Improving performance of broilers fed lower digestible protein diets

Shafqat Nawaz Qaisrani

Thesis committee

Promotor
Prof. Dr W.H. Hendriks
Professor of Animal Nutrition
Wageningen University

Co-promotors
Dr R.P. Kwakkel
Assistant professor, Animal Nutrition Group
Wageningen University

Dr M.M. van Krimpen
Senior researcher
Wageningen UR Livestock Research, Lelystad

Other members
Prof. Dr L. Babinszky, University of Debrecen, Hungary
Dr S. Steenfeldt, Aarhus University, Denmark
Prof. Dr H.A. Schols, Wageningen University
Prof. Dr R. Zijlstra, University of Alberta, Edmonton, Canada

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Thesis

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Shafqat Nawaz Qaisrani

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Contents

CHAPTER 1

General introduction

Background

The human population in the world (7.2 billion) is increasing rapidly and it is projected that in the next 12 years it will grow by 1 billion and reach 9.6 billion by 2050 (UNO, 2013). In conjunction, it is predicted that the increase in human population will be accompanied by an equivalent increase in animal production to meet the demand for animal derived foods (Boland et al., 2013). The expected increase in the number of animals will also increase demands for feed and feed ingredients. Among the animals used as human foods, poultry production is expected to increase more compared to the production of other animals. Modern-day broiler chickens have been selected to efficiently convert dietary protein/amino acids into body protein. Broilers have a high gain to feed ratio, a high growth rate and a relatively low carbon footprint (Leach et al., 2012). Over the last decade, poultry meat production and human consumption of poultry has increased rapidly and it is expected that, in many parts of the world, consumption of poultry meat per capita will continue to grow (Cavani et al., 2010). Poultry meat demand grows 3 times as fast as the world population (FAO, 2013). This increased demand reflects consumer preference for these high-quality products that are available for a relatively low price. Poultry meat has maintained its identity and has a high intrinsic value for several reasons. This value includes a relatively low and competitive price compared to other meats, the absence of cultural or religious obstacles, and dietary and nutritional properties. Regarding nutritional aspects, poultry meat fits well with the current consumer demand for a low-fat meat, with a high content of unsaturated fatty acids and with low sodium and cholesterol levels. Poultry meat may also be considered as a "functional food", because it provides bioactive substances with favorable effects on human health, like conjugated linoleic acid, vitamins, antioxidants, and a balanced n-6 to n-3 polyunsaturated fatty acid ratio (Givens, 2009).

Based on the trends in human population growth and the increased demand for animal products, as well as the competition for highly digestible diet ingredients between men and animal, it is likely that future poultry diets may contain lesser quality protein ingredients because of the price volatility of traditional protein sources such as soybean meal (SBM). In broiler chickens, protein digestion starts in the proventriculus where some proteins are broken down into polypeptides by pepsin and a low pH because of the secretion of hydrochloric acid (HCl) (Hinton Jr et al., 1990). In the gizzard, the feed is ground, refluxed back into the proventriculus and mixed more thoroughly with water, saliva, HCl and pepsin. In the duodenum, proteins and polypeptides are broken down into smaller peptides and amino acids by the activity of the enzymes such as trypsin, chymotrypsin and elastase. Further breakdown of peptides into tri and dipeptides as well as amino acids for absorption into the blood

occurs by the enzymes in the brush border of the small intestine. At the terminal ileum, between 65 to 93% of the dietary proteins has been absorbed in poultry. Maize gluten meal (93%), fish meal (88%), and SBM (86%) are well digestible protein sources, whereas rape seed meal (76%) and cotton seed extract (65%) are examples of poorly digestible protein sources for poultry (CVB, 2007). This variation in digestibility may be due to various factors, e.g. protein source (Parsons, 2004), protein concentration (Rist et al., 2011), antinutritional factors (Sarwar et al., 2012), feed processing conditions (Zhang and Parsons, 1996), genetics (Mignon-Grasteau et al., 2004) and age of the bird (Nir et al., 1994). A high dietary inclusion level of feed ingredients with low N-digestibility will result in more protein entering the hindgut. This undigested dietary protein as well as endogenous protein may be used by microbiota to derive energy and to synthesize N containing compounds, and incorporate these into microbial bodies. Protein fermentation is the anaerobic breakdown of protein by microbiota in the hindgut (Windey et al., 2012). Protein fermentation can yield a variety of intermediates and end-products such as short-chain fatty acids (SCFA), ammonia (NH_3), hydrogen (H_2), carbon dioxide, methane, branched chain fatty acids (BCFA), amines, volatile phenols, and indoles. An overview of hindgut fermentation in broilers is presented in Figure 1.

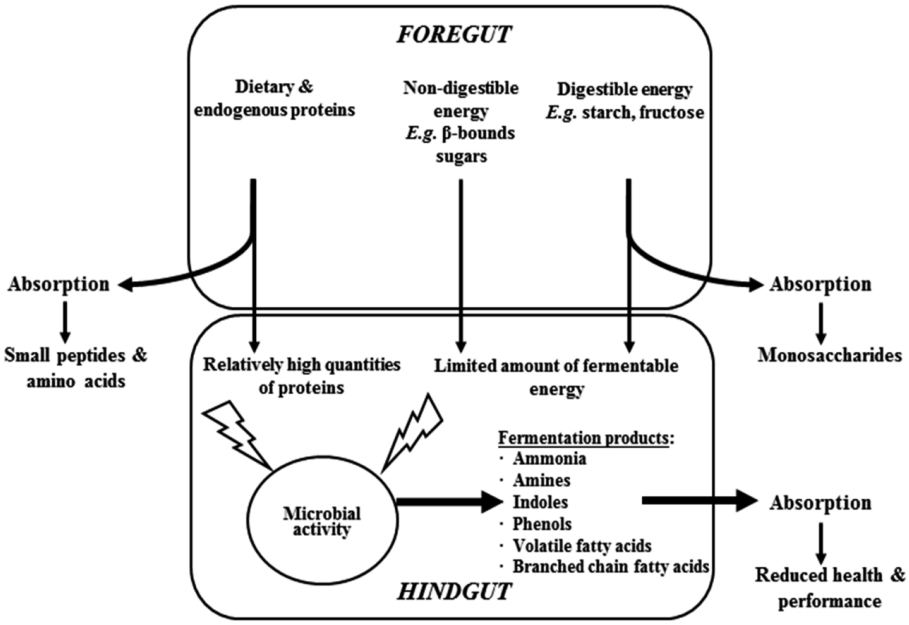

Figure 1. An overview of hindgut fermentation in broilers.

Compounds from protein fermentation may have deleterious effects on gut health and performance of animals (Thomke and Elwinger, 1998). The generated NH_3, for example, readily passes across the gut wall of the large intestine, and after transportation through the blood can affect the development of mucosa of the small intestine such as a reduction in villus height (Nousiainen, 1991). Furthermore, NH_3 needs to be eliminated from the body in the form of urea or uric acid, which requires energy. Eisemann and Nienaber (1990) estimated that NH_3 requires appropximately 7% of the total energy expenditure in livestock. Branched chain fatty acids, furthermore, can give a bad odour in feces and may cause post weaning diarrhea in pigs (Kim et al., 2008).

In chickens, fermentation mainly occurs in the ceca (Meyer et al., 2013) and is primarily influenced by the type of substrates available there. The amount of protein in the ceca depends on what is indigestible at ileal level and on the inclusion level of the dietary protein source. The imbalance between amount and type of carbohydrates and proteins entering the ceca may change the fermentation pattern into more carbohydrate or into more protein fermentation. A surplus of fermentable carbohydrates compared to protein in the ceca may lead to carbohydrate fermentation resulting in the production of volatile fatty acids (VFA) and utilization of NH_3 for microbial growth (Niba et al., 2009). These fermentable carbohydrates may provide energy to the gut microbes. The produced VFA may meet up to approximately 8% of the energy requirement of chickens (Saengkerdsub et al., 2006).

Protein source can alter the gut microbiota population towards more pathogenic species like *Clostridium perfringens* (Wilkie et al., 2005) and *Campylobacter* spp. (Wise and Siragusa, 2007). A wide variety of microorganisms including fungi, protozoa and bacteria (Gabriel et al., 2005) in the gut can be influenced by diet (Van der Hoeven-Hangoor et al., 2013), age of the bird, housing condition (Gabriel et al., 2005), and even litter material used (Torok et al., 2009). The microbiota differ in their substrate preferences and growth requirements which may be reflected by the diversity in different parts of the gastrointestinal tract (GIT) (Kiarie et al., 2013). There is competition for available nutrients in the gut between the resident microbiota and the host animal (Niba et al., 2009). This may decrease the availability of nutrients for the host and thus less growth (Ferrell, 1988).

Dietary particle size is an important factor controlling the passage rate in the GIT and its development. Dietary ingredients are ground into finer particles in the gizzard and then move towards the duodenum. Finely ground particles remain in the gizzard for a shorter period of time (Lott et al., 1992). This may result in impaired proventriculus, gizzard and gut function. This can have consequences for gut health. A coarse diet increases the chemical (pepsin in proventriculus) and physical (gizzard

muscle) functionality of the upper part of the GIT and, as a consequence, an enhanced growth performance (Gabriel et al., 2006). The slower digestion of large particles decreases feed passage rate, finally resulting in a high intestinal reflux and more exposure of feed particles to digestive enzymes leading to optimal absorption of nutrients and energy (Nir et al., 1994; Svihus, 2011).

Among the feeding strategies, short chain organic acid supplementation as a feed additive may be an important to improve ileal digestibility of protein. Feed additives play their role in maintaining gut health by numerous modes of actions such as: shifting gut pH, enhancement of pancreatic juice secretion, increasing nutrient intake, stimulating the humoral immune response, promoting beneficial microbiota or increasing fermentation acids (Van immerseel et al., 2005). Organic acids, furthermore, have bactericidal effects by disrupting the energy metabolism of bacteria (Ricke, 2003). Studies showed that the total bacterial population and in particular gram negative bacteria in broilers are less with organic acids supplementation (Gunal et al., 2006). Butyric acid is one of the most interesting organic acids used as a feed additive. It is assumed a very important energy source for the gut wall. Also other properties are important, such as a decrease in damage to epithelial cells because less toxic compounds from pathogenic bacteria are produced. Butyric acid is involved in providing energy to colonocytes and contributes to normal gut function but also reduces the formation of skatole, a metabolite of tryptophan degradation.

Aim of the Thesis

This thesis focusses on the use of low ileal digestible feed ingredients such as rapeseed meal, and their potential effect on hindgut protein fermentation in broilers. The phenomenon of hindgut protein fermentation, as well as an integrated approach to prevent this phenomenon in poultry has hitherto received little attention. As it can be expected that future diets of poultry may contain high levels of low ileal digestible protein, studies into hindgut fermentation and specifically protein fermentation are of value for diet formulation of future poultry diets.

The aim of the study is to find ways to improve protein digestibility of poor ileal digestible resources, to reduce potential hindgut protein fermentation by developing appropriate dietary strategies such as an adequate diet structure, the supplementation of organic acids and/or fermentable energy that improve nutrient availability at ileal level and gut health in broilers.

Outline of the Thesis

Chapter 2 contains a literature review about hindgut fermentation in broilers with special attention to protein fermentation, its detrimental effects on performance, health

and GIT microbiota population. The effects of protein source, dietary particle size, butyric acid and fermentable energy supplementation on performance and hindgut protein fermentation are described.

Dietary protein sources which differ in their protein digestibility in broilers are studied in **Chapter 3**. Performance, gut morphology, and cecal digesta characteristics are investigated using three different protein sources which had different enzymatic protein degradation characteristics. This study aims to find a model protein source for further experiments which, at an appropriate inclusion level, could be used as a standard low digestible protein source that potentially may generate hindgut protein fermentation.

Chapter 4 describes a study to investigate the gradual substitution of a highly digestible protein source with a low digestible protein source (as evaluated in Chapter 3), in interaction with diet structure on performance, gut morphology and cecal digesta characteristics. It is hypothesized that the effects of a poorly digestible dietary protein source on performance and gut health can be partly counteracted by feeding the diet in a coarse form. Coarse particles reside longer in the gizzard for proper grinding and mixing with digestive enzymes, before entering into the small intestine, which may enhance digestion. As a consequence, less protein will be available for fermentation in the hindgut by microbiota.

Effects of diet particle size with and without supplementation of butyric acid and fermentable energy are reported in **Chapter 5**. It is hypothesized, that the supplementation of coarse particles, combined with butyric acid and fermentable energy to a diet containing a poorly digestible protein source will counterbalance its negative consequences on overall performance of broilers. The aim of this study was to investigate the interaction effects of diet structure, butyric acid (as an organic acid) and fermentable energy supplementation on protein digestibility, gut health, performance, and hindgut fermentation characteristics in broilers.

In **Chapter 6**, effects of protein source, differing in digestibility, diet structure, supplementation with butyric acid (as an organic acid) and fermentable energy, as being investigated in the reported trials, on cecal microbiota composition and population were evaluated.

Finally in the General Discussion (**Chapter 7**), the results described in Chapters 3 to 6 are reviewed and evaluated. This chapter also provides the main conclusions and recommendations for further research.

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CHAPTER 2

Dietary factors affecting hindgut protein fermentation in broilers: a review

S.N. Qaisrani[1], M.M. van Krimpen[2], R.P. Kwakkel[1], M.W.A. Verstegen[1], and W.H. Hendriks[1]

[1]Animal Nutrition Group, Wageningen University, PO Box 338, NL-6700 AH Wageningen, The Netherlands

[2]Wageningen UR Livestock Research, PO Box 65, NL-8200 AB Lelystad, The Netherlands

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Abstract

High growth rates in modern-day broilers require diets concentrated in digestible protein and energy. In addition to affecting feed conversion efficiency, it is important to prevent surplus dietary protein because of greater amounts of undigested protein entering the hindgut that may be fermented by the resident microbiota. The latter may result in increased formation of a wide range of protein-derived compounds including ammonia, amines, indoles and phenols, in addition to secondary products (lactate, succinate) and gases such as methane, hydrogen and carbon dioxide. In poultry, studies have shown the presence of protein fermentation products such as biogenic amines and branched chain fatty acids (BCFA) in the ileal and cecal digesta. As a result, marked differences in protein digestibility when measured at the ileum and the total digestive tract can occur. The production and metabolism of nitrogenous waste products (as a result of protein fermentation) such as uric acid and ammonia may lead to a burden on the organism and cause additional energy losses. Absorbed ammonia can disturb villus heights and crypt depths, reduce nutrient absorption and thus growth performance of broilers. Although biogenic amines are important for normal gut development, greater concentrations may cause gizzard erosion, mortality and depressed growth rate in broilers. A decrease in indigestible protein reduces hindgut protein fermentation. In broilers, feeding diets with coarse particles (between 600 to 900 μm), can improve protein digestion and, thereby, may reduce hindgut protein fermentation. Feed additives, such as pre- and probiotics and organic acids, especially butyric acid, stimulate pancreatic juice secretion and improve protein digestion, thereby, potentially reducing hindgut protein fermentation. In light of future needs towards using poorer quality protein sources, studies are needed to determine the extent and importance of hindgut protein fermentation on performance and gut health in broilers.

Key words: gut health, protein fermentation, hindgut, broilers, feeding strategy, organic acids.

Introduction

The length of the growth period in broilers is decreasing year by year. Currently, meat chickens can attain 2.0 kg body weight after consuming 3.0 kg of feed within a 5 wk period (Choct, 2009). This rapid growth rate is related to a very high capacity for protein deposition combined with a high feed intake as a result of genetic selection over the past decades (Havenstein et al., 2003). Rapid growth requires diets that have a high content of digestible protein and energy. In practical feed formulation, diets high in digestible protein also contain appreciable quantities of undigested protein. The latter protein enters the hindgut where it can be used as a substrate for microbial degradation (Jeaurond et al., 2008). This undigested protein and unabsorbed endogenous protein may promote the proliferation of microbiota which uses the undigested amino acids as an energy source (Reid and Hillman, 1999). The latter can occur especially if insufficient fermentable carbohydrates are available. In an ideal situation, the unabsorbed dietary and endogenous amino acids in the large intestine will serve as building blocks for microbial protein synthesis with dietary carbohydrates used as an energy source. The extent to which hindgut protein fermentation occurs depends consequently, on the availability of protein in relation to the amount of fermentable carbohydrates as these carbohydrates are a preferred source of energy for gut microbes (Rehman et al., 2008). As such, the digestibility of dietary protein and carbohydrates, and their dietary inclusion level are important to determine the amounts of protein and carbohydrates entering the hindgut and thus the potential for protein and/or carbohydrate fermentation (Gill and Rowland, 2002).

Proteolysis is the first step in the utilization of protein by the microbiota (Jeaurond et al., 2008). Subsequent deamination and decarboxylation of amino acids delivers a substrate which can be used as an energy source. In poultry, ceca are the major sites for fermentation because they provide a stable environment for indigenous microbiota (Meyer et al., 2013) which may be due to a longer residence time of digesta there. Besides using dietary and endogenous amino acids for energy and protein synthesis, gut microbiota can produce different putrefactive compounds from undigested nitrogen (N) including indoles, phenols, sulphur-containing compounds, ammonia, and amines. In addition to the beneficial products like volatile fatty acids (VFA) and branched chain fatty acids (BCFA), some secondary products (lactate, succinate) and several gases such as methane, hydrogen, hydrogen sulfide (H_2S), and carbon dioxide, (Macfarlane et al., 1992) can be produced. Some of the BCFAs like isobutyrate, isovalerate and other compounds such as formate, valerate and capronate are produced in relative small proportions (10 to 20 mol/100 mol VFA) (Macfarlane et al., 1992). The nitrogenous waste products of hindgut protein fermentation such as ammonia, amines, indoles, phenol, and sulfides can be absorbed or may be transported back up

the gastrointestinal tract (GIT) by anti-peristaltic contractions (Macfarlane et al., 1988). These products may damage the mucosal surface in the small intestine as well as in the colon (Nousiainen, 1991) thereby reducing gut health.

The impact of protein fermentation on performance and gut health of modern-day broilers is becoming increasingly relevant in view of the growing demand for dietary protein sources which often have a low digestibility by poultry. The trend to search for cheaper sources of feed protein is due to the ever increasing price of highly digestible protein ingredients and the aim of cost-effective production. The goal of this review paper is to ascertain for available evidences for the occurrence of hindgut protein fermentation and its impact on broiler performance. Secondly, this review aims to describe the possibilities of reducing hindgut protein fermentation in broilers through the application of a number of potential nutritional strategies. Due to a lack of information in the poultry nutrition literature, some examples of other species are used in this review.

Protein Fermentation in Broilers

Fermentation is a breakdown of organic products by microbiota in the GIT. Fermentation of carbohydrates is considered as beneficial because it results in the production of VFAs which are used as an energy source (Guo et al., 2003), while protein fermentation is generally considered detrimental for health. The latter is due to the production of toxic compounds such as amines and ammonia (Macfarlane et al., 1988), and ultimately may result in poor performance of the birds. Rehman et al. (2007) in his review, reported the presence of lactate, ammonia and SCFA in the crop content of broilers, indicating that fermentation of dietary components already can occur in the crop. In poultry, due to a short residence time and low pH of digesta in the duodenum, there is a little microbial activity in the duodenum (Rehman et al., 2007). The SCFA concentration due to microbial activity is, therefore, also low (2-12 µmol/ g digesta) or sometimes even undetectable in the digesta in this gut segment (Rehman et al., 2007). In the ceca, bacterial fermentation activity can be high due to a long residence time of digesta and a high microbial density (Guo et al., 2003). To our knowledge, there are no studies in broilers that specifically studied the occurrence and importance of hindgut protein fermentation. In a number of studies, however, several metabolites which are indicative of the occurrence of hindgut protein fermentation in broilers have been quantified.

Gut Microbiota Population

The gut microbiota ecosystem is complex and is composed of fungi, protozoa and, most important, bacteria (Gabriel et al., 2005). Numbers of known bacterial species in

chickens has increased from 200 up to 640 in recent years (Apajalahti et al., 2004) due to advances in new techniques of microbiota analysis. The microbiome is affected by numerous factors including diet (Torok et al., 2011), genetics of the host (Mignon-Grasteau et al*.,* 2004), age of the bird, housing condition (Gabriel et al., 2005), and litter material used (Torok et al., 2009). Microbiota diversity in different parts of the GIT reflects the composition of the substrate present in that part intestinal segment as microbiota species differ in their substrate preferences and growth requirements (Kiarie et al., 2013). If in the small intestine the growth of pathogenic microbiota is increased, this may enhance endogenous losses and increase the maintenance requirements for replacing these losses thus compromising growth efficiency (Ferrell, 1988). A high inflow of undigested material in the large intestine allows microbes to use the digesta as a substrate in order to proliferate. The gut microbial community is influenced by dietary protein source favoring the growth of pathogenic bacteria like *Campylobacter* spp. (Wise and Siragusa, 2007) and *C. perfringens* (Wilkie et al., 2005). Similarly, increasing the level of dietary protein resulted in a higher *C. perfringens* counts independent of the protein source used (Drew et al., 2004). There is evidence in broilers that glycine (at levels above 3% in the diet) supports the growth of *C. perfringens* in ileum and cecum (Dahiya et al., 2005).

At day one after hatching, digesta from the ileum contains approximately 10^8 bacteria per gram and this increase to 10^9 at day 3 of life where after it remains constant till 30 days of age in broilers (Apajalahti et al., 2004). *Lactobacillus* (70%) is the major species found in the ileum, whereas the other species include *Clostridiaceae* (11%), *Streptococcus* (6.5%) and *Enterococcus* (6.5%) (Lu et al*.,* 2003). This microbiota population is influenced by dietary ingredients such as medium chain fatty acids which decreased the growth of gram-positive *Firmicutes* and some other species including *Lactobacillus*, *Micrococcaceae* and *Enterococcaceae*, whereas the growth of gram-negative bacteria increased (Van Der Hoeven-Hangoor et al*.,* 2013). The latter authors related the change in microbiota population with higher dietary concentration of medium chain fatty acid to the sensitivity of gram positive compared with gram negative bacteria.

In the ceca there is a higher diversity of microbiota compared to the ileum, with dominance of *Clostridium* (*Ruminococcus, Eubacterium*) spp. (Gabriel et al., 2005). The microbiota population in the ceca of broilers has been estimated at 10^{11} bacterial numbers per gram of cecal digesta and to mainly include obligate anaerobes (Barnes et al., 1972). In broilers, during the first few days after hatch, *Enterococcus faecalis* is usually dominant in ceca, but obligatory anaerobes become dominant as the bird grows. The cecal microbial population at 14 days of age contains anaerobic species comprising of gram-positive (*Clostridium* spp., *Eubacterium* spp., *Lactobacillus* spp.,

anaerobic cocci) and gram-negative (*Bacteriodes* spp., *Fusobacterium* spp. and *Gemmiger* spp.) bacteria (Barnes et al., 1972). This microbiota population reached a steady stage after 40 days of age and at this age consists mainly of gram-positive bacteria including *Bifidobacterium* spp., *Clostridium* spp., *Cocci, Streptococcus* spp., *E. coli*, *Bacteriods* spp. and *Lactobacillus* spp. (Barnes et al., 1972). An overview of different microbiota populations throughout the GIT of poultry is presented in Table 1.

Difference between Ileal and Excreta Digestibility

Nitrogen metabolism in the hindgut includes both catabolism and synthesis of proteins from dietary, endogenous and microbial origin as well as other N-containing compounds. Although there is some absorption of amino acids in the large intestine of monogastric animals, quantitatively it is insignificant compared to absorption in the small intestine (Hendriks et al., 2012). Dietary amino acid recovery in excreta will decrease as a result of net protein degradation in the large intestine (in the form of different compounds e.g. biogenic amines, ammonia, indole, phenols, cresol and skatole), thereby, overestimating amino acid digestibility. In contrast, the opposite will be the case and amino acid digestibility will be underestimated when synthesis results in a net increase of microbial amino acids in the hindgut. Kadim et al. (2002) did not find any difference in endogenous amino acid losses (except for Asp and Glu) when measured in ileal digesta or in excreta while comparing the ileal and excreta amino acid digestibility of different feed ingredients in broilers. The latter indicates that the large intestine does not appear to make a significant contribution to overall gut endogenous amino acid losses in broilers. As there is no significant absorption of amino acids in the hindgut (Hendriks et al., 2012), the difference between ileal and excreta digestibility can, therefore, be an important indicator of the occurrence of protein fermentation. If no protein fermentation occurs, digestibility values when measured using ileal digesta should not be significantly different from values determined in excreta, whether apparent or standardized digestibility values. There are a number of studies indicating significant differences between ileal and excreta digestibility values of different amino acids of various feed stuffs in broilers (Sebastian et al., 1997; Ravindran et al., 1999; Kadim et al., 2002). Some studies showed a net lower amino acid and total N recovery in the excreta compared with the ileal digesta (Ravindran et al., 1999; Sebastian et al., 1997), whereas others showed an increase in amino acid recovery in the excreta (Doeschate et al., 1993; Kadim et al., 2002). The difference between ileal and excreta digestibility values of various amino acids of different feed ingredients for poultry are shown in Table 2. For most amino acids, a negative values is observed although the range within ingredient can be highly variable

Table 1. Bacterial concentration in different segments of gastrointestinal tract (GIT) in broilers.

GIT segment	Bacterial species (log cfu/g of contents)								Reference
	Bacteroides	*Clostridium* spp.	*Bifidobacterium* spp.	*Enterococcus* spp.	*Streptococcus* spp.	*Enterobacteriaceae*	*Lactobacillus* spp.	*E. coli*	
Crop	ND	ND	ND	4.8	ND	7.6	7.9	ND	Kročko et al. (2012)
	ND	BDL	ND	6.5	ND	6.7	9.0	ND	Bjerrum et al. (2006)
	ND	ND	ND	ND	ND	5.6	8.9	ND	Zhang et al. (2003)
	ND	ND	ND	4.2	ND	6.6	ND	ND	Danicke et al. (1999)
	ND	ND	ND	ND	ND	5.8	7.6	ND	Rubio et al. (1998)
	10.8	8.9	7.2	3.7	8.6	10.1	8.4	ND	Takahashi et al. (1982)
	ND	ND	ND	ND	4.0	ND	8.7	1.7	Smith (1965)
Gizzard	ND	ND	ND	5.1	ND	ND	6.2	4.4	Bjerrum et al. (2005)
	ND	ND	ND	5.1	ND	ND	6.8	4.4	Engberg et al. (2004)
	ND	ND	ND	ND	ND	ND	6.4	3.4	Chen (2003)
	ND	ND	ND	4.3	ND	ND	6.6	4.3	Engberg et al. (2002)
	ND	ND	ND	5.0	ND	ND	7.5	4.8	Engberg et al. (2000)
	ND	ND	ND	ND	3.7	ND	7.3	ND	Smith (1965)
Jejunum	ND	4.8	ND	5.7	ND	ND	7.5	5.7	Engberg et al. (2004)
	ND	ND	ND	5.8	7.4	ND	8.0	5.5	Engberg et al. (2002)
	ND	ND	ND	5.9	ND	ND	8.1	6.3	Engberg et al. (2000)
	6.5	6.5	ND	ND	5.3	ND	6.5	5.7	Smits et al. (1998)
Ileum	ND	ND	ND	3.7	ND	5.9	7.4	ND	Kročko et al. (2012)
	ND	BDL	ND	6.5	ND	ND	9.0	6.7	Bjerrum et al. (2006)
	ND	6.4	ND	7.1	ND	ND	8.5	7.4	Engberg et al. (2004)
	ND	5.9	7.3	ND	ND	ND	7.5	6.9	Xia et al. (2004)
	ND	ND	ND	7.5	ND	ND	6.2	8.1	Alzueta et al. (2003)
	ND	ND	7.2	ND	ND	ND	7.5	7.0	Xu et al. (2003)
	7.7	7.7	ND	ND	6.6	ND	7.7	7.7	Smits et al. (1998)
Ceca	9.9	10.1	9.7	ND	ND	ND	9.4	ND	Jozefiak et al. (2010)
	ND	3.7	6.9	ND	ND	ND	6.4	3.6	Tiihonen et al. (2010)
	BDL	ND	ND	ND	6.7	ND	9.2	6.7	Bjerrum et al. (2006)
	11.2	4.3	8.4	ND	ND	ND	10.1	6.9	Cao et al. (2005)
	ND	6.5	ND	ND	7.1	ND	8.8	8.7	Engberg et al. (2004)
	ND	6.6	8.2	ND	ND	ND	8.4	7.5	Xia et al. (2004)
	6.7	ND	8.6	ND	7.4	ND	7.6	7.0	Guo et al. (2003)
	8.7	1.7	6.9	ND	6.7	ND	8.7	5.6	Smith (1965)

cfu = colony forming unit, ND = not determined, BDL = below detection level.

Table 2. Difference between ileal and excreta apparent digestibility (%) of different amino acids from different feed stuffs in broilers.

Ingredient	Essential amino acids									Reference
	Thr	Val	Met	Iso	Leu	Phe	His	Lys	Arg	
Blood meal	-14.2*	+ 0.4	+ 2.1	0	- 0.1	+ 3.8	+ 2.2	- 3.2	- 3.7	Kadim et al. (2002)
	- 5.0	0	0	+ 26.0	- 2.0	- 1.0	- 5.0	0	+ 3.0	Ravindran et al. (1999)
Canola meal	- 1.0	+ 2.0	+ 1.0	+ 2.0	+ 1.0	+ 2.0	+ 1.0	+ 1.0	- 2.0	Ravindran et al. (1999)
Corn-soya diet[1]	- 1.8	- 1.1	- 0.9	- 1.5	- 0.8	- 0.3	- 0.7	+ 1.1	+ 0.5	Sebastian et al. (1997)
Cotton seed meal	- 1.0	+ 3.0	+ 6.0*	+ 3.0*	+ 3.0*	+ 2.0	+ 1.0	+ 6.0*	- 1.0	Ravindran et al. (1999)
Feather meal	- 24.0*	- 14.0	- 12.0	- 12.0	- 14.0	- 8.0	- 12.0	- 20.0*	- 16.0*	Ravindran et al. (1999)
Fish meal	- 7.6*	- 0.6	+ 5.4	- 0.3	-5.4	- 0.3	- 0.3	- 1.3	- 1.3	Kadim et al. (2002)
	- 7.0*	+ 3.0*	0	+ 4.0*	0	0	- 4.0	+ 1.0	- 2.0*	Ravindran et al. (1999)
Maize	- 7.0	+ 6.0*	- 1.0	+ 8.0*	+ 1.0	+ 3.0	0	- 1.0	+ 1.0	Ravindran et al. (1999)
Meat meal	- 6.0	0	- 3.0	- 2.0	0	- 1.0	+ 3.0	- 1.0	- 3.0	Ravindran et al. (1999)
	- 19.0	- 11.0	- 11.0	- 10.0	- 11.0	- 3.0	- 6.0	- 16.0	- 13.0	Ravindran et al. (1999)
Meat and bone meal	- 16.7*	+ 5.1	+ 4.9	- 7.3*	- 8.6*	+ 2.2	- 3.6	- 8.0*	+ 0.7	Kadim et al. (2002)
	- 12.0*	- 5.0	- 6.0*	- 5.0	- 6.0	+ 3.0	+ 3.0	-14.0*	- 6.0	Ravindran et al. (1999)
Sorghum	- 8.7*	- 0.6	- 0.8	- 5.5	- 2.3	+ 1.2	+ 2.8	- 5.5	+ 3.9	Kadim et al. (2002)
	+ 3.0	+ 7.0	+ 1.0	+ 8.0	+3.0	+ 4.0	+ 12.0*	+10.0	+ 4.0	Ravindran et al. (1999)
Soybean meal	- 7.7*	- 4.4	+ 2.6	- 0.6	- 3.9	+ 1.3	- 3.1	- 1.0	- 1.9	Kadim et al. (2002)
	- 2.0*	+ 5.0*	+ 1.0	+ 5.0*	+ 1.0*	+ 1.0*	-	0	- 2.0	Ravindran et al. (1999)
	- 5.0*	+ 2.0	+ 9.0*	0	- 4.0	0	0	- 3.0	- 4.0*	Ravindran et al. (1999)
	- 3.0	0	+ 7.0	0	- 1.0	+ 1.0	0	+ 2.0	- 2.0	Ravindran et al. (1999)
	- 0.6	- 0.1	- 3.1	- 1.8	- 3.2	- 1.2	0	- 1.3	- 4.2	Doeschate et al. (1993)
Sunflower meal	- 1.0	+ 7.0*	+ 2.0	+ 7.0*	+ 5.0	+ 4.0*	0	+ 4.0	- 8.0	Ravindran et al. (1999)
Wheat	- 19.6*	- 11.3	- 1.6*	- 18.2*	- 20.4*	- 6.9*	- 16.0*	- 9.5*	- 20.3*	Kadim et al. (2002)
	+ 20.0*	+ 15.0*	+ 7.0*	+ 14.0	+ 11.0	+ 10.0	+ 18.0	+ 17.0*	+ 7.0*	Ravindran et al. (1999)
	- 3.3	+ 0.1	- 0.2	- 0.2	- 6.1	- 3.2	- 4.1	- 4.5	- 1.0	Doeschate et al. (1993)
Average	- 6.3	+ 0.3	+ 0.4	+ 0.5	- 2.7	+ 0.6	- 0.5	- 2.0	- 3.0	

	Non essential amino acids							
	Asp	Ser	Glu	Ala	Cys	Pro	Tyr	
Blood meal	- 16.9*	- 0.5	- 10.3*	+ 7.1*	ND	- 3.9	+ 3.3	Kadim et al. (2002)
	- 4.0	- 3.0	+ 2.0	0	ND	ND	+ 2.0	Ravindran et al. (1999)
Canola meal	- 2.0	- 3.0	+ 1.0	+ 4.0	ND	ND	0	Ravindran et al. (1999)
Corn-soya diet[1]	- 1.5	- 2.2	0	- 0.3	ND	- 0.1	- 1.2	Sebastian et al. (1997)
Cotton seed meal	- 1.0	- 2.0	+ 2.0*	+ 8.0*	ND	ND	+ 1.0	Ravindran et al. (1999)
Feather meal	- 37.0*	- 21.0*	- 18.0	- 12.0	ND	ND	- 13.0	Ravindran et al. (1999)
Fish meal	- 1.3	- 4.8	- 6.2	+ 1.3	ND	+ 0.2	+ 1.2	Kadim et al. (2002)
	- 9.0*	- 9.0*	0	+ 4.0	ND	ND	+ 2.0	Ravindran et al. (1999)

Maize	0	- 5.0	+ 2.0*	+ 5.0*	ND	ND	- 1.0	Ravindran et al. (1999)
Meat meal	- 18.0	- 10.0	- 4.0	0	ND	ND	- 8.0	Ravindran et al. (1999)
	- 30.0	- 21.0	- 14.0	- 13.0	ND	ND	- 8.0	Ravindran et al. (1999)
Meat and bone meal	- 17.1*	- 7.3*	- 10.0*	- 13.0*	ND	- 4.9	- 7.9*	Kadim et al. (2002)
	- 21.0*	- 13.0*	- 7.0	- 7.0	ND	ND	- 2.0	Ravindran et al. (1999)
Sorghum	- 2.9	- 8.8*	- 0.1	- 0.2	ND	- 0.1	- 2.1	Kadim et al. (2002)
	+ 4.0	0	+ 4.0	+ 8.0	ND	ND	- 4.0	Ravindran et al. (1999)
Soybean meal	- 2.5	- 5.8	- 6.6	- 4.8	ND	- 8.0*	- 4.5	Kadim et al. (2002)
	- 4.0*	- 4.0*	0	+ 9.0*	ND	ND	+ 1.0	Ravindran et al. (1999)
	- 5.0*	- 10.0	+ 2.0	+ 2.0	ND	ND	- 1.0	Ravindran et al. (1999)
	- 4.0*	- 5.0	+ 1.0	+ 4.0*	ND	ND	- 1.0	Ravindran et al. (1999)
	- 5.0	- 2.7	- 3.0	+ 0.7	- 2.6	+ 5.3	- 3.9	Doeschate et al. (1993)
Sunflower meal	+ 1.0	- 5.0*	+ 3.0*	+ 9.0*	ND	ND	+ 8.0	Ravindran et al. (1999)
Wheat	- 31.3*	- 6.5*	- 4.3	- 9.9*	ND	- 4.3	- 15.7*	Kadim et al. (2002)
	+ 18.0	+11.0*	+ 6.0*	+ 25.0*	ND	ND	+ 5.0*	Ravindran et al. (1999)
	- 1.5	+ 1.0	- 1.0	+ 0.9	- 3.4	+ 1.8	- 8.8	Doeschate et al. (1993)
Average	- 8.0	- 5.7	-2.6	+ 1.2	- 3.0	- 1.6	- 2.4	

Values with an * indicate significant (P<0.05) differences between ileal and excreta digestibilities, [1]Corn-soybean meal diet with normal P and normal Ca, ND = not determined, Difference = ileal digestibility-excreta digestibility.

e.g. wheat. The positive value for amino acids such as valine, methionine, isoleucine, phenylalanine, and alanine indicate a net synthesis of these amino acids in the hindgut of poultry. However, the negative values of amino acids such as threonine, leucine, histidine, lysine, arginine, aspartic acid, serine, glutamic acid, cysteine, proline, and tyrosine indicate a net catabolism of these amino acids in the large intestine of poultry. This may result in the formation of biogenic amines and BCFA which are discussed in detail in the respective sections of this review.

Metabolites of Hindgut Protein Fermentation

The main function of the GIT is the digestion and absorption of nutrients (Fasano and Shea-Donohue, 2005), the possibility for fermentation of indigestible ingredients such as NSP and proteins (Niba et al., 2009), and the excretion of waste products resulting from the digestive process (Cummings, 1983) and from metabolism. The end products of protein fermentation by resident microbiota in the hindgut are generally considered detrimental for the host animal (Nollet et al., 1999). Protein fermentation has been associated with the production of biogenic amines, BCFA, ammonia, phenols, indoles, cresol, skatole and hydrogen sulfide (H_2S). Although generally regarded as detrimental, some components (e.g. SCFA) will contribute to the energy supply to the host, whereas other compounds such as luminal polyamines are important local factors for growth and the development of the small intestinal and colonic mucosa (Löser et al., 1999).

Biogenic Amines

Amines are produced by decarboxylation of amino acids (Urlings et al., 1992) by different intestinal microbiota such as *Bacteroides*, *Clostridium*, *Bifidobacterium*, *Enterobacterium,* and *Streptococcus* spp. (Allison and Macfarlane, 1989). The amines formed by living organisms (called biogenic amines) include monoamines (tyramine) as well as polyamines such as cadaverine, putrescine, and spermine (Larqué et al., 2007). Fermentation of the amino acids histidine, ornithine, lysine, methionine, tyrosine, phenylalanine, tryptophan, and arginine results in the production of histamine or spermidine, putrescine or spermidine, cadaverine, spermidine, tyramine, phenylethylamine, tryptamine or serotonin, and agmatine or putrescine or spermidine, respectively. The polyamine, spermidine is formed from catabolism of amino acids including histidine, ornithine, methionine, and arginine. Spermidine may subsequently be converted into spermine. Polyamines, including putrescine, spermidine, and spermine, have been shown in rats to be beneficial protein catabolites required for repair of intestinal mucosal cells (Wang and Johnson, 1990). Löser et al. (1999) reported that rats fed polyamine-deficient diets long-term, had significant hypoplasia

of the small intestine and colonic mucosa. The total level of biogenic amines is important rather than the level of single amine because amines work synergistically to modify metabolism (Lyons et al., 1983). There is a scarcity of published data regarding the physiological role of biogenic amines in poultry.

Dietary supplementation of synthetic biogenic amines may result in a significant depression in growth and increased mortality in broilers. Shifrine et al. (1960) studied the dose response of dietary supplementation of histamine by feeding 0.25, 0.50 and 1.0% histamine in the diet. These authors reported a dose dependent decrease in the performance and increase in proventriculus enlargement. Addition of histamine (2.2 mg/kg of feed) to a broiler diet resulted in gizzard erosion, high mortality and depressed growth rate (Fossum et al., 1988). In contrast, putrescine supplementation at 0.2 and 0.4% of the diet resulted in a greater growth rate and improved feed efficiency in broilers compared with those on a diet without supplementation of putrescine, whereas supplementation of putrescine above 0.4% of the diet resulted in a decreased feed intake and poor feed conversion ratio (Smith, 1990). Similarly, Barnes et al. (2001) reported that supplementation of 0.1 and 0.2% histamine addition to broiler diets resulted in a 6.2 and 9.2% decrease in body weight gain, respectively. These authors, furthermore, reported a poor feed conversion ratio and lesions in the proventriculus of birds fed histamine supplemented diets. Some studies indicate that dietary supplementation of histamine resulted in increased gastric acid secretion leading to enlargement of the proventriculus (Shifrine et al., 1960; Harry et al., 1975). Tiihonen et al. (2010) studied the effects of essential oils on broiler performance and gut microbiota and reported an increased mortality at day 20 with a high concentration of total ileal biogenic amines (tryptamine, putrescine, cadaverine, histamine, tyramine, spermidine and spermine). In contrast, the authors reported improved body weight gain at day 41 with a high concentration (3150 vs. 2893 nmol/g wet weight) of total cecal biogenic amines (methylamine, tryptamine, putrescine, cadaverine, histamine, tyramine, spermidine and spermine) in broilers fed diets supplemented with essential oil compared with those fed the control diet. In addition, an inverse relation between the total biogenic amine concentration in the ileal digesta and the body weight of the broilers was observed at 41 day of age. Tiihonen et al. (2010), furthermore, reported a higher concentration of total biogenic amines (methylamine, tryptamine, putrescine, cadaverine, histamine, tyramine, spermidine and spermine) in the cecal compared with the ileal digesta (2893 vs. 776 nmol/kg wet digesta) which is consistent with a higher microbial proteolytic activity. This higher proteolytic activity may be due to a longer time period of digesta and/or also due to a greater microbial population in the ceca. Likewise, Rehman et al. (2008) reported a 9.5% greater cecal total biogenic amine concentration (tryptamine, putrescine, cadaverine, histamine, tyramine, spermidine,

and spermine) compared with ileal digesta in broilers fed a corn-soya diet. These authors reported greater concentrations of putrescine, cadaverine and tyramine in the ileal compared with the cecal digesta, whereas cecal digesta contained approximately a 4.4 times higher concentration of spermidine. The later results suggest greater fermentation of ornithine or arginine, lysine and tyrosine in the ileum compared to the cecum, whereas greater fermentation of methionine or histidine or ornithine or arginine in the cecum compared to the ileum.

Meyer et al. (2013) conducted a study in layers to explore the effects of feather pecking (FP) behavior in low and high FP breeds on different gut microbial metabolites and reported a 2.8 fold greater concentration of total biogenic amines (putrescine, cadaverine, histamine, spermidine and spermine) in the cecal digesta compared with the ileal digesta in the low FP breed. The latter indicates that feathers are fermented in the GIT of layers to form biogenic amines. In addition, a similar pattern of high concentrations of total biogenic amines in the cecum compared with the ileum was also observed in the high FP breed as well. This indicates greater microbial protein fermentation activity in the ceca compared to the ileum. The later authors, furthermore, reported greater concentration of putrescine in the ileal digesta, indicating more fermentation of ornithine or arginine there, whereas greater concentrations of spermidine in the cecal digesta were found indicating a higher microbial fermentation of methionine or histidine or ornithine or arginine in the ceca.

Branched Chain Fatty Acids

The degradation of the amino acids valine, isoleucine, and leucine in the intestinal tract of animals can results in the formation of the BCFA: isobutyrate, 2-methyl butyrate, and isovalerate, respectively. These BCFA have a pungent odour in comparison to straight short chain fatty acids which are formed from carbohydrate fermentation (Mackie et al., 1998). The presence of BCFA in the hindgut which are produced by many bacteria including *Bacteroides* spp., *Propionibacterium* spp., *Streptococcus* and *Clostridium* spp. (Rist et al., 2011), has been associated with post weaning diarrhea in pigs (Kim et al., 2008). The presence of n-valerate and BCFA (1.0 mol% of each) in cecal digesta and their absence in the crop and gizzard of broilers (Rehman et al., 2008) is indicative of proteolytic fermentation in the ceca and a lack thereof in the crop and gizzard. Tiihonen et al. (2010) reported approximately 12.9 times greater total BCFA concentrations in the ceca than the ileal digesta in broilers at 41 days of age. These authors found that isobutyric, isovaleric and 2-methyl butyric acid comprised 50.7, 29.2 and 20.1% of the total BCFA, respectively. This indicates a higher fermentation rate of valine, leucine and isoleucine in the ceca compared to the ileum. Greater levels of BCFA (19.7 vs. 9.5 mg acetic acid equivalents/g DM) have been reported by Guo et al. (2003) in intact mushroom contents compared with their

polysaccharides extracts while studying the *in vitro* fermentation characteristics using broiler cecal contents. The intact mushroom had a greater protein contents (155.5 vs. 80.5 g/kg DM) compared with their polysaccharide extracts. The greater concentration of BCFA indicates a higher proteolytic fermentation capability from the intact material compared with the extracts as BCFA are the only end products of protein fermentation (Macfarlane et al., 1992). Similarly, Faber et al. (2012) reported that broilers fed a soybean meal (SBM) diet had 2.7, 2.3, 3.0 and 2.5 folds greater isobutyrate, isovalerate, valerate, and total BCFA compared with those fed a soy protein isolate (SPI) diet. These data indicate that there is higher protein fermentation in protein rich diets in the ceca. Meyer et al. (2012) reported greater cecal molar ratios of isobutyrate and isovalerate (0.4 vs. 0.1 and 0.1 vs. 0.3 mol%, respectively) in layers fed 5% feather supplemented diets compared with those fed the control diet and attributed this greater concentration to protein fermentation of the dietary supplemented feathers in the ceca. The latter authors could not find significant differences in the concentrations of these microbial metabolites in the ileal digesta of birds fed feather supplemented diets. The concentration in the ileum was significantly lower compared with the cecal concentration. Similarly, in another study, Meyer et al. (2013) reported approximately 19% greater SCFA concentrations in the cecal compared with the ileal digesta in high compared with low FP breeds. These authors, however, found significantly greater molar percentages of cecal isobutyric and isovaleric acid (1.1 vs. 0.7 and 1.3 vs. 0.6, respectively) in low compared with high FP breed. The later metabolites were not detectable in the ileal digesta. This greater concentration of BCFA (isovaleric and isobutyric acid) in the ceca suggests enhanced microbial protein catabolic activity in the ceca compared with the ileum.

Ammonia

Ammonia is produced by deamination of amino acids and is a toxic waste product of microbial fermentation. It may be absorbed and excreted as uric acid in the urine by the birds (Salter and Fulford, 1974) or transformed into bacterial protein by the microbiota (Rist et al., 2011). According to Nousiainen (1991), a high concentration of ammonia in the large intestine of pigs potentially reflects a large quantity of undigested protein entering the hindgut which can negatively affect the growth of intestinal epithelial cells. This ammonia can pass through the gut wall and disturb intestinal mucosal development, as indicated by a reduced villus height. The absorption of ammonia from the gut wall has toxic effects on enterocytes (Macfarlane et al., 1992). Higher cecal ammonia levels resulted in an increase in cecal pH, providing a favourable environment for pathogenic microbiota and an increased risk for enteritis (Abdl-Rahman et al., 2011). Blood ammonia levels and excreta characteristics by lowering the dietary CP levels in broilers have been studied by

Namroud et al. (2008). These authors found a decrease in fecal nitrogen from 50.3 to 36.3 mg/g DM and uric acid from 113.5 to 101.1 mg/g DM of excreta digesta by lowering the dietary CP from 23 to 17%. The supplementation of 10% additional essential amino acids (Lys, Thr, Arg, and Trp) to a low (17%) CP diet resulted in an increased ammonia level (0.71 vs. 0.68 mg/100 mL) in the blood compared to the low CP diet without supplementation of essential amino acids. There can be a negative correlation between ammonia level and growth performance as has been reported in rats (Lardy and Fedott, 1950) and dogs (Russek, 1970). Ammonia is mainly converted into uric acid and the high levels of ammonia in blood may be reduced by enhancing the conversion of ammonia into uric acid which requires 1 mole of glycine to convert each molecule of uric acid in birds (Namroud et al., 2008). This may lead to amino acid loss resulting in a poor performance of the birds. The high concentration of ammonia in the blood will also result in more ammonia-nitrogen in the feces because there is a strong correlation between ammonia level in blood and its excretion in feces. The greater amount of blood ammonia resulted in reduced BW, feed intake, poor FCR and greater liver weight (3.2 vs. 2.4 % of the weight of visceral organs) in broiler fed a low (17%) CP diet supplemented with essential amino acids compared with broilers fed a high (23%) CP diet (Namroud et al., 2008). These authors assumed that lower BW and greater liver weight may be due to increased liver uric acid metabolism. In mammals, greater activity of urea cycle enzymes inhibits growth rate (Schimke, 1963). Also there is a linear relation between dietary protein and excretion of uric acid and ammonia (Teekell et al., 1968).

The results from the *in vitro* fermentation study of mushrooms, herb and their polysaccharides fraction by Guo et al. (2003) using broiler cecal contents shows a significantly greater ammonia level (370.6 vs. 249.3 mg/L) for the intact materials than for the extracts due to a greater protein and lower carbohydrate contents of the intact materials compared with their extracts. These authors, furthermore, reported a positive correlation between ammonia and BCFA in the cecal digesta. The greater levels of ammonia indicate increased protein and decreased carbohydrate fermentation of the intact material compared with the extracts. The greater levels of dietary indigestible protein especially in the absence of proper levels of fermentable carbohydrates should be avoided for optimum performance and gut health of the birds (Guo et al., 2003). Similarly, Khempaka et al. (2011) conducted a study to evaluate the effects of chitin (purified chitin composed of 83.9% chitin) addition at 1.07, 2.26, 3.34, and 4.53% of diet and four different levels of shrimp meal (SM; 5, 10, 15, and 20%) on growth performance, intestinal microbial populations, VFA, and ammonia production in broilers. These authors reported greater concentrations of ammonia in the cecal compared with the ileal digesta (0.67 vs. 0.13 g/100g of digesta) in broilers

fed 15% SM indicating more proteolytic activity in the cecum. These authors also assessed blood urea N and found the lowest concentration (1.87 vs. 2.15 mg/dL) in broilers fed 15% SM compared with the control group which resulted in improved performance of the broilers compared with other groups. Rehman et al. (2008) reported 5.0 and 3.9 folds greater cecal ammonia concentration compared with the gizzard and jejunum, respectively in broilers. This higher concentration may indicate more proteolytic fermentation in the ceca compared with the other mentioned GIT parts. Meyer et al. (2012) reported approximately 23% greater cecal ammonia concentration in layers fed a feather supplemented diet compared with those fed the control diet. These authors suggested that this greater ammonia level was due to higher bacterial protein catabolism because of feather protein degradation. In another study, however, Meyer et al. (2013) reported lower cecal ammonia in high feather pecking compared with low feather pecking layers (13.9 vs. 19.8 mmol/L). This was in contradiction with the expectations of the authors because high feather pecking birds consume more feathers and greater cecal fermentation of protein was expected in these birds resulting in greater ammonia level. These authors assumed that this lower amount of ammonia may be due to its greater use by the resident microbiota for their own body mass synthesis, which was confirmed by greater acetate levels in the cecal digesta of these birds.

Phenols, Indoles, Cresol and Skatole

Bacterial fermentation of aromatic amino acids such as phenylalanine, tryptophan, histidine and tyrosine can result in the production of phenols and indoles (Windey et al., 2012). These compounds are involved in carcinogenesis in the animal colon, whereas phenols are involved in nitrosation of secondary amines by nitrate (Kikugawa and Kato, 1988) and indoles can also enhance this nitrosation (Zuccato et al., 1993). Aerobic metabolism of aromatic amino acids results in the production of skatole, cresol and other phenolic compounds (Elsden et al., 1976). Skatole and methyl sulfide are mainly responsible for the manure like smell by excreta, whereas cresol is associated with noxious gases. Terada et al. (1993) reported the presence of phenol, p-cresol, indole and skatole at concentrations of 64.9, 64.1, 43.8 and 5.5 µg/g wet digesta, respectively, in the ceca of broilers at 56 days of age. The concentration of these compounds in the cecal digesta showed a decreasing trend with age of the broilers which suggests increase in protein digestibility with the age of broilers. Similarly, Terada et al. (1994) also conducted a study in broilers and reported the presence of phenol, p-cresol, indoles, and skatole (32.7, 91.3, 8.1, and 13.9 µg/g wet digesta, respectively) in the cecal digesta at 62 days of age. The presence of these putrefactive compounds in the ceca of broilers indicates the occurrence of fermentation of tyrosine and tryptophan, respectively. The presence of these

compounds was correlated with the higher cecal population of protein fermenting microbiota such as *Clostridia*, *Bacteriodaceae*, and *Staphylococci* spp. (Terada et al., 1994).

Hydrogen Sulfide

Hydrogen sulfide (H_2S) is one of the end products of fermentation of dietary and mucinous sulphate and sulphur-containing amino acids, such as methionine, cysteine and taurine by sulphate-reducing bacteria (Lewis and Cochrane, 2007). Hydrogen sulfide is a highly toxic agent for rodents comparable to cyanide (Reiffenstein et al., 1992). It has been reported that fecal sulfide concentration significantly correlates with dietary protein intake in humans (Hughes et al., 2000). The toxic potential of H_2S on colonic cells involves damage of the most important energy pathway for colonocytes by disrupting butyrate oxidation (De Preter et al., 2012). After exposure to sodium hydrogen sulfide, proliferation of epithelium cells in the rat intestinal crypt is increased (Deplancke and Gaskins, 2003). Sulfide provokes genomic DNA damage in colonic cancer cells at concentrations of 250 mM as evidenced by a modified comet assay in which DNA repair was inhibited (Attene-Ramos et al., 2006). In broilers, sulphur containing amino acids, such as Met and Cys are a major source of sulphur in feces and related to H_2S production by microbiota (Kadota and Ishida, 1972). Chavez et al. (2004) reported the presence of hydrogen sulfide, dimethyl disulfide and trimethyl trisulfide 224.4, 11.5 and 3.6 ng/g of feces, respectively, in the excreta of broilers fed diets supplemented with sodium methioninate. The later authors also reported the presence of hydrogen sulfide, dimethyl disulfide and trimethyl trisulfide, 49.6, 5.6 and 2.9 ng/g of feces, respectively, in the excreta of broilers fed the control corn-soybean based diet (CP: 21%) which may indicate the catabolism of amino acids. Similarly, Chen et al. (2012) reported the presence of (49 mg/L) H_2S in cecal digesta of broilers at 35 days of age, indicating that H_2S is a metabolite of protein degradation in poultry. The toxic effects of H_2S may not be as relevant in poultry as, in mammals, due to the high demand of the essential S-containing amino acids including methionine and cysteine for feather production. These amino acids are, therefore, limiting in most poultry diets and will be absorbed in the foregut.

Mode of Action of Dietary Factors to Reduce Hindgut Protein Fermentation

There are several dietary factors that affect hindgut protein fermentation. The mode of action of some of these will be described below.

Protein Source and Inclusion Level

Dietary CP content and its digestibility affect the formation and quantity of microbial metabolites resulting from hindgut protein fermentation (Hobbs et al., 1996).

Proteolytic microbial fermentation is directly related to CP and high contents of undigested dietary CP in the hindgut which may enhance the proliferation of pathogenic microbiota in the GIT (Ball and Aherne, 1987). The percentage of undigested amino acids of commonly used ingredients to formulate poultry diets, may vary between 8 to 35 of the total dietary CP content (CVB, 2007). A negative linear effect of increasing indigestible CP contents in the diet on feed conversion ratio (FCR) in broilers has been reported by De Lange et al. (2003). These authors found that FCR was increased by 0.080 with an increase of approximately 10 g/kg of indigestible CP contents (from 35.3 to 43.9 g/kg) of the diet using different levels of SBM, peas, hydrolyzed feather meal and meat and bone meal. A low carbohydrate: N ratio in the gut combined with a high dietary CP content is the basis for potentially harmful hindgut protein fermentation (Williams et al., 2001). Therefore, strategies that reduce the protein content or increase the fermentable carbohydrate contents in the hindgut may contribute to gut health. Highly digestible protein sources at appropriate inclusion levels in a broiler diet can be expected to reduce hindgut protein fermentation and achieve maximum growth performance. Improving foregut protein digestion, thereby, increasing protein digestion will reduce the inflow of indigested amino acids in the colon and reduce the potential for protein fermentation. Some strategies can be applied to increase the digestion and absorption of proteins in the foregut. As there is limited information available on poultry, also data from other species are discussed.

The first approach could be to limit dietary CP level, although this may have detrimental effects on performance in poultry (Bregendahl et al., 2002). Total dietary CP level, its amino acid composition and its digestibility determine the amount of protein arriving in the hindgut. A high number of proteolytic microbiota (such as *Campylobacter*, *Bacteroides*, *Clostridia*, and *Prevotella*) has been reported in finishing barrows fed a high CP (34%) corn-SBM based diet compared with those fed a low CP (15%) diet (Anugwa et al., 1989). In addition, Opapeju et al. (2009) reported a higher (414.9 vs. 182.7 mg/L) ammonia-N concentration in colonic digesta of piglets fed a high (22.5%) CP diet compared to those fed a low (17.6%) CP diet supplemented with essential amino acids. Similarly, these authors reported more carbohydrate fermenting bacteria (Roseburia) in colonic digesta of piglets fed low CP diet and suggested that this low CP diet may have shifted the microbiota population towards a more beneficial carbohydrate fermenting population. In birds, a low level of dietary CP, however, may negatively affect performance although it can have beneficial effects on the physical conditions in the gut (Table 3). The supplementation of proteolytic enzymes in feed may also improve N digestion and subsequently its retention. Keratinase, a high active proteolytic enzyme that works with a broad spectrum of substrates can increase protein digestibility and N retention (Wang et al., 2008). A low pH in the gizzard can result in

a better denaturation of dietary protein, leading to more accessible cleavage sites for proteolytic enzymes.

Table 3. Effects of lowering dietary crude protein on digesta characteristics and performance of poultry and pigs.

Crude protein level (%)	Species	Digesta[2, 3] characteristics (%)			Performance change (%)		Reference
		NH_3	VFA	BCFA	BW	FCR	
22.5[1] to 18.5	Poultry	ND	ND	ND	- 1.5	- 4.8	Laudadio et al. (2012)
22[1] to 10	Poultry	ND	ND	ND	- 32.7	+ 28.0	Buwjoom et al. (2010)
23[1] to 19	Poultry	- 15.6	ND	ND	- 17.5	+ 6.0	Namroud et al. (2009)
25.6[1] to 17.5	Pigs	- 19.0	- 15.3	- 1.9	- 6.7	- 2.6	Heo et al. (2009)
22.5[1] to 17.6	Pigs	- 66.0	+ 4.5	ND	- 5.2	- 1.9	Opapeju et al. (2009)
24[1] to 20	Pigs	- 32.3	- 27.5	- 52.0	- 3.5	0	Htoo et al. (2007)

[1]Control diet, [2]Cecal digesta in poultry, [3]Fecal digesta in pigs VFA = volatile fatty acids, BCFA = branched chain fatty acids, BW = body weight, FCR = feed conversion ratio, ND = not determined.

Diet Structure

There are no studies showing direct effects of dietary particle size on hindgut protein fermentation in poultry. However, there are studies from which can be inferred that particle size has the potential to reduce hindgut protein fermentation in broilers. A number of studies have shown that more coarsely ground diet improves broilers performance and villus heights in the small intestine (Table 4). This improved performance was due to greater digestibility of protein because of dietary coarseness (Liu et al., 2013; Pacheco et al., 2013). Extensive research has shown that the inclusion of a 'coarse' mash (with on average larger particles) results in a heavier gizzard which maximizes the grinding capacities of the GIT and enhances digestive capacity (Amerah et al., 2008; Svihus, 2011) as well as health by reducing pathogenic microbiota (Engberg et al., 2004). Nir et al. (1994) defined a fine diet as having a geometric mean diameter (GMD) of < 574 µm and a coarse diet having a GMD > 871 µm. A coarse diet increases pepsin secretion in the proventriculus, improves physical functionality of the gizzard muscles, feed intake and body weight gain (Gabriel et al., 2006). The addition of coarse particles to the diet increased gastric reflux exposing the feed to pepsin and hydrochloric acid in the gizzard (Gabriel et al., 2008; Pacheco et al., 2013). With coarse grinding, retention of particles in the gizzard is longer, which may result in better regulation of transit time. There are studies indicating a positive correlation between gizzard weight and ileal N digestion in broilers (Liu et al., 2013; Pacheco et al., 2013). This may be due to an improved functionality of the gizzard which provides more exposure of pepsin and hydrochloric acid to dietary proteins, thereby enhancing the initiation of protein digestion. It has been reported that whole wheat (coarse diet) feeding stimulates gizzard activity resulting in a high bile acid concentration in the gizzard (Svihus et al., 2004). This high bile acid concentration could be associated with a stimulation of pancreatic secretion due to an increase in gizzard activity (Hetland et al., 2003). In contrast, diets composed of fine particles increase the digesta viscosity (Amerah et al., 2007) which decreases nutrient

digestibility (Yasar, 2003). It can be assumed, therefore, that increasing feed particle size may help in decreasing the amount of indigestible protein reaching the hindgut due to an improved digestion and absorption in the foregut.

Pre- and Probiotics

Pre- and probiotics may shift the balance of the gut microbiota towards an increase in the numbers of potential health-promoting bacteria such as *Lactobacilli* and *Bifidobacteria* (De Preter et al., 2011). A prebiotic is defined as a food ingredient that is not hydrolyzed by the animal's own digestive enzymes in the upper GIT, but positively affects the host by stimulating the growth and activity of health beneficial microbiota in the colon (Gibson et al., 2004). Prebiotics are mostly carbohydrates and they can decrease proteolytic fermentation by (i) a rapid fermentation of the prebiotic substrate in the colon resulting in a lower colonic pH that reduces activity of peptides by bacterial proteases (Rastall, 2004), (ii) catabolite repression, by depressing the transcription of genes involved in amino acid catabolism in the presence of carbohydrates (Vince and Burridge, 1980), and (iii) an increased amino acid use for bacterial biosynthesis (Cummings and Bingham, 1987). The potential beneficial effects of prebiotics may include antagonism towards pathogens. Rehman et al. (2008) studied the effect of inulin (1%) supplementation as a prebiotic in a basal corn-soya based diet of broilers and reported a lower cecal pH (6.4 vs. 7.1), lower cecal ammonia contents (13.9 vs. 22.3 μmol/g of digesta), lower BCFA concentrations (1.0 vs. 0.6 mol % of total SCFA), greater concentration of butyric acid (15.0 vs. 7.4 mol % of total SCFA) and numerically lower concentration of total cecal biogenic amines (346.8 vs. 398.3 nmol/g of digesta) with inulin supplementation. These data suggest that inulin supplementation resulted in promoting saccharolytic instead of peptidolytic activity in the GIT as indicated by the fermentation products. These authors, furthermore, concluded that inulin can affect fermentation patterns as it was indicated by lower cecal ammonia and a greater concentration of butyrate. Mookiah et al. (2014) studied the effects of different levels (5 or 10 g/kg of basal diet) of isomalto-oligosaccharides, as a prebiotic, on performance, cecal microbiota population and cecal fermentation characteristics in broilers. These authors found that total cecal VFA, acetic, propionic and butyric acid levels were increased by 43, 46, 65 and 36%, respectively in broilers fed isomalto-oligosaccharides supplemented diets compared with those fed the control diet at 42 days of age. The greater concentrations of total cecal VFA were, however, observed in broilers fed higher levels (10 g/kg of diet) of isomalto-oligosaccharides. These authors suggested the greater concentration of total VFA in cecal digesta of broilers fed diets supplemented with a prebiotic may be due to higher density of *Lactobacilli* and *Bifidobacteria* and lower *E. coli* and total aerobe

Table 4. Changes in gizzard weight, body weight (BW), feed conversion ratio (FCR) and duodenal villus height in relation to diet structure.

Diet treatments	Gizzard weight	BW	FCR	Villus height	Reference
Fine[1] vs. coarse soybean meal	+ 11.0	- 9.8	- 4.1	ND	Pacheco et al. (2013)
Fine[1] vs. ground corn and soybean meal	+ 10.6	+ 2.0	+ 4.9	ND	Jacob et al. (2010)
Ground[1] vs. whole wheat	+ 2.8	0.0	0.0	ND	Amerah et al. (2009)
Fine[1] vs. coarse corn and soybean	ND	+ 4.9	+ 1.1	- 2.6	Zang et al. (2009)
Fine[1] vs. coarse	+ 34.0	+ 5.7	- 6.0	+ 3.0	Amerah et al. (2008)
Complete ground[1] vs. whole wheat	+ 25.8	+ 4.3	+ 12.0	+ 6.0	Gabriel et al. (2008)
Ground[1] vs. whole wheat	+ 50.0	- 5.0	+ 0.6	- 2.6	Williams et al. (2008)
Ground[1] vs. whole wheat	+ 72.0	- 1.1	- 5.8	ND	Engberg et al. (2004)
Ground[1] vs. whole wheat	+ 23.7	+ 1.5	- 2.0	ND	Svihus et al. (2004)
Complete ground[1] vs. whole wheat	+ 100.0	+ 14.0	0.0	ND	Gabriel et al. (2003)
Fine[1] vs. coarse	+ 7.2	- 1.7	0.0	ND	Engberg et al. (2002)
Hammer mill[1] vs. roller mill	+ 8.9	+ 7.2	- 2.9	ND	Nir et al. (1995)
Fine[1] vs. coarse corn, wheat and sorghum	+ 23.3	+ 9.9	+ 3.0	ND	Nir et al. (1994)

[1]Control diet, ND = not determined.

population in the ceca. Similarly, Faber et al. (2012) studied the effects of oligosaccharides in corn-soybean meal based diets on immune response in *Eimeria acervulina* challenged broilers. These authors reported improved performance, and greater SCFA production in SBM-fed birds compared with SPI diet fed broilers. Cecal acetate, propionate, butyrate and total VFA concentration was increased 3.6, 1.2, 3.9, and 3.4 folds in SBM fed broilers compared with those fed the SPI diet. These authors suggested that this increased concentration of VFA may be attributed to fermentable oligosaccharides present in SBM and these oligosaccharides are the main energy source for the intestinal epithelial cells and also stimulate cell growth and improve cecal health by improving cecal weight and producing more VFA.

Probiotics are live organisms, which are beneficial to the host when regularly provided in the diet in adequate quantities (Sanders, 2008). Probiotic administration may also influence the formation of SCFA. Analogue to prebiotics, probiotics may stimulate bacterial activity in the gut, resulting in an increased uptake of N, amino acids or metabolic products into the bacterial fraction (Salminen et al., 1998). Probiotics may exert an indirect effect on carbohydrate or protein fermentation and enrich the population of gut microbiota with those species that preferentially ferment carbohydrates and have little proteolytic activity. Mookiah et al. (2014) used a multi-strain probiotic (consisting of 11 *Lactobacillus* strains) and studied the performance and cecal fermentation characteristics in broilers. These authors reported that the total cecal concentration of acetic , propionic, butyric and VFA was increased by 38, 92, 11 and 34%, respectively, in broilers fed diet supplemented with multi-strain probiotics compared with those fed a control diet at 42 days of age. A similar pattern in the concentration of these fermentation products was observed at 21 days of age as well. Similarly, supplementation of *Lactobacillus acidophilus* as probiotics reduced intestinal putrefactive compounds such as ammonia and biogenic amines in poultry (Gallazzi, 2009). Chen et al. (2012) studied the effects of probiotics (*Bacillus subtillis* and *Lactobacillus acidophilus*) supplementation in broilers. These authors reported 33.1 and 28.7% lower fecal ammonia and 49.5 and 38.6% lower H_2S in 35 days old broilers fed a probiotic supplemented diet compared with those on control and antibiotic (flavomycoin) supplemented diets, respectively. According to the latter authors, probiotics reduced the pathogenic microbiota in the GIT by reducing the intestinal pH, which resulted in lower fecal ammonia and H_2S emission. Based on observations in poultry, it can be assumed that prebiotic and probiotic supplementation in diets may reduce hindgut protein fermentation by decreasing the pathogenic microbial count in the ceca.

Other Feed Additives

Feed additives can maintain gut health through different mechanisms such as shifting gut pH, enhancement in pancreatic juice secretion, increasing nutrient intake, motivating the humoral immune response, selecting beneficial microbiota or increasing fermentation acids, and consequently reducing the invasion of pathogenic microbiota (such as *Salmonella enteritidis* and *Escherichia coli* in the host) and increasing growth rate of the intestinal mucosa (Cummings and Macfarlane, 2002). Supplementation of organic acids, especially SCFA which have specific antimicrobial activity, is a promising strategy to improve gut integrity (Adil et al., 2010). Their supplementation also results in less damage to epithelial cells because of a reduced production of toxic compounds when numbers of pathogenic microbiota are reduced (Antongiovanni et al., 2009). Organic acids, due to their bactericidal effects, suppress protein fermenting microbiota, especially the gram negative population in broilers (Gunal et al., 2006) by disrupting their energy metabolism (Ricke, 2003) and decreasing the hindgut pH. Feed ingredients, such as oligosaccharides and NSPs, affect hindgut pH by the production of VFAs (Van Der Waaij and Nord, 2000). These VFAs have a positive effect on intestinal health by providing a readily available energy source. Energy requirements of the GIT are higher compared with other body tissues. The GIT comprises 6% of the total body mass, whereas it consumes about 25% of total oxygen consumed (Britton and Krehbiel, 1993). Reduced concentrations of SCFA, especially butyrate, may lead to ulcerative colitis, reduced gut mucosal barrier function and inflammatory conditions (Wächtershäuser and Stein, 2000). Butyric acid stimulates intestinal development, e.g. epithelial cell proliferation and differentiation (Dalmasso et al., 2008) and maintaining villus height (Hu and Guo, 2007). The effects of butyric acid supplementation on broiler performance are summarized in Table 5.

Table 5. Effects of dietary butyric acid inclusion on broiler performance and duodenal villus height.

Inclusion level range (%)	Optimal	Performance change[1] (%)			Reference
		BW	FCR	Villus height	
0.15	0.15	0	0	+ 15.9	Jerzsele et al. (2012)
2.0, 3.0	3.0	+ 9.3	- 8.4	+ 21.0	Adil et al. (2011)
0.2	0.2	+ 4.6	- 8.0	ND	Jang (2011)
2.0, 3.0	3.0	+ 6.8	- 8.9	+ 7.5	Adil et al. (2010)
0.2, 0.35, 0.5, 1.0	0.2	- 5.6	- 4.7	ND	Antongiovanni et al. (2009)
0.2, 0.4, 0.6	0.4	+ 5.6	- 8.0	+ 8.2	Panda et al. (2009)
0.2, 0.3	0.3	+ 8.0	- 17.5	ND	Taherpour et al. (2009)
0.05, 0.1, 0.2	0.2	- 3.0	+ 4.6	+ 10.0	Hu et al. (2007)
0.1, 0.2	0.2	+ 0.6	- 5.3	+ 9.4	Leeson et al. (2005)

[1]Related to optimal inclusion level, BW = body weight, FCR= feed conversion ratio, ND = not determined.

Jang (2011) reported an 8.6% improvement in FCR in broilers supplemented with 0.2% butyric acids glycerides. Butyric acid has several functions including stimulation and production of peptides, by attaching to specific G-protein-coupled receptors, especially GPR 41 and GPR 43 (Le Poul et al., 2003) in the hindgut (Tazoe et al.,

2008). Positive effects of some of these peptides have been reported for immunological development in health challenging situations, whereas some others have been reported to optimize gut motility (Tazoe et al., 2008). It also stimulates the immune system and reduces *Salmonella* colonization in the broiler GIT (Van Immerseel et al., 2005). In general, the use of SCFA (such as butyric acid) in the feed of poultry is considered a possible alternative to the use of antimicrobial growth promoters (Leeson et al., 2005).

Conclusions

There are no direct studies investigating the effects of protein fermentation on the performance of broilers. Metabolites of protein fermentation, however, have been reported in high concentrations in the digesta collected from the ileum and ceca in broilers. Greater concentration of biogenic amines, BCFA, H_2S, ammonia, indole, phenols, cresol and skatole in the cecal compared with the ileal digesta indicates more proteolytic fermentation. Low concentrations of some of the protein fermentation products including biogenic amines are necessary for the normal gut development. The precise effects of hindgut protein fermentation on gut health and performance in broilers remain poorly understood. Further studies should elucidate the impact of protein fermentation on performance of broilers. Nutritional strategies such as a reduction in dietary CP, dietary supplementation of pre- and probiotics and organic acids, or feeding diets with larger particle sizes may increase CP digestibility and, thereby, reducing the level of protein fermentation in the large intestine. Most of these nutritional interventions can potentially enhance protein digestion in the upper GIT and, therefore, less undigested protein will be available for fermentation in the hindgut.

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CHAPTER 3

Effects of protein source on performance, gut morphology and cecal fermentation characteristics in broilers

S.N. Qaisrani[1], M.M. van Krimpen[2], R.P. Kwakkel[1], M.W.A. Verstegen[1], and W.H. Hendriks[1]

[1]Animal Nutrition Group, Wageningen University, PO Box 338, NL-6700 AH Wageningen, The Netherlands

[2]Wageningen UR Livestock Research, PO Box 65, NL-8200 AB Lelystad, The Netherlands

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Abstract

The objective of this study was to determine the effects of three protein sources and their digestibility levels on performance, gut morphology and some fermentation characteristics in the hindgut of broilers. It was hypothesized that broilers fed a diet with high levels of indigestible protein, results in a reduced growth performance, lower villus heights, deeper crypts, and more protein fermentation products in cecal digesta. In total, 288 one-d-old male Ross 308 broilers were used in a completely randomized 3 × 2 factorial design. Three protein sources: soybean meal (SBM), rapeseed meal (RSM) and maize gluten (MG), and two digestible crude protein (DCP) levels: 15.8 and 17.2% were used. The treatments were randomly assigned to six replicate pens (3 × 2 × 6 = 36). Broilers fed SBM had greater feed intake ($P < 0.001$), greater BW gain ($P < 0.001$), and lower FCR ($P < 0.001$) compared with those fed RSM and MG diets. For most parameters, differences between RSM and MG were negligible. High DCP (17.2%) diet fed broilers showed better performance compared with those on low DCP (15.8%) diet. No significant effects of protein source as well as DCP level were found on gastrointestinal tract development, cecal ammonia and volatile fatty acid concentrations. Broilers fed SBM had 18.2 and 17.7% greater villus heights, 15.5 and 18.1% smaller crypts and 29.0 and 30.9% greater villus height to crypt depth ratio compared with those fed RSM and MG diets, respectively. Broilers fed RSM diet had a significant lower cecal pH ($P = 0.005$) and 16.5 and 14.9% greater branched chain fatty acid contents in cecal digesta compared with those fed SBM and MG diets, respectively, indicating more proteolytic fermentation. It is concluded that protein source as well as digestible crude protein level had an effect on performance, gut morphology and protein fermentation characteristics.

Key words: broilers, protein, cecal fermentation, performance, gut morphology.

Introduction

Broilers can attain a BW of 2.0 kg by consuming 3.0 kg of feed within 35 days (Choct, 2009). Such modern broilers have been selected to achieve a low feed conversion ratio and a high growth rate. This rapid growth rate requires a high concentration of digestible protein (e.g. digestible essential amino acids). Some dietary proteins are not well digested in the small intestine and consequently undigested dietary protein enters together with undigested endogenous proteins into the ceca and colon. Undigested proteins, peptides and amino acids are then a potential substrate for fermentation by microbiota in the large intestine. These undigested protein substances may stimulate the growth of N-utilizing microbiota (Reid and Hillman, 1999) leading to increased levels of toxic compounds (a.o. biogenic amines). These toxic compounds may be detrimental for bird performance and gut health (Thomke and Elwinger, 1998). Moreover, as a result of protein fermentation, higher levels of branched chain fatty acids and elevated levels of ammonia/g non-starch polysaccharides (NSP) may be produced (Bikker et al*.,* 2006).

Gut morphology is an important indicator of intestinal health (Zang et al*.,* 2009). There is a scarcity of published data regarding the effects of protein source on gut morphology in broilers. In addition, the available data show contradictory results. Buwjoom et al. (2010) found no significant effect of three different dietary CP levels (10, 16 and 22%) on villus height and crypt depth in broilers. Contrary to this, Laudadio et al. (2012) observed higher villus heights in broilers fed soybean meal based, medium and high CP diets compared with those fed a low CP diet and recommended a 20.5% dietary CP level to optimize growth performance. It is thought that feeding protein sources with similar amounts of essential amino acids but with different amounts of indigestible protein may have a different impact on broilers. It can be hypothesized, that increasing the dietary indigestible CP content by 25% will have negative effects on gut morphology and will decrease broiler performance. The current study investigated the effects of three dietary protein sources each at two indigestible CP levels on growth performance, gut morphology and hindgut protein fermentation characteristics in broilers.

Materials and Methods

Animal Ethics

Experimental procedures were in accordance with the Wageningen University and the Netherlands Animal Experimental Committee guidelines and code of practice. Ethical approval was granted before the conduct of the study.

Birds Management

In total, 288 male (Ross 308) one-d-old broilers were purchased from a commercial hatchery (Morren Breeds B.V., Lunteren, The Netherlands). Upon arrival, broilers were individually weighed, steel wing tagged, before being allotted to one of 36 floor pens (8 birds per pen), equally distributed over three identical climate-controlled rooms so that each pen had a similar initial total BW. Each pen (1.15 × 1.75 × 0.80 m) (L × W × H) had three drinking nipples with a cup underneath connected to a water tank of 10 L capacity. Pens were separated by solid walls to prevent contact between broilers from different treatment groups. A feeding tray was placed on the floor during wk 1 and replaced by a feeding trough thereafter. Feed and water were available *ad libitum* throughout the experiment. Wood shavings were used as litter material. The lighting schedule was maintained at 23L:1D for the first three days and, thereafter, maintained at 16L:8D with an intensity of approximately 20 lx at bird's level throughout the experimental period. During the first three days, room temperature was set at 32°C, and thereafter gradually decreased to a constant value of 22°C in wk 4 and maintained until the end of the experiment.

Experimental Design and Treatments

The study was conducted as a completely randomized block design with repeated measures (6 replicate pens per treatment and 8 birds per pen). All birds received a starter diet for the first 7 d of the experiment (CP: 21%; ME: 2895 kcal/kg). After wk 1, six dietary treatments were provided which contained three different protein sources: soybean meal (SBM), rapeseed meal (RSM) and maize gluten (MG) each at 15.8 and 17.2% digestible crude protein (DCP) levels. The diets were formulated to meet or exceed the nutrient recommendations for boilers (CVB, 2007). These dietary treatments were randomly assigned to the pens in three rooms (3 × 2 × 6 = 36). As shown in Table 1, the diets were formulated to contain different concentrations of indigestible CP based on calculated fecal digestible protein contents. Each diet was formulated to have similar concentrations of digestible essential amino acids and to be iso-energetic on an AMEn basis. Table 2 shows, for the six experimental diets, the amounts of analyzed CP entering the hindgut as calculated from apparent ileal CP digestibility values based on literature data. All six dietary treatments were offered as pellets with a pellet size of 2.5 mm for the starter and 4.0 mm for the grower diet. The diets did not contain antimicrobial growth promoters.

Traits Measured

Feed intake (FI), water intake (WI) and body weight (BW) gain per pen in each room were recorded at d 8, 15, 22, 29 and 33. Feed intake, BW gain and WI were

expressed per bird per d. Weights of dead birds were determined and their BW gain and FI included in the calculation of feed conversion ratio (FCR) per pen.

Table 1. Dietary ingredients and calculated nutrient composition of the experimental diets (g/kg, as-fed basis).

Protein source	Soybean meal		Rapeseed meal		Maize gluten	
Digestible CP level (%)	15.8	17.2	15.8	17.2	15.8	17.2
Ingredients						
Maize	312.8	241.2	300.0	459.6	300.0	305.7
Rapeseed	0.0	0.0	280.7	209.6	0.0	0.0
Maize gluten feed	0.0	0.0	0.0	0.0	185.6	165.5
Soybean meal (>48% CP)	212.5	247.9	20.0	20.0	130.6	118.2
Wheat	149.3	288.6	20.0	20.0	20.0	20.0
Peas	125.0	20.0	10.0	20.0	10.0	20.0
Soy oil	77.9	75.5	81.7	76.8	82.8	82.6
Lucerne	49.6	41.7	40.1	61.7	60.0	75.0
Maize starch	20.0	20.0	155.5	10.0	109.0	85.9
Potato protein (ash< 10)	17.0	20.0	63.7	37.3	70.9	73.9
Fish meal (63-68% CP)	0.0	15.0	0.0	70.8	0.0	31.9
Limestone	11.3	10.7	8.6	4.7	11.1	8.7
Monocalcium phosphate	6.8	5.3	6.0	0.1	6.0	3.4
Premix[1]	5.0	5.0	5.0	5.0	5.0	5.0
DL-Methionine	2.7	2.0	1.0	0.6	2.0	1.4
Sodium-bicarbonate	2.7	0.0	0.0	0.0	0.0	0.0
Phytase	2.0	2.0	2.0	2.0	2.0	2.0
L-Threonine	1.9	1.3	0.4	0.3	0.8	0.1
L-Lysine HCL	1.8	0.9	1.2	0.0	1.8	0.0
Salt	1.4	2.9	3.4	1.4	1.3	0.6
L-Tryptophan	0.3	0.0	0.1	0.1	0.2	0.1
L-Arginine	0.0	0.0	0.6	0.0	0.9	0.0
Calculated contents						
ME (MJ/kg)	12.6	12.6	12.6	12.6	12.6	12.6
CP	200.0	214.0	210.0	224.0	210.0	224.0
Digestible CP[2]	158.0	172.0	158.0	172.0	158.0	172.0
Indigestible CP	42.0	42.0	52.0	52.0	52.0	52.0
NSP[3]	154.0	147.9	167.2	171.5	185.7	185.2
Crude fiber	38.3	33.6	43.0	53.6	43.0	45.8
Digestible lysine	10.6	10.6	10.6	10.6	10.6	10.6
Digestible M + C	7.7	7.7	7.7	7.7	7.7	7.7
Digestible threonine	8.0	8.0	8.0	8.0	8.0	8.0
Digestible tryptophan	2.2	2.2	2.2	2.2	2.1	2.2

[1]Premix composition: 12,000 IE retinol, 2,400 IE cholecalciferol, 50 mg dl-a-tocopherol, 1.5 mg menadione, 2.0 mg thiamine, 7.5 mg riboflavin, 3.5 mg pyridoxine, 20 mcg cyanocobalamins, 35 mg niacin, 12 mg D-pantothenic acid, 460 mg choline chloride, 1.0 mg folic acid, 0.2 mg biotin; 80 mg iron, 12 mg copper, 85 mg manganese, 60 mg zinc, 0.40 mg cobalt, 0.8 mg iodine, 0.1 mg selenium, 125 mg anti-oxidant mixture.
[2]Based on data from CVB (2007). [3]NSP = non-starch polysaccharides, calculated by subtracting the crude protein, fat, starch, sugar, and ash content from the dry matter content.

Table 2. Analysed CP contents (g/kg), calculated ileal digestibility of CP and non-starch polysaccharide content of diets formulated with three different protein sources and two digestible crude protein levels (15.8 and 17.2%).

Protein source	Soybean meal		Rapeseed meal		Maize gluten		References[1]
Fecal digestible CP level (%)	15.8	17.2	15.8	17.2	15.8	17.2	
Nutrients							
Total CP							
Of the whole diet	201.9	213.9	200.9	217.9	199.8	214.8	1
Of the protein source[2]	104.5	121.1	104.6	79.1	42.6	38.3	2
Of the other ingredients[3]	97.4	92.8	96.3	138.8	157.2	176.5	
Ileal digestible CP							
Of the whole diet	175.8	188.9	162.5	176.5	164.7	176.8	3, 4
Of the protein source[2]	94.0	109.0	79.6	60.4	27.3	24.7	3, 4
Of the other ingredients[3]	81.8	79.9	82.9	116.1	137.4	152.1	3, 4
Of the essential amino acids	28.6	28.5	28.8	28.7	28.2	28.0	2
CP entering the hindgut							
Of the whole diet	26.1	25.0	38.4	41.4	35.1	38.0	
Of the protein source[2]	10.5	12.1	25.0	18.7	15.3	13.6	
Of the other ingredients[3]	15.6	12.9	13.4	22.7	19.8	24.4	
Total NSP[4] in diet	176.9	165.6	181.9	174.9	204.5	190.2	2
Soluble NSP	47.1	42.9	49.9	42.6	50.3	46.7	2
Insoluble NSP	129.8	122.7	132.0	132.3	154.2	143.5	2

[1]References: 1 = Analysed; 2 = CVB (2007); 3 = Lemme et al. (2004) for all ingredients except maize gluten; 4 = Scheele et al. (1992) for maize gluten.
[2]Protein sources were the soybean meal, rapeseed meal and maize gluten. [3]Other ingredients were maize, wheat, peas, fish meal, lucerne, and potato protein. [4]NSP = non-starch polysaccharides, calculated by subtracting the crude protein, fat, starch, sugar, and ash content from the dry matter content.

At the end of the experiment (d 34), 6 of the 8 birds per pen closest to the average weight of the pen were euthanized by an intravenous T-61 injection and the abdominal cavity opened. During the dissection day, all birds had access to feed and water up till the moment of euthanization. Birds in a pen were euthanized in course of replicate number. The different parts of gastrointestinal tract (GIT), i.e. the crop, proventriculus, gizzard, duodenum (from pyloric junction to pancreo-billiary duct), jejunum (from pancreo-billiary duct to Meckle's diverticulum), ileum (from Meckle's diverticulum to ileo-cecal junction), cecum (from ostium) and colon were segmented. The digesta contents from each segment were immediately removed by gentle squeezing and the empty segments weighed. Ceca content of the six birds in a pen were quantitatively pooled, thoroughly mixed and pH determined using a calibrated pH meter before the samples were freeze dried at -20°C pending volatile fatty acid (VFA) and ammonia analyses.

Tissue Collection and Histological Measurements

The gut segment (approximately 2 cm) in the middle of the duodenum was excised, rinsed with cold physiological saline (0.9%) and immediately placed in Bouin's fluid. Thereafter, the samples were transferred into 70% ethanol within 24 h. The samples were embedded in paraffin and sliced at 5µm thickness for histological examination. Six cross-sections per bird were processed using standard haematoxylin and eosin methods as described by Owusu-Asiedu et al. (2002). Villus heights and crypt depths were measured on 10 intact, well-oriented villi (from the 0.5 cm in the middle of the duodenum) per bird using a compound light microscope equipped with a video camera. Villus height was measured from the tip of the villous to the crypt-villous junction, whereas crypt depth was measured from the crypt-villous junction to the base.

Chemical Analysis

Dry matter, organic matter and N contents in the experimental diets were analyzed according to standard methods (AOAC, 2006). Ammonia-N in cecal digesta was analyzed by the indole phenol-blue method (Novozamsky et al., 1974). The samples were deprotonated by adding 10% (w/v) trichloroacetic acid solution followed by centrifugation. The ammonium was transformed by phenol and hypochlorite in an alkaline solution into a blue coloured indole phenol-blue by the Berthelot reaction and measured spectroscopically at 623 nm.

For the determination of VFAs, 5 g of cecal samples and 5 ml 0.1 M phosphoric acid were shaken at 100 rpm before being centrifuged (7000g) for 10 min. Residues were collected and the supernatants again centrifuged (20817g) for 10 min. Thereafter 600 µL of the supernatant was taken in a crimp vial and mixed with 600 µL of

phosphoric acid containing iso-capronic acid (2.29 g/L concentration) as an internal standard. Volatile fatty acids were separated by gas chromatography using an EM-1000 (30 m × 0.53 mm) column from Alltech (Deerfield, IL, USA) and helium as the mobile phase with detection by a fluorescent infrared detector. Quantification of VFAs was based on a chemical standard solution (Merck) after internal standard correction.

Statistical Analysis

The data were analyzed with the use of PROC MIXED in SAS (version 9.2; SAS Inst. Inc., Cary, NC) by using the following statistical model:

$$Y_{ijkl} = \mu + P_i + L_j + A_k + P_i \times L_j + P_i \times A_k + L_j \times A_k + P_i \times L_j \times A_k + e_{ijkl}$$

where Y_{ijkl} is the measured response, μ overall mean effect, P_i the i^{th} fixed protein source effect (i = SBM, RSM or MG), L_j the j^{th} fixed digestible CP level effect (j = 15.8 or 17.2%) and A_k the k^{th} age interval of measurement (k = 9 to 15, 16 to 22, 23 to 29 and 30 to 33). $P_i \times L_j$ the interaction between protein source and digestible CP level, $P_i \times A_k$ the interaction between protein source and age interval of the bird at the time of measurement, $L_j \times A_k$ the interaction between digestible CP level and age interval of the bird at the time of measurement, $P_i \times L_j \times A_k$ the interaction between protein source, digestible CP level and age interval of the birds at the time of measurement, and e_{ijkl} the error associated with j^{th} inclusion level assigned to the i^{th} protein source at day of measurement k, $e_{ijkl} \sim$ NID (0, σ^2_e). Differences were considered significant at a probability level of 5%.

Gastrointestinal tract development, morphometric indices of duodenum, ammonia, cecal pH, VFA and BCFA data were analyzed by PROC MIXED in SAS with the following linear model:

$$Y_{ijk} = \mu + P_i + L_j + P_i \times L_j + e_{ijk}$$

where Y_{ijk} is the measured response, μ overall mean effect, P_i the i^{th} fixed protein source effect, L_j the j^{th} fixed digestible CP inclusion level effect, $P_i \times L_j$ interaction between P_i and L_j and e_{ijk} the residual error.

Results

Bird Performance

Overall, mortality was low (1.7%) during the study. All performance data of broilers were corrected for mortality by day. Probability values for treatment effect on performance parameters are presented in Table 3. Protein source influenced ($P <$ 0.001) FI, with broilers fed the RSM and MG diets had a lower FI compared with those fed the SBM diet (Tables 3 and 4). There was an effect of DCP on FI with broilers fed the high DCP diet had a greater ($P < 0.001$) FI compared with those fed

Table 3. Probability values of main and interaction effects between protein source (PS), digestible crude protein (DCP, %) level and age (days) for different traits in broilers from 9 to 33 d of age.

Effects	Feed intake (g/bird/d)	Body weight gain (g/bird/d)	FCR[1] (g/g)	Water intake (ml/bird/d)	Water to feed ratio (g/g)
PS	< 0.001	< 0.001	< 0.001	0.002	0.006
DCP	< 0.001	< 0.001	0.001	< 0.001	< 0.001
Age	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
PS × DCP	0.310	0.431	0.521	< 0.001	0.013
PS × Age	0.004	0.263	0.999	< 0.001	0.072
DCP × Age	0.086	0.041	0.987	< 0.001	0.823

[1]Feed conversion ratio.

low DCP diet. A protein source × age interaction was observed for FI where differences in FI between protein sources became larger as broilers got older (P < 0.004; Figure 1).

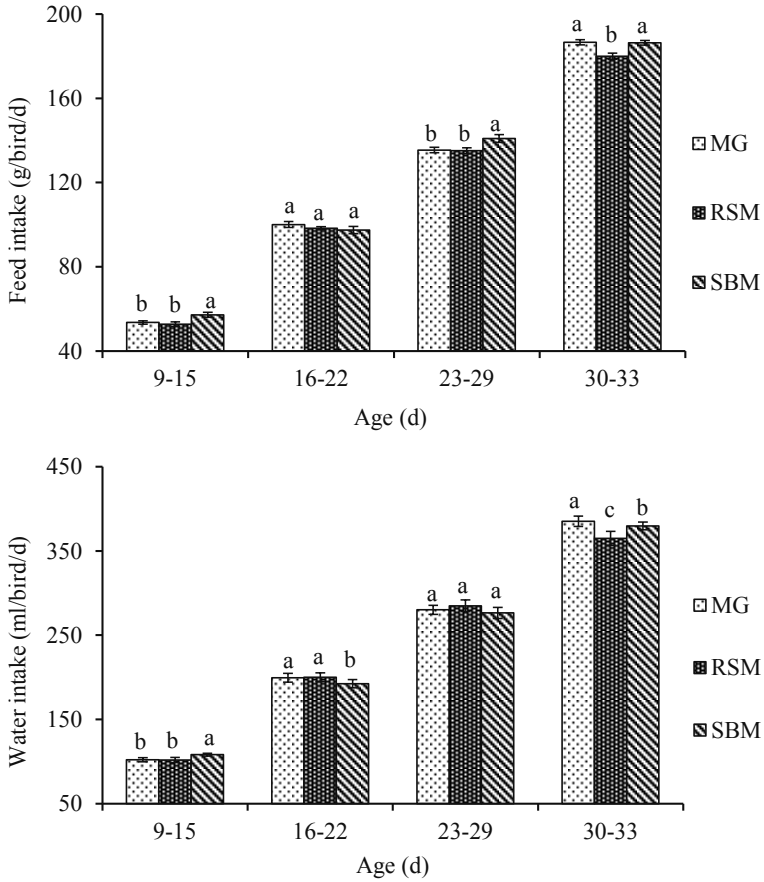

Figure 1. Least square means for traits of broilers that show a significant protein source (PS) × age (d) interaction.

The vertical bars represent standard error of means. Means with different superscripts within age significantly (P < 0.05) differs. (MG = maize gluten, RSM = rapeseed meal, SBM = soybean meal).

Protein source altered ($P < 0.001$) BW gain, with broilers fed the RSM and MG had a lower BW gain compared with those fed SBM diet. Broilers fed the high DCP diets had a greater ($P < 0.001$) BW gain compared with those fed the low DCP diets, regardless of the protein source. Body weight gain was affected by an interaction between DCP level and age of the broilers ($P = 0.041$; Table 3; Figure 2) where differences in BW gain between DCP levels became larger as broilers got older.

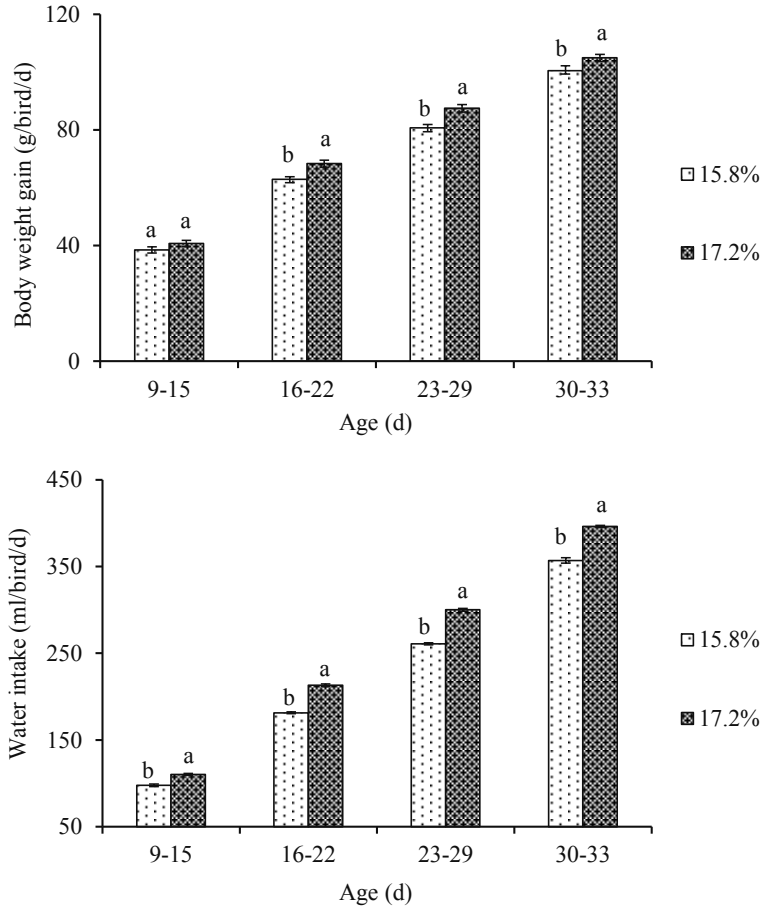

Figure 2. Least square means for traits of broilers that show a significant digestible crude protein level (DCP = 15.8 and 17.2%) × age (d) interaction.
The vertical bars represent standard error of means. Means with different superscripts within age significantly ($P < 0.05$) differs.

The FCR was affected ($P < 0.001$) by protein source with broilers fed the SBM diet had an improved FCR compared with those fed the RSM and MG diets. Broilers fed the high DCP diet showed an improved FCR ($P = 0.001$) compared with those fed the low DCP diet, regardless of dietary protein source (Tables 3 and 4).

Table 4. Least squares means[1] of performance parameters in broilers from 9 to 33 d of age as affected by protein source and digestible crude protein level.

Parameters	Treatment		Days			
	PS	DCP	9-15	16-22	23-29	30-33
Feed intake (g/bird/d)						
	SBM		57.2[a]	97.5	140.9[a]	186.4[a]
	RSM		52.7[b]	98.3	135.4[b]	180.0[b]
	MG		53.6[b]	100.1	135.5[b]	186.6[a]
	SEM		1.03	1.11	1.19	1.19
		15.8	54.1	96.6[b]	134.4[b]	183.8
		17.2	54.9	100.6[a]	139.9[a]	184.8
	SEM		0.84	0.91	0.98	0.97
Body weight gain (g/bird/d)						
	SBM		43.6[a]	68.2[a]	89.8[a]	108.1[a]
	RSM		37.0[b]	63.2[b]	80.5[b]	98.0[c]
	MG		38.1[b]	65.4[ab]	81.9[b]	102.1[b]
	SEM		1.03	1.13	0.93	1.26
		15.8	38.5[a]	62.9[b]	80.7[b]	100.5[b]
		17.2	40.7[a]	68.3[a]	87.5[a]	104.9[a]
	SEM		0.84	0.93	0.76	1.03
Feed conversion ratio (g/g)						
	SBM		1.31[b]	1.43[b]	1.57[b]	1.73[b]
	RSM		1.43[a]	1.56[a]	1.68[a]	1.84[a]
	MG		1.41[a]	1.53[a]	1.65[a]	1.83[a]
	SEM		0.03	0.03	0.02	0.03
		15.8	1.41	1.54	1.67[a]	1.83[a]
		17.2	1.36	1.48	1.60[b]	1.77[b]
	SEM		0.02	0.02	0.01	0.02
Water intake (ml/bird/d)						
	SBM		108.1[a]	192.4[b]	276.5[b]	379.8[b]
	RSM		101.9[b]	200.1[a]	284.9[a]	364.9[c]
	MG		102.2[b]	199.6[a]	280.1[b]	385.1[a]
	SEM		1.30	1.29	1.35	1.26
		15.8	97.8[b]	181.4[b]	260.9[b]	356.9[b]
		17.2	110.3[a]	213.3[a]	300.1[a]	396.2[a]
	SEM		1.06	1.05	1.10	1.03
Water to feed ratio (g/g)						
	SBM		1.90	1.97	1.96[b]	2.04
	RSM		1.94	2.04	2.11[a]	2.03
	MG		1.91	2.00	2.07[a]	2.06
	SEM		0.04	0.03	0.02	0.02
		15.8	1.81[b]	1.88[b]	1.94[b]	1.94[b]
		17.2	2.01[a]	2.13[a]	2.15[a]	2.14[a]
	SEM		0.03	0.02	0.02	0.01

[a-c]Means without a common superscript within a column and a parameter significantly ($P < 0.05$) differ, [1]Each value represents the mean of 6 replicates (8 birds per replicate).

Results of WI and water to feed (WF) ratio are also shown in Tables 3 and 4. Water intake and WF ratio were significantly affected by protein source and by DCP levels. Birds fed the MG diet had a greater WI compared to those fed the RSM and SBM diets. Similarly, high DCP diet resulted in a higher WI compared to low DCP diet fed birds. Water to feed ratio was greater in the RSM and MG diets fed birds compared to those fed the SBM diet. High DCP diet fed birds, likewise, had a higher WF ratio compared with those fed low DCP diet. Water intake showed an interaction between protein source and DCP level ($P < 0.001$; Table 3; Figures 1 and 2). In low DCP diet,

the RSM had a lower WI compared to the SBM and MG diets, whereas in high DCP diet the RSM and MG diets had higher WI compared to the SBM diet. The difference in WI between birds fed the low and high DCP level was 16.5% for the RSM diet, whereas this difference was 12.8 and 11.8% for birds fed the MG and SBM diets, respectively (Table 5). Water to feed ratio was affected by an interaction between protein source and DCP levels (P = 0.013; Table 3). The difference in WF ratio between birds fed the low and high DCP level was 13.7% for the RSM diet, whereas it was 7.9 and 11.6% for birds fed the SBM and MG diets, respectively (Table 5).

Table 5. Interaction effects of protein source and digestible crude protein level (%) on water intake and water to feed ratio in broilers from 9 to 33 d of age.

Protein source	Soybean meal		Rapeseed meal		Maize gluten	
Digestible CP level	15.8	17.2	15.8	17.2	15.8	17.2
Parameters						
Water intake (ml/bird/d)	225.9^{c}	252.5^{b}	219.8^{d}	256.1^{a}	272.2^{c}	256.4^{a}
Water to feed ratio (g/g)	1.89^{c}	2.04^{b}	1.90^{c}	2.10^{a}	1.90^{c}	2.12^{a}

$^{a-d}$Means without a common superscript within a row and a parameter significantly (P < 0.05) differ.

Digestive Tract Measurements

Protein source as well as their DCP levels did not influence the relative empty weights of the gut components and there were no interactions between protein source and DCP level (Table 6).

Table 6. Effects of protein source (PS) and digestible crude protein level (DCP, %) on mean relative weights[1] (g/100 g BW) of empty gastrointestinal segments in broilers from 1 to 33 d of age.

Effects	Crop	Proventriculus	Gizzard	Duodenum	Jejunum	Ileum	Ceca	Colon
Protein source								
Soybean meal	0.34	0.38	0.93	0.62	1.09	0.85	0.38	0.19
Rapeseed meal	0.36	0.40	1.04	0.68	1.22	0.95	0.41	0.18
Maize gluten	0.35	0.40	1.03	0.67	1.20	0.94	0.41	0.18
Pooled SE	0.01	0.01	0.04	0.21	0.05	0.03	0.01	0.01
Digestible CP level								
15.8	0.35	0.40	0.99	0.65	1.16	0.91	0.40	0.19
17.2	0.34	0.39	1.01	0.66	1.18	0.92	0.40	0.17
Pooled SE	0.01	0.01	0.03	0.02	0.04	0.03	0.01	0.01
P-value								
PS	0.198	0.126	0.112	0.098	0.117	0.109	0.120	0.860
DCP	0.512	0.693	0.680	0.815	0.653	0.699	0.720	0.240
PS × DCP	0.812	0.655	0.361	0.397	0.356	0.365	0.635	0.570

[1]Each value represents the mean of 6 replicates (6 birds per replicate).

Protein source, however, influenced (P < 0.001) duodenal morphology. Villus heights were 18.2 and 17.7% greater, whereas crypt depths were 15.5 and 18.1% smaller and villus height to crypt depth ratios were 29.0 and 30.9% greater in the SBM fed birds compared with those fed the RSM and MG diets, respectively (Table 7). Duodenal morphology was not significantly affected by DCP level, nor by the interaction between protein source and DCP level.

Table 7. Effects of protein source (PS) and digestible crude protein level (DCP, %) on villus height (μm), crypt depth (μm) and villus height to crypt depth ratio (VCR) in the duodenum of broilers from 1 to 33 d of age.

Effects	Villus height	Crypt depth	VCR
Protein source			
Soybean meal	1499[a]	277[b]	5.4[a]
Rapeseed meal	1226[b]	320[a]	3.8[b]
Maize gluten	1233[b]	327[a]	3.8[b]
Pooled SE	82.60	3.70	0.50
Digestible CP level			
15.8	1312	306	4.3
17.2	1327	309	4.3
Pooled SE	78.40	3.00	0.30
P-value			
PS	0.001	0.001	0.001
DCP	0.101	0.468	0.734
PS × DCP	0.490	0.853	0.344

[a-b]Means without a common superscript within a column significantly ($P < 0.05$) differ.

Cecal Digesta Characteristics

Protein source influenced ($P = 0.005$) cecal pH (Table 8). Birds fed the RSM diet had a lower pH in cecal contents compared to those fed the MG and SBM diets. Digestible CP level did not alter cecal pH. There were no interaction effects between level and protein source for cecal pH. Total VFA contents and ammonia concentration in cecal digesta were not influenced by either dietary protein source or DCP level. Branched chain fatty acids (BCFA) contents in cecal digesta, however, were altered by dietary protein source ($P = 0.030$; Table 9) and were 16.5 and 14.9% greater in RSM fed birds compared with those fed SBM and MG diets, respectively.

Discussion

The present study was designed to investigate the impact of three protein sources (which are known to differ in digestibility) on performance, gut morphology and cecal digesta characteristics in broilers. Each protein source was fed at two levels. It was hypothesized that an increased level of indigestible protein results in lower performance, a reduced gut health and elevated hindgut protein fermentation. Gut morphology and fermentation characteristics were studied as explanatory variables. The observed reduced FI in broilers fed the RSM diets is in agreement with several studies reporting adverse effects of RSM on FI if its inclusion level is more than 10% (Ahmad et al., 2007; Aftab, 2009) potentially due to its poor taste because of glucosinolates (Zeb, 1998) and its high fiber contents (Naseem et al., 2006). Rapeseed meal, additionally, contains a high amount of sulphur (1.14 vs. 0.44%) compared with SBM (Summers, 1995) which may interact with calcium due to an alteration in anion-cation balance, also resulting in a poor FI (Summers and Bedford, 1994). A lower FI in broilers fed the low DCP levels compared with those fed the high DCP levels is similar to results reported by Ferguson et al. (1998). The lower BW gain in RSM fed

birds compared to those on the other dietary treatments is related to the lower FI. Total levels of first limiting essential amino acids in the diets were similar in all treatments. Differences in performance may be partly associated with levels of non-essential amino acids in the low DCP diet (Corzo et al., 2005). Rapeseed meal, additionally, contains some toxic compounds such as glucosinolates, tannins, phytase, erucic acid, and sinapine (Khajali and Slominski, 2012) which may result in poor performance. The enzymatic degradation of these glucosinolates may lead to the production of goitrin which inhibits proper functioning of the thyroid glands and suppresses the secretion of thyroxin (Tripathi and Mishra, 2007). This improper functioning of thyroid gland may lead to a reduced weight gain in birds fed RSM. The isothiocyanates in rapeseed, likewise, may also result in a reduced FI and impaired growth (McNeill et al., 2004). For all protein sources, in the present study, birds on the low DCP level had a lower FI and impaired BW gain compared with the birds fed the high DCP level. A low digestibility level in a diet means less amino acids available for growth and potentially larger amounts of indigestible CP entering the hindgut leading to proteolytic fermentation. The latter findings are supported by results of De Lange et al. (2003). The processing and excretion of nitrogenous compounds will require more energy (Birkett and De Lange, 2001), resulting in less energy availability for growth. Low DCP levels as treatment factor, here, were accompanied by low dietary CP levels. Low CP levels reduced WI as well as the WF ratio. Such a significant reduction in WI of broilers fed low dietary CP levels was previously reported by Alleman and Leclercq (1997), and Ziaei et al. (2008). Lower WI in broilers fed a low (digestible) CP diet is associated with a low FI. It has been reported that 1% increase in protein content results in 3% more water consumption (Larbier and Leclercq, 1992). Difference in WI and WF ratio between the three proteins sources were, however, small.

Table 8. Effects of protein source (PS) and digestible crude protein (DCP, %) level on cecal digesta characteristics in broilers[1] from 1 to 33 d of age.

Effects	Cecal pH	NH_3 (g/kg DM)
Protein source		
Soybean meal	6.54[a]	4.95
Rapeseed meal	6.20[b]	4.55
Maize gluten	6.37[ab]	4.85
Pooled SE	0.07	0.18
Digestible CP level		
15.8	6.43	4.72
17.2	6.32	4.86
Pooled SE	0.05	0.14
P-value		
PS	0.005	0.252
DCP	0.187	0.495
PS × DCP	0.415	0.558

[a-b]Means without a common superscript within a column significantly ($P < 0.05$) differ. [1]Each value represents the mean of 6 replicates (6 birds per replicate).

Table 9. Effects of protein source (PS) and digestible crude protein (DCP, %) level on cecal volatile fatty acids (VFA (mmol/kg DM)) concentrations[1] in broilers from 1 to 33 d of age.

Effects	VFA	Acetic acid[2]	Propionic acid[2]	Butyric acid[2]	Valeric acid[2]	Total BCFA[2,3]	Iso-butyric acid[2]	Iso-valeric acid[2]
Protein source								
Soybean meal	130.4	73.80	6.52[ab]	16.01	1.49[c]	2.18[b]	1.00[b]	1.18
Rapeseed meal	126.1	74.12	5.90[b]	15.35	2.03[a]	2.61[a]	1.24[a]	1.37
Maize gluten	125.3	74.40	6.69[a]	14.92	1.74[b]	2.22[b]	1.06[b]	1.15
Pooled SE	3.30	0.58	0.24	0.44	0.06	0.12	0.06	0.07
Digestible CP level								
15.8	127.6	73.90	6.46	15.62	1.73	2.29	1.09	1.20
17.2	126.9	74.30	6.25	15.30	1.76	2.39	1.12	1.27
Pooled SE	2.70	0.47	0.20	0.36	0.05	0.10	0.05	0.06
P-value								
PS	0.510	0.620	0.046	0.207	< 0.001	0.030	0.023	0.072
DCP	0.862	0.533	0.440	0.572	0.508	0.459	0.667	0.366
PS × DCP	0.374	0.029	0.011	0.269	0.492	0.901	0.839	0.843

[a-c]Means without a common superscript within a column significantly ($P < 0.05$) differ. [1]Each value represents the mean of 6 replicates (6 birds per replicate). [2]Percentage of total VFA. [3]BCFA = branched chain fatty acid (sum of iso-butyric and iso-valeric acids).

Gut morphology is one of the markers for gut health and can be assessed by villus height and crypt depth (Awad et al., 2009). There is a scarcity of published data regarding the effect of CP sources and their digestible levels on villus height and crypt depth in broilers. The duodenum is the major site for digestion and absorption of nutrients in the small intestine. Duodenal histology, therefore, was measured to monitor the expected negative effects of nitrogenous substances on villus height (Nousiainen, 1991). The shorter villus height and greater crypt depth in broilers fed RSM and MG diets may be an indication of more damage to the gut by harmful compounds produced by microbial fermentation. These shorter villi indicate reduced intestinal health. A shorter villus height may decrease the surface area for absorption of nutrients from the gut as villi are the functional units of nutrient absorption (Zang et al*.,* 2009). A high crypt depth means an increased turnover rate of enterocytes and thus more protein and energy demand for this purpose. Crypt depth is an indicator of the number of crypt cells produced (Hampson, 1986). It has been reported that broilers spend approximately 12% of synthesized protein on GIT turnover (Choct, 2009). An increase in villus height may enhance nutrient transport across the villus surface, as suggested by Tufarelli et al. (2010) in rabbits. The absence of significant effects of DCP levels on villus height and crypt depth here, are confirmed by the findings of Buwjoom et al*.* (2010) in broilers.

Feeding a diet with a low protein digestible ingredient such as RSM, due to the fact that approximately 7% of the seed nitrogen (N) in RSM is tightly bound, will increase the amount of indigestible protein entering the ceca (Finlayson et al*.,* 1973). The low N digestibility of the RSM and MG diets compared to the SBM diet has led to an increase in N in the hindgut. This indigestible protein may stimulate protein fermentation (Hobbs et al*.,* 1996), if fermentable (insoluble) carbohydrates are present at low levels in the diet as with the RSM diet (Table 2). Fermentable (insoluble) carbohydrates provide additional energy to gut microbes and decrease the concentration of harmful compounds that are the result of protein fermentation (Swanson et al., 2002). A low level of fermentable carbohydrates with high indigestible protein levels in the diet may lead to specific protein fermentation. A high level of fermentable carbohydrates in such diets may result in microbial protein synthesis. The MG and RSM diets had similar high amounts of indigestible CP. Because of higher amounts of fermentable NSP in MG diets (154 and 143 g/kg) compared to SBM (130 and 123 g/kg) and RSM diets (132 and 132 g/kg), they differ in fermentation characteristics. This was confirmed by the data here. For example, greater cecal BCFA and lower propionic acid concentrations in birds fed the RSM diet compared to those fed the MG diet may indicate more protein and less carbohydrate fermentation in RSM fed birds, because propionic acid is suggested to be the result of

carbohydrate fermentation (Rodriguez et al*.,* 2013) and BCFA are supposed to be a marker for protein fermentation (Macfarlane et al*.,* 1992). Both RSM and MG diets had similar amounts of indigestible protein but different amounts of NSP. Moreover, the cecal pH in broilers increased from RSM < MG < SBM. This indicates more proteolytic fermentation in the distal GIT of broilers fed the RSM diets compared to the MG diets. Rapeseed meal contains more sulphur containing amino acids compared to SBM (Okrouhla et al*.,* 2012). Fermentation of sulphur containing amino acids (methionine, cysteine and taurine) by sulphate reducing bacteria results in the production of hydrogen sulfide (H_2S) which in turn lowers the pH (Lewis and Cochrane, 2007).

In conclusion, diets with RSM and MG as major protein source compared to SBM resulted in poorer performance, reduced villus heights, deeper crypts and greater BCFA concentrations. Greater cecal BCFA concentrations are indicative of proteolytic fermentation in the hindgut which may result in poorer gut morphology and an impaired FCR. Rapeseed meal with high levels of indigestible CP, therefore, could be used as a model for hindgut protein fermentation in broilers.

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CHAPTER 4

Protein source and diet structure influence growth performance, gut morphology and hindgut fermentation characteristics in broilers

S.N. Qaisrani[1], P.C.A. Moquet[1], M.M. van Krimpen[2], R.P. Kwakkel[1], M.W.A. Verstegen[1], and W.H. Hendriks[1]

[1]Animal Nutrition Group, Wageningen University, PO Box 338, NL-6700 AH Wageningen, The Netherlands

[2]Wageningen UR Livestock Research, PO Box 65, NL-8200 AB Lelystad, The Netherlands

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Abstract

An experiment with 210 male (Ross 308) 1-d-old broilers was conducted to test the hypothesis that a coarse diet improves performance of broilers fed a poorly digestible protein source. A highly digestible diet based on soybean meal was gradually replaced by a low digestible diet based on rapeseed meal (RSM) in five steps (RSM-0%, RSM-25%, RSM-50%, RSM-75% and RSM-100%). Two diet structures (fine and coarse) were used as an additional factor. These two factors were tested in a factorial design with ten dietary treatments. An increase in indigestible dietary protein negatively affected feed intake (FI) (P = 0.003), BW gain (P = 0.008) and feed conversion ratio (FCR) (P = 0.034). This increase in dietary indigestible protein contents resulted in a decrease (P = 0.001) in total cecal volatile fatty acid (VFA) concentration from 209.1 to 125.9 mmol/kg DM digesta in broilers with increasing RSM in diets. There was a trend (P = 0.067) for total cecal branched chain fatty acids (BCFA) to be influenced by protein source. Increase in the indigestible protein level, from RSM-0% to RSM-100%, resulted in a decrease (P = 0.042) in villus heights (1782 vs. 1574 μm), whereas crypt depths increased (P = 0.021; 237 vs. 274 μm). A coarse diet improved FI (P = 0.006), BW gain (P = 0.014) and FCR (P = 0.009). Broilers fed coarse diets had approximately 11, 24 and 10% lower relative empty weights of the crop, proventriculus and jejunum, respectively, and 15% heavier gizzard was found compared with those fed fine diets. Dietary coarseness resulted in approximately 16% lower gizzard pH, 21% greater villus heights, 27% lower crypt depths, 24% reduced BCFA, and 12% lower biogenic amines in the cecal digesta compared with broilers fed fine diets. It can be concluded that feeding coarse particles improve the performance of broilers even with a poorly digestible protein source. Hindgut protein fermentation can be reduced in diets with a low CP digestibility by coarse grinding of the diet.

Key words: broilers, cecal fermentation, particle size, gut health, biogenic amines.

Introduction

The percentage of undigested CP in various ingredients for poultry diets ranges from 8 to 35, depending on the protein source (CVB, 2007). A high inclusion level of protein sources with a relatively low amino acids digestibility such as RSM, therefore, will decrease performance. Moreover, non-digested protein at ileal level may increase the amount of undigested amino acids reaching the hindgut that in turn may enhance proteolytic fermentation by resident microbiota (Libao-Mercado et al., 2009). As a side effect, hindgut protein fermentation can even further negatively affect performance of the broilers due to the formation of toxic compounds such as amines, ammonia, skatole or indoles (Gabriel et al., 2005).

Diet structure plays an important role in controlling the passage rate of the digesta through the gastrointestinal tract (GIT). Dietary ingredients with, on average, large particles are normally ground into smaller particles in the gizzard and then transported to the duodenum. A coarse diet, remains for a longer time in the gizzard resulting in an enhanced muscular activity and gizzard weight (Jacobs and Parson, 2013). A large muscular gizzard can maximize the grinding capacities of the GIT. A longer retention time in the gizzard leads to more exposure of feed particles to gastric juices which improves the digestion of dietary proteins thereby contributing to a better feed efficiency (Liu et al., 2013; Pacheco et al., 2013). Less protein, consequently, will enter the hindgut resulting in low substrate availability for proteolytic fermentation and less production of toxic compounds.

Villus height and crypt depth in the small intestine are important indicators of gut development and animal health, and as such influence nutrient digestion and absorption (Wang and Peng, 2008). The beneficial effects of feeding a coarse diet on digestibility of nutrients arose from their influence on intestinal morphology. There is, however, a scarcity of published data regarding the effects of particle size on intestinal morphology in broilers fed partly ileal indigestible protein.

The objective of the current study was to determine the combined effects of diet structure and increased levels of (in) digestible dietary CP on performance, gut morphology and hindgut fermentation characteristics in broilers. It was hypothesized that the effects of a poorly digestible dietary protein source on performance and gut health can be partly counteracted by feeding the diet in a coarse form.

Materials and Methods

Animal Ethics

Experimental procedures were in accordance with the Wageningen University and the Netherlands Animal Experimental Committee guidelines and code of practice. Ethical approval was granted before the conduct of the study.

Birds and Husbandry

In total, 210 male (Ross 308) 1-d-old broilers were obtained from a commercial hatchery, individually weighed, steel wing tagged and randomly assigned to 30 floor pens (7 birds per pen) in a climate-controlled room. Each pen (1.15 × 1.75 × 0.80 m) (L × W × H) had three drinking nipples with a cup underneath connected to a water tank of 10 L capacity. Feed and water were available *ad libitum*. Soft Cells® were used as a bedding material in order to avoid confounding effects of consumed wood shavings on gut development. The lighting schedule was set at 23L:1D for the first three d and thereafter maintained to 16L:8D with an intensity of approximately 20 lx at bird's level throughout the experiment. During the first three days, room temperature was set at 32°C, and thereafter gradually decreased to a constant value of 22°C in wk 4 and maintained until the end of the experiment.

Experimental Design and Treatments

The study was conducted as a randomized complete block (5 × 2) orthogonal design. Five dietary treatments were used in which a good digestible protein source, soybean meal (SBM) was stepwise replaced by a less digestible protein source, rapeseed meal (RSM); containing diets with a very low amount of indigestible protein (RSM-0%), a low amount of indigestible protein (RSM-25%), a medium level of indigestible protein (RSM-50%), a high level of indigestible protein (RSM-75%) and very high level of indigestible protein (RSM-100%). These five diets were each fed in two different diet structures: finely and coarsely ground. All diets were fed to the birds from day one and randomly assigned to the pens in the room. Each treatment had three replicates resulting in 30 experimental units (5 × 2 × 3 = 30 pens). The diets were formulated to meet or exceed the nutrient recommendations for broilers (CVB, 2007), and fed as a starter (0 to 7 d of age) and a grower diet (8 to 34 d of age). The component composition and calculated chemical composition of the different dietary treatments are presented in Table 1. Diets were formulated to be iso-energetic on an ME basis and to have similar levels of essential amino acids. As a consequence, next to the stepwise substitution of SBM by RSM, also inclusion levels of ingredients such as maize starch, fish meal, potato protein and vegetable fat varied among the treatments and the differences between the dietary treatments were minimized by adding diamol (a binding material), which was exchanged by potato protein and vegetable fat. Each diet was processed with either a hammer mill with an opening screen of 3.0 mm (fine diet) or a roller mill with a roller distance of 1.6 mm (coarse diet). Table 2 shows the amounts of analyzed CP entering the hindgut as calculated from apparent ileal CP digestibility values based on literature data. The diets were offered as pellets with a pellet size of 2.5 mm for the starter and 4.0 mm for the grower diet. The diets did not contain antimicrobial growth promoters.

Table 1. Dietary ingredients, and analysed and calculated nutrients of the diets[1] (g/kg as-fed basis).

Item	Starter diet					Grower diet				
	RSM-0%	RSM-25%	RSM-50%	RSM-75%	RSM-100%	RSM-0%	RSM-25%	RSM-50%	RSM-75%	RSM-100%
Maize starch	117.5	111.1	104.8	98.5	92.1	64.2	58.6	53.1	47.4	41.9
Maize	300.0	300.0	300.0	300.0	300.0	300.0	300.0	300.0	300.0	300.0
Wheat	100.0	100.0	100.0	100.0	100.0	150.0	150.0	150.0	150.0	150.0
Fish meal	25.0	34.5	44.0	53.5	63.0	8.0	17.2	26.5	35.7	45.0
Soybean meal	350.0	262.5	175.0	87.5	0.0	350.0	262.5	175.0	87.5	0.0
Rapeseed meal	0.0	87.5	175.0	262.5	350.0	0.0	87.5	175.0	262.5	350.0
Potato protein	10.0	15.0	20.0	25.0	30.0	5.0	10.0	15.0	20.0	25.0
Vegetable fat	30.0	32.5	35.0	37.5	40.0	62.0	64.0	66.0	68.0	70.0
Diamol (binding material)	30.0	22.5	15.0	7.5	0.0	30.0	22.5	15.0	7.5	0.0
Premix[2]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Chalk	12.5	11.3	10.3	9.1	8.0	10.2	9.1	8.1	7.1	6.0
Mono-calcium phosphate	12.0	10.8	9.5	8.3	7.0	8.0	6.8	5.5	4.3	3.0
Salt	2.3	2.0	1.8	1.5	1.2	2.5	2.3	2.1	1.9	1.7
$NaHCO_3$	2.1	2.2	2.2	2.2	2.3	2.1	1.9	1.6	1.4	1.2
DL-methionine	2.0	1.6	1.1	0.6	0.2	1.7	1.3	0.8	0.4	0.0
L-threonine	0.9	0.7	0.4	0.2	0.0	0.7	0.5	0.4	0.2	0.0
L-valine	0.7	0.5	0.3	0.2	0.0	0.6	0.5	0.3	0.2	0.0
L-arginine	0.0	0.3	0.6	0.9	1.2	0.0	0.3	0.6	0.9	1.2
Calculated composition										
ME (kcal/kg)	2671.0	2673.5	2676.0	2678.5	2681.0	2835.0	2835.0	2835.0	2835.0	2835.0
CP	215.1	217.5	220.1	222.6	225.1	204.1	206.5	208.8	211.2	213.5
Digestible protein	178.3	177.6	176.9	176.3	175.6	168.5	167.7	166.9	166.1	165.3
Indigestible protein	36.8	39.9	43.2	46.3	49.5	35.6	38.8	41.9	45.1	48.2
NSP[3]	224.9	225.4	225.9	226.4	226.9	184.9	185.8	186.8	187.7	188.6
Crude fiber	35.3	39.3	43.2	47.2	51.1	36.4	40.4	44.3	48.3	52.2
Digestible Lys	10.6	10.6	10.6	10.6	10.6	9.6	9.6	9.6	9.6	9.6
Digestible M+C	7.7	7.7	7.7	7.7	7.8	7.2	7.2	7.2	7.2	7.2
Digestible Met	5.0	4.9	4.7	4.6	4.5	4.6	4.4	4.3	4.1	3.9
Digestible Thr	7.8	7.8	7.8	7.8	7.8	7.2	7.2	7.2	7.2	7.2

[1]RSM-0% = 100% soybean meal + 0% rapeseed meal, RSM-25% = 75% soybean meal + 25% rapeseed meal, RSM-50% = 50% soybean meal + 50% rapeseed meal, RSM-75% = 25% soybean meal + 75% rapeseed meal, RSM-100% = 0% soybean meal + 100% rapeseed meal.
[2]Premix composition: 12,000 IE retinol, 2,400 IE cholecalciferol, 50 mg dl-a-tocopherol, 1.5 mg menadione, 2.0 mg thiamine, 7.5 mg riboflavin, 3.5 mg pyridoxine, 20 mcg cyanocobalamins, 35 mg niacin, 12 mg D-pantothenic acid, 460 mg choline chloride, 1.0 mg folic acid, 0.2 mg biotin; 80 mg iron, 12 mg copper, 85 mg manganese, 60 mg zinc, 0.40 mg cobalt, 0.8 mg iodine, 0.1 mg selenium, 125 mg anti-oxidant mixture, [3]NSP = non-starch polysaccharides, calculated by subtracting the analysed content of CP, fat, starch, sugar, and ash from the analysed DM content.

Table 2. Analysed CP contents (g/kg) and calculated apparent ileal digestibility of CP of the four diets.

Diet	Starter		Grower		References[1]
RSM[a] inclusion level (%)	0	100	0	100	
Nutrients					
Total CP					
Of the whole diet	206.1	198.1	201.6	199.7	1
Of the protein source[2]	142.5	104.1	148.7	110.7	2
Of the other ingredients[3]	63.6	94.0	52.9	89.0	
Ileal digestible					
Of the whole diet	181.1	159.0	177.7	160.5	3
Of the protein source[2]	128.2	79.1	133.6	84.1	3
Of the other ingredients[3]	52.9	80.0	44.1	76.4	
Of the essential amino acids	27.3	25.1	26.2	24.7	
CP entering the hindgut					
Of the whole diet	25.0	39.1	23.9	39.2	
Of the protein source[2]	14.3	25.0	15.1	26.6	
Of the other ingredients[3]	10.7	14.0	8.8	12.6	
Total NSP[4]	161.9	189.5	166.9	188.5	2
Soluble NSP	58.6	54.7	59.7	54.1	2
Insoluble NSP	103.3	134.8	107.2	134.4	2

[a]RSM = rapeseed meal. [1]References: 1 = analysed, 2 = CVB (2007); 3 = Lemme et al. (2004) for all ingredients. [2]Protein sources were soybean meal and rapeseed meal. [3]Other ingredients were maize, wheat, fish meal, and potato protein. [4]NSP = non-starch polysaccharides, calculated by subtracting the CP, fat, starch, sugar, and ash content from the DM content.

Wet Sieve Analysis

Particle size distribution of the diets was analysed using the wet sieve method as described by Goelema et al. (1999) with minor modifications. Briefly, weighed samples of a diet were each subdivided into 2 subsamples. One subsample was dried overnight at 70°C in an oven until constant weight to determine the air DM content, whereas the other subsample was suspended in 500 ml of water for 45 min to ensure adequate hydration, before being washed through a set of sieves with decreasing mesh size 2.5, 1.25, 0.63, 0.315, 0.160 and 0.071 mm. The contents of each sieve were subsequently collected and dried overnight at 70°C in an oven to constant air DM weight. The dried weights of particles retained by each sieve and of the fines remaining in the bottom pan were expressed as percentages of total air DM recovered. Average particle size of the diets was calculated as (fraction < 0.071 mm × 0.035) + (fraction 0.071 - 0.16 mm × 0.115) + (fraction 0.16 - 0.315 mm × 0.237) + (fraction 0.315 - 0.630 mm × 0.472) + (fraction 0.630 - 1.25 mm × 0.940) + (fraction 1.25 - 2.50 mm × 1.65) + (fraction > 2.50 mm × 3.50)/100. Particle size distribution of the fine and the coarse diets is shown in Figure 1. For the fine RSM-0% diet, average particle size was 217 µm, whereas for the coarse RSM-0%, it was 482 µm. Average particle size for the fine RSM-100% diet was 208 µm, whereas it was 578 µm for the coarse RSM-100%.

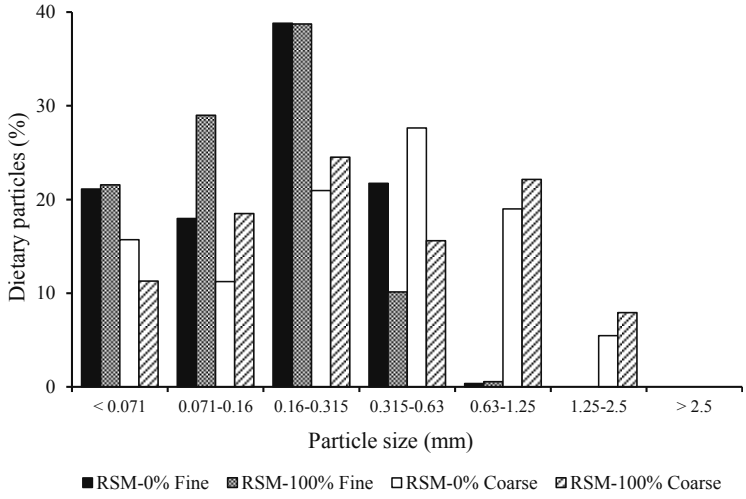

Figure 1. Particle size distribution of fine and coarse diets with different levels of rapeseed meal (RSM).

Pellet Durability

Pellet durability was determined in a Holmen Pellet Tester (New Holmen Pellet Tester, TekPro Ltd., Norfolk, UK) using the method described by Svihus et al. (2004). The pellet samples (100 g) were circulated pneumatically through a closed pipe for 30 s before being passed through a 3-mm sieve. The pellet durability index (PDI) was calculated as the relative proportion of pellets retained on the 3-mm sieve. The PDI values varied between 4.6 and 75.9% for the fine diets and 13.2 and 42.3% for the coarse diets (Figure 2). Although a difference existed, particularly between the fine and coarse pellets in the RSM-0% diets, birds were fed in such a way that all the nutrients were ingested and no leftovers were remained.

Traits Measured

Feed intake (FI), water intake (WI) and body weight gain (BWG) per pen were recorded at 7, 14, 21, 28 and 34 d of age, whereas mortality was recorded daily. Feed conversion ratio (FCR) was calculated by dividing total FI by weight gain of live plus dead birds. At the end of the experiment (d 35 and 36), 6 of the 7 birds from each replicate pen were randomly selected and killed by an intravenous T-61 injection, where after the abdominal cavity was opened. During the dissection day, all the birds had access to the feed until the moment of euthanization. The different parts of the GIT, i.e. the crop, proventriculus, gizzard, duodenum (from pyloric junction to pancreo-billiary duct), jejunum (from pancreo-billiary duct to Meckle's diverticulum), ileum (from Meckle's diverticulum to ileo-cecal junction), cecum (from ostium) and

colon were segmented. The digesta contents from each segment were immediately removed by gentle squeezing and the empty segments were weighed.

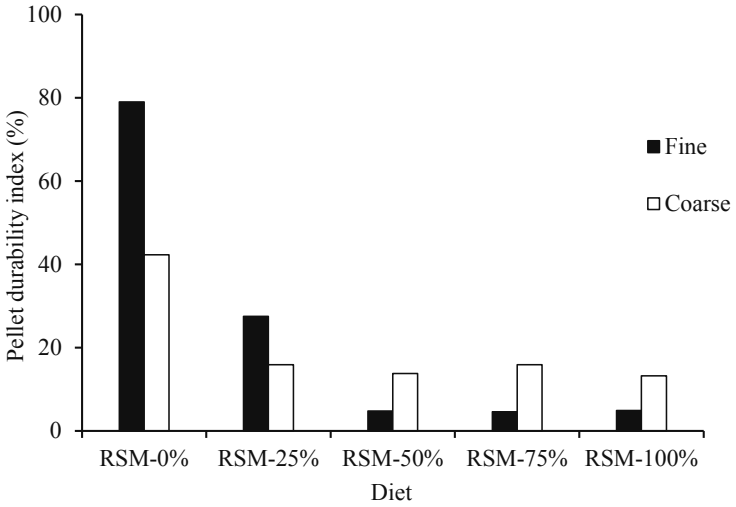

Figure 2. Pellet durability index of dietary treatments containing different levels of rapeseed meal (RSM).
Fine: Ingredients passed through an opening screen of 3.0mm in a hammer mill. Coarse: Ingredients passed through an opening screen of 1.6 mm in a roller mill.

Ceca content of the six birds in a pen were quantitatively pooled and mixed. The pH was determined using a calibrated pH meter before the samples were freeze dried at -20°C. The samples were frozen pending volatile fatty acids (VFA), biogenic amines and ammonia analyses.

Tissue Collection and Histological Measurements

For intestinal morphological examination, duodenal samples (approximately 2 cm in length) from the middle of the duodenum were collected, rinsed with cold physiological saline (0.9% saline) and immediately placed in Bouin's fluid. The samples, thereafter, were transferred into 70% ethanol within 24 h, embedded in paraffin and sectioned at 5 µm thickness. For histological examination, six cross-sections per bird were processed using standard haematoxylin and eosin methods as described by Owusu-Asiedu et al. (2002). Villus height (the distance from the apex of the villus to the junction of the villus and crypt) and crypt depth (the distance from the junction to the basement membrane of the epithelial cells at the bottom of the crypt) were measured on 10 intact, well-oriented villi (from the 2 cm in the middle of the duodenum) per bird using a compound light microscope equipped with a video camera.

Chemical Analysis

Dry matter, organic matter and N contents in the experimental diets were measured according to the standard methods (AOAC International, 2006). Ammonia in cecal digesta was measured by the indoles phenol-blue method (Novozamsky et al., 1974). The samples were deprotonated by adding 10% (w/v) trichloroacetic acid solution followed by centrifugation. The ammonium was transformed by phenol and hypochlorite in an alkaline solution into blue colored indoles phenol-blue by the Berthelot reaction. The ammonia content was measured spectroscopically at 623 nm.

For determination of VFAs, 5 g of cecal samples and 5 ml 0.1 M phosphoric acid were shaken at 100 rpm before being centrifuged (7000 g) for 10 min. Residues were collected and the supernatants again were centrifuged (20817 g) for 10 min. Afterwards, 600 µL of the supernatant was taken in a crimp vial and mixed with 600 µL of phosphoric acid containing isocapronic acid (2.29 G/L concentration) as an internal standard. Volatile fatty acids were separated by gas chromatography using an EM-1000 (30 m × 0.53 mm) column from Alltech (Deerfield, IL, USA) and helium as the mobile phase with detection by fluorescent infrared detector. Quantification of VFAs was based on a chemical standard solution (Merck) after internal standard correction.

Biogenic amines (histamine, putrescine, cadaverine, spermidine, spermine, and tyramine) in cecal digesta were determined by the method described by Meyer et al. (2013) with slight modifications. Briefly, 50 mg of freeze-dried bullet mill sample was added with 20 mg of sulphosalicylic acid and 1 ml of 0.1N hydrochloric acid. The samples were shaken for 15 min at a speed of 2000 g. Thereafter, the samples were placed in an ice-bath for 15 min, vortexed and transferred to a 1.5 ml Eppendorf tube. The supernatants were taken out and the tubes were centrifuged at 20000 g (14000 rpm) for 5 min, thereafter filtered over a 0.2 µm filter. A 20 µl filtered sample was analyzed on a column (Cation separation column LCAK17/K 4.6 × 30 mm) eluted with a combination of 0.4 N potassium citrate buffer (pH 5.75) and 2.5 N potassium citrate buffer (pH 8.4) followed by a post column ortho-phthalaldehyde fluorescent detection at 425 nm.

Statistical Analysis

The repeated statement within PROC MIXED of SAS (version 9.2; SAS Inst. Inc., Cary, NC) was used with pen being the random effect. The following statistical model was used:

$$Y_{ijkl} = \mu + PS_i + ST_j + PS_i \times ST_j + A_k + PS_i \times A_k + ST_j \times A_k + PS_i \times ST_j \times A_k + e_{ijkl}$$

where Y_{ijkl} is the measured response, μ overall mean effect, PS_i the i^{th} fixed protein source effect (i = soybean meal, rapeseed meal), ST_j the j^{th} fixed diet structure effect (j

= fine or coarse), $PS_i \times ST_j$ the interaction between PS_i and ST_j, A_k the k^{th} age interval of measurement (k = 1-7, 8-14, 15-21, 22-28 and 29-34), $PS_i \times A_k$ the interaction effect between protein source and age interval of the bird at the time of measurement, $ST_j \times A_k$ the interaction effect between diet structure and age interval of the bird at the time of measurement, $PS_i \times ST_j \times A_k$ the interaction effect between protein source, diet structure and age interval of the birds at the time of measurement, and e_{ijkl} the random error associated with the j^{th} diet structure assigned to the i^{th} protein source at d of measurement k, $e_{ijkl} \sim$ NID (0, σ^2_e). Differences were considered significant at a probability level of 5%. If significances of main effects or their interactions were detected, then means were compared using least squares means comparison. The factor 'age' was excluded from the model in case of GIT development, morphometric indices of duodenum, ammonia, cecal pH, VFAs, BCFA, biogenic amines, and gizzard pH.

Results

Bird Performance

Probability values for measured performance traits are presented in Table 3, whereas least square means of the performance parameters are presented in Table 4. Feed intake decreased (P = 0.003) with increasing amount of RSM in the diet. Coarseness of the diet also influenced (P = 0.006) FI with broilers fed the coarse diet having a greater intake than those fed the fine diet (Tables 3 and 4). Feed intake decreased (P = 0.003) with increasing amount of RSM in the diet. Coarseness of the diet also influenced (P = 0.006) FI with broilers fed the coarse diet having a greater intake than those fed the fine diet (Tables 3 and 4). A protein source × age interaction (P = 0.012) was observed for FI indicating that the differences in FI between protein sources became greater as broilers got older. Higher rapeseed protein inclusions in the diet decreased (P = 0.008) BW gain. Body weight gain was also influenced (P = 0.014) by diet structure, with broilers fed coarse diets having a greater gain compared to those fed finely ground diets. An interaction (P = 0.040) between protein source and age of the birds for BW gain (Tables 3 and 4) was also observed.

Table 3. Probability values of main effects and interactions between protein source (PS), diet structure (ST) and age (days) of broilers for different traits.

Effects	Feed intake (g/bird/d)	Body weight gain (g/bird/d)	FCR[1] (g/g)	Water intake (ml/bird/d)	Water to feed ratio (g/g)
PS	0.003	0.008	0.034	0.548	0.234
ST	0.006	0.014	0.009	0.798	0.002
Age	0.001	0.001	0.001	0.001	0.001
PS × ST	0.350	0.151	0.436	0.655	0.449
PS × Age	0.012	0.040	0.305	0.267	0.343
ST × Age	0.065	0.112	0.309	0.037	0.838

[1]FCR = feed conversion ratio.

Table 4. Least square means[1] of performance parameters in broilers from 1 to 34 days of age as affected by protein source (PS) and diet structure (ST; fine vs. coarse).

Parameters	Treatments		Days				
	PS	ST	0-7	8-14	15-21	22-28	29-34
Feed intake (g/bird/d)							
	RSM[2]-0%		27.5	47.2^{a}	93.4^{a}	135.4^{a}	188.3^{a}
	RSM-25%		28.9	45.8^{a}	91.5^{ab}	123.5^{b}	176.9^{ab}
	RSM-50%		29.7	41.9^{b}	86.9^{bc}	129.3^{ab}	176.9^{ab}
	RSM-75%		27.8	41.1^{b}	85.6^{c}	128.9^{ab}	175.5^{b}
	RSM-100%		25.9	41.8^{b}	82.4^{c}	120.7^{b}	165.3^{b}
	SEM		1.47	0.99	1.92	3.98	4.33
		Fine	28.1	43.1	86.9	122.7^{b}	170.9^{b}
		Coarse	27.8	43.9	88.9	132.4^{a}	182.2^{a}
	SEM		0.93	0.63	1.22	2.52	2.74
Body weight gain (g/bird/d)							
	RSM-0%		14.6	39.5^{a}	65.3^{a}	78.5^{a}	97.6^{a}
	RSM-25%		15.2	38.1^{a}	62.6^{ab}	71.9^{a}	86.2^{ab}
	RSM-50%		14.4	35.2^{b}	57.3^{bc}	73.2^{a}	88.4^{ab}
	RSM-75%		13.9	35.0^{b}	55.5^{cd}	73.3^{a}	84.3^{ab}
	RSM-100%		13.5	34.9^{b}	54.1^{d}	67.7^{b}	79.6^{b}
	SEM		0.91	0.87	2.00	2.70	4.40
		Fine	14.2	35.9	55.7	67.7^{b}	86.0^{b}
		Coarse	14.4	36.3	60.0	75.8^{a}	95.3^{a}
	SEM		0.57	0.55	1.27	1.71	2.93
Feed conversion ratio (g/g)							
	RSM-0%		1.88	1.19	1.43^{b}	1.72^{b}	1.93^{b}
	RSM-25%		1.90	1.20	1.46^{b}	1.72^{b}	2.05^{a}
	RSM-50%		2.06	1.19	1.52^{ab}	1.77^{b}	2.00^{a}
	RSM-75%		2.01	1.17	1.54^{ab}	1.76^{b}	2.08^{a}
	RSM-100%		1.92	1.20	1.52^{ab}	1.81^{a}	2.08^{a}
	SEM		0.06	0.02	0.05	0.04	0.06
		Fine	1.98	1.20	1.56	1.81	2.11^{a}
		Coarse	1.93	1.21	1.48	1.75	1.91^{b}
	SEM		0.05	0.01	0.03	0.03	0.05
Water intake (ml/bird/d)							
	RSM-0%		43.2	87.4	172.1^{a}	235.4	303.0
	RSM-25%		45.6	88.1	160.9^{b}	218.6	288.7
	RSM-50%		40.8	81.6	156.8^{b}	218.0	301.2
	RSM-75%		42.9	80.3	153.4^{b}	231.5	317.9
	RSM-100%		41.2	80.6	153.6^{b}	211.9	301.0
	SEM		2.10	2.79	3.48	9.36	11.08
		Fine	43.8	85.3	162.9^{a}	228.0	300.9
		Coarse	41.6	81.9	155.8^{b}	218.2	303.8
	SEM		1.32	1.77	2.19	5.92	7.01
Water to feed ratio (g/g)							
	RSM-0%		1.60	1.86	1.84	1.73	1.62^{b}
	RSM-25%		1.59	1.93	1.77	1.78	1.63^{ab}
	RSM-50%		1.38	1.95	1.80	1.67	1.71^{ab}
	RSM-75%		1.55	1.96	1.79	1.80	1.82^{a}
	RSM-100%		1.60	1.93	1.87	1.75	1.82^{a}
	SEM		0.08	0.07	0.04	0.04	0.06
		Fine	1.56	1.98	1.88^{a}	1.85^{a}	1.66
		Coarse	1.50	1.87	1.76^{b}	1.65^{b}	1.77
	SEM		0.06	0.04	0.02	0.03	0.05

$^{a-d}$ Means without a common superscript within a column and a parameter significantly ($P < 0.05$) differ. [1]Each value represents the mean of 3 replicates (7 birds per replicate). [2]RSM = rapeseed meal.

Differences in BW gain between diets containing different protein sources became greater as broilers got older. The increasing level of indigestible protein resulted in a poor (P = 0.034) FCR. Coarseness of the diet, however, resulted in an improved (P = 0.009) FCR. No interactions (P > 0.05) were observed for FCR (Tables 3 and 4). Water intake was neither affected by protein source (P = 0.548) nor by diet structure (P = 0.798; Tables 3 and 4). There was, however, an interaction (P = 0.037) between diet structure and age of the birds for WI indicating that a higher WI of broilers fed the finely ground diet relative to those fed the coarse diets increased when the birds became older up to 28 d of age. Water to feed ratio was not affected (P = 0.234) by protein source. Diet structure, however, influenced (P = 0.002) WF ratio with birds fed the fine diet had a greater WF ratio with respect to those fed the coarse diet.

Digestive Tract Measurements

The effects of protein source and diet structure on the relative tissue weights of various empty GIT segments are presented in Table 5. Protein source did not affect the relative empty weights of any of the GIT segments. In contrast, diet structure had a significant effect on relative empty weights of the crop, proventriculus, gizzard and jejunum. Broilers fed the coarse diets had, on average 11.1, 23.6 and 9.7% lower relative empty weights of the crop, proventriculus and jejunum, respectively, compared to broilers fed the fine diets. Relative empty gizzard weights, however, were 15.0% greater in broilers fed the coarse diet compared to those on the fine diet.

Table 5. Effects of protein source (PS) and diet structure (ST) on mean relative weights[1] (g/100 g BW) of empty gastrointestinal segments in broilers at 34 d of age.

Effect	Crop	Proventriculus	Gizzard	Duodenum	Jejunum	Ileum	Ceca	Colon
Protein source								
RSM[2]-0%	0.40	0.64	1.47	1.09	1.49	1.14	0.41	0.19
RSM-25%	0.39	0.63	1.43	1.05	1.50	1.16	0.38	0.18
RSM-50%	0.37	0.61	1.48	1.12	1.50	1.23	0.37	0.19
RSM-75%	0.36	0.59	1.48	1.11	1.51	1.20	0.37	0.19
RSM-100%	0.36	0.59	1.56	1.07	1.55	1.19	0.34	0.20
Pooled SE	0.03	0.03	0.06	0.04	0.05	0.03	0.03	0.03
Structure								
Fine	0.40[a]	0.68[a]	1.36[b]	1.11	1.58[a]	1.20	0.39	0.19
Coarse	0.36[b]	0.55[b]	1.60[a]	1.07	1.44[b]	1.16	0.37	0.19
Pooled SE	0.01	0.02	0.03	0.03	0.03	0.02	0.02	0.01
P-value								
PS	0.209	0.581	0.325	0.747	0.922	0.347	0.445	0.144
ST	0.005	0.001	< 0.001	0.229	0.003	0.120	0.273	0.673
PS × ST	0.332	0.677	0.083	0.988	0.110	0.837	0.927	0.335

[a-b] Means without a common superscript within a column and main effect significantly (P < 0.05) differ. [1]Each value represents the mean of 3 replicates (6 birds per replicate). [2]RSM = rapeseed meal.

The results of duodenal histology are shown in Table 6. Villus heights, crypt depths and villus height to crypt depth ratio (VCR) were significantly influenced by diet structure and protein source. There was a significant linear decrease in villus heights

and increase in crypt depths with the increase in RSM in the diet. This linear decrease in villus height was greater in broilers fed the fine diets relative to those receiving the coarse diets (Figure 3; panel A). Similarly, a linear increase in crypt depths was found in broilers fed the fine diets with increasing concentration of dietary RSM (Figure 3; panel B). Crypt depths, however, remained unaffected in broilers fed the coarse diets, despite the increasing dietary concentration of RSM, indicating a clear protein source × diet structure interaction ($P = 0.017$; Table 6). Crypt depths were, on average, 27.2% lower in broilers fed the coarse diets compared with those receiving the fine diets. An interaction ($P = 0.003$) was observed for VCR between protein source and diet structure, showing that VCR remained unaffected in broilers fed the coarse diets with an increasing level of RSM. On the contrary, VCR decreased in broilers fed the fine diets with an increasing level of RSM. Broilers fed the coarse diets had, on average a 17.5 and 33.2% greater villus heights and VCR, respectively, compared to those fed the fine diets.

Table 6. Effects of protein source (PS) and diet structure (ST) on villus height (μm), crypt depth (μm) and villus height to crypt depth ratio (VCR) in the duodenum of broilers at 34 d of age.

Effects	Villus height	Crypt depth	VCR
Protein source			
RSM[1]-0%	1782[a]	236.7[b]	7.6[a]
RSM-25%	1719[ab]	250.3[ab]	6.9[ab]
RSM-50%	1705[ab]	274.2[a]	6.4[bc]
RSM-75%	1610[b]	269.5[a]	6.1[c]
RSM-100%	1574[b]	274.1[a]	5.9[c]
Pooled SE	53.0	9.7	0.27
Structure			
Fine	1517[b]	292.2[a]	5.36[b]
Coarse	1840[a]	229.7[b]	8.06[a]
Pooled SE	36.0	7.0	0.20
P-value			
PS	0.042	0.021	0.001
ST	< 0.001	< 0.001	< 0.001
PS × ST	0.476	0.017	0.003

[a-c] Means without a common superscript within a column and main effect significantly ($P < 0.05$) differ. [1]RSM = rapeseed meal.

Digesta Characteristics

Gizzard pH remained unaffected ($P = 0.702$) by protein source. Diet structure, however, influenced gizzard pH ($P < 0.001$) with broilers fed the coarse diets having a lower pH compared to those fed the fine diets (Table 7). Cecal pH was neither influenced by protein source ($P = 0.454$) nor by diet structure ($P = 0.104$). Cecal ammonia contents were decreased ($P < 0.001$) with the increase in RSM. Broilers receiving RSM-0% had, on average 15, 11, 25 and 34% higher ammonia concentrations in cecal digesta compared to those fed RSM-25, 50, 75 and 100%. Cecal ammonia concentration was not altered ($P = 0.750$) by diet structure. The

percentage BCFA contents in cecal digesta tended to increase (P = 0.067) with an increase in RSM in the diet and it was significantly altered by diet structure (P = 0.027), mainly due to changes in isovaleric acid (Table 8).

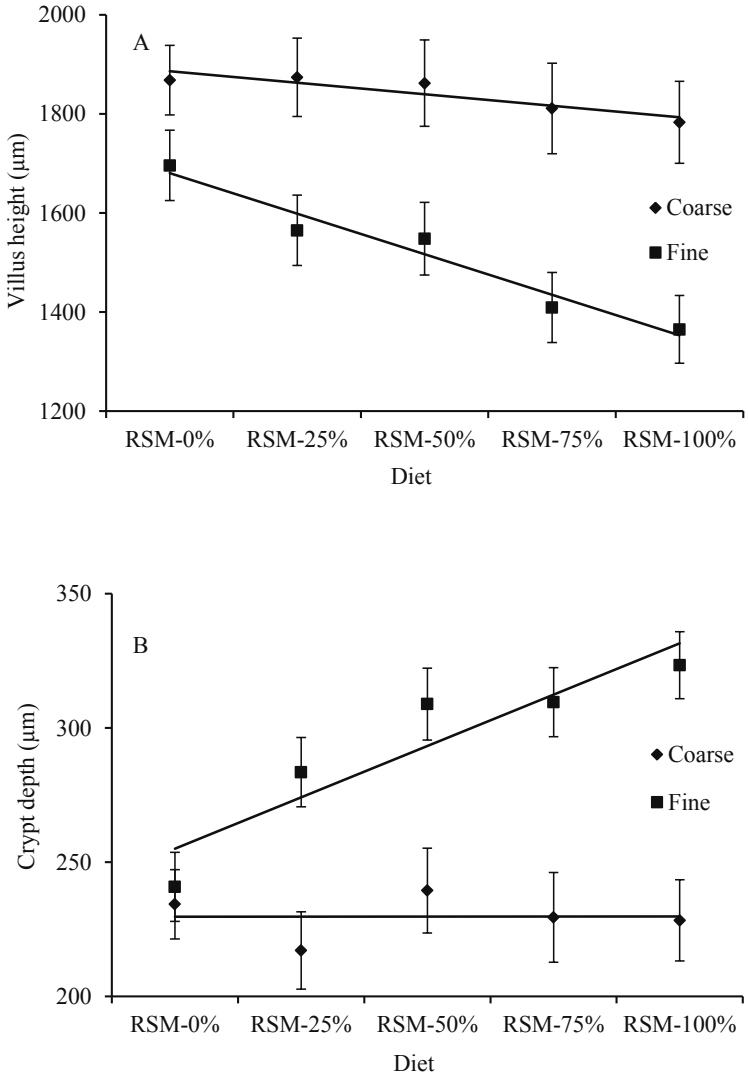

Figure 3. Effects of protein source and diet structure on duodenal villus heights (µm; panel A) and crypt depth (µm; panel B) in broilers fed different levels of rapeseed meal (RSM) diets.
The vertical bars represent standard error of means.

The total cecal biogenic amine concentration was greater (P = 0.032) in broilers fed RSM-0% compared to those fed RSM-100% (Table 9). Amongst the cecal biogenic amines, tyramine concentration decreased (P = 0.021) in broilers with increasing level

of RSM. Birds fed the coarse diets tended to have a decreased (P = 0.052) concentration of total cecal biogenic amines compared to those fed the fine diets. Diet structure also influenced (P < 0.05) the concentration of individual biogenic amines such as histamine, cadaverine and tyramine (Table 9). Broilers consuming the coarse diets had, on average a 37, 48, and 63% lower concentration of histamine, cadaverine and tyramine, respectively, in the large intestinal chyme compared with broilers fed the fine diets.

Table 7. Effects of protein source (PS) and diet structure (ST) on gizzard and cecal digesta characteristics[1] in broilers at 34 d of age.

Effects	Gizzard pH	Cecal pH	NH_3 (g/kg-DM)
Protein source			
RSM[2]-0%	3.67	6.28	5.38[a]
RSM-25%	3.54	6.22	4.60[bc]
RSM-50%	3.62	6.27	4.79[ab]
RSM-75%	3.55	6.06	4.02[cd]
RSM-100%	3.41	6.14	3.55[d]
Pooled SE	0.13	0.10	0.21
Structure			
Fine	3.88[a]	6.12	4.50
Coarse	3.24[b]	6.27	4.40
Pooled SE	0.08	0.06	0.13
P-value			
PS	0.702	0.454	< 0.001
ST	0.001	0.104	0.750
PS × ST	0.775	0.242	0.286

[a-d] Means without a common superscript within a column and main effect significantly (P < 0.05) differ. [1]Each value represents the mean of 3 replicates (6 birds per replicate). [2]RSM = rapeseed meal.

Discussion

The present study was designed to investigate the impact of indigestible protein level and particle sizes of a diet on performance, gut morphology and fermentation characteristics in broilers. It was hypothesized that performance on a lesser digestible protein source can be improved by feeding a coarsely ground diet instead of a finely ground diet. Diets were formulated to contain identical amounts of the digestible Lys, Met+Cys and Thr, and similar amounts of digestible protein, NSP and ME during the starter and grower phase. Indigestible protein increased with increasing levels of RSM. Digestive tract development, gut morphology, and cecal digesta characteristics were studied as explanatory variables. High villus heights and low crypt depths were used as indicators of intestinal health.

The observed lower FI reduced BW gain and poor FCR with increasing dietary RSM contents is in accordance with expectations and confirm earlier findings (Qaisrani et al., 2014, submitted). Poor performance in broilers which received high levels of RSM has been found also in other studies (McNeill et al., 2004, Tripathi and Mishra, 2007; Saleem, 2013). Protein fermentation in the GIT leads to the formation of

products such as BCFA, biogenic amines and ammonia (Macfarlane et al., 1992). Contrary to expectations, broilers fed the SBM diets had higher concentrations of these compounds than those fed the RSM diets (Tables 7, 8 and 9). The greater concentration of total VFA (including BCFA) in cecal digesta of broilers fed lower levels of RSM (and thus higher levels of SBM) may be due to a higher dietary concentration of fermentable oligosaccharides in SBM. Soybean meal contains approximately 15% oligosaccharides on a DM basis (Kocher et al., 2002) which are well fermented in broilers and may result in greater cecal VFA concentrations (Faber et al., 2012). Therefore, the poor performance on RSM diets may not be attributed to protein fermentation as such. There may be other factors which may have resulted in poor performance of the broilers consuming high levels of RSM. For instance, greater levels of NSP in RSM may increase the digesta viscosity and decrease the digestibility of some nutrients such as protein, starch and lipids (Smits et al., 1997). This poor digestion may result in less absorption and ultimately reduced performance of broilers fed high levels of RSM (Saleem, 2013). Another explanation of poor performance in RSM consuming birds may be due to the presence of antinutritional factors such as glucosinolates, tannins, phytic acid, and sinapine (Khajali and Slominski, 2012).

Glucosinolate contents of 8 μmol/g of diet resulted in severe depression of growth in broilers (Tripathi and Mishra, 2007). Presence of glucosinolates, phenolic compounds cause bitter taste and has been reported to reduce FI in broilers (Zeb, 1998). Similarly, tannins (1.9 to 6.2%) and phytic acid may affect protein digestion due to formation of complexes with protein and proteolytic enzymes in the GIT (Khajali and Slominski, 2012). This poor digestion of nutrients, and, as a consequence, poor performance of RSM fed birds may be related to lower villus heights and greater crypt depths (Table 6). High levels of digestible nutrients in the small intestine are associated with greater villi (Yamauchi, 2007). Performance data also support the presence of greater villi in broilers consuming low levels of RSM. Furthermore, high digesta viscosity increases the retention time of digesta in the GIT leading to more microbial activities and may lead to an increase in endogenous losses (Smits et al., 1997). This increases the amino acid requirements. With high inclusion levels of RSM (> 20%), the extra energy needed for gut wall renewal and for liver metabolic activities can result in reduced performance (Woyengo et al., 2011). Less protein, therefore, will be available for growth.

We observed poor pellet durability in the coarse diets. However, FI, BW and FCR were not negatively affected in broilers consuming coarse diets. To the contrary, broilers on coarse diets performed better than those consuming the fine diets as has been reported in other studies for broilers (Jacobs et al., 2010; Pacheco et al., 2013; Liu et al., 2013).

Table 8. Effects of protein source (PS) and diet structure (ST) on cecal volatile fatty acids (VFA) concentrations[1] (mmol/kg DM) in broilers at 34 d of age.

Effects	VFA	Acetic acid[2]	Propionic acid[2]	Butyric acid[2]	Valeric acid[2]	Total BCFA[2,3]	Isobutyric acid[2]	Isovaleric acid[2]
Protein source								
RSM[4]-0%	209.1^{a}	68.70^{b}	5.64	22.52^{a}	1.34^{bc}	1.80	0.75	1.05^{a}
RSM-25%	220.4^{a}	71.70^{ab}	5.41	20.24^{b}	1.15^{c}	1.50	0.59	0.92^{a}
RSM-50%	176.2^{b}	71.60^{ab}	6.43	19.04^{b}	1.24^{bc}	1.69	0.75	0.94^{a}
RSM-75%	168.9^{b}	72.98^{a}	4.05	20.00^{b}	1.45^{ab}	1.52	0.74	0.78^{ab}
RSM-100%	125.9^{c}	73.90^{a}	4.33	19.01^{b}	1.65^{a}	1.10	0.59	0.52^{b}
Pooled SE	8.34	1.1	0.81	0.75	0.08	0.18	0.08	0.12
Structure								
Fine	175.8	71.7	5.20	20.02	1.38	1.71^{a}	0.73	0.97^{a}
Coarse	184.5	71.9	5.29	20.15	1.35	1.30^{b}	0.59	0.71^{b}
Pooled SE	5.27	0.68	0.51	0.47	0.05	0.12	0.05	0.07
P-value								
PS	0.001	0.030	0.273	0.013	0.004	0.067	0.135	0.049
ST	0.257	0.829	0.965	0.780	0.679	0.027	0.092	0.021
PS × ST	0.723	0.828	0.531	0.078	0.075	0.293	0.312	0.337

[a-c] Means without a common superscript within a column and main effect significantly ($P < 0.05$) differ. [1]Each value represents the mean of 3 replicates (6 birds per replicate). [2]Percentage of total VFA. [3]BCFA = branched chain fatty acid (sum of isobutyric and isovaleric acids). [4]RSM = rapeseed meal.

Table 9. Effects of protein source (PS) and diet structure (ST) on total and individual biogenic amine concentrations (mmol/kg DM) in the cecal digesta of broilers[1] at 34 d of age.

Effects	Histamine	Putrescine	Cadaverine	Spermidine	Spermine	Tyramine	Total
Protein source							
RSM[2]-0%	0.66	1.68	2.07	22.67	0.80	4.15[a]	32.06[a]
RSM-25%	0.80	1.35	1.52	22.55	0.82	1.60[b]	28.63[ab]
RSM-50%	0.72	3.04	2.56	23.32	1.14	1.60[b]	32.38[a]
RSM-75%	0.86	1.34	2.55	20.29	1.01	1.37[b]	27.42[ab]
RSM-100%	0.97	1.04	0.55	17.84	1.11	1.20[b]	22.70[b]
Pooled SE	0.16	0.57	0.61	1.57	0.14	0.64	2.18
Structure							
Fine	0.99[a]	1.98	2.43[a]	21.21	0.98	2.88[a]	30.49[a]
Coarse	0.62[b]	1.40	1.27[b]	21.46	0.97	1.08[b]	26.79[b]
Pooled SE	0.10	0.36	0.38	0.99	0.08	0.40	1.38
P-value							
PS	0.670	0.148	0.146	0.123	0.305	0.021	0.032
ST	0.017	0.265	0.043	0.864	0.955	0.005	0.052
PS × ST	0.795	0.138	0.238	0.408	0.786	0.548	0.252

[a-b] Means without a common superscript within a column and main effect significantly ($P < 0.05$) differ. [1]Each value represents the mean of 3 replicates (6 birds per replicate). [2]RSM = rapeseed meal.

Poor pellet durability in coarse diets is in agreement with the findings of Svihus et al. (2004) and Amerah et al. (2008), and this inverse relation between coarseness of the diet and pellet durability has already been reported by Angulo et al. (1996). The inclusion of large particles in pellets weakens the feed structure probably due to less contact points between the particles. The significantly improved FCR in broilers fed the coarse diet may be explained by a better functioning gizzard which optimizes gut motility and especially gastro-duodenal reflux. The lower rate of passage with coarse particles may, furthermore, result in an extended time available for mixing of feed particles with enzymes. As a result optimal digestion and availability of the nutrients to the birds may be reached. Chyme reflux between proventriculus and gizzard may explain the low gizzard pH in broilers fed the coarse diets compared with those on the fine diets. This low pH improves denaturation and hydrolysis of protein by pancreatic enzymes and this may enhance protein digestion (Pacheco et al., 2013). This improved protein digestion may reduce the undigested dietary protein reaching the hindgut, which is in agreement with the cecal fermentation indices data (Tables 8 and 9). The significant low concentration of cecal BCFA, biogenic amines, and numerically lower ammonia in broilers fed the coarse diets may be attributed to a better ileal protein digestion. The positive effect of coarseness of a diet on protein digestibility in broilers has been previously reported in the literature (Pacheco et al., 2013; Liu et al., 2013). The greater cecal concentration of histamine, cadaverine and tyramine in broilers fed the fine diets suggests a poorer digestibility of protein in the foregut and greater cecal fermentation of the amino acids histidine, lysine and tyrosine, respectively.

The significant greater villus height and VCR in broilers fed the coarse diets compared with those fed the fine diets, is in agreement with the findings of Zang et al. (2009). These authors reported significantly greater villus height (1451 vs. 1353 μm) and higher VCR (10.93 vs. 10.53) in coarse and fine diet fed broilers, respectively. The greater villus height, VCR and less deeper crypts in broilers fed the coarse diets may have stimulated nutrient digestion and absorption because of an increased surface area. Deeper crypts, furthermore, are an indication of more cell division at crypts and of increased tissue renewal of the villus resulting in more energy consumption in the gut wall itself. The greater relative empty weights of the crop and proventriculus in the broilers fed the fine diets can be attributed to a longer residence time of the diets with fine particles in these organs. In the broilers fed the coarse diets, the phenomenon of over-eating is observed less frequently (Amerah and Ravindran, 2009). The effects of coarse structures on GIT development and relative weight of the gizzard, observed here, are in agreement with results of previous studies (Gabriel et al., 2003; Jacobs et al., 2010; Pacheco et al., 2013). These effects may be due to the stimulatory properties of larger particles, which lead to the mechanical stimulation of the gizzard and will

result in heavier gizzards (Jacobs et al., 2010). The low relative weights of the jejunum in the broilers fed the coarse diets suggest that the chyme in these broilers may require less peristaltic activity from the jejunum (Duke, 1986). The intestines of broilers fed coarse particles may, therefore, have more effective action of enzymes to the chyme (Gabriel et al., 2003).

The results of the present study indicate that higher levels of indigestible dietary protein result in poorer performance and impaired gut morphology. Inclusion of dietary coarse particles improved gut development. Heavy gizzards, enhanced gut morphology, greater BW gain, and improved FCR as well as significantly lower concentrations of hindgut fermentation products (BCFA and biogenic amines) were found in broilers fed coarse diets. It can be concluded that feeding coarse particles may improve the performance of broilers even with a poorly digestible protein source. The negative effects of moderate inclusion of indigestible protein can thus be counteracted to some extend by coarse grinding the diet.

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CHAPTER 5

Diet structure, butyric acid, and fermentable energy influence growth performance, gut morphology and cecal fermentation characteristics in broilers

S.N. Qaisrani[1], M.M. van Krimpen[2], R.P. Kwakkel[1], M.W.A. Verstegen[1], and W.H. Hendriks[1]

[1]Animal Nutrition Group, Wageningen University, PO Box 338, NL-6700 AH Wageningen, The Netherlands

[2]Wageningen UR Livestock Research, PO Box 65, NL-8200 AB Lelystad, The Netherlands

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Abstract

An experiment with 288 male (Ross 308) 1-d-old broilers was conducted to test the hypothesis that a coarse diet supplemented with butyric acid (BA) and fermentable energy (FE) improves performance of broilers on a poorly digestible protein source. The interaction effects of diet structure (fine vs. coarse), FE (with vs. without) and BA supplementation (with vs. without) in a poorly digestible diet based on rapeseed meal (RSM) were tested in a factorial arrangement of 8 (2 × 2 × 2) dietary treatments. Coarseness of the diet affected feed intake (FI) ($P < 0.001$), BW gain ($P = 0.001$) and feed conversion ratio (FCR) ($P = 0.001$) positively. Broilers fed the coarse diets had, on average 14% heavier gizzard, and 11, 7, 5 and 6% lower relative empty weights of the crop, duodenum, jejunum and ileum, respectively, compared with those fed the fine diets. Dietary coarseness resulted in, on average a 6% greater ileal protein digestibility, 20% lower gizzard pH, 19% greater villus heights, 18% lower crypt depths, and 23% reduced cecal branched chain fatty acids (BCFA) compared with those fed the fine diets. Broilers fed BA supplemented diets had an improved ($P = 0.004$) FCR and decreased crypt depths ($P < 0.001$) compared with those fed the diets without BA. Fermentable energy supplementation did not influence ($P > 0.05$) performance nor gut development and contents of total BCFA and total biogenic amines in the cecal digesta. Supplementation with FE, however, decreased ($P = 0.002$) the cecal concentration of spermine by approximately 31% compared with those fed diets without FE. In conclusion, feeding a coarse diet supplemented with BA improve performance of broilers if they are fed a diet containing a poorly digestible protein source. The negative effects of a low digestible protein source can thus be partly counterbalanced by coarse grinding and BA supplementation in the diet.

Key words: diet structure, butyric acid, cecal fermentation, broilers, fermentable energy.

Introduction

Feeding diets with a lower protein digestibility will result in more protein reaching the hindgut (Wiernusz et al., 1995) potentially resulting in increased protein fermentation. The latter results in the production of ammonia, amines, phenols, indoles, sulphide, branched-chain fatty acids (BCFA), volatile fatty acids (VFA), and gases (hydrogen, carbon dioxide, and methane), as well as intermediate products, such as lactate and succinate (Macfarlane et al., 1992). Some of these compounds may have detrimental effects on health and production of the host (Bikker et al., 2007).

Feeding coarse particles may be an effective nutritional strategy to enhance ileal protein digestibility in poultry. Coarse particles are ground into finer particles in the gizzard before entering the duodenum, which prolongs retention time in the gizzard (Amerah et al., 2007). This extended retention time enhances the effectiveness of hydrochloric acid and digestive enzymes that are secreted from the proventriculus into the gizzard (González-Alvarado et al., 2008). It has been shown to have positive effects on gizzard function and development resulting in improved protein denaturation and digestion (Liu et al., 2013; Pacheco et al., 2013). It can be assumed, therefore, that diet structure can decrease the amount of undigested protein reaching the hindgut and reduce the potential detrimental effects of hindgut protein fermentation.

Organic acids, such as butyric acid (BA), supplemented as a feed additive may be another strategy to improve ileal protein digestibility of low digestible protein sources. Butyric acid is a readily available energy source for gut epithelial cells and stimulates their multiplication and differentiation (Dalmasso et al., 2008) consequently improving feed efficiency (Adil et al., 2010). Butyric acid, moreover, stimulates the development of gut associated lymphoid tissue, functional development of the gastrointestinal tract (GIT) in terms of digestion and absorption of nutrients, and increases peptide production in the distal GIT (Cox et al., 2009). It may also reduce hindgut protein fermentation, because it suppresses protein fermenting microbiota, especially the gram negative population in broilers (Gunal et al., 2006) by disrupting their energy metabolism (Ricke, 2003) and decreasing hindgut pH. In addition, BA decreases bacterial colonization of the intestinal wall (Langhout et al., 1999), and as a consequence, less toxic compounds are produced by pathogenic microbiota, resulting in less damage to the epithelial cells (Antongiovanni et al., 2009).

The provision of sufficient fermentable carbohydrates shifts microbial proteolytic fermentation into more carbohydrate fermentation (Awati et al., 2006), because cecal resident microbiota prefer carbohydrates as their main energy source (Rehman et al., 2008). As such supplementation of diets with fermentable carbohydrates, therefore,

could decrease hindgut protein fermentation, improve gut health and promote beneficial microbiota, all which may affect growth performance positively.

It is hypothesized, therefore, that coarse particles, combined with butyric acid and/or fermentable energy supplementation to a diet containing a poorly digestible protein source will counterbalance the negative consequences on overall performance of broilers. The aim of this study was to investigate the interacting effects of diet structure, butyric acid and fermentable energy supplementation on performance, gut morphology and cecal fermentation characteristics in broilers.

Materials and Methods

Animal Ethics

Experimental procedures were in accordance with the Wageningen University and the Netherlands Animal Experimental Committee guidelines and code of practice. Ethical approval was granted before the conduct of the study.

Birds Husbandry and Diets

In total, 288 male (Ross 308) 1-d-old broilers were housed in three identical, environmentally controlled rooms, individually weighed, steel wing-banded and randomly assigned to 36 floor pens (8 birds per pen). Each pen (1.15 × 1.75 × 0.80 m) (L × W × H) had a perch and three drinking nipples with a cup underneath connected to a water tank of 10 L capacity. Pens were separated by solid walls to prevent contact between broilers from different treatment groups. Soft Cells®, fibreless bedding was used as a litter material. Each dietary treatment was randomly applied to 4 replicate pens and broilers were allowed *ad libitum* access to feed and water. For the first three d, broilers were exposed to a 23L:1D cycle and reduced thereafter to 16L:8D with approximately 20 lx intensity at bird's level throughout the experiment. The temperature was set at 32°C for the first three d and subsequently gradually decreased to a constant value of 22°C in wk 4 and maintained until the end of the experiment.

A 2 × 2 × 2 factorial arrangement of 8 treatments was employed to test the interaction effects of particle size (fine or coarse), FE level (with or without) and BA supplementation level (with or without) in a rapeseed meal (RSM) based diets. A finely ground positive control diet based on soybean meal (SBM) as the main protein source, was added as an additional treatment to the 8 treatments. Each diet was processed with either a hammer mill with an opening screen of 3.0 mm (fine diets) or a roller mill with a roller distance of 1.6 mm (coarse diets). The high fermentable energy level was obtained by substituting maize starch by potato starch. Butyric acid, as sodium butyrate (Adimix®, INVE Nutri-Ad, Kasterlee, Belgium), was mixed with the feed in a dosage of 2 kg/ton. All experimental diets (Table 1) were formulated to meet or exceed the recommendations for broiler diets (CVB, 2007). The diets were offered

as 2.5 mm pellets for the starter and 4 mm for the grower diet. The diets did not contain antimicrobial growth promoters.

Table 1. Dietary ingredients and calculated nutrients of the diets (g/kg as-fed basis).

Items	Basal RSM diet	FE-RSM diet	SBM diet
Maize starch	92.1	32.1	117.5
Maize	300.0	300.0	300.0
Wheat	100.0	100.0	100.0
Fish meal	63.0	63.0	25.0
Soybean meal	0.0	0.0	350.0
Rapeseed meal	350.0	350.0	0.0
Potato protein	30.0	30.0	10.0
Potato starch dry	0.0	50.0	0.0
Vegetable oil	40.0	50.0	30.0
Binding material	0.0	0.0	30.0
Premix[1]	5.0	5.0	5.0
Limestone	8.0	8.0	12.5
Mono-calcium phosphate	7.0	7.0	12.0
Salt	1.2	1.2	2.3
Sodium bicarbonate	2.3	2.3	2.1
DL-Methionine	0.2	0.2	2.0
L-Threonine	0.0	0.0	0.9
L-Valine	0.0	0.0	0.7
L-Arginine	1.2	1.2	0.0
Total	1000.0	1000.0	1000.0
Calculated contents			
ME (Kcal/kg)	2681.0	2680.0	2671.0
CP (Analyzed)	225.1	224.9	215.2
Digestible protein[2]	178.9	178.9	184.6
Indigestible protein	46.2	46.0	30.6
NSP[3]	177.5	182.4	158.5
Crude fiber	51.1	51.0	35.3
Digestible Lys	10.6	10.6	10.6
Digestible Met + Cys	7.8	7.8	7.7
Digestible Thr	7.8	7.8	7.8
Digestible Trp	2.2	2.2	2.2

RSM = rapeseed meal, FE = fermentable energy, SBM = soybean meal.
[1]Premix composition: 12,000 IE retinol, 2,400 IE cholecalciferol, 50 mg dl-a-tocopherol, 1.5 mg menadione, 2.0 mg thiamine, 7.5 mg riboflavin, 3.5 mg pyridoxine, 20 mcg cyanocobalamins, 35 mg niacin, 12 mg D-pantothenic acid, 460 mg choline chloride, 1.0 mg folic acid, 0.2 mg biotin; 80 mg iron, 12 mg copper, 85 mg manganese, 60 mg zinc, 0.40 mg cobalt, 0.8 mg iodine, 0.1 mg selenium, 125 mg anti-oxidant mixture. [2]Based on data from CVB (2007). [3]NSP = Non-starch polysaccharides, calculated by subtracting the CP, fat, starch, sugar, and ash content from the DM content.

Wet Sieve Analysis

Particle size distribution of the diets was analyzed using the wet sieve method as described by Goelema et al. (1999) with minor modifications. Weighed samples of a diet were each subdivided into 2 subsamples. One subsample was dried overnight at 70°C in an oven until constant weight to determine the air DM content, whereas the other subsample was suspended in 500 ml of water for 45 min to ensure adequate hydration, before being washed through a set of sieves with decreasing mash size 2.5, 1.25, 0.63, 0.315, 0.160 and 0.071 mm. The contents of each sieve were subsequently collected and dried overnight at 70°C in an oven to constant air DM weight. The dried weights of particles retained by each sieve and of the fines remaining in the bottom

pan were expressed as percentages of total air DM recovered. Average particle size of the diets was calculated as (fraction < 0.071 mm × 0.035) + (fraction 0.071 - 0.16 mm × 0.115) + (fraction 0.16 - 0.315 mm × 0.237) + (fraction 0.315 - 0.630 mm × 0.472) + (fraction 0.630 - 1.25 mm × 0.940) + (fraction 1.25 - 2.50 mm × 1.65) + (fraction > 2.50 mm × 3.50)/100. Particle size distribution of the fine and coarse diets is shown in Figure 1. For the fine and coarse RSM diets, the average particle size was 190 and 368 µm, respectively, whereas for the SBM (control) diet it was 279 µm.

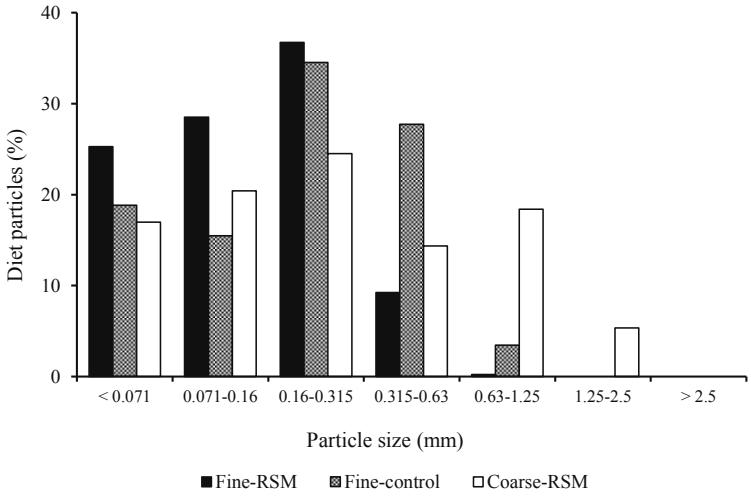

Figure 1. Particle size distribution of the control soybean meal (SBM) and experimental rapeseed meal (RSM) coarse and fine diets.

Traits Measured

Feed intake (FI), water intake (WI) and BW gain per pen were recorded at 7, 14, 21, 28 and 34 d of age, whereas mortality was recorded daily. Feed conversion ratio (FCR) was calculated by dividing total FI by weight gain of live plus dead birds. At the end of the experiment (d 35 and 36), 6 of the 8 birds from each replicate pen were randomly selected and euthanized by an intravenous T-61 injection, where after the abdominal cavity was opened.

Pellet Durability

Pellet durability was determined in a Holmen Pellet Tester (New Holmen Pellet Tester, TekPro Ltd., Norfolk, UK) using the method described by Svihus et al. (2004). The pellet samples (100 g) were circulated pneumatically through a closed pipe for 30 s before being passed through a 3 mm sieve. The pellet durability index (PDI) was calculated as the relative proportion of pellets retained on the 3 mm sieve. The PDI

values varied between 33.5 and 62.7% for the fine and 17.8 and 20.3% for the coarse diets (Figure 2).

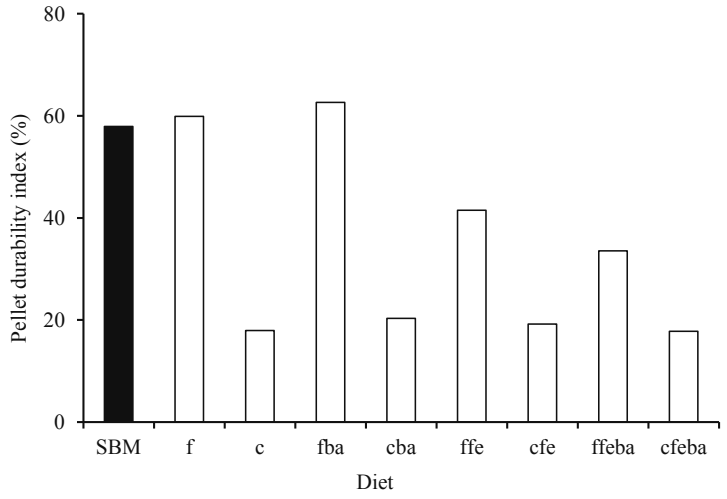

Figure 2. Pellet durability index of the control and experimental diets.
SBM = soybean meal diet, f = fine diets, c = coarse diets, fba = fine diet supplemented with butyric acid, cba = coarse diet supplemented with butyric acid, ffe = fine diet supplemented with fermentable energy, cfe = coarse diet supplemented with fermentable energy, ffeba = fine diet supplemented with fermentable energy and butyric acid, cfeba = coarse diet supplemented with fermentable energy and butyric acid.

The different segments of the GIT, i.e. the crop, proventriculus, gizzard, duodenum (from pyloric junction to pancreo-billiary duct), jejunum (from pancreo-billiary duct to Meckle's diverticulum), ileum (from Meckle's diverticulum to ileo-cecal junction), cecum (from ostium) and colon were segmented. The digesta contents from each segment were immediately removed by gentle squeezing and the empty segments were weighed. The terminal ileum, defined as the segment of the GIT from 15 to 2 cm anterior to the ileo-cecal junction of birds from each pen was fixed to avoid contamination. The ileal digesta was immediately collected from birds within a pen by gently squeezing into a plastic container, pooled, and frozen at -20°C in airtight containers till further chemical analysis. Ceca content of the six birds in a pen were quantitatively pooled and mixed. The pH was determined using a calibrated pH meter before the samples were freeze dried at -20°C. The samples were frozen pending volatile fatty acids (VFA), biogenic amines and ammonia analyses. During the dissection days, all birds had access to the feed until the moment of euthanization.

Tissue Collection and Histological Measurements

For intestinal morphological examination, duodenal samples (approximately 2 cm in length) from the middle of the duodenum were collected, rinsed with cold physiological saline (0.9% saline) and immediately placed in Bouin's fluid. The samples, thereafter, were transferred into 70% ethanol within 24 h, embedded in

paraffin and sectioned at 5 µm thickness. For histological examination, six cross-sections per bird were processed using standard haematoxylin and eosin methods as described by Owusu-Asiedu et al. (2002). Villus height (the distance from the apex of the villus to the junction of the villus and crypt) and crypt depth (the distance from the junction to the basement membrane of the epithelial cells at the bottom of the crypt) were measured on 10 intact, well-oriented villi (from the 2 cm in the middle of the duodenum) per bird using a compound light microscope equipped with a video camera.

Chemical Analysis

Dry matter, organic matter and N contents in the experimental diets were measured according to the standard methods (AOAC International, 2006). Ammonia in cecal digesta was measured by the indoles phenol-blue method (Novozamsky et al., 1974). The samples were deprotonated by adding 10% (w/v) trichloroacetic acid solution followed by centrifugation. The ammonium was transformed by phenol and hypochlorite in an alkaline solution into blue colored indoles phenol-blue by the Berthelot reaction. The ammonia content was measured spectroscopically at 623 nm.

For determination of VFAs, 5 g of cecal samples and 5 ml 0.1 M phosphoric acid were shaken at 100 rpm before being centrifuged (7000 g) for 10 min. Residues were collected and the supernatants again were centrifuged (20817 g) for 10 min. Afterwards, 600 µL of the supernatant was transferred to a crimp vial and mixed with 600 µL of phosphoric acid containing isocapronic acid (2.29 g/L concentration) as an internal standard. Volatile fatty acids were separated by gas chromatography using an EM - 1000 (30 m × 0.53 mm) column from Alltech (Deerfield, IL, USA) and helium was used as the mobile phase with detection by fluorescent infrared detector. Quantification of VFAs was based on a chemical standard solution (Merck) after internal standard correction.

Biogenic amines (histamine, putrescine, cadaverine, spermidine, spermine, and tyramine) in cecal digesta were determined by the method described by Meyer et al. (2013) with slight modifications. Briefly, 50 mg of freeze-dried bullet mill sample was added with 20 mg of sulphosalicylic acid and 1 ml of 0.1 N hydrochloric acid. The samples were shaken for 15 min at a speed of 2000 g. Thereafter, the samples were placed in an ice-bath for 15 min, vortexed and transferred to a 1.5 ml Eppendorf tube. The supernatants were taken out and the tubes were centrifuged at 20000 g (14000 rpm) for 5 min, thereafter filtered over a 0.2 µm filter. A 20 µl filtered sample was analyzed on a column (Cation separation column LCAK17/K 4.6 × 30 mm) eluted with a combination of 0.4 N potassium citrate buffer (pH 5.75) and 2.5 N potassium citrate buffer (pH 8.4) followed by a post column ortho-phthalaldehyde fluorescent detection at 425 nm.

Protease Activity

The extraction and dilution was conducted following the method described by Khoa (2007), with analysis performed following the method described by OJEC (1972). Briefly, the proventriculus was weighed, thawed and placed in a 250 ml beaker. Thereafter, 3 ml phosphate buffer (PBS, pH 7) per gram of proventriculus tissue was added, homogenized with ultra turrax at 0°C (place the 250 ml beaker containing the organ in a 1 L beaker filled with ice and water) and was centrifuged at 12400 g for 60 min at 4°C. The supernatants were extracted with a pasteur pipette and transferred into a 2.5 ml eppendorf cup. Thereafter, 1 ml of the supernatant was transferred into a plastic tube and 4 ml of PBS was added, mixed with a vortex and stored at -20°C. Upon chemical analysis, diluted proventriculus extract was brought to room temperature and 100 µl was mixed with 4 ml of ice-cold acidified hemoglobin solution and 6.8 ml of trichloroacetic acid. The contents were filtered on Whatman paper filter and 2.5 ml of the filtrate was pipetted in a 12 ml tube. Five ml of sodium hydroxide solution and 3 ml of dilute Folin-Ciocalteu reagent was added and the tube vortexed. After 5-10 min, the absorbance was measured at 750 nm against water and data expressed as the amount (µmole) of tyrosine released per min per g of tissue.

Apparent Ileal Digestibility of Crude Protein

Crude protein contents in the diet and ileal digesta were calculated as N × 6.25 with nitrogen (N) being measured by the Kjeldahl method with $CuSO_4$ as a catalyst (ISO 5983). Apparent ileal CP digestibility (AID) was calculated using the following equation (Stein et al., 2001):

AID = [100 - (Crude protein$_d$ / Crude protein$_f$) × (Ti$_f$ / Ti$_d$)], where crude protein$_d$ and Ti$_d$ were the concentrations of CP and titanium in the ileal digesta, respectively, and crude protein$_f$ and Ti$_f$ were the concentrations of the same dietary components in the feed, respectively, all expressed on a DM basis.

Statistical Analysis

The repeated statement within PROC MIXED of SAS (version 9.2; SAS Inst. Inc., Cary, NC) was used for data analysis. The following statistical model was used for the factorial analysis of the eight treatments:

$$Y_{ijkl} = \mu + ST_i + BA_j + FE_k + ST_i \times BA_j + ST_i \times FE_k + BA_j \times FE_k + ST_i \times BA_j \times FE_k + e_{ijkl}$$

where Y_{ijkl} is the measured response, µ overall mean effect, ST_i the i^{th} fixed diet structure effect (i = fine or coarse), BA_j the j^{th} fixed BA supplementation effect (j = with or without BA), FE_k the k^{th} fixed FE supplementation effect (k = with or without FE), $ST_i \times BA_j$ the interaction between diet structure and BA, $ST_i \times FE_k$ the interaction between diet structure and FE, $BA_j \times FE_k$ the interaction between BA and FE supplementation, whereas $ST_i \times BA_j \times FE_k$ the interaction between diet structure, BA

and FE supplementation, e_{ijkl} ~ NID (0, σ2e). Differences were considered significant at a probability level of 5%. The interactions were ignored, if the main effect for a particular trait was found non-significant. Analysis of the data showed that there were no three way interactions except for FI. It was decided, therefore, not to report three way interactions in the results section. The SBM diet was added as the 9th treatment, used as a positive control group and disregarded from statistical analysis.

Results

Bird Performance

The effects of the dietary treatments on the performance of the broilers are presented in Table 2. Over the entire experimental period (0 to 34 d of age), broilers fed the eight experimental (RSM) diets had, on average 13, 26, 9, 15 and 3% lower FI, BW gain, FCR, WI, and WF ratio, respectively, compared with those fed the SBM diet (not statistically tested).

Broilers fed the coarse RSM diets had, on average 6% greater FI, 11% greater BW gain, 5% improved FCR, and 7% lower WF ratio over the 34 d experimental period compared with those fed the fine RSM diets. Body weight gain and FCR were affected by BA supplementation during the starter (0 to 14 d of age) as well as during the 34 d experimental period. Broilers fed BA supplemented diets showed a 7 and 4% greater BW gain and 5 and 3% improved FCR during both periods, respectively, compared with those fed the diets without BA. None of the other parameters were affected by BA supplementation. Water to feed ratio was affected by FE supplementation during the starter as well as over the entire experimental period. Broilers fed FE supplemented diets showed, on average 9 and 3% lower WF ratio during both periods, respectively, compared to those fed the diets without FE supplementation. During the 34 d experimental period, an interaction (P = 0.032) between ST × BA was observed for FI, indicating that broilers fed the coarse diets supplementation with BA increased FI, whereas in broilers fed the fine diets, FI did not increase with BA supplementation. For both periods (0 to 14 and 0 to 34 d of age), interactions between ST × BA were observed for WI (P = 0.012 and P = 0.013) and WF ratio (P = 0.003 and P = 0.003), indicating that broilers fed the coarse diets supplemented with BA had greater WI and WF ratio, whereas broilers fed the fine diets supplemented with BA, had a lower WI and WF ratio. During the entire experimental period, interactions between ST × FE were observed for FI (P = 0.009) and BW gain (P = 0.009), indicating that broilers fed the coarse diets supplemented with FE, FI and BW gain was decreased, whereas broilers fed the fine diets supplemented with FE, FI and BW gain was increased.

Table 2. Effects of diet structure (ST), butyric acid (BA) and fermentable energy (FE) supplementation on performance parameters in broilers from 0 to 14 and 0 to 34 d of age.

Effects	Parameters[1]									
	FI (g/bird/d)		BW gain (g/bird/d)		FCR (g/g)		WI (ml/bird/d)		WF ratio (ml/bird/d)	
Age (d) →	0-14	0-34	0-14	0-34	0-14	0-34	0-14	0-34	0-14	0-34
Control SBM diet[2]	38.2	117.2	31.6	84.5	1.23	1.40	97.1	224.7	2.59	1.92
Factorial analysis of the experimental RSM diets[3]										
ST: Fine										
With BA										
With FE	35.4	98.4^{c}	27.9^{abc}	65.8^{cd}	1.31^{ab}	1.50^{bc}	76.6^{cde}	176.1^{b}	2.16^{bcd}	1.79^{b}
Without FE	34.7	99.4^{c}	26.3^{cd}	63.6^{de}	1.38^{a}	1.56^{ab}	85.2^{abc}	196.2^{ab}	2.40^{abc}	1.97^{a}
Without BA										
With FE	36.4	104.1^{ab}	26.9^{cd}	65.1^{cd}	1.36^{ab}	1.60^{a}	90.9^{a}	202.3^{a}	2.46^{ab}	1.94^{ab}
Without FE	33.1	96.8^{c}	25.4^{d}	60.5^{e}	1.33^{ab}	1.60^{a}	87.5^{abc}	194.7^{ab}	2.60^{a}	2.01^{a}
ST: Coarse										
With BA										
With FE	35.7	106.2^{ab}	29.2^{ab}	72.6^{a}	1.23^{b}	1.46^{c}	75.3^{cde}	187.7^{ab}	2.08^{cd}	1.77^{c}
Without FE	35.5	107.2^{a}	29.6^{a}	71.8^{a}	1.21^{b}	1.49^{bc}	88.1^{ab}	205.6^{a}	2.48^{ab}	1.92^{b}
Without BA										
With FE	36.4	103.5^{b}	27.1^{bcd}	67.7^{bc}	1.35^{a}	1.53^{bc}	72.4^{e}	186.1^{ab}	1.96^{d}	1.82^{c}
Without FE	35.9	105.4^{ab}	26.5^{cd}	71.1^{ab}	1.36^{a}	1.48^{bc}	74.0^{de}	176.9^{b}	2.04^{d}	1.68^{d}
Pooled SE	0.86	1.19	0.78	1.19	0.04	0.02	4.35	7.37	0.11	0.07
P-value										
ST	0.114	< 0.001	0.014	0.001	0.867	0.001	0.021	0.536	0.003	0.004
BA	0.856	0.715	0.003	0.009	0.006	0.004	0.979	0.792	0.823	0.891
FE	0.063	0.354	0.145	0.223	0.812	0.597	0.126	0.318	0.013	0.020
ST × BA	0.513	0.032	0.116	0.576	0.005	0.379	0.012	0.013	0.003	0.003
ST × FE	0.174	0.009	0.210	0.009	0.602	0.269	0.455	0.859	0.768	0.352
BA × FE	0.253	0.037	0.674	0.587	0.470	0.038	0.071	0.014	0.195	0.065

[a-d] Means without a common superscript within a column significantly ($P < 0.05$) differ. [1]FI = feed intake, BW = body weight, FCR = feed conversion ratio, WI = water intake, WF ratio = water to feed ratio. [2]Control soybean meal, n = 4 replicates. [3]Experimental rapeseed meal, n = 32 replicates. For each replicate in all the treatments n = 8 birds.

Over the entire experimental period, interactions between BA × FE were observed for FI ($P = 0.037$), FCR ($P = 0.038$) and WI ($P = 0.014$), indicating that for broilers fed a BA supplemented diet, FE supplementation did not influence FI, whereas FE supplementation increased FI in broilers fed diets without BA. Fermentable energy supplementation improved FCR in broilers fed BA supplemented diets, whereas FCR was decreased by FE supplementation in broilers fed the diet without BA. Water intake was decreased in broilers fed FE in BA supplemented diets, whereas FE supplementation increased WI in broilers fed diets without BA.

Digestive Tract Measurements

The effects of dietary treatments on relative empty weights of the GIT segments are presented in Table 3. Broilers fed the eight experimental (RSM) diets had, on average 14% heavier gizzard and 11% lower relative weights of the colon compared with broilers fed the SBM diet. Other GIT segments were similar between broilers fed the eight RSM and SBM diets. Broilers fed the coarse RSM diets had, on average 11, 7, 5 and 6% lower ($P < 0.05$) relative empty weights of the crop, duodenum, jejunum and ileum, respectively, compared with those fed the fine RSM diets. The gizzard was, however, on average 12% heavier ($P < 0.001$) in broilers fed the coarse diets compared with those fed the fine diets. An interaction ($P = 0.021$) between ST × BA was observed for the empty relative weight of the gizzard, indicating that in the coarse diet fed broilers, BA supplementation resulted in a lower gizzard weight, whereas in the fine diets fed broilers BA supplementation increased the gizzard weight.

The effects of dietary treatments on duodenal villus heights, crypt depths and VCR are presented in Table 4. Broilers fed the eight RSM diets had, on average 11, 49, and 42%, lower villus heights, crypt depths, and VCR, respectively, compared to broilers fed the SBM diet. Villus heights, crypt depths and VCR were affected by diet structure, where coarse RSM diets fed broilers had, on average 19% greater villus heights, 18% deeper crypts and 48% greater VCR compared to broilers fed the fine RSM diets. Villus heights were not, but crypt depths and VCR were also affected by BA supplementation, whereas broilers fed BA supplemented diets showed, on average 10% deeper and 14% greater crypt depths and VCR, respectively, compared to broilers fed diets without BA supplementation. Fermentable energy supplementation resulted in 5 and 6% lower villus heights and deeper crypts, respectively, compared with those fed diets without FE. Interactions between ST × BA were observed for villus heights ($P = 0.045$), crypt depths ($P = 0.041$), and VCR ($P < 0.001$), indicating that in broilers fed the coarse diets, BA supplementation did not influence villus heights, whereas in those fed the fine diets, BA supplementation decreased villus heights. Crypt depths were decreased and VCR increased in broilers fed BA supplemented coarse diets, whereas in those fed the fine diets, both parameters remained unaffected by BA

Table 3. Effects of diet structure (ST), butyric acid (BA) and fermentable energy (FE) supplementation on mean relative weights (g/100 g BW) of empty gastrointestinal segments in broilers from 0 to 34 d of age.

Effects	Crop	Proventriculus	Gizzard	Duodenum	Jejunum	Ileum	Ceca	Colon
Control SBM diet[1]	0.32	0.62	1.00	0.91	1.23	1.10	0.37	0.18
Factorial analysis of the experimental RSM diets[2]								
ST: Fine								
With BA								
With FE	0.34^{ab}	0.68	1.08^{de}	1.00^{ab}	1.32^{ab}	1.13^{ab}	0.37	0.20
Without FE	0.34^{ab}	0.65	1.13^{bcd}	0.96^{ab}	1.24^{ab}	1.15^{ab}	0.37	0.22
Without BA								
With FE	0.32^{ab}	0.56	0.98^{e}	0.93^{ab}	1.26^{ab}	1.19^{a}	0.35	0.20
Without FE	0.36^{a}	0.58	1.07^{de}	1.02^{a}	1.34^{a}	1.15^{ab}	0.39	0.21
ST: Coarse								
With BA								
With FE	0.31^{ab}	0.55	1.24^{ab}	0.91^{ab}	1.22^{ab}	1.09^{bc}	0.38	0.20
Without FE	0.28^{b}	0.63	1.13^{bcd}	0.87^{b}	1.19^{b}	1.04^{c}	0.33	0.19
Without BA								
With FE	0.31^{ab}	0.56	1.25^{a}	0.92^{ab}	1.22^{ab}	1.10^{bc}	0.36	0.21
Without FE	0.31^{ab}	0.56	1.25^{a}	0.91^{ab}	1.23^{ab}	1.11^{b}	0.35	0.19
Pooled SE	0.01	0.05	0.04	0.05	0.05	0.03	0.02	0.01
P-value								
ST	0.001	0.208	< 0.001	0.029	0.047	0.006	0.153	0.064
BA	0.623	0.098	0.803	0.753	0.611	0.195	0.702	0.674
FE	0.760	0.676	0.891	0.947	0.902	0.573	0.683	0.434
ST × BA	0.550	0.380	0.021	0.592	0.922	0.765	0.877	0.417
ST × FE	0.145	0.532	0.052	0.441	0.862	0.685	0.038	0.019
BA × FE	0.150	0.829	0.189	0.199	0.162	0.947	0.166	0.894

[a-d] Means without a common superscript within a column significantly ($P < 0.05$) differ. [1]Control soybean meal, n = 4 replicates. [2]Experimental rapeseed meal, n = 32 replicates. For each replicate in all the treatments n = 6 birds.

supplementation. An interaction between ST × FE for crypt depths (P = 0.041) was observed which indicated that FE supplementation did not affect crypt depths in broilers fed the coarse diets, whereas crypt depths was decreased in those fed the fine diets supplemented with FE. Interactions were observed between BA × FE for crypt depths (P = 0.006) and VCR (P = 0.010), indicating that FE supplementation in broilers fed BA supplemented diets did not influence crypt depths, whereas FE supplementation decreased crypt depths in broilers fed diets without BA. The FE diet supplemented with BA decreased VCR, compared to the diet without BA supplementation.

Table 4. Effects of diet structure (ST), butyric acid (BA) and fermentable energy (FE) supplementation on duodenal villus heights (μm), crypt depths (μm) and villus height to crypt depths ratio (VCR) in broilers at 34 d of age.

Effects	Villus height	Crypt depth	VCR
Control SBM diet[1]	1413.4	163.6	9.59
Factorial analysis of the experimental RSM diets[2]			
ST: Fine			
With BA			
With FE	1028.2^{c}	258.5^{b}	4.11^{d}
Without FE	1197.0^{b}	267.2^{b}	4.62^{d}
Without BA			
With FE	1146.5^{bc}	254.7^{b}	4.66^{d}
Without FE	1205.0^{b}	298.8^{a}	4.30^{d}
ST: Coarse			
With BA			
With FE	1380.4^{a}	207.5^{d}	6.92^{b}
Without FE	1396.7^{a}	198.7^{d}	7.64^{a}
Without BA			
With FE	1334.8^{a}	231.5^{c}	5.94^{c}
Without FE	1332.2^{a}	247.8^{bc}	5.62^{c}
Pooled SE	41.83	8.04	0.249
P-value			
ST	< 0.001	< 0.001	< 0.001
BA	0.890	< 0.001	0.002
FE	0.041	0.006	0.454
ST × BA	0.045	0.041	< 0.001
ST × FE	0.070	0.041	0.721
BA × FE	0.273	0.006	0.010

[a-d] Means without a common superscript within a column significantly (P < 0.05) differ. [1]Control soybean meal, n = 4 replicates. [2]Experimental rapeseed meal, n = 32 replicates. For each replicate in all the treatments n = 6 birds.

Digesta Characteristics

The effects of dietary treatments on digesta characteristics are presented in Table 5. Broilers fed the eight RSM diets had, on average a 19, 6, and 26% lower gizzard and cecal pH and cecal ammonia, respectively, compared to broilers fed SBM diet. As a result of the factorial analysis of the experimental groups, broilers fed the coarse diets

had, on average 20% more acidic gizzard pH and a 6% improved ileal apparent protein digestibility compared with those fed the fine diets. No interactions were found for these traits. Dietary treatments did not influence ($P > 0.05$) protease activity in the proventriculus. There was a trend ($P = 0.056$) for BA supplementation to increase protease activity in the proventriculus.

The effects of dietary treatments on cecal VFA concentrations are presented in Table 6. Broilers fed the SBM diet had, on average 9% greater total VFA concentrations. Percentages of propionic acid (7.0 and 5.5%), total BCFA (2.66 and 1.78%) and valeric acid (1.63 and 2.05%) relative to total VFA differed between the broilers fed the SBM and RSM diets, respectively. Broilers fed the coarse RSM diets had a lower percentage of isovaleric acid ($P < 0.026$) and total BCFA ($P < 0.037$) in the total VFAs measured compared with broilers fed the fine RSM diets (1.56 vs. 2.01%).

The effects of dietary treatments on cecal biogenic amines are presented in Table 7. Broilers fed the SBM diet had approximately 16% greater total biogenic amine concentrations in the cecal digesta compared with those fed the RSM diets. Diet structure or BA supplementation did not affect ($P > 0.05$) the cecal biogenic amine concentrations. A trend ($P = 0.055$), however, for BA supplementation to decrease cadaverine concentration in the cecal digesta was observed. Broilers fed FE supplemented diets had, on average a 31% lower ($P < 0.02$) concentration of spermine in the cecal digesta compared with those fed diets without FE.

Discussion

The present study was conducted to investigate the impact of diet structure, BA and FE supplementation on performance, gut morphology and hindgut fermentation characteristics in broilers. It was hypothesized that a poor performance of broilers due to a low digestible protein source (RSM) could be counterbalanced by feeding a coarsely ground diet supplemented with BA and FE. Performance, GIT development and cecal digesta characteristics were, therefore, studied as explanatory variables. Increased villus heights and decreased crypt depths in the duodenum were used as an indicator of intestinal health. The observed poorer performance (lower FI, reduced BW gain and poor FCR) of the broilers fed the RSM diets compared with those fed the SBM diet is in accordance with expectations (Montazer-Sadegh et al., 2008; Chiang et al., 2010; Saleem, 2013). This reduced performance on the RSM diets in the present study cannot be attributed to hindgut protein fermentation. Greater levels of BCFA, biogenic amines and ammonia are considered evidence of the occurrence of protein fermentation (Macfarlane et al., 1992). These fermentation products were not increased in broilers fed the RSM diets in the present study. Other factors such as

Table 5. Effects of diet structure (ST), butyric acid (BA) and fermentable energy (FE) supplementation on gizzard and cecal pH, cecal NH_3 (g/kg DM), apparent ileal protein digestibility (%), and protease activity in the proventriculus (μmol tyrosine released/min/g of tissue) in broilers at 34 d of age.

Effects	Gizzard pH	Cecal pH	NH_3	Protein digestibility	Protease activity
Control SBM diet[1]	3.96	6.45	3.20	72.9	84.6
Factorial analysis of the experimental RSM diets[2]					
ST: Fine					
With BA					
With FE	3.93^{a}	5.98	2.07	70.8^{ab}	105.5
Without FE	3.10^{bcd}	6.11	2.30	66.9^{b}	104.0
Without BA					
With FE	3.78^{ab}	6.14	2.67	67.1^{b}	88.3
Without FE	3.44^{abc}	6.03	2.33	68.6^{ab}	94.4
ST: Coarse					
With BA					
With FE	3.02^{cd}	5.98	2.48	74.9^{a}	97.2
Without FE	2.89^{cd}	6.15	2.32	69.4^{ab}	101.2
Without BA					
With FE	2.66^{d}	6.10	2.22	72.9^{ab}	92.2
Without FE	2.79^{cd}	6.08	2.49	72.0^{ab}	94.3
Pooled SE	0.246	0.10		2.53	6.80
P-value					
ST	0.004	0.849	0.740	0.038	0.705
BA	0.707	0.671	0.236	0.851	0.056
FE	0.103	0.523	0.997	0.225	0.586
ST × BA	0.368	0.892	0.116	0.719	0.442
ST × FE	0.108	0.633	0.627	0.588	0.938
BA × FE	0.293	0.136	0.752	0.175	0.774

[a-d] Means without a common superscript within a column significantly ($P < 0.05$) differ.[1]Control soybean meal, n = 4 replicates. [2]Experimental rapeseed meal, n = 32 replicates. For each replicate in all the treatments n = 6 birds.

Table 6. Effects of diet structure (ST), butyric acid (BA) and fermentable energy (FE) supplementation on cecal volatile fatty acids (VFA (mmol/kg DM)) concentrations in broilers at 34 d of age.

Effects	Total VFA	Acetic acid[1]	Propionic acid[1]	Butyric acid[1]	Valeric acid[1]	Isobutyric acid[1]	Isovaleric acid[1]	Total BCFA[2]
Control SBM diet[3]	92.6	71.6	7.0	17.08	1.63	1.28	1.38	2.66
Factorial analysis of the experimental RSM diets[4]								
ST: Fine								
With BA								
With FE	86.9	72.2	4.72	19.93	1.97	1.18	1.01[a]	2.18[a]
Without FE	78.6	73.8	5.75	16.89	2.16	1.07	1.06[a]	2.12[ab]
Without BA								
With FE	94.4	72.8	5.71	18.23	2.00	0.95	0.79[ab]	1.74[ab]
Without FE	73.7	73.6	5.69	16.65	2.28	0.99	0.99[ab]	1.98[ab]
ST: Coarse								
With BA								
With FE	90.3	73.1	5.38	17.97	2.00	0.89	0.66[b]	1.55[ab]
Without FE	87.7	72.3	6.27	17.41	2.09	1.10	0.82[ab]	1.92[ab]
Without BA								
With FE	79.9	74.3	5.15	17.58	1.95	0.72	0.59[b]	1.31[b]
Without FE	79.8	72.1	5.61	18.91	1.94	0.78	0.66[b]	1.44[ab]
Pooled SE	8.86	1.20	0.44	1.25	0.16	0.19	0.17	0.29
P-value								
ST	0.871	0.833	0.672	0.962	0.360	0.206	0.026	0.037
BA	0.537	0.684	0.979	0.818	0.898	0.139	0.295	0.122
FE	0.218	0.888	0.072	0.287	0.234	0.708	0.329	0.419
ST × BA	0.417	0.882	0.161	0.398	0.424	0.722	0.918	0.867
ST × FE	0.304	0.125	0.793	0.139	0.412	0.517	0.947	0.705
FE × BA	0.695	0.529	0.247	0.352	0.981	0.992	0.901	0.937

[a-b] Means without a common superscript within a column significantly ($P < 0.05$) differ. [1]Percent of total VFA (acetic acid + propionic acid + butyric acid + valeric acid + isobutyric acid + isovaleric acid). [2]BCFA = branched chain fatty acids (sum of isobutyric and isovaleric acids). [3]Control soybean meal, n = 4 replicates. [4]Experimental rapeseed meal, n = 32 replicates. For each replicate in all the treatments n = 6 birds.

Table 7. Effects of diet structure (ST), butyric acid (BA) and fermentable energy (FE) supplementation on cecal biogenic amines (mmol/kg DM) concentration in broilers at 34 d of age.

Effects	Histamine	Putrescine	Cadaverine	Spermidine	Spermine	Tyramine	Total
Control SBM diet[1]	0.45	1.71	0.43	21.65	0.60	1.31	27.42
Factorial analysis of the experimental RSM diets[2]							
ST: Fine							
With BA							
With FE	0.36	1.27	0.59	17.50	0.61^{c}	0.75	24.42
Without FE	0.40	1.46	0.65	20.41	1.00^{ab}	0.85	24.76
Without BA							
With FE	0.66	1.73	1.04	17.89	0.79^{bc}	0.84	22.94
Without FE	0.37	1.33	0.87	18.08	1.00^{ab}	0.82	22.42
ST: Coarse							
With BA							
With FE	0.38	1.18	0.82	16.71	0.72^{bc}	0.92	20.72
Without FE	1.17	1.58	0.61	18.72	0.88^{abc}	0.91	23.88
Without BA							
With FE	0.43	1.49	0.96	17.90	0.71^{bc}	0.85	21.84
Without FE	0.55	1.46	0.91	19.11	1.24^{a}	0.81	24.08
Pooled SE	0.17	0.18	0.19	1.42	0.13	0.19	2.13
P-value							
ST	0.143	0.879	0.800	0.727	0.612	0.668	0.498
BA	0.549	0.320	0.055	0.930	0.168	0.841	0.675
FE	0.187	0.747	0.503	0.129	0.002	0.964	0.384
ST × BA	0.101	0.786	0.674	0.390	0.588	0.699	0.391
ST × FE	0.026	0.254	0.791	0.979	0.707	0.818	0.352
BA × FE	0.052	0.055	0.879	0.388	0.657	0.784	0.765

[a-c] Means without a common superscript within a column significantly ($P < 0.05$) differ.[1]Control soybean meal, n = 4 replicates.[2]Experimental rapeseed meal, n = 32 replicates. For each replicate in all the treatments n = 6 birds.

the presence of antinutritional factors, glucosinolates, tannins, phytic acid, and sinapine may have resulted in a poor performance of broilers fed the RSM diets (Khajali and Slominski, 2012; Rutkowski et al., 2012; Ahmed et al., 2014). A high fiber content is another factor increasing digesta viscosity and digesta passage rate in the gut (Choct et al., 2010), resulting in a poor digestibility of nutrients (Smits et al., 1997). Reduced villus heights, deeper crypts and lower VCR in broilers fed RSM compared with those fed SBM may be due to the above mentioned antinutritional factors (ANF). Greater villus heights and VCR are indicative for proper digestion and absorption of nutrients (Chiang et al., 2010). Some ANFs result in gut wall damage and increased endogenous protein losses (Smits et al., 1997). High inclusion levels of RSM (> 20%) increased the energy needed for gut wall repair and for liver metabolic activities which may result in reduced performance (Woyengo et al., 2011). Improved performance in broilers fed the coarse diets compared with those fed the fine diets is in accordance with expectation and confirms some recent studies in broilers (Jacobs et al., 2010; Rodgers et al., 2012; Jacobs and Parsons, 2013; Pacheco et al., 2013). The decreased gizzard pH in broilers fed the coarse diet can be explained by a greater stimulatory activity of the gizzard, allowing more hydrochloric acid secretion. The greater protein digestibility with coarse particles, in our study, is also in accordance with the findings of recent studies for broilers (Pacheco et al., 2013; Liu et al., 2013). This improved digestibility may be attributed to a more functional gizzard, increased gastric reflux between proventriculus and gizzard resulting in more time for gastric enzyme activity and even for more protease activity in the duodenum (Benedetti et al., 2011). Pacheco et al. (2013) reported greater protein digestibility in broilers fed coarsely ground corn compared with those fed fine corn (86.1 vs. 84.8%). In addition, fine particles can increase digesta viscosity (Amerah et al., 2007), which may have a negative effect on nutrient digestibility.

The lower weights of the empty crop in broilers fed the coarse diets indicates less feed accumulation in the crop compared with those fed the fine diets. The greater relative weights of the gizzard in broilers fed the coarse diets compared with those fed the fine diets, in the current study is supported by the findings of others in broilers (Benedetti et al., 2011; Bhuiyan et al., 2012; Rodgers et al., 2012; Jacobs and Parsons, 2013). Jacob and Parson (2013) reported a 47 and 22% heavier gizzard in broilers fed whole sorghum and coarse corn, respectively, compared to those fed fine diets. The improved gizzard weight in broilers fed the coarse diets may be due to a more functional gizzard compared with those fed the fine diets. The greater relative weights of the duodenum, jejunum and ileum in broilers fed the fine diets can be explained by their increased activity as a result of a poorer developed and smaller non-functional gizzard.

Greater duodenal villus heights and VCR and lower crypt depths in broilers fed the coarse diets suggest an improved digestion of nutrients because of a proper pre-digestion in the foregut (Pacheco et al., 2013). Greater villus heights in broilers fed coarse diets may also be due to less abrasive action by digesta in the duodenum. Correspondingly, Sogunle et al. (2013) reported 89% greater duodenal villus heights in broilers fed diets with, on average, a dietary particle size of 2 mm compared with those fed particle size of 1 mm.

Broilers fed the coarse diets were expected to show decreased concentrations of cecal BCFAs and biogenic amines because of an improved ileal protein digestibility due to enhanced gizzard weights. In the present work, however, coarse diet feeding had no influence on cecal biogenic amines concentrations, but there was a decrease in total BCFA, mainly isovaleric acid, which is produced as a result of bacterial fermentation of leucine. The improved performance in broilers fed BA supplemented diets is in accordance with previously reported studies for broilers (Antongiovanni et al., 2009; Adil et al., 2010; Pouraziz et al., 2013). Pouraziz et al. (2013) reported approximately 8 and 6% improved BW and FCR, respectively, in broilers fed a diet with 0.004g/g of BA glycerides compared with broilers fed a control diet in both starter and grower phases. These positive effects of BA supplementation may be due to an improved digestion and absorption of nutrients (Mansoub, 2011), as a consequence of increased pancreatic enzyme secretion, and because of their effects on gut mucosa and their antimicrobial activity (Adil et al., 2010). Improved gut morphology in broilers fed BA supplemented diets may be due to the provision of energy to enterocytes, because BA is one of the major energy sources of these cells (Czerwiński et al., 2012). Antongiovanni et al. (2009) hypothesized BA to be the main growth promoter of the gut wall in broilers. Histological changes in the intestine as a result of BA supplementation may increase the surface area for absorption of nutrients in the intestine, enhancing growth performance, thereby reducing the amount of substrate available for fermentation by microbiota in the hindgut of the broilers. This was confirmed by the improved growth performance in broilers fed the diets supplemented with BA compared with those fed the diets without BA in this study. Due to improved gut health, as illustrated by enhanced gut morphology, BA supplementation was expected to increase ileal digestibility of protein. In the present study, however, no effect on ileal digestibility of protein was found in broilers fed the BA supplemented diets. Therefore, it did not affect the concentration of cecal total VFA, BCFA and biogenic amines. A tendency of a lower cecal cadaverine concentration in broilers fed the BA supplemented diets can be explained by a decrease in the number of pathogenic microbiota such as *C. perfringens* (Qaisrani et al., unpublished data) as *C. perfringens* seems to be involved in protein fermentation in the hindgut (Richardson et

al., 2013). The decreased cecal concentration of spermine in broilers fed FE supplemented diets may indicate a lower concentration of spermidine present in the ceca. Spermine is produced due to the catabolism of spermidine, which in turn is produced as a result of fermentation of amino acids such as histidine, ornithine, arginine, or methionine. This indicates less fermentation of the above mentioned amino acids in the ceca of broilers fed the FE supplemented diets.

The overall results of the present study indicate that RSM resulted in a poor performance and impaired gut morphology. Inclusion of dietary coarse particles improved gut development. Heavier gizzards, enhanced gut morphology, better protein digestibility, and improved FCR as well as significantly lower concentrations of hindgut fermentation products, BCFA, were found in broilers fed the coarse diets. Supplementation of BA had an additional positive effect to feeding a coarse diet on performance of broilers. In conclusion, feeding a coarse diet supplemented with BA improves performance of broilers fed a poorly digestible protein source.

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CHAPTER 6

Influence of protein source, diet structure, butyric acid and fermentable energy on cecal microbiota population in broilers

S.N. Qaisrani[1], Y. Feng[1], M.M. van Krimpen[2], R.P. Kwakkel[1], M.W.A. Verstegen[1], J. Zhang[3], H. Smidt[3], and W.H. Hendriks[1]

[1]Animal Nutrition Group, Wageningen University, PO Box 338, NL-6700 AH Wageningen, The Netherlands

[2]Wageningen UR Livestock Research, PO Box 65, NL-8200 AB Lelystad, The Netherlands

[3]Laboratory of Microbiology, Wageningen University, PO Box 338, NL-6700 AH Wageningen, The Netherlands

Abstract

Three experiments were conducted to investigate the main and interaction of protein source, diet structure, butyric acid (BA) and fermentable energy (FE) supplementation on cecal microbiota population in broilers. In total, 128, 210 and 288 male Ross (308) 1-d-old broilers were used in three experiments. In Experiment (Exp.) 1, none of the dietary treatments influenced ($P > 0.05$) the average relative contributions of the observed cecal microbial species. In Exp. 2, protein source influenced ($P < 0.05$) the average relative contribution of health beneficial *L. paracasei* and *C. lactifermentans* spp., with broilers fed the soybean meal (SBM) diets having a greater relative contributions of these species compared with those fed the rapeseed meal (RSM). The average relative contribution of *L. paracasei* was uninfected in all fine diets, but increased with higher levels of SBM if the diet was fed as coarse. Broilers fed the coarse diets had a reduced ($P = 0.028$) average relative contribution of *E. coli* compared with those fed the fine diets. In Exp. 3, BA supplementation promoted ($P < 0.05$) the average relative contribution of *C. lactifermentans* and *R. bromii*, and suppressed ($P = 0.038$) the pathogenic *C. perfringens*. Fermentable energy, in contrast, promoted ($P = 0.036$) *C. perfringens*. The overall results indicate that dietary coarseness, BA supplementation and a good digestible protein source (SBM) resulted in decreased cecal microbial diversity and an improved performance. Soybean meal resulted in a greater relative contribution of health beneficial microbiota. Dietary coarseness and BA supplementation suppressed pathogenic and promoted health beneficial microbiota in the cecal digesta. Feeding a poorly digestible protein source such as RSM in a coarsely ground diet and supplemented with BA, can be an effective strategy to promote commensal and suppress pathogenic microbiota in cecal digesta, thereby, potentially enhancing performance of broilers.

Key words: broilers, protein, cecal microbiota, butyric acid, fermentable energy.

Introduction

In modern-day broilers, commensal microbiota play an important role in maintaining an active immune system (Kohl, 2012), gut function, and in safeguarding against an overgrowth of pathogenic microbiota (Kelly and King, 2001). This prevention against pathogenic bacteria may be due to the production of short chain fatty acids (SCFA) such as acetate, propionate and butyrate as a result of carbohydrate fermentation in broilers. These SCFA provide energy to the gut wall and decrease cecal pH resulting in inhibition of acid sensitive microorganisms (Apajalahti, 2005). Cecal microbiota develops fully within 6 to 7 wks after hatch (Coloe et al., 1984) and are highly dense and diverse compared to other parts of the GIT in broilers (Gong et al., 2007).

There are several factors including age, dietary treatment, structure and physical appearance of the diet, feed additives, and disease load which affect microbiota population (Engberg et al., 2004; Rehman et al., 2008; Torok et al., 2011; Van der Hoeven-Hangoor et al., 2013). Microbiota diversity in different parts of the GIT is related to the type of substrates available where species differ in their substrate preferences and growth requirements (Kiarie *et al.*, 2013). At the end of the ileum, undigested dietary ingredients are potential substrates for gut microbiota in the hindgut (Apajalahti et al., 2004). For instance, undigested CP may stimulate the growth of N-utilizing microbiota (Reid and Hillman, 1999). A linear relation between dietary CP level and *C. perfringens* population in the ceca of broiler has been reported earlier by Elwinger et al. (1998). This surplus of protein in the hindgut in combination with a low concentration of fermentable carbohydrates can lead to more proteolytic microbiota such as *Clostridium* spp., *Enterococcus* spp., and *Bacteriodes* spp. which may produce NH_3 and produce biogenic amines, phenols and indoles (Macfarlane et al., 1992).

The ceca, where substantial fermentation occurs, contain approximately 10^{11} bacteria per gram of fresh cecal contents in broilers (Adil and Magray, 2012). These microbiota mainly consists of *Clostridium coccoides* (27.1%), *Sporomusa* (21.2%), *Enterics* (20.8%), *Clostridium Ieptum* (20.2%), *Atopobium* (3.6%), *Bacteroides* (1.9%) and others (5.2%) (Zhu et al., 2002). These cecal microbiota perform several functions including nitrogen recycling by break down of uric acid (Karasawa, 1999), production of essential amino acids (Obst, 1989) and supply of B vitamin (McNab, 1973) in broilers.

Extensive studies on gut microbiota in broilers are based on culturing and fingerprinting techniques (Van der Hoeven-Hangoor et al., 2013). The major disadvantage of these techniques is the underestimation of the microbiota population and their composition (Kohl, 2012). Approximately 90% of the microbiota species

cannot be cultured under ordinary lab conditions (Apajalahti et al., 2004). There have been studies indicating that fingerprinting techniques cannot identify taxonomically specific microbiota (Engberg et al., 2004; Torok et al., 2011). Therefore, the degree to which gut microbiota undergo changes due to dietary manipulation is largely unknown (Kohl, 2012). There are many limitations in these molecular techniques, including that DNA extraction and amplification may be biased in favour of certain bacteria and DNA sequencing (Jozefiak et al., 2010). A number of these disadvantages can be overcome by applying DNA microarray techniques. An example of this technique is the Chicken intestinal tract chip (ChickenChip), a DNA microarray containing 2018 probes targeting bacterial 16S rRNA gene sequences of 98 groups of bacteria, which is designed based on the human intestinal tract chip (Rajilić-Stojanović et al., 2009). This technique presents an extensive profile of the intestinal microbiota community and also provides the relative contribution of 98 individual species.

To the author's knowledge, no studies have been published using this DNA microarray technique to investigate the main and interaction effects of protein source, particle size, butyric acid and fermentable energy supplementation on cecal microbiota population in broilers. The primary objective of the study, therefore, was to investigate the influence of all the above mentioned factors on changes in the cecal microbiota population.

Materials and Methods

Experimental procedures were in accordance with the Wageningen University and the Netherlands Animal Experimental Committee guidelines and code of practice. Ethical approval was granted before the conduct of the study.

Birds Management

In total, 128, 210 and 288 male Ross (308) 1-d-old broilers were purchased from a commercial hatchery for experiments (Exp.) 1, 2 and 3, respectively. Upon arrival, broilers were individually weighed and wing banded, before being randomly allotted to one of 16 floor pens for Exp. 1 (8 broilers per pen), one of 30 floor pens for Exp. 2 (7 broilers per pen), and one of 36 floor pens for Exp. 3 (8 broilers per pen). Within experiments, each pen had a similar initial total body weight. Each pen (1.15 × 1.75 × 0.80 m) (L × W × H) had three drinking nipples with a cup underneath connected to a water tank of 10 L capacity. Pens were separated by solid walls to prevent contact between broilers from different treatment groups. Feed and water were available *ad libitum* throughout the experiment. Wood shavings were used as litter material in Exp. 1, whereas Soft Cells® were used as litter material in Exp. 2 and 3. The lighting schedule was set at 23L:1D for the first three days and, thereafter, maintained at 16L:8D with an intensity of approximately 20 lx at bird's level throughout the

experimental period. On d 1, the temperature in climate-controlled rooms was set at 32°C with a relative humidity of 70 to 80%. The temperature was, thereafter, reduced by 2 to 3°C weekly until it reached 22°C in wk 4 and this setting was maintained until the end of the experiment.

Experimental Design and Treatments

Cecal contents of broilers from these three experiments were used to assess the microbial profiles. Exp. 1 (Chapter 3) comprised a randomized complete block design (2 × 2) with four dietary treatments comprising of two different protein sources: soybean meal (SBM) and rapeseed meal (RSM) at two different digestible crude protein (DCP) levels: 15.8 and 17.2%, with similar concentrations of first limiting essential amino acids per diet. Each treatment had four replicates resulting in 16 experimental units (2 × 2 × 4 = 16).

In Exp. 2 (Chapter 4), had a randomized complete block (5 × 2) orthogonal design with five dietary treatments in which a good digestible protein source, SBM, was stepwise replaced by a poorer digestible protein source i.e. RSM. Treatments included diets with a very low level of indigestible protein (RSM-0%), a low level of indigestible protein (RSM-25%), a medium level of indigestible protein (RSM-50%), a high level of indigestible protein (RSM-75%) and a very high level of indigestible protein (RSM-100%). These five diets were each fed in two different diet structures: finely and coarsely ground. As a consequence, next to the stepwise substitution of SBM by RSM, also inclusion levels of ingredients such as maize starch, fish meal, potato protein and vegetable fat varied among the treatments. Each treatment had three replicates resulting in 30 experimental units (5 × 2 × 3 = 30).

Experiment 3 (Chapter 5) comprised a 2 × 2 × 2 factorial arrangement of 8 treatments where the interaction effects of particle size (fine vs. coarse), fermentable energy level (with vs. without) and butyric acid supplementation level (with vs. without) in RSM based diets were tested. A finely ground positive control diet based on SBM as the main protein source was added as treatment 9 to these 8 treatments. The highly fermentable energy level was attained by substituting maize starch by potato starch. Butyric acid was added in the premix in a dosage of 2 kg/ton of feed.

In all three experiments, the diets were fed as a starter (0 to 7 d of age) and a grower diet (8 to 34 d of age). The diets were formulated to meet or exceed the recommendations for broilers (CVB, 2007; Table 1). Fine and coarse diets were processed with a hammer mill through an opening screen of 3.0 mm and a roller mill with a roller distance of 1.6 mm, respectively. The diets were offered as pellets with a pellet size of 2.5 mm for the starter and 4.0 mm for the grower diet.

Table 1. Overview of the experimental factors and nutrient contents of the grower diets in the three experiments.

Item	Experiment 1				Experiment 2					Experiment 3	
Protein source	SBM		RSM		RSM					SBM	RSM
Digestible CP inclusion level (%)	15.8	17.2	15.8	17.2							
Inclusion level (%)					0	25	50	75	100		
Particle size (fine vs. coarse)	No	No	No	No	Yes	Yes	Yes	Yes	Yes	No	Yes
Butyric acid (with vs. without)	No	No	No	No	No	No	No	No	No	No	Yes
Fermentable energy (with vs. without)	No	No	No	No	No	No	No	No	No	No	Yes
Calculated composition											
ME (Kcal/kg)	3014.3	3014.3	3014.3	3014.3	2835.0	2835.0	2835.0	2835.0	2835.0	2671.0	2680.5
CP	200.0	214.0	210.0	224.0	204.1	206.5	208.8	211.2	213.5	215.2	225.0
Indigestible CP	42.0	42.0	52.0	52.0	35.6	38.8	41.9	45.1	48.2	30.6	46.1
Digestible CP[1]	158.0	172.0	158.0	172.0	168.5	167.7	166.9	166.1	165.3	184.6	178.9
NSP[2]	154.0	147.9	167.2	171.5	184.9	185.8	186.8	187.7	188.6	158.5	180.0
Crude fiber	38.3	33.6	43.0	53.6	36.4	40.4	44.3	48.3	52.2	35.3	51.0
Digestible Lys	10.6	10.6	10.6	10.6	9.6	9.6	9.6	9.6	9.6	10.6	10.6
Digestible Met + Cys	7.7	7.7	7.7	7.7	7.2	7.2	7.2	7.2	7.2	7.7	7.8
Digestible Thr	8.0	8.0	8.0	8.0	7.2	7.2	7.2	7.2	7.2	7.8	7.8
Digestible Trp	2.2	2.2	2.2	2.2	2.5	2.5	2.5	2.5	2.5	2.2	2.2

[1]Based on data from CVB (2007). [2]NSP = Non-starch polysaccharides, calculated by subtracting the CP, fat, starch, sugar, and ash content from the DM content.

Sampling and DNA Isolation

At the end of the experimental period in all three experiments, 6 broilers per pen closest to the average weight of the pen were euthanized by an intravenous T-61 injection, where after the abdominal cavity was opened. The individual digesta contents from the ceca were removed by gentle squeezing in an eppendorf of 2 ml. The samples, thereafter, were freeze dried at -20°C pending microbiota analysis. Samples of the 6 individual broilers from the same pen were first homogenized for DNA isolation. Thereafter, 200 µl defrosted pooled digesta was pipetted into a 2 ml tube, and mixed sufficiently using the pipet. The DNA was extracted by the method described by Salonen et al. (2010) and purified using QIAmp DNA Mini Kit (Qiagen, Germany).

Microarray Hybridization and Analysis

The microbiota composition in the cecal contents was determined by ChickenChip, a DNA microarray containing 2018 probes targeting bacterial 16S rRNA gene sequences of 98 groups of bacteria, which design is based on the human intestinal tract chip (Rajilić-Stojanović et al., 2009). Primers T7 prom-Bact-27-F (5'-TGA ATT GTA ATA CGA CTC ACT ATA GGG GTT TGA TCC TGG CTC AG–3') and Uni-1492-R (5'-CGG CTA CCT TGT TAC GAC-3') were employed for the amplification of bacterial 16S rRNA gene fragments from 10 ng of DNA extracted from cecal samples (Lane, 1991). The PCR program was initiated for 5 min heating at 95°C, then 35 cycles were conducted described as follows: 95°C for 30 sec, 52°C for 40 sec and 72°C for 1 min, followed by a final step at 72°C for 10 min. A High Pure PCR cleanup micro kit (Roche Diagnostics GmbH, Mannheim, Germany) was used for purification, while the final concentration of 16S rRNA was determined using NanoDrop-ND-2000 spectrophotometer (NanoDrop® Technologies, Wilmington, DE 19810 USA).

In vitro transcription of DNA into RNA was performed using a RNAMAXX T7 transcription kit (Agilent Technologies, Stratagene, CA) and 5-(3-Aminoallyl)-UTP (Life Technologies Corporation, Ambion®, USA). After a 60 min incubation at room temperature, RNase-free DNase (Promega Corporation®, Madison, USA) was included for another 15 min incubation to digest any remaining DNA. For purification of RNA products, an RNeasy Minielute Cleanup Kit (Quiagen, Hilden, Germany) was used and finally the RNA concentration was measured using a NanoDrop-ND-2000 spectrophotometer (NanoDrop® Technologies, Wilmington, DE 19810 USA).

For fluorescent labeling of RNA samples, containing modified aminoallyl nucleotides, the CyDye Post-labeling reactive Dye (GE Healthcare, Buckinghamshire, UK) dissolved in 84 µl of DMSO (Sigma-Aldrich, St Louis, USA) was used. Each labeling reaction contained labeling dye (20 µl), RNA (2000 ng), and 0.1 M $NaHCO_3$ (sodium bicarbonate, 10 µl) with the pH adjusted to 8.7. Additional, 15 µl of NH_2OH

(hydroxylamine) was added to the sample after 90 min. The labeling reaction was terminated by performing an additional dark incubation for 15 min. Labeled RNA was, thereafter, purified using a RNeasy Minielute clean-up kit, and stored at -80°C until use after measurement of concentrations with a NanoDrop-2000.

The hybridization mixture of each array (49 µl), including two labeled samples as described above with Cy3 and Cy5 dye, was pipetted to 8 × 15 K ChickenChip slides (Agilent technologies, Palo Alto, CA, USA). The reaction in a rotate hybridization chamber (Agilent) lasts 18 h at 62°C. Subsequently, slides were first washed by 1 × SSC, 0.3% SDS solution for 10 min at room temperature, gently transferred to 0.1 × SSC, 0.3% SDS solution (water bathed overnight at 38°C) followed by washing in the hybridization chamber for 10 min at 45°C. Lastly, a solution containing 0.06 × SSPE was used for another 5 min at room temperature (Sambrook, 1989) and cleaned by acetonitrile (Sigma-Aldrich, GmbH, Mannheim, Germany). ChickenChip slides were eventually scanned using Agilent Feature Extraction software (version 9.5, http://www.agilent.com). Data were retrieved from the MySQL (version 5.1) database and processed using R as described by Rajilic-Stojanovic et al. (2009).

Statistical Analysis

Shannon's diversity index was generated in statistical software R (2.15.2) to show the ecological changes of the cecal microbiota. The data were analyzed using PROC MIXED in SAS (version 9.2; SAS Inst. Inc., Cary, NC). The data from Exp. 1 were analyzed by the following model;

$$Y_{ijk} = \mu + P_i + L_j + P_i \times L_j + e_{ijk}$$

where Y_{ijk} is the measured response, μ overall mean effect, P_i the i^{th} fixed protein source effect (i = SBM or RSM), L_j the j^{th} fixed digestible CP inclusion level effect (j = 15.8 or 17.2%), $P_i \times L_j$ the interaction between protein source and digestible CP inclusion level, and e_{ijk} the random error.

The data from Exp. 2 were analyzed by the following model;

$$Y_{ijk} = \mu + PS_i + ST_j + PS_i \times ST_j + e_{ijk}$$

where Y_{ijk} is the measured response, μ overall mean effect, PS_i the i^{th} fixed protein source effect (i = RSM-0%, RSM-25%, RSM-50%, RSM-75% and RSM-100%), ST_j the j^{th} fixed diet structure effect (j = fine or coarse), $PS_i \times ST_j$ the interaction between protein source and diet structure, and e_{ijk} the random error.

The data from Exp. 3 were analyzed by the following model;

$$Y_{ijkl} = \mu + ST_i + BA_j + FE_k + ST_i \times BA_j + ST_i \times FE_k + BA_j \times FE_k + ST_i \times BA_j \times FE_{k+} e_{ijkl}$$

where Y_{ijkl} is the measured response, μ overall mean effect, ST_i the i^{th} fixed diet structure effect (i = fine or coarse), BA_j the j^{th} fixed BA supplementation effect (j = with or without BA), FE_k the k^{th} fixed FE supplementation effect (k = with or without FE), $ST_i \times BA_j$ interaction between diet structure and BA supplementation, $ST_i \times FE_k$ the interaction between diet structure and FE supplementation, $BA_j \times FE_k$ the interaction between BA and FE supplementation level, whereas $ST_i \times BA_j \times FE_k$ the interaction between diet structure, BA and FE supplementation, $e_{ijkl} \sim$ NID (0, σ2e). Differences were considered significant at a probability level of 5%. Interactions were ignored, if the main effect for a particular trait was found non-significant. By analyzing the data, no three way interactions were found. It was decided, therefore, not to show the three way interactions in the results section. The SBM diet was added as the 9th treatment, used as a positive control group but disregarded from statistical analysis.

Results and Discussion

Analysis of the cecal contents of broilers fed different dietary treatments using the ChickenChip provides insight into the diversity of the microbiota between broilers due to diet changes. Due to similar good hygienic environment-controlled conditions during the broiler trials and the absence of a pre-set challenged exposure to any pathogenic microbiota to the broilers, little variation in the cecal microbial population were expected.

Relative Contribution of Microbial Classes

The overall relative contribution of different microbial classes in the cecal digesta of the broilers in the three experiments is presented in Table 2. Although the average relative contribution of different microbiota classes was almost similar, variation, however, existed among the different experiments. For example, the share of *Bacteroidetes* was 3.78, 5.11 and 3.97%, *Bacilli* was 18.5, 14.2, and 14.5%, *Clostridium* cluster IV was 25.7, 28.4, and 28.9% and of *Clostridium* cluster XIVa was 13.4, 17.5, and 16.7% of the total microbiota population in the cecal digesta of broilers in Exp. 1, 2 and 3, respectively. This variation in different microbial classes between different experiments may be due to diet structure, as protein sources were almost similar in all experiments. There are studies that showed that diet structure influences cecal microbial population in broilers (Engberg et al., 2004). These differences may also be due to, among others, flock of origin, hygienic conditions and health status of the broilers.

Table 2. Average relative contribution (%) of different classes of microbiota in the cecal digesta of broilers in three experiments based on ChickenChip[1] data[2,3].

Phyla	Class	Experiment 1	Experiment 2	Experiment 3
Actinobacteria	*Actinobacteria* (*a.o. Bifidobacterium*)	1.78 ± 0.08	1.65 ± 0.07	1.58 ± 0.03
Bacteroidetes	*Bacteroidetes*	3.78 ± 0.08	5.11 ± 0.04	3.97 ± 0.04
	Flavobacteria	0.03 ± 1.13	0.03 ± 0.53	0.03 ± 0.47
Chlamydiae	*Chlamydiae*	0.03 ± 0.37	0.02 ± 0.24	0.02 ± 0.13
Cyanobacteria	*Unclassified*	1.03 ± 0.07	0.93 ± 0.06	0.98 ± 0.05
Deferribacteres	*Deferribacteres*	0.40 ± 0.00	0.38 ± 0.00	0.41 ± 0.00
Deinococcus-Thermus	*Deinococci*	0.45 ± 0.03	0.44 ± 0.04	0.45 ± 0.02
Firmicutes	*Bacilli (a.o.Enterococcus, Lactobacillus paracasei, Streptococcus bovis)*	18.54 ± 1.18	14.23 ± 0.69	14.46 ± 0.68
	Clostridium cluster I (*a.o. Clostridium perfringens*)	0.70 ± 0.10	0.57 ± 0.05	0.60 ± 0.04
	Clostridium cluster IV (*a.o. Ruminococcus bromii*)	25.73 ± 0.16	28.44 ± 0.04	28.86 ± 0.11
	Clostridium cluster IX	1.92 ± 0.55	1.58 ± 0.36	1.67 ± 0.36
	Clostridium cluster XI	2.36 ± 0.16	1.50 ± 0.10	2.45 ± 0.09
	Clostridium cluster XIVa (*a.o. Clostridium lactifermentans*)	13.38 ± 0.04	17.47 ± 0.02	16.73 ± 0.02
	Clostridium cluster XIVb	7.40 ± 0.14	6.39 ± 0.08	6.69 ± 0.04
	Clostridium cluster XVI	0.77 ± 0.01	0.61 ± 0.01	0.67 ± 0.01
	Clostridium cluster XVIII	2.44 ± 0.02	2.35 ± 0.01	2.14 ± 0.01
	Mollicutes	5.20 ± 0.03	4.57 ± 0.01	4.60 ± 0.01
Lentisphaerae	*Victivallales*	0.38 ± 0.02	0.36 ± 0.04	0.35 ± 0.02
Proteobacteria	*Alphaproteobacteria*	2.79 ± 0.00	2.73 ± 0.00	2.78 ± 0.00
	Betaproteobacteria	3.22 ± 0.14	3.08 ± 0.06	3.26 ± 0.07
	Deltaproteobacteria	0.78 ± 0.11	0.70 ± 0.05	0.72 ± 0.05
	Epsilonproteobacteria	0.53 ± 0.03	0.77 ± 0.01	0.51 ± 0.01
	Gammaproteobacteria (a.o. Escherichia coli)	5.34 ± 0.03	5.09 ± 0.01	5.10 ± 0.02
Spirochaetes	*Spirochaetes*	0.62 ± 0.01	0.50 ± 0.06	0.53 ± 0.03
Verrucomicrobia	*Verrucomicrobia*	0.40 ± 0.02	0.50 ± 0.01	0.44 ± 0.01
Total		100.0	100.0	100.0

[1]ChickenChip = Chicken Intestinal Tract Chip.[2]Values are mean ± SE. [3]n = 16, 30 and 36 for Experiment 1, 2 and 3, respectively.

Microbial Diversity and Performance

The cecal microbial diversity index of the four experimental diets used in Exp 1 is presented in Figure 1 and related to observe feed conversion ratio (FCR) and duodenal villus heights. The results indicate that the dietary treatments did not significantly affect the cecal microbial diversity. There were, however, numerical differences in diversity values among the broilers fed the different diets. Broilers fed the low (15.8%) DCP level had a greater cecal microbiota diversity compared with those fed diets with the high (17.2%) DCP level. Broilers fed the SBM diets, similarly, showed greater microbial diversity compared with those fed the RSM diets. No clear relationship between cecal microbiota diversity and FCR or duodenal villus heights could be detected from the data of Exp. 1.

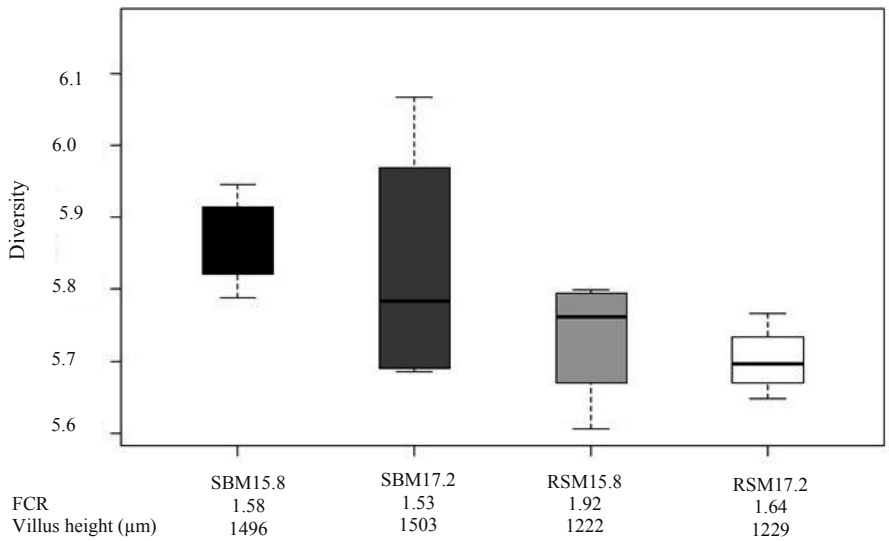

Figure 1. Boxplots of Shannon diversity index of the experimental diets, with horizontal lines representing the group means inside the box, whereas vertical bars indicating the standard errors.
SBM15.8: soybean meal with 15.8% digestible CP; SBM17.2: soybean meal with 17.2% digestible CP; RSM15.8: rapeseed meal with 15.8% digestible CP; RSM17.2: rapeseed meal with 17.2% digestible CP.

For Exp. 2, the cecal microbial diversity index of the 10 experimental diets is summarized in Figure 2. The results indicate a greater cecal microbial diversity in broilers fed the fine diets at all inclusion levels of RSM. In contrast, cecal microbial diversity in broilers fed coarse diets was low in the SBM diet and gradually increased if SBM was replaced by RSM. It was observed that cecal microbial diversity was reduced in broilers fed coarse diets compared with those fed fine diets. If cecal microbial diversity is related to performance traits, it seems that reduced microbial diversity is accompanied with better FCR and longer villi.

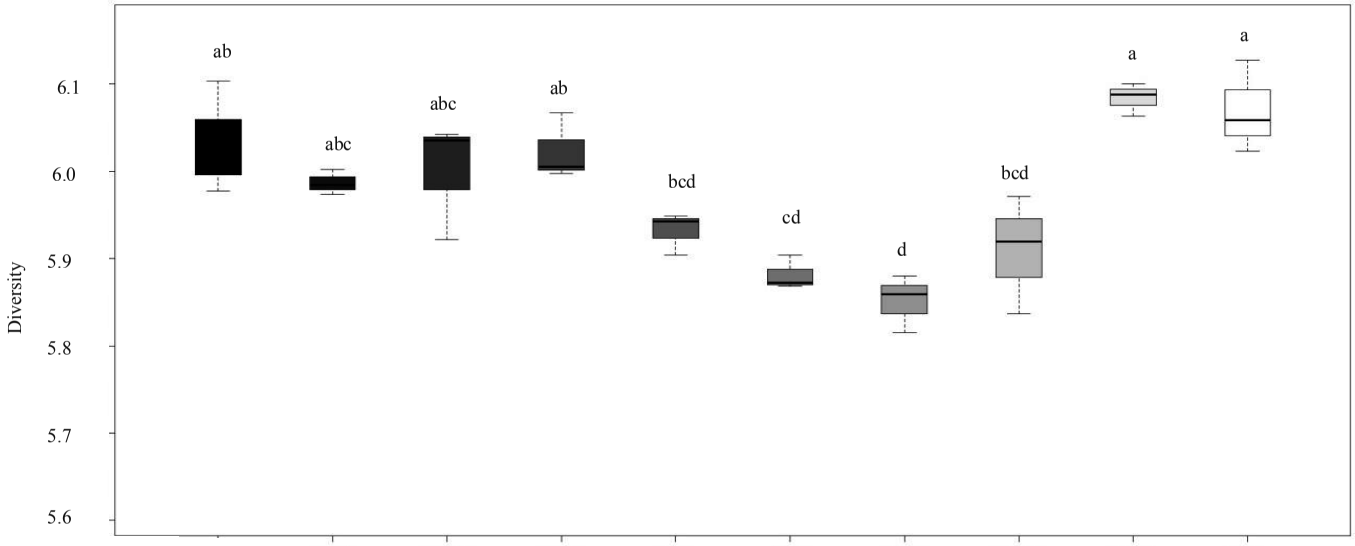

Figure 2. Boxplots of Shannon diversity index of the experimental diets, with horizontal lines representing the group means inside the box, whereas vertical bars indicating the standard errors.

RSM: rapeseed meal; F: fine; C: coarse. [a-d]Means without a common superscript significantly ($P < 0.05$) differ.

For Exp. 3, the cecal microbial diversity index of the 9 dietary treatments is summarized in Figure 3. Broilers fed the fine diets numerically showed a greater cecal microbial diversity compared with those fed the coarse diets. Dietary coarseness, however, did not significantly affect the cecal microbial diversity (5.90 in coarse vs. 5.96 in fine diets). Supplementation with BA and FE suppressed cecal microbial diversity in broilers fed both coarse and fine diets. The SBM diet suppressed cecal microbial diversity compared with those fed the experimental RSM diets (5.78 vs. 5.93). Again, a reduced microbiota diversity index seems to be related to an improved FCR and greater villus heights. These results seem to contradict those reported by Santos et al. (2007). These authors reported greater microbial diversity in the ileum of broilers fed a coarse triticale or corn based diet compared with those fed the fine diets. This contradiction may be due to different sites of observation (ceca vs. ileum) and/or differences in the dietary treatments.

Summarizing, a greater cecal diversity may be detrimental for health and performance of broilers because of the presence of different pathogenic microbial species that consume extra energy and nutrients for their development which may otherwise be used for the host growth. The greater cecal microbial diversity may indicate greater fermentation activities in the ceca of broilers fed fine diets compared with those on the coarse diets as fermentation in the ceca increases microbial diversity in broilers (Perez Mendoza, 2011). Another possible reason of a reduced cecal microbial diversity in broilers fed coarse diets may be due to the well-functioning gizzard, because there are studies indicating suppressed populations of different cecal microbial species such as *Salmonella* (Huang et al., 2006) and *C. jujeni* (Skånseng et al., 2013) in broiler ceca with well-developed gizzards.

Cecal Microbiota Populations

The effects of protein source and digestible CP levels on the average relative contribution of different cecal microbiota species in broilers from Exp. 1 are shown in Table 3. There were no statistically significant differences among the treatments (SBM vs RSM, and high vs low digestible CP levels) regarding the average relative contribution of different cecal microbiota species. There was a trend for *R. bromii* (P = 0.060) where a low DCP (15.8%) promoted their relative contribution in the cecal digesta.

The effects of protein source and diet structure on the relative contribution of different cecal microbiota species in broilers from Exp. 2 are presented in Table 4. There were no statistically significant differences among dietary treatments regarding the average relative contribution of *Bifidobacteria*, *Enterococcus*, *C. perfringens*, and *R. bromii* spp.

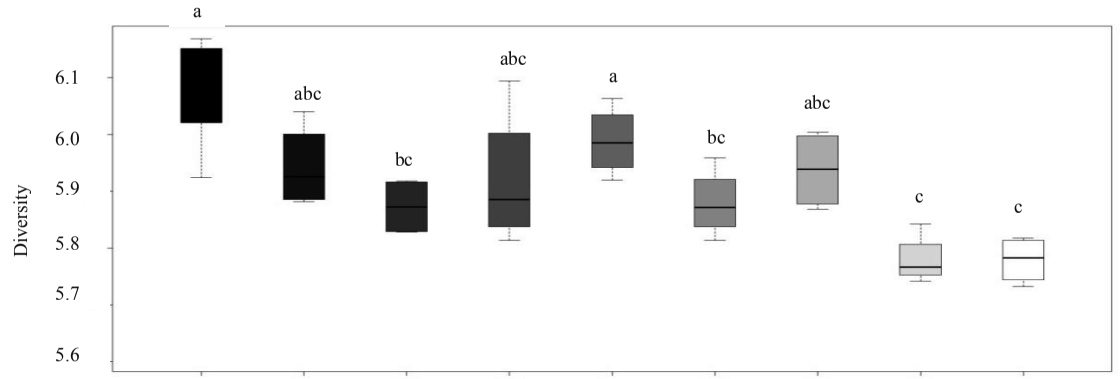

Figure 3. Boxplots of Shannon diversity index of the experimental diets, with horizontal lines representing the group means inside the box, whereas vertical bars indicating the standard errors.

BA- = without butyric acids, BA+ = with butyric acids FE- = without fermentable energy, FE + = without fermentable energy [a-c]Means without a common superscript significantly ($P < 0.05$) differ.

Table 3. Effects of protein source (PS) and digestible crude protein (DCP) levels on average relative contribution[1] (%) of cecal microbial selective phylogenetic groups in broilers.

Effects	*Bifidobacteria*	*Enterococcus*	*L. paracasei*	*Streptococcus*[2]	*C. lactifermentans*	*C. perfringens*	*E. coli*	*R. bromii*
SBM[3]								
DCP level (%)								
15.8	0.26	1.53	0.34	0.43	0.89	0.41	1.15	1.14
17.2	0.22	1.40	0.32	0.48	0.83	0.40	1.19	0.82
RSM[4]								
DCP level (%)								
15.8	0.24	1.32	0.35	0.60	0.83	0.43	1.06	1.26
17.2	0.19	1.92	0.37	0.82	0.84	0.47	1.08	0.94
Pooled SE	0.040	0.413	0.05	0.32	0.077	0.039	0.12	0.152
P-value								
PS	0.476	0.707	0.408	0.268	0.722	0.291	0.274	0.437
DCP	0.293	0.577	0.899	0.555	0.721	0.684	0.753	0.060
PS × DCP	0.863	0.397	0.682	0.720	0.691	0.562	0.874	0.981

[1]Each value is based on observations of 4 replicates (6 birds per replicate). [2]*Streptococcus* represents *S. bovis* and *S. suis.* [3]SBM = soybean meal. [4]RSM = rapeseed meal.

Protein source, however, resulted in an increased population of beneficial *C. lactifermentans* (P = 0.007) spp. with broilers fed the SBM diets having a greater relative contribution in the cecal digesta compared with those fed the RSM diets. An interaction (P < 0.001) existed for *L. paracasei*, indicating that in coarse diets the share of *L. paracasei* in the cecal digesta of broilers increased with decreasing level of dietary RSM, whereas *L. paracasei* was not affected by dietary RSM level in fine diets.

The dominance of *L. paracasei* and *C. lactifermentans* in the ceca of broilers fed large amounts of SBM in the diet may be due to the fact that less substrate is available for proliferation of pathogenic microbiota, because of the assumed better protein digestibility, particularly if the diet was coarsely ground. *L. paracasei* belongs to *Lactobacillus* spp. which is considered an excellent probiotic and is used for controlling necrotic enteritis caused by *C. perfringens* in broilers (Stephenson et al., 2010). Supplementation of *L. paracasei* as a probiotic in broilers, furthermore, increased phagocytic and bacterial activity in the distal GIT (Barug, 2006). This suppression of pathogenic microbiota resulted in favorable conditions for beneficial microorganisms. These findings were accompanied by an observed improved villus height and FCR in broilers fed the SBM diets compared with those fed the RSM diets (Figure 2). *Clostridium lactifermentans*, likewise, is a health beneficial microbiota found in the GIT of broilers, which can produce short chain fatty acids. Van der Wielen et al. (2002) reported that *C. lactifermentans* ferments lactate to acetate and propionate which inhibits the *in vitro* growth of *Salmonella*. Both acetate and propionate have the potential to reduce the growth of *E. coli* (Van der Wielen et al., 2000).

Dietary coarseness suppressed (P = 0.028) the average relative contribution of *E. coli,* where broilers fed the coarse diets had a lower average relative contribution compared with those fed the fine diets. This reduced contribution of gram-negative (*E. coli*) bacteria may be due to their acid intolerance. Optimum pH for *E. coli* growth is between 6.4 and 7.2 (Holt et al., 1994) and their growth is reduced at pH 4.6 (Glass et al., 1992). A reduction in gizzard pH from 3.88 to 3.24 in the fine diets compared with those fed the coarse diet, respectively, (Qaisrani et al., unpublished data) may have had an effect on the survival of *E. coli* when passing through the gizzard. This reduction in pH is due to greater stimulation of the gizzard and more acid secretion in the coarse diets fed broilers compared with those fed the fine diets. The gizzard, therefore, can be a critical control point for reducing *E. coli* in the ceca of broilers. These results are in accordance with those reported by Engberg et al. (2004), as these authors reported reduce populations of acid intolerant gram-negative bacteria (*Salmonella* and *Compylobacter*) in broilers fed whole wheat compared with those fed a pelleted diet.

Table 4. Effects of protein source (PS) and diet structure (ST) on average relative contribution[1] (%) of cecal microbial selective phylogenetic groups in broilers.

Effects	*Bifidobacteria*	*Enterococcus*	*L. paracasei*	*Streptococcus*[2]	*C. lactifermentans*	*C. perfringens*	*E. coli*	*R. bromii*
RSM[3] fine diets								
0%	0.34	1.05	0.26^{cd}	1.16	0.86^{abc}	0.31	1.23^{ab}	0.97
25%	0.45	1.00	0.27^{c}	1.19	0.85^{abc}	0.34	1.29^{a}	0.87
50%	0.44	1.12	0.26^{cd}	0.64	0.79^{cd}	0.66	1.17^{ab}	0.83
75%	0.37	1.12	0.24^{cde}	0.83	0.82^{bcd}	0.29	1.20^{ab}	1.00
100%	0.54	1.06	0.28^{bc}	0.93	0.81^{bcd}	0.34	1.16^{ab}	0.94
RSM coarse diets								
0%	0.71	0.95	0.28^{bc}	1.94	0.90^{a}	0.34	1.20^{ab}	0.91
25%	0.38	1.28	0.34^{a}	1.54	0.95^{a}	0.41	1.08^{b}	1.19
50%	0.45	1.24	0.31^{ab}	1.14	0.84^{abc}	0.39	1.12^{b}	0.99
75%	0.45	1.19	0.21^{e}	1.81	0.71^{d}	0.26	1.13^{b}	0.86
100%	0.36	1.00	0.22^{de}	1.34	0.78^{cd}	0.26	1.15^{ab}	0.88
Pooled SE	0.109	0.131	0.014	0.462	0.037	0.116	0.051	0.059
P-value								
PS	0.837	0.566	< 0.001	0.674	0.007	0.252	0.628	0.250
ST	0.603	0.472	0.325	0.051	0.659	0.453	0.028	0.268
PS × ST	0.165	0.632	< 0.001	0.951	0.082	0.619	0.336	0.004

[a-d] Means without a common superscript within a column and main effect significantly ($P < 0.05$) differ. [1]Each value is based on observations of 3 replicates (6 birds per replicate). [2]*Streptococcus* represents *S. bovis* and *S. suis.*[3]RSM = rapeseed meal.

Similarly, various studies in broilers fed coarse diets showed reduced pathogenic gut microbiota including *C. perfringens* (Bjerrum et al., 2005), and greater counts of beneficial microbiota and reduced *Coliform* (Gabriel et al., 2003). Dietary coarseness tended ($P = 0.051$) to promote the average relative contribution of *Streptococcus* spp. compared with those fed the fine diets. This S*treptococcus* spp. has health beneficial effects and improves performance and is used as a probiotic in broiler nutrition (Kabir, 2009). Reduced populations of pathogenic and enhanced populations of health beneficial microbiota may result in improved performance and the data in the present study also support these findings regarding the changes in cecal microbiota. Singh et al. (2014) in their review concluded that coarse grinding encourages the commensal microbiota and discourages the pathogenic and detrimental microbiota in the GIT by several mechanisms including better grinding by the gizzard, more hydrochloric acid secretion, and competitive exclusion.

The effects of diet structure, BA and FE supplementation on the relative contribution of cecal microbiota species in broilers of Exp. 3 are presented in Table 5. The results indicate that, contrary to Exp. 2, diet structure in this experiment did not affect ($P > 0.05$) the average relative contribution of any of the cecal microbiota species. Butyric acid supplementation, however, promoted the average relative contribution of *C. lactifermentans* ($P = 0.005$) and *R. bromii* ($P = 0.002$) spp., whereas BA suppressed the relative contribution of *C. perfringens* ($P = 0.003$). Fermentable energy supplementation, in contrast, promoted ($P = 0.036$) the average relative contribution of cecal *C. perfringens* compared with the diets without FE. *Ruminococcus bromii* plays a fundamental role in the fermentation of resistant starch in the human colon (Ze et al., 2012). However, its role in the broiler hindgut, to the knowledge of the authors, has not yet been thoroughly investigated. Fermentable energy supplementation was expected to enhance the growth of *R. bromii* due to their key role in fermentation of resistant starch. The present data, however, do not support these findings. The greater proportion of *R. bromii* in the cecal digesta of broilers fed BA supplemented diets may be due to the probiotic effects of BA because it has been suggested that populations of anaerobes such as *Eubacterium, Roseburia, or Ruminococcus* spp. can be enhanced through pre- and probiotic treatment in humans (Duncan et al., 2003). Similarly, higher populations of *C. lactifermentans* in broilers fed BA supplemented diets indicate that BA supplementation promoted the health beneficial microbiota because *C. lactifermentans* are non-pathogenic *Clostridium* spp. and are involved in the metabolism of a wide range of organic molecules, such as carbohydrates, amino acids, amines and organic acids (Leja et al., 2014). Reduced population of *C. perfringens* in broilers fed BA supplemented diets may be due to its bactericidal activity, as reported by Timbermont et al. (2010). *Clostridium perfringens*

Table 5. Effects of diet structure (ST), butyric acid (BA) and fermentable energy (FE) supplementation on average relative contribution[1] (%) of cecal microbial selective phylogenetic groups in broilers.

Effects	*Bifidobacteria*	*Enterococcus*	*L. paracasei*	*Streptococcus*[2]	*C. lactifermentans*	*C. perfringens*	*E. coli*	*R. bromii*
Control SBM diet[3]	0.19	2.20	0.36	0.51	1.00	0.42	1.27	0.98
Factorial analysis of the experimental RSM diets[4]								
ST: Fine								
With BA								
Without FE	0.23	1.66	0.34	0.58	0.88^{a}	0.29^{c}	1.09	0.95^{bc}
With FE	0.26	1.29	0.26	0.63	0.90^{a}	0.38^{ab}	1.05	1.13^{a}
Without BA								
Without FE	0.37	1.37	0.25	0.59	0.77^{b}	0.39^{ab}	1.08	0.89^{c}
With FE	0.35	1.66	0.32	0.50	0.86^{ab}	0.33^{bc}	1.14	0.98^{bc}
ST: Coarse								
With BA								
Without FE	0.23	1.36	0.29	0.51	0.93^{a}	0.32^{bc}	1.07	1.05^{ab}
With FE	0.20	1.72	0.35	0.50	0.94^{a}	0.36^{b}	1.11	0.99^{bc}
Without BA								
Without FE	0.28	1.40	0.26	0.61	0.83^{ab}	0.35^{bc}	1.09	0.95^{bc}
With FE	0.20	1.70	0.31	0.51	0.86^{ab}	0.44^{a}	1.11	0.88^{c}
Pooled SE	0.052	0.227	0.021	0.048	0.038	0.025	0.034	0.043
P-value								
ST	0.059	0.760	0.377	0.197	0.202	0.225	0.819	0.491
BA	0.068	0.855	0.093	0.907	0.005	0.038	0.289	0.002
FE	0.530	0.381	0.115	0.284	0.210	0.036	0.413	0.250
ST × BA	0.245	0.933	0.436	0.106	0.843	0.490	0.584	0.964
ST × FE	0.452	0.261	0.063	0.587	0.556	0.191	0.695	0.003
BA × FE	0.517	0.364	0.058	0.120	0.375	0.126	0.476	0.460

[a-c] Means without a common superscript within a column significantly ($P < 0.05$) differ. [1]Each value is based on observations of 4 replicates (6 birds per replicate). [2]*Streptococcus* represents *S. bovis* and *S. suis*. [3]Soybean meal, n = 4 replicates. [4]Rapeseed meal, n = 32 replicates.

is a gram positive, spore forming bacterium commonly found in the GIT of broilers and is associated with necrotic enteritis (Gholamiandekhordi et al., 2006). Their lower relative abundance may result in improved performance. This is confirmed by the present data that show a better performance of broilers fed BA supplemented diets compared with those fed diets without BA. Undissociated sodium butyrate lowers cell pH by penetrating inside the bacterial cell by dissociation into hydrogen and anion ions, which leads to energy deficiency in the bacterial cell. This finally results in bacteriostatic or bactericidal effects (Dahiya et al., 2006).

The overall results of the present study indicate that a coarse diet suppresses cecal microbial diversity, resulting in an improved villus heights and performance of broilers. A better digestible protein source (SBM) results in a greater relative contribution of health beneficial microbiota including *L. paracasei* and *C. lactifermentans*. Dietary coarseness was found (Exp. 2) to reduce pathogenic microbiota including *E. coli* and *C. perfringens* in cecal digesta. Butyric acid supplementation, similarly, promoted the relative contribution of commensal microbiota including *C. lactifermentans* and *R. bromii*, and suppressed the pathogenic *C. perfringens* in cecal digesta. Feeding a coarse diet to broilers, supplemented with BA, therefore, may be an effective strategy to promote health beneficial and suppress pathogenic microbiota in the ceca, thereby, enhancing villus heights and overall performance of broilers.

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CHAPTER 7

General discussion

Introduction

Demand for Poultry Meat

There is a growing demand for poultry meat throughout the world. The expected increase in meat demand, particularly in developing countries, is due to an increase in the population and also in the income and nutritional, and taste preferences of the customers. The world population is expected to increase to 7.51 billion by 2020 (USDA, 2013). Meat demand is also expected to increase by 16% during this period and especially poultry meat consumption is predicted to increase rapidly (20%) (Table 1).

Table 1. World meat production projections (Mt).

Meat type	2012	2020	% Increase
Cattle meat	66	74	12.8
Pig meat	112	127	13.7
Poultry meat	**102**	**122**	**19.8**
Sheep meat	13	16	17.2

Source: OEDC-FAO Outlook, 2011.

In order to accompany this growing demand for poultry meat, broilers are used due to their rapid growth rate because of a high capacity for protein deposition combined with a high feed intake as a result of genetic selection (Havenstein et al., 2003). To meet the higher demands for growth, broilers need highly digestible dietary protein and energy rich diets. An overview of different protein source used in broiler rations with their digestibility coefficients are presented in Table 2.

Table 2. Protein source and their apparent fecal digestibility (%) as used in a broiler rations.

Ingredient	Digestibility
Good digestible	
Lupin	92
Sesame seed	88
Soybean meal	85
Peas	85
Sunflower seed extract	83
Poorly digestible	
Rapeseed meal	74
Sorghum	73
Maize gluten	67

Source: CVB, 2007.

Among others, soybean meal (SBM) is the main vegetable protein source used in broiler rations due to its high crude protein content (44 to 50%), a balanced essential amino acid composition and a high digestibility of amino acids including lysine (91%). Its recent price volatility, however, suggests an urgent need for an alternative cost-effective protein source. Rapeseed meal (RSM), a by-product obtained from the

process of extracting the oil from rape seed contains somewhat less CP (35 to 40% CP; Zeb, 1998) compared to SBM. On the other hand, RSM has a high level of sulfur containing amino acids and these amino acids have an almost twice as high digestibility compared to SBM (CVB, 2007). That makes it quite useable for poultry diets. Because of its availability and lower price compared with SBM, it can be a good alternative vegetable protein source for broilers. However, its high levels of non-starch polysaccharides (NSP) approximately 20 to 40% of the meal (Slominski et al., 1999), limit its utility in broiler diets because of their negative effects on digestion of other nutrients by increasing digesta viscosity (Smits and Annison, 1999). Besides the disadvantage of its poor digestibility, several other reasons, including antinutritional factors such as glucosinolates, saponin and phytate limits the use of RSM in broiler diets (Khajali and Slominski, 2012; Ahmed et al., 2014). Glucosinolates reduce nutrient availability and break down into toxic aglucons. There are a number of glucosinolates, each of which breaks down into different products.

Protein Fermentation

Feeding a protein source with a lower digestibility, such as RSM, in a broiler diet may result in more undigested protein reaching the hindgut. This undigested protein along with endogenous amino acids enters in the ceca where these substrates are fermented by gut microbiota. In poultry, the ceca are major sites for fermentation (Meyer et al., 2013) probably due to longer residence time of digesta and high microbial density (Guo et al., 2003). Preferentially, carbohydrates are fermented by resident microbiota in the GIT but relatively large amount of undigested protein, combined with a low amount of fermentable carbohydrates, may lead to protein fermentation. Proteolysis is the first step in the utilization of protein by microbiota (Jeaurond et al., 2008). Subsequent deamination and decarboxylation of amino acids delivers a substrate which can be used as an energy source. In addition to the beneficial products like volatile fatty acids (VFA) and branched chain fatty acids (BCFA), some secondary products (lactate, succinate) and several gases such as methane, hydrogen, hydrogen sulfide (H_2S), and carbon dioxide, (Macfarlane et al., 1992) can be produced. Some of them can be detrimentral for the health of the host animal (Nollet et al., 1999). This fermentation changes cecal microbiota towards more proteolytic species including *Clostridium*, *Lactobacillus*, *Enterococcus*, *Enterobacteriaceae* and *Staphylococcus* spp. (Ladero et al., 2010). A high inflow of undigested material in the large intestine allows microbes to use the digesta as a substrate in order to proliferate. More specifically related to poultry, dietary protein source can influence microbial community by favoring the growth of (pathogenic) bacteria like *Campylobacter* spp. (Wise and Siragusa, 2007) and *C. perfringens* (Wilkie et al., 2005). This increase in pathogenic microbiota may enhance endogenous

losses and increase the maintenance requirements for replacing these losses thus compromising growth efficiency. Among other proteolytic fermentation products, an excess of biogenic amines are detrimental for the health of the host. In pigs, studies showed that greater concentrations of these protein fermentation products such as ammonia, BCFA, biogenic amines, indoles and phenols may lead to post weaning diarrhoea resulting in reduced growth rate (Gaskins, 2001; Kim et al., 2008). On the other hand, in humans, biogenic amines play an important role in brain activity, immune response, regulate body temperature, stomach pH, gastric acid secretion and cell growth and differentiation (Ladero et al., 2010). Some compounds (e.g. SCFA) contribute to the energy supply of the host, and also low concentrations of polyamines are important for enhancing gut development and colonic mucosa (Löser et al., 1999). However, there are studies indicating that greater levels of biogenic amines may result in disturbances in humans. A harmful intake level of different biogenic amines varies with specific biogenic amines. For instance, histamine intake > 50 ppm per person may lead to rash and itching, whereas above 100 ppm may lead to headache and nausea, and its intake > 1500 ppm may be lethal (Pieper et al., 2012). For tyramine, an intake of 600 mg by a healthy person is safe (Hazards, 2011).

To the author's knowledge, there are no published data for broilers showing the detrimental level of biogenic amines produced in the ceca. There are, however, studies indicating that dietary additions of different biogenic amines are detrimental for health and performance of broilers. Shifrine et al. (1960) studied the dose response of dietary supplementation of histamine in broilers by feeding 0.25, 0.50 and 1.0% histamine in the diet and reported a dose dependent decrease in performance and increase in proventriculus dilatation. Similarly, Barnes et al. (2001) studied the supplementation of 0.1 and 0.2% histamine in broiler diets and reported a 6.2 and 9.2% decrease in body weight gain, respectively, a poor feed conversion ratio (FCR) and lesions in the proventriculus. Some studies indicate that dietary supplementation of histamine resulted in increased gastric acid secretion leading to enlargement of the proventriculus (Shifrine et al., 1960; Harry et al., 1975). In contrast, putrescine supplementation at 0.2 and 0.4% of the diet resulted in a greater growth rate and improved feed efficiency in broilers compared with those on a diet without supplementation of putrescine, whereas supplementation of putrescine above 0.4% of the diet resulted in a decreased feed intake and poor feed conversion ratio (Smith, 1990). Tiihonen et al. (2010) reported an increased mortality (6.67 vs. 2.22%) at day 20 when broilers had a high concentration (698 vs. 455 nmol/g wet weight) of biogenic amines (tryptamine, putrescine, cadaverine, histamine, tyramine, spermidine and spermine) in the ileum.

Ammonia is formed from the deamination of amino acids, and is a toxic waste product of microbial fermentation. A high concentration in the large intestine of pigs can negatively affect the growth of intestinal epithelial cells (Nousiainen, 1991). The absorption of ammonia from the gut wall has toxic effects on enterocytes (Macfarlane et al., 1992). The increased level of ammonia in blood is converted into uric acid which requires 1 mole of glycine for each mole of ammonia in birds (Namroud et al., 2008). This may lead to amino acid loss resulting in a poor performance of the birds. Increased amount of blood ammonia resulted in a reduced BW, feed intake, poor FCR and greater liver weight (3.2 vs. 2.4 % of the weight of visceral organs) in broilers fed a low (17%) CP diet supplemented with essential amino acids compared with broilers fed a high (23%) CP diet (Namroud et al., 2008), which may be due to increased liver uric acid metabolism (Chapter 2).

Carbohydrate Fermentation

In contrast, fermentation of carbohydrates is considered beneficial because it results in the production of VFAs, which are used as an energy source, and utilization of ammonia (Guo et al., 2003). Fermentable carbohydrates can provide additional energy to microbes and may indirectly decrease the concentration of harmful compounds in the gut. If microbes receive energy from fermentation of carbohydrates, they will use protein as a substrate for their own growth and this will result in less protein fermentation. In an ideal situation, the unabsorbed dietary and endogenous amino acids in the large intestine will serve as building blocks for microbial protein synthesis with dietary carbohydrates used as an energy source. However, if there are insufficient carbohydrates available, the undigested protein and unabsorbed endogenous protein may promote the proliferation of microbiota which uses undigested amino acids as an energy source (Reid and Hillman, 1999). The extent to which hindgut protein fermentation occurs depends consequently on the availability of protein in relation to the amount of fermentable carbohydrates as these carbohydrates are a preferred source of energy for gut microbes (Rehman et al., 2008). As such, the digestibility of dietary protein and carbohydrates, and their dietary inclusion level are important to determine the amounts of protein and carbohydrates entering the hindgut and thus the potential for protein and/or carbohydrate fermentation (Gill and Rowland, 2002). The greater levels of dietary indigestible protein especially in the absence of proper levels of fermentable carbohydrates should be avoided for optimum performance and gut health of broilers (Guo et al., 2003). Protein fermentation in the hindgut can be altered by the availability of carbohydrates there (Younes et al., 1995). There are studies in pigs that indicate that the supplementation of fermentable carbohydrates increases carbohydrate fermentation and decrease protein fermentation products in the colon: fermentable carbohydrates supplementation (5% beet pulp) reduced the concentration of ammonia

(154 vs. 193 µg/ mL), tyramine (140 vs. 304 nmol/g DM) and spermidine (174 vs. 219 nmol/g DM) in the colon indicating lower fermentation of protein due to fermentable carbohydrate supplementation (Jeaurond et al., 2008). The supplementation of fermentable carbohydrates, therefore, may reduce the amount of undigested protein and result in beneficial modulation of microbiota in the hindgut. This may lessen hindgut protein fermentation as well as its harmful effects.

Aim of the Thesis

To the author's knowledge, no study in broilers has been published to dates which investigate hindgut protein fermentation by feeding poorly, ileal digestible protein sources. The impact of protein fermentation on performance and gut health of modern-day broilers is becoming increasingly relevant in view of the growing demand for dietary protein sources which are less digestible at an ileal level in poultry. The trend to search for cheaper sources of feed protein is due to the ever increasing price of highly digestible protein ingredients mainly used for humans and the aim of cost-effective broiler production. A low amount of fermentable energy is, furthermore, reaching the hindgut due to the use of highly digestible energy sources in poultry diets. Therefore, all the conditions in the hindgut may favour proteolytic fermentation. Therefore, the aim of the research reported in this thesis was to find ways to improve protein digestibility of poorly ileal digestible protein resources, to reduce potential hindgut protein fermentation by developing appropriate dietary strategies such as an adequate diet structure, the supplementation of organic acids and/or fermentable energy that may improve nutrient availability at ileal level and gut health in broilers.

From the literature, it could be concluded that some nutritional strategies may improve the ileal digestibility of protein, thereby, reducing hindgut protein fermentation (Chapter 2). The present study was performed to test the hypothesis that nutritional factors such as a coarsely ground diet and supplementation of butyric acid, and fermentable carbohydrates may improve ileal digestibility of poorly digestible protein, thereby improving gut development and overall performance of broilers.

A study was performed using three protein sources, soybean meal (SBM), rapeseed meal (RSM), and maize gluten (MG), with different ileal digestibility coefficients in Experiment 1 (Chapter 3). On the basis of the results of this study, two protein sources (SBM and RSM) were selected for further observations in Experiments 2 and 3 (Chapters 4 and 5). An overview of the experimental diets used in the research reported in this thesis is presented in Table 3. All experiments reported were conducted with one-day-old male Ross (308) broilers.

Table 3. Overview of the experimental factors and nutrient contents of the diets in three different experiments

	Experiment 1						Experiment 2					Experiment 3	
Ingredients													
Protein source	SBM		RSM		MG		RSM					SBM	RSM
Digestible CP inclusion level (%)	15.8	17.2	15.8	17.2	15.8	17.2							
Inclusion level (%)							0	25	50	75	100		
Particle size (fine vs. coarse)	No	No	No	No	No	No	Yes	Yes	Yes	Yes	Yes	No	Yes
Butyric acid (with vs. without)	No	No	No	No	No	No	No	No	No	No	No	No	Yes
Fermentable energy (with vs. without)	No	No	No	No	No	No	No	No	No	No	No	No	Yes
Calculated composition													
ME (Kcal/kg)	3014.3	3014.3	3014.3	3014.3	3014.3	3014.3	2835.0	2835.0	2835.0	2835.0	2835.0	2671.0	2680.5
CP	200.0	214.0	210.0	224.0	210.0	224.0	204.1	206.5	208.8	211.2	213.5	215.2	225.0
Indigestible CP	42.0	42.0	52.0	52.0	52.0	52.0	35.6	38.8	41.9	45.1	48.2	30.6	46.1
Digestible CP[1]	158.0	172.0	158.0	172.0	158.0	172.0	168.5	167.7	166.9	166.1	165.3	184.6	178.9
NSP[2]	154.0	147.9	167.2	171.5	185.7	185.2	184.9	185.8	186.8	187.7	188.6	158.5	180.0
Crude fiber	38.3	33.6	43.0	53.6	43.0	48.5	36.4	40.4	44.3	48.3	52.2	35.3	51.0
Digestible Lys	10.6	10.6	10.6	10.6	10.6	10.6	9.6	9.6	9.6	9.6	9.6	10.6	10.6
Digestible Met + Cys	7.7	7.7	7.7	7.7	7.7	7.7	7.2	7.2	7.2	7.2	7.2	7.7	7.8
Digestible Thr	8.0	8.0	8.0	8.0	8.0	8.0	7.2	7.2	7.2	7.2	7.2	7.8	7.8
Digestible Trp	2.2	2.2	2.1	2.2	2.1	2.2	2.5	2.5	2.5	2.5	2.5	2.2	2.2

MG = maize gluten, [1]Based on data from CVB (2007). [2]NSP = Non-starch polysaccharides, calculated by subtracting the CP, fat, starch, sugar, and ash content from the DM content.

Major Findings of the Thesis

It was expected that a RSM diet would result in more hindgut protein fermentation due to its poorer digestibility compared with a SBM diet. In the first experiment (Chapter 3), greater levels of protein fermentation products such as BCFA were found in the cecal digesta of broilers fed the RSM diet compared with those fed the SBM diet and results showed poor performance. The results from the subsequent Experiments 2 and 3 (Chapters 4 and 5), however, did not support these findings. Although performance of the broilers fed RSM diets was impaired in all three experiments. This poor performance in broilers fed RSM diets, however, may thus not be attributed to protein fermentation *per se*. There may be some other reasons including antinutritional factors and a high fiber content (Rutkowski et al., 2012).

Another major finding was that one of the dietary strategies, coarse grinding of the diet, helped to overcome part of the negative effects of a low digestible protein diet. In agreement with the expectation, lower concentrations of protein fermentation products (BCFA and biogenic amines) in the cecal digesta of broilers fed coarse diets compared with those fed the fine diets were observed. Other parameters such as growth performance was improved by coarse grinding of the diets due to its positive effects on gastrointestinal tract (GIT) development, especially enlargement of the gizzard. The improved gizzard was most likely related to enhanced digestibility of dietary ingredients such as protein (Chapter 5). The latter results were also confirmed by some recent studies in broilers (Liu et al., 2013; Pacheco et al., 2013). The improved gut development may have had effects on the digestibility of dietary ingredients, such as protein, fat, carbohydrates, resulting in lower substrate availability for fermentation in the hindgut. The results suggest that digestibility of dietary ingredients can be improved by coarse grinding of the diet for broilers.

Effects of Indigestible Protein Intake on Performance

In the present study, the amount of indigestible protein intake was inversely related to FCR (Figure 1; see also Table 3). Rapeseed meal fed broilers consumed more indigestible protein compared with those fed SBM diets. Broilers fed RSM diets had, therefore, poorer performance. There are several possible reasons, e.g. increased availability of glucosinolates and other antinutritional factors such as tannins (1.5 to 3%), phytic acid (3 to 6%), sinapine (0.6 to 1.8%) and NSP (18%) which may reduce amino acid digestibility at ileal level (Khajali and Slominski, 2012). Furthermore, these antinutritional factors bind minerals and can damage the gut wall (Bell, 1993). Although digestible essential amino acid levels were the same in all the diets, and glucosinolate contents has been reduced to one-twelfth of the original contents (10 vs.

120 µmol/g) in double zero RSM. Rapeseed meal inclusion still resulted in poorer performance in broilers.

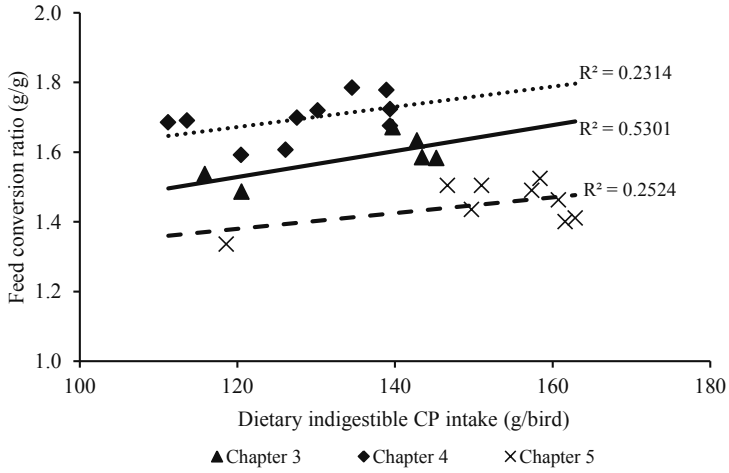

Figure 1. Relation between indigestible CP intake and feed conversion ratio of broilers.

It has been reported that poultry diets with 10% RSM contain 2.07 µmol/g glucosinolates. It is believed that levels in poultry diet should be less than 2.5µmol/g of diet (Payvastagan et al., 2012). A glucosinolate contents of 8 µmol/g of diet resulted in a severe growth depression in broilers (Tripathi and Mishra, 2007). Similarly, tannins (1.9 to 6.2%), phytic acid and sinapine (approximately1% of RSM) may affect the nutritive values of RSM. For instance, tannins and phytic acid affect protein digestion by the formation of insoluble complexes with protein and several minerals in the GIT (Khajali and Slominski, 2012). Also the high levels of NSP in RSM may increase digesta viscosity resulting in less absorption of nutrients (Saleem, 2013).

Effects of Villus Heights on Performance

Gastrointestinal development is partly responsible for the utilization efficiency of nutrients. This development can be assessed by the measurements of villus heights. Villus heights are functional units of the small intestine for absorption and digestion of nutrients, and their structural changes will affect absorption and digestion of nutrients (Zang et al., 2009). In the crypts, new cells are formed, and the villi and crypts are also the areas where activities of membrane-bound digestive enzymes in the small intestine can be found (Swatson et al., 2002).

In the present study, an inverse correlation was observed between villus heights and FCR of the broilers (Figure 2). Broilers fed highly digestible SBM diets (Chapters 3, 4, and 5) and those fed coarse diets (Chapters 4 and 5) had greater villus heights. Greater

villus height increases the surface area for absorption and digestion of nutrients resulting in improved performance, as shown by a reduction in FCR. The improved performance with greater villus heights is also supported by some recent findings in broilers as well (Zang et al., 2009).

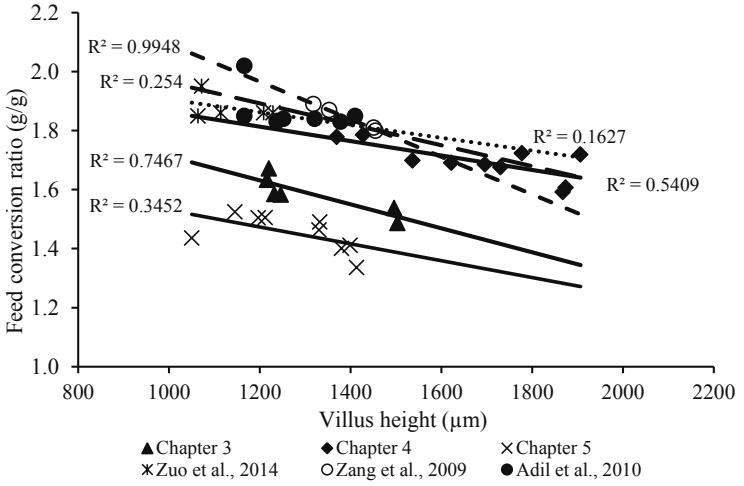

Figure 2. Relation between duodenal villus heights and performance in broilers.

Effects of Diet Structure on Gut Development and Proteolytic Fermentation

The effects of diet structure on gut development are presented in Table 4. Coarse diets improved the foregut, especially the gizzard, due to a more intense grinding of the coarse diets. The present study showed that the concentration of protein fermentation products including BCFA and biogenic amines linearly decreased with an increase in gizzard weight (Figure 3). This may be due to a better functioning of the gizzard in broilers fed the coarse diets. Coarse particles are retained in the gizzard for longer periods, resulting in a reduction in pH due to more hydrochloric acid secretion and an increase of reflux of the digesta, thereby improving protein digestibility (Liu et al., 2013; Pacheco et al., 2013). Dietary coarseness increases the gizzard size (Chapters 4 and 5) potentially due to increased frequency of gizzard contractions to grind the coarse particles. It has been shown that the gizzard selectively retains feed particles and gradually releases them into the small intestine for further digestion (Svihus, 2011). These results of a greater gizzard weight in coarse diet feeding are consistent with previously published data on coarse corn feeding (Jacobs and Parsons, 2013; Singh et al., 2014), and whole wheat feeding in broilers (Amerah and Ravindran, 2008). This better developed gizzard does increase gut motility and peristaltic movements between gizzard and proventriculus (Duke et al., 1992) which may improve nutrient digestibility.

Table 4. Effects (%) of diet structure on gut development in broilers.

Diet structure[1]	Age (d)	Gizzard weight	Duodenal villus height	Reference
Fine vs. coarse	34	+ 15	+ 18	Chapter 4
Fine vs. coarse	34	+ 14	+ 16	Chapter 5
Fine vs. coarse soybean meal	49	+ 11	-	Pacheco et al. (2013)
Fine vs. ground corn and soybean meal		+ 11	-	Jacob et al. (2010)
Fine vs. coarse	21	+ 34	+ 3	Amerah et al. (2008)
Complete ground vs. whole whet	44	+ 26	+ 6	Gabriel et al. (2008)
Pellet vs. 40% whole wheat	21	+ 39	No effect	Williams et al. (2008)
Pellet vs. 55% barley	28	+ 35	-	Svihus et al. (1997)

[1]fine, completely ground and pellet in these studies were referred to as a control diet.

It was confirmed in the present study that coarsely ground diets fed to broilers had improved ileal digestibility of protein. This improved digestibility resulted in lower concentration of fermentation products such as cecal BCFA and biogenic amines (Figure 3). Lower concentrations of isovaleric acid indicate lower availability of leucine

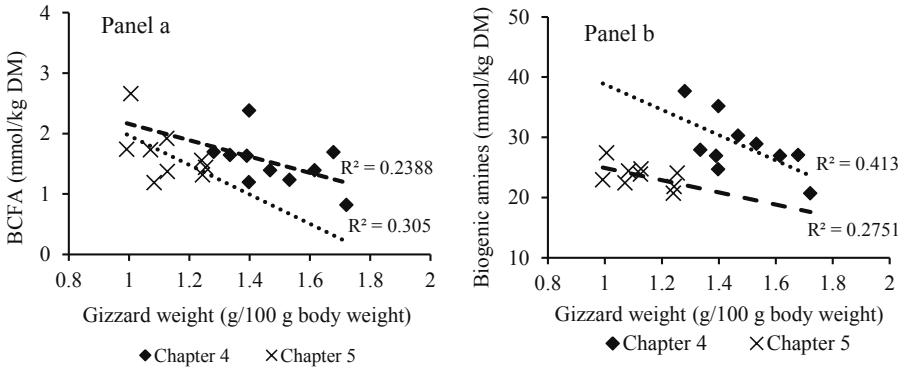

Figure 3. Relation between gizzard weight and branched chain fatty acids (BCFA; panel a) and biogenic amines (panel b) in the cecal digesta of broilers.

in the hindgut for proteolytic fermentation (Chapter 5). As BCFA and biogenic amines are distinctive end products of protein fermentation (Macfarlane et al., 1992), their lower concentration in broilers with a well-developed gizzard may indicate decreased protein fermentation. Dietary coarseness will result in a low pH in the gizzard probably due to more grinding activities because of coarse particles resulting in an extended retention time (Chapters 4 and 5). Previously, it has been reported that gut motility was improved in a well-developed gizzard (Ferket, 2000). A well-developed gizzard will increase cholecystokinin release (Svihus et al., 2004) which may stimulate pancreatic enzyme secretion and duodenal reflux (Duke, 1992).

Cecal Microbial Diversity and Performance

In the present study, greater cecal microbial diversity was associated with reduced villus heights leading to a poorer performance of broilers. A negative correlation was found between cecal microbial diversity and FCR (Figure 4). A greater cecal microbial diversity indicated an impaired gut health and performance of broilers. Broilers fed coarse diets had lower values of cecal diversity compared with those fed fine diets. This may be due to a better digestibility of dietary ingredients due to a well-developed gizzard which reduces the amount of substrate available for fermentation in the hindgut.

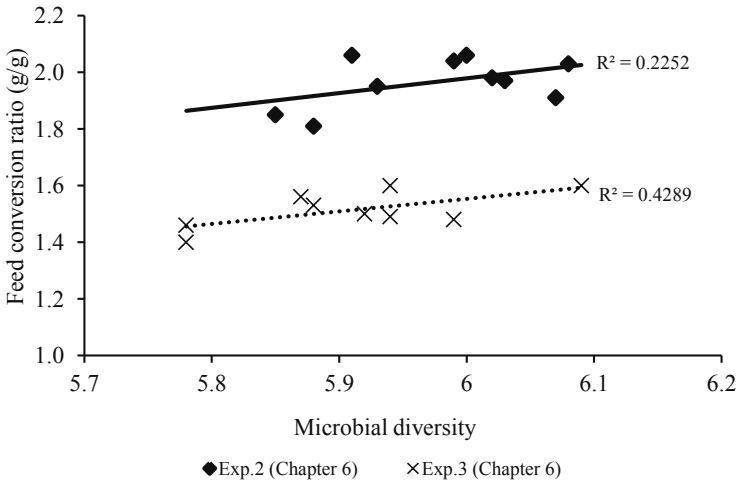

Figure 4. Relation between cecal microbial diversity and feed conversion ratio in broilers.

Furthermore, the greater cecal microbial diversity may indicate greater fermentation activities in the ceca of broilers fed fine diets compared with those on the coarse diets as fermentation in the ceca increases microbial diversity in broilers (Perez Mendoza, 2011). A reduced cecal microbial diversity in broilers fed coarse diets may be due to the well-functioning gizzard, because there are studies indicating suppressed populations of different cecal microbial species such as *Salmonella* (Huang et al., 2006) and *C. jujeni* (Skånseng et al., 2013) in broiler ceca with well-developed gizzards.

Effects of Butyric Acid Supplementation

It was expected that butyric acid (BA) supplementation improves apparent ileal digestibility of protein, thereby, lowering substrate availability for proteolytic fermentation in the hindgut. The results reported in the present study, however, do not support this. There was no effect of BA supplementation on ileal protein digestibility.

Butyric acid supplementation, however, improved the gut integrity resulting in less deeper crypts and leads to a greater villus height to crypt depth ratio and thus improved performance. This was in accordance with expectations. There are studies indicating that BA improves gut health and performance in broilers (Antongiovanni et al., 2009; Pouraziz et al., 2013). This improved gut integrity in broilers fed BA may be due to provision of more energy to gut cells because BA is a major source of energy for gut maintenance and cell proliferation (Smulikowska et al., 2010). The results of the present study encourage the supplementation of BA in a broiler diet.

Practical Implications of the Research

In broiler production, feed costs amount to approximately 70% of the total cost of production. The use of RSM as a protein source reduces broiler performance, thereby increasing production costs compared to the use of SBM. Application of coarse grinding and supplementation with BA, on the basis of the results reported in this thesis, will reduce these increased costs of broiler production. These nutritional strategies may allow the use of RSM (up to approximately 17% inclusion level) as an alternative protein source for broilers instead of more costly vegetable protein source such as SBM. Eliminating the grinding steps in feed processing will also save some energy, thereby reducing the total cost of feed production and hence the total cost of broiler production. Some economic calculations were performed, assuming that an increase of 1 g of BW gain/d and a decrease of 0.01 (g/g) FCR improves profitability by € 0.32/100 birds and € 0.58/100 birds, respectively (Vermeij, 2014; personal communication). The present data show approximately 7.0 g BW gain improvement and 0.075 (g/g) reduced FCR in broilers fed a coarse diet resulting in a net profit of approximately € 6.5/100 broilers (7×0.32 + 0.075×0.58 = 6.5) compared with those fed a fine diet. Similarly, BA supplementation shows approximately 2.4 g BW gain improvement and 0.05 (g/g) reduced FCR resulting in a net profit of approximately € 1.6/100 broilers (2.4×0.32 + 0.05×0.58 = 3.7 - 2.1 (cost of BA) = 1.6) compared with those fed a diet without BA.

Conclusions

The main conclusion of the work reported in this thesis is that RSM diets reduce performance and gut health, but these negative effects can be partially counterbalanced by coarse grinding and butyric acid supplementation. Some specific conclusions based on the results of the studies described in this thesis are:

1. Rapeseed meal is a low digestible protein source for broilers compared to SBM and results in a reduced growth performance and gut morphology.

2. Coarse diet feeding enhances the development of the foregut, in particular the gizzard, and it increases villus heights and reduces crypts depths in the duodenum.
3. The negative effects of a moderate inclusion of indigestible protein can be ameliorated to some extent by feeding a coarse diet.
4. The improved foregut development and villus height in the duodenum is associated with an enhanced ileal digestibility of protein, thereby allowing less protein available for fermentation in the hindgut.
5. Butyric acid supplementation improves growth performance and gut morphology.
6. Both SBM and dietary coarseness suppress cecal microbial diversity.
7. Dietary coarseness suppresses pathogenic bacteria and promotes health beneficial microbiota in the ceca.
8. Coarsely ground diet with a moderate inclusion of poorly digestible protein source, supplements with butyric acid, is a good strategy to improve the apparent ileal digestibility of protein and thus growth performance of broilers.

Suggestions for Future Research

The main recommendations for potential follow up work are:

1. Analysis of antinutritional factors especially glucosinolates in RSM may provide a better understanding of the diets.
2. Other low digestible protein sources, such as sorghum and cotton seed, should also be investigated in combination with certain coarseness in terms of protein fermentation, gut health and performance in broilers.
3. Measurement of ammonia concentration in blood as well as in digesta may give an appropriate estimate of ammonia produced in the ceca during fermentation and the quantity absorbed in the blood. This may provide a better idea of excreted ammonia as well.
4. Other products such as β-glucans, pro- and prebiotics can also be tested for their effects of improving protein digestibility and gut health when broilers are fed lower digestible protein diets.
5. The measurement of cholecystokinin in the blood with certain coarseness of the diet is suggested because it is believed that a well-developed gizzard increase its release.

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Summary

Background and Problem Statement

There has been, and still is, a great interest to replace soybean meal by cost effective protein sources such as rapeseed meal and maize gluten in animal feed. In poultry, this replacement, however, may result in a reduced performance due to, amongst others, lower ileal amino acid digestibility of alternatives, which may lead to hindgut protein fermentation. This hindgut protein fermentation can result in the production of harmful compounds such as ammonia, branched chain fatty acids, biogenic amines and different sulfur-containing compounds such as skatole, indole and phenolic compounds. This can negatively affect the cost of broiler meat production. When digestibility is low, gut health may be compromised affecting feed to gain ratios and overall performance. Apart from the economic losses, good digestible protein sources such as soybean protein may in the future be more and more used for human consumption. Thus, the challenge in poultry production is to develop new feeding strategies that meet the nutrient requirements of modern-day broilers, especially when they are fed a low ileal digestible protein source, i.e. rapeseed meal and maize gluten, thereby minimizing the interruption of their production performance.

Objectives of the Study

The aim of the studies reported in this thesis was to find ways to improve protein digestibility of poor digestible resources, to reduce potential hindgut protein fermentation by developing appropriate dietary strategies such as an adequate diet structure, the supplementation of organic acids and/or fermentable energy that improve nutrient availability at an ileal level and gut health in broilers.

The specific objectives of the present work were to:

1. Review various factors that influence hindgut protein fermentation in broilers and nutritional strategies that may reduce hindgut protein fermentation (Chapter 2).
2. Determine the effects of protein source, differing in fermentation characteristics, and of digestible dietary protein content on performance, gut morphology and cecal fermentation characteristics in broiler (Chapter 3).
3. Investigate the effects of diet structure combined with different levels of indigestible dietary protein source on performance and gut morphology in broilers (Chapter 4).
4. Study the main and interactive effects of protein source, diet structure, butyric acid and fermentable energy supplementations on performance, gut morphology and cecal fermentation characteristics (Chapter 5).
5. Analyze the effects of protein source, diet structure, butyric acid and fermentable energy supplementation on cecal microbiota population and composition (Chapter 6).

Major Findings of the Thesis

This thesis contains a literature review and describes the results of three experiments in which different nutritional strategies were tested. All experiments were conducted with the same strain of broiler (Ross-308).

Hindgut fermentation in broilers with special attention to protein fermentation, its detrimental effects on performance, gut health and on gut microbiota population was reviewed in Chapter 2. A wide range of harmful products such as ammonia, branched chain fatty acids, biogenic amines and different sulfur-containing compounds such as skatole, indole and phenols can be produced as a result of protein fermentation in the GIT. Greater concentrations of biogenic amines, branched chain fatty acids, hydrogen sulfide, ammonia, indole, phenols, cresol and skatole in the ceca indicate more proteolytic fermentation in this part of the gastrointestinal tract in broilers. Low concentrations of some of the protein fermentation products including biogenic amines are necessary for a normal gut development. This may result in distinct differences in protein digestibility when measured at the ileum and the total digestive tract. It was concluded that there is a limited research on the influence of protein fermentation in broilers and that the extent to which protein fermentation can influence performance of broilers is unknown. In addition, nutritional strategies, such as a reduction in dietary CP, supplementation of pre- and probiotics and organic acids, or feeding diets with coarse particles holds potential to increase ileal CP digestibility, thereby reducing the amount of substrate available for fermentation in the lower gastrointestinal tract.

In Chapter 3, effects of three major protein sources, soybean meal, rapeseed meal and maize gluten at two different digestible CP levels (15.8 and 17.2%) were studied to test the hypothesis that broilers fed a diet with high levels of indigestible protein, will result in a reduced growth performance, lower villus heights, deeper crypts, and more protein fermentation products in cecal digesta. In total, 288 one-day-old male broilers were used for this study. Broilers fed the soybean meal diet showed a better performance compared with those fed rapeseed meal and maize gluten diets due its greater ileal digestibility compared with the other two protein sources. High digestible CP (17.2%) diet fed broilers showed better performance compared with those on a low digestible CP (15.8%) diet. No significant effects of protein source as well as digestible CP level were found on gastrointestinal tract development, cecal ammonia and volatile fatty acid concentrations. Broilers fed soybean meal had improved duodenal morphology compared with those fed the rapeseed meal and maize gluten diets. A lower cecal pH and greater branched chain fatty acids concentrations in the cecal digesta were observed in broilers fed the rapeseed meal diet compared with those fed the soybean meal and maize gluten diets, indicating more proteolytic fermentation.

This study showed that protein source as well as digestible CP level affected growth performance, gut morphology and protein fermentation characteristics in broilers.

In Chapter 4, a hypothesis that a coarse diet improves performance of broilers fed a poorly digestible protein source was tested using 210 one-day-old broilers. A highly digestible protein diet based on soybean meal was gradually replaced by a low digestible protein diet based on rapeseed meal (RSM) in five steps (RSM-0%, RSM-25%, RSM-50%, RSM-75% and RSM-100%) with two diet structures (fine vs. coarse). An increase in indigestible dietary protein decreased the performance of broilers. Total cecal volatile fatty acid concentrations decreased from 209 to 126 mmol/kg DM digesta in broilers with increasing rapeseed meal in diets. Increase in the indigestible protein level, from RSM-0% to RSM-100%, decreased villus heights (1782 vs. 1574 μm), whereas crypt depths increased (237 vs. 274 μm). A coarse diet improved the performance with a 15% heavier empty gizzard weight and changed gut morphology. Coarseness of the diet reduced the empty weights of the crop, proventriculus and jejunum, and reduced gizzard pH by 16%. Protein fermentation indices such as branched chain fatty acids and biogenic amines were reduced by 24 and 12%, respectively, in the cecal digesta of broilers fed coarse diets compared with those fed fine diets. This contribution showed that feeding coarse particles improves the performance of broilers even with a poorly digestible protein source. Hindgut protein fermentation was reduced in broilers fed diets with a low CP digestibility by coarse grinding of the diet.

In Chapter 5, the effects of protein source, diet structure and supplementation of butyric acid and fermentable energy on growth performance and cecal digesta characteristics were investigated to test the hypothesis that a coarse diet supplemented with butyric acid and fermentable energy improves growth performance of broilers fed a poorly digestible protein source. The interaction effects of diet structure (fine vs. coarse), fermentable energy (with vs. without) and butyric acid supplementation (with vs. without) in a poorly digestible diet based on rapeseed meal were evaluated. Coarseness of the diet positively affected performance and improved relative empty gizzard weights by 14%, on average. The relative empty weights of the crop, duodenum, jejunum and ileum, were reduced in coarse diets fed broilers compared with those fed fine diets. Broilers fed coarse diet showed a 6% greater ileal protein digestibility, 20% lower gizzard pH, improved gut morphology, and 23% reduced cecal branched chain fatty acids compared with those fed the fine diets. Broilers fed butyric acid supplemented diets had improved performance and gut morphology compared with those fed the diets without butyric acid. Fermentable energy supplementation did not influence growth performance nor gut development and contents of total branched chain fatty acids and total biogenic amines in the cecal

digesta. Supplementation with fermentable energy, however, decreased the concentration of spermine in cecal digesta by approximately 31%. Feeding a coarse diet supplemented with butyric acid improves growth performance of broilers even if they are fed a diet containing a poorly digestible protein source. The negative effects of a low digestible protein source can thus be partly counterbalanced by coarse grinding and butyric acid supplementation in the diet.

In Chapter 6, the effects of protein source, diet structure, butyric acid and fermentable energy supplementations on cecal microbiota population and composition in broilers were evaluated. Cecal digesta samples collected in experiments 1, 2 and 3 were, therefore, analysed for some microbiota. The results indicated that cecal microbial diversity was suppressed by dietary coarseness. Similarly, butyric acid and fermentable energy supplementation also resulted in a lower microbial diversity. Soybean meal promoted the average relative contribution of health beneficial *L. paracasei* and *C. lactifermentans* spp. compared with those fed rapeseed meal. Dietary coarseness reduced the average relative contribution of *E. coli*. Butyric acid supplementation promoted the average relative contribution of *C. lactifermentans* and *R. bromii*, and suppressed the pathogenic *C. perfringens* in the cecal digesta. Fermentable energy, in contrast, promoted *C. perfringens*. Feeding a poorly digestible protein source, with coarse grinding and supplemented with butyric acid may be an effective strategy to promote health beneficial and suppress pathogenic microbiota in the cecal digesta.

Conclusions

The main conclusion of the work is that rapeseed meal diets reduce performance and gut health, but these negative effects can be partially counterbalanced by coarse grinding and butyric acid supplementation. Some specific conclusions based on the results of the studies described in this thesis are:

- Rapeseed meal is a poorer digestible protein source for broilers compared to soybean meal and results in reduced growth performance and gut morphology (Chapter 3).
- Coarse diet feeding enhances the development of the foregut, specially the gizzard, and it increases villus heights and reduces crypts depths in the duodenum (Chapters 4 and 5).
- The negative effects of moderate inclusion of indigestible protein can be ameliorated to some extent by feeding a coarse diet (Chapters 4 and 5).
- The improved foregut development and villus height in the duodenum is associated with an enhanced ileal digestibility of protein, thereby allowing less protein available for fermentation in the hindgut (Chapter 5).

- Butyric acid supplementation improves growth performance and gut morphology (Chapter 5).
- Both soybean meal and dietary coarseness suppress cecal microbial diversity (Chapter 6).
- Dietary coarseness suppresses pathogenic bacteria and promotes health beneficial microbiota in the ceca (Chapter 6).
- Coarsely ground diet with a moderate inclusion of poorly digestible protein source, supplements with butyric acid, is a good strategy to improve the ileal digestibility of protein and thus growth performance of broilers (Chapters 4 and 5).

Practical Implementations

Rapeseed meal can be used as a protein source in a broiler ration. Coarsely ground rapeseed meal, supplemented with butyric acid (approximately €2.1/100 broilers), can potentially replace soybean meal. It will not only provide a cheaper protein source but will also reduce the cost of feed production by reducing the steps associated with grinding the feed ingredients or using a roller mill instead of a hammer mill. These coarse particles will improve gut morphology and growth performance of broilers as well as increase the profitability of broiler feed producers and also broiler farmers (approximately €6/100 broilers). The most perspective feeding strategy to enhance the growth performance of broilers fed a poorly digestible protein source is the use of coarsely ground supplemented with butyric acid.

Samenvatting

Achtergrond en beschrijving van het probleem

Al sinds langere tijd is er een grote belangstelling voor het vervangen van sojaschroot in diervoeders door kostprijs-technisch gelijkwaardige alternatieven, zoals raapzaadschroot en maisglutenvoermeel. Deze vervanging kan bij pluimvee echter leiden tot verminderde dierprestaties als gevolg van onder andere een relatief lagere ileale aminozuurverteerbaarheid van de alternatieve eiwitten, wat kan resulteren in een toename van eiwitfermentatie in het laatste deel van het maagdarmkanaal. Bij deze eiwitfermentatie kunnen schadelijke stoffen vrijkomen, zoals ammonia, vertakte vetzuren, biogene aminen en verschillende zwavel bevattende componenten, zoals skatol, indol en fenolverbindingen. Deze componenten kunnen de productiekosten van pluimveevlees negatief beïnvloeden, omdat deze negatieve bijwerkingen van een lage eiwitverteerbaarheid de darmgezondheid aantasten en de dierprestaties, met name de voederbenutting, verminderen. Ondanks de economische aantrekkelijkheid zullen hoogwaardige eiwitbronnen, zoals sojaeiwit, in de toekomst meer en meer gebruikt worden voor humane consumptie. De uitdaging voor de pluimveehouderij is daarom om nieuwe voedingsstrategieën te ontwikkelen die voorzien in de nutriëntenbehoefte van hedendaagse vleeskuikens die eiwitbronnen verstrekt krijgen met een relatief lage ileale eiwitverteerbaarheid, zoals raapzaadschroot en maisglutenvoermeel, zodanig dat er een minimale verstoring is van de dierprestaties.

Doel van de studie

Het doel van de in dit proefschrift beschreven studie is het zoeken naar manieren om de eiwitverteerbaarheid van matig verteerbare eiwitbronnen te verbeteren en de mogelijke eiwitfermentatie aan het einde van het maagdarmkanaal te verminderen door het ontwikkelen van geschikte voedingsstrategieën, zoals gewenste voerstructuur, het verstrekken van organische zuren en/of fermenteerbare energie voor verbetering van de nutriëntbeschikbaarheid op ileaal niveau en voor verbetering van de darmgezondheid van vleeskuikens.

De specifieke doelstellingen van de huidige studie waren:

1. Via een literatuuronderzoek na te gaan welke factoren van invloed zijn op de eiwitfermentatie in het laatste deel van het maagdarmkanaal en welke voedingsstrategieën deze eiwitfermentatie kunnen verminderen (Hoofdstuk 2).
2. Vast te stellen wat de effecten zijn van eiwitbronnen die verschillen in fermentatiekarakteristieken, en van het gehalte aan verteerbaar eiwit in het voer op dierprestaties, morfologie van de darmwand en fermentatiekarakteristieken in de blinde darmen van vleeskuikens (Hoofdstuk 3).

3. Het onderzoeken van de gecombineerde effecten van voerstructuur en niveaus van onverteerbaar eiwit in het voer op dierprestaties en morfologie van de darmwand van vleeskuikens (Hoofdstuk 4).
4. Het bestuderen van de hoofd- en interactie-effecten van eiwitbron, voerstructuur en verstrekking van boterzuur en fermenteerbare energie op dierprestaties, darmmorfologie en fermentatiekarakteristieken in de blinde darmen van vleeskuikens (Hoofdstuk 5).
5. Het analyseren van de effecten van eiwitbron, voerstructuur en verstrekking van boterzuur en fermenteerbare energie op de microbiota populatie en samenstelling in de blinde darmen van vleeskuikens (Hoofdstuk 6).

Belangrijkste bevindingen van deze studie

Dit proefschrift bevat een literatuurstudie en beschrijft de resultaten van drie dierexperimenten, waarin verschillende voedingsstrategieën zijn onderzocht. In alle dierexperimenten is gebruik gemaakt van hetzelfde ras vleeskuiken (Ross-308).

Hoofdstuk 2 bevat een literatuurstudie naar het optreden van fermentatie in het laatste deel van het maagdarmkanaal van vleeskuikens, met speciale focus op eiwitfermentatie, en de schadelijke effecten ervan op de dierprestaties, de darmgezondheid en de microbiota populatie. Bij eiwitfermentatie kan in het maagdarmkanaal een heel scala aan schadelijke producten gevormd worden, zoals ammonia, vertakte vetzuren, biogene aminen en verschillende zwavelhoudende componenten, zoals skatol, indol en fenol. Hogere concentraties van biogene aminen, vertakte vetzuren, waterstofsulfide, ammonia, indol, fenol, cresol en skatol in de blinde darmen duiden op meer eiwitfermentatie in dit deel van het maagdarmkanaal van vleeskuikens. Lage concentraties van sommige van deze fermentatieproducten, zoals biogene aminen, zijn nodig voor een normale darmontwikkeling. Als gevolg van eiwitfermentatie verschilt de eiwitverteerbaarheid gemeten op ileaal niveau van die gemeten op fecaal niveau. Uit het literatuuronderzoek blijkt dat er slechts een beperkte hoeveelheid studies is uitgevoerd naar de effecten van eiwitfermentatie bij vleeskuikens en dat niet duidelijk is in welke mate eiwitfermentatie van invloed is op de dierprestaties van vleeskuikens. Wel is duidelijk geworden dat bepaalde voedingsstrategieën, zoals het verminderen van het ruw eiwitgehalte, het toevoegen van pre- of probiotica en organische zuren, of het verstrekken van voer met grove delen kunnen bijdragen aan het bevorderen van de ileale eiwitverteerbaarheid, zodat er minder substraat overblijft voor fermentatie in het laatste deel van het maagdarmkanaal.

In hoofdstuk 3 zijn de effecten van drie belangrijke eiwitbronnen, sojaschroot, raapzaadschroot en maisglutenvoermeel, en van twee niveaus van verteerbaar ruw

eiwit in het voer (15.8 en 17.2%) onderzocht om de hypothese te testen dat het verstrekken van voer met een hoog gehalte aan onverteerbaar eiwit aan vleeskuikens zal resulteren in verminderde dierprestaties, lagere villushoogte, diepere crypten en meer fermentatieproducten in de digesta van de blinde darmen. In dit onderzoek werden in totaal 288 mannelijke eendagskuikens ingezet. Vleeskuikens die voer met sojaschroot kregen presteerden beter dan vleeskuikens die voer met raapzaadschroot of maisglutenvoermeel kregen, wat samenhing met een hogere ileale eiwitverteerbaarheid van sojaschroot in vergelijking met de andere twee eiwitbronnen. Vleeskuikens die voer met een hoog gehalte aan verteerbaar eiwit (17.2%) kregen, hadden betere dierprestaties dan de kuikens die het voer met het lage gehalte aan verteerbaar eiwit (15.8%) kregen. Er waren geen aantoonbare effecten van de eiwitbronnen en van het verteerbaar eiwitgehalte van het voer op de ontwikkeling van het maagdarmkanaal en de gehalten aan ammonia en vluchtige vetzuren in de blinde darmen. Vleeskuikens die voer met sojaschroot kregen hadden een verbeterde darmmorfologie in vergelijking met kuikens die voer met raapzaadschroot of maisglutenvoermeel kregen. De blinde darminhoud van de kuikens die voer met raapzaadschroot kregen had een lagere pH en een hogere concentratie vertakte vetzuren ten opzichte van de kuikens die voer met sojaschroot of maisglutenvoermeel kregen, wat duidt op meer eiwitfermentatie. Dit onderzoek toont aan dat zowel de eiwitbron als het gehalte aan verteerbaar eiwit van invloed is op de dierprestaties, de darmmorfologie en de eiwitfermentatie-karakteristieken van vleeskuikens.

In hoofdstuk 4 is de hypothese onderzocht dat het toevoegen van grove delen aan voer dat een matig verteerbare eiwitbron bevat zorgt voor verbetering van de dierprestaties van vleeskuikens. In dit onderzoek werden 210 eendagskuikens ingezet. In het voer werd een goed verteerbare eiwitbron, sojaschroot, in toenemende mate vervangen door een matig verteerbare eiwitbron, raapzaadschroot (RSM). Dit resulteerde in vijf niveaus aan raapzaadschroot: RSM-0%, RSM-25%, RSM-50%, RSM-75% en RSM-100%. Elk niveau van raapzaadschroot werd gecombineerd met twee voerstructuren (fijn versus grof). Een toename van het gehalte aan onverteerbaar eiwit in het voer leidde tot een afname van de dierprestaties. Naarmate het aandeel raapzaadschroot in het voer toenam, daalde het gehalte aan totale vluchtige vetzuren in de blinde darmen van 209 naar 126 mmol/kg droge stof digesta. Bij een toenemend aandeel raapzaadschroot in het voer daalde de lengte van de villi van 1782 naar 1574 µm, terwijl de diepte van de crypten toenam van 237 naar 274 µm. Het verstrekken van voer met grove delen verbeterde de dierprestaties, zorgde voor een 15% zwaardere lege spiermaag en verbeterde de darmmorfologie. Grof voer verminderde het gewicht van de lege kliermaag, proventriculus en jejunum, en verlaagde de pH in de spiermaag met 16%. Als gevolg van het verstrekken van voer met grove delen daalde de

eiwitfermentatie. De gehalten van vertakte vetzuren en biogene aminen in de digesta van de blinde darmen, die als indicatoren van eiwitfermentatie gezien worden, daalden met respectievelijk 24 en 12% als grof voer in plaats van fijn voer werd verstrekt. Uit deze studie blijkt dat het verstrekken van grof voer in staat is om de dierprestaties van vleeskuikens te verbeteren, zelfs als dit voer een matig verteerbare eiwitbron bevat. Eiwitfermentatie in het laatste deel van het maagdarmkanaal nam af als vleeskuikens een voer met een matige eiwitbron gekregen, waaraan grove delen waren toegevoegd.

In hoofdstuk 5 zijn de effecten onderzocht van eiwitbron, voerstructuur en toevoeging van boterzuur en fermenteerbare energie aan het voer op dierprestaties en karakteristieken van de blindedarm digesta. In dit onderzoek werd de hypothese getest dat een voer met grove delen, dat verrijkt is met boterzuur en fermenteerbare energie, resulteert in verbeterde dierprestaties van vleeskuikens die voer krijgen met een matig verteerbare eiwitbron. In deze studie werden de interactie effecten onderzocht van voerstructuur (fijn vs. grof), fermenteerbare energie (met en zonder) en boterzuur (met en zonder) bij vleeskuikens die voer met een matig verteerbare eiwitbron (raapzaadschroot) kregen. Grof gemalen voer verbeterde de dierprestaties en zorgde voor een gemiddeld 14% hoger gewicht van de lege spiermaag. Het relatieve gewicht van de lege krop, duodenum, jejunum en ileum nam af als kuikens grof gemalen voer kregen in vergelijking met kuikens die fijngemalen voer kregen. Kuikens die grof gemalen voer kregen lieten een toename zien van 6% in ileale eiwitverteerbaarheid, hadden een 20% lagere pH in de kliermaag, hadden een verbeterde darmmorfologie en een 23% verlaagd gehalte aan vertakte vetzuren in de blinde darmen in vergelijking met kuikens die fijn gemalen voer kregen. Kuikens die voer kregen waaraan boterzuur was toegevoegd hadden verbeterde dierprestaties en een betere darmmorfologie ten opzichte van kuikens die geen boterzuur verstrekt kregen. Het toevoegen van fermenteerbare energie aan het voer had geen effect op de dierprestaties, de darmontwikkeling en het gehalte aan vertakte vetzuren en biogene aminen in de blinde darminhoud. Het verstrekken van fermenteerbare energie resulteerde echter wel in een 31% verlaagd sperminegehalte in de blinde darminhoud. Het verstrekken van een grof gemalen voer waaraan tevens boterzuur was toegevoegd verbeterde de dierprestaties van de kuikens, zelfs als deze voer kregen die een matig verteerbare eiwitbron bevatte. De negatieve effecten van een matig verteerbare eiwitbron kunnen dus deels worden opgeheven door het verstrekken van grof gemalen voer, waaraan tevens boterzuur is toegevoegd.

In hoofdstuk 6 komen de effecten van eiwitbron, voerstructuur, boterzuur en fermenteerbare energie op de microbiota populatie en samenstelling van vleeskuikens aan de orde. Hiervoor zijn darmmonsters, die verzameld zijn in de blinde darmen van de kuikens uit de experimenten 1, 2 en 3, geanalyseerd op de aanwezigheid van

bepaalde darmbacteriën. De resultaten tonen aan de diversiteit van de microbiota in de blinde darmen verminderde door het verstrekken van grof gemalen voer. Ook het verstrekken van boterzuur en fermenteerbare energie resulteerde in een lagere microbiële diversiteit in de blinde darmen. Het toevoegen van sojaschroot als eiwitbron aan het voer zorgde voor een relatief hoger aandeel van de gunstige bacteriën *L. paracasei* en *C. lactifermentans* spp. in vergelijking met voer dat raapzaadschroot als eiwitbron bevatte. Door grof gemalen voer te verstrekken nam het relatieve aandeel van de *E. coli* bacteriën af. Toevoeging van boterzuur bevorderde het relatieve aandeel *C. lactifermentans* en *R. bromii* en verlaagde het relatieve aandeel van de pathogene bacterie *C. perfringens* in de digesta van de blinde darmen. Het verstrekken van fermenteerbare energie, daarentegen, zorgde juist voor een toename van het relatieve aandeel *C. perfringens*. Het verstrekken van een matig verteerbare eiwitbron in combinatie met grof malen en het toevoegen van boterzuur kan een effectieve strategie zijn om de darmgezondheid te bevorderen en pathogenen bacteriën in de blinde darmen te onderdrukken.

Conclusies

De belangrijkste conclusie van dit onderzoeksproject is dat raapzaadschroot in het voer resulteert in verminderde dierprestaties en darmgezondheid. Deze negatieve effecten kunnen echter ten dele worden opgeheven door het verstrekken van grof gemalen voer, waaraan boterzuur is toegevoegd. Enkele specifieke conclusies, gebaseerd op de resultaten van dit onderzoek dat beschreven is in dit proefschrift, zijn.

- In vergelijking met sojaschroot is raapzaadschroot voor vleeskuikens een slechter verteerbare eiwitbron en verstrekking ervan resulteert in verminderde dierprestaties en darmmorfologie (Hoofdstuk 3).
- Het verstrekken van grof gemalen voer bevordert de ontwikkeling van het voorste deel van het maagdarmkanaal, in het bijzonder de spiermaag, en het vergroot de lengte van de villi en vermindert de diepte van de crypten in het duodenum (Hoofdstuk 4 en 5).
- De negatieve effecten van een beperkte hoeveelheid onverteerbaar eiwit in het voer kunnen tot op zekere hoogte gecompenseerd worden door het verstrekken van grof gemalen voer (Hoofdstuk 4 en 5).
- De verbeterde ontwikkeling van het voorste deel van het maagdarmkanaal, wat onder andere blijkt uit langere villi, hangt samen met een verhoogde ileale eiwitverteerbaarheid, waardoor minder eiwit beschikbaar blijft voor fermentatie in het laatste deel van het maagdarmkanaal (Hoofdstuk 5).

- Boterzuur toevoeging verbetert de dierprestaties en de darmmorfologie (Hoofdstuk 5).
- Zowel sojaschroot als grof gemalen voer verlagen de microbiële diversiteit in de blinde darmen en bevorderen het aandeel gezondheid-bevorderende bacteriën (Hoofdstuk 6).
- Grof gemalen voer onderdrukt het aandeel pathogene bacteriën en stimuleert de gezondheid-bevorderende bacteriën in de blinde darmen (Hoofdstuk 6).
- Het grof malen in combinatie met toevoeging van boterzuur aan een voer dat een beperkte hoeveelheid van een matig verteerbare eiwitbron bevat is een goede strategie om de ileale verteerbaarheid van eiwit te verbeteren en daarmee de dierprestaties van vleeskuikens op niveau te houden (Hoofdstuk 4 en 5).

Praktische toepassingen

Raapzaadschroot kan gebruikt worden als eiwitbron in voer voor vleeskuikens. Grof gemalen raapzaadschroot, aangevuld met boterzuur (waarvan de kosten ca. € 2.10/100 kuikens bedragen) kan tot op zekere hoogte sojaschroot vervangen. Niet alleen is raapzaadschroot een goedkopere eiwitbron. Ook de kosten voor het produceren van het grove voer zijn lager, omdat er minder energie nodig is voor fijn malen van de grondstoffen. In plaats van een hamermolen kan dan gebruik gemaakt worden van een rollermolen. De grove deeltjes hebben een stimulerend effect op de darmmorfologie en de dierprestaties van de vleeskuikens en bevorderen daarmee de winstgevendheid van de vleeskuikenhouderij (met ca. € 6,00/100 vleeskuikens). De meest perspectiefvolle voedingsstrategie om de dierprestaties van vleeskuikens die voer met een matig verteerbare eiwitbron krijgen op niveau te houden, is het gebruik van grof gemalen voer waaraan boterzuur is toegevoegd.

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Shafqat Nawaz Qaisrani
Wageningen University, September 10, 2014.

Curriculum vitae

Shafqat Nawaz Qaisrani was born on April 3, 1980 in Taunsa Sharif, District Dera Ghazi Khan, Pakistan. After completing higher secondary school education in 1998, he started to study at the University of Agriculture Faisalabad (UAF) Pakistan, where he obtained his 4-year B.Sc. (Hons.) Animal Husbandry degree in 2002. He continued his study at the University of Veterinary & Animal Sciences, Lahore, Pakistan for Doctor of Veterinary Medicine (DVM) degree in 2004. He joined Livestock and Dairy Development Department (L&DD) Govt. of the Punjab as a Veterinary Officer (Health) in 2005 and served there for 2 years. In 2007, he was awarded an "Overseas MS leading to PhD scholarship" by the Higher Education Commissions (HEC) of Pakistan in collaboration with the Netherlands organization for international cooperation in higher education (NUFFIC). In September 2008, he came to the Netherlands where he joined the MSc programme in Animal Sciences with a specialization in Animal Nutrition at Wageningen University, the Netherlands. In September 2010, he got admission to the PhD programme under the supervision of Dr. M.M. van Krimpen, Dr. R.P. Kwakkel, and Prof. Dr. W.H. Hendriks at Wageningen University. The main target of his research was to improve protein digestibility of poor ileal digestible resources to reduce potential hindgut protein fermentation by developing appropriate dietary strategies such as adequate diet structure, supplementation of organic acid and fermentable energy. This PhD research work resulted into this thesis.

Email: qaisrani111@yahoo.com

: qaisranino.1@gmail.com

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- **Qaisrani, S.N**., M. van Krimpen, R.P. Kwakkel, M.W.A. Verstegen and W.H. Hendriks (2014). Diet structure, Butyric acid, and Fermentable Energy Influence Growth Performance, Gut morphology and Cecal Fermentation Characteristics in Broilers. In: Proceedings of the 14th European Poultry Conference, Stavanger, Norway, 23-27 June, 2014.

Training and Supervision Plan		
Name	**Shafqat Nawaz Qaisrani**	
Group	Animal Nutrition	
Daily supervisors	Dr. ir. Marinus van Krimpen, Dr. ir. Rene P. Kwakkel	
Supervisor	Prof. dr. ir. Wouter H. Hendriks	
The Basic Package	**Year**	**Credits***
WIAS introduction course	2011	1.5
Ethics and philosophy of life sciences	2010	1.5
International conferences		
19th European symposium on poultry nutrition, Potsdam, Germany	2013	1.2
XIVth European poultry conference, Stavanger, Norway	2014	1.5
Seminars and workshops		
Dietary lysine and the importance of processing of food and feedstuffs, Wageningen, The Netherland	2010	0.15
Nutrition and welfare issues in poultry production, Wageningen, The Netherlands	2011	0.3
Scientific research in animal welfare; Do we make a difference? Wageningen, The Netherlands	2011	0.15
How to write a world class article?	2011	0.15
100 Doctors of Philosophy in Animal Nutrition; Which questions can possibly remain?	2012	0.3
WIAS Science Days (4x)	2010-14	1.2
Presentations		
Poster in XXIV Poultry congress, Brazil	2012	1.0
WIAS science day (Poster presentation)	2012 & 13	2.0
Poster presentation at 19th European symposium on poultry nutrition, Potsdam, Germany	2013	1.0
Oral presentation at XIVth European poultry conference, Stavanger, Norway	2014	1.0
Disciplinary and interdisciplinary courses		
Tropical farming systems with livestock	2013	2.0
Feed evaluation science	2013	1.5
Advanced statistics courses		
Design of experiment	2010	1.0
Statistics for life sciences	2011	2.0
Professional Skills Support Courses		
Project and time management	2010	1.5
Effective behaviour in your professional surroundings	2011	0.7
Information literacy for PhD and introduction to Endnote	2011	0.6
Moral dilemmas in daily scientific practices	2011	1.2
Competence assessment	2011	0.3
Techniques for writing and presenting scientific papers	2012	1.2
High impact writing in science	2012	1.3
Stress identification and management	2012	0.15
Reviewing a scientific paper	2012	0.15
Voice matters: voice and presentation skills training	2012	0.3
Carrier perspective	2013	1.6
Research Skills Training		
Preparing own PhD research proposal	2010	6.0
Supervising practical and excursions		
Supervising M.Sc. students (2)	2012-13	4.0
Practical demonstration: B.Sc. course “Principles of Animal Nutrition”	2013	1.5
Total		38.8

* One ECTS credit equals a study load of approximately 28 hours.

Colophon

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Mirian Hendriks

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