent and produces primary sequence data that can be compared to known microbial isolates. As well, the sequence information that is obtained can be used to design new 16S rRNA probes for competitive or real-time PCR assays that can be used to investigate the numbers and activities of uncultivated organisms.

Such 16S rRNA gene cloning experiments have shown that as little as 6–7% of the cloned sequences represent cultivated organisms with known 16S rRNA sequences

(Whitford et al., 1998; Tajima et al., 1999). Some of the new sequences may represent organisms that have been cultivated and placed in culture collections but never sequenced, perhaps because they are phenotypically similar to organisms that have been sequenced, but this is nonetheless a stunning demonstration of the diversity found within the rumen, and certainly an illustration of the inadequacy of culture- and phenotype-based approaches for examining diversity. The functional properties of these newly recognized ruminal bacteria is a matter of conjecture and will undoubtedly be the subject of future investigations.

In contrast to studies of ruminal bacteria, relatively few nucleic acid-based studies have been conducted to characterize rumen protozoal or fungal populations. Comparative sequence analysis of 18S rRNA has been used in classifying ruminal protozoa and fungi. *Polyplastron multivesiculatum* and *Dasytricha ruminantium* were both classified among the members of hydrogenosome protozoa using this procedure (Embley et al., 1995). Although development of signature probes for *Entodinium caudatum*, *Epidinium caudatum* and *P. mutivesiculatum* has been explored (Wright et al., 1997), this technique has not been widely used in ecological studies. Similar to protozoa, comparative sequence analysis of 18S rRNA was used to confirm anaerobic ruminal fungi as members of the Chytridomycetes (Doré and Stahl, 1991), but did not allow differentiation between members within this group. Restriction fragment length polymorphisms (RFLP) of the ribosomal large subunit gene (Hausner et al., 2000) and ribosomal intergenic (ITS1) sequence (Brookman et al., 2000) were far more effective for differentiating between *Neocallimastix* and *Piromyces*. These studies clearly demonstrated that molecular classification tools are pivotal to the establishment of fully functional classification systems for ruminal protozoa and fungi.

Molecular methods are also providing new information on archaeal populations in the rumen, and it is now apparent that these are more diverse than previously believed. A phylogenetic analysis of cloned 16S rDNA sequences obtained from the bovine rumen showed the presence of two subclusters of *Methanobrevibacter* spp., a cluster of *Methanosphaera* spp., and a cluster of *Methanosarcina* spp. different from *Ms. barkeri* (Whitford et al., 2001b). A similar analysis has shown that the ovine rumen contained *Methanomicrobium mobile* and a range of *Methanobrevibacter* spp. (Yanagita et al., 2000). As well, novel archaeic sequences similar to those of *Thermoplasma* spp. and unrelated to those of known methanogens have been found in DNA from the bovine rumen (Tajima et al., 2001b).

Denaturing gradient gel electrophoresis (DGGE) is another approach that has been used to identify bacterial diversity in pure cultures of fibrolytic bacteria (Cann et al., 1996) as well as in ruminal contents collected from steers fed different diets (Kocherginskaya et al., 2001). This procedure allows DNA fragments of the same length but different sequence to be separated on the basis of differences in their migration within polyacrylamide gels containing a linear gradient of denaturant. Cann et al. (1996) used the technique to characterize the sequence diversity of the V3 region of the 16S rRNA gene from *Ruminococcus albus*, *R. flavefaciens* and *Fibrobacter succinogenes*. The V3 region was highly conserved in nine strains of *R. flavefaciens*, but exhibited a high degree of diversity and phylogenetic heterogeneity in nine strains of *R. albus*. Mixtures of the PCR-amplified V3 region from strains of *F. succinogenes*, *R. albus* and *R. flavefaciens* were easily resolved in DGGE gels. Application of this technique to total genomic DNA from samples collected *in vivo* demonstrated that DGGE profiles differed between animals fed corn-based vs. forage-based diets and that profiles also differed among animals fed corn-based diets (Kocherginskaya et al., 2001). The high degree of diversity demonstrated in these studies supports electron microscopic observations showing that the types of bacteria involved in digestion may differ even between feeds with similar chemical compositions, such as corn and barley (McAllister et al., 1990b).

3. MOLECULAR CHARACTERIZATION AND QUANTIFICATION OF RUMEN MICROBIAL POPULATIONS

The traditional methods used to enumerate ruminal bacteria are to culture samples on semi-defined media, and count, purify and characterize each colony using an array of techniques including microscopy, substrate utilization and fermentation product assays, enzyme production, and membrane fatty acid analysis. These methods are inaccurate and cumbersome due to the failure of most of the population to grow, the large number of colonies required to be picked to attain statistical significance, and the poor discrimination offered by conventional characterization methods. Therefore, investigators have looked for new techniques to evaluate populations of microbes in gastrointestinal environments.

Primers and probes based on the 16S rRNA gene sequence have been developed as an aid in detecting and quantitating a variety of ruminal microorganisms. One of the first cultivation-independent studies of ruminal ecology used 16S rRNA probes targeting *Fibrobacter* and *Lachnospira* spp. (Stahl et al., 1988) and demonstrated how this technique could be used to track changes in rumen bacterial populations when the diet was modified with monensin. Consequently, 16S rRNA probes were designed for a large number of cultivated ruminal bacteria, including *Ruminococcus* (Krause et al., 1999), *Butyrivibrio* (Forster et al., 1997), *Prevotella* (Avgustin et al., 1994) and methanogens (Lin et al., 1997). Such probes have been used in a semiquantitative format to estimate numbers of the fiber-degrading bacterium *Ruminococcus albus* SY3 (Krause et al., 2000). Dilutions of DNA from a known number of *R. albus* cells were serially diluted and amplified with the SY3-specific PCR probe to act as a standard curve for quantitation of cells in ruminal samples. However, the assay was only semiquantitative because it measured the amount of PCR product at the end of the assay.

There are several factors that seriously limit the ability of the 16S rRNA probe technique to accurately quantify bacteria in environmental samples. Significant error is introduced as a result of variation in RNA binding on nylon membranes and signal quantitation (Raskin et al., 1997). More importantly, it has been noted that the intensity of signal obtained from perfectly matching target sequences can vary between different bacterial species (Forster et al., 1999). This phenomenon was especially evident in the large variation in the intensity of the signal observed when a Gram-positive probe was employed on bacterial 16S rRNA obtained from the bovine rumen (MacGregor et al., 2001). Thus, 16S rRNA probe hybridization may be more appropriately applied when a qualitative assessment of signal is needed, such as in fluorescent *in situ* hybridization (FISH). Use of the FISH technique with ruminal samples has been limited by background autofluorescence problems (Forster et al., 2000). Employment of FISH in conjunction with flow cytometry (Rigottier-Gois et al., 2003) may alleviate this difficulty.

Competitive PCR (CPCR) assays based on 16S rRNA or other sequences have proven useful for determining the level of a target organism within a population. These assays estimate the number of copies of a target DNA sequence in relation to the amplification of an internal control sequence, the concentration of which is known. The amplification of the target sequence is inversely proportional to the amount of competitive template added to the reaction. To be quantitative, the internal standard must be amplified against a range of samples of known concentration to generate the standard curves, from which the concentration of an unknown sample can be derived. The internal control can be designed by slightly modifying the target sequence, by either the deletion or insertion of a short sequence between the specific primer sites. Because the internal control acts as a competitive template, it must be designed so that its amplification efficiency is as similar to the target as possible, but still allows for its differentiation from the target DNA.

In recent years, CPCR assays have been developed for a number of ruminal bacteria, including strains of *Butyrivibrio*, *Clostridium proteoclasticum, Ruminococcus, Fibrobacter, Prevotella*, *Streptococcus* and *Eubacterium* (Reilly and Attwood, 1998; Koike and Kobayashi, 2001; Mrazek and Kopency, 2001; Reilly et al., 2002). Koike et al. (2003) recently used this technique to quantify the attachment of cellulolytic bacteria to plant fragments in the rumen. Currently many more CPCR assays are being developed, including assays for higher taxonomic groups of ruminal bacteria, such as the Gram-positive bacteria and the *Prevotella-Bacteroides* group, and for groups defined solely on the basis of cloned sequences.

Competitive PCR is still subject to the same biases that affect all PCR reactions that are used to study microbial communities. These include unequal isolation of template DNA due to variations in cell lysis, the presence of PCR inhibitors, differential amplification and the formation of artefact or chimeric PCR products. As well, CPCR assays are technically demanding to design, and significant time is required to scan and analyze the results.

Real-time PCR assays are quickly becoming the standard for molecular quantification of genes and for gene expression analysis. In the simplest form of this assay, the amplified target sequence is detected in real-time as the PCR reaction proceeds using a fluorescent intercalating dye (commonly SYBR Green®). In the more robust 5′-nuclease assay, a specific probe is used to hybridize to the amplified target. The oligonucleotide probe is labeled with a reporter fluorophore on the 5′ end and a quenching molecule on the 3′ end. The reporter fluorophore is released by the nuclease activity of the polymerase during each round of amplification and is subsequently detected in proportion to the number of amplified molecules. The 5′ nuclease assay is more sensitive than SYBR Green assays if primer dimers or nonspecific amplification is a problem. Real-time PCR assays have been shown to be quicker and easier to perform than 16S rRNA hybridization, are very specific and sensitive, and have low coefficients of variation (Malinen et al., 2003).

A number of specific PCR primers for ruminal bacteria have been designed for use in SYBR Green real-time PCR assays (Tajima et al., 2001a). As well, 5′-nuclease assays have been developed for *Megasphaera elsdenii* (Ouwerkerk et al., 2002), *Streptococcus bovis* and *Butyrivibrio* spp. (Klieve et al., 2003). The latter researchers used specific assays to track a probiotic *M. elsdenii* inoculant during transition of cattle from a high-forage to a high-grain diet. The study demonstrated establishment of *M. elsdenii* in the rumen and in addition, successfully detected microbial changes associated with clinical acidosis in one animal. Development of a more comprehensive set of assays for the major groups of ruminal bacteria will allow accurate and statistically valid monitoring of changes in rumen microbial ecology.

4. MOLECULAR TECHNIQUES FOR ASSESSING GENE FUNCTION

4.1. Differential display RT-PCR

Differential display reverse transcriptase PCR (RT-PCR) has proven to be a powerful tool in assessing the impact of environmental stimuli (e.g. acid, stress, starvation, substrate type) on gene expression in pathogenic bacteria (Liang, 2002). Despite the frequent use of this procedure in the biomedical research community, there appears to be only one report of its use in ruminal ecology. Larson and Morrison (1999) used the procedure to study differential gene expression in *R. albus* grown in the presence of phenylacetic and phenylpropionic acid, compounds that have been shown to stimulate production of cellulases and enhance the rate of cellulose digestion (Stack and Hungate, 1984). Data from cDNA sequence analysis suggested that production of several transcripts that encoded enzymes and transport and regulatory proteins involved in cellulose digestion increased in the presence of these compounds. In addition to

determining responses of ruminal bacteria to various environmental stimuli, e.g. substrate availability, differential display RT-PCR should also have applications in other areas such as unraveling the mechanisms of cell-to-cell communication that regulate the development of microbial biofilms within the rumen.

4.2. Expression arrays

The expression of thousands of genes identified from large-scale genome-sequencing efforts can be monitored using macro- or microarrays (Lockhart et al., 1996). This technique will become very useful when whole genome sequences of ruminal bacteria become available. Expression array methodology can also be used to monitor rRNA abundance from microorganisms in the environment as well as DNA homology to known genes (Ye et al., 2001). High-density microarrays of rRNA probes have been used to monitor microbial populations in a number of environments including air (Wilson et al., 2002) and feces (Wang et al., 2002). Recently, methodology was developed to monitor rRNA from environmental samples using oligonucleotide microarrays without prior sequence amplification (El Fantroussi et al., 2003). However, detection limits for expression of individual genes using microarrays have been shown to be five times higher than conventional membrane hybridization, thereby restricting the sensitivity of this technology for detecting those genes with limited expression in environmental samples (Cho and Tiedje, 2002).

4.3. Metagenomics

The development of bacterial artificial chromosome (BAC) vectors capable of stably maintaining very large (>100 kb) DNA fragments (Asakawa et al., 1997; Zhao et al., 2000) has made it feasible to generate clone libraries that encompass the collective genome (i.e., metagenome) of complex microbial communities and allow their sequencing and analysis. Such a library is capable of defining the membership and structure of the community and providing ordered access to the community's genetic resources without isolation and cultivation of the community members. This approach has been employed to generate libraries that contain more than 10^9 basepairs of genomic DNA from soil and marine planktonic microbial communities (Beja et al., 2000; Rondon et al., 2000). Subsequent phylogenetic analysis of 16S rRNA genes in the libraries demonstrated that they contained a wide diversity of microbial phyla. Expression and screening of the libraries in *E. coli* revealed clones expressing a wide range of activities including lipase, amylase and antibacterial activities. The use of this technique to isolate novel antibiotics from soil microbial communities (Gillespie et al., 2002) further demonstrates the value of this approach. Similar approaches with the rumen metagenome would provide immediate access to the genetic resources of the community, including the complete hydrolytic enzyme complement of the rumen as well as entire metabolic pathways. Such a strategy would facilitate the discovery of new pharmaceutical agents and enzymes and aid in elucidating the mechanisms of microbe–microbe and host–microbe interactions, and the intricacies of regulation of metabolic pathways. Moreover, it would provide ordered access to the genomes of uncultivated organisms for sequencing.

4.4. Complete genome sequencing

In 2001, the North American Consortium for the Genomics of Fibrolytic Ruminal Bacteria initiated projects to sequence the complete genomes of the three principal fibrolytic bacteria, *Fibrobacter succinogenes* S85, *Ruminococcus albus* 8 and *R. flavefaciens*, and of one

proteolytic bacterium, *Prevotella ruminicola* 23. The random sequencing phase of the project for *F. succinogenes* was completed in January 2002 and closure has progressed to the point that only 14 gaps remain in the genome assembly (Morrison et al., 2003a). Despite the abundance of work that has been completed on genes coding for polysaccharide hydrolysis in *F. succinogenes*, an incredible 23 additional genes encoding xylanases and related enzymes have been identified. Sequencing of the genome of *R. albus* 8 is also proceeding but is proving to be more challenging, and a total of 200 physical gaps remain in the genome sequence (Morrison et al., 2003b). Nonetheless, a number of previously unidentified coding sequences involved in nitrogen fixation and C_1 metabolism in this organism have been identified. Undoubtedly, complete genome sequencing will provide the most comprehensive information on the genetic nature of processes such as fiber digestion and gene flow. Given the diversity of the microbial ecosystem and the still rudimentary state of the community catalog, however, it will be a considerable time before the genomes of even the most commonly cultured bacteria are fully annotated.

5. THE RUMEN AS A NOVEL SOURCE OF ENZYMES

The rumen microbial population represents a rich source of novel enzymes with tremendous potential for industrial applications, but it has not been fully exploited. The enzyme activities confirmed to exist in the rumen are diverse, encompassing those involved in plant cell wall polymer degradation (e.g. cellulases, xylanases, β-glucanases, pectinases), amylases, proteases, phytases and specific plant toxin-degrading enzymes (e.g. tannases). This variety of enzyme activities arises not only because the microbial community is phylogenetically diverse, but also because individual microorganisms produce multiple fibrolytic enzymes (Ali et al., 1995; Forsberg et al., 2000; Morrison et al., 2003a).

5.1. Fibrolytic activity

Recognition of the ruminal microbiota as a particularly promising source of superior fibrolytic enzymes is increasing, and the majority of research to date has focused in this area. Cellulases and xylanases produced by ruminal fungi are among the most active fibrolytic enzymes described thus far (Gilbert et al., 1992; Trinci et al., 1994). In attempting to determine the mechanisms of fiber digestion and to find the most effectual enzymes for industrial use, researchers have cloned a growing number of genes from ruminal bacteria, fungi and protozoa. Over 100 different genes encoding primarily enzymes with role(s) in fiber digestion have been cloned from ruminal microorganisms. Most of these are from a small group of bacterial species including *Butyrivibrio fibrisolvens, F. succinogenes, P. ruminicola, R. albus* and *R. flavefaciens* (Wallace, 1994; Selinger et al., 1996; Teather et al., 1997; Forsberg et al., 2000).

Study of the genetics of anaerobic fungi and protozoa isolated from the rumen is more recent. Nonetheless, interest in the genetics of anaerobic fungi from the rumen has been stimulated, in part, by their high fibrolytic activities capable of degrading plant cell wall polymers generally recalcitrant to bacterial fibrolytic activities (Chen et al., 1997; Li et al., 1997; Qiu et al., 2000). Sequences of at least 48 cDNAs and genes relating to fiber degradation have been determined from more than ten isolates and registered with GenBank (NCBI, 2003), including sequences coding for three acetylxylan esterases, four cellulases and seven xylanases from *Neocallimastix* species; one acetylxylan esterase, 15 cellulases, one feruloyl esterase, one lichenase, one mannanase and one xylanase from *Orpinomyces* species; and eight cellulases, three mannanases and two xylanase from *Piromyces* species.

Although ruminal protozoa do play a role in fiber digestion, the genes of these microorganisms remain largely uncharacterized. To date, nine fibrolytic genes have been cloned – four from *Epidinium ecaudatum* and five from *Polyplastron multivesiculatum* (Devillard et al., 1999; Takenaka et al., 1999). These genes were found to exhibit homology with other genes coding for fibrolytic enzymes in the rumen and in some instances with protozoal genes of nonruminal origin.

5.2. Amylase activity

Amylase activity has been widely described among the ruminal bacteria (Cotta, 1988; McAllister et al., 1990a) and fungi (Mountfort and Asher, 1988; McAllister et al., 1993). With the exception of a gene encoding an amylase from *Butyrivibrio fibrisolvens* H17c (Rumbak et al., 1991), cloning of amylases of ruminal origin has focused almost exclusively on those present in *Streptococcus bovis* (Clark et al., 1992; Cotta and Whitehead, 1993; Satoh et al., 1993). One of the genes coding for amylase from *S. bovis* 148 was used to demonstrate the value of a transformation system for the extracellular production of enzymes in the yeast starter stains *Streptococcus thermophilus* and *Lactobacillus delbrueckii* (Satoh et al., 1997).

5.3. Protease activity

Although considerable research effort has been expended towards defining ruminal nitrogen metabolism (Morrison, 2000), the first cloning and characterization of a gene involved in this process was reported only recently (i.e., a dipeptidyl peptidase IV (DPP-IV) from *Prevotella albensis* M384; Walker et al., 2003). The *P. albensis* DPP-IV shares many properties with mammalian DPP-IV, such as preferential hydrolysis of peptide bonds on the carboxyl side of proline. Notably, the activity of this enzyme increases in the presence of amino acids, and peptides are not required to induce its activity. It was concluded, therefore, that activity of DPP-IV in *P. albensis* may be coordinated with other enzymes involved in nitrogen metabolism. Further cloning and characterization of the enzymes responsible for the broad spectrum of proteolytic activities observed in the rumen may identify proteases with novel activities that have applications outside of the ruminal environment.

5.4. Phytase activity

Phosphorus pollution is a major problem in many areas of the world where intensive livestock production systems are located. Up to 90% of the phosphate in cereal grains and oilseeds is present in the form of phytate (Graf, 1986), which passes though the digestive tract intact. Thus, poultry and swine must be supplemented with inorganic phosphate, even though large amounts of phosphorus are excreted (as phytate) in their feces, with the negative consequence of contributing to eutrophication of surface waters (Van Gorcom et al., 1995). Efforts to minimize this problem have focused on using enzyme feed additives (i.e., phytases), that liberate phytate phosphorus and make it available to the nonruminant animal. Currently available commercial phytase supplements are produced using genetically modified strains of the soil fungus, *Aspergillus niger,* from which three phytase-encoding genes have been characterized. Information from these characterizations has enabled phytase production to be enhanced through gene amplification and promoter substitution (Ehrlich et al., 1993; Piddington et al., 1993; Van Hartingsveldt et al., 1993).

Phytate degradation by ruminal bacteria has been recognized for over 40 years, but a definitive assessment of the phytase activity of these microorganisms was not undertaken until the

Table 1

Phytase activity of ruminal bacteria

Phytase activity[a]	Microorganism	Number of isolates
Very strong (>125 mU/ml)	*Mitsuokella multiacidus*	1
	Selenomonas ruminantium	8
Strong (25–125 mU/ml)	*Prevotella ruminicola*	5
	S. ruminantium	16
Moderate (5–24.9 mU/ml)	*Megasphaera elsdenii*	5
	P. ruminicola	2
	S. ruminantium	18
Weak (< 5 mU/mL)	*Bacillus* sp.	1
	M. elsdenii	2
	P. ruminicola	3
	S. ruminantium	19
Negative	*Anaerovibrio lipolytica*	2
	Bacillus sp.	4
	Butyrivibrio fibrisolvens	44
	Clostridium sp.	1
	Coprococcus sp.	3
	Enterococcus sp.	4
	Eubacterium sp.	7
	Fibrobacter succinogenes	8
	Lachnospira multiparus	4
	Lactobacillus sp.	20
	M. elsdenii	7
	Peptostreptococcus sp.	1
	P. ruminicola	38
	Ruminobacter amylophilus	4
	Ruminococcus albus	6
	Ruminococcus flavefaciens	11
	S. ruminantium	2
	Staphylococcus sp.	6
	Streptococcus sp.	49
	Succinovibrio dextrisolvens	12
	Treponema sp.	11
	Unidentified	8
	Total isolates screened	**332**

Adapted from Yanke et al. (1998).

[a]One Unit = release of 1 μmol P_i from sodium phytate per minute.

late 1990s (Cheng et al., 1997; Yanke et al., 1998). This activity was detected in 24% of the 332 Lethbridge Research Centre culture collection isolates, including 61 of 63 isolates (97%) of *Selenomonas* (table 1). The only other genera in which phytase activity was common were *Megasphaera* (50% of isolates) and *Prevotella* (21%). Thirty cultures (24 *S. ruminantium* isolates, five *P. ruminicola* and one *Mitsuokella multiacidus*) exhibited over 25 mU/ml of phytase activity, where one unit was defined as release of 1 μmol of phosphorus from sodium phytate per minute. The phytase activity of nine of the isolates (eight *S. ruminantium* and one *M. multiacidus*) was more than double that measured in the other strains. In all cases, the phytase activity was cell-associated. A phytase gene and gene product from *S. ruminantium* strain JY35 have been characterized, and are found to represent a novel family of phytases characterized by a protein-tyrosine-phosphatase motif.

Subsequent to the work in Canada, a phytase-producing isolate, *Mitsuokella jalaludinii*, was isolated from the rumen of cattle in Malaysia (Lan et al., 2002b). Studies are presently underway to optimize culture conditions in order to maximize phytase production from this isolate for possible commercial applications (Lan et al., 2002a).

5.5. Enzymatic detoxification

Many plants produce astringent or toxic secondary metabolites for defense against herbivores. Ruminants consume these compounds during grazing, or when toxic plants are inadvertently consumed (Gregg, 1995). These toxins cost producers millions of dollars annually as a result of animal deaths and decreased growth performance. The most widely studied toxins include mimosine T-2 toxins, nitrotoxins, pyrrolizidine alkaloids, *trans*-aconitate and tannins. They reduce animal productivity by reducing intake, feed palatability, enzyme activity, ruminal fermentation, nutrient availability, meat and wool growth, or by inducing toxicosis (Gregg, 1995; Hammond, 1995; McMahon et al., 1999).

In many areas of the world, indigenous ruminants have evolved the ability to ingest native plants that are toxic to nonruminant animals (Jones, 1981). Often this ability can be traced to the activities of ruminal microorganisms that convert toxic ingredients to harmless or even beneficial compounds (Jones and Lowry, 1984). Well-documented examples of this phenomenon include *Leucaena* toxin resistance in ruminants in Hawaii (Hammond, 1995) and ovine resistance to pyrrolizidine alkaloids (Craig et al., 1992).

Leucaena leucocephala is a legume grown in the tropics for a variety of uses (including feed for livestock). This plant produces mimosine, a free amino acid that is toxic to nonruminants and to ruminants in which neither mimosine nor its ruminal degradation product, 3-hydroxy-4(1H)-pyridone (3,4-DHP), is degraded in the rumen (Jones, 1981). The involvement of ruminal microorganisms in mimosine detoxification was demonstrated by Jones and Lowry (1984). Mimosine-sensitive Australian goats were able to tolerate ingested *Leucaena* following an infusion of ruminal fluid from tolerant Indonesian goats. Subsequent to this work, the bacterium responsible for degrading mimosine and 3,4-DHP, *Synergistes jonesii*, was identified, which led to the development of a mixed ruminal inoculum that can be administered to susceptible cattle (Klieve et al., 2002). The advantage of this approach is that *S. jonesii* only needs to be administered to a few members of a group, as it appears to be rapidly disseminated throughout the herd (Klieve, personal communication).

To date there has been only a single example of successful employment of recombinant techniques to overcome adverse effects of a plant toxin in ruminants. Many shrubs and trees native to Australia accumulate monofluoroacetate. The LD50 of this toxin in ruminants is 0.3 mg/kg body weight (Annison et al., 1960). In contrast to mimosine and 3,4-DHP, however, indigenous bacteria capable of degrading fluoroacetate have not been isolated, thus the need existed for alternative approaches to prevent toxicosis. A transcriptional fusion between the fluoroacetate dehalogenase gene from *Moraxella* sp. strain B and the *erm* promoter from pAMβ1 was ligated to a *B. fibrisolvens/E. coli* shuttle vector (Gregg et al., 1994). Under nonselective conditions, isolates of *B. fibrisolvens* OB156 carrying the dehalogenase expression plasmid (pBHf) were able to detoxify fluoroacetate *in vitro* and this trait was maintained for up to 500 generations. A mixture of four recombinant fluoroacetate-degrading strains of *B. fibrisolvens* was subsequently introduced into sheep and maintained a combined population in the rumen of 10^6–10^7 cells/ml of ruminal fluid (Gregg et al., 1998). Sheep inoculated with the recombinant strains exhibited substantially reduced toxicological symptoms from fluoroacetate poisoning as compared to uninoculated controls.

The discovery of ruminal detoxification of plant metabolites has prompted a more detailed examination of the bacteria that effect the degradations. Enrichment and selection techniques have been utilized to isolate anaerobic bacteria able to degrade 3,4-DHP (Allison et al., 1992), tannins (McMahon et al., 1999; Odenyo et al., 2001), *trans*-aconitate (Cook et al., 1994) and nitrotoxins (Anderson et al., 1993). It is widely recognized that ruminants are less sensitive to mycotoxins than are other nonruminant livestock species, due to degradation of these toxins by ruminal bacteria (Hussein and Brasel, 2001). Continued characterization of the ruminal bacteria responsible for mycotoxin degradation, as reported by Matsushima et al. (1996), may identify target genes that can be introduced into expression systems to generate products that reduce the threat posed by these toxins to other livestock such as swine and poultry.

6. THE RUMEN AS A NOVEL SOURCE OF ANTIMICROBIAL AGENTS

The ratio of production of acetate and propionate in the rumen appears to be a primary determinant of the efficiency of feed utilization by ruminants. This A:P ratio is determined by the relative numbers of acetate- and propionate-producing bacteria in the rumen. Feeding strategies that alter the relative populations of these bacteria in favor of propionate production have been shown to increase the overall efficiency of feed conversion and to reduce the level of production of waste products such as methane. Administration of the ionophore monensin (Rumensin®) is one example of such a strategy that is widely employed in ruminant production.

6.1. Bacteriocins

Bacteriocins, which are antimicrobial proteins produced by bacteria, offer promise as a means of altering the relative numbers of acetate- and propionate-producers in the rumen (Callaway et al., 1997). Similar to ionophores, bacteriocins are membrane-active compounds that interfere with energy metabolism and transduction in microbial cells (Bruno and Montville, 1993).

Bacteriocins offer a number of potential advantages over ionophores for use in animal production. They are often very specific, which affords the possibility of improved performance through targeting more defined bacterial populations, as opposed to the broad-spectrum activity of ionophores against Gram-positive bacteria. Creation of bacteriocin variants to overcome resistance, should it develop, among target strains is straightforward, because the primary structure of bacteriocins is encoded directly by a gene. As proteins, bacteriocins are digestible by the animal, giving rise to no residues. A number of bacteriocins have established a history of safe use in the food industry, particularly in dairy processing (Delves-Broughton et al., 1996) and because bacteriocins are not used in the treatment of disease in humans, their use in animal production is not expected to be restricted by regulatory requirements.

A large number of bacteriocin-like inhibitory substances (BLIS) of ruminal origin have so far been identified (Kalmokoff et al., 1997, 1999; Pattnaik et al., 2001; Whitford et al., 2001a; Mantovani et al., 2002). Twenty-five of 49 *B. fibrisolvens* isolates examined were found to produce BLIS (Kalmokoff et al., 1997), and BLIS-like activity was readily apparent in 13 of 16 isolates of *B. fibrisolvens* in a deferred antagonism assay using *B. fibrisolvens* OB156 as the indicator strain (fig. 3). Similarly, approximately 20% of 35 strains of *S. bovis* produced BLIS (Whitford et al., 2001a), as did 5% of *Ruminococcus* spp. isolates (Whitford and Teather, unpublished data). In general, the incidence of BLIS production among ruminal bacteria examined to date is higher than that observed in most other microbial environments, which may reflect the competitive nature of the ruminal environment. Several of the bacteriocins

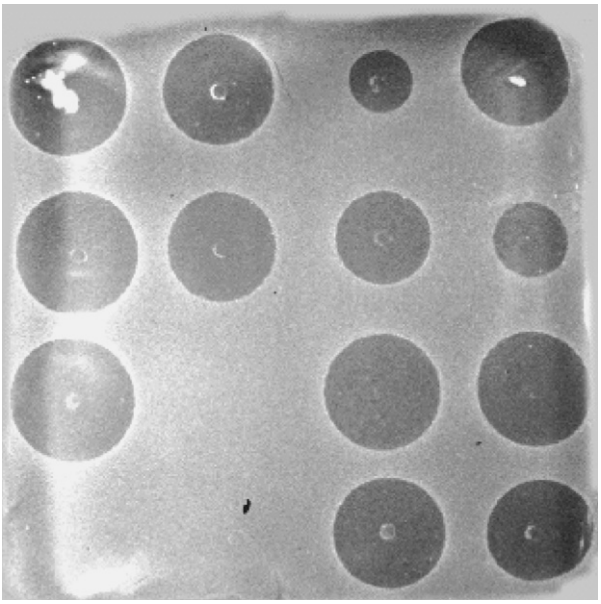

Fig. 3. Deferred antagonism assay demonstrating activity against the indicator strain, *Butyrivibrio fibrisolvens* OB156, by 13 of 16 bacteriocin-producing *Butyrivibrio* isolates.

from *B. fibrisolvens* have been cloned into other organisms and their gene structure and regulation of expression have been studied. One of the characterized bacteriocins, named butyrivibriocin AR10, was found to be a type II bacteriocin, whereas *B. fibrisolvens* OR79 produces two different type I (lantibiotic) bacteriocins that are related to previously studied bacteriocins produced by lactic acid bacteria (Kalmokoff et al., 1999).

In general, bacteriocins of ruminal origin are resistant to most gastric proteases, as might be expected for proteins that function in the ruminal environment. It appears that a number of these bacteriocins have a range of activities that mimics that of ionophores, which suggests that they could serve as effective alternatives to these antibiotics for modifying rumen microbial populations and fermentation products. For example, BLIS from *B. fibrisolvens* has been shown to inhibit the growth of obligate amino acid-fermenting bacteria in a manner similar to monensin, and as a result may have applications in the regulation of ruminal nitrogen metabolism (Rychlik and Russell, 2002). Similarly, a bacteriocin produced by *S. bovis* HC5 has been shown to be a potent inhibitor of methanogens and consequently may have applications in reducing methane production in ruminants. Other bacteriocins are more specific in their range of activity, and may allow specific species within bacterial populations to be targeted. A number of BLIS derived from ruminal bacteria also have activity against foodborne pathogens such as *Listeria* spp. and *Clostridium* spp. and consequently may have applications in food preservation (Kalmokoff et al., 1997).

6.2. Bacteriophage

Ruminal bacteriophage with activity against organisms including *Prevotella* spp. (Ambrozic et al., 2001), *S. bovis* (Klieve and Bauchop, 1991), *S. ruminantium* (Cheong and Brooker, 1998), *Propionibacterium* spp. (Cheong and Brooker, 1999) and a ruminal acetogen

(Jiang et al., 1995) have been identified and characterized. Most of the bacteriophage isolated to date exhibit a narrow host range, affecting only a few strains of a given bacterial species. Consequently, identifying bacteriophage with a host range sufficiently broad to alter genetically heterogeneous groups of bacteria such as methanogens or ammonia-producing bacteria could prove difficult. It seems more likely that bacteriophage therapy would prove fruitful for use in excluding specific pathogens from the ruminant digestive tract. For example, a single bacteriophage effectively hastened disappearance of *E. coli* O157:H7 from continuous ruminal cultures, although this approach did not reduce fecal shedding of the organism by experimentally challenged lambs (Bach et al., 2003). Because bacteriophage resistance can develop readily in bacteria (Joerger, 2003), it is likely that a mixture of phage will be necessary for effective exclusion of specific pathogens from the ruminant digestive tract.

7. APPLICATIONS FOR GENETIC MATERIAL FROM RUMINAL MICROORGANISMS

The cost of production of performance-enhancing, biologically active proteins for livestock can be reduced by using more effective expression and delivery systems (Hodgson, 1994). Genes encoding unique feed or toxin-degrading enzymes or BLIS could be used to fortify existing production systems or to create new microbial and plant systems aimed at improving the use of low-quality forage, eliminating toxins, or targeting potentially undesirable or pathogenic microorganisms.

7.1. Conventional microbial expression systems

Conventional methods for producing recombinant proteins rely on the cultivation of a few species of fungi (e.g. *Aspergillus* or *Trichoderma* spp.) and bacteria (e.g. *Bacillus subtilis*, *Bacillus licheniformis*). Single strains are inoculated into large-scale, deep-tank fermentors (>10 000 L) and the resultant culture is subjected to downstream processing (e.g. centrifugation, filtration, dehydration) to remove extraneous materials such as cells, cell fragments and/or water. The protein of interest is then processed into liquid or powdered formulations, which may include glycerol, skimmed milk powder or other stabilizing agents. Molecular strategies, such as increasing gene copy number and/or use of high-activity promoters, are being employed to enhance protein yield. For example, the phytase gene from *A. niger* has been characterized and phytase production was increased over 1400-fold through the use of the *A. niger* glucoamylase promoter and gene amplification (Ehrlich et al., 1993; Van Gorcom et al., 1995).

7.2. Transgenic plants

Recent advances in plant biotechnology promise to revolutionize the production of biologically active proteins by offering alternative, cost-effective methods for production and delivery of these products (Fischer and Emans, 2000). The existing agricultural infrastructure allows inexpensive production of large quantities of plant biomass. Expression of enzymes in plant species commonly used for animal feed will minimize downstream processing, as the whole or parts of the bioprotein-producing plants are fed directly to livestock.

At the Lethbridge Research Centre, we are combining the utility of plant expression systems with superior fibrolytic enzymes produced by ruminal fungal and bacteria. In collaboration with researchers at the University of Calgary, an oleosin (oil-body membrane

protein)-*N. patriciarum* xylanase gene construct was introduced into canola via *Agrobacterium*-mediated transformation (Liu et al., 1997) and xylanase activity was detected in crude oil-body protein preparations from transgenic seeds. Similarly, we have successfully effected expression of a β-glucanase from *F. succinogenes,* under the control of the cauliflower mosaic virus 35S promoter, in potato (Armstrong et al., 2002). Including the transgenic potato as a source of β-glucanase in barley-based diets for broiler chickens at 0.6 kg/tonne improved feed efficiency by 9%; including it at 1.2 kg/tonne reduced the viscosity of intestinal digesta by 42% (table 2).

8. CONSTRAINTS TO USING GENETIC MATERIAL FROM THE RUMEN

At this point it is still uncertain if regulatory agencies will approve the release of genetically modified microbes or plants expressing genes of ruminal origin. There is ample evidence that gene transfer takes place within the rumen (Barbosa et al., 1999; Garcia-Vallve et al., 2000) and the possibility that similar transfer events could occur with novel genes introduced into or exported from the ruminal environment warrants consideration. Concern over regulatory issues has prevented the commercialization of *B. fibrisolven* strains engineered to degrade fluoroacetate (Gregg et al., 1994). Although numerous strategies involving genetic modification of ruminal microbes have been proposed (Teather, 1985), it seems that few of these approaches will come to fruition until the regulatory issues are resolved. Numerous genetically modified crops expressing herbicide- and pest-resistant traits are currently fed to ruminants in several countries. Present data suggest that even in intact plant material, the likelihood of uptake of transgenic DNA by ruminal microbes would be minimal, given its rapid degradation upon coming into contact with ruminal nucleases (Alexander et al., 2002). Introduction, into plants, of relatively innocuous genes, such as those coding for carbohydrases, should pose minimal risk when one considers that many of these same genes are expressed by soil microorganisms, which exist in intimate contact with plants. Further studies into the fate of transgenic DNA within the rumen are required to provide the scientific information that is necessary to establish a regulatory framework.

Table 2

Effects of including untransformed or transformed[a] potato tuber in barley-based diets for broiler chicks[b] on DM intake, growth performance, and intestinal digesta viscosity

		Untransformed potato		Transformed potato		
	Control	0.06%	0.12%	0.06%	0.12%	SEM[c]
Feed intake per bird (g/d)	33.6	33.6	34.8	33.4	32.5	2.49
Body weight at d 17 (g)	291.3	286.9	313.7	309.4	295.6	24.43
Weight gain per bird (g/d)	16.5	16.1	16.1	17.9	16.8	1.80
Feed conversion (DMI/gain)	2.05^{B}	2.09^{B}	1.93^{A}	1.87^{A}	1.94^{AB}	0.13
Digesta viscosity (cP)	260.5^{B}	230.0^{B}	161.8^{A}	207.5^{AB}	152.3^{A}	23.57

Adapted from Baah et al. (2002).

[a]Transformed potato (cv. Desiree) expressed a β-glucanase from *Fibrobacter succinogenes* (Armstrong et al. 2001), and provided 30 and 60 Units of β-glucanase per kg diet, when included at 0.6 and 1.2 g/kg, respectively. Untransformed potato was cv. CDC Silky.

[b]Potato tuber replaced barley in the diets in the proportions shown (DM basis).

[c]Standard error of the mean ($n = 4$). Each block (cage) contained six chicks.

A–C: Within a row, means followed by different letters differ ($P < 0.05$).

9. FUTURE PERSPECTIVES

The rumen is the most thoroughly studied anaerobic ecosystem, but biotechnological approaches have only recently provided a glimpse into the true diversity and potential that this unique environment offers. It is apparent that the ruminal ecosystem is far more diverse than was originally surmised on the basis of traditional laboratory techniques. Developments in molecular biology have broadened our current knowledge of this environment on both an ecological and a functional level. Procedures such as real-time PCR have enabled population responses to environmental stimuli (e.g. dietary changes, antimicrobial agents) to be monitored with a degree of sensitivity and precision that was previously impossible. Metagenomics and complete genome sequencing offer the promise of identifying additional genes that code for proteins with potential applications in industry, livestock production and human medicine. To date, myriad biotechnological techniques and strategies have been proposed or developed as a means of manipulating the ruminal ecosystem, but few, if any, have been implemented at a commercial level. It is imperative that a regulatory framework anchored in science be established in order that the benefits of those approaches that do not compromise food or environmental safety can be realized.

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20 Manipulation of the ecosystem of pigs through biotechnology

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Biotechnology is providing new methods for improving the food conversion efficiency of pigs and their ability to utilize specific nutrients. Such improvements will be reflected in reduced effluent production, in some cases targeted to reduction of specific wastes such as phosphorous. Novel mapping methods for identifying genes involved in variation in food conversion efficiency will ultimately lead to identification of genes, biochemical pathways and physiological processes, amenable to manipulation by selection, transgenesis or by nonheritable approaches, such as RNA interference or use of other small molecules for controlling gene expression. Taken in conjunction with improved approaches to processing and exploiting the valuable components of effluent as well as in modifying feeds, the biotechnological approach to modifying animals promises both economic and environmental benefits for pig production.

1. INTRODUCTION

There is a worldwide trend for reduction in numbers of pig producers while pig populations are generally increasing or static. The outcome is a far greater concentration of pigs and their associated wastes in larger production enterprises. Consequently, pigs are a major source of effluent and their environmental impact is of increasing concern (Jongbloed and Lenis, 1998). In Western Europe, where more than 200 million pigs are slaughtered per year, the industry is often further concentrated in geographically small countries with dense human populations like the Netherlands, further exacerbating the problem of waste disposal and its environmental impact. Jongbloed and Lenis (1998) described legislation introduced in the Netherlands to control and reduce environmental contamination by nitrogen and phosphorous from animal effluent, including requirements for detailed accounting by farmers of the production and disposal of effluent. The legislation included strict limits on the amounts of these pollutants per animal and a strategy of reducing the overall pig population in the Netherlands by up to 25% between 1998 and 2000. There is some debate about the future viability of pig farming under such conditions. Even in countries like Australia, where availability of land and open space is less of an issue, piggeries are becoming much larger and effluent disposal is an

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increasing problem. As environmental standards become more stringent throughout the world, new and better ways of managing effluent are being sought.

Numerous and frequently very effective *post hoc* methods have been developed for handling piggery effluent and minimizing environmental impact. These include effluent lagoons for trapping waste and retaining the most environmentally damaging nitrogen and phosphorous, either by assimilation into algal biomass, which sediments out in the ponds, and/or by chemical precipitation. These ponds can dramatically reduce the levels of phosphorous and nitrogen in water released from the ponds. When one considers that high-quality phosphate for fertilizer and other uses is a limited resource, with known reserves of phosphate rock unlikely to last more than 100 years at current rates of usage (Driver, 1998), reduction in phosphate use and recovery of phosphate waste will become increasingly important from the perspective of management of a diminishing resource, let alone from avoidance of pollution of groundwater, surface-water and soils. As is often the case, what is a waste from one perspective can often be a valuable resource from another. Struvite, magnesium ammonium phosphate ($MgNH_4PO_4$-$6H_2O$), tends to spontaneously precipitate from effluent wastewater and the precipitation is augmented with increasing pH and Mg concentration. This precipitation can reduce P concentrations to as low as 2 mg/l and seems particularly useful for dealing with piggery wastes. Struvite is a valuable fertilizer, which can be recovered from piggery effluent. It can provide an effective slow-release nitrogen and phosphorus fertilizer (Johnston and Richards, 2003).

However, there are other ways to minimize environmental problems caused by pig effluent. Pigs utilize only 30–35% of ingested dietary N and P (Jongbloed and Lenis, 1992) and improvement in the efficiency of digestion, either general or specific, would necessarily reduce the levels and volumes of these wastes. Use of feed additives and genetic improvement of pigs have already been shown to have great potential to reduce these wastes (table 1). Biotechnology raises alternative ways of reducing the total levels of these wastes by manipulation of the animals or their feeds. Conventional selective breeding programs have been used for many years to improve numerous traits in pigs and other agriculturally important animal species, often with very large cumulative effects accrued over time. For example, performance-based selection for improvement in food conversion efficiency has always been attractive to pig breeders, since such improvements can substantially improve profitability, even though food conversion efficiency can be a difficult and expensive trait to measure. A corollary of the improved profitability from selective breeding for food conversion efficiency is a reduction in effluent, since less feed input per unit of production will necessarily mean less waste output. Performance-based selection has been a feature of pig breeding for many years, but modern

Table 1

Trends in feed conversion efficiency (kg/kg) and average levels of excretion of P and N (kg/pig) of grower-finisher pigs from 25–110 kg in the Netherlands (adapted from Jongbloed and Lenis, 1998)

Year	Feed conversion ratio	P	N
1973	3.37	1.62	4.74
1983	3.08	1.18	4.30
1988	2.94	0.85	4.64
1992	2.86	0.77	4.46
1996	2.74	0.67	4.13

biotechnology, especially in quantitative trait locus (QTL) mapping and genomics, is now allowing us to find the locations and ultimately the identity of the genes responsible for variation in feed conversion efficiency. This knowledge can be implemented in marker-assisted breeding programs and also in furthering our understanding of the biological processes affecting feed conversion efficiency, thus assisting in the development of genetic and even nongenetic means for further improving efficiency and reducing wastes. For example, pigs could be directly treated with a gene product, discovered via gene mapping or expression analysis, such as a peptide hormone, whose expression is known to impact on food conversion efficiency. Thus, the pigs need not be genetically modified, but the gene product could be used to exert a transient favorable effect on their growth and efficiency.

Pigs and other nonruminants are incapable of using certain nutrients or forms of nutrients, which consequently pass into the waste stream and become an important source of pollution. In some cases, these nutrients must be made available in an alternative form, but at considerable expense. In one case described below, it has already been established that a specific genetic modification can be made to pigs to introduce a biochemical function from a microbial source, which enables them to utilize an otherwise unavailable nutrient.

The major feed grains like barley, oats, rye and wheat used by the pig industry can contain antinutritive factors, which interfere with nutrient assimilation and markedly reduce the efficiency of feed utilization. Biotechnology provides the means to selectively target antinutrients, either by treating the feeds or by genetically altering the animal.

Thus, attempts to modify pigs to improve efficiency and reduce waste production can be targeted at an overall general improvement of food conversion efficiency using conventional selective breeding, augmented by gene discoveries arising from QTL mapping and genomics, or can target specific aspects of nutritional efficiency or impediments to nutritional efficiency, targeting specific nutrients or antinutrients. Gene discovery also opens the possibility of transient treatments of animals with purified gene products to manipulate efficiency of production. Taken together, they provide reason for optimism about reduction of wastes and environmental impact of piggeries.

2. GENETIC IMPROVEMENT OF FOOD CONVERSION EFFICIENCY

2.1. Conventional genetic improvement

Genetic improvement can be obtained by performance-based selection of breeding stock. However, food conversion efficiency is difficult and expensive to measure, since individual feed intake must be recorded. Traditionally this has involved individual housing and thus the trait could be measured only on very limited numbers of animals. Clutter and Brascamp (1998) have reviewed published estimates of the heritability of food conversion ratio (the inverse of food conversion efficiency), ranging from 0.12–0.58 and averaging 0.30. Clearly there is substantial genetic variation for this trait but the difficulty of its measurement and the extra costs of individual housing have inhibited its widespread incorporation into selective improvement programs. For this reason, growth rate (average daily gain) is frequently utilized as an indirect selection criterion for improving feed efficiency, because of the relatively large and favorable genetic correlation between food conversion ratio (FCR) and average daily gain (–0.53, range –1.24 to –0.34 for *ad libitum* feeding; Clutter and Brascamp, 1998).

Feeding devices, which electronically identify each animal and record individual intake on group-housed animals, provide one answer to this problem. Such devices have been designed and used in a very large pig-breeding operation in Australia and genetic and phenotypic

parameters have been estimated. In this population analyzed by Hermesch et al. (2000a, 2000b), average FCR was 2.85 (±0.58), the phenotypic variance was 0.265 and h^2 for FCR was 0.15 (±0.04). This heritability is at the low end of the published estimates, probably due to a high environmental variance resulting from a short period of measurement of feed intake and inaccuracy in its measurement using this type of device. Nevertheless, it is quite clear that the implementation of performance-based selection will gradually improve food conversion ratio and the ability to more widely measure animal performance and implement such selection will necessarily accelerate the rate of improvement. Although the main justification for this type of selection is increased profitability from reduced feed costs, inevitably the amount of effluent produced per pig will also decrease. Table 1 shows trends in feed conversion ratio in grower-finisher pigs in the Netherlands where the improvement in feed conversion ratio is accompanied by a decrease in phosphorous and nitrogen excretion per pig.

2.2. Mapping genes for efficiency

An alternative or adjunct to direct measurement of a trait and performance-based selection involves identifying the genes responsible for variation in feed conversion efficiency. Since the pioneering study of Andersson et al. (1994) on pigs, the use of genetic markers to perform genome scans to detect chromosomal regions affecting various traits has become well established for many domestic animal species. If a chromosomal region (or better still a gene within that region) responsible for variation in a trait can be identified, then it may be possible to choose superior parents for breeding on the basis of a molecular test for that gene or chromosomal region, that is by marker-assisted selection, as an alternative or a supplement to performance-based selection. This has elicited much commercial interest and for economically important traits like FCR, the results of genomic mapping studies on pigs are generally commercially confidential and thus unavailable for review. Bidanel and Rothschild (2002) reviewed all available published QTL studies in the pig, finding very few reports of QTLs affecting food conversion efficiency, probably reflecting the commercial sensitivity of the research on this trait. Rohrer (2000) found suggestive evidence for an efficiency QTL on the short arm of chromosome 1. In their 1994 study, Andersson et al. found evidence for a QTL for intestine length on chromosome 4 in a Large White × Wild Boar resource pedigree. Subsequently, Knott et al. (1998) found evidence for multiple QTLs for this trait. It has been speculated that variation in intestine length could affect food conversion efficiency – Wild Boar have much shorter intestines than domestic pigs. Although some growth QTLs map to the same positions as the intestine length QTLs, no food conversion efficiency QTL are known to co-locate with them.

Professor Herman Geldermann of the University of Hohenheim has co-ordinated a large international collaborative effort to map QTLs for many commercially relevant traits in pigs using a large F2 resource based on crosses between Pietrain, a lean and efficient European breed, Meishan, a fat but highly fecund Chinese breed and European Wild Boar, the ancestor of European domestic pigs, which has low food conversion efficiency (Geldermann et al., 2003). Numerous QTLs have been discovered in this work, some with very high levels of statistical support and explaining a very substantial proportion of the F2 phenotypic variance. The statistical support for QTLs for food conversion efficiency was not overwhelmingly strong, but there was evidence at the 5% chromosome wide level or better of significant QTLs for this trait on six different chromosomes (table 2).

The additive effects of several of these QTLs are very small. Only the best statistically supported QTL on pig chromosome 18 has a substantial additive effect and explains a reasonably large proportion of the F2 variance. Of course, it is possible that none of these QTLs are

Table 2

Chromosomal location and effects of QTLs for food conversion ratio detected in the Hohenheim wide cross study

Chromosomal location	F2 family[a]	Effect (a, d, %F2 variance[b])	Reference
SSC4*	W×P	0.11 ± 0.04 0.12 ± 0.06 3.6	Cepica et al., 2003
SSC5*	M×P	–0.01 ± 0.07 –0.35 ± 0.10 3.7	Lee et al., 2003
SSC6*	W×M	–0.14 ± 0.05 –0.13 ± 0.07 3.8	Yue et al., 2003
SSC8*	M×P	–0.01 ± 0.08 0.43 ± 0.12 3.5	Beeckmann et al., 2003
SSC18**	W×M	–0.20 ± 0.05 –0.12 ± 0.08 5.3	Dragos-Wendrich et al., 2003
SSCX*	W×P	0.10 ± 0.04 - 3.3	Cepica et al., 2003

[a]F2 families were made by crossing Wild Boar (W), Meishan (M) and Pietrain (P) parental stocks.
[b]a, additive effect of QTL; d, dominance effect; %F2 variance, percentage of F_2 phenotypic variance explained by the QTL.
**, significant at $P < 0.05$ genome-wide threshold; *, significant at $P < 0.05$ chromosome-wide threshold.

segregating in commercial populations of pigs, but they do give us an indication of where to look in commercial populations for QTLs where marker-assisted selection might be applied and they also give us an excellent indication of where to look to begin to explain the large differences in traits between these diverse breeds.

At this stage, no QTLs for food conversion efficiency have been characterized at the molecular level. That is, in no case has the gene and mutation within it underlying the QTL effect been identified. However, there is no doubt that such discoveries will occur in the reasonably near future. The results will lead not only to better selective breeding programs but to a better understanding of the physiological processes influencing food conversion efficiency. And, of course, as argued previously, any improvement in food conversion efficiency will be reflected in lower levels of effluent.

3. REDUCING PHOSPHOROUS IN EFFLUENT

Phosphorous is an essential nutrient for animal growth. Although there is plenty of phosphorous in plant-based diets, monogastric animals are unable to utilize phytate, the organic form of phosphorous normally present in plants, and inorganic phosphate must be added to achieve satisfactory growth. As a consequence, effluent from poultry and pig production is a leading source of phosphorous pollution, both from undigested phytate and unutilized inorganic phosphate additives in the diet. If some way could be found to reduce or preferably make the phosphorous in phytate available, it would go a long way to reducing phosphate pollution from piggeries, with the added bonus of reducing or eliminating the need to add inorganic phosphate to the diet.

There are three approaches to overcoming this problem: modification of feed during processing, genetic modification of the plants and genetic modification of the animal. All have been tried and all shown to be successful.

3.1. Phytase from microorganisms

Many microorganisms produce phytase, which hydrolyzes phytate. For example, mutant strains of *Aspergillus niger* have been identified producing approximately 7.9 g/l of phytase in the medium, an increase of 1440-fold compared with wild-type strains (Wodzinski and Ullah, 1996). Adding purified phytase of microbial origin to feed at the appropriate level can remove any need to supplement pig diets with inorganic phosphate (Murry et al., 1997; O'Quinn et al., 1997) and will reduce phosphorous in effluent. The disadvantages are cost of production and inactivation of phytase during feed treatment or storage. Phytase treatment of feed can reduce fecal phosphorous levels by up to 56% (Wodzinski and Ullah, 1996). Wodzinski and Ullah (1996) have estimated that if phytase treatment were applied to all monogastric feeds in the United States, this would make available phosphorous equivalent in value to US$1.68 $\times$ 10^8 per year and would prevent 8.23 $\times$ 10^7 kg of phosphorous from entering the environment each year. Clearly the impact both on profitability and effluent production would be enormous.

3.2. Low-phytate plants

Mutations have been identified in maize which reduce the phytate concentration in seeds by 33–66% but do not affect the overall amount of phosphorous. Pigs fed on diets formulated from maize from these lines were found to have no requirement for inorganic phosphate supplementation, and phosphorous levels excreted were significantly reduced (Spencer et al., 2000). This approach is believed to be superior to using high-phytase plants like wheat or transgenic plants overexpressing phytase, as the phytase may be destroyed during processing of the grain. Breeding low-phytate lines of plants has the advantage of eliminating the cost of phytase treatment. The limitation is that similar mutations would have to be identified in all plant species contributing to the diets of monogastrics.

3.3. Modifying animals to express phytase

Finally, animals can be genetically modified to enable them to utilize phytate by providing them with the capacity to produce their own phytase. Initially, Golovan et al. (2001a) produced transgenic mice expressing bacterial phytase in their saliva, and evaluated the mice using diets containing both phytate and inorganic phosphate. They found that expression of the transgene in these mice could lower fecal phosphorous levels by 11%. Subsequently Golovan et al. (2001b) used a recombinant construct consisting of the parotid secretory protein promoter and the *E. coli* phytase coding region to make lines of transgenic pigs. In the best of these lines, high levels of phytase were produced in the saliva. Such pigs could thrive on diets where the only source of phosphorous was phytate, and fecal phosphorous output was reduced by up to 75% (fig. 1). Clearly, such animals could have a profound impact on the levels of phosphorous pollution from pig effluent, since the reduction in fecal levels is even greater than for phytase treatment of feed, does not involve the cost of the phytase treatment and has the added economic incentive of eliminating the need for inorganic phosphate supplementation of the diets of pigs.

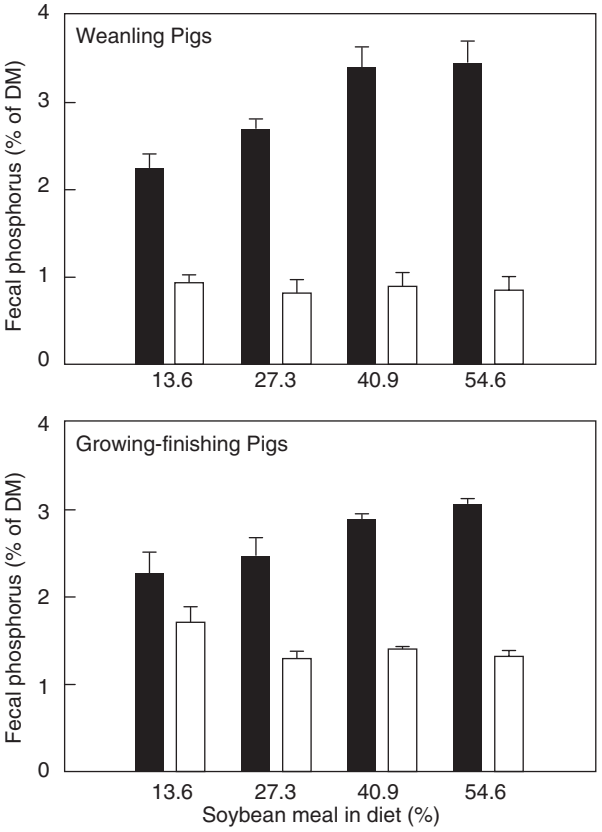

Fig. 1. Total phosphorous content in feces (on a dry matter basis) of transgenic pigs expressing salivary phytase (white) and nontransgenic controls (black). All pigs were fed a diet in which soybean meal containing 53% phytate phosphorous was the sole source of phosphorous. (Adapted from Golovan et al., 2001b, with permission of Nature Publishing Group.)

The promoter used in the transgenic pigs directed expression of phytase in these pigs, as expected, to the salivary glands. Transgenic pigs showed high levels of phytase in parotid, sublingual and submaxillary salivary glands, with low but substantial levels in tissues from the fundus region of the stomach and duodenum (Golovan et al., 2001b). Phytase activity levels were quite high in the contents of the stomach, duodenum and ileum, but were much lower in the contents of the cecum or colon of weaners. Negligible levels were found in nontarget tissues like skin, muscle, heart and liver.

Interestingly, the phytase expressed in the transgenic pigs was N-glycosylated and consequently had a higher molecular mass than the native *E. coli* enzyme, but this had no adverse impact on enzyme activity or stability. Like the unglycosylated enzyme, the salivary phytase expressed by these pigs retained more than 90% of its activity after incubation with 1000-fold excess of the digestive enzyme pepsin for 6 hours in acid conditions (pH 2.5) mimicking stomach conditions. The expression of salivary phytase in the transgenic pigs tended to decline with age, with growers showing lower levels of expression than weaners. Such age-dependent declines in expression of transgenes have been found in other species and could be exacerbated by the high copy number of the transgene. Golovan et al. (2001b) reported

numerous other transgenic pigs produced but not evaluated at that time. It is possible that these additional lines produced by microinjection of embryos, or others that could be produced using *in vitro* transformation and *in vitro* evaluation, prior to cloning by nuclear transfer, will overcome this or any other problems related to gene expression.

4. OVERCOMING ANTINUTRIENTS

Glucans are soluble fiber polysaccharides found in the major feed grains like wheat, barley, oats and rye. They are not digested in the stomach or small intestine of monogastric animals and reduce nutrient assimilation and food conversion efficiency, mainly by increasing the viscosity of the gut contents. Endo-β-1,4-glucanases digest β-glucans, overcoming the digestive problems and providing an additional benefit in the release of glucose subunits. Glucanases and xylanases are added to poultry diets to overcome the digestive problems created by such polysaccharides in cereals (Classen, 1996).

A transgenic mouse model has been made by Zhang et al. (1997) to evaluate the potential for modifying animals to overcome the problems created by glucans in the diet. Initially, Zhang et al. (1997) created a transgene construct consisting of the endo-β-1,4-glucanase gene C6.5 coding sequence from *Bacillus subtilis* with mouse amylase (Amy-2.2) signal peptide coding sequence and driven by the SV40 promoter/enhancer for evaluation in Chinese hamster ovary cells and with the Amy2.2 promoter/enhancer for evaluation in a pancreatic cell line. Both types of cells expressed the protein in secreted form. The glycosylated form of the enzyme expressed in these cultured mammalian cells had the same activity, protease resistance and cellulose binding as the unglycosylated form expressed in bacteria. Subsequently, Zhang et al. (1999) made transgenic mice expressing this bacterial endoglucanase in the pancreas, where it is secreted into the small intestine (table 3). The enzyme was reduced by proteolysis to an active form resistant to further protease degradation, and high levels of enzyme activity could be detected in the pancreas and contents of the small intestine.

Table 3

Expression of endoglucanase is targeted to the pancreas of transgenic mice and delivered to the small intestine where the enzyme will break down antinutritive glucans impeding digestion (modified from Zhang et al., 1999)

	Endoglucanase activity (mU/mg) Mean ± SD (number of animals tested)	
Tissue	Transgenic	Nontransgenic
Pancreas	0.221 ± 0.062 (6)	0.009 ± 0.023 (3)
Small intestine (tissue)	<0.001 ± 0.024 (5)	<0.001 ± 0.027 (3)
Small intestine (contents)	0.113 ± 0.057 (5)	0.021 ± 0.022 (3)
Spleen	0.014 ± 0.018 (4)	0.003 ± 0.011 (3)
Kidney	<0.001 ± 0.001 (4)	<0.001 ± 0.010 (3)
Heart	<0.001 ± 0.006 (4)	0.005 ± 0.003 (3)
Brain	<0.001 ± 0.004 (4)	0.002 ± 0.008 (3)
Muscle	<0.001 ± 0.006 (4)	<0.001 ± 0.003 (3)
Liver	<0.001 ± 0.007 (4)	<0.001 ± 0.004 (3)

Since the promoter and enhancer sequences used to drive expression of the transgene were taken from an amylase gene, the transgene was inducible by starch in the diet. No assessment of the effect on viscosity of gut contents was reported and no follow-up studies in mice or other species have been published. Although further work is required to determine whether this will be a fruitful approach to improving overall food conversion efficiency in monogastrics and reducing the levels of effluent produced, these initial results are very promising. The study has established proof of principle in targeting transgene expression of an exogenous enzyme to a specific organ, from where it was secreted into the small intestine to attack a specific antinutrient found in many cereals. Studies are required in pigs to further evaluate the potential of this approach in a production animal.

5. USING ANIMAL GENE DISCOVERIES FOR INCREASING FOOD CONVERSION EFFICIENCY

Discovery of gene variants or variation in gene expression causally related to variation in feed conversion efficiency immediately raises the possibility of manipulation of the animals directly, either by treating them with the purified gene product or with small molecules that regulate gene expression. For example, animals can already be treated with a purified peptide hormone, with a very short half-life, to augment food conversion efficiency. Future possibilities for this technology include the use of emerging techniques, like RNA interference (RNAi) (McManus and Sharp, 2002), where small RNA molecules can be targeted against specific genes to down-regulate their expression. Although this technique is still at a very experimental stage, numerous possibilities are emerging for manipulating biochemical pathways and physiological processes to improve efficiency.

5.1. Treatment with porcine somatotropin (PST)

Treatment of growing pigs by injection with porcine somatotropin (PST), growth hormone, is known to improve growth rates and feed conversion efficiency by 15–20% (Boyd and Bauman, 1989; Campbell et al, 1989, 1991; Harrell et al, 1997). In Australia, recombinant PST (rPST) has been legally available as a safe and effective growth promotant for grower pigs since 1993. (Currently, Alpharma Animal Health Pty Ltd markets rPST as Reporcin® http://www.reporcin.com/). One of the selling points is the environmental advantage of reducing per animal levels of effluent production. Dunshea et al. (1999, 2002) have shown a strong beneficial effect on food conversion efficiency of the use of Reporcin® under commercial conditions (table 4) especially for gilts.

Table 4

Effect of rPST treatment on performance of finisher pigs under commercial conditions (extracted from Dunshea et al., 2002. In: Aust. J. Agric. Res. 53(3), 287–293, http://www.publish.csiro.au/journals/ajar, with permission of CSIRO Publishing and the authors)

	Boar		Gilt		
Treatment	Control	PST	Control	PST	Significance of PST effect
Initial weight (kg)	79.4	79.1	78.6	77.9	0.31
Final weight (kg)	114.7	115.3	107.7	113.5	0.023
Feed conversion ratio	2.43	2.11	2.90	2.12	<0.001

5.2. Transgenic modification of pigs for improved overall efficiency

Early attempts to modify pigs to overexpress growth hormone or other hormones in the growth hormone axis met with equivocal results (reviewed in Murray, 1999). Although the animals were generally leaner and more feed efficient, there were serious complications resulting from unregulated overexpression of circulating growth hormone. The promoter sequences used initially did not regulate growth hormone secretion in a tightly controlled, inducible fashion. More recently, Nottle et al. (1999) have shown that selection for transgenic animals showing no elevated basal levels of the transgene, but stimulation of the metallothionein promoter and thus PST expression in response to zinc, resulted in favorable effects on growth without any of the attendant abnormalities previously seen. Similarly, Pursell et al. (1999) were able to target insulin-like growth factor 1 (IGF1) expression specifically to skeletal muscle using a skeletal muscle α-actin promoter, avoiding elevating plasma IGF1 levels, and thus were able to achieve favorable growth and composition effects without the unfavorable effects on animal health of elevated plasma IGF1 levels seen using other promoters. Thus, there remains hope that this approach to transgenic improvement of efficiency of pigs may yet bear fruit.

6. FUTURE PERSPECTIVES

Biotechnology can provide quite effective solutions to the problems of effluent creation and disposal affecting intensive animal production systems such as for pigs. Furthermore, developments in genomics and genetic research will provide new avenues for improving food conversion efficiency, with immediate collateral benefits on effluent production, including novel genetic approaches to identifying superior animals. Moreover, discovering the genes underlying variations in important digestive and physiological processes may lead to environmental manipulation of gene expression, in addition to the application of the knowledge in genetic improvement. The opportunities for application of such technology extend beyond manipulation of gross food conversion efficiency. Already it has been demonstrated that gene technology can be targeted at specific nutrients, such as the phosphate in phytate, and against specific antinutritive factors such as glucans.

The eventual impact of biotechnology will depend ultimately on consumer and public acceptance. While the public in some parts of the world is currently quite GMO-averse, it will be interesting to see how long this will be sustained in the face of elegant demonstrations of the power of transgenic technology to provide environmental benefits, such as the salivary phytase-expressing pigs. It is already clear that such genetic modifications could provide substantial economic and environmental advantages. It will be very interesting to see how attitudes to GMO animals will evolve over time and whether this technology will get an opportunity to provide environmental benefits.

In some cases, permanent genetic modification of pigs by transgenesis may be unnecessary. Direct administration of PST or other gene products to pigs may provide more efficient control of the delivery and will avoid the current GMO impasse, subject to meeting welfare and product quality assurance requirements.

Now that a complete genomic sequence for the pig is a fast-approaching reality, novel gene discovery and improved understanding of gene regulation open endless possibilities for improving efficiency and reducing waste production for this very important species. The future discovery of small molecules, including RNAi molecules, capable of regulating and controlling expression of genes implicated in food conversion efficiency, promises novel and

efficient opportunities for "environmental" (i.e. nongenetic) manipulations of pig performance, in addition to permanent genetic improvements.

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21 Manipulation of the poultry ecosystem through biotechnology

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This chapter reviews a number of biotechnological tools or products, which may influence the poultry ecosystem, including new diagnostic methods and the development of vaccines through genetic engineering of bacteria and viruses, the introduction of genetic resistance to infectious pathogens in lines of poultry used in production, the development of more effective feed enzymes and probiotics which may directly or indirectly influence the microflora of the gastrointestinal tract, and the effect of plant products modified by means of biotechnology. It is concluded, that even though the new biotechnological products might have a positive impact on poultry ecosystems, more experimental evidence should be accumulated, before permitting farmers to use these new technologies, to prove that their widespread use will not have a negative impact on the human ecosystem.

1. INTRODUCTION

Poultry production will continue to play a major role in feeding the fast-growing world population, supplying meat and eggs that are inexpensive and nutritious. The rapid expansion of the poultry industry in the last 75 years has been due to intensive research and development in different fields. First, it witnessed spectacular progress in selective breeding, leading to improvements of both layer and broiler traits. Second, the nutrient requirements of poultry and the nutrient (and antinutrient) composition of the main feed ingredients have been firmly established. Other no less important fields, are the application of intensive and fully automated indoor housing systems and the development of medication systems and vaccines, which provide protection against the most dangerous poultry pathogens. Thanks to developments in all of these fields, the number of eggs per hen per year has increased 3-fold, and the growth rate (days to 1.5 kg live weight) 4-fold during this period. Poultry production has become the most economically efficient field of animal production.

Success in breeding has nonetheless been accompanied by a higher incidence of undesirable traits, including reduced resistance to infectious diseases and poor vaccine response (Albers, 1998; Burt, 2002). Medication systems, used to maintain the ecosystem of poultry at

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a level ensuring profitability (use of antibiotic growth promoters and coccidiostatics in feed), do not fully prevent outbreaks of foodborne bacterial diseases (Van Immerseel et al., 2002), while creating the possibility of transferring antibiotic resistance to the human ecosystem (Lange and Ek, 1995). The great concentration of poultry production creates threats to the environment (pollution of soil and water with nitrogen (N) and phosphorus (P) connected with the disposal of manure). At present, the major concerns of health-conscious consumers in western societies have shifted towards the safety and quality of food (with regard to human health), the quality of production methods (with regard to animal health and welfare), and the quality of the natural environment aimed at limiting the negative impact of poultry production on agro-ecosystems. Addressing these concerns requires that the levels of nonpathogenic and pathogenic microflora in the ecosystem of poultry be controlled, without a negative impact on human ecosystems.

Further progress in the safety of poultry production without compromising efficiency will be possible only through application of the modern tools of biotechnology. The scope and potential of biotechnology have dramatically increased in recent decades through developments in the fields of molecular and cell biology, which resulted in recombinant DNA technology and genetic engineering. The development of multivalent and multipathogen vaccines through genetic engineering, improvement of resistance to infectious diseases in poultry populations by means of genomics, inexpensive production of novel feed additives that may help in better utilization of feed ingredients, and of probiotics able to eliminate harmful microflora by competitive exclusion, may all help to reduce the transfer of foodborne pathogens from poultry feed to the human food chain. The reduction of pollution of soil and groundwater sources by nitrogen, phosphorous and other undigested feed components contained in poultry manure, and reduction in pollution of the atmosphere inside and around large poultry units by such noxious gases as ammonia, may be also easier with the use of bacterial strains or products developed by means of modern biotechnology.

2. GENETIC ENGINEERING OF BACTERIA AND VIRUSES AND PATHOGENIC AGENTS IN THE POULTRY ECOSYSTEM

The application of intensive and fully automated indoor housing systems and the high concentration of poultry production in certain regions of the world make poultry populations susceptible to infections with pathogens such as bacteria, viruses and parasites. Poultry products are also an important source of bacterial pathogens that can enter the food chain, among the most common of which are *Salmonella, Campylobacter* and *Listeria* spp. (Mead, 2002; Van Immerseel et al., 2002). For human health it will be also important to monitor poultry populations for some viruses, such as influenza viruses, which, while not adversely influencing carrier birds, might be of great importance to human influenza epidemics and pandemics. New diagnostic methods, using advanced techniques such as enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), and reverse transcriptase PCR (RT-PCR), will enable identification of the emerging agents and the development of effective vaccines against them (Nagy, 2001). Molecular descriptions of host–pathogen interactions, in terms of differential gene expression, will not only provide insights on the molecular basis of disease pathogenesis, pathogen virulence and host immunity, but will be important for the design and development of effective immunomodulators and vaccines (Munir and Kapur, 2003).

Bacteria and viruses could be genetically engineered, by adding novel foreign genes or deleting normally present ones, and used directly as vaccines. Recombinant DNA technology has made it possible to generate “vectored vaccines”, utilizing viruses as vectors for the

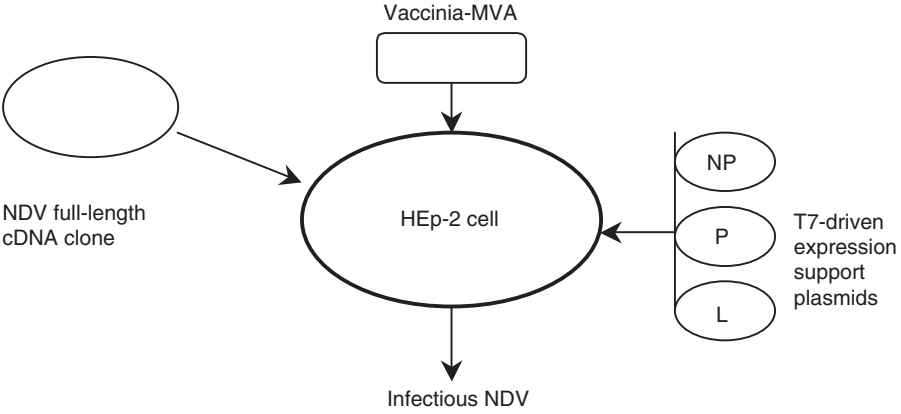

Fig. 1. The reverse genetics protocol employed for generating recombinant Newcastle disease viruses (NDV). The full-length antigenomic cDNA of NDV and support plasmids encoding the nucleocapsid protein (NP), the phosphoprotein (P) and the large polymerase protein (L) were transfected into human epidermoid carcinoma (HEp-2) cells that were preinfected with recombinant vaccinia virus (MVA), to recover the recombinant virus. The recombinant virus was then amplified in embryonated chicken eggs post-transfection. (Adapted from Huang et al., 2003, with permission of the Poultry Science Association.)

expression of protective antigens of other viruses (fig. 1). Thus, Newcastle disease virus (NDV) has great potential as a vaccine vector for the next generation of vaccines for veterinary use in poultry production (see review by Huang et al., 2003).

Bacteria and viruses can also be manipulated to provide proteins for use as specialized subunit vaccines or as diagnostic reagents (Nagy, 2001). Genetically engineered vaccines against coccidiosis are also promising, because protection against coccidiosis is more related to T-cell immunity than to antibody-based immunity. Most important in the development of this type of vaccine is the choice of the candidate antigen, derived from a certain developmental stage of the parasite, and the choice of the vector. However, at present no genetically engineered vaccines against coccidiosis are available for use in the field (Jeurissen and Veldman, 2002). The development of multivalent and multipathogen vaccines through genetic engineering would reduce the infection pressure of specified zoonotic agents in poultry ecosystems. This would reduce the use of chemotherapeutics in poultry production and diminish the amount of chemotherapeutics that enter the environment with poultry excreta.

3. FUNCTIONAL GENOMICS AND THE POULTRY ECOSYSTEM

Albers (1998) concluded that despite the great progress in poultry breeding, the biological limits have still not been reached. In broiler production there is the possibility of improving the feed conversion ratio by about 20%, while in egg production the laying rate may grow by about 15% in the next 15–20 years. Genetic selection directed towards increasing efficiency, increasing resistance to pathogens, and reducing the main negative traits, will not only reduce costs but positively affect bird welfare and poultry ecosystems. Application of genomics in the poultry industry may increase its efficiency. Genomics does not create genetic variation, but is a precise tool that detects natural genetic variation in breeding populations. According to Burt (2002), there are four areas of interest in poultry genomics: isolation and mapping of genetic markers, mapping of quantitative trait loci (QTL), candidate-gene identification, and identification of genes controlling quantitative traits of interest. This new era of functional genomics requires access to arrayed cDNA and bacterial artificial chromosome (BAC)

libraries, expressed sequence tags (ESTs) databases, and high-throughput technologies (sequencing, DNA microarrays, etc.). Work in the area of complete genome sequencing is fairly advanced, while discovering gene functions is still far from completion. Schmid et al. (2000) listed a genetic linkage map of over 2000 loci in chickens and a number of QTL have been mapped. Zhou et al. (2003) reported that approximately 2400 genes and genetic markers have been mapped in the chicken, with the majority of these being anonymous genetic markers. An international consortium constructed and normalized five tissue-specific chicken cDNA libraries, which yielded 30 609 EST for chickens, and assembled a preliminary Chicken Genome Index from the world's public collection of 407 602 chicken EST (Cogburn et al., 2003). The draft sequence of the Chicken Genome was expected in March 2004 (Burt and Pourquie, 2003).

Beside the QTLs for body weight (Groenen et al., 1997), muscling (Lander and Kruglyak, 1995) and growth and feed efficiency (Van Kaam et al., 1999), QTLs affecting susceptibility to Marek's disease (Yonash et al., 1999) or resistance to salmonellosis (Burt, 2002) have been identified. With the use of DNA microsatellite markers Yonash et al. (2001) found three markers associated with the immune response and disease resistance (table 1). They concluded that poultry immunocompetence may be improved by marker-assisted selection. The results presented by Zhou et al. (2003) suggest that regions on chromosomes 3, 5, 6 and Z contain QTLs that affect antibody kinetics in the hen.

Furthermore, a transgenic mouse has been developed that secretes phytase in its saliva (Golovan et al., 2001), which suggests that it should also be possible to directly manipulate poultry to express genes which enable an increase in the array of enzymes produced by the internal organs.

The introduction of genetic resistance to infectious pathogens in the lines of poultry used in production might reduce the pressure of infection with pathogenic agents in poultry ecosystems, which might be an alternative to the overuse of chemotherapeutics. The development of transgenic birds producing enzymes that are active against most noxious antinutrients, can

Table 1

The effects[a] and significance[b] of microsatellite markers on antibody response to *Escherichia coli*, ship red blood cells (SRBC), and Newcastle disease virus (NDV) and on survival rate following pathogenic *E. coli* challenge in meat-type chickens, estimated from genotyping analyses of the entire resource population[c] (adapted from Yonash et al., 2001, with permission of the Poultry Science Association)

Linkage group[d]	Marker	Size of sire alleles	Trait	n^e	Effect[a]	R[b](%)	P(t)[b]
Ch.2	ADL0146	152/164	SRBC	156	0.38	4.4	0.009
			NDV	157	0.39	3.8	0.014
Ch.8	ADL0258	162/169	SRBC	156	0.02	0.0	0.889
			NDV	157	0.11	0.3	0.494
Ch.5	ADL0298	102/106	*E. coli*	155	0.34	4.4	0.009
			Survival, %	145	0.24	2.8	0.027
E31	ADL0290	174/189	*E. coli*	154	0.11	0.5	0.385
			NDV	155	0.30	2.7	0.043

[a]Differences between carriers of each of the sire alleles, given in phenotypic SD.
[b]Significance of the effect: *t*-test for antibody level; chi-square test for survival rate.
[c]Resource population = the F_2 + backcross (BC_1) family.
[d]Ch. = chromosome; E31 = linkage group 31.
[e]Number of birds genotyped with the particular marker that were informative for the analysis of the sire alleles.

help in stabilizing gastrointestinal tract microflora without using antibiotics or other feed additives.

4. THE IMPORTANCE OF GASTROINTESTINAL TRACT (GIT) MICROFLORA TO THE HEALTH AND PERFORMANCE OF BIRDS

The most important part of the poultry ecosystem is the GIT microflora, which comprises several hundred species and up to 10^{12} bacteria per gram of colonic contents. The intestine of the bird is sterile before hatching, but over 400 microbial species live in the environment of the hatchery and henhouse (Snel et al., 2002). Immediately after hatching and later during their life cycle, birds are continually exposed to microbes and parasites found in the environment; some of which permanently colonize the intestinal tract. Though the microflora compete with the host for nutrients (according to Muramatsu et al. (1994), conventional birds retain 10% less energy from the diet than germfree ones) it is very important to the bird's health status and gut function. If the bird and its GIT microflora are in a state of equilibrium, most of the GIT microbes represent bacteria that are capable of using undigested feed residues, are harmless to the host, and are capable of inhibiting the growth of pathogens. Indigenous bacteria colonize the intestinal tract permanently, multiplying in particular intestinal niches at a rate that equals or exceeds their rate of washout or elimination at the site. Certain species of bacteria reduce colonization by other species. Thus, the presence of *Bacteroides* may reduce colonization of such members of *Enterobacteriaceae* as *E. coli* and *Salmonella*. The variety of species, as well as the microbial numbers in the gut, are determined by such factors as pH of intestinal contents, intestinal motility, immune activity and feed composition (Snel et al., 2002). After exposure of the bird to different stress conditions, the composition of microbiota may change and their numbers may increase, negatively affecting the performance and wellbeing of the bird as well as the environment of the poultry unit.

Different strategies have been developed to stabilize the GIT ecosystem and to suppress the activity of pathogenic bacteria. They include supplementation of animal feeds with subtherapeutic levels of antibiotics, feed enzymes, mono- or mixed cultures of live microorganisms (probiotics and competitive exclusion cultures), prebiotics, minerals or organic acids.

Antibiotic growth promoters directly modify the GIT microflora by killing some species, or by interfering with their ability to replicate. They suppress both nonpathogenic and pathogenic microflora (Engberg et al., 2000), which is related to a decrease in production of growth-depressing bacterial toxins, with reduced competition for nutrients and with less stimulation of the immune system of the bird. This positively affects the health status and performance, an effect which is most visible in very young birds. However, the extensive use of antibiotics in animal farming has resulted in the emergence of resistance and multiple resistance of microbes (Lange and Ek, 1995; Wegener et al., 1998; Logue et al., 2003). Transmission of antibiotic resistance among bacterial strains is well documented, and occurs by transfer of plasmids, small circular extrachromosomal pieces of DNA, or by insertion of intact antibiotic resistance genes from the genomic DNA of one bacteria to that of another (Beever and Kemp, 2000). It may lead to the emergence of new strains of foodborne pathogens displaying increased resistance to therapeutic antimicrobials. Logue et al. (2003) recorded significant resistance levels for antimicrobials such as ampicillin, gentamicin, tetracycline (50%), sulfomethazole, streptomycin (35%) and kanamycin among the *Salmonella* isolated from freshly processed turkey carcasses.

Potentially, integrons might also be major agents in the dissemination of multidrug resistance among Gram-negative bacteria. The integron contains an integrase gene and a

Table 2

An example of PCR primers and oligonucleotides used to probe for class 1 integrons and integron-associated antibiotic resistance cassettes among DNA sequences extracted from the bacterial microflora of broiler chicken litter. Integron gene cassettes were amplified by PCR by using consensus sequences flanking the integration site *attC*. Oligonucleotide probes were used in a cocktail of capture probes in PCR-Elisa to detect gene(s) that confer resistances (adapted from Lu et al., 2003)

Gene cocktail and target gene(s)	Oligonucleotide probe sequence (5′-3′)	Presence or absence of antibiotic resistance genes in litter
Aminoglycoside resistance		+
aadA1, aadA2	GCAGCGCAATGACATTCTTG	
aacA7	TTTCGATCCGCCCGTATG	
Chloramphenicol resistance		+
catB2, catB3, catB4, catB5, catB6	GCCATGRTYATGCCCGGVATCAA	
CmlA	CTAGGTTTGGGCATGATCGC	
Trimethoprim resistance		–
dfrA14(Ib), dfr5	CCTTCGAAGTTGTTTTTGAGCAA	
dfrB1, dfrB2	TCCTGTKGCKGCRCTTGAAC	

site-specific integration site, where the integrase can link antibiotic resistance gene cassettes in the integration site if the circular cassette molecules possess a 59-base element. Lu et al. (2003), using PCR primers and an oligonucleotide probe, detected cassettes encoding aminoglycoside resistance and chloramphenicol resistance in litter from broiler houses, even though antibiotics of these classes were not administered to the flocks of birds in the study (table 2).

Indeed, the use of most antibiotics in animal feed has now been banned in the EU, and they have instead been replaced by other bioactive components.

Bedford (2000) listed many treatments currently in use or considered for use in antibiotic-free poultry diets, among which a few might be more effective with the use of modern biotechnology tools. These include improvement in the immunity of birds to disease (discussed above), and supplementation of feed mixtures with feed enzymes and probiotics.

The most commonly used feed enzymes in poultry production are carbohydrases (enzymes hydrolyzing viscous and nonviscous nonstarch polysaccharides). In chickens, rapid feed passage, low pH in the proventriculus-gizzard, high oxygen tension and the presence of bile salts in the duodenum limit bacterial growth in the upper regions of the GIT. Further, along the small intestine the environment changes and becomes more favorable for bacterial growth. When digestion and absorption in the upper regions of the small intestine are optimal, little starch and protein remain for digestion by the microbiota in the lower part of the GIT and the small intestinal and cecal microbial populations utilize mainly fermentable fiber. Soluble nonstarch polysaccharides (NSP), such as β-glucans of barley or arabinoxylans of wheat are viscous. When digestion and absorption in the upper part of the small intestine are slowed down by the high viscosity of digesta, more nutrients enter the lower parts of the small intestine, supplying more substrate to the microflora. The effect is similar when the potentially digestible components are interconnected with, or encapsulated by, the indigestible NSP (cage effect), which makes them resistant to digestion by the bird's own enzymes (Bedford, 2000).

Carbohydrases (β-glucanases and arabinoxylanases) added to cereal-based diets partially degrade β-glucans and arabinoxylans, helping to release nutrients encapsulated by this NSP and to decrease digesta viscosity. Due to this the flow of fermentable substrates into the ileum is reduced, preventing proliferation of microflora. Due to their smaller size, the NSP-degradation products can pass the ileo-cecal filters and are fermented by cecal microflora to short-chain fatty acids (SCFA). Choct et al. (1996) reported that in birds fed diets supplemented with viscous arabinoxylans, SCFA production in the ileum markedly increased, while after supplementation of the diet with arabinoxylanase, the concentration of SCFA in the ileal content was reduced and that of the cecum increased. The increase in the availability of fermentable sugars in the ceca results in growth of *Bifidobacteria* and other species, and in reductions in the relative proportions of the populations of *Salmonella, Campylobacter* and *Clostridium* (Bedford, 2000). The last species is involved in necrotic enteritis in poultry, while the first two are important for the safety of poultry products. Currently used industrial feed enzymes are produced with the use of advanced biotechnological methods and presently phytases (enzymes liberating phosphorous from plant phytate), as well as carbohydrases, are being used with success in the poultry industry.

Enzymes added to poultry feed should withstand the low gastric pH and the proteolytic activity of endogenous enzymes in the GIT of the bird, and if used in pelleted feed, high heat tolerance is also needed. Enzymes used in the poultry industry are produced by different microorganisms (bacteria, yeasts, fungi and molds). Biotechnological tools have been used to determine the molecular characteristics of enzymes, genes and the regions coding for expression of enzymes. Recombinant strains of different microorganisms are used to enhance the secretion of enzymes, to increase enzyme thermostability, and to improve the cost-effectiveness of feed enzyme production (Vohra and Satyanarayana, 2003). Commonly used microbial phytase is produced by overexpressing the *Aspergillus ficuus* phytase gene in *Aspergillus niger* (Zhang et al., 2000). Genetic engineering of microorganisms is focused on production of enzymes with improved thermostability and substrate specificity. Thus, Pasamontes et al. (1997) cloned the heat-stable phytase-encoding gene *(phyA)* from *Aspergillus fumigatus* and overexpressed it in *Aspergillus niger* (fig. 2). They obtained phytase able to withstand temperatures of up to 100°C for a period of 20 min with minimal (10%) loss of initial enzymatic activity.

In studies on the effects of different pro- and prebiotics and other feed additives on GIT microflora and poultry ecosystems, accurate means to enumerate and evaluate the various

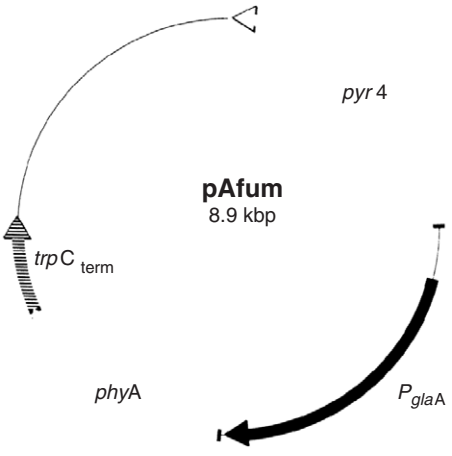

Fig. 2. Expression plasmid containing the *phy*A gene of *A. fumigatus* used to transform *A. niger. pyr-4*, orotidine-5′-phosphorate decarboxylase gene of *N. crassa*; P_{glaA}, glucoamylase promoter of *A. niger, phyA, A. fumigatus* phytase gene; $trpC_{term}$, *A. nidulans* tryptophan C terminator. (Adapted from Pasamontes et al., 1997, with permission of the American Society for Microbiology.)

microbial populations are needed. There is also a need for simple, rapid and inexpensive methods for the detection of zoonotic agents. Biotechnology supplies very effective molecular tools for analysis of microflora. In a review, Snel et al. (2002) listed such tools as fluorescent *in situ* hybridization (FISH), quantitative polymerase chain reaction (Q-PCR), and denaturing gradient gel electrophoresis (DGGE). These tools, based on detection of ribosomal RNA and DNA (rRNA and rDNA) are increasingly used to determine the effect of dietary interventions on microfloral composition (Hume et al., 2003), selection of optimal probiotic strains (Ehrmann et al., 2002) and detection of zoonotic agents (Lund et al., 2003). They constitute a valuable contribution to such methods as cumulative gas production and measurements of the production rate of microbial protein or fermentation end-products.

The efficiency of probiotics could be enhanced by genetic modifications that increase plant cell wall hydrolase production in bacterial strains. Birds could benefit from GM strains only if they have a significant impact on the microbial populations within the GIT. Netherwood et al. (1999) reported that application of an *Enterococcus faecium* strain (constructed by transforming erythromycin-resistant plasmid pVACMC1 containing the *Ruminococcus flavefaciens* β-1-4-glucanase) in the diet decreased, while application of the parent strain increased the relative amount of *E. faecalis* in the total eubacterial population of the GIT of chickens, in comparison with an untreated group (fig. 3).

However, neither the health status nor the performance of chickens were significantly affected due to treatment with either the GM or the non-GM strain. When the probiotics were removed from the diet, the relative amount of *E. faecalis* returned to the level observed in the untreated control group (Netherwood et al., 1999).

5. THE EFFECT ON POULTRY ECOSYSTEMS OF PLANT PRODUCTS MODIFIED BY BIOTECHNOLOGICAL MEANS

According to Flachowsky and Aulrich (2001), from the nutritional point of view, feed plants modified by means of biotechnology (genetically modified organisms; GMOs), may be distinguished as first- and second-generation GMOs. First-generation GMOs are characterized by changed tolerance or resistance to insects, herbicides or pesticides, with minor changes in nutrient contents. Second-generation GMOs are characterized by substantial changes in the content of nutrients, antinutrients or active components. Substantial equivalence with isogenic plants must be proven before commercial release of each first-generation crop of GMOs, so they should not affect poultry ecosystems to a greater extent than isogenic plants.

Examples of second-generation GMOs able to positively affect the microbial populations in the GIT of birds are those with enhanced levels of some enzymes that are not produced by the intestinal organs of birds. It was reported (Baah et al., 2002) that when a freeze-dried potato, capable of expressing 1,3-1,4-β-D-glucan 4-glucanohydrolase from *Fibrobacter succinogenes* was included at a level of 0.6 kg/ton or 1.2 kg/ton of a barley-based diet (it provided 30 units β-glucanase/kg and 60 units β-glucanase/kg in the diets) it improved feed conversion and substantially reduced ileal digesta viscosity in broiler chickens. The production of transgenic tobacco (Pen et al., 1993), alfalfa (Vohra and Satyanarayana, 2003), soybean (Denbow et al., 1998) and oilseed rape (Beudeker and Pen, 1995; Zhang et al., 2000) with enhanced levels of phytase has been reported. Detailed descriptions of gene constructs used for transformation of tobacco have been published by Pen et al. (1993). The phytase gene from *Aspergillus niger* was placed under the control of a modified CaMV 35S promoter and AMV RNA4 leader sequence, the *npt II* (neomycin phosphotransferase II) gene was used as a selectable marker and secretion of the protein into the intercellular fluid was established by

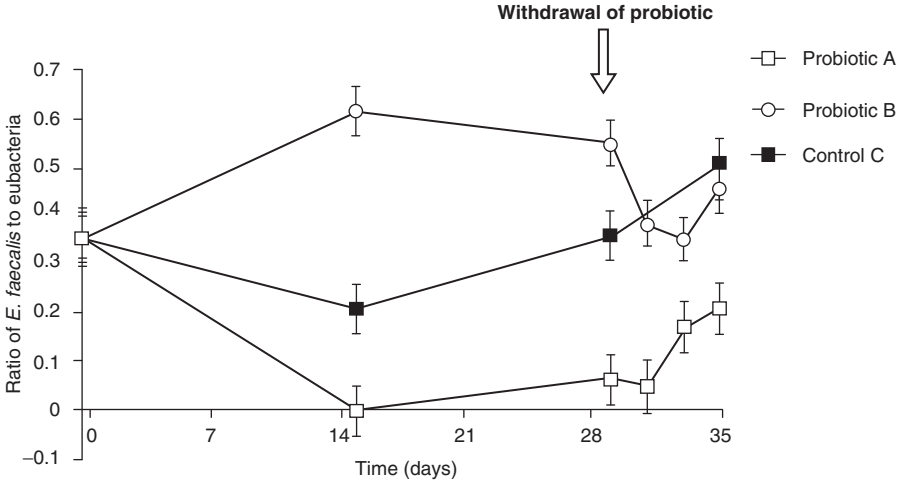

Fig. 3. Change in the ratio of *E. faecalis* to eubacteria in the three treatment groups. The ratio of *E. faecalis* to total bacteria was estimated by measuring the levels of probe hybridization to PCR-amplified regions of 16S DNA for samples treated with probiotic A (GM strain) and probiotic B (unmodified strain) and control (untreated) samples. The values are means ± standard errors of the means based on three replicates. Probiotics were removed from the diets after 28 days. (Adapted from Netherwood et al., 1999, with permission of the American Society for Microbiology.)

use of the signal sequence from tobacco PR-S protein. However, the yield of phytase in the transformed seeds may differ greatly. Thus, Beudeker and Pen (1995) reported that in tobacco seeds 1% of the soluble protein was expressed as phytase, while in oilseed rape, expression levels were up to 10% of soluble protein.

A new plant source of phytase, Phytaseed®, has been produced in canola seed by expressing the same phytase gene that was expressed in *Aspergillus niger* for the production of the microbial phytase Natuphos®. The efficacy of both sources of phytase was found to be similar in broiler chickens (Zhang et al., 2000). Furthermore, transformed soybeans containing *A. niger* phytase gene was equally as effective as the commercial microbial phytase Natuphos®, as shown in table 3 (Denbow et al., 1998).

Table 3

Effect of feeding phytase transformed soybeans (TSB) or Natuphos® phytase (N) added to corn-soybean meal diet on various growth, bone or phosphorus measurements (adapted from Denbow et al., 1998, with permission of the Poultry Science Association)

Diet, phytase supplement	BWG, g	g feed/g BWG	P digestibility, % intake	P excretion, g/kg DM intake	Toe ash, % of DM
Basal (B)	407	1.52	47.2	2.56	8.88
B + 400U phytase TSB	435	1.48	55.8	2.15	9.65
B + 800U phytase TSB	451	1.58	60.4	1.98	10.86
B + 1200U phytase TSB	474	1.52	61.6	1.82	11.45
B + 400U phytase N	416	1.48	50.6	2.34	9.92
B + 800U phytase N	440	1.56	53.3	2.24	10.62
B + 1200U phytase N	454	1.56	55.1	2.10	10.77

An example of a second-generation GMO with a lower content of antinutrients is a highly available phosphorous corn (HAP), which was developed by Raboy et al. (2000) using the low phytic acid 1-1 (*1 pal-1*) allele of the corn LPA1 gene and which contained 0.27% P and 0.17% nonphytate P (NPP). Snow et al. (2003) and Ceylan et al. (2003) compared this corn in layer diets to near isogenic normal corn, which (in the second study) contained 0.23% P and 0.05% NPP. HAP corn in both studies allowed less dicalcium phosphate supplementation in layer diets and lowered P level in excreta compared to normal corn while supporting equal egg production. However, in the study of Ceylan et al. (2003) the retention of Ca, Zn, Cu and Mn was reduced in HAP corn groups compared to normal corn. This negative effect was alleviated by phytase supplementation to the HAP corn diet, what indicates the presence of incomplete inositol phosphate in the HAP corn with potential binding sites available for cations. This study of Ceylan et al. (2003) emphasizes the need to very rigorously check for side effects and for long-lasting effects with second-generation GMOs in birds.

A general concern connected with feeding GM plants is the possibility of transmission of antibiotic resistance into the feed-to-food chain, e.g. by transmission to bacterial strains in the GIT or litter (Phipps and Beever, 2000). This concerns mainly plants in which resistance genes are engineered through incorporation of vector DNA from bacterial constructs. A commonly used selection marker in plant transformation is resistance to kanamycine or other antibiotics, encoded by the bacterial *npt II* gene (Beever and Kemp, 2000). So far, experimental studies have not demonstrated the uptake and function of a complete plant *npt II* gene by GIT microbes. However, Gebhard and Smalla (1998) have shown that plant DNA containing the *npt II* gene, which encodes resistance to neomycin and kanamycin, can at low frequency rescue *Acinetobacter* sp. (soil bacteria) that already have an *npt II* gene. Chambers et al. (2002) examined the fate of an antibiotic resistance marker incorporated into transgenic maize, when fed to chickens. They found plant-derived marker genes only in the crop and stomach contents of a few birds, but found none in the intestines or feces. They concluded that it is very unlikely that bacterial populations will be transformed to antibiotic-resistant ones in the gut of chickens fed transgenic maize. On the other hand, kanamycine resistance among 22% of *Salmonella* isolates recovered from freshly processed turkey carcasses in a US plant suggests that transgenic feed may be a factor influencing the creation of antimicrobial resistance (Logue et al., 2003).

According to Knock (2002), animal feed has not been subject to special legislation in respect to GMOs and GM material. The concept of traceability, introduced by EP Directive 2001/18/EC specifically for GMOs, requires that operators keep records and transmit specified information that a product is produced from GMOs to the next operator in the production and distribution chain. However, there is an exemption for the accidental presence of GM material in food and feed products at a threshold of 1% or lower, if the GMOs are approved in the EU or have undergone a safety assessment by a recognized EU scientific body. The proposal requires all food and feed ingredients derived from GMOs to be labeled. Chesson and Flachowsky (2003), reviewing the use of transgenic plants in poultry nutrition, concluded that at present, consumers in Europe are not willing to accept the widespread use of GM products in food production.

6. FUTURE PERSPECTIVES

At present, only a few biotechnological products derived from genetically engineered microorganisms (feed enzymes, vaccines) are generally accepted in poultry production. The biotechnological methods of engineering poultry genes and poultry GIT microbiota genes, as

well as GM crops, are currently available or well advanced in the research. The development and use of biotechnological products may have a positive impact on the poultry ecosystem, may greatly enhance the safety and quality of poultry products and may improve the quality of production methods. However, before farmers are permitted to use these new technologies, it seems prudent that more experimental evidence should be accumulated to prove that the widespread use of biotechnology in poultry production is safe and will not have a negative impact on the human ecosystem.

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