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Caecal protein fermentation in broilers: a review

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SUMMARY

Protein fermentation (PF) is the degradation of protein by microbiota in the gastro-intestinal tract. It results from high intake of indigestible protein and/or increased endogenous losses, and it may be the cause of gut health issues. This is important as the use of less digestible protein sources for poultry is expected to increase as a consequence of the food-feed discussion. Here we review the relations between dietary protein, caecal PF and gut health and identify critical knowledge gaps. Finally, we suggest methods for the investigation of caecal PF. The majority of the microorganisms have never been cultured, however, through cultivation-independent molecular approaches, many new taxa have been identified. Researchers have identified taxa that are enriched in healthy/ unhealthy birds. The mechanisms underlying these associations remain unclear. PF results in the production of potentially detrimental metabolites. This generally results in a higher pH, further encouraging PF. Studies on the effects of PF (metabolites) on gut health in poultry are limiting. For the *in vivo* evaluation of PF an increase in protein flow into the caeca is required, which can result from an increased level of dietary indigestible protein. Heat damage reduces protein digestibility and can therefore be used to create a within ingredient contrast for *in vivo* studies. A remaining challenge is that the relation between indigestible protein level and subsequent PF is not straightforward, as fractional separation of digesta occurs in poultry, allowing part of the digesta to bypass the caeca. To further study the extent to which microorganisms will ferment the protein fraction flowing into the caeca, *in vitro* studies can be applied. However, their application depends on the ability to separate the fraction of pre-digested feed that is likely to enter the caeca. Altogether, an increase in PF will affect microbiota composition, metabolite production, and potentially gut health.

KEYWORDS

Protein fermentation; caeca; microbiota; gut health; *in vivo*; *in vitro*

Introduction

Modern day broilers grow fast. In 2017, broilers required only 36 d to reach a live weight of 2.3 kg, while in 1995 broilers required 52 d (Aftab 2019). To support such growth broilers require a high amount of digestible protein intake. However, due to the growing world population, sources of highly digestible protein will become less available for animal nutrition and less digestible proteins will likely be used in

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animal feed instead. Nutrients that remain undigested and unabsorbed in the small intestine will move towards the caeca and colon and may become available for fermentation by microbes (Apajalahti and Vienola 2016). Microbial fermentation of protein may lead to the production of ammonia, sulphur containing compounds, biogenic amines and phenols, compounds which have possible negative effects on gut health and growth (Qaisrani et al. 2015b), raising the use of prophylactic and curative antibiotic treatments.

Antibiotic use increases the selective pressure towards resistance in bacterial populations, which then might spread to humans (Chang et al. 2015). Indeed, consumption of antibiotics by food-producing animals such as broilers is associated with increased occurrence of resistant bacteria in both humans and animals (ECDC, EFSA, and EMA 2017). This poses a risk to public health, as infections with these resistant bacteria are increasingly difficult to treat. Due to this public health risk, worldwide regulations on the use of antibiotics in livestock production are becoming more restrictive. In 2006, the European Union banned the nonmedicinal use of antibiotics in animals, and efforts to reduce antibiotic use continues. Indeed, from 2011 to 2016, the overall veterinary antibiotic use of 25 European countries decreased with 20% (EMA 2018). Unfortunately, restricting antibiotics for animals is not without consequence. In the past, the prophylactic use of antibiotics enhanced growth and feed efficiency in broilers by reducing subclinical infections and colonisation of opportunistic pathogenic bacteria (Hume 2011). Restrictions on antibiotics have allowed for the re-emergence of diseases such as necrotic enteritis (NE) in broilers (Van Immerseel et al. 2009). Possible nutritional support to reduce antibiotics use such as, prebiotics, probiotics and bacteriophages have been reviewed by Hume (2011) and Kogut (2019). However, the prospect that less digestible protein sources will be fed to poultry warrants the evaluation of protein fermentation (PF) on gut health.

Gut health issues in broilers and other meat-producing animals that require high levels of protein in their diets, might result from the occurrence of PF, which occurs when high levels of protein are present in the hindgut and fermentable carbohydrates are limited. When the carbohydrate to protein ratio decreases, microbes will ferment the proteins as energy source instead of the preferred carbohydrates (Rehman, Böhm, and Zentek 2008). These proteins entering the caeca can originate from the diet, endogenous protein production or from microbial protein (Apajalahti and Vienola 2016). Hence, a poor protein digestibility of the diet as well as factors contributing to endogenous production and microbial activity in the small intestine might increase caecal PF. Higher levels of indigestible dietary protein above adequate levels of digestible amino acid (AA) requirements reduced broiler performance, which might very well be due to PF (De Lange, Rombouts, and Oude Elferink 2003).

In order to feed poultry with future (less-digestible) protein sources without hampering gut health, knowledge on the relation between PF and gut health and its underlying mechanisms, is required. The current paper aims to review these relations and identify current knowledge gaps. Furthermore, we suggest methods for the investigation of caecal PF to close these knowledge gaps.

Protein fermentation, causes and effects

Fermentation is the anaerobic degradation of feed components by microbes, and the fermentation of protein is sometimes also referred to as putrefaction (Windey, De Preter, and Verbeke 2012). Fermentation of feed components may take place throughout the gastro-intestinal tract (GIT), but the major sites for fermentation in poultry are the crop and the caeca (Adil and Magray 2012). However, *ad libitum* feeding of broilers appears to encourage feed to bypass the crop (Svihus 2014), in which case no or little fermentation will occur. The current paper, therefore, focusses on PF in the caeca.

The caeca

The caeca are blind-ending distal gut segments. Chickens have two caeca with a sack-like appearance, that are found in-situ alongside the ileum. They are attached to the beginning of the colon just beyond the ileal-colon valve, creating a four-way crossroad for digesta (Ferrer et al. 1991; Svihus, Choct, and Classen 2013). After digesta from the ileum enters the colon, the digesta can be pushed into the caeca via anti-peristaltic movement of the gut (Svihus, Choct, and Classen 2013). The proximal part of the caeca contain villi, although the density and height of the villi are much lower than in the small intestine (only 24 villi/mm^2 in proximal caecum compared to 46 villi/mm^2 in jejunum and a villus height of 364 µm in the proximal caecum compared to a height of 525 µm in the distal jejunum (yolk-sac region)). The mid and distal part of the caecum have poorly developed villi similar to low mounds (Ferrer et al. 1991).

Unlike most mammals, the caeca and not the colon are considered the main sites for fermentation in the hindgut of poultry (Adil and Magray 2012). The colon is short as is retention time of digesta there. In the literature estimations of the retention time in the colon vary from 4 to 56 min, the average being about 30 min (Van Krimpen et al. 2011; Van der Klis, Verstegen, and De Wit 1990; Danicke et al. 1999). In the caeca, on the other hand, digesta retention is expected to be long, due to infrequent emptying (Svihus, Choct, and Classen 2013). Even after 24 hours of fasting, Hinton Jr, Buhr, and Ingram (2000) and Warriss et al. (2004) still found little reduction in digesta present in the caeca of broilers.

The caeca also play a role in N-cycling of the bird. Urine containing uric acid is excreted in the colon and via anti-peristalsis enters the caeca, where it is used as an N-source for microbial growth. It has been shown in chicken that microbial protein and AA can be absorbed directly from the caeca, providing the host with new protein from recycled N (Karasawa and Maeda 1995). This kind of N-recycling, however, only seems to contribute to the total N-balance of chickens fed protein deficient diets (Karasawa and Maeda 1994).

Any feed component that is not absorbed in the small intestine flows into the hindgut and partially into the caeca and may become a substrate for microbial fermentation. The fermentation of carbohydrates is commonly considered to be beneficial due to the production of volatile fatty acids (VFA), which contribute to the energy supply for the host (Windey, De Preter, and Verbeke 2012). Hindgut fermentation of proteins and AA, however, are considered detrimental to the host as

compounds such as biogenic amines, phenols, indoles, cresol, sulphur-containing compounds and ammonia are produced (Apajalahti and Vienola 2016; Qaisrani et al. 2015b). Some of these compounds can have detrimental effects on gut epithelial cells (Gilbert et al. 2018).

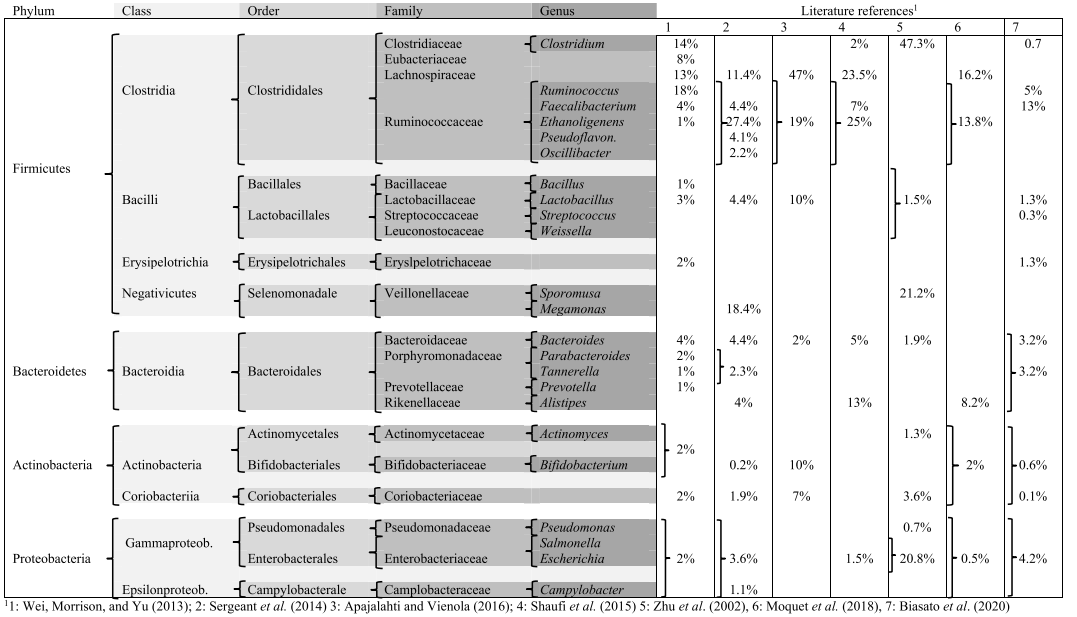
The caecal microbiome

The caecal microbiome is unique and dynamic. It consists mostly of bacteria (approx. 98%), but also contains *Archaea, Eukarya* (e.g. parasites, fungi) and viruses (Qu et al. 2008; Glendinning et al. 2020). The focus of most of the literature and therefore this review, as well, goes to the bacteria.

The caecal microbiome generally increases in richness and diversity as birds age (Modh Shaufi et al. 2015). Large differences in caecal microbiota composition are found between studies (Figure 1). Even within flocks of the same broiler strain fed the same diet large differences in microbiota can be found (Stanley et al. 2012a, 2013). Even within birds, one caecum or the other, considerable microbiota differences exist (Sergeant et al. 2014). That the caecal microbiota of broilers varies so much might in part be due to the high hygiene in hatcheries resulting in the lack of colonisation by maternally derived microorganisms (Stanley et al. 2013). Moreover, the housing condition has impact on how broiler microbiota may respond to a dietary intervention. To this end, Kers et al. (2019) found that a diet intervention with a blend of medium-chain fatty acids explained only 10% of the caecal microbiota variation between broilers, while the three different housing conditions investigated explained 28%.

Characterisation of GIT microbiota composition increasingly relies on the application of cultivation-independent approaches, in most cases employing approaches targeting the 16S rRNA gene through PCR amplification and sequence analysis (e.g. Wei, Morrison, and Yu 2013; Sergeant et al. 2014; Modh Shaufi et al. 2015; Kers et al. 2019). This approach can uncover the caecal bacteria for a large part down to the taxonomic rank of genus, and irrespective of the ability to culture them (Modh Shaufi et al. 2015). Also some methanogens from the domain of *Archaea* are identified using this technique (Saengkerdsub et al. 2007). It should be noted, however, that not all primers commonly used for 16S rRNA gene-targeted prokaryotic community profiling consistently provide sufficient coverage of *Archaea*. Furthermore, amplicon-sequencing-based approaches for determining the composition and relative abundance of fungi similarly suffer from qualitative and quantitative biases and are still being developed (De Filippis et al. 2017). Figure 1 shows an overview of most bacterial groups found in the caeca of broilers and the composition (%) in different investigations.

In the caeca, 50 to 96% of the bacteria belong to the phylum Firmicutes (Wei, Morrison, and Yu 2013; Moquet et al. 2018; Sergeant et al. 2014; Modh Shaufi et al. 2015; Biasato et al. 2020; Glendinning et al. 2020). The second most predominant phylum is Bacteroidetes (0.2 to 21%). Also other phyla such as Proteobacteria (0.3 to 14%), Actinobacteria (0.2 to 2.5%), Tenericutes (1.7 to 2.6%), Cyanobacteria (0.2 to 2.5%), Verrucomicrobiota (0.4%) and Lentisphaerae (0.1%) have been detected in the caeca (Wei, Morrison, and Yu 2013; Moquet et al. 2018; Sergeant et al. 2014; Modh Shaufi et al. 2015; Biasato et al. 2020; Glendinning et al. 2020).



[1]1: Wei, Morrison, and Yu (2013); 2: Sergeant *et al.* (2014) 3: Apajalahti and Vienola (2016); 4: Shaufi *et al.* (2015) 5: Zhu *et al.* (2002), 6: Moquet *et al.* (2018), 7: Biasato *et al.* (2020)

Figure 1. Phylogenetic tree and composition of the caecal bacteria in broilers in different studies. 1: Wei, Morrison, and Yu (2013) 2: Sergeant et al. (2014) 3: Apajalahti and Vienola (2016) 4: Modh Shaufi et al. (2015) 5: Zhu et al. (2002) 6: Moquet et al. (2018) 7: Biasato et al. (2020)

The microbiome might play an important role in maintaining intestinal health of the host. *Clostridium*, one of the most abundant genera found in poultry caeca (Stanley et al. 2012a; Zhu et al. 2002), is of particular interest. One of its species, *C. perfringens*, is notorious for causing NE in broilers. However, not all *Clostridium* species are pathogenic. In fact, a study by Stanley et al. (2012b) demonstrated that while broilers with induced NE had higher abundance of *C. perfringens*, other *Clostridium* species, such as *C. leptum*, were reduced in comparison to healthy control birds. Hence, analyses of the microbiome down to the level of genus, might not be sufficient to determine if a diet results in an overall more pathogenic microbial composition.

The phylum Proteobacteria appears to be a small group in healthy birds, although high contributions of 10 to 21% were seen in some studies (Zhu et al. 2002; Sergeant et al. 2014; Biasato et al. 2020). Potentially pathogenic genera such as *Escherichia, Salmonella* and *Campylobacter* are found within the Proteobacteria. An increased level of Proteobacteria is therefore sometimes considered to be a sign of microbial disbalance (Moquet et al. 2018; Biasato et al. 2020).

The large group of still unidentified bacterial species might be of great importance to health as well. Stanley et al. (2012a) found microbes that strongly differed in abundance between high and low performance broilers, but these were mostly still unclassified. Moreover, a principal component analysis included in the latter study showed a grouping of the poorly performing birds, but not of the high performing birds, indicating that birds are likely more affected by particular bacteria that reduce performance rather than bacteria that promote performance. Interestingly, Siegerstetter et al. (2017) found more bacterial 16S rRNA gene copies by quantitative PCR in the caeca of birds that were more feed efficient.

Particular bacteria (groups) have been associated with health or productivity. In a study of Stanley et al. (2012b) with NE induced broilers, the phylotype *Weissella confusa* and relatives was found only in healthy birds. In addition, some species of *Lactobacillus* were decreased in diseased birds (*L. johnsonii* and *L. ferementum*), while others were increased (*L. crispatus, L. pontis, L. ultunese* and *L. salivarius*). Siegerstetter et al. (2017) found a positive correlation between *Faecalibacterium* and *Ruminococcus* and feed conversion ratio (FCR), indicating that birds with poorer growth performance had increasing levels of these genera. In turn, an unclassified genus-level group within the Ruminococaceae showed a negative correlation with FCR, indicating a possible contribution of this unknown genus to a better growth performance. An overview of dietary protein interventions in broilers and their effects on specific gut bacteria or bacterial clusters is provided in Table 1.

In conclusion, although a number of researchers have associated changes in chicken microbiota composition with the gut health and productivity of the chickens, exactly which bacteria or groups of bacteria are important for improving or reducing health and through which mechanisms remains largely unclear.

Dietary factors regulating caecal fermentation

Many factors such as age, breed, sex, climate or housing impact caecal fermentation. These non-dietary effects have recently been reviewed by Kers et al. (2018). In the following paragraph, the effects of diet, and in particular that of protein, on caecal fermentation are reviewed.

Table 1. Effects of dietary protein interventions on different bacteria in the gastrointestinal tract of broilers.

Dietary intervention[1]	Bacterial group	Site	Effect[2]	Ref[3]
Increasingly replacing SBM with RSM in finely ground diet	*L. paracasei* et rel.	Caecum	NE	a
	C. lactifermentans et rel.	Caecum	NE	
	L. paracasei et rel.	Caecum	-	
Increasingly replacing SBM with RSM in coarsely ground diet	*C. lactifermentans* et rel.	Caecum	-	
SBM control diet (CP=22.1%) vs:	*Lactobacillus*	Jejenum	-	b
(1)– 50.93% SBM diet (CP=28.7%)		Ileum	+	
		Caecum	NE	
	Bacteriodes	Jejenum	-	
	Prevotella	Ileum	+	
		Caecum	NE	
	Enterobacteriaceae	Ileum	NE	
		Caecum	-	
(2)– 1.091% added EAA (CP=22.6%)	*Lactobacillus*	Jejenum	-	
		Ileum	NE	
		Caecum	NE	
Diets with increasing glycine level	*C. perfringens*	Ileum	+	c/d
		Caecum	+	
	Lactobacillus	Ileum	-	
		Caecum	-	
300.8 g/kg SBM control diet vs.	*Lactobacillus*	Crop	+	e
(1)– 400 g/kg lupin meal & 63.4 g/kg casein diet		Ileum	+	
		Caecum	+	
(2)– 320 g/kg dehulled lupin meal		Crop	NE	
		Ileum	+	
		Caecum	+	
(3)– 400 g/kg lupin meal		Crop	NE	
(all diets had 210 g/kg protein)		Ileum	NE	
		Caecum	+	
Increasing supplemented methionine levels (from 0 to 0.8%, CP = 23%)	*C. perfringens*	Ileum	-	f
		Caecum	-	
	Lactobacillus	Ileum	NE	
		Caecum	+	
Diet with cottonseed meal (7.5%) vs. fermented cottonseed meal (7.5–15.1%)	*Lactobacillus*	Caecum	+	g
Diets with increasing fish waste silage (0, 6 or 12%) replacing SBM	*Bifidobacterium* spp.	Caecum	+	h
	Lactobacillus spp.	Caecum	+	
	Escherichia coli	Caecum	-	

[1]Abbreviations used are: SBM = soybean meal, RSM = rapeseed meal, CP = crude protein level, EAA = essential amino acids.
[2]Effect: decrease is -, increase is +, no effect is NE.
[3]References: a: Qaisrani et al. (2014a), b: Nakphaichit et al. (2014), c: Wilkie et al. (2005) d: Dahiya et al. (2005), e: Rubio et al. (1998), f: J. Dahiya et al. (2007), g: Wang et al. (2017), h: Shabani et al. (2019)

High levels of protein in the caeca might be a prepositioning factor for disease, as infection of *C. perfringens* alone is not enough to cause NE. High levels of the amino acid glycine increases the level of *C. perfringens* in the caeca (Wilkie et al. 2005; Dahiya et al. 2005), and therefore fishmeal (a protein source rich in glycine) is often used in studies in which NE is induced. Fishmeal supplementation causes large shifts in microbiota and increases the pH in the caeca to neutral (Wu et al. 2014).

Recent work from Shabani et al. (2019) showed that fish waste silage (fermented with molasses by *Bacillus subtilis*) may be a very promising protein ingredient for broilers to be used instead of fishmeal. Feeding 6 or 12% of this silage increased caecal butyric and

lactic acid contents, decreased digesta pH throughout most of the GIT including the caeca, decreased excreta ammonia concentration, increased caecal *Bifidobacterium* spp. and *Lactobacillus* spp. and decreased *Escherichia coli* counts. These effects are likely due to the fermentation of carbohydrates in the silage resulting in the production of lactic acid, reducing the pH.

Generally a more acidic pH in the GIT is considered beneficial to the host, as this protects the animal from colonisation by pH-sensitive pathogenic bacteria (Donoghue et al. 2006) and promotes the growth of beneficial bacteria (Raninen et al. 2011). For example, *in vitro* studies have shown that VFAs in a slightly acidic environment (pH = 6) protect an avian intestinal epithelial cell line against invasion *Salmonella enteritidis* (Van Immerseel et al. 2003) and *S. typhimurium* (Durant et al. 1999). Probiotics such as *Lactobacillus plantarum, L. fermentum, Pediococcus acidilactici, Enterococcus faecium* and *Saccharomyces cerevisiae* reduced the pH in the ileum of broilers and improved their resistance towards *Pasteurella multocida* (a pathogen from the phylum Proteobacteria) and enterobacteria, but pH in the caecum remained unaffected (Reuben et al. 2021).

The pH may both be a cause as well as an effect of different types of fermentation. Fermentation of both carbohydrates and protein leads to the production of butyrate and other VFAs which decrease pH, whereas an excess of AA, that are then deaminated, may increase pH via the production of ammonia. In this case, the change in pH is an effect of increasing proteolytic fermentation. On the other hand, Smith and Macfarlane (1998) demonstrated in an *in vitro* trial that increasing pH from 5.5 to 6.8 favoured peptide and amino acid fermentation by faecal bacteria from humans, hence, a change in pH caused a change in fermentation.

Intestinal microbes are known to have a preference for using carbohydrates as energy source over protein (Apajalahti and Vienola 2016; Smith and Macfarlane 1998). Therefore, feeding fibres (pre-caecally undigested carbohydrates) may prevent PF and keep the environment slightly acidic. Fibres such as inulin and fructooligosaccharides (FOS) have been extensively studied as feed additives to promote gut health. Studies in broilers have shown that dietary inulin or FOS improve growth performance, increase intestinal villi length, increase the counts of *Bifidobacterium* and *Lactobacillus* and decrease the numbers of *Escherichia coli, Clostridium perfringens, Campylobacter* and *Salmonella* (Kozlowska, Marc-Pienkowska, and Bednarczyk 2016).

When feeding wheat-based diets, xylanase supplementation may also be a strategy to increase the level of carbohydrates in the caeca. Lee et al. (2017) demonstrated that an increased level of sugars in the caeca as a result of xylanase supplementation encouraged the colonisation of *Bififdobacterium* and a higher production of butyric and acetic acid, while reducing branched-chain fatty acid (BCFA) concentration (the latter being PF metabolites).

Another nutritional strategy for improving gut health has been investigated by Qaisrani et al. (2015a). A coarse dietary structure improved performance of broilers fed a poorly digestible protein source (rapeseed meal) and reduced PF metabolites such as BCFA and biogenic amines in the caeca of broilers (Qaisrani et al. 2014b). This might have been the result of improved pre-caecal digestion due to more refluxing activity, which unfortunately was not determined.

Overall, PF depends on both a high level of protein as well as a low level of carbohydrates in the caeca. It is associated with relatively high pH and might be a prepositioning factor for disease, although clear proof of the latter is missing.

Known protein fermentation metabolites

A number of known protein fermentation metabolites are ammonia, hydrogen sulphide, nitric oxide, biogenic amines (such as: tyramine, histamine, cadaverine, putrescine, spermine), BCFA (isobutyrate, 2-methyl butyrate, isovalerate), phenols and indoles (Qaisrani et al. 2015b). Table 2 shows from which proteinaceous components these metabolites can be formed. Some, but not all, of the metabolites can be detrimental to the health of the host. Ammonia, produced upon amino acid deamination, is very toxic, but quickly absorbed and converted to and excreted as uric acid. It can also be used by bacteria for their own protein synthesis (Smith and Macfarlane 1997).

Phenol, produced by the fermentation of aromatic amino acids, is associated with cancer as its reaction with nitrite produces the mutagen Diazoquinone (Kikugawa and Kato 1988). Hydrogen sulphide is an end-product of the breakdown of sulphur-containing amino acids and is very toxic, as it inhibits cellular respiration (Leschelle et al. 2005). Nitric oxide is both a fermentation metabolite, produced by microbiota from arginine and nitrate, and an endogenously produced signalling molecule with antibiotic

Table 2. An overview of PF metabolites and the amino acids from which they can be derived.

Metabolite	Amino acid(s)	Ref
Ammonia (NH_3)*	Deamination of all amino acids	1
Hydrogen sulphide (H_2S)*	Sulphur AA: methionine, cystine, cysteine or taurine	1,2
Nitric oxide (NO)*	Arginine	3
Branched- & short chain fatty acids		
isobutytate	Valine	1
2-methyl-butyrate	Isoleucine	2
isovalerate	Leucine	2
Acetate*	Alanine, aspartate, arginine, cysteine, glutamate, glycine, histidine, lysine, serine, threonine	1
Propionate*	Alanine, aspartate, cysteine, methionine, threonine.	1
Butyrate*	Alanine, arginine, cysteine, glutamate, histidine, lysine, methionine.	1
Indoles:		
3-methyl-indole (skatole)	Tryptophan	2
indole	Tryptophan	2
Phenols:		
Phenol	Tyrosine	2
4-ethyl-phenol	Tyrosine	2
p-cresol	Tyrosine	2
Phenylpropionate	Phenylalanine	2
Phenylacetate	Phenylalanine	2
Biogenic Amines:		
tyramine	Tyrosine	4
histamine	Histidine	4
putrescine	Arginine, ornithine	5
spermidine	Arginine, ornithine	5
spermine	Arginine, ornithine	5
2-methylbutylamine	Valine	1

*These metabolites can also derive from other components than amino acids. References: 1 = Smith and Macfarlane (1997), 2 =Windey, De Preter, and Verbeke (2012), 3 = Vermeiren et al. (2009), 4 =Pessione and Cirrincione (2016), 5 = Larqué, Sabater-Molina, and Zamora (2007)

properties (Vermeiren et al. 2009). As reviewed by Gilbert et al. (2018), metabolites such as ammonia, phenols, hydrogen sulphide and nitric oxide affect gut mucosa by reducing cellular respiration, increasing permeability and affecting the expression of genes involved in transport or cell maintenance. It should be noted that the studies on which the latter is based have been performed on cell-lines and tissues of humans, rodents or pigs. Very little research has been performed on fermentation metabolites and their effects in chicken.

Methods for determining caecal protein fermentation

In vivo

Absorption of peptides and AA mainly occurs in the small intestines of livestock. The size of the fraction from dietary origin is directly dependent on the digestibility of protein in the diet. Hence a low true ileal protein digestibility may indicate a high potential for microbial PF in the caeca. Feeding birds diets contrasting in protein digestibility may be useful in the study of PF.

One method for this is to feed protein ingredients with a different digestibility level, as Qaisrani et al. (2014b), did by comparing a soybean meal (SBM) based diet (high protein digestibility) with a rapeseed meal (RSM) based diet (low protein digestibility). In the latter study a loss in growth performance in the RSM fed birds was not related to an increase in PF metabolites in the caeca. This might have been due to the higher level of fibre in RSM compared with SBM, confounding the effect of poor protein digestibility. Another method without this disadvantage is to use heat damage to reduce protein digestibility, which can be used to create a within ingredient contrast for *in vivo* studies.

Animal diets generally undergo different hydrothermal processing steps such as the removal of oil (used in the food industries) from soybeans and sunflower seeds resulting in meals, which can be used in animal feed. After mixing of the various ingredients, the ultimate diet is often pelleted which is an additional hydrothermal step.

When hydrothermal processing is too severe it can negatively affect the ileal digestibility of dietary proteins for animals, as aggregation of proteins occurs after denaturation and a decrease in lysine and available lysine occurs as a result of Maillard reactions (Sergio Salazar-Villanea et al. 2016). Maillard reactions, which are browning reactions between reducing sugars and AA or other feed components that occur during heating, can decrease digestibility. The level of available lysine is strongly affected by hydrothermal processing, as availability decreases quickly in the first stages of the Maillard reaction (Hulshof et al. 2016a). Total lysine consists of lysine with a free ε-amino-group, which is available, and lysine reacted into an early Maillard reaction product. This reaction product is converted back to free lysine under the acidic test conditions of conventional AA analysis, hence overestimating the lysine available to the animal (Hulshof et al. 2016a).

Table 3 summarises the effects of (hydro)thermal processing on ileal digestion in animals in different studies. This information might be useful for designing *in vivo* trials with contrasts in protein digestibility using similar ingredients.

Table 3. Effects of (hydro)thermal processing of ingredients on digestibility in animals.

Ingredient[1]	Process	Effect[2]	Reference
SBM RSM	Lignosulfonate added and toasted for 30 min at 95°C (all pelleted)	Standardised ileal digestibility CP (pigs): -12.3 (71.6 vs. 83.9) -10.3 (64.6 vs. 74.9)	Hulshof et al. (2016a)
SBM RSM	Lignosulfonate added and toasted for 30 min at 95°C (all pelleted)	Apparent ileal digestibility CP (pigs): -17.8 (63.0 vs. 80.8) -19 (43.2 vs. 62.2)	Hulshof et al. (2016b)
SBM	Autoclaved at 125°C for: - 15 min - 30 min Oven dried at 125°C: - 30 min	Apparent ileal digestibility CP (pigs): -4.6 (80.0 vs. 84.6) -9.3 (75.3 vs. 84.6) -1.9 (82.7 vs. 84.6)	González-Vega et al. (2011)
RSM	Desolventizer toasting at 100–110°C for: -60 min (mash) -120 min (mash) Pelleted at conditioning temp 80°C after toasting: -60 min -120 min	Apparent ileal digestibility CP (pigs): -2.1 (65.2 vs. 67.3) -11.0 (56.3 vs. 67.3) +1.3 (67.5 vs. 66.2) -0.6 (65.6 vs. 66.2)	Salazar-Villanea et al. (2018)
Sunflower meal	Autoclaved at 130°C for: -20 min -40 min -60 min	Apparent ileal digestibility CP (pigs): +0.6 (70.1 vs. 69.5) -4.8 (64.7 vs. 69.5) -11.6 (57.9 vs. 69.5)	Almeida et al. (2014)
Hipro SBM	Unprocessed Autoclaved at 121°C for 80 min	FCR (in broilers, no digestion measured) 1.34 1.55	Nell et al. (, unpublished pilot study)

[1]SBM = Soybean meal, RSM = Rapeseed meal.
[2]Unless otherwise stated, the effect show the difference in digestibility caused by the (hydro)thermal processing of the reference ingredient.

As shown in Table 3, hydrothermal processing must be done at considerably high temperatures (+130°C) or long durations (+120 min), to cause a decrease in digestibility of pre-processed ingredients such as SBM and RSM, when done without the addition of a reducing sugar (e.g.: ligonosulfonate) to induce Maillard reactions (Hulshof et al. 2016a, 2016b).

In order to determine the effect of pressurised steam toasting on protein digestibility of SBM, dehulled sunflower meal (dSFM) and dehulled rapeseed meal (dRSM), these ingredients were toasted for different durations, and the lysine and available lysine content were analysed (unpublished data; Table 4). Toasting rapidly reduced the level of lysine and available lysine, in all three meal types, particularly in the dSFM and dRSM. Toasting these meals could therefore be an interesting method to create contrasts in protein digestion for *in vivo* trials in which poorly digestible protein sources are used to induce protein fermentation.

To what extent a poor ileal digestion of protein leads to PF, hence to what extent these undigested proteins can be consumed by the microbiota in the caeca, is unclear and might depend strongly on the protein source (and the degree of toasting). Fractional separation of digesta occurs in poultry as the more soluble and finer particles enter the caeca and the larger insoluble particles remain in the colon (De Vries et al. 2014). If the fraction of undigested and unabsorbed proteins consists of large insoluble particles, it might not be subject to caecal fermentation as it will not enter the caeca. To what extent

Table 4. Effects of toasting Soybean meal (SBM), dehulled Rapeseed meal (dRSM) and dehulled sunflower meal (dSFM) on lysine and available lysine content.

	Lysine (g/kg)	Available Lysine (g/kg)	Reactive Lysine as % of total Lysine
SBM as is	27.51	27.58	100.25
SBM 30 min toasted	22.35	19.50	87.25
SBM 60 min toasted	17.57	9.81	55.83
SBM 90 min toasted	15.02	7.93	52.80
dRSM as is	23.84	25.05	105.08
dRSM 30 min toasted	13.69	8.74	63.84
dRSM 60 min toasted	9.70	4.35	44.85
dRSM 90 min toasted	7.20	1.88	26.11
dSFM as is	16.16	14.56	90.10
dSFM 30 min toasted	11.76	8.55	72.70
dSFM 60 min toasted	8.19	4.41	53.85
dSFM 90 min toasted	6.47	2.54	39.26

Source: Elling-Staats et al. (unpublished data).

using toasting ingredients such as SBM and RSM would reduce the solubility of its protein, resulting in little change of PF in the caeca, remains unclear. Toasting, however, does not need to reduce protein solubility of SBM or RSM. Hulshof et al. (2016b) demonstrated that toasting lead to poorer digestion in the small intestine of pigs without decreasing the N solubility of the digesta.

A method to determine if poorer protein digestion may lead to more PF in the hindgut, could be by measuring the difference between pre-caecal and total tract digestion. Differences between digestibility of AA when measured at pre-caecal level or in the faeces can be substantial and differs between protein sources (Ravindran et al. 1999; Kadim, Moughan, and Ravindran 2002). If the fraction of undigested protein by-passes the caeca the difference between faecal apparent digestibility and ileal apparent digestibility would be negligible. A negative difference between ileal and faecal apparent digestibility would indicate a net synthesis of protein (bacterial growth) while a positive difference in digestibility would indicate a net catabolism of proteins (proteins used for energy expenditure) (Qaisrani et al. 2015b).

Certain characteristics of the avian GIT could complicate the method described above. Chickens can move digesta from the colon back into the small intestine and further via reverse peristalsis movement of the gut, and this reflux appears to be part of their normal gut motility (Sacranie et al. 2007). It is therefore likely that also caecal protein and AA are refluxed into the small intestine and digested and absorbed there. It has also been shown that direct absorption of protein and AA from the caeca could occur in chickens (Karasawa and Maeda 1995) and this too complicates the method. A decrease in AA concentration found in the faeces compared with the ileal digesta is expected to indicate a net catabolism of AA by bacteria, but could also be the result of absorption. This absorption is, however, expected to be low.

More specific information on the type of fermentation occurring in the caeca can be determined by measuring typical PF metabolites, such as the examples mentioned earlier in Table 2. Ammonia is the end-product of all AA catabolism and is therefore expected to increase with higher level of overall AA fermentation. However, as ammonia is assimilated rapidly by the AA fermenting bacteria themselves, ammonia yield would unlikely quantitatively reflect total PF (Smith and Macfarlane 1997). Another complication of using ammonia as a measure for PF is the fact that it can also be derived from uric acid or

other N-containing components and might very well be quickly absorbed from the gut. BCFA are produced by bacteria from the AA valine, leucine and isoleucine (Windey, De Preter, and Verbeke 2012), and therefore specifically reflecting fermentation of these AA. Still, also BCFA are absorbed, and therefore their concentration in the caeca is not necessarily equal to their production but just an indication.

In conclusion, contrasts in pre-caecal protein digestibility of some ingredients can be created by hydrothermal processing and may be useful in the study of PF in chickens, despite complicating factors such as reverse peristalsis and the separation of digesta entering the caeca. The above-mentioned methods to determine PF in chicken, taking the difference between pre-caecal and total tract protein digestion or measuring PF metabolites in the caeca are useful indicators of PF, but these do not fully predict PF.

In vitro

In vitro gas production methods (Cone et al. 1996), which were originally developed to estimate fermentation kinetics of substrates in the rumen of ruminants, are also used to investigate fermentation in the hindgut of various species including chicken using faeces or caecal digesta as inoculum. In some studies, substrates are pre-digested by *in vitro* simulation of digestion up to the end of the ileum before fermentation using the gas production method (Cone et al. 2005; Bosch, Vervoort, and Hendriks 2016). To our knowledge this kind of method has not yet been used to look into PF in poultry caeca.

Cone et al. (2005) used a slightly adjusted gas production method to determine protein fermentation kinetics of different feedstuffs in pigs. Briefly, substrates were pre-digested using a modified method of Babinszky et al. (1990) and after filtration, the dried residue was used for incubation in a N-free buffer solution with excess of rapidly fermentable carbohydrates to ensure N was the limiting factor for microbial growth. Therefore, gas production profiles reflected the availability of N from the pre-digested substrates for microbial growth. This method could also be used to determine which protein source is highly available for chicken caecal microbiota, but the process is not the same as PF.

PF is known to occur in the hindgut of animals when there is an excess of proteinaceous components and carbohydrates are limiting, in which case microbes catabolise protein to meet their energy requirements and produce harmful metabolites (Windey, De Preter, and Verbeke 2012). Hence, limiting N is not helpful when using the gas production technique to estimate the potential of a substrate to allow for microbial protein catabolism and the production of potentially harmful metabolites. For this, it would be more appropriate to have an excess of the N source of interest and samples of the fluid should be analysed for the production of metabolites.

Pre-digesting substrates prior to *in vitro* fermentation using the gas production method is considered necessary as the substrate that is ingested by the animal is no longer the same when it enters the hindgut. The difficulty, however, of this kind of *in vitro* digestion (Babinszky et al. 1990; Boisen and Fernández 1997) is that these are an oversimplification of the digestive and absorption processes that occur in the animal. The pre-digestion methods consist of steps in which substrates are mixed into fluids and with digestive enzymes at body temperature and pH corresponding to the gut segment and

allowed to solubilise for a certain amount of time after which the mixture is filtrated. The residue, consisting of particles that were not solubilised, is considered the undigested fraction.

This insoluble fraction is, however, not the fraction of interest when mimicking caecal fermentation in poultry, as the caeca are likely to contain soluble components due to the fractional separation occurring at this point of the GIT in poultry (De Vries et al. 2014). This fractional separation effect was determined by adding soluble and insoluble indigestible markers into the feed. The ratio insoluble:soluble marker changed from 1.2 in the feed to 0.08 in the caecal digesta. Older studies confirm this type of separation (Vergara et al. 1989). Simple filtration to separate the 'undigested' from the 'digested' fraction will not provide researchers with the correct substrate for gas production, as this is likely to contain particles which do not enter the caeca. It would be more representative to use a two-step separation in which first the fraction that is likely absorbed before leaving the ileum is removed, after which the soluble/small particles and non-soluble/large particles of the remaining fraction are separated. The composition of these fractions in the bird have, unfortunately, not yet been quantified.

Conclusions

The chicken caeca are considered to be the most important sites for microbial fermentation and contain many different microbial groups that vary considerably in composition and relative abundance among birds and trials. Both the composition and the activity of the caecal microbiome are affected by the proteins and carbohydrates present as substrate. PF occurs when high levels of proteins and few carbohydrates are present. This generally results in a higher pH (close to neutral) in the caeca, further encouraging PF, as protein degrading microbes appear to favour this higher pH. Exactly which microbes are of importance to gut health has not yet been fully elucidated. PF is generally considered to be detrimental to the host, because of the metabolites produced. However, research on the effects of PF (metabolites) on gut health in poultry is limiting.

To investigate this relation, PF should be induced, which can be achieved by feeding high levels of dietary indigestible protein. Contrasts in dietary indigestible protein for *in vivo* studies of PF can be created within ingredient types by hydrothermal processing (resulting in less digestible Maillard products). However, the effect of hydrothermal processing on protein digestibility may differ among ingredients, and therefore, not all ingredients may be suitable. Another remaining challenge is that the relation between the level of indigestible protein and subsequent PF is not straightforward, as fractional separation of digesta occurs in poultry, allowing part of the digesta (potentially containing protein fractions) to bypass the caeca.

Altogether, in light of future poultry diets, an increase in PF can be expected, which will affect microbiota composition and metabolite production, and potentially gut health. A combination of *in vivo* and *in vitro* techniques allow to adequately evaluate the effects of PF on gut health in the unique digestive system of poultry.

Future research prospects

The future prospect of feeding low digestible protein sources to poultry and the lack of knowledge on the potential negative effects of PF warrants the need for studies evaluating the impact of PF on gut health in poultry. To this end, diets with contrasting protein digestibility levels can be used to investigate the effects of PF and PF metabolites on health and productivity in broilers. We believe that a within ingredient contrast in protein digestibility by applying heat damage will provide a suitable model for this. Furthermore, the fractional separation of digesta entering the caeca will need to be determined to evaluate whether lowering the digestibility will actually lead to an increase in protein flow towards the caeca. The use of multiple digesta markers following both solid and soluble digesta fractions could be a solution to this (De Vries et al. 2014; Martens et al. 2019). Also, when the flow of protein towards the caeca is increased, measuring the concentration of typical PF metabolites in the caeca can provide insight into the extent to which the proteins are actually fermented, as some of these metabolites are produced when specific amino acids are fermented. Furthermore, future research should combine microbiota analysis not just with performance, but also with metabolites produced and effects on gut physiology. This should shed light on which microbial groups possibly benefit from indigestible protein and what the effects on gut health are.

In vitro fermentation methods, such as the gas-production method of Cone et al. (2005) may be helpful to determine N availability of a indigestible protein to bacteria and even PF metabolite production from protein sources. The usefulness of such methods for evaluating PF in poultry will depend on the possibility to separate *in vitro* the fraction of pre-digested feed that is likely to enter the caeca *in vivo*. Besides simple filtration, techniques which separate soluble fractions (e.g. dialysis) would be required.

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References

Adil, S., and S. N. Magray. 2012. "Impact and Manipulation of Gut Microflora in Poultry: A Review." *Journal of Animal and Veterinary Advances* 11 (6): 873–877. doi:10.3923/javaa.2012.873.877.

Aftab, U. 2019. "Energy and Amino Acid Requirements of Broiler Chickens: Keeping Pace with the Genetic Progress." *World's Poultry Science Journal* 75 (4): 507–514. doi:10.1017/S0043933919000564.

Almeida, F. N., J. K. Htoo, J. Thomson, and H. H. Stein. 2014. "Digestibility by Growing Pigs of Amino Acids in Heat-Damaged Sunflower Meal and Cottonseed Meal." *Journal of Animal Science* 92 (2): 585–593. doi:10.2527/jas.2013-6769.

Apajalahti, J., and K. Vienola. 2016. "Interaction between Chicken Intestinal Microbiota and Protein Digestion." *Animal Feed Science and Technology* 221: 323–330. doi:10.1016/j.anifeedsci.2016.05.004.

Babinszky, L., J. M. Van Der Meer, H. Boer, and L. A. Den Hartog. 1990. "An in-Vitro Method for Prediction of the Digestible Crude Protein Content in Pig Feeds." *Journal of the Science of Food and Agriculture* 50 (2): 173–178. doi:10.1002/jsfa.2740500205.

Biasato, I., I. Ferrocino, S. Dabbou, R. Evangelista, F. Gai, L. Gasco, L. Cocolin, M. T. Capucchio, and A. Schiavone. 2020. "Black Soldier Fly and Gut Health in Broiler Chickens: Insights into the Relationship between Cecal Microbiota and Intestinal Mucin Composition." *Journal of Animal Science and Biotechnology* 11 (1): 1–12. doi:10.1186/s40104-019-0413-y.

Boisen, S., and J. A. Fernández. 1997. "Prediction of the Total Tract Digestibility of Energy in Feedstuffs and Pig Diets by in Vitro Analyses." *Animal Feed Science and Technology* 68 (3–4): 277–286. doi:10.1016/S0377-8401(97)00058-8.

Bosch, G., J. J. M. Vervoort, and W. H. Hendriks. 2016. "In Vitro Digestibility and Fermentability of Selected Insects for Dog Foods." *Animal Feed Science and Technology* 221: 174–184. doi:10.1016/j.anifeedsci.2016.08.018.

Chang, Q., W. Wang, G. Regev-Yochay, M. Lipsitch, and W. P. Hanage. 2015. "Antibiotics in Agriculture and the Risk to Human Health: How Worried Should We Be?" *Evolutionary Applications* 8 (3): 240–247. doi:10.1111/eva.12185.

Cone, J. W., A. W. Jongbloed, A. H. Van Gelder, and L. De Lange. 2005. "Estimation of Protein Fermentation in the Large Intestine of Pigs Using a Gas Production Technique." *Animal Feed Science and Technology* 123: 463–472. doi:10.1016/j.anifeedsci.2005.04.020.

Cone, J. W., A. H. van Gelder, G. J. W. Visscher, and L. Oudshoorn. 1996. "Influence of Rumen Fluid and Substrate Concentration on Fermentation Kinetics Measured with a Fully Automated Time Related Gas Production Apparatus." *Animal Feed Science and Technology* 61 (1–4): 113–128. doi:10.1016/0377-8401(96)00950-9.

Dahiya, J. P., D. Hoehler, A. G. Van Kessel, and M. D. Drew. 2007. "Effect of Different Dietary Methionine Sources on Intestinal Microbial Populations in Broiler Chickens." *Poultry Science* 86 (11): 2358–2366. doi:10.3382/ps.2007-00133.

Dahiya, J. P., D. Hoehler, D. C. Wilkie, A. G. Van Kessel, and M. D. Drew. 2005. "Dietary Glycine Concentration Affects Intestinal Clostridium Perfringens and Lactobacilli Populations in Broiler Chickens." *Poultry Science* 84 (12): 1875–1885. doi:10.1093/ps/84.12.1875.

Danicke, S., W. Vahjen, O. Simon, and H. Jeroch. 1999. "Effects of Dietary Fat Type and Xylanase Supplementation to Rye-Based Broiler Diets on Selected Bacterial Groups Adhering to the Intestinal Epithelium on Transit Time of Feed, and on Nutrient Digestibility." *Poultry Science* 78 (9): 1292–1299. doi:10.1093/ps/78.9.1292.

De Filippis, F., M. Laiola, G. Blaiotta, and D. Ercolini. 2017. "Different Amplicon Targets for Sequencing-Based Studies of Fungal Diversity." *Applied and Environmental Microbiology* 83 (17). doi:10.1128/AEM.00905-17.

De Lange, L., C. Rombouts, and G. Oude Elferink. 2003. "Practical Application and Advantages of Using Total Digestible Amino Acids and Undigestible Crude Protein to Formulate Broiler Diets." *World's Poultry Science Journal* 59 (4): 447–457. doi:10.1079/WPS20030028.

De Vries, S., R. P. Kwakkel, A. M. Pustjens, M. A. Kabel, W. H. Hendriks, and W. J. J. Gerrits. 2014. "Separation of Digesta Fractions Complicates Estimation of Ileal Digestibility Using Marker Methods with Cr2O3 and Cobalt-Ethylenediamine Tetraacetic Acid in Broiler Chickens." *Poultry Science* 93 (8): 2010–2017. doi:10.3382/ps.2013-03845.

Donoghue, A. M., M. B. Farnell, K. Cole, and D. J. Donoghue. 2006. "Mechanisms of Pathogen Control in the Avian Gastrointestinal Tract." In *Avian Gut Function in Health and Disease*, edited by G. Perry. Wallingford, UK: CABI Int. doi:10.1079/9781845931803.0000.

Durant, J. A., V. K. Lowry, D. J. Nisbet, L. H. Stanker, D. E. Corrier, and S. C. Ricke. 1999. "Short-chain Fatty Acids Affect Cell-association and Invasion of HEp-2 Cells by Salmonella Typhimurium." *Journal of Environmental Science and Health, Part B* 34 (6): 1083–1099. doi:10.1080/03601239909373246.

ECDC, (European Centre for Disease Prevention and Control), EFSA (European Food Safety Authority), and EMA (European Medicines Agency). 2017. "ECDC/EFSA/EMA Second Joint Report on the Integrated Analysis of the Consumption of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Bacteria from Humans and Food-Producing Animals: Joint Interagency Antimicrobial Consumption and Resistance Analysis (JIACRA) Report." *EFSA Journal* 15 (7): 4872, 135. doi:10.2903/j.efsa.2017.4872.

EMA (European Medicines Agency). 2018. "Sales of Veterinary Antimicrobial Agents in 30 European Counties in 2016. Trends Form 2010 to 2016. Eighth ESVAC Report." EMA/275982/2018. doi:10.2809/807372.

Ferrer, R., J. M. Planas, M. Durfort, and M. Moreto. 1991. "Morphological Study of the Caecal Epithelium of the Chicken (Gallus Gallus Domesticus L.)." *British Poultry Science* 32 (4): 679–691. doi:10.1080/00071669108417394.

Gilbert, M. S., N. Ijssennagger, A. K. Kies, and S. W. C. van Mil. 2018. "Protein Fermentation in the Gut; Implications for Intestinal Dysfunction in Humans, Pigs and Poultry." *American Journal of Physiology-Gastrointestinal and Liver Physiology* 315: G159–G170. doi:10.1152/ajpgi.00319.2017.

Glendinning, L., R. D. Stewart, M. J. Pallen, K. A. Watson, and M. Watson. 2020. "Assembly of Hundreds of Novel Bacterial Genomes from the Chicken Caecum." *Genome Biology* 21 (1): 1–16. doi:10.1186/s13059-020-1947-1.

González-Vega, J. C., B. G. Kim, J. K. Htoo, A. Lemme, and H. H. Stein. 2011. "Amino Acid Digestibility in Heated Soybean Meal Fed to Growing Pigs." *Journal of Animal Science* 89 (11): 3617–3625. doi:10.2527/jas.2010-3465.

Hinton Jr, A., R. J. Buhr, and K. D. Ingram. 2000. "Physical, Chemical, and Microbiological Changes in the Ceca of Broiler Chickens Subjected to Incremental Feed Withdrawal." *Poultry Science* 79 (4): 483–488. doi:10.1093/ps/79.4.483.

Hulshof, T. G., P. Bikker, A. F. B. Van der Poel, and W. H. Hendriks. 2016a. "Assessment of Protein Quality of Soybean Meal and 00-Rapeseed Meal Toasted in the Presence of Lignosulfonate by Amino Acid Digestibility in Growing Pigs and Maillard Reaction Products." *Journal of Animal Science* 94 (3): 1020–1030. doi:10.2527/jas.2015-9700.

Hulshof, T. G., A. F. B. Van der Poel, W. H. Hendriks, and P. Bikker. 2016b. "Processing of Soybean Meal and 00-Rapeseed Meal Reduces Protein Digestibility and Pig Growth Performance but Does Not Affect Nitrogen Solubilization along the Small Intestine." *Journal of Animal Science* 94 (6): 2403–2414. doi:10.2527/jas.2015-0114.

Hume, M. E. 2011. "Historic Perspective: Prebiotics, Probiotics, and Other Alternatives to Antibiotics." *Poultry Science* 90 (11): 2663–2669. doi:10.3382/ps.2010-01030.

Kadim, I. T., P. J. Moughan, and V. Ravindran. 2002. "Ileal Amino Acid Digestibility Assay for the Growing Meat Chicken-Comparison of Ileal and Excreta Amino Acid Digestibility in the Chicken." *British Poultry Science* 43 (4): 588–597. doi:10.1080/0007166022000004507.

Karasawa, Y., and M. Maeda. 1994. "Role of Caeca in the Nitrogen Nutrition of the Chicken Fed on a Moderate Protein Diet or a Low Rotein Diet Plus Urea." *British Poultry Science* 35 (3): 383–391. doi:10.1080/00071669408417703.

Karasawa, Y., and M. Maeda. 1995. "In Situ Degradation and Absorption of [15N] Urea in Chicken Ceca." *Comparative Biochemistry and Physiology Part A: Physiology* 111 (2): 223–227. doi:10.1016/0300-9629(95)00010-5.

Kers, J. G., F. C. Velkers, E. A. J. Fischer, G. D. A. Hermes, D. M. Lamot, J. A. Stegeman, and H. Smidt. 2019. "Take Care of the Environment: Housing Conditions Affect the Interplay of Nutritional Interventions and Intestinal Microbiota in Broiler Chickens." *Animal Microbiome* 1 (1): 1–14. doi:10.1186/s42523-019-0009-z.

Kers, J. G., F. C. Velkers, E. A. J. Fischer, G. D. A. Hermes, J. A. Stegeman, and H. Smidt. 2018. "Host and Environmental Factors Affecting the Intestinal Microbiota in Chickens." *Frontiers in Microbiology* 9: 235. doi:10.3389/fmicb.2018.00235.

Kikugawa, K., and T. Kato. 1988. "Formation of a Mutagenic Diazoquinone by Interaction of Phenol with Nitrite." *Food and Chemical Toxicology* 26 (3): 209–214. doi:10.1016/0278-6915(88)90121-4.

Kogut, M. H. 2019. "The Effect of Microbiome Modulation on the Intestinal Health of Poultry." *Animal Feed Science and Technology* 250: 32–40. doi:10.1016/j.anifeedsci.2018.10.008.

Kozlowska, I., J. Marc-Pienkowska, and M. Bednarczyk. 2016. "Beneficial Aspects of Inulin Supplementation as a Fructooligosaccharide Prebiotic in Monogastric Animal Nutrition-A Review." *Annals of Animal Science* 16 (2): 315–331. doi:10.1515/aoas-2015-0090.

Larqué, E., M. Sabater-Molina, and S. Zamora. 2007. "Biological Significance of Dietary Polyamines." *Nutrition* 23 (1): 87–95. doi:10.1016/j.nut.2006.09.006.

Lee, S. A., J. Apajalahti, K. Vienola, G. González-Ortiz, C. M. G. A. Fontes, and M. R. Bedford. 2017. "Age and Dietary Xylanase Supplementation Affects Ileal Sugar Residues and Short Chain Fatty Acid Concentration in the Ileum and Caecum of Broiler Chickens." *Animal Feed Science and Technology* 234: 29–42. doi:10.1016/j.anifeedsci.2017.07.017.

Leschelle, X., M. Goubern, M. Andriamihaja, H. M. Blottière, E. Couplan, C. Petit, A. Pagniez, et al. 2005. "Adaptative Metabolic Response of Human Colonic Epithelial Cells to the Adverse Effects of the Luminal Compound Sulfide." *Biochimica Et Biophysica Acta (Bba)-general Subjects* 1725 (2): 201–212. doi:10.1016/j.bbagen.2005.06.002.

Martens, B. M. J., T. Flécher, S. De Vries, H. A. Schols, E. M. A. M. Bruininx, and W. J. J. Gerrits. 2019. "Starch Digestion Kinetics and Mechanisms of Hydrolysing Enzymes in Growing Pigs Fed Processed and Native Cereal-Based Diets." *British Journal of Nutrition* 121 (10): 1124–1136. doi:10.1017/S0007114519000503.

Modh Shaufi, M. A., C. C. Sieo, C. W. Chong, H. M. Gan, and Y. W. Ho. 2015. "Deciphering Chicken Gut Microbial Dynamics Based on High-Throughput 16S rRNA Metagenomics Analyses." *Gut Pathogens* 7 (1): 4. doi:10.1186/s13099-015-0051-7.

Moquet, P. C. A., J. van Baal, L. Kruijt, H. Smidt, R. Ducatelle, W. H. Hendriks, and R. P. Kwakkel. 2018. "Chapter 6. Unprotected Butyrate Induces Bacterial Dysbiosis and Inflammation in the Distal Gastrointestinal Tract of Broilers." In *Butyrate in Broiler Diets: Impact of Butyrate Presence in Distinct Gastrointestinal Tract Segments on Digestive Function, Microbiota Composition and Immune Responses*, edited by Moquet. PhD diss. Wageningen University. doi:10.18174/449961.

Nakphaichit, M., C. Phraephaisarn, S. Keawsompong, O. Sukpiriyagul, and S. Nitisinprasert. 2014. "Effect of Increasing Dietary Protein from Soybean Meal on Intestinal Microbiota and Their Fatty Acids Production in Broiler Chicken." *Advances in Animal and Veterinary Sciences* 2 (6): 337–343. doi:10.14737/journal.aavs/2014/2.6.337.343.

Pessione, E., and S. Cirrincione. 2016. "Bioactive Molecules Released in Food by Lactic Acid Bacteria: Encrypted Peptides and Biogenic Amines." *Frontiers in Microbiology* 7: 876. doi:10.3389/fmicb.2016.00876.

Qaisrani, S. N., Y. Feng, M. M. Van Krimpen, R. P. Kwakkel, M. W. A. Verstegen, J. Zheng, H. Smidt, and W. H. Hendriks. 2014a. "Influence of Protein Source, Diet Structure, Butyric Acid and Fermentable Energy on Cecal Microbiota Population in Broilers." In *Improving Performance of Broilers Fed Lower Digestible Protein Diets*, edited by Qaisrani. PhD diss. Wageningen University. http://edepot.wur.nl/313161

Qaisrani, S. N., P. C. A. Moquet, M. M. Van Krimpen, R. P. Kwakkel, M. W. A. Verstegen, and W. H. Hendriks. 2014b. "Protein Source and Dietary Structure Influence Growth Performance, Gut Morphology, and Hindgut Fermentation Characteristics in Broilers." *Poultry Science* 93 (12): 3053–3064. doi:10.3382/ps.2014-04091.

Qaisrani, S. N., M. M. Van Krimpen, R. P. Kwakkel, M. W. A. Verstegen, and W. H. Hendriks. 2015a. "Diet Structure, Butyric Acid, and Fermentable Carbohydrates Influence Growth Performance, Gut Morphology, and Cecal Fermentation Characteristics in Broilers." *Poultry Science* 94 (9): 2152–2164. doi:10.3382/ps/pev003.

Qaisrani, S. N., M. M. Van Krimpen, R. P. Kwakkel, M. W. A. Verstegen, and W. H. Hendriks. 2015b. "Dietary Factors Affecting Hindgut Protein Fermentation in Broilers: A Review." *World's Poultry Science Journal* 71 (1): 139–160. doi:10.1017/S0043933915000124.

Qu, A., J. M. Brulc, M. K. Wilson, B. F. Law, J. R. Theoret, L. A. Joens, M. E. Konkel, et al. 2008. "Comparative Metagenomics Reveals Host Specific Metavirulomes and Horizontal Gene Transfer Elements in the Chicken Cecum Microbiome." *PloS One* 3 (8): e2945. doi:10.1371/journal.pone.0002945.

Raninen, K., J. Lappi, H. Mykkänen, and K. Poutanen. 2011. "Dietary Fiber Type Reflects Physiological Functionality: Comparison of Grain Fiber, Inulin, and Polydextrose." *Nutrition Reviews* 69 (1): 9–21. doi:10.1111/j.1753-4887.2010.00358.x.

Ravindran, V., L. I. Hew, G. Ravindran, and W. L. Bryden. 1999. "A Comparison of Ileal Digesta and Excreta Analysis for the Determination of Amino Acid Digestibility in Food Ingredients for Poultry." *British Poultry Science* 40 (2): 266–274. doi:10.1080/00071669987692.

Rehman, H., J. Böhm, and J. Zentek. 2008. "Effects of Differentially Fermentable Carbohydrates on the Microbial Fermentation Profile of the Gastrointestinal Tract of Broilers." *Journal of Animal Physiology and Animal Nutrition* 92 (4): 471–480. doi:10.1111/j.1439-0396.2007.00736.x.

Reuben, R. C., L. S. Shovon, I. Habiba, M. A. A. Setu, P. C. Roy, and I. K. Jahid. 2021. "Novel Multi-Strain Probiotics Reduces Pasteurella Multocida Induced Fowl Cholera Mortality in Broilers." *Scientific Reports* 11 (1): 1–16. doi:10.1038/s41598-021-88299-0.

Rubio, L. A., A. Brenes, I. Setien, G. De La Asuncion, N. Duran, and M. T. Cutuli. 1998. "Lactobacilli Counts in Crop, Ileum and Caecum of Growing Broiler Chickens Fed on Practical Diets Containing Whole or Dehulled Sweet Lupin (Lupinus Angustifolius) Seed Meal." *British Poultry Science* 39 (3): 354–359. doi:10.1080/00071669888890.

Sacranie, A., P. A. Iji, L. L. Mikkelsen, and M. Choct. 2007. "Occurrence of Reverse Peristalsis in Broiler Chickens." In Proceedings of the 19th Australian Poultry Science Symposium, Sydney, New South Wales, Australia, February 12–14, 161–164.

Saengkerdsub, S., R. C. Anderson, H. H. Wilkinson, W. K. Kim, D. J. Nisbet, and S. C. Ricke. 2007. "Identification and Quantification of Methanogenic Archaea in Adult Chicken Ceca." *Applied and Environmental Microbiology* 73 (1): 353–356. doi:10.1128/AEM.01931-06.

Salazar-Villanea, S., E. M. A. M. Bruininx, H. Gruppen, W. H. Hendriks, P. Carré, A. Quinsac, and A. F. B. van der Poel. 2016. "Physical and Chemical Changes of Rapeseed Meal Proteins during Toasting and Their Effects on in Vitro Digestibility." *Journal of Animal Science and Biotechnology* 7 (1): 62. doi:10.1186/s40104-016-0120-x.

Salazar-Villanea, S., E. M. A. M. Bruininx, H. Gruppen, W. H. Hendriks, P. Carré, A. Quinsac, and A. F. B. van der Poel. 2018. "Pelleting and Extrusion Can Ameliorate Negative Effects of Toasting of Rapeseed Meal on Protein Digestibility in Growing Pigs." *Animal* 12 (5): 950–958. doi:10.1017/S1751731117002476.

Sergeant, M. J., C. Constantinidou, T. A. Cogan, M. R. Bedford, C. W. Penn, and M. J. Pallen. 2014. "Extensive Microbial and Functional Diversity within the Chicken Cecal Microbiome." *PloS One* 9 (3): e91941. doi:10.1371/journal.pone.0091941.

Shabani, A., V. Jazi, A. Ashayerizadeh, and R. Barekatain. 2019. "Inclusion of Fish Waste Silage in Broiler Diets Affects Gut Microflora, Cecal Short-Chain Fatty Acids, Digestive Enzyme Activity, Nutrient Digestibility, and Excreta Gas Emission." *Poultry Science* 98 (10): 4909–4918. doi:10.3382/ps/pez244.

Siegerstetter, S. C., S. Schmitz-Esser, E. Magowan, S. U. Wetzels, Q. Zebeli, P. G. Lawlor, N. E. O'Connell, and B. U. Metzler-Zebeli. 2017. "Intestinal Microbiota Profiles Associated with Low and High Residual Feed Intake in Chickens across Two Geographical Locations." *PloS One* 12 (11): e0187766. doi:10.1371/journal.pone.0187766.

Smith, E. A., and G. T. Macfarlane. 1997. "Dissimilatory Amino Acid Metabolism in Human Colonic Bacteria." *Anaerobe* 3 (5): 327–337. doi:10.1006/anae.1997.0121.

Smith, E. A., and G. T. Macfarlane. 1998. "Enumeration of Amino Acid Fermenting Bacteria in the Human Large Intestine: Effects of pH and Starch on Peptide Metabolism and Dissimilation of Amino Acids." *FEMS Microbiology Ecology* 25 (4): 355–368. doi:10.1111/j.1574-6941.1998.tb00487.x.

Stanley, D., S. E. Denman, R. J. Hughes, M. S. Geier, T. M. Crowley, H. Chen, V. R. Haring, and R. J. Moore. 2012a. "Intestinal Microbiota Associated with Differential Feed Conversion Efficiency in Chickens." *Applied Microbiology and Biotechnology* 96 (5): 1361–1369. doi:10.1007/s00253-011-3847-5.

Stanley, D., M. S. Geier, R. J. Hughes, S. E. Denman, and R. J. Moore. 2013. "Highly Variable Microbiota Development in the Chicken Gastrointestinal Tract." *PloS One* 8 (12): e84290. doi:10.1371/journal.pone.0084290.

Stanley, D., A. L. Keyburn, S. E. Denman, and R. J. Moore. 2012b. "Changes in the Caecal Microflora of Chickens following Clostridium Perfringens Challenge to Induce Necrotic Enteritis." *Veterinary Microbiology* 159 (1–2): 155–162. doi:10.1016/j.vetmic.2012.03.032.

Svihus, B. 2014. "Function of the Digestive System." *Journal of Applied Poultry Research* 23 (2): 306–314. doi:10.3382/japr.2014-00937.

Svihus, B., M. Choct, and H. L. Classen. 2013. "Function and Nutritional Roles of the Avian Caeca: A Review." *World's Poultry Science Journal* 69 (2): 249–264. doi:10.1017/S0043933913000287.

Van der Klis, J. D., M. W. A. Verstegen, and W. De Wit. 1990. "Absorption of Minerals and Retention Time of Dry Matter in the Gastrointestinal Tract of Broilers." *Poultry Science* 69 (12): 2185–2194. doi:10.3382/ps.0692185.

Van Immerseel, F., J. De Buck, F. Pasmans, P. Velge, E. Bottreau, V. Fievez, F. Haesebrouck, and R. Ducatelle. 2003. "Invasion of Salmonella Enteritidis in Avian Intestinal Epithelial Cells in Vitro Is Influenced by Short-Chain Fatty Acids." *International Journal of Food Microbiology* 85 (3): 237–248. doi:10.1016/S0168-1605(02)00542-1.

Van Immerseel, F., J. I. Rood, R. J. Moore, and R. W. Titball. 2009. "Rethinking Our Understanding of the Pathogenesis of Necrotic Enteritis in Chickens." *Trends in Microbiology* 17 (1): 32–36. doi:10.1016/j.tim.2008.09.005.

Van Krimpen, M. M., R. P. Kwakkel, C. M. C. Van Der Peet-Schwering, L. A. Den Hartog, and M. W. A. Verstegen. 2011. "Effects of Dietary Energy Concentration, Nonstarch Polysaccharide Concentration, and Particle Sizes of Nonstarch Polysaccharides on Digesta Mean Retention Time and Gut Development in Laying Hens." *British Poultry Science* 52 (6): 730–741. doi:10.1080/00071668.2011.638620.

Vergara, P., C. Ferrando, M. Jimenez, E. Fernandez, and E. Gonalons. 1989. "Factors Determining Gastrointestinal Transit Time of Several Markers in the Domestic Fowl." *Quarterly Journal of Experimental Physiology* 74 (6): 867–874. doi:10.1113/expphysiol.1989.sp003357.

Vermeiren, J., T. Van de Wiele, W. Verstraete, P. Boeckx, and N. Boon. 2009. "Nitric Oxide Production by the Human Intestinal Microbiota by Dissimilatory Nitrate Reduction to Ammonium." *Journal of Biomedicine and Biotechnology* Article ID 284718. doi:10.1155/2009/284718.

Wang, Y., Q. Deng, D. Song, W. Wang, H. Zhou, L. Wang, and A. Li. 2017. "Effects of Fermented Cottonseed Meal on Growth Performance, Serum Biochemical Parameters, Immune Functions, Antioxidative Abilities, and Cecal Microflora in Broilers." *Food and Agricultural Immunology* 28 (4): 725–738. doi:10.1080/09540105.2017.1311308.

Warriss, P. D., L. J. Wilkins, S. N. Brown, A. J. Phillips, and V. Allen. 2004. "Defaecation and Weight of the Gastrointestinal Tract Contents after Feed and Water Withdrawal in Broilers." *British Poultry Science* 45 (1): 61–66. doi:10.1080/0007166041668879.

Wei, S., M. Morrison, and Z. Yu. 2013. "Bacterial Census of Poultry Intestinal Microbiome." *Poultry Science* 92 (3): 671–683. doi:10.3382/ps.2012-02822.

Wilkie, D. C., A. G. Van Kessel, L. J. White, B. Laarveld, and M. D. Drew. 2005. "Dietary Amino Acids Affect Intestinal Clostridium Perfringens Populations in Broiler Chickens." *Canadian Journal of Animal Science* 85 (2): 185–193. doi:10.4141/A04-070.

Windey, K., V. De Preter, and K. Verbeke. 2012. "Relevance of Protein Fermentation to Gut Health." *Molecular Nutrition & Food Research* 56 (1): 184–196. doi:10.1002/mnfr.201100542.

Wu, S. B., D. Stanley, N. Rodgers, R. A. Swick, and R. J. Moore. 2014. "Two Necrotic Enteritis Predisposing Factors, Dietary Fishmeal and Eimeria Infection, Induce Large Changes in the Caecal Microbiota of Broiler Chickens." *Veterinary Microbiology* 169 (3–4): 188–197. doi:10.1016/j.vetmic.2014.01.007.

Zhu, X. Y., T. Zhong, Y. Pandya, and R. D. Joerger. 2002. "16S rRNA-Based Analysis of Microbiota from the Cecum of Broiler Chickens." *Applied and Environmental Microbiology* 68 (1): 124–137. doi:10.1128/AEM.68.1.124-137.2002.