Impact of dietary protein on microbiota composition and activity in the gastrointestinal tract of piglets in relation to gut health: a review

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(Received 4 July 2012; Accepted 10 December 2012; First published online 15 February 2013)

In pigs, the microbial ecosystem of the gastrointestinal tract (GIT) is influenced by various factors; however, variations in diet composition have been identified as one of the most important determinants. Marked changes in fermentation activities and microbial ecology may occur when altering the diet, for example, from milk to solid feed during weaning. In that way, access of pathogens to the disturbed ecosystem is alleviated, leading to infectious diseases and diarrhea. Thus, there is increasing interest in improving intestinal health by use of dietary ingredients suitable to beneficially affect the microbial composition and activity. For example, fermentable carbohydrates have been shown to promote growth of beneficial Lactobacillus species and bifidobacteria, thereby enhancing colonization resistance against potential pathogens or production of short-chain fatty acids, which can be used as energy source for epithelial cells. On the other hand, fermentation of protein results in the production of various potentially toxic products, such as amines and NH₃, and is often associated with growth of potential pathogens. In that way, excessive protein intake has been shown to stimulate the growth of potentially pathogenic species such as Clostridium perfringens, and to reduce fecal counts of beneficial bifidobacteria. Therefore, it seems to be a promising approach to support growth and metabolic activity of the beneficial microbiota by developing suitable feeding strategies. For example, a reduction of dietary CP content and, at the same time, dietary supplementation with fermentable carbohydrates have proven to successfully suppress protein fermentation. In addition, the intestinal microbiota seems to be sensible to variations in dietary protein source, such as the use of highly digestible protein sources may reduce growth of protein-fermenting and potentially pathogenic species. The objective of the present review is to assess the impact of dietary protein on microbiota composition and activity in the GIT of piglets. Attention will be given to studies designed to determine the effect of variations in total protein supply, protein source and supplementation of fermentable carbohydrates to the diet on composition and metabolic activity of the intestinal microbiota.

Keywords: intestinal microbiota, protein fermentation, piglet

Implications

Protein fermentation, as a result of excessive supply of dietary protein to piglets’ intestinal microbiota, could be regarded as one factor for the development of enteric diseases. Although some results are inconsistent, it appears that a lower amount of protein reaching the lower gastrointestinal tract (GIT) may reduce the incidence of diarrhea and inhibit proliferation of pathogenic bacteria especially in stress situations, such as a high pressure of infections. Furthermore, dietary inclusion of fermentable carbohydrates shows the potential to reduce detrimental protein fermentation and growth of potential pathogens.

Introduction

The GIT of mature pigs harbors a quite stable microbiota, mainly consisting of beneficial members such as lactobacilli but also potential pathogenic bacteria, for example, Escherichia coli (Gaskins, 2001). However, there is a critical period around weaning because of the transition from liquid (sow milk) to solid feed, accompanied by morphological, histological, physiological and microbial changes in the GIT of young mammals (Pluske et al., 1997), often in addition to environmental and social stress on the animal (Etheridge et al., 1984). This period is often associated with a high incidence of post-weaning diarrhea (PWD) caused by potential pathogens such as E. coli (Hampson, 1994). Until 2006, in-feed antibiotics have been frequently used in pig production to suppress gastrointestinal...
proliferation of pathogenic bacterial strains, thereby reducing the incidence of enteric diseases during the weaning period. Nowadays, it is increasingly being realized that GIT health is largely influenced by the composition of the microbial community and diverse end-products of bacterial metabolism (Klose et al., 2010). In this context, diet composition has been identified as a major factor being responsible for differences in composition and function of the microbiota harboring the GIT of pigs (Mosenthin et al., 1999). Thus, nutritional strategies to modulate the intestinal microbial ecosystem appear to be a useful tool to improve GIT health. For example, enhancing growth and metabolic activity of beneficial members of the gastrointestinal microbiota and therefore promoting a stable GIT ecosystem by specific dietary ingredients provides one major option (Bauer et al., 2006). Accordingly, fermentable carbohydrates such as different types of resistant starch, non-starch polysaccharides (NSP) and nondigestible oligosaccharides have been shown to enhance proliferation of beneficial bacteria in the small intestine (Bikker et al., 2007). As a result, there was an increased production of short-chain fatty acids (SCFA) that may be used as energy source by the host (Gaskins, 2001), but which are also known to exert selective antimicrobial effects against certain pathogens (Dongowski et al., 2002). On the other hand, excessive fermentation of dietary protein should be avoided because of the production of detrimental substances, including ammonia, amines and phenols (Jensen, 2001). These substances have been associated with various detrimental effects in the GIT of the host animal, such as cytotoxic effects on epithelial cells (e.g. Gaskins, 2001). In addition, fermentation of protein often coincides with the growth of potential pathogens, such as Bacteroides and Clostridium species, thereby increasing the risk for infectious diseases (Macfarlane and Macfarlane, 1995). Depending on the source of protein in the diet but also the total amount of dietary protein fed to the animal, the availability of fermentable protein for the gastrointestinal microbiota may vary, thereby furnishing different amounts of substrate for bacterial proliferation and metabolic activity (Etheridge et al., 1984). Thus, by selecting feed ingredients containing protein of rather high ileal digestibility, a surplus of protein reaching the large intestine and serving there as substrate for protein-fermenting bacteria, could be avoided (Pluske et al., 2002). In that way, both the proliferation of protein-fermenting potential pathogens and the production of detrimental metabolites of protein fermentation can be suppressed. On the other hand, reducing the amount of dietary protein reaching the large intestine by lowering the total supply of dietary protein to the pig has also been described as an alternative option to avoid excessive protein fermentation (Heo et al., 2008). It is acknowledged that this approach potentially contributes to improvements in pigs’ health conditions and growth performance (Williams et al., 2005); however, the implementation of this strategy is limited by the animals’ minimum requirement for protein and amino acids (AA; Nyachoti et al., 2006).

There is currently considerable interest in scrutinizing the potentials and limitations of different strategies to minimize detrimental protein fermentation and the number of protein-fermenting bacteria in the GIT of pigs (e.g. Heo et al., 2008; de Lange et al., 2010). The main focus of the present review is directed to the discussion and comparative evaluation of these strategies.

The microbial ecosystem of the GIT: composition and interactions with the host

The GIT of monogastric animals including humans and pigs harbors a complex, numerically dense and metabolically active community of microbes (Macfarlane and Macfarlane, 2007). In pigs, the gastrointestinal microbiota is primarily composed of not only Gram-positive bacteria, such as aerotolerant Streptococcus, microaerobe or obligate anaerobe Lactobacillus, Bifidobacterium, obligate anaerobe Peptostreptococcus, Clostridium and Ruminococcus, facultative anaerobe Escherichia, but also obligate anaerobe Gram-negative bacteria such as Fusobacterium, Bacteroides, Selenomonas, Butyrivibrio and Prevotella. Detailed reports on the composition of the pigs’ gastrointestinal microbiota have been published elsewhere (e.g. Gaskins, 2001). Both, the relative proportion of species and the absolute quantity of bacteria, vary considerably along the digestive tract (Savage, 1977), with bacterial densities generally increasing from the proximal to the distal parts of the GIT (Gaskins 2001; Jensen, 2001). In contrast to the human GIT, the pigs’ GIT also harbors a substantial number of indigenous bacteria, mainly lactobacilli, in proximal sections, that is, in the stomach (Conway, 1994) and small intestine (Jensen and Jørgensen, 1994). In addition to a proximal to distal gradient in bacterial density within the GIT of pigs, there is also a radial distribution of microbes within each segment of the gut. In total, there are four microhabitats colonized by the commensal microbiota, which include the gastrointestinal lumen, the unstirred mucus layer, the deep mucus in the crypts and the surface of the intestinal epithelial cells (Pluske et al., 2002). Consequently, the microbiota present in digesta and feces is not fully representative for the entire GIT microbiota, even though digesta will likely contain microbes from mucosa that have been shed into the digesta as a result of mucosal sloughing or release of attached microbes (Richards et al., 2005).

There exist mutual interactions between host organism and gastrointestinal microbiota. This has been shown for different species, for example, men, rats (Collinder et al., 2003) and pigs (Hopwood and Hampson, 2003). For example, the composition and activity of the gastrointestinal microbiota is influenced by immune and stress status of the host, pathogen pressure originating from the environment and competition for substrates of endogenous and exogenous origin, such as carbohydrates, AA or minerals (Stewart et al., 1993). Moreover, the presence of local defense systems such as specific immune response, as well as gut receptors to which certain bacteria may attach, will also affect the prevailing microbial community (Stewart et al., 1993). On the other hand, the gastrointestinal microbiota supports the host animal through immune modulation (Fanning et al., 2012), colonization
resistance against pathogens via competition for nutrients and adhesion sites or production of antimicrobial substances (Rolfe, 1997). According to an in vitro study by Roselli et al. (2007) using porcine intestinal epithelial cells, some lactobacilli strains are able to exert protective effects against damages of the epithelial barrier induced by enterotoxigenic E. coli. Furthermore, the gastrointestinal microbiota provides nutrients to the host, for example, by production of straight-chain SCFA, which are rapidly absorbed from the large intestine as energy source (Gaskins, 2001). There are also metabolic costs to the host because of bacterial degradation of dietary or endogenous protein resulting in lower availability of AA and energy for maintenance and protein synthesis (Columbus et al., 2010). Furthermore, bacteria can have a direct impact on the genetic programming of the GIT (Kelly and King, 2001) such as gene expression of epithelial cells, for example, of genes coding for brush border digestive enzymes (Willing and van Kessel, 2009).

Carbohydrate fermentation

Fermentable carbohydrates reaching the GIT are the major source of energy for the gastrointestinal microbiota. They consist of different types of resistant starch, NSP, nondigestible oligosaccharides, such as fructo- or galactooligosaccharides, but also inulin (Houdijk et al., 2002). Fermentation of carbohydrates is considered to be most pronounced in the proximal colon where higher concentrations of SCFA and a more acidic pH have been determined (Williams et al., 2005). However, certain carbohydrates including starch, some soluble β-glucans, oligosaccharides and short-chain inulin may also be fermented in the small intestine. The saccharolytic activity of the gastrointestinal microbiota is considered to be beneficial for the host (Geboes et al., 2006) because of the formation of various straight-chain SCFA including acetic, propionic and butyric acid. In addition, different fermentation gases (H₂, CO₂, and methane) are being produced (Varel and Yen, 1997). The trophic effect of SCFA on the intestinal epithelium has been reviewed elsewhere (e.g. Bauer et al., 2006). Moreover, SCFA create a slightly acidic environment (Unger and Viernstein, 2004), thereby preventing the growth of acid-sensitive detrimental bacteria, including pathogenic Salmonella and E. coli (Wells et al., 2005). SCFA are rapidly absorbed from the hindgut and may provide up to 30% of the maintenance energy requirements for growing pigs (Morita et al., 2004). The promotion of specific bacterial groups or strains with beneficial properties for the host is mainly directed to the proliferation of Lactobacillus and Bifidobacterium, also referred to as probiotic bacteria. These genera are known for playing an important role in inhibiting the establishment of pathogenic bacteria via a mechanism known as competitive exclusion, also described as colonization resistance, for example, by better competing for nutrients or adhesion sites (Van der Waaij et al., 1991). However, it has to be considered that the microbiota of the porcine GIT appears to be highly individual (Loh et al., 2006), and that in the intestinal contents of about 60% of the assessed pigs no bifidobacteria were detectable (e.g. Loh et al., 2006). However, according to Loh et al. (2006), the role of bifidobacteria in the GIT of pigs is underestimated, maybe because of the analytical detection methods, such as fluorescent in situ hybridization, where the detection limit is comparably high, which may partly explain the low number of bifidobacteria.

Furthermore, lactic acid, as produced during carbohydrate fermentation by many intestinal bacteria, such as lactobacilli, bifidobacteria or enterococci, is utilized in the GIT of pigs by Megasphaera, Selenomonas and Veillonella, resulting in the formation of acetate, propionate but also butyrate (Duncan et al., 2004), the preferred energy source of colonocytes (Loh et al., 2006). Eubacterium spp. and Clostridium spp. also seem to be able to utilize lactate, but need extracellular acetate to produce butyrate (Ushida et al., 2002). This cross-feeding between intestinal bacteria plays an important role in preventing intestinal diseases, otherwise lactate may assimilate in the large intestine and cause diarrhea because of dyspepsia (Ushida et al., 2002).

Protein fermentation

In addition to carbohydrates, protein both from exogenous and endogenous sources can be used as a fermentable substrate by the gastrointestinal microbiota (Macfarlane and Macfarlane, 1995). Although for the pig, a considerable part of the carbohydrate fraction is fermented in the proximal parts of the GIT, protein fermentation takes place more distally, in particular, when there are less fermentable carbohydrates available (Williams et al., 2005). However, as protein is present throughout the whole GIT in the form of nondegradable and endogenous sources (Williams et al., 2005), some fermentation may also occur in the small intestine, mainly of AA and peptides. The degradation of protein results – unlike the fermentation of carbohydrates – in a number of additional metabolites such as branched-chain fatty acids (BCFA), but also potentially toxic products including ammonia, amines, phenols and indoles are being formed (Williams et al., 2005; Bikker et al., 2007). The BCFA originate from the deamination of branched-chain AA such as valine, isoleucine and leucine, and include isobutyrate, 2-methyl-butyrate and iso-valerate (Macfarlane and Macfarlane, 1995). These substances are formed by many bacteria such as Bacteroides spp., Propionibacterium spp., Streptococcus and Clostridium species. For example, BCFA are produced in large amounts by clostridia via the Stickland reaction (Macfarlane and Macfarlane, 1995). This chemical reaction involves the coupled oxidation and reduction of AA to organic acids. Thus, intestinal concentrations of BCFA may be used as indicator for the extent of protein fermentation (Macfarlane et al., 1992).

Ammonia. Ammonia present in the GIT of pigs is a toxic catabolite of microbial AA deamination, or it originates from urea hydrolysis due to the action of bacterial urease (Mosenthin et al., 1992). Ammonia is rapidly absorbed into the portal blood, converted to urea in the liver and subsequently excreted in the urine. A second pathway is that ammonia is assimilated by bacteria to synthesize bacterial
protein, provided that fermentable carbohydrates are available to be used as energy source. Such a shift of urinary to fecal nitrogen excretion may help to reduce ammonia emission (Mosenthin et al., 1992). Ammonia is one of the major odor components of pig manure contributing to environmental pollution (Otto et al., 2003). The concentration of ammonia in the large intestine depends on the balance between AA deamination and bacterial protein synthesis (Mosenthin et al., 1992; Hughes et al., 2000). Therefore, a reduction of ammonia emission from pig manure seems to be possible by lowering dietary protein (Otto et al., 2003).

**Amines.** A number of gastrointestinal bacteria such as *Bacteroides*, clostridia and bifidobacteria but also enterobacteria, lactobacilli and streptococci are capable of producing amines through decarboxylation of AA. Therefore, high levels of amines in pigs’ digesta and feces eventually indicate an increased activity of these bacteria in the GIT (Gaskins, 2001). Amines include monoamines such as tyramine, dimethylamine but also polyamines including cadaverine, agmatine, histamine, spermidine, putrescine and spermine (Pietrzak et al., 2002). As was reviewed by Pietrzak et al. (2002), they may exert varying physiological effects on the host. For example, some polyamines, such as spermidine and spermine, have been shown to be essential for somatic cell growth. On the other hand, above certain threshold levels, amines present in the GIT may produce detrimental effects such as increasing the incidence of diarrhea (Pietrzak et al., 2002). In humans, amines are rapidly absorbed from the GIT mucosa or liver, or excreted via urine (Hughes et al., 2000). These findings have been confirmed for histamine in pig’s colon by Aschenbach et al. (2006).

**Phenolic compounds.** Phenolic and indolic compounds are microbially produced from the aromatic AA phenylalanine, tyrosine and tryptophan. Major genera that have been identified to break down aromatic AA are *Bacteroides*, clostridia and bifidobacteria (Macfarlane and Macfarlane, 1995). In the healthy GIT of humans, phenolic compounds are absorbed in the colon. Thereafter, they are detoxified by mono- and diamine oxidases in the gut mucosa or liver, or excreted via urine (Hughes et al., 2000). A positive correlation between dietary protein intake and urinary phenol excretion has been reported in pigs (Otto et al., 2003).

**Impact of dietary protein on the incidence of PWD**

Abrupt weaning of young piglets is characterized by a sudden transit from a milk-based to a solid feed-based diet (Kluess et al., 2010). As a result, a marked decrease in voluntary feed intake may occur before the piglets adapt to solid feed (e.g. Hopwood and Hampson, 2003). Changes in feeding behavior and diet composition are also reflected in considerable alterations in the composition of the gastrointestinal microbiota adapting to the new environment (Kluess et al., 2010). This period is often associated with a growth check because of a high incidence of gastrointestinal disorders such as PWD (Aherne et al., 1992). Commonly, PWD is characterized by an increase in microbial fermentation of undigested protein, resulting in the formation of watery feces in combination with growth impairment, morbidity and even mortality (Wellock et al., 2008a). Some studies have been conducted to assess the detrimental effect of protein fermentation on piglets’ GIT health. For example, Heo et al. (2008) reported elevated plasma urea levels, together with a higher incidence of PWD when feeding a high- (243 g/kg) compared with a low- (173 g/kg) CP diet to weaned piglets. Similarly, Bikker et al. (2006) determined higher ammonia concentrations in the small intestine of weaned piglets fed a high- (216 g/kg) compared with a low- (152 g/kg) CP diet. They concluded that protein fermentation was reduced because of the lower supply of dietary protein; however, there was no effect on intestinal morphology, such as villus height and crypt depth. In general, an increase of ammonia concentration is assumed to exert detrimental effects on the health of the GIT, as ammonia can negatively affect growth and differentiation of intestinal epithelial cells (Gaskins, 2001), and may cause disturbances of the intestinal microbial balance during weaning (Bertschinger et al., 1979).

It appears that fermentation of protein is often linked to the growth of potential pathogenic bacteria (Ball and Aherne, 1987), such as β-hemolytic enterotoxigenic strains of *E. coli* (ETEC; Heo et al., 2008), but rather indirect. They may proliferate in the small intestine and release enterotoxins (Hampson, 1994). However, during weaning, the initially predominant lactobacilli are decreasing; therefore, their beneficial metabolic activities such as the stimulation of GIT immunity and the formation of SCFA are suppressed (Jensen, 1998). As a consequence of these cascading effects, proliferation of *E. coli* is more facilitated. In addition, diets with a high-CP content have a high buffering capacity (Partanen and Mroz, 1999) and will increase small intestinal pH, thereby favoring the proliferation of pathogenic bacteria (Htoo et al., 2007). Therefore, *E. coli* has been suggested to be an opportunist rather than a primary pathogen (Li et al., 1990).

**Strategies to reduce protein fermentation and potential pathogenic proteolytic bacteria in the GIT**

Principally, dietary protein becomes available for bacterial fermentation when it escapes digestion by host enzymes (Libao-Mercado et al., 2009). Consequently, with decreasing protein digestibility and/or increasing supply of dietary protein to the pig, more protein will become available to the microbiota in the large intestine. This may lead to an increase in protein fermentation, possibly associated with an enhanced proliferation of protein-fermenting bacteria (Libao-Mercado et al., 2009). The source of protein, its quality and level in the diet may have an influence on the site of fermentation within the GIT. It is generally accepted that
highly digestible protein sources such as casein are digested by host enzymes in the proximal GIT. As a result, they are not available for microbial fermentation (Pluske et al., 2002). On the other hand, plant protein sources are usually not completely digested by host enzymes, which makes them available for microbial fermentation more distally, especially at higher dietary protein levels. According to Rist et al. (unpublished data), no effects of protein source (i.e. soybean meal (SBM) vs. casein) on bifidobacteria, lactobacilli and clostridia could be observed in ileal digesta of weaned piglets, whereas in feces these bacterial groups were increased for piglets fed the SBM-based diet. This effect was even more pronounced when piglets were fed diets with elevated protein levels, suggesting increased availability of nutrients to the large intestinal microbiota. It has to be acknowledged that plant protein sources such as SBM also contain relatively high amounts of fermentable carbohydrates. As a result, with any increase in dietary inclusion level of plant proteins, a greater part of these carbohydrates will pass into the distal parts of the GIT. In addition, protein quality may have an influence on the site of fermentation. As determined in humans by Scott et al. (2012), heat-damaged pea protein is less digestible by host enzymes in the small intestine and is therefore reaching the more distal parts of the GIT where protein fermentation dominates. In conclusion, the amount and composition of fermentation metabolites in the different sites of the GIT, mainly in the large intestine, depend on the digestibility of the dietary proteins, which in turn is influenced by the quality, source and level of dietary protein (Windey et al., 2012). Thus, in the following, dietary strategies will be described, aiming to reduce protein fermentation through reduction of growth and metabolic activities of potential protein-fermenting pathogens.

Source of dietary protein

In piglets fed highly digestible protein, for example, casein, the protein is almost completely digested and absorbed before reaching the large intestine (Morita et al., 2004). Therefore, it has been suggested that diets formulated to contain highly digestible protein ingredients would be appropriate to reduce the amount of substrate available for protein fermentation, thereby reducing the frequency of PWD in piglets (Pliske et al., 2002). In several studies on pigs, the effect of different dietary protein sources on intestinal protein fermentation including formation of detrimental metabolites, such as ammonia or phenols, has been described (e.g. Blasco et al., 2005). In addition, the effect of source of dietary protein on the incidence of protein fermentation-induced diarrhea has been investigated, however, with controversial results. For example, in a challenge study by Owusu-Asiedu et al. (2003), piglets were orally infected with ETEC K88 and either fed a diet based on plant protein (pea protein) or animal protein (spray-dried porcine plasma). Piglets fed the pea protein diet had a higher incidence of diarrhea, and mortality was increased compared with piglets fed the plasma diet. In contrast, Wellock et al. (2006) observed no different effects of a plant protein source (SBM) in comparison with an animal protein source (dried skim milk powder) on fecal scores of piglets. It can be speculated whether these differences in response to plant and animal protein have been biased by the challenge situation in the aforementioned study by Owusu-Asiedu et al. (2003). Another factor that affects fecal consistency, but not mentioned in the study by Owusu-Asiedu et al. (2003), may be the temporal hypersensitivity against soy proteins as determined by Li et al. (1990). In the study of Li et al. (1990), piglets fed a SBM-based diet had shorter villi and a higher immunoglobulin level than piglets fed a dried skim milk-based diet, indicating the antigenic property of SBM. According to Li et al. (1990), the formation of complexes between antigens present in the SBM such as glycinin and β-conglycinin and immunoglobulin antibodies may be responsible for the damage to villi. Furthermore, plant protein sources often contain anti-nutritive factors including elevated levels of soluble NSP present in SBM that may increase the incidence of diarrhea in weaned piglets (Choc et al., 2010).

As increased intestinal protein fermentation suggests proliferation of potential pathogens (Hughes et al., 2000), several studies were designed to examine the effect of dietary protein source on composition (Table 1) of the intestinal microbiota. For example, in a study by Etheridge et al. (1984), piglets at 7 days of age were fed either a corn–SBM starter diet or an oat–casein diet or they remained with the sow (control treatment). The control animals were weaned at 21 days of age. At this time, piglets fed the corn–SBM diet showed a lower growth performance associated with a higher incidence of diarrhea than the other treatments. According to the authors, the microbial breakdown of undigested proteins in the lower digestive tract might have been responsible for the observed growth check. In addition, although this was not within the scope of these studies, the occurrence of immune reactions against dietary antigens such as soy protein has to be considered as a possible factor that may negatively affect fecal consistency (Li et al., 1990). However, the observed lower growth performance and higher incidence of diarrhea for the corn–SBM group did not correspond to fecal coliform counts, which did not differ from those of the control treatment, but were highest for the oat–casein treatment. In addition, in this study, the counts of total bacteria were higher in piglets fed the corn–SBM or oat–casein diet between days 23 and 27 of age, compared with the control treatment (Etheridge et al., 1984). Higher fecal counts of total bacteria might reflect an increase in microbial diversity, which would rather be associated with enhanced stability of the microbial community and therefore an increased resistance to diseases (Konstantinov et al., 2004). Kellogg et al. (1964) also observed differences in fecal microbial counts in newly weaned piglets as influenced by the protein source in the diet (SBM, dried skim milk, fish meal, meat meal or cottonseed meal). In this study, the plant protein sources resulted in a marked reduction in fecal counts of pathogenic coliforms and also of staphylococci. This observation suggests a reduced activity of the proteolytic microbiota upon feeding plant protein sources to newly
weaned piglets. In the study by Kellogg et al. (1964), SBM and dried skimmmed milk increased total anaerobes and total aerobes, whereas the other protein sources resulted in lower fecal counts of these bacteria. Wellock et al. (2006) could show that newly weaned piglets fed a diet based on dried skimmed milk powder consumed more feed, resulting in higher BW gain and improved feed conversion ratio than those offered a SBM-based diet. Although all piglets remained clinically healthy, in piglets fed the SBM-based diet, the lactobacilli to enterobacteria ratio was raised from 100 to 200 g/kg, and counts of lactobacilli increased when the protein level of the diets was increased from 100 to 200 g/kg. Beyond these levels, total counts of aerobes and total anaerobes in fecal samples of newly weaned piglets as protein level in a corn–SBM-based diet increased beyond a level of 200 g/kg (Kellogg et al., 1964). In more recent studies with newly weaned piglets, a SBM-based diet from 230 to 130 g/kg was associated with lower fecal water content. In addition, fecal samples contained lower concentrations in the small intestine (Bikker et al., 2006), lower fermentation activity as indicated by lower ammonia concentrations in the small intestine (Bikker et al., 2006), lower plasma urea levels and reduced contents of SCFA in ileal digesta (Nyachoti et al., 2006) compared with high-protein diets. However, studies in piglets revealed inconsistent results on the effect of protein level in the diet on composition of the gastrointestinal microbiota (Table 2).

### Table 1 Effect of dietary protein source on the composition of the intestinal and fecal microbiota of weaned piglets

<table>
<thead>
<tr>
<th>Protein sources</th>
<th>Sampling site</th>
<th>Response</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBM4</td>
<td>Feces</td>
<td>Total bacteria ↑; yeast and molds ↓</td>
<td>Etheridge et al. (1984)</td>
</tr>
<tr>
<td>Casein</td>
<td>Feces</td>
<td>Total bacteria ↑; yeast and molds ↓</td>
<td>Kellogg et al. (1964)</td>
</tr>
<tr>
<td>SBM</td>
<td>Feces</td>
<td>Lactobacilli, total aerobes, total anaerobes, streptococci ↑; coliforms, staphylococci ↓</td>
<td>Kellogg et al. (1964)</td>
</tr>
<tr>
<td>Dried skimmed milk</td>
<td>Feces</td>
<td>Lactobacilli, total aerobes, total anaerobes, streptococci ↑; coliforms, staphylococci ↓</td>
<td>Kellogg et al. (1964)</td>
</tr>
<tr>
<td>Fish meal</td>
<td>Distal jejunum</td>
<td>Enterobacteria, lactobacilli, lactobacilli: Enterobacteriaceae ↔</td>
<td>Kellogg et al. (2009)</td>
</tr>
<tr>
<td>SBM, dried skimmed milk</td>
<td>Ileum, colon, feces</td>
<td>Coliforms, lactobacilli, lactobacilli to coliforms ratio ↔</td>
<td>Kellogg et al. (2006)</td>
</tr>
</tbody>
</table>

1↑increase, ↓decrease, ↔no effect, SBM — soybean meal.
2Microbial plate counts.
3Compared with sow milk.
4Compared each with the other.
5Piglets showed clinical signs of diarhoea.

#### Dietary protein intake

In rats, the total protein intake rather than the source of dietary protein has been identified as a major factor eventually affecting the extent of protein fermentation in the GIT, and also intestinal bacterial composition (Le Leu et al., 2006). In addition, experiments in which weaned piglets were fed a low-protein diet resulted in a reduction of protein fermentation activity as indicated by lower ammonia concentrations in the small intestine (Bikker et al., 2006), lower plasma urea levels and reduced contents of SCFA in ileal digesta (Nyachoti et al., 2006) compared with high-protein diets. However, studies in piglets revealed inconsistent results on the effect of protein level in the diet on composition of the gastrointestinal microbiota (Table 2).
lower counts of pathogenic bacteria, such as coliforms, and a higher ratio of lactobacilli to coliforms, indicating a decreased risk for the development of PWD.

In several studies with newly weaned piglets, protein-reduced diets were formulated and supplemented with graded levels of crystalline AA to meet piglets’ AA requirements (e.g. Heo et al., 2008). Although the supplementation of essential AA to pig diets is becoming increasingly common in commercial practice, some restrictions may apply in their use in protein-reduced starter diets. There is evidence that a decrease in dietary CP content may create serious deficiencies of nonessential AA, resulting in reduced growth performance (Opapeju et al., 2008). For example, Nyachoti et al. (2006) observed a decline in weaned piglets’ growth performance if the dietary CP content was 190 g/kg or less. At the same time, the authors reported no effect on ileal digesta counts of total coliforms, anaerobic sporeformers, aerobic sporeformers, Enterobacteriaceae, enterococci and E. coli when weaned piglets were fed diets with graded levels of CP ranging from 170 to 230 g/kg. However, feeding protein-reduced diets was associated with lower contents of ammonia in ileal digesta, indicating reduced fermentation activity of protein in the small intestine but without affecting the composition of the bacterial community. These findings were confirmed by Pieper et al. (2012) who measured lower ammonia concentrations in piglets’ colon when feeding 147 g/kg CP compared with 200 g/kg diets. However, in this study, the high-CP diet increased counts of the Clostridium leptum group in the proximal colon of weaned piglets, but had no effect on total bacteria, lactobacilli, enterobacteria, Bacteroides and the Clostridium coccoides group in the proximal colon. According to a study of Bikker et al. (2007), no effect on counts of lactobacilli and coliform bacteria were found in jejunum and colon of newly weaned piglets fed a potato protein-based diet supplemented with wheat and maize gluten meal with 220 g/kg CP in comparison with piglets fed the basal diet with 150 g/kg CP. However, the authors observed a decrease in ammonia concentration in jejunal and colonic digesta samples of piglets fed the low-protein diet, suggesting reduced protein fermentation.

### Table 2 Effect of dietary protein level on the composition of the intestinal and fecal microbiota of weaned piglets

<table>
<thead>
<tr>
<th>CP levels (g/kg, as fed)</th>
<th>Protein source</th>
<th>Sampling site</th>
<th>Response</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>225, 176</td>
<td>Milk protein</td>
<td>Colon</td>
<td>Lactic acid bacteria ↓↓</td>
<td>Opapeju et al. (2009)1,2,4</td>
</tr>
<tr>
<td>130, 180, 230</td>
<td>Milk protein</td>
<td>Colon</td>
<td>Coliforms ↑↑ until 230 g/kg</td>
<td>Wellock et al. (2006)1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lactobacilli ↓ until 230 g/kg</td>
<td></td>
</tr>
<tr>
<td>230, 130</td>
<td>Milk protein</td>
<td>Colon</td>
<td>Lactobacilli to coliforms ratio ↓ until 230 g/kg</td>
<td>Wellock et al. (2008b)1,4</td>
</tr>
<tr>
<td>197, 217</td>
<td>Poultry meal</td>
<td>Colon</td>
<td>Coliforms ↓↓</td>
<td>Jeaurond et al. (2008)1</td>
</tr>
<tr>
<td>150, 220</td>
<td>SBM</td>
<td>Jejunum, colon</td>
<td>Lactic acid bacteria ↓↓</td>
<td>Bikker et al. (2006 and 2007)1</td>
</tr>
<tr>
<td>154, 194</td>
<td>SBM</td>
<td>Feces</td>
<td>Escheria coli, enterococci (plate counts) ↓↓</td>
<td>Hermes et al. (2009)1,2</td>
</tr>
<tr>
<td>100, 150, 200, 250, 300</td>
<td>SBM</td>
<td>Feces</td>
<td>Enterobacteria, lactobacilli (PCR) ↓↓</td>
<td>Kellogg et al. (1964)1</td>
</tr>
<tr>
<td>170, 190, 210, 230</td>
<td>SBM + fish meal</td>
<td>Ileum</td>
<td>Lactic acid bacteria ↓↓</td>
<td>Nyachoti et al. (2006)1</td>
</tr>
<tr>
<td>147, 200</td>
<td>SBM</td>
<td>Colon</td>
<td>Enterobacteriaceae ↓↓</td>
<td>Pieper et al. (2012)2</td>
</tr>
</tbody>
</table>

†increase, ↓ decrease, ↔ no effect, SBM = soybean meal.
*Microbial plate counts.
1PCR.
2Piglets showed clinical signs of diarrhea.
3Piglets were challenged with E. coli.
Obviously, a reduction in dietary protein content may result in a lower intestinal ammonia concentration, but not necessarily in changes of the intestinal bacterial community, maybe because of the ability of the microbiota to adapt to a certain extent to changes in the availability of nutrients (Heo et al., 2012). In contrast, in studies with weaned piglets experimentally infected with pathogens, dietary CP supply had more pronounced effects on the composition of the intestinal microbiota. For example, in a study by Opapeju et al. (2009), newly weaned piglets were fed corn–SBM–fish meal diets, containing either 225 or 176 g/kg CP. They were supplemented with crystalline AA to meet the animals’ AA requirement. Eight days post weaning, piglets were challenged with ETEC K88. Thereafter, they were slaughtered on days 1, 3 and 7 post challenge. Three days after being challenged, ETEC was not present in ileal digesta of piglets fed the 176 g/kg CP diet, but could be found in ileal digesta of 80% of piglets fed the 225 g/kg CP diet. Moreover, piglets fed the 225 g/kg CP diet had a higher ammonia-N concentration in colonic digesta on days 1 and 7 post challenge, indicating a higher protein fermentation activity. On the other hand, in piglets fed the 176 rather than the 225 g/kg CP diet, counts of carbohydrate-fermenting, butyrate-producing *Roseburia* were higher in colonic digesta on day 7. According to the authors, a reduction of dietary protein supply may inhibit the proliferation of those pathogens (e.g. ETEC), which preferentially ferment protein. As a result, there is a shift in intestinal microbial composition toward higher counts of beneficial bacteria, which preferentially ferment carbohydrates (Opapeju et al., 2009). In addition, in another challenge study with newly weaned piglets, which were orally infected with ETEC, a reduction of dietary protein content from 230 to 130 g/kg in SBM-based or dried skimmed milk powder-based diets was associated with lower fecal excretion of ETEC (Wellock et al., 2008a and 2008b).

In a study by Hermes et al. (2009) on 35-day-old piglets, the effects of diets with either 190 or 150 g/kg CP on bacterial composition in feces were assessed. In this study, no differences in fecal samples were found between treatments, on counts of coliforms, enterococci, enterobacteria and lactobacilli. When using finisher boars, O’Shea et al. (2010) also observed no differences in total counts of *Lactobacillus* spp. and enterobacteria both at the ileal and fecal level, when the protein content of a wheat–SBM-based diet was decreased from 200 to 150 g/kg CP. Given the stable intestinal microbiota of an adult mammal, one might assume that it would be rather difficult to influence microbial composition by dietary means when compared with a weaner piglet harboring an instable microbiota (Konstantinov et al., 2004).

Altogether, results in studies with piglets are rather inconsistent, though it appears that feeding diets with high levels of protein is in favor of increasing the number of protein-hydrolyzing bacteria (e.g. Wellock et al., 2006). Furthermore, it appears that, although this must not necessarily be reflected in the intestinal bacterial composition, a reduction in dietary protein content may lower detrimental protein fermentation as indicated, for example, by decreased intestinal ammonia concentrations (Bikker et al., 2007; Pieper et al., 2012). Furthermore, in piglets challenged with pathogens, changes in dietary protein content are reflected in the composition of the intestinal microbiota, with reduced counts of pathogens for lower protein content. Thus, a reduction of dietary CP in diets for weaned piglets is suggested, in particular in stress situations such as increased pressure of pathogens and increased risk of infections.

**Dietary supplementation of fermentable carbohydrates**

As was reviewed by de Lange et al. (2010), the use of fermentable carbohydrates in pigs’ diet seems to be the most promising approach to beneficially affect the composition and activity of the intestinal microbiota. Therefore, in an effort to possibly reduce protein fermentation in the GIT, some authors suggested to add fermentable carbohydrates to the diet (Awati et al., 2006; Jeaurond et al., 2008), thereby shifting fermentation processes toward carbohydrate rather than protein fermentation (Kim et al., 2008). Such a shift in fermentation activity is reflected in lower levels of ammonia and BCFA as indicators of reduced protein fermentation (e.g. Williams et al., 2005). To stimulate beneficial bacteria, fermentable carbohydrates must be neither hydrolyzed nor absorbed in the upper GIT of the animal (Branner et al., 2004). Fermentable carbohydrates that have been frequently used in pig diets include NSP, such as cellulose, hemicellulose or pectin, resistant starches and nondigestible oligosaccharides (Bauer et al., 2006). For example, after inclusion of sugar beet pulp, inulin, lactulose and wheat starch in diets for newly weaned piglets, Awati et al. (2006) determined lower fecal concentrations of ammonia and BCFA. Obviously, owing to the preferential fermentation of these carbohydrates, the formation of potentially harmful metabolites originating from protein fermentation was reduced. In addition, in some studies designed to limit protein fermentation in the GIT of pigs, the effect of fermentable carbohydrates on composition of the intestinal microbiota was examined (Table 3).

According to Konstantinov et al. (2004), the addition of nondigestible, fermentable carbohydrates (sugar beet pulp and fructooligosaccharides) to the diet of weaned piglets increased the number of lactobacilli in the small intestine, and enhanced stability and diversity of the bacterial community in the porcine colon, thereby eventually suppressing potential pathogenic proteolytic bacteria. The authors suggest that the inclusion of fermentable carbohydrates improved gut health during weaning because of a shift from protein to carbohydrate fermentation. According to Houdijk et al. (2002), the supplementation of nondigestible oligosaccharides to the diet of newly weaned piglets affected the microbial population at the ileal, but not at the fecal level. In this study, total aerobes and enterococci were lower upon dietary inclusion of fructo- and transgalactooligosaccharides compared with piglets fed the control diet. Moreover, lactobacilli and *Bacteroides* spp. were not affected, but supplemental oligosaccharides had a reducing effect on potential harmful proteolytic *E. coli* (Houdijk et al., 2002). Furthermore, Lynch et al. (2009) reported that the inclusion...
of inulin (15 g/kg) and lactose (230 g/kg) to a low- (160 g/kg) and a high- (200 g/kg) CP diet for piglets increased *Lactobacillus* spp. and decreased Enterobacteria spp. in feces of weaned piglets. In addition, supplementation of inulin together with high lactose (230 g/kg) decreased BCFA, compared with piglets offered inulin-supplemented diets containing only 50 g/kg lactose. Obviously, a combination of inulin and high lactose was able to beneficially affect microbial composition and reduce harmful protein fermentation. However, different concentrations of inulin in the diets, the degree of polymerization of inulin and other factors affect microbial degradation, which may explain varying effects on the fermentative activity of the intestinal microbiota (Paßlacket al., 2012).

Hermes et al. (2009) added two native sources of NSP, wheat bran and sugar beet pulp to a low- (160 g/kg) and a high- (200 g/kg) protein diet fed to piglets. The counts of coliforms in feces were lower, whereas the lactobacilli to enterobacteria ratio tended to be higher upon supplementing the diets with NSP. There was a lower incidence of diarrhea when NSP was supplemented to the high-protein diet but a higher incidence of diarrhea when NSP was supplemented to the low-protein diet. According to the authors, the higher-dietary CP content was associated with enhanced protein fermentation in the colon, whereas the lower concentration of NSP in the diet. However, different concentrations of inulin in the diets, the degree of polymerization of inulin and other factors affect microbial degradation, which may explain varying effects on the fermentative activity of the intestinal microbiota (Paßlacket al., 2012).

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### Table 3

<table>
<thead>
<tr>
<th>Carbohydrates</th>
<th>Sampling site</th>
<th>Response</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat bran, sugar beet pulp</td>
<td>Feces</td>
<td>Coliforms ↓</td>
<td>Hermes et al. (2009)¹,²</td>
</tr>
<tr>
<td>Fructo-oligosaccharides, transgalactos</td>
<td>Ileum</td>
<td>Total aerobes ↔</td>
<td>Houdijk et al. (2002)³</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Enterococci ↓</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total anaerobes ↔</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Bacteroides</em> ↔</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Lactobacilli</em> ↔</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Bifidobacteria</em> ↔</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Escherichia coli</em> ↔</td>
<td></td>
</tr>
<tr>
<td>Inulin, lactulose, wheat starch, sugar beetle pulp</td>
<td>Colon</td>
<td><em>Lactobacilli</em> ↑</td>
<td>Konstantinov et al. (2003)²</td>
</tr>
<tr>
<td>Lactose, inulin</td>
<td>Feces</td>
<td><em>Lactobacilli</em> spp. ↑</td>
<td>Lynch et al. (2009)¹</td>
</tr>
<tr>
<td></td>
<td>Colon</td>
<td><em>Enterobacteria</em> spp. ↓</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Colon</td>
<td><em>Bacteroides</em> ↑</td>
<td>Pieper et al. (2012)²</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Clostridium leptum</em> ↑</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Clostridium coccoides</em> ↑</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total bacteria ↔</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Lactobacilli</em> ↔</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Enterobacteria</em> ↔</td>
<td></td>
</tr>
</tbody>
</table>

¹ increase, ² decrease, ↔ no effect.

*Microbial plate counts.

PCR.

The authors also determined the lowest incidence of diarrhea for the low-protein diet without supplemental NSP, whereas cell counts of lactobacilli in the proximal colon were lowest for the low-protein diet supplemented with NSP. Moreover, they observed increased numbers of potential proteolytic *Bacteroides* and *Clostridium*. However, despite this increase putrescine and ammonia levels in the proximal colon were lower in piglets fed the NSP supplemented diet, likely due to a reduced protein fermentation activity. Obviously, shifts in microbial composition as observed are not always reflected in changes of metabolic activity as anticipated; however, overall there is growing evidence that supplementation of piglets’ diets with fermentable carbohydrates seems to be a promising approach to reduce both intestinal protein fermentation and potential pathogenic bacteria.

### Conclusion

Diet appears to be an important factor controlling the composition and metabolic activities of the gastrointestinal microbiota of single-stomached animals. Thus, it is essential to understand the interactions between the host, diet and intestinal microbiota. Although results were not always consistent, it appears that avoiding excessive amounts of protein reaching the lower GIT may reduce the incidence of PWD and inhibit proliferation of pathogenic bacteria, especially in piglets reared under nutritional and environmental stress conditions. In addition, although not always associated with changes in bacterial composition, a moderate reduction of dietary protein level may reduce formation of detrimental fermentation products, for example, ammonia. Moreover, there is evidence that the inclusion of fermentable carbohydrates seems to be a promising approach to reduce both intestinal protein fermentation and potential pathogenic bacteria.
carbohydrates seems to be promising in reducing detrimental protein fermentation and proliferation of potential pathogenic proteolytic bacteria. However, studies designed to assess the effect of protein level, protein source or fermentable carbohydrates on occurrence of proteolytic bacteria in the GIT are rather limited. Furthermore, owing to the wide variety of methods used, the results of these studies are sometimes difficult to compare. For example, most of the studies used culture-based bacterial count techniques, whereas only few studies were based on molecular methods for quantitative assessment of bacteria. By using more sensitive and culture-independent methods, at least some of the variation between results of different studies could be reduced. Furthermore, results between studies pertaining to assess the effect of different levels or sources of dietary protein on microbial composition and metabolic activity are often confounded by the use of varying ingredients in the basal diet. For example, supplementation of fermentable carbohydrates to a basal diet, already rich in contents of fermentable carbohydrates (e.g. cereal-based diet), may mask any potential effects of varying protein levels or sources on bacterial composition and activity. Thus, for a better comparability between studies, further research should take into consideration the use of standardized basal diets containing well-defined feed ingredients. Finally, it has to be emphasized that some studies have rather limited practical implications. For example, some experimental designs are based on the use of casein or dried skimmed milk as dietary protein sources. Although these feed proteins are almost completely digested in the small intestine and therefore hardly available for protein fermentation in the large intestine, their use in pigs’ diets is rather limited because of their low cost-efficiency.

Acknowledgments
The authors are grateful to AHRHOFF GmbH, Bönen, Germany for the financial support of Vanessa T. S. Rist. They thank Silke Hörner for her technical assistance.

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