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Review

Health relevance of intestinal protein fermentation in young pigs

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Abstract

The physiological role of the gastrointestinal microbiota has become an important subject of nutrition research in pigs in the past years, and the importance of intestinal microbial activity in the etiology of disease is doubtless. This review summarizes the recent knowledge related to the microbial ecology of protein fermentation and the appearance of protein-derived metabolites along the pig intestine. The amount of fermentable protein depends on factors such as dietary protein concentration, protein digestibility due to secondary or tertiary structure, the interaction with dietary compounds or anti-nutritional factors, and the secretion of endogenous proteins into the gut lumen. High protein diets increase the luminal concentrations and epithelial exposure to putatively toxic metabolites and increase the risk for post-weaning diarrhea, but the mechanisms are not yet clarified. Although the use of fermentable carbohydrates to reduce harmful protein-derived metabolites in pigs is well-established, recent studies suggest that the inclusion of fermentable carbohydrates into diets with low protein digestibility or high dietary protein level may not ameliorate all negative effects with regard to epithelial response. Based on the current knowledge, the use of diets with low levels of high-quality protein may help to reduce the risk for intestinal disease in young pigs.

Keywords: protein fermentation, amines, ammonia, short chain fatty acids, pigs, post weaning diarrhea.

Introduction

The gastrointestinal tract (GIT) of pigs is colonized by a highly diverse microbiota with distinct longitudinal, radial and timely distribution. Within a relatively short time, our knowledge about the pig intestinal microbiota community composition (Kim *et al.*, 2011a; Isaacson and Kim, 2012), the importance of early-life bacterial colonization patterns on immune system development (Mulder *et al.*, 2011; Lewis *et al.*, 2012), the composition of the mucosa-associated bacterial communities (Looft *et al.*, 2014; Mann *et al.*, 2014), or the long-term impact of microbial colonization patterns on the metabolic phenotype later in life (Merrifield *et al.*, 2015) has emerged substantially in the past years. A further understanding of the complex interaction of the three-component system nutrition – microbiota – host is pivotal to establish successful feeding and management strategies. It is well established that the content of fermentable substrates in pig diets drives the microbial ecology in the GIT and can thereby possibly exert health promoting or detrimental

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effects (Aumiller *et al.*, 2014). The promotion of lactic acid bacteria and short chain fatty acid production (e.g. specifically butyrate) is considered beneficial for the host and may help to maintain a 'balanced' intestinal ecosystem. On the other hand, bacterial protein fermentation taking place mainly in the hindgut, represents a potential risk factor for a compromised intestinal barrier function and increased enteric disease caused by pathogens. However, in pigs, the etiology of diarrhoea associated with higher intestinal protein fermentation is still highly unclear. Understanding the factors influencing intestinal bacterial protein fermentation, the formation of toxic metabolites and subsequent influence on the host is crucial to develop dietary strategies to maintain GIT health. In complement to recent reviews covering parts of this important topic (Rist *et al.*, 2013; Jha and Berrocoso, 2016), the present review will summarize recent advances in the understanding of microbial ecology of protein fermentation and discuss possible consequences for dietary interventions in pigs.

Factors influencing fermentable protein concentration in the gut

Outbalanced physiological conditions

External and endogenous factors that can influence protein digestion and absorption under normal and outbalanced physiological conditions along the GIT and some putative consequences for intestinal bacterial protein fermentation are summarized in Table 1. Generally, an efficient pre-caecal protein digestion and absorption is critical for both the supply of the animal with essential amino acids for protein synthesis and a reduced risk of excessive protein fermentation in the large intestine. Under normal physiological conditions, protein digestion begins in the stomach with the initiation of proteolysis through digesta acidification and the autocatalytic activation of pepsin from pepsinogen at low pH. This results in the release of a mix of polypeptides, oligopeptides and some free amino acids. The gastric phase of protein digestion may play only a minor role with regard to total pre-caecal protein digestibility (Erickson and Kim, 1990). As indicated below, there are some indications that dietary (i.e. digesta viscosity, dietary particle size, buffering capacity, antinutritional factors, diet changes) as well as endogenous factors (i.e stress or other hormonal signals affecting gastric emptying rate and feed intake) can lead to conditions that may favor bacterial fermentative activity already in the stomach and the onset of putrefactive fermentation (Table 1). In the small intestine, protein is further digested by pancreatic enzymes and intestinal brush border membrane enzymes to free amino acids and small oligopeptides, which can then be taken up through brush border amino acid transport systems (Ganapathy, 2012). It seems therefore clear that dietary (i.e. digesta viscosity and transit, diet changes, antinutritional factors) or animal-derived factors (i.e. gut immaturity at weaning, hormonal signals) or primary enteric infections (e.g. viruses) may interfere with these normal conditions and lead to inflammatory reactions, insufficient enzyme release or activation and endogenous (protein and mucus) secretions (Table 1). An increased flow of undigested protein into the distal parts of the GIT and promotion of bacterial protein fermentation are the logical consequence of these imbalances. In addition, dietary protein levels and fermentability may also favor the growth of specialized proteolytic bacteria (e.g. clostridia). Whether protein fermentation, the promotion of certain bacteria or host inflammatory signals may trigger further bacterial signals (i.e. quorum sensing) and the activation of bacterial pathogenic factors is yet unclear. In the large intestine, non-digested dietary compounds as well as secreted endogenous substrates can be fermented by the indigenous bacteria. As outlined below, the substrate availability is a major driver for bacterial fermentation pathways. An impaired pre-caecal protein digestion and absorption, high dietary protein level in general or higher endogenous secretions will therefore likely increase the amount of fermentable protein entering the large intestine, promote putrefactive fermentation and selective growth of proteolytic bacteria (Table 1). In turn, protein-derived metabolites and dysbioses may interfere with normal physiological conditions of the large intestine such as absorption of bacterial metabolites, electrolytes and fluids as outlined below.

Feed-derived factors influencing protein digestibility

Knowledge about the undigested protein fractions and their fermentability throughout the different parts of the intestine is still scarce. Usually, protein in animal feed is determined roughly as $N \times 6.25$ and further characterized by the standardized ileal amino acid digestibility, whereas the relationship between structural elements and susceptibility to proteolysis has not been clarified so far. Nonetheless, it is well established that in feedstuffs of lower quality, more undigested dietary proteins will enter the hindgut (Kambashi *et al.*, 2014). Variations observed in individual amino acid digestibility within a feedstuff may be greater than the variation among feedstuffs (Moughan *et al.*, 2014). For example, the quality of fishmeal may vary due to the animal species and tissues used and to the type of processing (Rojas and Stein, 2013). In plant ingredients, the structural properties of proteins may play a major role in the resistance to denaturation and gastrointestinal digestion. For example, the salt-soluble protein content rather than differences on the vitreousness of corn grains provides a better indication of susceptibility of the protein matrix to enzymatic hydrolysis (Gehring *et al.*, 2012). On one hand, the β-sheet structures of raw legume proteins and the intermolecular β-sheet aggregates, arising upon heating were highly negatively correlated with feed digestibility values ($r = -0.980$) (Carbonaro *et al.*, 2012). For soybean and barley seeds, β-sheet arrangement accounts for at least 30% of the whole secondary structure. In contrast, animal feed ingredients have a low content of the β-band: 10.7 and 6.9% for milk and chicken meat, respectively. The decrease in protein digestibility as dependent on the β conformations can be explained by the high hydrophobic character of these structures, which involves aromatic amino acids (phenylalanine, tyrosine, and tryptophan) and β-branched amino acids (leucine, isoleucine, and valine). Hydrophobic interactions may adversely affect the solubility of vegetable proteins by promoting protein–protein interaction and aggregate

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Table 1. Protein digestion and absorption under normal physiological conditions and factors modulating protein digestion and proteolytic fermentation along the gastrointestinal tract of pigs

Site of the GIT	Normal physiological conditions	Outbalanced physiological conditions	Contributing factors	Consequences for bacterial activity and metabolites production
Stomach	Acidic protein denaturation, pepsinogen release and autocatalytic activation through low pH	Reduced gastric emptying rate, gastric ulcers, limited digesta acidification, reduced proteolysis, reduced mucus secretion	High viscosity, reduced feed intake, stress, sudden dietary changes, endocrine signals, feed particle size (e.g. small particles), high buffering capacity of the feed (e.g. minerals, protein level), antinutritional factors (e.g. tannins, phytate, lectins)	Bacterial fermentative activity ↑ Organic acids ↑ Activation of bacterial amino acid deaminase and decarboxylase systems Ammonia ↑ Biogenic amines ↑ (nitrosation reactions)
Small intestine	Digesta alkalization, pancreatic proenzyme release and activation (trypsin, chymotrypsin, elastase, carboxypeptidases, proteases), brush border peptidases (endo-, amino-, carboxy-, di-), amino acid and small peptide absorption by enterocytes some bacterial amino acid synthesis	Exocrine pancreatic insufficiency, reduced activity of digestive enzymes, inflammatory processes, excessive endogenous (mucus) secretions	Reduced digesta transit (e.g. high viscosity and NSP), gut hormones, gut immaturity, antinutritional factors (e.g. protease inhibitors, lectins), weaning stress, dietary changes and composition, primary enteric infections (e.g. viruses)	Bacterial fermentative activity ↑ Ammonia ↑ Biogenic amines ↑ Proliferation of proteolytic bacteria Bacterial toxins and LPS ↑ Sulfur metabolism, H_2S production ↑
Large intestine	Acidic pH, Intense carbohydrate fermentation, production and absorption of SCFA, bacterial amino acid and protein synthesis and lysis of endogenous and dietary protein (distal colon) fluid and electrolyte absorption,final condensation of digesta (distal colon)	Neutral or alkaline pH, inflammatory processes, malabsorption of organic acids, electrolytes and fluids, dysbiosis	Low level of dietary fiber, high dietary protein level, high level of indigestible protein, small intestinal maldigestion and/or malabsorption, high secretion of endogenous protein in proximal GIT	Proteolysis ↑ Bacterial amino acid fermentative activity ↑ Accumulation of organic acids Ammonia ↑ Biogenic amines ↑Phenolic compounds ↑ Proliferation of proteolytic bacteria (e.g. some clostridia) Bacterial toxins and LPS ↑ Sulfur metabolism, H_2S production ↑ (nitrosation reactions)

formation likely reducing accessibility of susceptible sites to proteases (Carbonaro *et al.*, 1993, 1997). On the other hand, the presence of random coil or unordered secondary structures in animal food proteins has been related to the increment in digestibility.

Differences in protein digestibility have also been related to the interaction of protein with dietary heat-stable (phytic acid, tannins, calcium, non-starch polysaccharides) and heat-labile compounds (protease inhibitors and lectins). It is generally accepted that the negatively charged phytate molecules may form binary, salt-like protein–phytate complexes with proteins carrying a net positive charge, or unhindered basic amino acid residues at the outer surface of the protein, at a pH lower than their isoelectric point (Cosgrove, 1966). The protein molecules become closely packed around the phytate anion forming insoluble aggregates (Rajendran and Prakash, 1993) that are refractory to pepsin digestion (Knuckles *et al.*, 1989). At a pH above their isoelectric point, proteins carrying a net negative charge may establish cationic bridges (usually Ca^{2+}) with phytate molecules in ternary complexes. However, it is generally accepted that ternary complexes are not as important as binary complexes in respect of phytate-reducing protein availability (Selle *et al.*, 2012). The negative ionic properties of phytate may also indirectly stabilize proteins and reduce their solubility mainly by changing the hydrogen-bonding properties of water in the surrounding medium.

Processing of feedstuffs such as soy meal improves its digestibility and inactivates protease inhibitors, but may also damage several amino acids (e.g. lysine, arginine, methionine, cysteine, and tryptophan) (González-Vega *et al.*, 2011). Protein-bound lysine and free lysine, both having an epsilon amino group, can react with reducing sugars in Maillard reactions, fats and their oxidation products, polyphenols, and various dietary additives. In particular, the reaction between lysine and reducing sugars may take place under mild conditions of processing to form the deoxy-ketosyl derivative (the Amadori compound), while it may induce a brown pigment formation at advanced stages of Maillard reactions when lysine is completely altered. The Amadori compound may be reverted by the hydrochloric acid hydrolysis during amino acid analysis, but reversion does not occur under the milder conditions encountered in the pig digestive tract (Moughan *et al.*, 2014). Based on reactive lysine analyses, Fontaine *et al.* (2007) characterized different soybean meal and dried distillers grains with solubles (DDGS) samples, and determined that 10–20% lysine damage is typical for normal soybean meals and that overheated batches had lost up to 67% of the reactive lysine.

As indicated, the secreted endogenous proteins may also contribute to the flow of undigested protein into the hindgut. Endogenous protein is considered to consist of two components: a basal endogenous flow, which is related to food dry matter intake, and a dietary-variable flow, referred to as the specific endogenous protein loss (Stein *et al.*, 2007). The specific loss is affected by dietary factors such as type and concentration of fiber and the level of various antinutritional factors (e.g. trypsin inhibitor, tannins, lectins, phytate). Cellulose, lignin, arabinoxylans and pectin can increase the sloughing of intestinal mucosal cells and enhance viscosity of digesta and mucus production (De Lange *et al.*, 1989; Choct and Annison, 1992). Thus, it is likely that the inclusion of dietary fiber into pig diets will also increase the flow of undigested endogenous proteins to the large intestine (Souffrant, 2001).

Bacterial protein fermentation in the gastrointestinal tract

Bacterial amino acid metabolism occurs via oxidative and reductive reactions including deamination, decarboxylation, and alpha and beta-elimination. Deaminases are more active at neutral or even slightly alkaline pH, whereas decarboxylase activity is higher under acidic conditions (Blachier *et al.*, 2007). Thus, bacterial protein catabolism is closely related to pH and the availability of fermentable carbohydrates (Smith and MacFarlane, 1998; Davila *et al.*, 2013). In the GIT of the pig, the continuous digestion and bacterial fermentation of carbohydrates favors the metabolic shift of the microbiota from the extracellular hydrolysis of protein into amino acids and peptides for further incorporation in bacterial cells towards true amino acid fermentation for energy metabolism in the distal colon, where the pH again increases above 6 as a consequence of the release of alkaline ammonia. However, as indicated above (Table 1), bacterial protein fermentation may not be limited to the large intestine and can also occur to some extent in the proximal GIT. The major amino acid fermenting bacteria in the GIT include proteolytic members of *Fusobacteria, Firmicutes* (*Streptococcaceae, Veillonellaceae, Megasphera, Selenomonas*), *Proteobacteria*, and *Bacteroidetes* (Dai *et al.*, 2011). This comprises a large variety of phylogenetically distinct species but includes also putatively pathogenic species such as *E. coli, Klebsiella* spp., *Campylobacter* spp., *Streptococcus* spp., *C. perfringens, C. difficile* or *Bacteroides fragilis*, and may partly explain why high protein diets in pigs have been associated with gastrointestinal dysbioses and diarrhea (Ball and Aherne, 1987; Wellock *et al.*, 2008; Opapeju *et al.*, 2009).

Deamination of amino acids leads to formation of ammonia and the remaining carbon skeleton can be further metabolized to yield short chain fatty acids (SCFAs) and branched chain fatty acids (BCFAs) from branched chain amino acids. Thus, bacterial amino acid utilization also contributes to considerable amounts of acetate (e.g. from alanine, aspartate, glycine, threonine, lysine), propionate (e.g. from alanine, threonine) and butyrate (from lysine, glutamate). BCFAs are formed by deamination reaction from branched chain amino acids such as valine (isobutyrate), isoleucine (2-methylbutyrate) and leucine (isovalerate) and are thus indicators of microbial amino acid metabolism. Decarboxylation of amino acids yields several biogenic amines and occurs usually at a pH between 4 and 6. Examples for typical amines are cadaverine (lysine), histamine (histidine), tyramine (tyrosine), tryptamine (tryptophane), ethylamine (alanine) or agmatine (aspartate). Polyamines, which can be found in higher concentrations in the large intestine, include mainly putrescine, spermine and spermidine. Obviously, the decarboxylation (and to some extent also the deamination) of amino acids in the pig intestinal tract can already occur in the small

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intestine (Table 1). For example, increased concentrations of ammonia and biogenic amines can already be found in the stomach and ileum of pigs (Pieper *et al.*, 2014). The production of amines such as cadaverine, in turn, reflects a bacterial adaptation in order to buffer a low pH under high concentration of SCFA and lactate (Pieper *et al.*, 2014). Lysine decarboxylase positive bacteria can increase cadaverine production under co-cultivation conditions with lactic acid bacteria (Kuley *et al.*, 2012). Amines also act as precursors for *N*-nitrosation but little is yet known about the formation of nitrosamines and their pathophysiological function in the pig gut. Many intestinal bacteria (including bifidobacteria, lactobacilli, and enterobacteria) are capable of reducing nitrate to nitrite as prerequisite for nitrosation reactions. Using an *in vitro* model of the pig cecum, it was shown that nitrate reduction was accompanied with *N*-nitrosamine production (Engemann *et al.*, 2013). Since nitrosamine formation occurs under low pH, a higher production of these compounds may also take place in the stomach.

A number of phenolic and indolic compounds may also be produced from the metabolism of aromatic amino acids (Blachier *et al.*, 2007; Davila *et al.*, 2013). Tyrosine leads to the formation of 4-ethylphenol, phenol and p-cresol, whereas tryptophane is metabolized to indole, skatole (3-methylindole) (Blachier *et al.*, 2007). Urinary metabolomic profiles of germfree and conventionally colonized rats were largely different and in conventional rats characterized by products from tryptophane and tyrosine metabolism indicating the profound role of the GIT microbiota in phenol and indole formation (Wikoff *et al.*, 2009).

Finally, sulfur-containing amino acids and intestinal sulfomucins are converted to a number of sulfur containing metabolites including hydrogen sulphide, methanethiol and dimethyltrisulphide (Geypens *et al.*, 1997). A broad range of different bacterial species including *Clostridiales, Bacteroides, Prevotella, Enterobacteriaceae* or *Streptococcaceae* have been identified for their role in metabolism of sulphur containing substrates such as methionine, cysteine, taurine, sulfomucins and bile acids in the intestine (Carbonero *et al.*, 2012). Sulfate reducing bacteria were identified to play a major role in H_2S formation in the pig, with the genus *Desulfovibrio* being the most important.

Host response to microbial fermentation products

Short chain fatty acids

Generally, SCFA (derived from carbohydrate or protein fermentation) are rapidly absorbed from the gut lumen and serve as energy substrates. Acetate acts mainly as precursor for fatty acids synthesis, whereas propionate is mainly used for gluconeogenesis in the liver. Butyrate, in turn, is mainly metabolized by epithelial cells and has been proposed as the main energy source for colonocytes (Hamer *et al.*, 2008). In the pig, the utilization of SCFA can contribute considerably to the daily energy supply depending on the diet and the physiological status of the animal, adults being more able to extract energy from intestinal fermentation than young pigs (Kambashi *et al.*, 2014). In addition, SCFA stimulate epithelial proliferation and barrier function, and modulate immune response and satiety through receptor mediated signaling (Hamer *et al.*, 2008; Willing and Van Kessel, 2010). For example, the monocarboxylate transporter 1 (*MCT1*) can be stimulated by butyrate leading to increased butyrate uptake (Borthakur *et al.*, 2012). The entire mechanism is yet not clear but as butyrate is an important energy source for colonocytes, both stimulation and inhibition of *MCT1* may have important health implications. Interestingly, the pro-inflammatory cytokine TNF-α reduced *MCT1* expression and impaired butyrate uptake (Villodre Tudela *et al.*, 2015). SCFAs have been shown to improve epithelial barrier function, which has been attributed – at least in part – to higher expression of tight junction proteins ZO-1 and occludin through butyrate-induced signaling cascades (Plöger *et al.*, 2012). On the other hand, TNF-α and other pro-inflammatory cytokines can decrease barrier function through reduced claudin-1 and 2 expression (Plöger *et al.*, 2012). SFCA also influence mucus production by goblet cells: propionate directly increases MUC2 expression through a SCFA-responsive regulatory element, whereas butyrate regulates gene expression via effects on histone acetylation (Burger-van Paassen *et al.*, 2009). Propionate, acetate, and to a lesser extent butyrate, can act as signaling molecules through free fatty acid receptors (FFAs) such as *FFA2R* (GPR43) or *FFA3R* (GPR41) (Ulven, 2012). The *FFA2R* is highly expressed in neutrophils, macrophages and monocytes and SCFA have been shown to promote neutrophil chemotaxis through this receptor (Vinolo *et al.*, 2011). Both, *FFA2R* and *FFA3R* have been associated with activation of mitogen-activated protein kinase (MAPK) signaling and pro-inflammatory cytokine expression in mice (Kim *et al.*, 2013). Both receptors are also expressed in large intestinal enteroendocrine cells, which release peptide YY and glucagon-like peptide-1 (GLP-1). Whereas PYY reduces gastric emptying and increases satiety, GLP-1 (and the co-released GLP-2) promotes small intestinal proliferation and improves barrier function (Liu *et al.*, 2013). This could be also interpreted as reversed large to small intestinal signaling, as reduced feed intake and gastric emptying accompanied with increased absorptive capacity in the small intestine would reduce large intestinal SCFA concentrations. Finally, it has been hypothesized that the excessive production of SCFA is causative for intestinal inflammation and the onset of necrotizing enterocolitis in young piglets (Di Lorenzo *et al.*, 1995; Lin, 2004). Thus, positive effects of SCFA on intestinal epithelia seem to be dose- and also age-dependent.

Biogenic amines

Biogenic amines have several effects on the host. Polyamines can be either produced by bacteria in the GIT or synthetized by colonocytes through ornithine decarboxylase. The role of polyamines such as spermidine, spermine and putrescine has been studied in detail due to their enhancing effect on cell proliferation (Seiler and Raul, 2007). However, studies in gnotobiotic rats did not reveal any significant *in vivo* effects of bacteria-derived polyamines on intestinal morphology (Slezak

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et al., 2013). Amines are rapidly absorbed from the intestine and either further metabolized in the epithelium via specific mono- or diamine oxidase and excreted via urine (Hughes *et al.*, 2000). For example, histamine has several biological functions including induction of chloride secretion into the gut lumen, which may promote diarrhea (Ahrens *et al.*, 2003). Histamine is derived from exogenous L-histidine or released endogenously by mast cells and is metabolized by diamine oxidase and histamine *N*-methytransferase in the colon epithelium (Aschenbach *et al.*, 2009). Higher concentration of luminal histamine increased the epithelial capacity to metabolize this compound in the pig colon (Kröger *et al.*, 2013). Little is yet known about the role of other biogenic amines. Cadaverine and putrescine may reduce the metabolism of histamine in the epithelium as both are also converted by diamine oxidase, thus possibly enhancing negative effect of histamine. Although speculative, this may explain why increased incidence of diarrhea was associated with high intestinal concentrations of amines such as cadaverine and putrescine (Pietrzak *et al.*, 2002). There might also be a link between lysine decarboxylation to cadaverine and pathogenicity of *E. coli* and *Salmonella*. For example, the absence of the responsible *cadA* gene, which encodes L-lysine decarboxylase, dramatically increases enterobacterial virulence (Torres, 2009). Whether cadaverine itself can provide feedback signals influencing the expression of pathogenic factors is yet not clear. Finally, as indicated above, little is yet known about the formation and function of nitrosamines in the pig gut.

Ammonia

Protein fermentation products such as ammonia have been primarily associated with toxigenic and damaging effects on the intestinal epithelium in human beings (Blaut and Clavel, 2007; Davila *et al.*, 2013). Ammonia can interfere with the oxidative metabolism of SCFA in colonocytes, likely inducing energy deficiency in the cell (Blachier *et al.*, 2007). Moreover, increased apoptosis and higher proliferation may occur through activation of caspases and mitosis, respectively (Blachier *et al.*, 2007; Willing and Van Kessel, 2010). Whether this is due to direct toxigenic effect on the cells or mediated through specific receptors is not yet clear. In human beings, higher ammonia concentrations in the distal colon have been associated with tumor promotion (Hughes *et al.*, 2000; Blaut and Clavel, 2007). Recent results suggest that ammonia and other protein-derived metabolites present in the lumen may entail inflammatory responses in the colonic mucosa, which negatively influence the expression of *MCT1* (Villodre Tudela *et al.*, 2015). Ammonia and BCFA impaired barrier function and promoted pro-inflammatory signaling expression through NFkB-mediated signaling in Caco-2 cells, and the co-incubation with butyrate did not ameliorate these reactions (Villodre Tudela *et al.*, 2016). This may provide an interesting link between impaired protective effects of butyrate on the colon epithelium and pro-inflammatory conditions in the colon when high concentrations of ammonia are present. In addition, ammonia increased permeability towards macromolecules in Caco-2 monolayers (Hughes *et al.*, 2008). Upon absorption, ammonia is mainly detoxified in the liver to urea and plasma urea nitrogen can be used to some extent to assess the degree of intestinal protein degradation in pigs fed diets with balanced amino acid profile. In turn, higher plasma urea levels were associated with increased urea flux from the serosal to the mucosal site in pig cecum and this effect was related to cecal luminal SCFA levels as compensatory pH-dependent mechanism (Stumpff *et al.*, 2013). Although yet not clearly established, this may have implications when higher levels of SCFA (e.g. by feeding higher levels of non-digestible carbohydrates) are present in the gut lumen, as transports of urea into the gut lumen will in turn increase toxic ammonia levels.

Hydrogen sulphide

Hydrogen sulphide is a toxic gas and has been attributed to both beneficial and deleterious effects in the intestine (Blachier *et al.*, 2010). High intestinal concentrations have been considered detrimental for the host due to its effect on cell respiration and genomic DNA damage (Davila *et al.*, 2013). H_2S can inhibit butyrate β-oxidation in human epithelial colonic cells by inhibition of short chain acyl-CoA dehydrogenase activity as well as glutamine and acetate oxidation (Leschelle *et al.*, 2002). In addition, NaHS can stimulate chloride secretion in isolated colonic tissue of pigs (Kröger *et al.*, 2013). On the other hand, H_2S at lower concentrations is also considered as a signaling molecule associated with physiological and pathophysiological functions including inhibition of insulin secretion, vasoconstrictive effects as well as effects on mononuclear cell infiltration (Blachier *et al.*, 2010; Wallace *et al.*, 2012). Thus, the positive or detrimental effects of hydrogen sulphide on intestinal epithelial cells seem to be largely dose-dependent.

Phenolic compounds

Upon absorption in the large intestine (as the main site of phenol and indole formation), phenolic compounds are detoxified through glucuronidation and sulfate conjugation and excreted via urine, mainly as *p*-cresol. Thus, total phenol and *p*-cresol in urine, besides their concentration in the large intestinal digesta, can serve as markers for increased intestinal amino acids catabolism (Geypens *et al.*, 1997). Phenol has been shown to increase epithelial permeability (Hughes *et al.*, 2008). In the GIT, phenols might be involved in *N*-nitrosation of dimethylamine, and reaction with nitrite produces the toxic metabolites, p-nitrosophenol and diazoquinone (Kikugawa and Kato, 1988). Finally, from a consumers point of view, the role of 3-methylindole (skatole) is of high importance in intact male pigs as it is involved in 'boar taint' of the meat (Jensen *et al.*, 1995).

The role of protein fermentation in diarrhea in pigs

It is well established for decades that increased protein fermentation in the GIT can lead to intestinal disorders including post-

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weaning diarrhea (PWD). In fact, it has already been reported >35 years ago that diets high in protein (21 versus 13% CP) predispose for enteropathogenic *E.coli* infections and diarrhea in piglets (Prohaszka and Baron, 1980). The etiology and mechanisms behind this are not entirely clear. Table 2 summarizes some putative factors related to bacterial protein utilization that may contribute to different types of pathophysiological conditions and diarrhea in pigs. It has been previously assumed that a higher protein level may change the buffering capacity in the stomach, thereby reducing the gastric barrier and favoring the proliferation of enterobacteria or clostridia (Prohaszka and Baron, 1980). This effect may be further amplified through a lower gastric acid production due to (weaning) stress. Although not clearly established, the hygienic quality of the feed might be considered as additional factor. It has been assumed that protein fermentation may selectively favor the growth of enteropathogenic *E. coli* (Rist *et al.*, 2013). However, *E. coli*-induced secretory diarrhea affects mainly the small intestine (Fairbrother *et al.*, 2005), whereas the site of excessive microbial protein fermentation is usually the large intestine. Protein-derived metabolites formed by other proteolytic bacteria such as clostridia may also induce impaired barrier function through altered mucus composition and tight junctions, thereby favoring the translocation of enterobacteria as secondary opportunists. Although not directly related to bacterial protein fermentation, foreign proteins of plant origin (e.g. legumes) can induce small intestinal epithelial damage due to overwhelming immune reactions and episodes of diarrhea (Stokes *et al.*, 1987). Large intestinal epithelial damage by protein-derived metabolites or bacterial toxins may reduce the ability for fluid re-absorption, which otherwise could mask small intestinal hyper-secretion (Table 2). Interestingly, diets high in fermentable protein reduced the activity of the large intestinal epithelial ameloride-sensitive sodium channel ENaC, which was associated with more liquid feces in piglets (Richter *et al.*, 2014). Indirect effects of protein-derived metabolites include higher formation of histamine, which contribute to increased chloride secretion and fluid loss into the large intestinal lumen through receptor-mediated signaling (Kröger *et al.*, 2013, 2015). Other factors such as digesta viscosity or digesta transit may promote the ability of enterobacteria to adhere to specific mucus motifs in the small intestine. Intestinal metabolites or bacterial quorum sensing molecules may also induce the expression of virulence factors in enterobacteria, which has yet not been studied in detail.

It has been proposed that the dietary protein level rather than the protein source may predispose piglets to intestinal diseases (Rist *et al.*, 2013). This is in good agreement with a recent between-experiment study showing that fecal ammonia concentration and blood urea levels were increased with higher dietary protein levels and, in turn, increased the incidence of PWD (Heo *et al.*, 2015). Thus, a common strategy to reduce the risk of PWD is through a low (<18%) dietary protein level or total daily intake of less than 60 g (Heo *et al.*, 2012, 2015). Feeding such low (13%) versus high (23%) protein diets may also increase the fecal lactobacilli to enterobacteria ratio (Wellock *et al.*, 2006). However, other studies showed no effect of protein level on the number of different microbial groups (Bikker *et al.*, 2006; Nyachoti *et al.*, 2006; Hermes *et al.*, 2009). As indicated above, numerous different types of bacteria are able to utilize dietary and endogenous protein sources and thus the measured response in terms of bacterial counts can vary widely. Diets low in dietary protein have been associated with reduced incidence of diarrhea in ETEC challenged and non-challenged piglets (Heo *et al.*, 2008, 2009; Opapeju *et al.*, 2009; Kim *et al.*, 2011b). In turn, lower protein levels were accompanied with increased levels of bacterial species (*Roseburia*/*E. rectale*-like), which are specialized for carbohydrate utilization and butyrate formation (Opapeju *et al.*, 2009). Although no differences in total SCFA were observed in this study, it shows that a low protein level and likely also the flow of fermentable carbohydrates may beneficially affect the large intestinal environment.

Intestinal bacteria may switch between complete or partial utilization of carbohydrates and protein (amino acids) as substrates to derive energy. Thus, dietary inclusion of a variety of fermentable carbohydrates may be a promising approach to reduce harmful protein fermentation in the porcine GIT. The fermentation of these carbohydrates induces a shift on nitrogen excretion from urine to feces (Bindelle *et al.*, 2007, 2009; Jha and Berrocoso, 2016). In fact, inulin, resistant starch, cereal fibers and sugar beet pulp have been shown to decrease the abundance of protein fermentation products (Awati *et al.*, 2006; Bikker *et al.*, 2006; Nyachoti *et al.*, 2006; Pieper *et al.*, 2012, 2014). However, although the effects of fiber inclusion on bacterial metabolites in high protein diets were often statistically significant, the concentration of toxic metabolites such as ammonia was by far not reduced to similar levels as with low protein diets. For example, although dietary carbohydrate inclusion (wheat bran, SBP) reduced the formation of intestinal protein-derived metabolites in the large intestine of piglets, blood urea nitrogen was higher in high protein diets regardless of carbohydrate level (Pieper *et al.*, 2012; Stumpff *et al.*, 2013). In addition, the expression of pro-inflammatory cytokines, mucus genes and oxidative stress indicators was largely unaffected by dietary carbohydrate level and increased with protein level in the colon epithelium (Pieper *et al.*, 2012). Linking the site of dietary protein fermentation with the effectiveness of different carbohydrate sources (insoluble, partly soluble), it was shown that SBP mainly increased SCFA and lactate and decreased protein-derived metabolites in the large intestine, whereas cellulose was partly fermented in the distal large intestine and reduced mainly phenols, indoles and cadaverine, but not ammonia (Pieper *et al.*, 2014). This may have health relevance as it was recently shown that protein-derived metabolites (i.e. ammonia, putrescine) were negatively correlated with the *MCT1* gene expression in the intestine of pigs (Villodre Tudela *et al.*, 2015). The *MCT1* expression was reduced in high protein diets regardless of dietary carbohydrate inclusion. Further analyses revealed that ammonia (through TNF-α-mediated signaling) leads to a down-regulation of the *MCT1* gene linking impaired protective effects of butyrate and pro-inflammatory conditions in the colon of pigs. This raises the question about optimum inclusion levels of different types of fermentable carbohydrate sources that direct microbial fermentation patterns towards carbohydrates

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Table 2. Pathophysiological conditions leading to diarrhea and their putative relation to bacterial protein utilization in different parts of the porcine GIT

Site	Pathophysiological reactions	Cause	Consequence	Putative relation to dietary protein and bacterial protein fermentation
Stomach	Low acidification, inflammatory reactions (?)	Buffering capacity of the feed, secretory dysfunction, overload with feed, stress or interfering diseases (?), spoiled feedstuff (protein sources)	Bacterial overgrowth, insufficient inactivation of exogenous bacteria (feed pathogens), inflammatory reactions	Pathogen intrusion in the small intestine, feed poisoning, bacterial toxins and metabolites (e.g. biogenic amines)
Small intestine	Hypersecretory	Bacterial toxins (e.g. *E.coli, Shigella* toxins), viral infections, biogenic amine production (histamine), tissue damage and inflammation	Fluid, chloride, sodium, HCO_3 secretion, motoric dysfunction	Selective growth of toxin producing strains, toxin activation (?), overgrowth and translocation of secondary opportunistic bacteria, protein-derived metabolites (e.g. ammonia, biogenic amines) ↑
	Hyperosmotic	Lactose, minerals	High fluid secretion and net fluid losses	Proteolysis and protein digestibility in SI ↓, Fermentable protein entering the large intestine ↑
	Hypersensitivity to allergens	Legumes, eg. glycinin, conglycinin	Leucocyte infiltration, cytokine release and inflammatory reactions, impaired barrier function, hypersecretory reactions	Direct (foreign/antigenic) protein effect
Large intestine	Hypersecretory	Bacterial toxins (e.g. *C. perfringens, Salmonella*), viral infections, protozoans/ metazoans, biogenic amine production (histamine), tissue damage and inflammation	Fluid, chloride, sodium, HCO_3 secretion, motoric dysfunction	Selective growth of toxin producing strains, Toxin activation (?), protein-derived metabolites (e.g. ammonia, biogenic amines)
	Hyperosmotic	organic acid accumulation	High fluid secretion and net fluid losses	Excessive fermentation of easily accessible substrates (low SI digestibility)
	Malabsorption	Dysfunction or limited capacity of epithelial sodium channels	Fluid malabsorption	indirect through cytokine signaling and protein-induced inflammatory reactions

and promote bacterial biomass formation along the entire GIT (Pieper *et al.*, 2015). As an example, the addition of non-starch polysaccharides to diets containing resistant starch shifted the site of starch and fiber fermentation towards more distal parts of the colon (Govers *et al.*, 1999). Otherwise, even at high inclusion rates of fermentable carbohydrates, it seems that protein fermentation is not sufficiently reduced or just shifted distally without protective effects on intestinal functionality.

Summary and outlook

The porcine GIT is colonized by a highly diverse microbial community, which is increasingly recognized for its role in nutrient utilization and influence on host health. Our understanding of this ecosystem has emerged during the past years but some aspects including the complex nutrition – microbe – host interactions have yet received less attention. New concepts related to the mucosal microbiota and bacterial functionality rather than studying the community composition in general may gain more attention in the near future. Targeting the intestinal microbial ecosystem by means of nutritional manipulation offers great potential to maintain gut health. Fermentation of undigested proteins may impair epithelial functionality and set the scene for intestinal disorders through a variety of toxic metabolites. Reducing the dietary protein content, inclusion of different types of fermentable fiber and choice of highly digestible protein sources are likely to limit negative effects of bacterial protein fermentation. The interaction of protein and fiber fermentation along the intestinal tract and the influence on the host have still to be defined further in order to formulate appropriate 'healthy' pig diets.

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