Protein digestion kinetics in pigs and poultry

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Chapter 1

General introduction

Importance of sustainable feeding of pigs and poultry

The global human population is expected to increase to 9.1 billion by 2050 and to 11.2 billion by 2100 (UN, 2015). To feed the rapid growing global population, 70 to 100 % more food is required compared to the current production to provide global food security (Godfray et al., 2010). By 2050, meat production is expected to increase to 455 billion kg, of which pig and poultry meat accounts for approximately 31 and 40 %, respectively (Steinfeld et al., 2006). To meet this increasing worldwide demand for animal meat, more feed ingredients, in absolute quantity, are needed to pig and poultry feed manufacturers. The competition between human food and animal feed ingredient cultivation on the limited arable land, however, will likely result in a shortage of animal feed ingredients in the future (FAO, 2009). To overcome the challenge of a global shortage and increasing prices of current protein sources, a multi-targeted approach is needed. Increasing the utilization efficiency of current protein sources as well as development of alternative protein sources are considered as main strategies for sustainable feeding of pigs and poultry in the future. Insects, for example, are potential alternative protein sources which can be used in pig and poultry diets (Veldkamp et al., 2012). They not only contribute to the protein fraction in the diet but also are a good source of fat, minerals and vitamins. In addition to insects, seaweeds, algae and yeast are also considered potential alternative protein sources. To increase the utilization efficiency of protein sources, it is necessary to further elucidate the factors limiting protein digestion as well as factors influencing the post-absorption utilization of dietary protein in monogastric farm animals.

Digestion of dietary protein in pigs and poultry

In the gastrointestinal tract (GIT) of pigs and poultry, dietary protein requires to be broken down into di- and tri-peptides or free amino acids (AAs) prior to absorption by enterocytes in the small intestinal mucosa (Webb et al., 1992; Ganapathy et al., 2000). In pigs, digestion of dietary protein starts in the stomach by the action of pepsin and hydrochloric acid. In poultry, digestion of dietary protein can already take place in the crop by microbial fermentation (Rehman et al., 2007), followed by the hydrolysis by pepsin and hydrochloric acid in the proventriculus and gizzard. Hydrolysis of protein by pepsin is affected by the AA residue at the amino group end of peptide bonds. In general, pepsin rarely cleaves the carboxyl end of histidine and lysine (Hamuro et al., 2008). After gastric digestion, dietary protein is further digested by pancreatic proteases

in the small intestine (i.e. trypsin, chymotrypsin, elastase, and carboxypeptidase A and B). Trypsin and chymotrypsin are endopeptidases, Trypsin cleaves the carboxyl end of arginine and lysine, and chymotrypsin cleaves the carboxyl end of phenylalanine, tyrosine, tryptophan, valine and leucine (Riviere and Tempst, 2001). Elastase and carboxypeptidase A and B are exopeptidases, which release AAs from the carboxyl terminal side of peptides. Elastase releases alanine, glycine and serine, and carboxypeptidase A and B releases aromatic AAs and basic AAs, respectively (Folk et al., 1960; Riviere and Tempst, 2001). The final stage of dietary protein digestion occurs at the brush border membrane of the small intestinal mucosa, which involves several peptidases including endopeptidases, carboxypeptidases, and aminopeptidases (Erickson and Kim, 1990).

Dietary protein can also be fermented by the commensal microbiota in the GIT. Proteolytic fermentation predominantly occurs in the colon of pigs and the caeca of poultry. Proteolytic fermentation not only produces volatile fatty acids (VFAs), which can be used as an energy source by animals, but also potentially toxic metabolites such as ammonia, amines, volatile phenols and indoles, which have a negative impact on gut health and animal performance (Williams et al., 2001). For example, ammonia produced by proteolytic fermentation can disturb the development of the intestinal mucosa and reduce villus height (Visek, 1984; Nousiainen, 1991).

Ileal and faecal protein digestibility differ substantially among commonly used feed ingredients in pigs and poultry diets (Table 1.1). The extent of protein digestion can be affected by the intrinsic characteristics of proteins present in protein sources. The number and accessibility of cleavage sites for proteases largely depend on the AA sequence of polypeptide chains (chemical composition) and their conformation, which is determined by the AA sequence. For instance, rapeseed albumins (napin) showed a higher resistance to pepsin hydrolysis *in vitro* compared to rapeseed globulins (cruciferin) (Malabat and Rabiller, 2001). This is likely due to the compact conformation of napins due to the presence of disulphide bonds (Schwenke et al., 1988). Moreover, *in vitro* protein digestibility was negatively correlated to the proportion of intramolecular β-sheet structures but positively correlated to the ratio between α-helix and β-sheet structures in plant protein sources (Carbonaro et al., 2012; Rubio et al., 2014). With respect to physicochemical properties, a water-soluble form of casein (i.e. Na^+-caseinate) was digested approximately 2-fold faster *in vitro* than water-insoluble casein at pH 8.0 (Tonheim et al., 2007). The extent of digestion, however, did

not differ for both soluble and insoluble caseins. In rapeseed meal, the soluble protein fraction showed a faster hydrolysis rate but a lower extent of hydrolysis than the insoluble protein fraction (Salazar-Villanea et al., 2017). The non-protein constituents can also interact with the proteins in the protein source and, therefore, affect protein digestion. For instance, tannins present in legume seeds and phytate present in soybeans and other protein sources of plant origin can bind to proteins. The bound proteins are not susceptible to enzymatic hydrolysis by digestive enzymes, leading to a reduced protein digestibility in pigs (Mangan, 1988; Selle et al., 2012).

Table 1.1. Protein content and protein digestibility of commonly used feed ingredients in pig and broiler diets.

Feed ingredient	Protein content (g/kg)	Digestibility (%)[1]			
		Pig		Broiler	
		SID	AID	SID	ATTD
Cereal grains					
Maize	64 - 88	82	69	90	83
Wheat	85 - 139	89	80	88	81
Barley	76 - 124	80	70	90	70
Rice	69 - 87	95	82	-	82
Sorghum	66 - 108	84	73	86	76
Oat	66 - 138	76	66	-	75
Plant protein sources					
Pea	170 - 236	79	74	76	87
Lupins	284- 440	87	84	86	90
Soybean meal (fibre < 4.5 %)	438 - 498	88	85	90	87
Soybean meal (fibre > 4.5 %)	390 - 485	86	83	-	85
Rapeseed meal	308 - 403	72	70	76	76
Sunflower meal	324 - 438	80	78	84	85
DDGS-maize	238 - 292	73	69	-	-
DDGS-wheat	246 - 402	77	74	-	-
Animal protein sources					
Fish meal	506 - 749	85	83	80	88
Meat bone meal	413 - 497	59	57	65	73

[1] SID= standardized ileal digestibility; AID= apparent ileal digestibility; ATTD= apparent total tract digestibility. Reference: Lemme et al. (2004); CVB (2016).

Apart from the intrinsic characteristics of protein sources, protein digestion is also significantly affected by the digestive capacity of animals. Broilers showed a higher (i.e. 2-10 %) ileal protein digestibility than pigs for most cereal grains and vegetable protein sources (Table 1.1). This is likely due to the fact that broilers have a greater size of the GIT relative to their body than pigs (Table 1.2). In addition, protein digestion requires adequate interactions between digestive enzymes and dietary protein. A fast

passage rate of digesta along the GIT, therefore, might hinder protein digestion. On average, pigs have a longer retention time of digesta in the stomach and the small intestine than poultry (Weurding et al., 2001; Wilfart et al., 2007; Liu et al., 2013). The passage rate of digesta along the GIT depends also on the feeding pattern (i.e. feeding frequency and meal quantity) and the physicochemical properties (e.g. solubility, viscosity, and water binding capacity) of digesta. A large volume of a meal (Hunt and Stubbs, 1975), a high solubility of digesta (Low et al., 1978) and an increase in dietary fibre content (Wilfart et al., 2007) decrease the passage rate of digesta along the GIT.

Table 1.2. Comparison of the dimensions of the gastrointestinal tract (GIT) of pigs and broilers.

Item	Pig	Broiler
Body weight (kg)	50	1.5
Weight relative to live body weight (%)		
GIT	3.5	7.8
Stomach	0.5	3.0[1]
Small intestine	1.6	3.3
Length of small intestine (cm)	1750	163
Length relative to small intestine length (%)		
Duodenum	4	20
Jejunum	91	35
Ileum	4	45

[1] The weight of proventriculus and gizzard. Reference: Barea et al. (2011); Mabelebele et al. (2014).

Even though several factors related to both intrinsic characteristics of protein sources and to digestive physiology of animals have been attributed to the differences in the extent of *in vivo* protein digestion, current knowledge, however, is still limited and has not elucidated complicated mechanisms causing differences in protein digestion among protein sources.

Protein evaluation in pig and poultry diets

In current feed evaluation systems, the nutritional value of protein sources in pig and poultry diets is based on digestible AAs at the end of the ileum as dietary protein degraded in the hindgut by microbial fermentation, does not significantly contribute to AA supply for animals (Lemme et al., 2004; Ravindran et al., 2005; NRC, 2012; CVB, 2016).

Protein and AA digestibility of protein sources can be evaluated via both *in vitro* and *in vivo* approaches. *In vivo* ileal protein and AA digestibility are determined and calculated using ileal digesta collected via ileal-cannulated animals or sampled under anaesthesia of animals. Compared to *in vivo* approaches, *in vitro* approaches are relatively fast and cheap methods, that can be used to obtain an estimate for the protein digestibility of different feed ingredients. Various *in vitro* methods have been developed to estimate protein and AA digestibility of feed ingredients for pigs (Hsu et al., 1977; Babinszky et al., 1990; Boisen and Fernández, 1995; Huang et al., 2000). In these methods, feed ingredients are incubated sequentially with different enzymes, such as pepsin, pancreatin, trypsin, chymotrypsin and peptidases, to simulate gastric and intestinal protein digestion. Generally, these methods are static methods. They generate a single *in vitro* digestibility value for nitrogen (N) or crude protein (N × 6.25) after a fixed period of incubation. The *in vitro* digestibility of protein is determined as the solubility of N in the sample, assuming that N present in soluble form is absorbed in the GIT *in vivo*. However, around 50 % of unabsorbed N was present as soluble N in ileal digesta of pigs (Hulshof et al., 2016), indicating that this assumption is incorrect. Moreover, endogenous protein secretions in the GIT and the transition of digesta along the GIT are not taken into account in these static methods. As a consequence, *in vitro* digestibility values are not well-correlated with *in vivo* digestibility values (Table 1.3).

Compared to static models, a multicompartmental and dynamic model, such as the TNO intestinal model (TIM), might have a more accurate simulation of protein digestion *in vivo*. This model is able to simulate peristaltic mixing and transition of digesta, continuous addition of endogenous secretions, and absorption of digestion end products via dialysis (Minekus et al., 1995). However, it can still not fully mimic *in vivo* digestion due to the limitations on simulating responses of the intestinal mucosa and commensal microbiota to dietary compounds.

Table 1.3. Overview of *in vitro* protein digestibility of feed ingredients using different incubation conditions.

Reference	Enzymes	pH	Time	Temperature	Ingredient	*In vitro*	*In vivo*
Hsu et al., 1977	Trypsin Chymotrypsin Peptidase	8.0	10 min	37 °C	Wheat flour	82	86
					Soy concentrate	90	90
					Cottonseed meal	88	87
					Full lactose whey	85	81
Babinszky et al., 1990	Pepsin	1.0	1.5 h	40 °C	Soybean meal	88	90
	Pancreatin	6.8	1.0 h	40 °C	Linseed expeller		
					Lupin	83	91
					Rapeseed meal	77	81
Boisen and Fernández, 1995	Pepsin Pancreatin	2.0 6.8	6h 18h	39 °C 39 °C	Soybean meal	93	78
					Rapeseed meal	84	69
					Sunflower meal	91	73
					Peas	96	80
					Meat and bone meal	85	56
					Skim milk powder	100	86
Huang et al., 2000	Pepsin Pancreatin	2.0 7.6	4h 24h	37 °C 37 °C	Fish meal (Peru)	58	83
					Fish meal (China)	48	81
					Rapeseed meal	56	89
					Cottonseed meal	52	86

Classification of fast and slow protein sources

The classification of fast and slow protein sources was first proposed by Boirie et al. (1997) when feeding casein and whey protein to human subjects. Both whey protein and casein are considered highly digestible in humans (Hambraeus and Lönnerdal, 2003). They, however, displayed differences in the timing and the extent of postprandial increase of plasma AAs. Whey protein induced a pronounced but transient postprandial increase of plasma AAs, whereas casein induced a smaller but more prolonged postprandial increase of AAs and peptides in plasma (Boirie et al., 1997). Thus, based on the timing and the extent of postprandial increase of plasma AAs and peptides, protein sources can be categorised into fast and slow protein sources (Boirie et al., 1997; Bos et al., 2003; Tang et al., 2009) (Figure 1.1). Usually, categorisation of fast and slow protein sources is done based on the judgement of the postprandial increase in plasma AA concentration. Analysis of the postprandial plasma AAs curves by calculating increase and elimination rates using mathematical equations are rarely done in studies comparing fast and slow protein sources. As a result, quantitative information on differences in protein digestion kinetics among protein sources is hardly available.

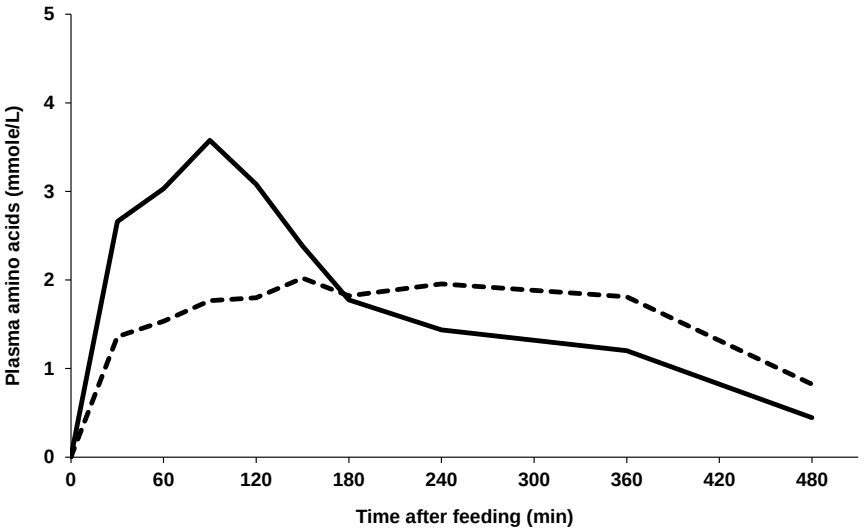

Figure 1.1. Example of postprandial increase of plasma amino acids of fast protein (solid line) and slow protein (dashed line) after meal ingestion based on the results of the present thesis (Chapter 3).

Factors affecting postprandial increase of plasma amino acids

The timing and the extent of the postprandial increase of plasma AAs are related to four aspects: 1) the passage rate of digesta along the GIT, 2) the hydrolysis rate of dietary protein, 3) the absorption rate of AAs and peptides by the small intestinal mucosa and 4) the metabolism of AAs and peptides by the small intestinal mucosa. Studies have shown that a more rapid and pronounced postprandial increase of plasma AAs could be related to a fast gastric emptying of dietary protein. In the study of Boirie et al. (1997), the rapid and pronounced postprandial increase of plasma leucine observed in human subjects ingesting whey protein might be due to the fast gastric emptying of whey protein. Whey protein remains soluble in the stomach, whereas casein coagulates. The liquid fraction is emptied faster from the stomach to the small intestine than the solid fraction (Low, 1990). Similar results were observed in a pig study, where milk fed in a gel form showed a delayed N flow from the stomach to the small intestine compared to pigs fed milk in liquid form, resulting in a delayed and less pronounced postprandial increase of plasma AAs (Barbé et al., 2013).

Apart from the gastric emptying, a fast hydrolysis of dietary protein and absorption of AAs can also be attributed to a more rapid increase of plasma AAs after ingestion of a

meal. It is demonstrated in human studies that ingestion of protein hydrolysates result in a faster and greater postprandial increase of AAs than ingestion of their non-hydrolysed equivalents (Calbet and Holst, 2004; Koopman et al., 2009; Morifuji et al., 2010). This can be related to protein hydrolysates requiring less hydrolysis in the small intestine prior to absorption in the form of peptides and free AAs. Another example is that in pigs, soy protein concentrate showed a more rapid portal appearance of AAs than a mixture of untoasted and toasted soybean meal after meal ingestion (Jansman et al., 1997). The difference might be partly explained by a higher trypsin inhibitor activity in the diet with a mixture of untoasted and toasted soybean meal, resulting in a lower rate of hydrolysis of dietary protein.

The intestinal mucosa also plays an important role in regulating the timing and determining the extent of postprandial appearance of plasma AAs due to its extensive metabolism of AAs. Amino acids are the major energy source for the intestinal enterocytes, of which glutamate is the main fuel for the intestinal enterocytes via glutaminolysis. In piglets, as much as 95 % of glutamate is metabolized by the intestinal enterocytes (Stoll et al., 1998). Apart from glutamate, glutamine and branched-chain AAs can be metabolised into glutamate and, therefore, are also highly catabolised in the intestinal mucosa with the purpose of energy production (Wu, 1998; Chen et al., 2007). In addition to energy production, AAs are also metabolised in the intestinal enterocytes for maintaining intestinal mucosal mass and for the synthesis of metabolites, such as glutathione and nitric oxide, which are critical for regulating integrity of intestinal mucosa (Wu, 1998).

Effects of fast and slow protein sources on post-absorption protein metabolism

An efficient utilisation of dietary AAs for muscle protein synthesis is economically important in production animals, particularly with the forthcoming global protein scarcity. Amino acids are used by organs and tissues, either or not after transformation into other AAs, to synthesise proteins or as an energy source after deamination. The efficiency of protein utilisation depends on the balance between these two processes, in which a higher efficiency relates to a higher body protein synthesis (Figure 1.2).

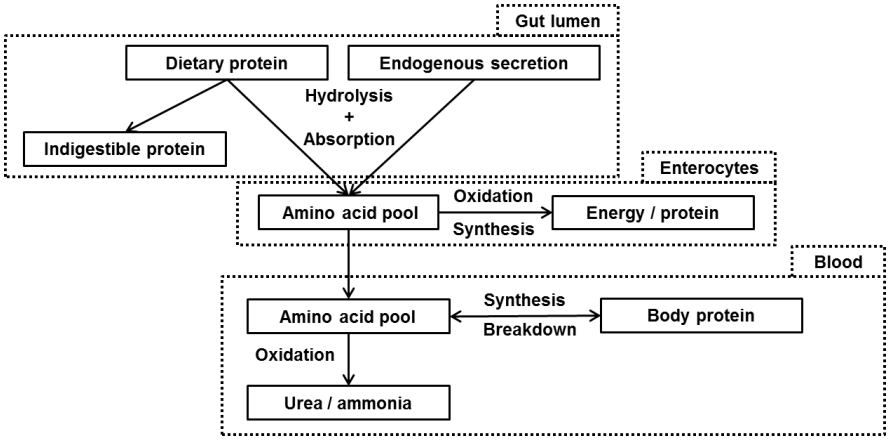

Figure 1.2. Schematic overview of post-absorption metabolism of dietary protein (adapted from Frühbeck, 1998 and Sauer et al., 2000).

Muscle protein synthesis is significantly stimulated after meal ingestion. This is due to the action of insulin, which increases protein synthesis in skeletal muscle (Davis et al., 1996; Svanberg et al., 1996). The effect of insulin on skeletal muscle protein synthesis, however, is 4.5 times higher in neonatal pigs than in weaning pigs (Davis et al., 1996). In addition to insulin, dietary essential AAs, especially leucine, exert a significant regulatory effect on muscle protein synthesis via the mammalian target of rapamycin (mTOR) pathway in both pigs and poultry (Suryawan et al., 2008; Deng et al., 2013).

The simultaneous availability of energy and AAs are prerequisite for protein synthesis (Geiger, 1950). Starch, provided by cereal grains such as wheat, maize and barley, is the main energy source in pig and poultry diets. Starch hydrolysis and glucose absorption mainly take place in the duodenum and proximal jejunum (Riesenfeld et al., 1980; Knudsen et al., 2006), whereas protein hydrolysis and AA absorption mainly take place in the jejunum and is completed in the ileum (Low, 1980; Sklan and Hurwitz, 1980). This suggests that dietary starch on average is digested faster than dietary protein along the GIT, which might lead to an asynchronous supply of energy and protein in the case of meal fed animals or humans. Indeed, pigs fed pea starch, a slowly digestible source, showed an increased essential AA flux into portal circulation by 12 % compared to pigs fed maize starch, a fast digestible starch (van der Meulen et al., 1997). The increase in AA appearance in the portal vein was suggested to be related to the release of glucose in the distal part of the small intestine from slowly digestible starch, thereby sparing AAs from being catabolised to produce energy. Broilers fed diets with more slowly

digestible starch increased body weight gain with 5 % and decreased feed conversion ratio by 2 % (Weurding et al., 2003). In addition, N retention was negatively correlated to the starch digestion rate in broilers fed sorghum-based diets (r = -0.39), indicating slowly digestible starch increased protein retention in broilers (Liu et al., 2013). Thus, the fate of dietary AAs in the post absorptive metabolism depends on the kinetics of protein digestion relative to the digestion kinetics of energy providing nutrients in the diet such as starch.

Formulation of the knowledge gaps

Protein digestion involves many different processes, including enzymatic hydrolysis of proteins and peptides, secretion of digestive enzymes, transit of digesta, absorption of peptides and AAs, and protein fermentation by intestinal microbiota. Most currently applied *in vitro* and *in vivo* techniques for determining protein digestibility provide end-point values related to the quantity of proteins absorbed from the GIT up to the end of the ileum, with or without the correction for basal endogenous protein loss. They, however, do not provide information on the kinetics of protein digestion, which could significantly affect the post-absorption metabolism of AAs originating from dietary protein. Although some studies focusing on protein digestion kinetics have been performed in humans, they mainly focused on milk proteins. The kinetics of protein digestion of feed ingredients used in pig and poultry diets remains largely unknown. Information on protein digestion kinetics of protein sources can be used to further develop the concept of synchronising the dietary supply of energy and protein, which could improve protein retention and overall protein utilisation efficiency in pigs and poultry.

Aim and outline of the thesis

The aim of the present thesis was to provide further insight into the digestion kinetics of dietary protein along the GIT of pigs and poultry. First, a modified two-step *in vitro* approach was applied to screen the kinetics of N solubilisation and the release of low molecular weight peptides (< 500 Da) for various protein sources (Chapter 2). Based on the *in vitro* results, five protein sources (i.e. soybean meal, rapeseed meal, wheat gluten, dried porcine plasma protein and black solder fly larvae) were selected for

further investigation. The *in vivo* protein digestion kinetics of these five protein sources were determined in both pigs (Chapter 3) and broiler chickens (Chapter 4). The *in vivo* protein digestion kinetics was evaluated with respect to the apparent disappearance of dietary crude protein fraction from the small intestine and the change in molecular weight distribution of proteins and peptides present in digesta. After the differences in protein digestion kinetics among protein sources, both *in vitro* and *in vivo*, were identified, the effects of synchronising dietary protein and starch using information on their kinetics of digestion on the growth performance and carcass characteristics in broilers were investigated (Chapter 5). Finally, the results presented in this thesis are discussed (Chapter 6).

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Chapter 2

In vitro protein digestion kinetics of protein sources for pigs

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ABSTRACT

The objective of the present study was to determine the *in vitro* protein digestion kinetics of different protein sources (soybean meal (SBM), wheat gluten (WG), rapeseed meal (RSM), whey powder (WP), dried porcine plasma protein (DPP), yellow meal worm larvae (MW), and black soldier fly larvae (BSF)). Protein sources were incubated with pepsin at pH 3.5 for 0-90 min and subsequently with pancreatin at pH 6.8 for 0-210 min at 39 °C. The *in vitro* protein digestion kinetics were described as the kinetics of nitrogen (N) solubilisation and the release of low molecular weight peptides (< 500 Da). The N solubilisation rate ranged from 0.025 min^{-1} for BSF to 0.685 min^{-1} for WP during the incubation with pepsin, and from 0.027 min^{-1} for RSM to 0.343 min^{-1} for WP during the incubation with pancreatin. The release rate of low molecular weight peptides ranged from 0.027 min^{-1} for WG to 0.093 min^{-1} for WP during the incubation with pepsin, and from 0.029 min^{-1} for SBM to 0.385 min^{-1} for WP. At the end of the sequential incubation with pepsin (90 min) and pancreatin (210 min), WG and WP showed the highest percentage of N present in low molecular weight peptides relative to total N (78 and 79 %, respectively), whereas SBM showed the lowest (35 %). In conclusion, protein sources for pig diets show substantial differences in *in vitro* protein digestion kinetics as measured by the kinetics of N solubilisation and the release of low molecular weight peptides. The rate of release of low molecular weight peptides was not correlated to the rate of N solubilisation for each of the protein sources evaluated.

IMPLICATION

The animal feed industry is facing the challenge of contributing to the provision of sufficient food of animal-origin to the growing world population while simultaneously improving environmental sustainability of animal production systems. Therefore, there is a need to use feed ingredients more efficiently in animal production. Information on protein digestion kinetics of protein sources using *in vitro* approaches can be used to further develop the concept of synchronising the dietary supply of energy and protein, which could improve protein retention and efficiency in production animals.

INTRODUCTION

In current feed evaluation systems, the nutritional value of protein sources in diets for pigs is based on the concentration and ratio of indispensable amino acids, and their digestibility up to the end of ileum (CVB, 2016; NRC, 2012). The ileal digestibility of protein and amino acids only provides information on the quantity of proteins and amino acids apparently absorbed from the gastrointestinal tract (GIT) up to the end of the ileum. Such data do not account for the kinetics of protein digestion along the GIT. Additional information on protein digestion kinetics may help to understand the timing of delivery of amino acids and peptides from dietary proteins along the GIT.

Protein sources with comparable ileal protein digestibility can differ in the kinetics of protein digestion, thereby affecting the postprandial appearance of amino acids and peptides in blood and their post-absorptive metabolism (Mahé et al., 1995; Boirie et al., 1997; Dangin et al., 2001). In humans, fast digestible dietary proteins such as whey protein show an earlier postprandial appearance of amino acids and peptides in blood compared to more slowly digestible sources such as casein (Boirie et al., 1997). The timing of postprandial appearance of amino acids and peptides in blood may relate to the release kinetics of free amino acids or di- and tri-peptides during the process of protein digestion. The release kinetics of free amino acids or di- and tri-peptides can be affected by the chemical composition, the structure of constituting proteins, and the physicochemical properties (e.g. solubility) of protein sources. For instance, the *in vitro* hydrolysis rate of a water-soluble form of casein (i.e. Na^+-caseinate) was almost twice as high compared to that of water-insoluble casein at pH 8.0 (Tonheim et al., 2007).

Protein digestion along the GIT can be simulated *in vitro* by incubating protein sources with pepsin and pancreatin sequentially using incubation conditions as prevailing in the GIT. Various *in vitro* methods have been developed to estimate protein digestibility of feed ingredients for pigs (Babinszky et al., 1990; Cone and van der Poel, 1993; Boisen and Fernández, 1995). Generally, these methods are end point methods, meaning that they generate a single *in vitro* digestibility value for nitrogen (N) or crude protein (N × 6.25) after incubation for a fixed period of time. The *in vitro* digestibility of protein is determined as the solubility of N in the sample, assuming that N present in soluble form is absorbed in the GIT *in vivo*. However, *in vivo* dietary proteins need to be hydrolysed into free amino acids or di- and tri-peptides before they can be absorbed by enterocytes in the small intestinal mucosa (Webb et al., 1992; Ganapathy et al., 2000). Protein sources with a similar *in vitro* protein digestibility based on determination of N

solubility can differ in the extent of release of free amino acids or di- and tri-peptides. Current *in vitro* methods for estimating protein digestibility do not take this into account. Rather than measuring only the change in N solubility *in vitro*, determination of the change of molecular weight distribution of soluble proteins and peptides before and during enzymatic incubation might improve the understanding of both *in vitro* and *in vivo* digestion of proteins in feed ingredients.

The objective of the present study was to determine *in vitro* protein digestion kinetics of different protein sources. The *in vitro* protein digestion kinetics were described as the change in N solubility and in molecular weight distribution of the soluble protein and peptide fraction during *in vitro* simulation of gastric and intestinal protein digestion in pigs.

MATERIALS AND METHODS

Experimental design

Each protein source was incubated in triplicate with pepsin and pancreatin sequentially. Per protein source, values were averaged and expressed as mean ± SEM.

Protein sources and enzymes used for in vitro *incubations*

The protein sources evaluated were five batches of soybean meals (SBMs) (four obtained from Nutreco, Boxmeer, the Netherlands; one as a commodity batch obtained via Research Diet Services, Wijk bij Duurstede, the Netherlands), and single batches of wheat gluten (WG), rapeseed meal (RSM), whey powder (WP) (all commodity batches obtained via Research Diet Services, Wijk bij Duurstede, the Netherlands), dried porcine plasma protein (DPP) (obtained from Darling Ingredients Inc., Irving, TX, USA), yellow meal worm larvae (MW) (obtained from Kreca, Ermelo, the Netherlands), and black soldier fly larvae (BSF) (obtained from the Laboratory of Entomology, Wageningen University, the Netherlands).

Porcine pepsin (2000 FIP U/g, Merck, Darmstadt, Germany), porcine pancreatin (P1750, Sigma-Aldrich, St. Louis, MO, USA) and porcine bile extract (B8631, Sigma-Aldrich, St. Louis, MO, USA) were used in the peptic and pancreatic incubations, respectively.

Chemical analysis

All chemical analyses were performed according to standard laboratory methods. Protein sources were analysed for DM (method ISO 6496; ISO, 1999b), ash (method ISO 5984; ISO, 2002), acid-hydrolysed ether extract (method ISO 6492; ISO 1999a) and N by Kjedahl method (method ISO 5983-1; ISO, 2005). The N content of samples obtained during the sequential incubation with pepsin and pancreatin was determined using the Dumas method (method ISO 16634-1; ISO, 2008) and a Flash EA 1112 NC Analyzer (Thermo Fisher Scientific, Waltham, MA, USA).

In vitro *incubations with pepsin and pancreatin*

The protein sources were hydrolysed according to a two-step method described by Boisen and Fernández (1997) with modifications. All protein sources were ground using an ultracentrifugal mill with a 1 mm sieve (ZM200, Retsch GmbH, Hann, Germany). For the simulation of protein digestion in the stomach, 1 g of sample was incubated in a 100 mL plastic centrifuge tube with a phosphate buffer (25 mL, 0.1 M, pH 6.0) and an HCl solution (2 mL, 1 M). The pH was adjusted to 3.5 with 1 M HCl or 1 M NaOH. Freshly prepared pepsin solution (1 mL, 10 g/L) was added and each centrifuge tube was covered with a plastic lid and placed in the heating chamber at 39 °C under constant magnetic stirring. The incubation times with pepsin were 0, 30, 60 and 90 min. Following the 90-min incubation with pepsin, the protein digestion in the small intestine was simulated by adding 10 mL phosphate buffer (0.2 M, pH 6.8) and 3 mL, 1 M, NaOH to the samples and the pH was adjusted to 6.8 with 1 M HCl or 1 M NaOH. Freshly prepared pancreatin solution (1 mL, 100 g/L) and bile solution (1 mL, 150 g/L) were added and the incubation with pancreatin was continued in the heating chamber at 39 °C under constant magnetic stirring. The incubation times with pancreatin were 0, 30, 60, 90, 120, 150, 180 and 210 min. To correct for the N of the enzymes added, blanks (triplicates) without protein sources were analysed. The samples taken during the sequential incubation with pepsin and pancreatin and from blanks were cooled on ice for 10 min and then centrifuged (30 min, 20,000 g, 4 °C) to separate the insoluble protein fraction (IPF) and soluble fraction (SPF). After centrifugation, the supernatant containing the soluble fraction was transferred to a 50 mL volumetric flask and the flask was made up to 50 mL with de-mineralized water. One mL of soluble fraction was transferred to a 2 mL Eppendorf tube (Eppendorf AG, Hamburg, Germany) and 20 % sulfosalicylic acid was added to the sample in a 1:1 (v/v) ratio. The sample was centrifuged (10 min, 12000 g, 4 °C) to separate the soluble high molecular weight

(> 500 Da) peptides (HMW) and the soluble low molecular weight (< 500 Da) peptides (LMW) (Greenberg and Shipe, 1979). The IPF was freeze-dried and ground using an ultracentrifugal mill with a 1 mm sieve (ZM200, Retsch GmbH, Hann, Germany) prior to chemical analysis.

Size exclusion chromatography

The molecular weight distribution of hydrolysates in the SPF was analysed using an ÄKTA micro system (GE Healthcare, Uppsala, Sweden) with a Superdex Peptide PC 3.2/30 column (GE Healthcare). The eluent used was a 10 mM potassium phosphate buffer with 150 mM NaCl and 2 % SDS. All samples were first boiled for 5 min to inactivate the enzymes and then diluted with the eluent in a 1:1 (v/v) ratio. Thereafter, samples were centrifuged (10 min, 20000 g, 20 °C), and 50 µL of the sample were injected onto the column. The absorbance was measured at 214 nm. For the calibration curve, β-lactoglobulin (18,360 Da), aprotinin (6,512 Da), vitamin B12 (1,335 Da), glutathione (307 Da) and glycine (75 Da) were used. The chromatograms obtained were separated into molecular weight ranges of >10 kDa, 10-5 kDa, 5-3 kDa, 3-1 kDa and <1 kDa by calculating the eluent volumes based on the calibration curve.

Calculations and statistical analysis

The N solubility was calculated by equation 1:

$$\text{N solubility (\%)} = \frac{N_{sample} - (N_{IPF} - N_{blank})}{N_{sample}} \times 100\ \% \quad (1)$$

where N_{sample} (mg) is the amount of N in 1 g of protein source, N_{IPF} (mg) is the amount of N in the IPF during the sequential incubation with pepsin and pancreatin, and N_{blank} (mg) is the amount of N in the IPF of blank samples during the sequential incubation with pepsin and pancreatin.

The N present in LMW was calculated by equation 2:

$$\text{Soluble low molecular weight peptides fraction (\%)} = \frac{N_{LMW} - N_{blank}}{N_{sample}} \times 100\ \% \quad (2)$$

where N_{LMW} (mg) is the amount of N in the LMW during the sequential incubation with pepsin and pancreatin, N_{blank} (mg) is the amount of N in the LMW of blank samples during the sequential incubation with pepsin and pancreatin, and N_{sample} (mg) is the amount of N in 1 g of protein source.

The kinetics of N solubilisation and the release of LMW for different protein sources during the incubations were described using an exponential equation (Ørskov and McDonald, 1979) (equation 3):

$$D_t = D_0 + \Delta D \times (1 - e^{-kt}) \quad (3)$$

where D_t (%) is the N solubility or the N present in LMW at incubation time t (min), D_0 (%) is the N solubility or the N present in LMW at 0 min, ΔD (%) is maximum N solubility or the N present in LMW (asymptote) corrected for D_0, and k is the rate constant.

The kinetics of N solubilisation of MW during pepsin hydrolysis and the kinetics the release of LMW of MW during pancreatin hydrolysis were described using a linear equation (equation 4) as these data did not fit the exponential equation.

$$D_t = D_0 + kt \quad (4)$$

where D_t (%) is the N solubility or the N present in LMW at incubation time t (min), D_0 (%) is the N solubility or the N present in LMW at 0 min, and k is the rate constant.

Data on N present in the IPF, HMW and LMW as percentage of total N during the sequential incubation with pepsin and pancreatin were analysed by analysis of variance using the GLM procedure of SAS (version 9.3, SAS Institute Inc., Cary, NC) with protein source, time and the interaction between protein source and time as fixed effects. All dependent variables were tested for normality and non-normal distributed data were log transformed. Probability levels of less than 5 % were considered to be statistically significant, and levels between 5 to 10 % were considered a trend.

RESULTS

Chemical composition of protein source

The analysed chemical composition of the protein sources is presented in Table 2.1. The SBMs had a mean crude protein content of 46.5 ± 2.2 % (standard deviation). The crude protein content of the other protein sources ranged from 24.9 % for WP to 80.0 % for DPP.

Table 2.1. Chemical composition (g/100 g sample) and apparent ileal protein digestibility of protein sources[1].

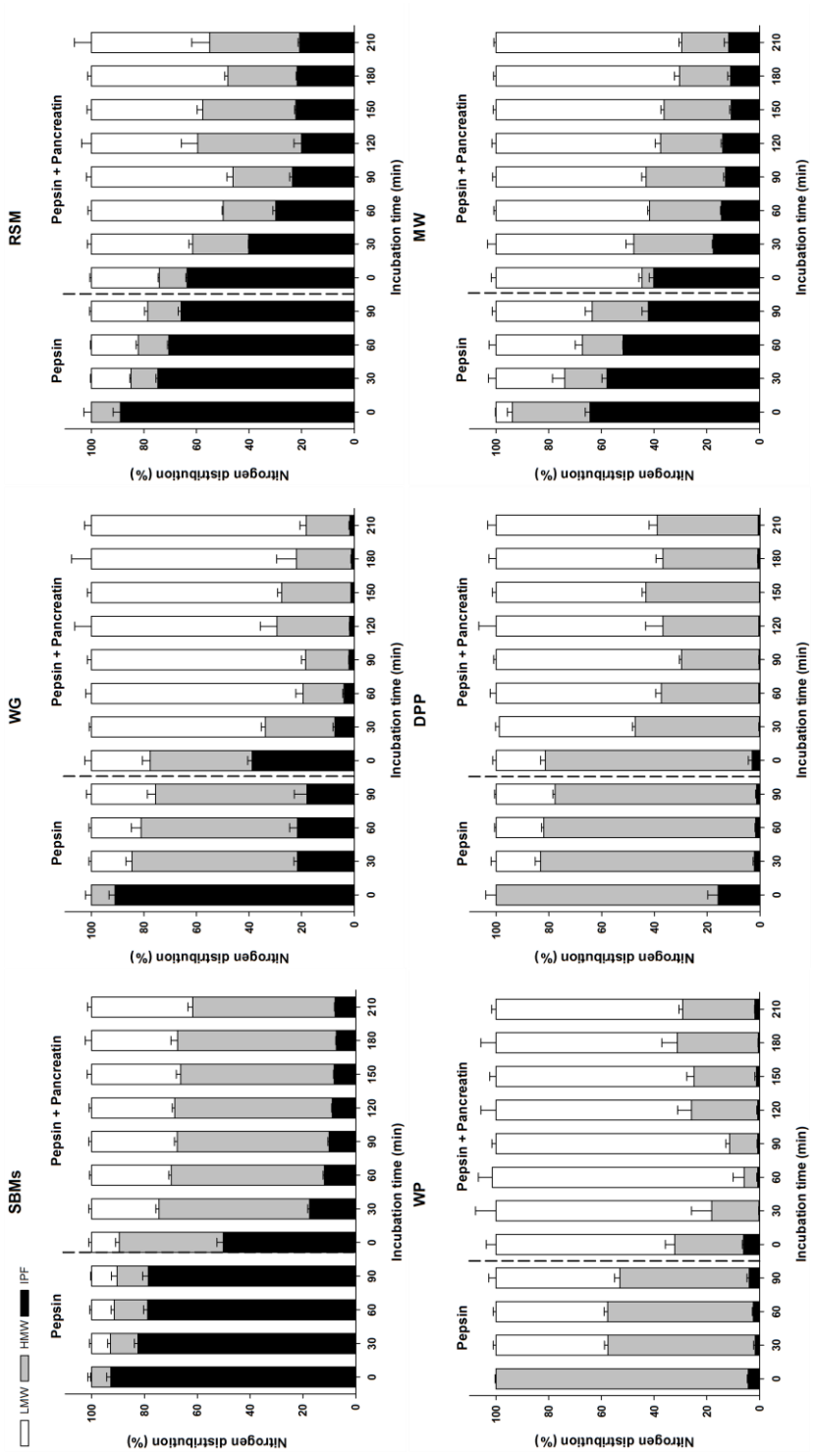
Item	Feed ingredient										
	SBM 1	SBM 2	SBM 3	SBM 4	SBM 5	WG	RSM	WP	DPP	MW	BSF
Moisture [2]	12.7	11.3	10.9	10.3	10.4	7.2	11.6	5.5	8.5	4.1	2.3
Crude protein[2]	45.1	47.6	47.6	43.4	48.7	76.7	33.2	24.9	80.0	45.7	53.4
Crude fat[2]	1.7	-	-	-	3.1	5.8	3.2	2.1	0.5	25.9	14.3
Ash[2]	6.4	6.7	6.0	5.1	5.9	0.9	-	17.4	7.1	3.3	8.8
CVB (2016) [3]				SBM[4]	SBM[5]	WG	RSM	WP	DPP	MW	BSF
Moisture [3]				11.3±0.8	12.8±0.5	8.9±2.6	11.1±0.7	4.4±0.6	n.a[6]	n.a	n.a
Crude protein[3]				46.8±1.0	48.5±0.3	78.1±1.8	34.4±1.2	25.2±3.2	n.a	n.a	n.a
Crude fat[3]				1.6±0.3	1.9±0.6	-	3.2±1.2	-	n.a	n.a	n.a
Ash[3]				6.4±0.2	6.5±0.3	0.9±0.1	6.7±0.3	17.7±1.2	n.a	n.a	n.a
Ileal digestibility coefficient of crude protein (%)[3]											
Standardized				88	88	100	72	92	n.a	n.a	n.a
Apparent				85	85	98	70	88	n.a	n.a	n.a

[1] SBM= soybean meal; WG= wheat gluten; RSM= rapeseed meal; WP= whey powder; DPP= dried porcine plasma protein; MW= meal worm; BSF= black soldier fly larvae. [2] Analysed values. [3] Mean values and standard deviations as reported by CVB (2016). [4] Soybean meal with a crude fibre content smaller than 4 % and a crude protein content smaller than 48 %. [5]Soybean meal with a crude fibre content smaller than 4 % and a crude protein content larger than 48 %. [6] Not available.

Separation of soluble nitrogen into high molecular weight fraction and low molecular weight fraction

Prior to the start of incubation with pepsin, the five batches of SBM showed a mean N solubility of 7 ± 6 %. The soluble N was only present in HMW (Table 2.2). Between 0 and 30 min of the incubation with pepsin, the N solubility of SBMs increased from an average of 7 to 18 %. At the end of incubation with pepsin (90 min), the SBMs showed a mean N solubility of 21 ± 2 %, of which 10 % was present in LMW and 11 % in HMW. After the incubation with pepsin, pH of the incubation solutions was adjusted from 3.5 to 6.8. This change of pH resulted in an increased N solubility of SBMs from an average of 21 to 50 %, mainly related to an increased solubility of HMW (from an average of 11 to 39 %). During the incubation with pancreatin, the N solubility of SBMs increased from an average of 50 to 83 % between 0 and 30 min. At the end of incubation with pancreatin (210 min), SBMs showed a mean N solubility of 92 ± 1 %, of which 38 % was present in LMW and 54 % in HMW.

For the other protein sources, prior to the start of incubation with pepsin, the N solubility ranged from 9 % for WG to 95 % for WP. For MW and BSF, 6 and 11 % of total N was present in LMW, respectively. For the other protein sources, soluble N was only present in HMW (Figure 2.1). The N solubility of WG, RSM, MW and BSF increased during the sequential incubation with pepsin and pancreatin and was affected by the interaction between protein source and incubation time ($P < 0.001$) (Table 2.3). The N solubility of these four protein sources ranged from 34 % for RSM to 82 % for WG at the end of incubation with pepsin, and from 79 % for RSM to 98 % for WG at the end of incubation with pancreatin. Similar to what was observed in SBMs, the changes in N solubility occurred mainly in the initial phase (between 0 to 30 min) of both the incubation with pepsin and with pancreatin. In contrast, the N solubility for WP and DPP remained high with values 98 and 99 %, respectively, during the sequential incubation with pepsin and pancreatin.


LMW
HMW
IPF
SBMs
WG
RSM
WP
DPP
MW
Pepsin
Pepsin + Pancreatin
Nitrogen distribution (%)
Incubation time (min)
0
20
40
60
80
100
0
30
60
90
0
30
60
90
120
150
180
210

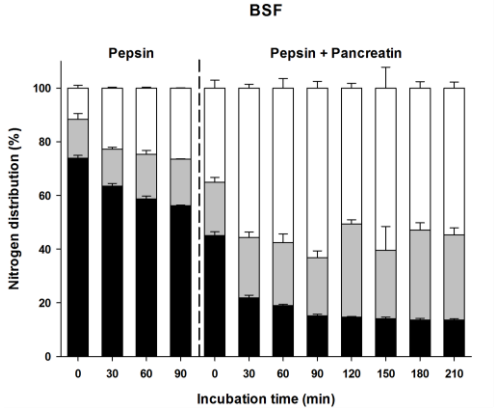


Figure 2.1. Nitrogen present in the insoluble protein fraction (IPF), the soluble high molecular weight peptides fraction (HMW) and the soluble low molecular weight peptides fraction (LMW) of soybean meal (SBM), wheat gluten (WG), rapeseed meal (RSM), whey powder (WP), dried porcine plasma protein (DPP), meal worm (MW) and black soldier fly larvae (BSF) as percentage (%) of total N at different time points during the sequential incubation with pepsin and pancreatin. Values are means ± SEM calculated for triplicate (n = 3).

Table 2.2. Nitrogen (N) solubility (%) and N present in the soluble high molecular weight peptides fraction (HMW) and the soluble low molecular weight peptides fraction (LMW) as a percentage (%) of total N of five different batches of soybean meal at different time points during the sequential incubation with pepsin and pancreatin.

Item	N solubility (%)				HMW (%)				LMW (%)			
	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max
Pepsin (min)												
0	7	6	2	17	7	6	2	17	0	0	0	0
30	18	4	15	24	9	2	7	13	8	2	7	12
60	21	3	16	31	12	4	8	19	9	2	8	12
90	21	2	19	23	11	2	9	13	10	2	8	12
Pepsin + pancreatin (min)												
0	50	2	47	52	39	2	36	41	11	1	9	11
30	83	2	80	85	57	4	53	64	25	3	22	30
60	88	2	85	90	58	4	54	63	30	3	26	34
90	90	1	89	91	58	3	53	61	32	4	29	37
120	91	1	90	92	60	1	59	61	31	2	29	33
150	92	1	91	93	58	5	51	64	34	5	28	42
180	93	1	92	93	58	2	56	60	35	2	32	38
210	92	1	91	93	54	6	46	61	38	5	32	44

The N present in the HMW and LMW as percentage of total N during the sequential incubation with pepsin and pancreatin showed an interaction between protein source and time of incubation ($P < 0.001$) (Table 2.3). The differences in the percentage of N present in the HMW and LMW were large among protein sources (Figure 2.1). At the end of incubation with pepsin, N present in LMW ranged from 21 % for RSM to 44 % for WP. Rape seed meal, MW and BSF showed a higher percentage of N in the LMW compared to the HMW. At the end of incubation with pancreatin, N present in the LMW ranged from 45 % for RSM to 82 % for WG. All protein sources showed a higher percentage of N in the LMW compared to the HMW at the end of incubation with pancreatin with the exception of SBMs.

Table 2.3. Effect of protein source, incubation time and their interaction on the nitrogen present in the insoluble protein fraction (IPF), the soluble high molecular weight peptides fraction (HMW) and the soluble low molecular weight peptides fraction (LMW) as percentage of total N during the sequential incubation with pepsin and pancreatin.

Fraction	Pepsin			
		P-value		
	Pooled SEM	Protein source	Incubation time	Interaction
IPF	2.0	<0.001	<0.001	<0.001
HMW	2.2	<0.001	<0.001	<0.001
LMW	1.1	<0.001	<0.001	<0.001
Fraction	Pepsin + pancreatin			
		P-value		
	Pooled SEM	Protein source	Incubation time	Interaction
IPF	0.8	<0.001	<0.001	<0.001
HMW	3.4	<0.001	<0.001	<0.001
LMW	3.3	<0.001	<0.001	<0.001

Changes in molecular weight distribution of soluble proteins and peptides during in vitro *digestion based on size exclusion chromatogram (SEC)*

An increase of total absorbance at 214 nm was observed from the SEC chromatogram of SBMs during the sequential incubation with pepsin and pancreatin (Figure 2.2). During the incubation with pepsin, the amount of peptides with a molecular weight range of 5-10 kDa increased (Figure 2.3). During the incubation with pancreatin, the amount of peptides with a molecular weight range of 5-10 kDa decreased, whereas the amount of peptides with a molecular weight smaller than 1 kDa increased.

Wheat gluten, RSM, MW and BSF behaved similar to the SBMs, with respect to total absorbance as well as the changes in molecular weight distribution of soluble proteins and peptide. In contrast, the total absorbance at 214 nm of WP and DPP stayed constant during the sequential incubation with pepsin and pancreatin. During the sequential incubation with pancreatin, the amount of peptides with a molecular weight range of 5-10 kDa decreased, whereas the percentage of peptides with a molecular weight range of 1-5 kDa increased.

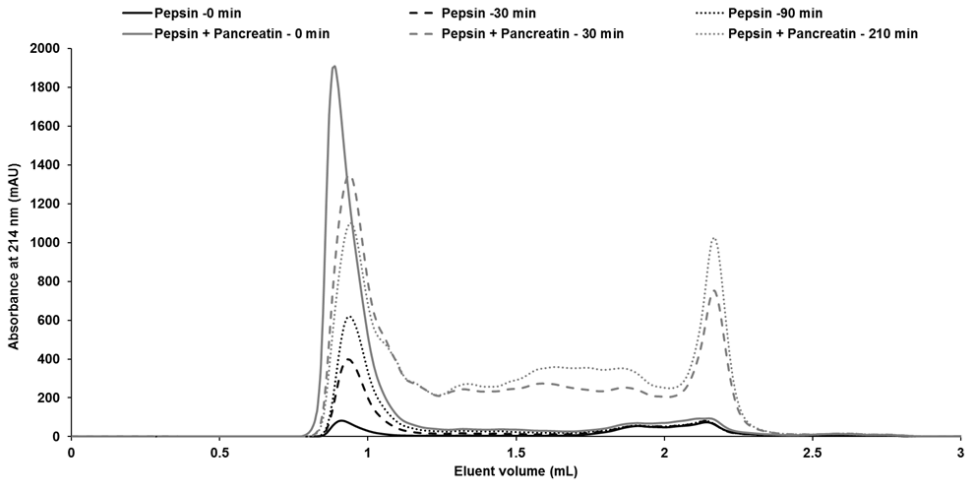
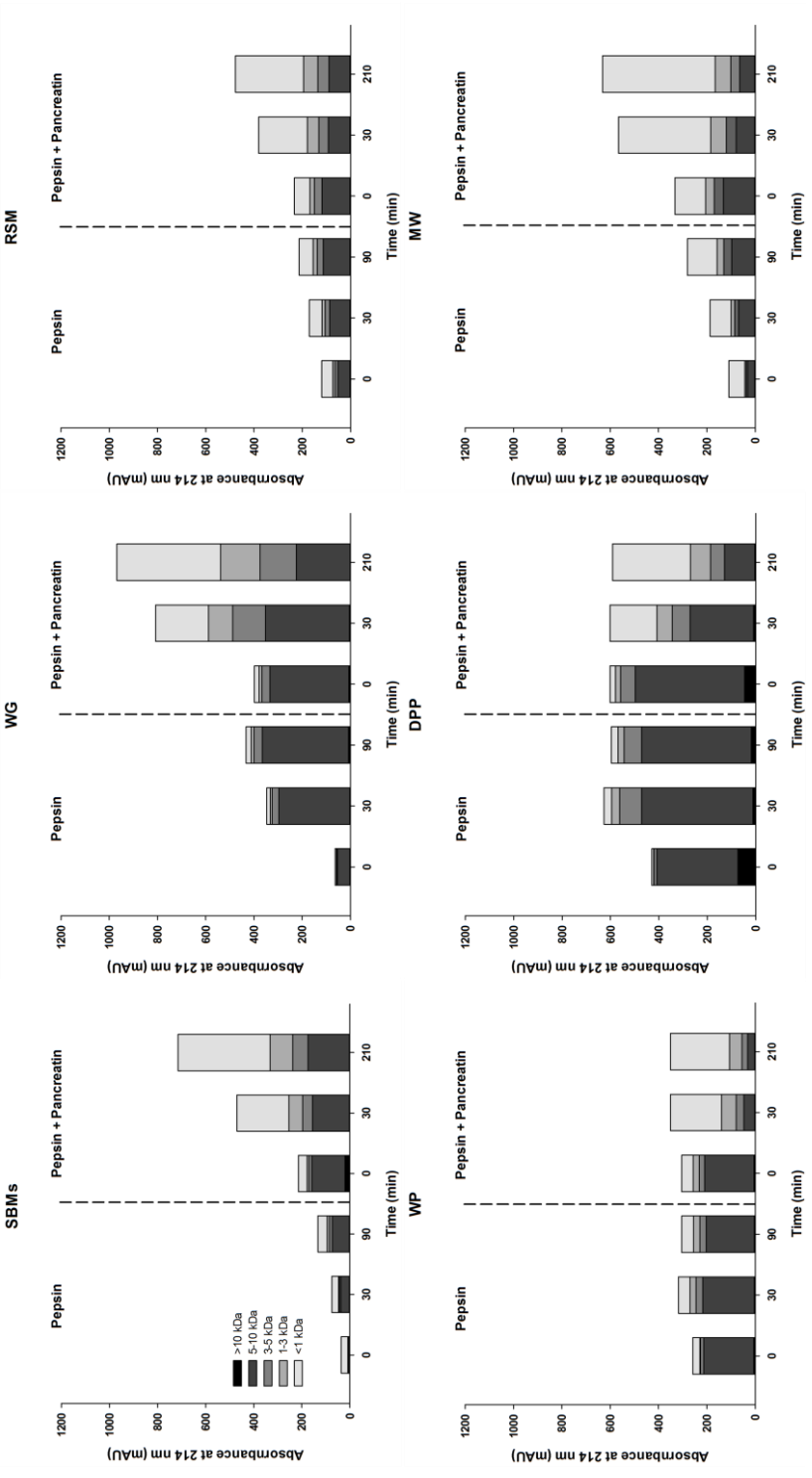


Figure 2.2. Size-exclusion chromatograms of soluble proteins and peptides of one batch of soybean meal at different time points during sequential incubation with pepsin and pancreatin.

Kinetics of nitrogen solubilisation and the release of low molecular weight peptides

During the incubation with pepsin, SBMs had a mean N solubilisation rate of 0.042 ± 0.007 min^{-1} with a mean maximum increase (ΔD) of 15 ± 2 % (Table 2.4). As for the kinetics of the release of low molecular weight peptides, SBMs had a mean rate of 0.065 ± 0.011 min^{-1} with a mean maximum increase of 10 ± 1 %. During the incubation with pancreatin, SBMs had a mean N solubilisation rate of 0.049 ± 0.003 min^{-1} with a mean maximum increase of 42 ± 1 %. As for the kinetics of the release of low molecular weight peptides, SBMs had a mean rate of 0.029 ± 0.005 min^{-1} with a mean maximum increase of 24 ± 1 %.

There were large differences between the kinetics of N solubilisation and the release of LMW for the different protein sources (Figure 2.4). During the incubation with pepsin, the N solubilisation rate ranged from 0.025 min^{-1} for BSF to 0.685 min^{-1} for WP, and the release rate of LMW ranged from 0.027 min^{-1} for WG to 0.093 min^{-1} for WP. During the incubation with pancreatin, the N solubilisation rate ranged from 0.027 min^{-1} for RSM to 0.343 min^{-1} for WP, and the release rate of low molecular weight peptides ranged from 0.046 min^{-1} for RSM to 0.385 min^{-1} for WP (Table 2.4).


SBMs
WG
RSM
WP
DPP
MW
Pepsin
Pepsin + Pancreatin
Time (min)
Absorbance at 214 nm (mAU)
>10 kDa
5-10 kDa
3-5 kDa
1-3 kDa
< 1 kDa

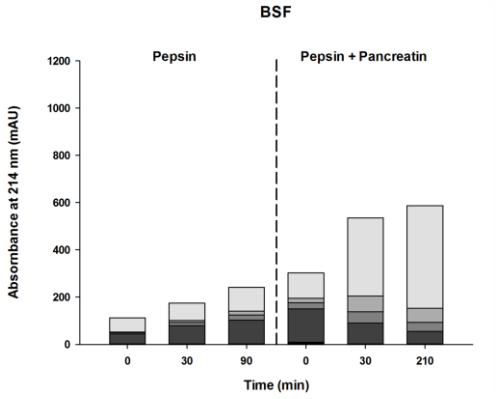
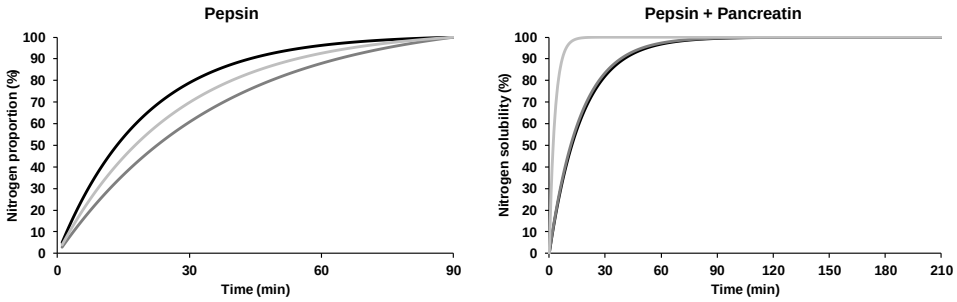


Figure 2.3. Molecular weight distribution (>10 kDa; 5-10 kDa; 3-5 kDa; 1-3 kDa; <1 kDa) of the soluble proteins and peptides of the soybean meals (SBMs), wheat gluten (WG), rapeseed meal (RSM), whey powder (WP), dried porcine plasma protein (DPP), meal worm (MW) and black soldier fly larvae (BSF) at different time points during the sequential incubation with pepsin and pancreatin.

(A) N solubilisation



(B) Release of LMW

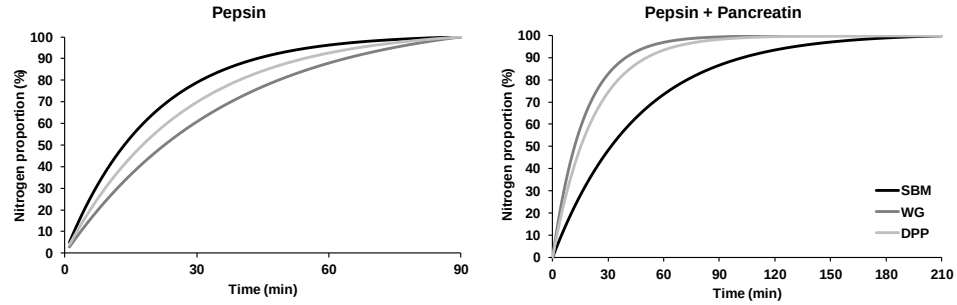


Figure 2.4. Rescaled kinetics curves (using D_0 as 0 % and D_{max} as 100 %) of nitrogen (N) solubilisation (A) and the release of low molecular weight peptides (LMW) (B) of one batch of soybean meal (SBM), wheat gluten (WG) and dried porcine plasma protein (DPP) during sequential incubation with pepsin and pancreatin.

Table 2.4. Parameter estimates[1] characterising the kinetics of nitrogen (N) solubilisation and the kinetics of the release of low molecular weight peptides (LMW) and high molecular weight peptides (HMW)[2] of protein sources[3] during the sequential incubation with pepsin and pancreatin.

Source	Pepsin											
	N solubilisation					LMW					HMW	
	D_0 (%)	ΔD_N (%)	D_{max} (%)	k (min^{-1})	$t_{1/2}$ (min)	D_0 (%)	ΔD_{LMW} (%)	D_{max} (%)	k (min^{-1})	$t_{1/2}$ (min)	D_0 (%)	D_{max} (%)
SBMs	7±3	15±2	23±1	0.042±0.007	17±3	0±0	10±1	10±1	0.065±0.011	11±3	7	13
WG	9	71	80	0.122	6	0	26	26	0.027	26	9	54
RSM	11	25	36	0.026	27	0	21	21	0.038	18	11	14
WP	96	2	98	0.685	1	0	45	45	0.093	7	96	52
DPP	84	14	98	0.104	7	0	21	21	0.037	19	84	77
MW	35	-	-	0.242[4]	-	6	32	38	0.032	22	29	-
BSF	26	20	46	0.025	28	12	15	27	0.046	15	15	20

Source	Pepsin + pancreatin											
	N solubilisation					LMW					HMW	
	D_0 (%)	ΔD_N (%)	D_{max} (%)	k (min^{-1})	$t_{1/2}$ (min)	D_0 (%)	ΔD_{LMW} (%)	D_{max} (%)	k (min^{-1})	$t_{1/2}$ (min)	D_0 (%)	D_{max} (%)
SBMs	50±1	42±1	92±0	0.049±0.003	14±1	11±0	24±1	35±1	0.029±0.005	24±4	39	57
WG	61	37	98	0.060	12	22	55	78	0.059	12	39	21
RSM	36	43	80	0.027	26	25	22	47	0.046	15	11	32
WP	94	5	99	0.343	2	68	11	79	0.385	2	26	20
DPP	97	3	100	0.330	2	25	22	47	0.046	15	72	52
MW	60	28	88	0.051	14	52	-	-	0.085[4]	-	8	-
BSF	55	31	86	0.041	17	35	20	55	0.329	2	20	31

[1] D_0 (%) is the N percentage at 0 min; ΔD (%) maximum N solubility or the N present in LMW (asymptote) corrected for D_0; D_{max} (%) is the sum of D_0 and ΔD (asymptote); k (min^{-1}) is the rate constant; $t_{1/2}$(min) is the time where half of ΔD is reached. [2] Values for HMW were calculated by the difference between values for N solubilisation and values for LMW. [3] SBMs= soybean meals; WG= wheat gluten; RSM= rapeseed meal; WP= whey powder; DPP= dried porcine plasma protein; MW= meal worm; BSF= black soldier fly larvae. [4] Data were fitted using a linear equation: $Dt = D_0 + kt$.

DISCUSSION

In the present study, *in vitro* protein digestion kinetics of protein sources differing in origin, chemical composition and *in vivo* protein digestibility was studied using a two-step *in vitro* method with sequential incubation with pepsin and pancreatin. Multiple batches of SBM as well as single batches of WG, RSM, WP, DPP, MW and BSF were evaluated. The chemical composition of the batches of SBMs, WG, RSM and WP was close to the tabulated values (CVB, 2016), indicating that the batches used in the current study were representative for these ingredients as protein sources in animal feeds with respective to the chemical composition.

In the present study, *in vitro* protein digestibility values are represented by the values for maximum N solubility after the incubation with pancreatin. Values for SBM, RSM, MW and BSF (92, 80, 88 and 86 %, respectively) are comparable to those reported in literature in which similar *in vitro* methods were applied (93, 84, 90 and 91 %, respectively) (Cone and van der Poel, 1993; Boisen and Fernández, 1995; Bosch et al., 2014). The kinetics of N solubilisation was established only for the potentially soluble fraction, corrected for the soluble fraction at the start of the *in vitro* incubations (D_{0N}). ΔD_N differed greatly among protein sources, ranging from 2 for WP to 71 % for WG. During the sequential incubation with pepsin and pancreatin, WP showed a higher rate of N solubilisation than the other protein sources. The N solubility of WP, however, was already high prior to the start of enzymatic incubations, resulting in a low ΔD_N for WP (2 and 5 % during the incubations with pepsin and pancreatin, respectively). A rate estimated based on a small ΔD fraction provides limited information compared to a rate based on a large ΔD fraction. The N solubilisation rates of WP should, therefore, be viewed with caution. In the present study, WG showed a greater rate of N solubilisation than SBM during the incubation with pepsin. This result is not in agreement with data from (Wilfart et al., 2008), who found no difference in the rate of N solubilisation for SBM and wheat during the incubation with pepsin.

The kinetics of the release of LMW was established only for the potentially releasable fraction corrected for the fraction already present at the start of the *in vitro* incubations (D_{0LMW}). This fraction also differed largely among protein sources, ranging from 11 for WP to 55 % for WG. Whey protein showed a faster release of LMW than other protein sources. Wheat gluten showed the slowest release during the incubation with pepsin and SBMs during the incubation with pancreatin. At the end of sequential incubation with pepsin and pancreatin, WG and WP showed the highest percentage of LMW in

the soluble fraction and SBMs the lowest. Under *in vivo* conditions, the release of LMW is expected to be higher than measured *in vitro*. This relates to the action of brush border aminopeptidases, which are involved in the final step of protein digestion. The brush border aminopeptidases contribute to a substantial release of LMW (Silk et al., 1985; Picariello et al., 2015).

Luo et al. (2015) showed that the *in vitro* hydrolysis of whey protein using pepsin followed the "zipper" type of protein hydrolysis mechanism, meaning that proteins are hydrolysed into peptides with a very wide range of molecular weights during the various stages of the hydrolysis process (Adler-Nissen, 1976). Assuming the hydrolysis of protein sources using pepsin and pancreatin also follows the "zipper" type of protein hydrolysis mechanism, a substantial quantity of peptides with intermediate molecular weight were expected during the *in vitro* incubations in the present study. Such a substantial quantity of intermediate peptides, however, was not observed. Instead, a decrease of intact proteins and HMW and a concomitant and immediate increase of LMW were observed. These results suggest that intact proteins from the protein sources are hydrolysed in one sequence to low molecular weight peptides and free amino acids by the proteolytic action of pepsin and proteases in pancreatin.

It should be emphasized that in the present study, a high rate of N solubilisation did not correlate to a fast release of LMW for each of the protein sources evaluated. For instance, WG showed a high rate of N solubilisation but a slow release of LMW during the incubation with pepsin. Compared to the *in vitro* protein digestion kinetics based on N solubilisation, the *in vitro* protein digestion kinetics based on the release of LMW might be a better prediction of *in vivo* protein digestion kinetics. *In vivo*, dietary proteins need to be hydrolysed into free amino acids and di- and tri-peptides prior to absorption as these are the main forms of N that can be absorbed by enterocytes in the small intestine (Webb et al., 1992; Ganapathy et al., 2000). Therefore, a fast release of free amino acids and di- and tri-peptides from the protein sources results in a fast absorption in the GIT, thereby leading to a more rapid postprandial appearance of amino acids and peptides in blood after ingestion of a meal. There is limited published information available on the correlation between *in vitro* and *in vivo* protein digestion kinetics of feed ingredients for pigs. In contrast to protein digestion kinetics, starch digestion kinetics, both *in vitro* and *in vivo*, for pigs has been extensively studied. In pigs, portal glucose appearance is strongly related to *in vitro* release of glucose (R^2 = 0.89) by starch digestion. Fast digestible starch, such as rice starch, showed a fast release of glucose *in vitro* and a rapid portal appearance of glucose *in vivo*. In contrast,

slow digestible starch, such as pea starch, showed a slower release of glucose *in vitro* and a delayed portal appearance of glucose *in vivo* (van der Meulen et al., 1997; van Kempen et al., 2010). Similar to glucose as the end product of starch digestion, free amino acids and di- and tri-peptides are the end products of protein digestion. Thus, the kinetics of the release of LMW might better reflect *in vivo* protein digestion kinetics than the kinetics of N solubilisation. Digestion kinetics of protein can influence postprandial appearance of free amino acids and peptides in blood in time. Protein sources with a fast release of LMW during the sequential incubation with pepsin and pancreatin can be expected to be digested faster *in vivo*, resulting in an early appearance of amino acids and peptides in the blood after a meal. Information on protein digestion kinetics can be used to further develop the concept of nutrient (i.e. energy and protein) synchronisation, which could improve overall protein utilisation (van den Borne et al., 2007; Drew et al., 2012).

The differences in the extent and the kinetics of protein digestion among protein sources can be related to various factors. Pepsin, trypsin and chymotrypsin are specific enzymes, meaning they can only cleave certain peptide bonds. The number and accessibility of these cleavage sites for enzymes depend on the amino acid sequence of polypeptide chains and their conformation. For instance, rapeseed albumins showed a higher resistance to pepsin hydrolysis *in vitro* compared to rapeseed globulins (Malabat and Rabiller, 2001). This is likely due to the compact conformation of rapeseed albumins due to the presence of disulphide bonds (Schwenke et al., 1988). The accessibility of the cleavage sites for enzymes also depends on the physicochemical properties of the protein. A soluble form of casein (i.e. Na^+-caseinate) was hydrolysed faster *in vitro* than native casein because soluble proteins have a better accessibility to enzymes than insoluble proteins (Tonheim et al., 2007). The non-protein constituents can interact with the proteins in the protein source. For instance, tannins present in legume seeds and phytate present in soybeans can bind to proteins. The bound proteins are not susceptible to enzymatic hydrolysis by digestive enzymes, leading to a reduced protein digestibility in pigs (Mangan, 1988; Selle et al., 2012). Thermal treatment is commonly involved in the processing of ingredients or complete diets for pigs to improve nutrient digestibility (Johnston et al., 1998) or to eliminate anti-nutritional factors (van der Poel, 1990). Severe thermal treatment, however, induces protein aggregation, resulting in a higher resistance to digestive enzymes (Sánchez-Rivera et al., 2015).

CONCLUSION

The present study showed substantial differences in *in vitro* protein digestion kinetics among protein sources, as measured via kinetics of N solubilisation and release of free amino acids and soluble low molecular weight peptides (LMW). The rate of release of LMW was not correlated to the rate of N solubilisation for each of the protein sources evaluated. Whey protein showed a fast rate of both N solubilisation and release of LMW. In contrast, wheat gluten and dried porcine plasma protein showed a fast rate of N solubilisation but a slow release of LMW. *In vitro* protein digestion kinetics as determined by the kinetics of release of LMW might be a preferred method for the prediction of *in vivo* protein digestion kinetics.

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Chapter 3

Protein sources differ in digestion kinetics in the small intestine of growing pigs and affect postprandial appearance of amino acids in blood

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ABSTRACT

The aim of the present study was to determine the *in vivo* protein digestion kinetics up to the end of the small intestine (SI) of five protein sources (soybean meal (SBM), wheat gluten (WG), rapeseed meal (RSM), dried porcine plasma protein (DPP), and black soldier fly larvae (BSF)), and its influence on the postprandial appearance of amino acids (AAs) and peptides in systemic blood in growing pigs (body weight 35 kg). Forty pigs were randomly allocated to one of the five experimental diets containing the respective protein sources as the only source of protein. Four pigs per experimental diet were fitted with an ear-vein catheter and blood samples were collected before and after a morning meal. At dissection, digesta samples from the stomach and the SI, divided into four segments of equal length, were quantitatively collected. Apparent digestibility (AD) of crude protein (CP), and retention time (RT) of the solid fraction of digesta along the stomach and the SI were determined to calculate protein digestion kinetics. The RT in the stomach and in the 1st, 2nd, and 3rd SI segment did not differ among diets ($P > 0.05$). The AD_{CP} was affected by the diet in the 3rd and 4th SI segment and over the total gastrointestinal tract ($P < 0.001$). The CP digestion rate ranged from 0.010 min^{-1} for the RSM diet to 0.035 min^{-1} for the DPP diet. The postprandial concentration of AAs and peptides in systemic blood after a meal showed an interaction between time and diet ($P < 0.001$). The rate of increase of the concentration of AAs and peptides ranged from 0.0046 min^{-1} for the SBM diet to 0.0330 min^{-1} for the RSM diet. Over all diets, the increase of the postprandial concentration of AAs and peptides in systemic blood plasma after a meal was positively correlated with the amount of apparent digested protein up to the end of the small intestine ($r = 0.73$; $P < 0.001$). In conclusion, WG and DPP can be regarded as fast digestible protein sources while SBM, RSM and BSF as more slowly digestible protein sources in growing pigs.

INTRODUCTION

Currently pig diets are formulated taking into account the requirement of ileal digestible essential amino acids (AAs) of the animal, which only accounts for the total quantity of dietary essential AAs that is apparently absorbed in the gastrointestinal tract (GIT) up to the end of the ileum (NRC, 2012, CVB, 2016). In addition to ileal digestibility of protein and AAs, growing attention is given to the kinetics of protein digestion along the GIT since it provides information on the timing of release and absorption of AAs along the GIT after ingestion of a meal, e.g. in broilers (Liu and Selle, 2015). Information on the kinetics of protein digestion in pigs is scarce.

Based on the timing and the extent of postprandial increase of plasma AAs and peptides, protein sources can be categorised into fast and slowly digestible proteins (Boirie et al., 1997; Bos et al., 2003; Tang et al., 2009). Fast digestible proteins, which induce a pronounced but transient postprandial increase of plasma AAs and peptides, stimulate protein synthesis but at the same time might increase AA oxidation. In contrast, slowly digestible proteins, which induce a small but prolonged postprandial increase of AAs and peptides in plasma, inhibit body protein degradation and, therefore, can increase overall protein retention (Boirie et al., 1997; Dangin et al., 2001). Utilization of AAs and peptides in the post absorptive metabolism can be influenced by the kinetics of protein digestion relative to the digestion kinetics of energy providing nutrients in the diet such as starch. The simultaneous availability of AAs and glucose as energy source in blood increased body protein retention in pigs, resulting in a higher post absorptive utilization of absorbed AAs and peptides (van den Borne et al., 2007; Drew et al., 2012). Information on protein digestion kinetics as affected by protein sources can be used to further develop the concept of synchronising the supply of energy and protein, which could improve protein retention and overall protein utilisation efficiency in pigs.

The overall kinetics of dietary protein digestion is related to three aspects: 1) the passage rate of digesta along the GIT, 2) the hydrolysis rate of dietary proteins, and 3) the absorption rate of AAs and peptides by the intestinal mucosa. The passage rate of digesta along the GIT depends on the feeding pattern (i.e. feeding frequency and meal quantity) and the physicochemical properties (e.g. solubility, viscosity, water binding capacity) of digesta. A large volume of a meal (Hunt and Stubbs, 1975), a high solubility of digesta (Low, 1979) and an increase in dietary fibre content (Wilfart et al., 2007) decrease the passage rate of digesta along the GIT. The hydrolysis rate of dietary

proteins is affected by protein conformation (Malabat and Rabiller, 2001), protein solubility (Tonheim et al., 2007), and by the interaction between proteins and non-protein constituents in protein sources (Selle et al., 2012). The AA and peptide transport capacity across the intestinal mucosa was shown to be regulated by the luminal concentration of AAs and peptides (Stevens, 1992).

In humans and pigs, the postprandial increase of AAs in blood was found to be more rapid and pronounced for proteins with a high gastric passage rate (e.g. whey protein soy protein isolate) than for proteins with a delayed gastric emptying (e.g. casein) (Boirie et al., 1997; Tang et al., 2009). In pigs, soy protein concentrate showed a more rapid portal appearance of AAs than a mixture of untoasted and toasted soybean meal after meal ingestion. The difference might be due to a higher trypsin inhibitor activity in the diet with a mixture of untoasted and toasted soybean meal, resulting in a lower rate of hydrolysis of the dietary proteins (Jansman et al., 1997). It was demonstrated in a study with humans that protein hydrolysates show a faster and greater postprandial increase of plasma AAs than their non-hydrolysed equivalents. This might be due to that protein hydrolysates only require limited additional hydrolysis before being able to be absorbed in the small intestine (SI) (Morifuji et al., 2010).

The objectives of the present study were: 1) to determine the *in vivo* protein digestion kinetics up to the end of the SI in pigs of common and alternative protein sources used as feed ingredient, and 2) to evaluate the effect of protein digestion kinetics on the postprandial appearance of AAs and peptides in systemic blood. Protein digestion kinetics was studied by determining digesta retention time, protein digestibility and molecular weight distribution of proteins and peptides in digesta along the SI of growing pigs. It was hypothesized that: 1) highly digestible protein sources are digested faster than less digestible sources in the SI of pigs, and 2) protein sources with a fast digestion kinetics (i.e. fast gastric emptying, fast hydrolysis and/or absorption) induce a more rapid and pronounced postprandial appearance of amino acids and peptides in systemic blood of pigs.

MATERIALS AND METHODS

Protein sources and experimental diets

The protein sources evaluated were soybean meal (SBM), wheat gluten (WG), rapeseed meal (RSM) (all commodity batches obtained via Research Diet Services, Wijk bij Duurstede, the Netherlands), dried porcine plasma protein (DPP) (obtained from Darling Ingredients Inc., Irving, TX, USA), and black soldier fly larvae meal (BSF) (obtained from Protix, Dongen, the Netherlands). All five experimental diets were formulated to be isoproteineous (CP, 160 g/kg as-fed basis). Free AAs were added so the diets met at least 65 % of the requirement of the first limiting amino acids of pigs (CVB, 2008). For BSF, information on the AA profile and ileal AA digestibility was obtained from literature (Veldkamp et al., 2012; Bosch et al., 2014). The amount of additional free AAs ranged from 0 g/kg diet (as-fed basis) for RSM diet to 6.3 g/kg diet (as-fed basis) for WG diet. In addition, a protein-free diet was formulated. Titanium dioxide (TiO_2) was included in all the diets as an indigestible maker at 2.5 g/kg diet (as-fed basis). All diets were produced by Research Diet Services (Wijk bij Duurstede, the Netherlands). The ingredient composition of the six experimental diets is presented in Table 3.1.

Design, animals and housing

This study was approved by the Animal Care and Use Committee of Wageningen University & Research (Wageningen, the Netherlands). A total of 43 growing pigs (boars) (Topigs 20 × Tempo from van Beek, Lelystad, the Netherlands) with an average initial body weight of 34.9 ± 3.4 kg on the day of arrival were used. Forty out of 43 pigs were blocked on litter (eight blocks, five pigs per block) and pigs within a block were randomly allocated to one of the five experimental diets (n = 8). The remaining three pigs were allocated to the protein-free diet. Twenty pigs, four pigs per experimental diet excluding protein-free diet, were fitted with an ear-vein catheter at day 13, 14 and 15. Pigs were housed individually in metabolic cages (1.3 × 1.3 m or 2.0 × 1.0 m) with a tender foot floor in one large temperature controlled room. The ambient temperature was kept at 24 °C on day 1 and 2, at 23 °C on day 3, and constant at 22 °C from day 4 onwards. From day 1 to 27, the lights were turned on between 5.30 h till 19.00 h. From day 28 to 30, the lights were turned on between 2.30 h till 19.00 h.

Table 3.1. Composition of experimental diets (g/kg, as-fed basis).

	Diets containing the various protein source[1]					
Ingredients	SBM	WG	RSM	DPP	BSF	PF
Soybean meal	342.7	-	-	-	-	-
Wheat gluten	-	194.7	-	-	-	-
Rapeseed meal	-	-	489.6	-	-	-
Dried porcine plasma protein	-	-	-	196.0	-	-
Insect protein meal	-	-	-	-	305.0	0.0
Maize starch	376.2	527.9	258.0	521.4	451.0	712.8
Sucrose	100.0	100.0	100.0	100.0	100.0	100.0
Dextrose	50.0	50.0	50.0	50.0	50.0	50.0
Arbocel	50.0	50.0	50.0	50.0	50.0	50.0
Soy oil	43.3	17.6	30.0	32.6	6.4	30.0
Mineral and vitamin premix[2]	5.0	5.0	5.0	5.0	5.0	5.0
Limestone	14.4	15.9	5.1	15.7	0.0	16.3
Salt	4.1	2.0	4.0	-	-	2.0
Potassium chloride	-	-	-	-	4.8	2.9
Monocalcium phosphate	10.1	13.6	4.6	14.2	11.7	15.5
Potassium carbonate	0.0	10.6	0.0	11.2	5.0	8.4
Sodium bicarbonate	1.4	3.9	1.2	-	6.7	4.6
L-Lysine HCL	-	5.8	-	-	-	-
DL-Methionine	0.3	-	-	1.4	1.3	-
L-Threonine	-	0.5	-	-	-	-
L-Tryptophan	-	-	-	-	0.6	-
TiO_2	2.5	2.5	2.5	2.5	2.5	2.5
Calculated nutrient composition						
NE, MJ/kg	10.7	10.7	9.2	10.7	10.7	11.2
Calcium	8.2	8.2	7.0	8.2	11.8	8.5
Available phosphorus	2.8	2.8	2.4	2.8	2.8	2.9
Sodium	2.0	2.0	2.0	4.7	2.0	2.0
Digestible Lys	8.7	7.0	6.2	12.5	9.0	0.0
Digestible Met + Cys	4.2	5.7	5.2	7.2	4.2	0.0
Digestible Thr	5.1	4.1	4.6	7.3	5.0	0.0
Digestible Trp	1.8	1.3	1.4	2.1	1.3	0.0

[1] SBM= soybean meal; WG= wheat gluten; RSM= rapeseed meal; DPP= dried porcine plasma protein; BSF= black soldier fly larvae; PF= protein free. [2] The mineral and vitamin premix supplied per kilogram of diet: Vitamin A, 7,000 IU; Vitamin D3, 1,700 IU; vitamin E, 20 IU; vitamin K3, 1.5 mg; vitamin B1, 1.5 mg; vitamin B2, 4 mg; pantothenic acid, 11 mg; niacin, 18 mg; vitamin B12, 18 µg; folic acid, 1 mg; vitamin B6, 1 mg; choline chloride, 100 mg; Fe, 75 mg, as $FeSO_4$; Cu, 10 mg, as $CuSO_4 \cdot H_2O$; Zn, 65 mg, as $ZnSO_4$; Mn, 30 mg, as MnO; Co, 0.15 mg, as $CoCO_3$; I, 0.75 mg, as KI; Se, 0.3 mg, as Na_2SeO_3; anti-oxidant, 50 mg.

Feeding

From day 1 to 6, starting with 100 % of a commercial diet, pigs were gradually fed increasing amounts of the experimental diets from day 7 onwards, from which point pigs were fed only the experimental diets. The experimental diets were provided in a mash form and mixed with water at a ratio of 1 : 2 (feed : water, w/w basis). Additional water consumption was limited to 0.3 L which was provided after each feeding. The feeding level was 2.5 times NE requirement for maintenance (293 kJ NE/kg $BW^{0.75}$). During day 7 to 26, the feed allowance was divided into two equal amounts, fed at 8.00 h and 16.00 h. During day 27 to 30, feed allowance was divided into 6 equal amounts with feeding starting at 5.30 h in the morning at intervals of three hours. On dissection days, all pigs were fed at least three of their six daily portions 2, 4 and 6 hours prior to dissection, starting at 8.30 h in the morning and finishing at 16.30 h in the afternoon. Pigs that were dissected in the afternoon received up to three additional portions of feed in the morning.

The three pigs fed the protein-free diet received the SBM diet from day 1 to 21 in two equal portions at 8.00 h and 16.00 h. These pigs were gradually adapted to the protein-free diet from day 22 to 24, and were completely fed the protein-free diet from day 25 to 27. During day 25 to 27, the daily feed allowance was divided into six equal portions, provided from 5.30 h in the morning at 3 h intervals.

Sample collection and dissection procedure

From day 14 to 16, blood samples (1 mL per time point) were collected at 1 and 0.5 h before and 0.5, 1, 1.5, 2, 3, 4 ,6, and 8 h after the morning meal ingestion via the ear-vein catheter fitted one day before the blood collection. Blood samples were collected in tubes containing lithium-heparin and immediately centrifuged at 3000 g for 10 min at 4 °C. Supernatants were stored at -80 °C for further analysis on the total plasma AA and peptide concentration. Faeces were collected using a faeces collection bag system (Wageningen UR Livestock Research, The Netherlands) attached to the rear end of the pigs from day 21 to 23. The collection bags were emptied each day and the faeces for four days collection were pooled per pig and freeze-dried for nitrogen analysis to calculate faecal digestibility of crude protein (CP). At the dissection days, pigs were anesthetised by injecting pentobarbitone in the jugular vein, followed by exsanguination through the carotid artery. The pigs were placed on their right lateral side and the body cavity was opened before the GIT from the stomach to the anus was carefully removed. The SI was carefully placed horizontally on the table without

disturbing the digesta and separated from the stomach and the large intestine. The SI was then dissected from the mesentery and divided into four segments of equal length (1st, 2nd, 3rd and 4th SI segment, starting from the stomach to the large intestine). Throughout the entire dissection, care was taken to prevent the movement of digesta. The 1st SI segment comprised the entire duodenum and approximately the proximal quarter of the jejunum. The 2nd and 3rd SI segment comprised only the jejunum. The 4th SI segment comprised approximately the distal quarter of the jejunum and the entire ileum. Digesta samples from the stomach and four segments of the SI were quantitatively collected by gentle stripping. The collected digesta samples were freeze-dried for nitrogen and titanium analysis to calculate digestibility of CP and retention time of the solid fraction of digesta along the GIT.

Chemical analysis

All chemical analyses were performed according to standard laboratory methods. The experimental diets were analysed for dry matter (DM) (method ISO 6496; ISO, 1999a), ash (method ISO 5984; ISO, 2002), acid-hydrolysed ether extract (method ISO 6492; ISO 1999b), starch (method ISO 15914; ISO, 2004), total sugars as reducing sugars (van Vuuren et al., 1993) and nitrogen by the Kjedahl method (method ISO 5983-1; ISO, 2005a). The protein sources were analysed for AA composition (method ISO 13903; ISO, 2005b). The nitrogen content of digesta and faecal samples was determined using the Dumas method (method ISO 16634-1; ISO, 2008) using a Flash EA 1112 NC Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). The experimental diet, digesta and faecal samples were analysed for Ti content (Myers et al., 2004). Amino acids and peptides concentration in the plasma samples was determined using the ninhydrin method (Lee and Takahashi, 1966). In short, plasma samples were first deproteinized with 8 % sulphosalicylic acid and then incubated with ninhydrin for 1 h at 100 °C. The amino groups in the plasma samples reacted with ninhydrin and the purple colour was measured at 570 nm using an UV-visible light spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The total concentration of AAs and peptides in the plasma samples was calculated using a calibration curve using leucine.

Size exclusion chromatography

The molecular weight distribution of proteins and peptides in digesta samples was analysed using an ÄKTA micro system (GE Healthcare, Uppsala, Sweden) with a Superdex Peptide PC 3.2/30 column (GE Healthcare). The eluent used was a 10 mM potassium phosphate buffer with 150 mM NaCl and 2 % SDS. Freeze-dried digesta

samples (~ 20 mg) were weighed and solubilised in a 2 % SDS solution with 100 mM dithiothreitol. The samples were heated (100 °C) for 30 min and then centrifuged (10 min, 20,000 g, 20 °C). The supernatants were diluted with the eluent in a 1:1 (v/v) ratio. Samples were then centrifuged, and 50 µL of the supernatant was injected on the column. The absorbance was measured at 214 nm and corrected for the signals from the SDS solution and dithiothreitol solution in the chromatograms. For the calibration curve, β-lactoglobulin (18,360 Da), vitamin B12 (1,335 Da), glutathione (307 Da) and glycine-proline-glycine (229 Da), phenylalanine (165 Da) and alanine (89 Da) were used. The chromatograms obtained were separated into molecular weight ranges of >10 kDa, 10-5 kDa, 5-3 kDa, 3-1 kDa and <1 kDa by calculating the eluent volumes based on the calibration curve. The mass-based extinction coefficient (mAU/mg) was calculated by dividing the absorbance measured at 214 nm by the protein content of the samples.

Calculations and statistical analysis

The area under the curve (AUC) (mmole · min/L) of the postprandial plasma AAs and peptides curve was calculated using the trapezoidal method. The postprandial curve was described by a one-compartment pharmacokinetic model, from which a $k_{increase}$ (increase rate) and a $k_{elimination}$ (elimination rate) were calculated.

Apparent digestibility (AD) of DM and CP was calculated by equation 1:

$$\text{Apparent digestibility (\%)} = \frac{(\text{Nutrient}_{diet}/\text{Ti}_{diet}) - (\text{Nutrient}_{digesta}/\text{Ti}_{digesta})}{(\text{Nutrient}_{diet}/\text{Ti}_{diet})} \times 100\ \% \quad (1)$$

where Nutrient_{diet} and $\text{Nutrient}_{digesta}$ (%) are the nutrient (DM or CP) content in the experimental diets (as-fed basis) and in the freeze-dried digesta samples, respectively, and Ti_{diet} and $\text{Ti}_{digesta}$ (%) are the Ti content in the same samples of experimental diets and digesta, respectively.

Retention time (RT) of the solid fraction of digesta in the stomach and four segments of the SI was calculated by equation 2:

$$\text{Retention time (min)} = \frac{1440 \times \text{Ti}_{digesta} \times \text{W}_{digesta}}{\text{FI}_{24h} \times \text{Ti}_{diet}} \quad (2)$$

where $\text{Ti}_{digesta}$ (%) is the Ti content in the freeze-dried digesta samples, $\text{W}_{digesta}$ (g) is the weight of freeze-dried digesta samples from the stomach or four segments of the SI, FI_{24h} (g) is the feed intake over 24 h before sampling, Ti_{diet} (%) is the Ti content in the experimental diets (as-fed basis), and the factor 1440 is used to convert time from days to minutes.

The nutrient digestion kinetics of different protein sources were calculated by relating the apparent digestibility coefficient for DM and CP at each segment of the SI with the sum of RT up to that segment. The curve was fitted using a first order reaction rate equation (equation 3):

$$D_t = D_{max} (1- e^{-kt}) \quad (3)$$

where D_t (%) is the digestibility coefficient of DM or CP at time t (min), D_{max} (%) is the potentially digestible DM or CP (%) (asymptote), k is the rate constant. The equation was fitted using the MODEL procedure of SAS (version 9.3, SAS Institute Inc., Cary, NC) and the parameter estimates and the approximate standard error of the estimates were modelled.

The RT, AD_{DM} and AD_{CP} in each segment of the SI, and the AUC at each of the various time points were analysed by analysis of variance using the GLM procedure of SAS with protein source as a fixed effect. The postprandial concentration of AAs and peptides in plasma was analysed using the MIXED procedure with protein source, time and the interaction between protein source and time as fixed effects and pig as a random effect. The residuals of the dependent variables were tested for normality and non-normal distributed data were log transformed. Correlation between AUC and digestible proteins was analysed using the CORR procedure of SAS. Probability levels of less than 5 % were considered to be statistically significant, and levels between 5 to 10 % were considered a trend.

RESULTS

One pig from the DPP diet died during the installation of the ear-vein catheter and was replaced by one of the other four remaining pigs within the group. The other pigs remained healthy throughout the experiment.

Chemical composition of experimental diets

The CP content of the experimental diets containing the various protein sources ranged from 17.2 % for the BSF diet to 18.3 % for the SBM diet (DM basis) (Table 3.2). The WG and DPP diets had higher starch contents (50.1 and 74.2 %, respectively) and lower fat contents (2.1 and 2.2 %, respectively) than the other diets.

Table 3.2. Analysed chemical composition of the experimental diets and the protein sources (%, dry matter basis).

	Diets containing the various protein source [1]					
	SBM	WG	RSM	DPP	BSF	PF
Dry matter (% as-fed)	90.4	90.4	91.1	91.2	91.9	89.6
Ash	5.8	4.9	5.7	6.2	5.2	5.3
Crude protein	18.3	17.3	17.9	17.3	17.2	0.3
Crude fat	3.4	2.1	5.3	2.2	4.9	1.8
Starch	32.8	50.1	25.9	47.2	41.9	63.0
Sugar	19.4	16.9	21.9	17.8	16.3	17.8
	Protein source[1]					
Dry matter (% as-fed)	88.7	92.4	89.8	92.0	95.1	
Ash	7.3	1.0	8.0	7.6	8.7	
Crude protein	52.9	86.8	36.3	88.4	56.1	
Crude fat	1.7	5.0	3.9	1.6	11.8	
Indispensable AA						
His	1.5	1.8	1.1	3.0	1.6	
Ile	2.5	3.1	1.5	3.4	2.0	
Leu	4.1	5.7	2.6	8.4	3.2	
Lys	3.2	1.3	1.9	7.3	2.6	
Met	0.7	1.3	0.7	0.7	0.8	
Phe	2.8	4.3	1.5	5.3	4.0	
Thr	2.1	2.1	1.7	5.1	1.9	
Trp	0.6	0.7	0.5	1.5	0.7	
Val	2.6	3.3	2.0	5.8	2.9	
Dispensable AA						
Ala	2.4	2.2	1.6	4.7	3.3	
Arg	3.4	2.5	1.9	4.4	1.9	
Asx	6.0	2.5	2.7	8.3	4.1	
Cys	0.7	1.5	0.8	2.7	0.4	
Glx	9.5	29.4	6.1	12.1	4.4	
Gly	2.3	2.7	1.9	3.1	2.4	
Pro	2.7	10.4	2.3	5.5	2.6	
Ser	2.6	3.6	1.6	4.9	1.9	
Tyr	2.0	3.0	1.3	4.5	3.2	

[1] SBM= soybean meal; WG= wheat gluten; RSM= rapeseed meal; DPP= dried porcine plasma protein; BSF= black soldier fly larvae; PF= protein free.

Digestion kinetics of dry matter and crude protein along the SI

The retention time (RT) of the solid fraction of digesta differed among the GIT segments ($P < 0.001$) (Table 3.3). The RT in the stomach did not differ among the diets ($P > 0.05$) with an average of 128 ± 41 min. The RT increased along the SI for all diets. The averaged RT from all diets in the 1st, 2nd, 3rd and 4th SI segment was, 10 ± 13, 36 ± 25, 63 ± 37, and 181 ± 72 min, respectively, with an estimated mean RT over the entire SI of 282 ± 89 min. The RT in the 1st, 2nd, and 3rd SI segment did not differ among the diets ($P > 0.05$). The RT in the 4th SI segment was higher for the WG diet than for the SBM, RSM and BSF diets ($P < 0.05$).

The AD_{DM} and AD_{CP} increased along the SI for all diets ($P < 0.001$). The AD_{DM} did not differ among the diets in the 1st and 2nd SI segment ($P > 0.05$). The AD_{DM} was affected by the diet in the 3rd and 4th SI segment and over the total GIT ($P < 0.001$). In the 4th SI segment and consequently over the total GIT, the AD_{DM} of WG and PM diets was highest and for RSM diet lowest. The DPP diet tended to have a higher AD_{CP} in the 1st and 2nd SI segment than the RSM diet ($P = 0.09$ and 0.07, respectively). The AD_{CP} was affected by the diet in the 3rd and 4th SI segment and over the total GIT ($P < 0.001$). In the 4th SI segment and over the total GIT, the WG diet showed the highest and the RSM diet the lowest AD_{CP}.

The AD_{DM} and AD_{CP} at each SI segment was related to the sum of the RT of digesta in that segment to determine the digestion rate of each diet. Large differences in DM and CP digestion rate were observed among diets (Table 3.3). The DM digestion rate ranged from 0.0128 min^{-1} for the WG diet to 0.0377 min^{-1} for the DPP diet. The CP digestion rate ranged from 0.0109min^{-1} for the RSM diet to 0.0354 min^{-1} for the DPP diet.

Table 3.3. Digesta retention time (RT, min) and apparent digestibility (%) (AD) of dry matter (DM) and crude protein (CP) of the experimental diets containing a single protein source[1] along the small intestine (SI) and at faecal level in growing pigs.

	Diets containing the various protein source										Pooled SEM	*P*-value	
	SBM		WG		RSM		DPP		BSF			Diet	Segment
RT (min)													<0.001
Stomach	151	(8)[2]	110	(8)	135	(8)	115	(7)	129	(8)	14.3	0.254	
1st SI	8^{ab}	(7)	7^{ab}	(8)	23^{a}	(8)	5^{b}	(4)	6^{b}	(8)	4.7	0.021	
2nd SI	31	(8)	42	(7)	42	(8)	33	(7)	34	(8)	9.5	0.966	
3rd SI	62	(8)	93	(8)	43	(7)	68	(7)	50	(8)	12.6	0.083	
4th SI	140^{b}	(8)	251^{a}	(7)	148^{b}	(8)	222^{ab}	(7)	157^{b}	(8)	21.7	0.003	
SI	239^{b}	(8)	356^{a}	(8)	250^{ab}	(8)	325^{ab}	(7)	246^{ab}	(8)	28.2	0.015	
Stomach + SI	391	(8)	466	(8)	386	(8)	440	(7)	374	(8)	30.1	0.239	
AD_{DM} (%)													<0.001
1st SI	17.2	(7)	16.6	(8)	24.8	(8)	42.3	(4)	17.8	(7)	8.5	0.327	
2nd SI	53.1	(8)	38.0	(7)	35.1	(8)	53.1	(7)	45.1	(8)	6.7	0.212	
3rd SI	65.7^{ab}	(8)	62.4^{ab}	(8)	51.2^{b}	(7)	76.4^{a}	(7)	57.1^{b}	(8)	3.6	<0.001	
4th SI	75.8^{b}	(8)	83.8^{a}	(7)	58.9^{c}	(8)	85.8^{a}	(7)	76.4^{b}	(8)	0.9	<0.001	
Faecal	90.5^{b}	(8)	92.9^{a}	(8)	78.3^{c}	(8)	93.2^{a}	(7)	89.3^{b}	(8)	0.3	<0.001	
AD_{CP} (%)													<0.001
1st SI	11.9	(7)	34.0	(8)	4.0	(8)	57.0	(4)	17.6	(7)	12.7	0.086	
2nd SI	25.8	(8)	38.4	(7)	19.4	(8)	59.0	(7)	37.6	(8)	9.6	0.068	
3rd SI	54.8^{bc}	(8)	82.0^{a}	(8)	43.6^{c}	(7)	75.6^{ab}	(7)	48.9^{c}	(8)	4.5	<0.001	
4th SI	74.4^{c}	(8)	91.3^{a}	(7)	60.0^{e}	(8)	86.6^{b}	(7)	67.6^{d}	(8)	1.0	<0.001	
Faecal	83.4^{b}	(8)	94.4^{a}	(8)	69.8^{c}	(8)	95.9^{a}	(7)	80.9^{b}	(8)	0.7	<0.001	

[1] SBM= soybean meal; WG= wheat gluten; RSM= rapeseed meal; DPP= dried porcine plasma protein; BSF= black soldier fly larvae. [2] Values in brackets indicated the number of pigs of which samples could be collected and analysed. [a-e] Means within a row without a common superscript differ ($P < 0.05$).

Table 3.4. Parameter estimates characterising digestion kinetics of dry matter (DM) and crude protein (CP) in the small intestine, and postprandial appearance of amino acids and peptides in systemic blood of pigs fed experimental diets containing a single dietary protein source[1].

Parameter	Diets containing the various protein source									
	SBM		WG		RSM		DPP		BSF	
DM digestion[2]										
D_{max} (%)	75.0	(5.6)[4]	85.2	(5.0)	60.5	(2.8)	85.4	(3.7)	77.9	(5.5)
k (min^{-1})	0.0313	(0.0117)	0.0128	(0.0030)	0.0208	(0.0133)	0.0377	(0.0125)	0.0193	(0.0047)
$t_{1/2}$ (min)	22.1		54.1		33.4		18.4		35.9	
CP digestion[2]										
D_{max} (%)	78.4	(9.0)	93.8	(4.7)	61.2	(12.9)	86.0	(3.5)	68.0	(5.2)
k (min^{-1})	0.0120	(0.0035)	0.0154	(0.0027)	0.0109	(0.0059)	0.0354	(0.0112)	0.0180	(0.0041)
$t_{1/2}$ (min)	57.9		45.1		63.4		19.6		38.5	
Amino acids and peptides[3]										
$k_{increase}$ (min^{-1})	0.0046		0.0319		0.0330		0.0062		0.0188	
$k_{elimination}$ (min^{-1})	0.0039		0.0053		-		0.0056		0.0019	
$t_{1/2increase}$ (min)	151.2		21.8		21.0		112.5		36.9	
$t_{1/2elimination}$ (min)	177.2		130.3		-		123.9		373.2	

[1] SBM= soybean meal; WG= wheat gluten; RSM= rapeseed meal; DPP= dried porcine plasma protein; BSF= black soldier fly larvae. [2] D_{max} (%) is potentially digestible DM or CP (asymptote); k (min^{-1}) is the rate constant; $t_{1/2}$ (min) is the time where half of D_{max} is reached. [3] $k_{increase}$ (min^{-1}) is the rate constant at which amino acids and peptides appear in systemic blood; $k_{elimination}$ (min^{-1}) is the rate constant at which amino acids and peptides are cleared from systemic blood; $t_{1/2increase}$ (min) is the time where half of maximal concentration of amino acids and peptides in systemic blood is reached; $t_{1/2elimination}$ (min) is the time where half of maximal concentration of AAs and peptides in systemic blood is removed. [4] Values in brackets indicated the approximate standard error of the estimate.

Molecular weight distribution of protein and peptides in digesta of the stomach and SI

The 2 % SDS solution with 100 mM dithiothreitol solubilised 85 ± 3 % of the proteins and peptides in the digesta samples. Proteins and peptides in the diets predominantly had a molecular weight > 5 kDa (Figure 3.2). In the stomach, the amount of peptides with a molecular weight between 5 to 10 kDa increased for all diets. In digesta of the SI, proteins and peptides had molecular weights either > 5 kDa or < 1 kDa. A substantial quantity of peptides with a molecular weight < 1 kDa was present in the 4th SI segment of pigs fed the SBM, RSM and BSF diets. The presence of protein in digesta of pigs fed the protein free diet showed that endogenous proteins and peptides (mostly with molecular weight > 5 kDa) were secreted in the stomach and SI. The average molecular weight distribution of proteins and peptides in digesta over the SI was similar for each of the dietary treatments (Figure 3.2). The molecular weight fractions > 5 kDa accounted for the majority of protein in digesta in all treatments.

Postprandial concentration of amino acids and peptides in systemic blood

The postprandial concentration of AAs and peptides in systemic blood showed an interaction between time and diet ($P < 0.001$) (Figure 3.3). Two types of response were observed: a peak response for the WG and DPP diets and a plateau response for the SBM, RSM and BSF diets. The peak response showed a higher increase of plasma AAs and peptides after feeding than the plateau response. In addition, the clearance of AAs and peptides from plasma was faster in case of the diets with a peak response, whereas the plasma concentration of AAs and peptides remained close to its maximum for a longer period of time in case of the diets with a plateau response. The rate of increase ranged from 0.0046 min^{-1} for the SBM diet to 0.0330 min^{-1} for the RSM diet and the elimination rate ranged from 0.0019 min^{-1} for the BSF diet to 0.0056 min^{-1} for the DPP diet. Up to 30, 60, 90, 120 and 150 min after feeding, the WG diet showed the highest AUC in comparison to the other diets ($P < 0.001$) (Table 3.5). Up to 180 min, the WG and DPP diets showed a higher AUC compared to the SBM and RSM diets ($P < 0.05$). Over all treatments, AUC in time after a meal was positively correlated ($r = 0.73$, $P < 0.001$) with the amount of apparent digested protein in the SI (Figure 3.4).

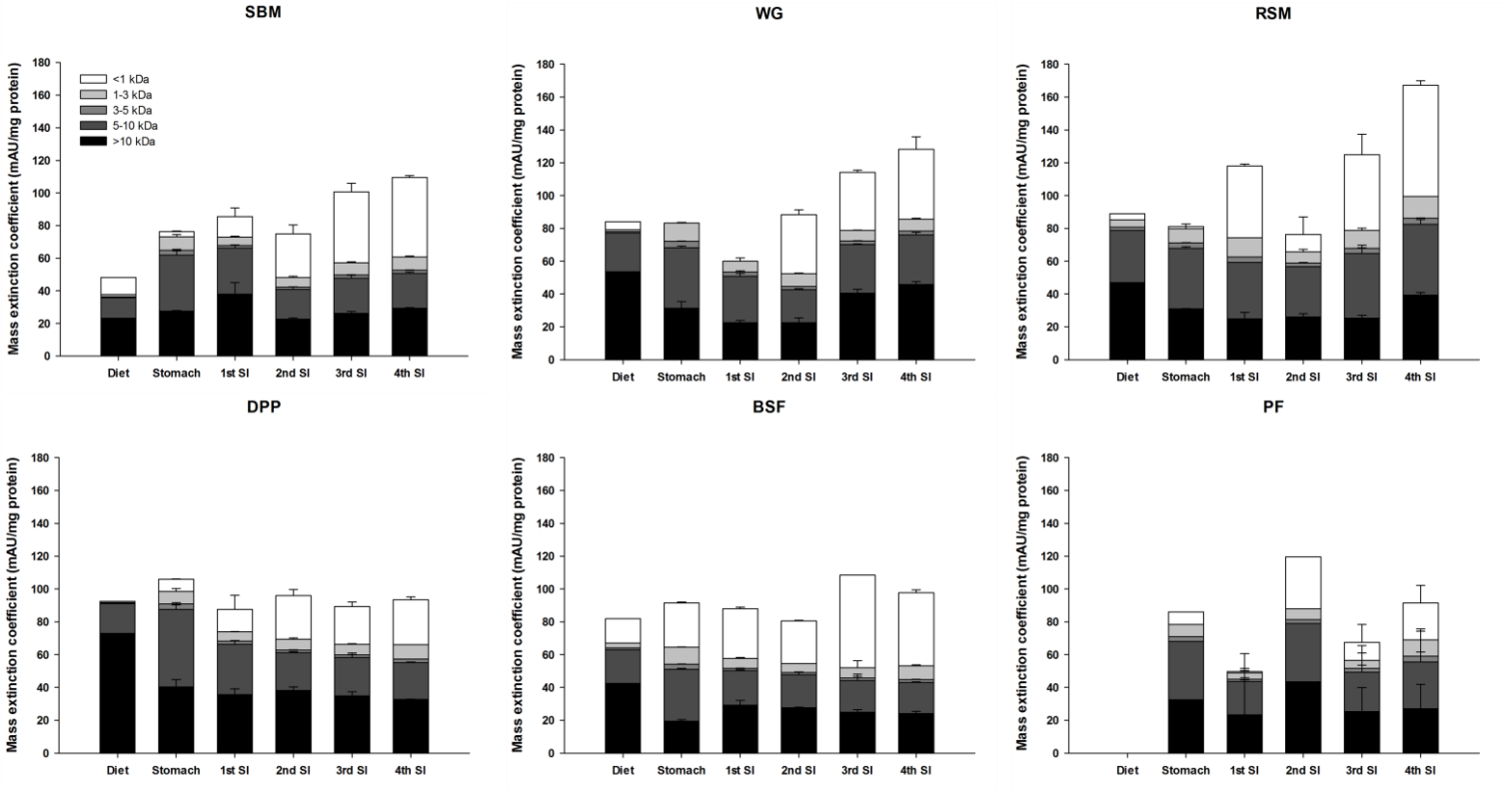

Figure 3.2. Molecular weight distribution (>10 kDa; 5-10 kDa; 3-5 kDa; 1-3 kDa; <1 kDa) of the protein component of the experimental diets and digesta samples from the stomach and the small intestine (SI) of growing pigs fed experimental diets containing soybean mean (SBM), wheat gluten (WG), rapeseed meal (RSM), dried porcine plasma protein (DPP) or black soldier fly larvae (BSF) as a single protein source, or no protein (PF). Values are means ± SE with n = 2 except for values of the stomach and 25-50 % SI segment sample for the PF diet where n = 1. Mass extinction coefficients of peptides smaller than 1 kDa could be underestimated after the correction for the signals from the SDS and dithiothreitol solution used for extraction as the signal was mainly found in the range of the low molecular weight (i.e. <1 kDa) fraction in the chromatogram.

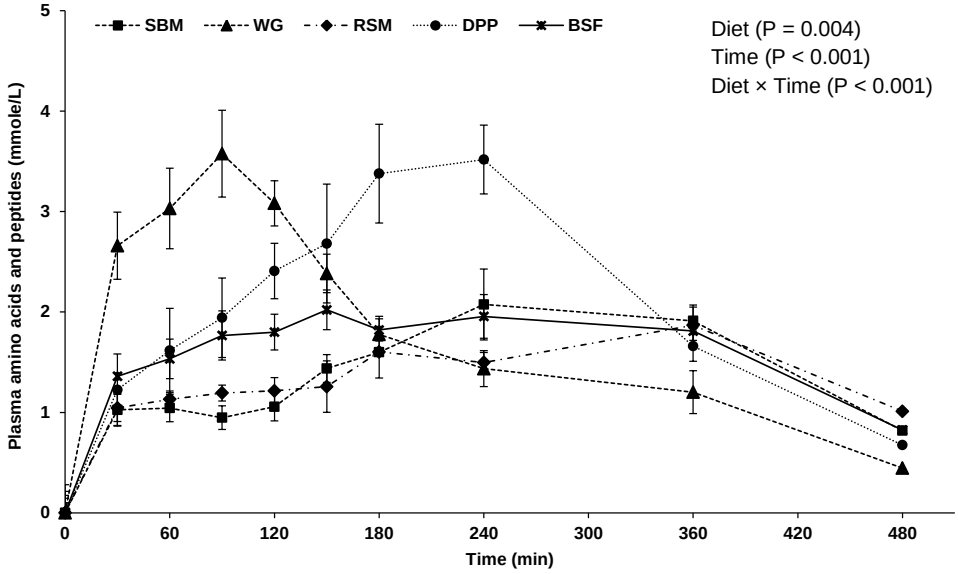

Figure 3.3. Postprandial concentration of amino acids and peptides in systemic blood of growing pigs fed experimental diets containing soybean mean (SBM), wheat gluten (WG), rapeseed meal (RSM), dried porcine plasma protein (DPP) or black soldier fly larvae (BSF) as the only dietary protein source. Values are means ± SEM with n = 4 per diet.

Table 3.5. Quantitative postprandial concentration (expressed as area under the curve, mmole · min/L) of amino acids and peptides in systemic blood of growing pigs fed experimental diets containing a single protein source[1].

Time period	Diets containing the various protein source					Pooled SEM	*P*-value
	SBM	WG	RSM	DPP	BSF		
n[2]	4	4	4	4	4		
0-30 min	15.4^{b}	39.9^{a}	9.9^{b}	18.4^{b}	20.4^{b}	3.1	<0.001
0-60 min	46.4^{b}	125.2^{a}	42.6^{b}	61.0^{b}	63.8^{b}	8.2	<0.001
0-90 min	76.3^{b}	224.3^{a}	77.4^{b}	114.4^{b}	113.3^{b}	14.3	<0.001
0-120 min	106.4^{b}	324.2^{a}	113.6^{b}	179.7^{b}	166.8^{b}	20.9	<0.001
0-150 min	143.8^{b}	406.2^{a}	150.6^{b}	256.0^{b}	224.1^{b}	26.0	<0.001
0-180 min	189.3^{c}	468.6^{a}	193.5^{c}	346.9^{ab}	281.7^{bc}	29.4	<0.001

[1] SBM= soybean meal; WG= wheat gluten; RSM= rapeseed meal; DPP= dried porcine plasma protein; BSF= black soldier fly larvae. [2] Number of pigs of which samples were collected and analysed. [abc] Means within the row without a common superscript differ ($P < 0.05$).

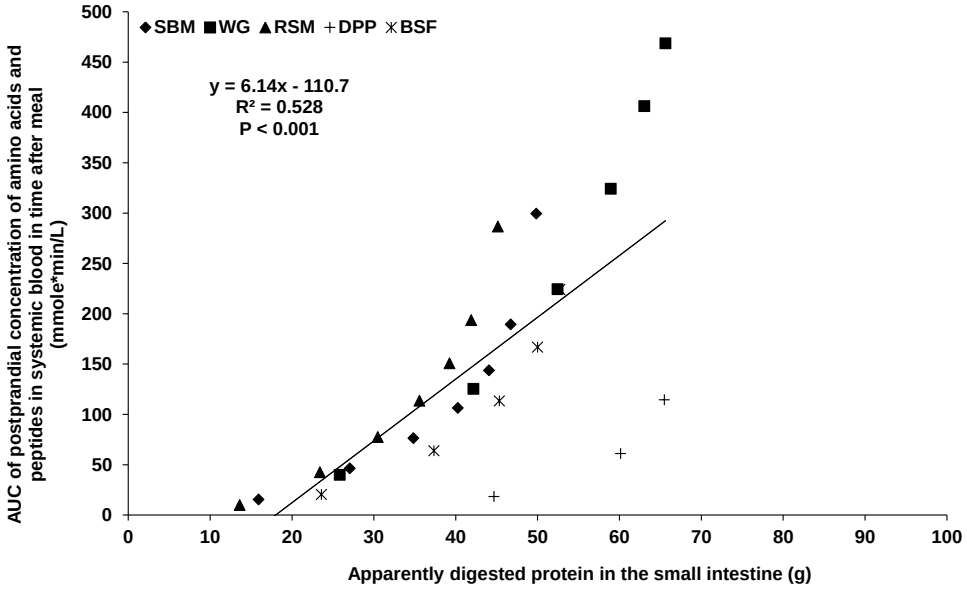

Figure 3.4. The relationship between the amount of apparently digested protein (g) up to the end of the small intestine and the appearance of AA in systematic blood using the area under the curve (AUC) method (mmole · min/L) of experimental diets containing different protein sources.

DISCUSSION

The aim of the present study was to determine the *in vivo* protein digestion kinetics up to the end of the SI of different protein sources, and its influence on the postprandial appearance of AAs and peptides in systemic blood in pigs. Free AAs were supplemented to the diets in order to avoid severe deficiencies in the supply of essential AAs from the diets. The level of supplementation, however, was low (ranging from 0 to 6.3 g/kg) compared to the content of protein in the experimental diets (160 g/kg). The differences in protein digestion kinetics of the experimental diets were, therefore, attributed to the protein sources which were included as a single protein source. Among protein sources, WG and DPP had a higher CP content than the other protein sources. Since all experimental diets were formulated isoproteineous, the inclusion level of WG and DPP in the diets was lower than the other protein sources. Maize starch was used to fill up the gap of non-protein components present in SBM, RSM and BSF in the diets with WP and DPP, resulting in a higher starch content of these diets than that of the SBM, RSM and BSF diets. The apparent ileal protein digestibility

was approximated by the AD_{CP} measured in the 4th SI segment. Values for SBM, WG and RSM diets (74, 91, and 60 %, respectively) were lower than the tabulated values for apparent ileal protein digestibility in CVB (2016) (85, 98, and 70 %, respectively). This could be due to the fact that digesta samples were collected from the 4th SI segment, comprising the distal quarter of the jejunum and the entire ileum, instead of only from the end of the ileum. More undigested protein is likely to be present in the former, resulting in a lower AD_{CP}. The ranking of protein sources based on the AD_{CP} measured in the 4th SI segment, however, is in accordance with the ranking based on data on ileal digestibility as presented by CVB (2016). These results suggest that the differences in the extent and the kinetics of protein digestion among evaluated protein sources are as expected and can be related to the nature (e.g. chemical composition, protein conformation, physicochemical properties) of protein sources.

In pig diets, WG and DPP are considered as highly digestible protein sources while the ileal protein digestibility of RSM is relatively low (CVB, 2016). Proteins present in highly digestible protein sources are assumed to be more susceptible to hydrolysis by digestive enzymes. As a result, high molecular weight proteins and peptides are assumed to be hydrolysed into low molecular weight peptides and should be present in low quantities in digesta at the end of the SI. In contrast, proteins present in low digestible protein sources are assumed to be more resistant to enzymatic hydrolysis, resulting in the higher presence of high molecular weight proteins and peptides in digesta at the end of the SI. Assuming the former, the molecular weight distribution of proteins and peptides in digesta of pigs fed high and low digestible protein sources were expected to be different. The molecular weight distribution of proteins and peptides in digesta throughout the GIT, however, was comparable among protein sources in the present study. These results indicate that the mechanism of hydrolysis and absorption of proteins was rather similar among protein sources, although the nature of proteins present in protein sources is different, resulting in various protein digestion kinetics and digestibility. In addition, the results indicate that the hydrolysis of protein sources by digestive enzymes followed a “one-by-one” type of hydrolysis mechanism, meaning intact proteins from the protein sources are hydrolysed to low molecular weight peptides and free AAs and are absorbed by the intestinal mucosa in one sequence (Adler-Nissen, 1976) .

In pigs, the absorption of AAs, and di- and tri-peptides by intestinal enterocytes takes place in the jejunum and ileum, of which the proximal jejunum is the major site of

absorption (Low, 1979; Bröer, 2008). A slow hydrolysis of dietary proteins in the proximal part of the SI could lead to the release of AAs and di- and tri-peptides in the more distal part of the SI (i.e. 3rd and 4th SI segment). Since the absorption of AAs and di- and tri-peptides is less efficient in the distal part of the SI than in the proximal part of the SI, there is a high chance that these potentially digestible AAs, and di- and tri-peptides are passed to the colon and considered indigestible in the small intestine. In the present study, a relatively large amount of peptides with a molecular weight < 1 kDa was found in the digesta from the end of the SI of pigs fed the SBM, RSM and BSF diet. This might be due to the formation of indigestible peptide aggregates during enzymatic hydrolysis (Fischer et al., 2007), or to the low AD_{CP} in the proximal part of the SI (i.e. 1st and 2nd SI segment) for the SBM, RSM and BSF diets observed in the present study. The WG diet also showed a low AD_{CP} in the 2nd SI segment. However, in the 3rd SI segment, the AD_{CP} of the WG diet was largely much higher than that of the SBM, RSM and BSF diets. This might be due to the longer RT of the WG diet in that SI segment. These results indicate that a prolonged RT in the SI may compensate the effect of slow release of AAs, and di- and tri-peptides and result in a quantitatively higher digestibility of dietary proteins up to the end of the small intestine.

The digestion kinetics of dietary proteins was evaluated by combining data on the AD_{CP} and the cumulative RT of digesta up to that SI segment. The DPP diet showed the highest fractional protein digestion rate. Although the WG diet showed a similar AD_{CP} in the 4th SI segment to the DPP diet (91 and 87 %, respectively), the fractional protein digestion rate of the WG diet was 2.3 times lower than that of the DPP diet (0.0154 and 0.0354 min^{-1}, respectively). In addition, the WG and BSF diet showed a comparable fractional protein digestion rate (0.0154 and 0.0180 min^{-1}, respectively) while the AD_{CP} in the 4th SI segment was higher for the WG diet (91 %) than for the BSF diet (68 %). These results opposed the hypothesis that highly digestible protein sources are digested faster in the SI of pig. The fractional protein digestion rate is not related to the extent of protein digestion up to the end of small intestine over the protein sources evaluated in the present study.

Blood samples were collected from the ear vein to follow the postprandial appearance of AAs and peptides in systemic blood. The WG diet showed a more rapid and pronounced postprandial appearance of AAs and peptides in systemic blood than SBM, RSM and BSF diets. A more rapid and pronounced postprandial appearance can be related to a high passage rate of digesta through the stomach (Gaudichon et al., 1994), a high hydrolysis rate of dietary proteins in the stomach and small intestine

(Guan et al., 2016), and/or a high absorption rate of AAs and peptides by the intestinal mucosa. Indeed, the WG diet showed a numerically lower RT in the stomach and a numerically higher AD_{CP} in the proximal SI than SBM, RSM and BSF diets. The differences were not statistically significant due to the relatively large animal variation within each treatment. Although the DPP diet also showed a similar RT in the stomach and AD_{CP} in the proximal SI to the WG diet, a delay in the postprandial appearance of AAs and peptides in systemic blood of pigs fed the DPP diet was observed. This might be due to differences in the extent of AA metabolism in the intestinal mucosa and the liver of protein sources related to differences in their AA profile. It should be noted that TiO_2 was used as an indigestible marker to estimate RT of digesta along the GIT. This is a water-insoluble marker and therefore the RT calculated based on TiO_2 represents primarily the RT of the solid fraction of digesta (Solà-Oriol et al., 2010). Studies in pigs have shown that the solid fraction of digesta had a longer RT in the stomach than the liquid fraction (Gregory et al., 1990; Johansen et al., 1996; Davis et al., 2001). In our previous study, WG and DPP showed a high protein solubility under the conditions as prevailing in the stomach (Chapter 2). The use of TiO_2, therefore, might hinder the determination of RT for soluble proteins. As a consequence the RT for soluble proteins present in the WG and DPP diet in the stomach could be overestimated.

The linear relationship between data on the kinetics of protein digestion up to the end of the SI and the kinetics of postprandial appearance of AAs and peptides in systemic blood was determined. Studies on digestion kinetics of nutrients commonly provide information on the potentially digestible fraction (i.e. the extent) and the fractional digestion rate, similar to results presented in the present study. The digestion rate of a nutrient in an ingredient or diet is basically independent of its extent of digestion. In quantitative nutrition research, comparison of parameters accounting for both the fractional rate and the extent is preferred over comparison of fractional rates only. An approach, therefore, was taken to relate the quantitative disappearance of AAs and peptides from the lumen of the SI to the quantitative appearance of AAs and peptides in systemic blood. Over all dietary treatments, the concentration of AAs and peptides in systemic blood after a meal was positively correlated to the amount of apparent digested protein up to the end of the SI.

It should be noted that leucine was used as a standard to determine the concentration of total AAs and peptides in blood plasma in the ninhydrin assay. The molar extinction coefficient of individual AA in this assay, however, was shown to range from 0 for

proline to 1.08 for lysine relative to leucine (Friedman, 2004). Moreover, one peptide molecule gives a similar molar extinction coefficient to one molecule of free AA. The concentration of AAs in plasma, therefore, could be underestimated in the case of high presence of peptides relative to free AAs using this method. The concentration and profile of AAs and peptides in systemic blood could be different from that of AAs and peptides absorbed from the intestinal lumen by enterocytes because of the metabolism of AAs in the intestinal mucosa and in the liver and other organs and tissues (Stoll et al., 1998a;b). In addition, it was shown that the extent of intestinal and hepatic metabolism of absorbed AAs can be affected by dietary protein source (Nunes et al., 1991). The postprandial appearance of AAs and peptides in systemic blood, therefore, is the net result of AAs and peptides being absorbed from the lumen of the SI and AAs and peptides being removed from the blood circulation for metabolism in organs and tissues, and as a result does not completely resemble the apparent disappearance of AAs and peptides from the lumen of the SI. Despite this, the results suggest that observations on changes in AA and peptide concentration after a meal in systemic blood cab be used to study protein digestion kinetics.

CONCLUSIONS

The kinetics of protein digestion and postprandial appearance of AAs and peptides in systemic blood of pigs differed among diets containing different protein source. WG and DPP can be regarded as fast digestible sources while SBM, RSM and BSF as more slowly digestible protein sources. The kinetics of appearance of AAs and peptides in blood was positively correlated to the kinetics of disappearance of AAs and peptides from the lumen of the SI. A more rapid and pronounced postprandial appearance of AAs and peptides in blood can be explained by a high passage rate of digesta through the stomach and/or a high rate of enzymatic hydrolysis of proteins and absorption of AAs and peptides in the SI.

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Chapter 4

Protein digestion kinetics in the small intestine of broilers differs among protein sources

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ABSTRACT

The aim of the present study was to determine the *in vivo* protein digestion kinetics up to the end of the small intestine (SI) of six protein sources (soybean meal (SBM), soy protein isolate (SPI), wheat gluten (WG), rapeseed meal (RSM), dried porcine plasma protein (DPP), and black soldier fly larvae (BSF)) in broilers. A total of 378 26-day-old male broilers with average body weight of 1430 ± 48 g were randomly allocated to 42 pens. Pens were randomly allocated to one of the seven diets (i.e. a basal diet and six experimental diets with the respective protein sources). At dissection, digesta samples from the crop, gizzard, duodenum, proximal jejunum, distal jejunum, and ileum were quantitatively collected. Apparent digestibility (AD) of dry matter (DM) and crude protein (CP), and retention time (RT) of the solid and liquid fractions of digesta along the crop, gizzard and small intestine (SI) were determined to calculate protein digestion kinetics. The AD_{CP} was affected by the diet in the SI ($P < 0.001$), of which the WG diet showed the highest and the RSM diet showed the lowest AD_{CP} along the SI. Over all experimental diets, the solid fraction of digesta showed a longer RT in the gizzard ($P < 0.01$) and tended to show a longer RT in the SI than the liquid fraction of digesta ($P = 0.052$). The RT for the solid fraction of digesta was affected by the diet in the crop, gizzard and duodenum ($P < 0.05$) but not in the proximal jejunum, distal jejunum and ileum. The initial rate of CP digestion ranged from 4 $g \cdot min^{-1}$ per kg diet for the RSM diet to 67 $g \cdot min^{-1}$ per kg diet for the SPI diet. In conclusion, SPI, WG and DPP can be regarded as fast digestible protein sources while SBM, RSM and BSF as more slowly digestible protein sources in broilers.

INTRODUCTION

An efficient utilisation of dietary protein is economically important in modern-day broiler production particularly in light of the forthcoming global protein scarcity. Amino acids (AAs) and peptides provided via dietary proteins are used to synthesise body proteins or as an energy source after deamination. The efficiency of protein utilisation depends on the balance between these two processes, of which a higher efficiency relates to a higher body protein synthesis. The simultaneous availability of AAs and energy increases protein synthesis in organs and tissues (Geiger, 1950; van den Borne et al., 2007). As such, the fate of dietary AAs and peptides in the post absorptive metabolism depends on the kinetics of protein digestion relative to the digestion kinetics of energy providing nutrients in the diets such as starch. The kinetics of starch digestion for various feed ingredients, both *in vitro* and *in vivo*, has been extensively studied in broilers (Weurding, 2002). The digestion rate of starch in the small intestine (SI) of chickens varied substantially among feed ingredients ranging from 0.009 min^{-1} for raw potato starch to 0.071 min^{-1} for tapioca starch (Weurding et al., 2001). Information on the digestion kinetics of protein in different feed ingredients in broilers is limited. Such data are required to ensure the synchronisation of the supply of dietary energy and protein, which could improve protein retention and efficiency of protein utilisation in broiler production (Liu and Selle, 2015).

The overall kinetics of dietary protein digestion is related to three aspects: 1) the passage rate of digesta along the gastrointestinal tract (GIT), 2) the hydrolysis rate of dietary proteins in the GIT, and 3) the absorption rate of AAs and peptides by the intestinal mucosa. In broiler chickens, the passage rate of digesta along the GIT can be affected by diet type (i.e. purified and non-purified diets) and composition. For example, a semi-purified diet with dextrose and distillers dried grains with solubles showed a 8 % lower digesta retention time along the GIT than a corn-soybean meal (SBM)-based diet in broilers (5.13 vs. 5.58 h) (Rochell et al., 2012). Moreover, it has been shown that an increase in the concentration of dietary insoluble non-starch polysaccharides tended to increase digesta retention time in the crop and gizzard of laying hens by 20 % (van Krimpen et al., 2011). As for the hydrolysis rate of dietary proteins, it is affected by protein conformation (Malabat and Rabiller, 2001), protein solubility (Tonheim et al., 2007), and by the interaction between proteins and non-protein constituents in protein sources (Selle et al., 2012). In addition, diet composition could affect digestive enzymes activity and secretion, thereby influencing the rate of protein hydrolysis. For example,

a diet with high tannin concentration reduced the activity of trypsin in the ileum of pigs compared to a diet with a low tannin concentration. This likely contributed to a lower ileal protein digestibility for the diet with high tannin concentration (Jansman et al., 1994). In addition, in broilers, eight peptidases were shown to be involved in the digestion of a SBM diet, whereas only six peptidases were involved for a rapeseed meal (RSM) diet (Recoules et al., 2017). This could possibly partly explain why SBM shows a higher ileal protein digestibility than RSM in broilers (CVB, 2016). With regard to the absorption rate of AAs and peptides by the intestinal mucosa, it was shown to be regulated by the luminal concentration of AAs and peptides in mice and pigs (Stevens, 1992).

The objective of the present study was to determine the *in vivo* protein digestion kinetics up to the end of the SI of different common and alternative protein sources used as feed ingredients in diets for broilers. Protein digestion kinetics were studied by determining digesta retention time, protein digestibility and molecular weight distribution of soluble proteins and peptides in digesta along the SI of broilers. It was hypothesized that protein sources with a higher ileal protein digestibility are digested faster (i.e. having a higher initial digestion rate) in the SI of broilers than protein sources with a lower ileal protein digestibility.

MATERIALS AND METHODS

Protein sources and experimental diets

The protein sources evaluated were SBM, soy protein isolate (SPI), wheat gluten (WG), RSM (all commodity batches obtained via Research Diet Services, Wijk bij Duurstede, the Netherlands), dried porcine plasma protein (DPP) (obtained from Darling Ingredients Inc., Irving, TX, USA), and black soldier fly larvae meal (BSF) (obtained from Protix, Dongen, the Netherlands). Initially, all six experimental diets were formulated isoproteineous (CP, 168 g/kg as-fed basis) and protein sources were included as a single protein source in the experimental diets. Free AAs were added so the diets to meet at least 80 % of the requirement of the limiting amino acids in broilers (CVB, 2009). For BSF, information on the AA profile and ileal AA digestibility was obtained from (Veldkamp et al., 2012; De Marco et al., 2015). The total level of supplemented free AAs ranged from 0 g/kg diet (as-fed basis) for the RSM diet to 17.5 g/kg diet (as-fed basis) for the WG diet. However, feed intake over experimental day 7 to 12 appeared to be

low for birds receiving the SPI, WG, DPP and BSF diets, which was possibly related to the fine physical structure of these diets, compared to the SBM and RSM diets. To further limit differences in feed intake between birds of the different experimental treatments, from day 13 onwards, 100 g/kg (additional) SBM was included on top of all experimental diets. In addition, a basal diet with low protein content, containing only 91 g/kg SBM, was used as a reference diet in the experiment.

Titanium dioxide (TiO_2) was included in all diets as an indigestible solid marker at 2.1 g/kg diet (as-fed basis). In addition, chromium-ethylenediamine tetraacetic acid (Cr-EDTA) was included as an indigestible soluble marker at 1.7 g/kg diet (as-fed basis). All diets were produced by Research Diet Services (Wijk bij Duurstede, the Netherlands). The ingredient composition of the experimental diets is presented in Table 4.1.

Design, animals and housing

This study was approved by the Animal Care and Use Committee of Wageningen University & Research (Wageningen, the Netherlands). The experiment followed a randomized complete block design. Seven diets were used: a basal diet and six experimental diets with tested protein sources. Pens were blocked on the location in the experimental room (six blocks, seven pens per block) and pens within a block were randomly allocated to one of the seven experimental diets. A total of 378, 26-day-old male broilers (Ross 308, Aviagen Group, Newbridge, UK) with average body weight of 1430 ± 48 g were used. Broilers were housed in 42 pens (1.00 × 0.75 m) with nine broilers in a pen. A plate was connected to the pen which allowed for excreta collection. Wood shavings were used as bedding material but were removed during the period of excreta collection. The ambient temperature was kept constant at 21° C. From experimental day 1 to 15, lighting schedule followed a 16L : 8D regime. From day 16 to 20, lights were continuous on (24L : 0D). The relative humidity was between 40 and 70 % throughout the experimental period.

Feeding

From day 1 to 6, broilers were fed a commercial diet and gradually adapted to the experimental diets. From day 7 to 12, broilers were fed the experimental diets containing the respective protein sources. From day 13 to 20, broilers were fed the experimental diets including the additional 100g/kg of SBM. The experimental diets were provided in a mash form. Throughout the entire experimental period, broilers had unlimited access to feed and water. The broilers fed the basal diet received the SBM

diet from day 1 to 12 and were gradually adapted to the basal diet from day 13 to 16. At day 17 and 18, these broilers were only fed the basal diet.

Sample collection and dissection procedure

Total feed intake and body weight per pen were recorded at day 7, 12 and 20. Average daily feed intake (ADFI) and body weight gain (ADG) were calculated between day 12 and 20. Feed conversion ratio (FCR) was calculated by dividing total feed intake by total body weight gain, including dead birds. At the dissection days (day 19 and 20), broilers were euthanized by electrocution being placed on their posterior side and the body cavity was opened, after which the GIT from the crop to the cloaca was carefully removed. The GIT was carefully located horizontally on the table without disturbing the digesta and separated into crop, proventriculus, gizzard, duodenum, proximal jejunum (1st half of the jejunum), distal jejunum (2nd half of the jejunum), ileum, caeca and colon. The jejunum and the ileum were separated at the Meckel's diverticulum. Throughout the entire dissection, care was taken to prevent the movement of digesta. Digesta from these GIT segments was quantitatively collected by gentle stripping. The collected digesta samples were freeze-dried and analysed for DM, nitrogen (N), and markers to calculate digestibility of DM and CP, and retention time of the solid and liquid fraction of digesta along the GIT.

Chemical analysis

All chemical analyses were performed according to standard laboratory methods. The experimental diets were analysed for dry matter (DM) (method ISO 6496; ISO, 1999a), ash (method ISO 5984; ISO, 2002), acid-hydrolysed ether extract (method ISO 6492; ISO 1999b), starch (method ISO 15914; ISO, 2004), total sugars as reducing sugars (van Vuuren et al., 1993) and N by the Kjedahl method (method ISO 5983-1; ISO, 2005a). A factor of 6.25 was used to calculate the crude protein (CP) content from analysed N. The protein sources were analysed for AA composition (method ISO 13903; ISO, 2005b). The N content of digesta and excreta samples was determined using the Dumas method (method ISO 16634-1; ISO, 2008) using a Flash EA 1112 NC Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). The experimental diets, digesta and excreta samples were analysed for their Ti (Myers et al., 2004) and Cr concentration (Williams et al., 1962; van Bussel et al., 2010).

Table 4.1. Composition of the experimental diets (g/kg, as-fed basis otherwise stated).

	Diets containing the various protein source[1]						
Ingredients	SBM	SPI	WG	RSM	PP	BSF	Basal
Soybean meal	404.2	90.9	90.9	90.9	90.9	90.9	90.9
Soy protein isolate	0.0	177.2	0.0	0.0	0.0	0.0	0.0
Wheat gluten	0.0	0.0	165.7	0.0	0.0	0.0	0.0
Rapeseed meal	0.0	0.0	0.0	454.5	0.0	0.0	0.0
Dried porcine plasma protein	0.0	0.0	0.0	0.0	178.5	0.0	0.0
Insect protein meal	0.0	0.0	0.0	0.0	0.0	262.0	0.0
Maize starch	259.3	426.7	425.8	130.1	424.7	331.5	599.5
Sucrose	82.6	82.6	82.6	82.6	82.6	82.6	82.6
Dextrose	41.3	41.3	41.3	41.3	41.3	41.3	41.3
Oat hulls	82.6	82.6	82.6	82.6	82.6	82.6	82.6
Arbocel	41.3	41.3	41.3	41.3	41.3	41.3	41.3
Soy oil	55.7	12.1	7.2	60.7	12.1	8.0	13.8
Mineral and vitamin premix[1]	4.1	4.1	4.1	4.1	4.1	4.1	4.1
Limestone	9.3	16.5	10.5	1.2	9.8	16.5	10.2
Salt	3.1	3.4	0.8	3.1	0.0	1.7	3.4
Monocalcium phosphate	9.4	10.7	12.7	2.3	13.6	11.2	14.5
Potassium carbonate	0.0	4.6	10.2	0.2	10.8	8.6	10.6
Sodium bicarbonate	0.0	0.3	4.4	1.2	0.0	3.2	1.3
L-Lysine HCL	0.0	0.0	6.9	0.0	0.0	2.4	0.0
DL-Methionine	1.7	0.0	1.1	0.0	2.0	3.0	0.0
L-Threonine	0.0	1.8	2.1	0.0	0.0	0.9	0.0
L-Tryptophan	0.0	0.0	0.2	0.0	0.0	0.0	0.0
L-Arginine	0.6	0.0	3.6	0.0	1.0	3.6	0.0
L-Isoleucine	0.9	0.0	0.7	0.0	0.7	0.7	0.0
L-Valine	0.0	0.0	1.5	0.0	0.0	0.1	0.0
TiO_2	2.1	2.1	2.1	2.1	2.1	2.1	2.1
Cr-EDTA	1.7	1.7	1.7	1.7	1.7	1.7	1.7
Calculated nutrient composition							
ME[2], MJ/kg as-fed	11.5	11.5	12.0	10.0	11.7	11.5	11.9
Ash	5.6	5.9	5.3	5.4	5.9	7.7	5.4
Crude protein	19.5	19.5	19.5	19.5	19.5	19.5	4.7
Starch	22.6	36.4	37.4	12.0	36.2	29.5	50.8
Sugars	17.5	14.2	14.6	18.4	14.2	14.2	14.2
Non-starch polysaccharides	19.2	14.0	12.7	28.4	13.3	17.7	13.3
Calcium	6.3	8.3	6.5	5.4	6.3	16.8	6.6
Available phosphorus	2.9	2.9	3.0	2.5	2.9	2.9	3.0
Sodium	1.4	1.7	1.7	1.8	4.3	1.7	1.7
Digestible Lys	11.9	11.9	10.4	11.0	15.2	11.2	2.9
Digestible Met + Cys	7.3	7.0	7.4	8.0	9.5	7.1	1.3
Digestible Thr	7.5	7.5	7.1	8.3	9.6	7.3	1.8
Digestible Trp	2.5	2.4	2.0	2.5	2.8	2.3	0.6

[1] SBM= soybean meal; SPI= soy protein isolate; WG= wheat gluten; RSM= rapeseed meal; DPP= dried porcine plasma protein; BSF= black soldier fly larvae. [2]The mineral and vitamin premix supplied per kilogram of diet: Vitamin A, 12,000 IU; cholecalciferol, 0.6 mg; vitamin E, 50 mg; vitamin B2, 7.5 mg; vitamin B6, 3.5 mg; vitamin B1, 2.0 mg; vitamin K, 1.5 mg; vitamin B12, 20 µg; choline chloride, 460 mg; anti-oxidant, 125 mg; niacin, 35 mg; pantothenic acid, 12 mg; biotin, 0.2 mg; folic acid, 1 mg; Mn, 85 mg as MnO; Fe, 80 mg as $FeSO_4$; Zn, 60 mg as $ZnSO_4$; Cu, 12 mg as $CuSO_4$; I, 0.8 mg as KI; Co, 0.4 mg as $CoSO_4$; Se, 0.15 mg as Na_2SeO_3. [2]Metabolisable energy.

Size exclusion chromatography

The molecular weight distribution of soluble proteins and peptides in digesta from the different segments of the GIT was analysed using an ÄKTA micro system (GE Healthcare, Uppsala, Sweden) with a Superdex Peptide PC 3.2/30 column (GE Healthcare). The eluent used was a 10 mM potassium phosphate buffer with 150 mM NaCl and 2 % SDS. Freeze-dried digesta samples (20 mg) were weighed and solubilised in a 2 % SDS solution with 100 mM dithiothreitol. The samples were heated (100 °C) for 30 min and then centrifuged (10 min, 20,000 g, 20 °C). The supernatants were diluted with the eluent in a 1:1 (v/v) ratio. Samples were then centrifuged, and 50 µL of the supernatant was injected on the column. The absorbance was measured at 214 nm. The absorbance of the samples was corrected for the signals from the SDS and dithiothreitol solution in the chromatograms. For the calibration curve, β-lactoglobulin (18,360 Da), vitamin B12 (1,335 Da), glutathione (307 Da), glycine-proline-glycine (229 Da), phenylalanine (165 Da) and alanine (89 Da) were used. The chromatograms obtained were separated into molecular weight ranges of >10 kDa, 10-5 kDa, 5-3 kDa, 3-1 kDa and <1 kDa by calculating the eluent volumes based on the calibration curve. The mass-based extinction coefficient (mAU/g) was calculated by dividing the absorbance measured at 214 nm by the amount of protein in the samples.

Calculations and statistical analysis

Apparent digestibility (AD) of DM and CP was calculated by equation 1:

$$\text{Apparent digestibility of nutrient (\%)} = \frac{(\text{Nutrient}_{diet}/\text{Ti}_{diet}) - (\text{Nutrient}_{digesta}/\text{Ti}_{digesta})}{(\text{Nutrient}_{diet}/\text{Ti}_{diet})} \times 100\ \% \quad (1)$$

where Nutrient_{diet} and $\text{Nutrient}_{digesta}$ (%) are the nutrient (DM or CP) content in the experimental diets (as-fed basis) and in the freeze-dried digesta samples, respectively, and Ti_{diet} and $\text{Ti}_{digesta}$ (%) are the Ti content in the same samples of experimental diets and the digesta, respectively.

Retention time of the solid (RT_{solid}) and liquid fraction (RT_{liquid}) of digesta in the GIT segments was calculated by equation 2:

$$\text{Retention time (min)} = \frac{1440 \times \text{Marker}_{digesta} \times W_{digesta}}{FI_{24h} \times \text{Marker}_{diet}} \quad (2)$$

where $\text{Marker}_{digesta}$ (%) is the marker (Ti or Cr) content in the freeze-dried digesta samples, $W_{digesta}$ (g) is the weight of freeze-dried digesta samples from the stomach or the four segments of the SI, FI_{24h} (g) is the feed intake over 24 h prior to digesta sampling,

$Marker_{diet}$ (%) is the marker (Ti or Cr) content in the experimental diets (as-fed basis), and the factor 1440 is used to convert time from days to minutes.

The nutrient digestion kinetics up to the end of the SI of the diets containing the different protein sources were calculated by relating the AD coefficient for DM and CP at each segment of the SI to the sum of RT_{solid} up to that segment (Ørskov and McDonald, 1979). The curve was fitted using a first order reaction rate equation (equation 3):

$$D_t = D_{max} (1 - e^{-kt}) \quad (3)$$

where D_t (g/kg diet) is the digestible of DM or CP per kg of diet at time t (min), D_{max} (g/kg diet) is the potentially digestible DM or CP per kg of diet (asymptote), and k is the rate constant. The equation was fitted using the MODEL procedure of SAS (version 9.3, SAS Institute Inc., Cary, NC) and the parameter estimates and the approximate standard error of the estimates were calculated.

All variables (initial BW, final BW, ADG, ADFI, FCR, RT_{solid}, RT_{liquid}, AD_{DM} and AD_{CP} in each GIT segment) were analysed by analysis of variance using the GLM procedure of SAS with dietary protein source as a fixed effect. Residuals of all dependent variables were tested for normality and non-normally distributed data were log transformed. Probability levels of less than 5 % were considered to be statistically significant, and levels between 5 to 10 % were considered a trend.

RESULTS

One bird from the treatment of SBM, RSM, DPP, BSF and basal diet, and two birds from the SPI treatment died during the experiment. The other broilers remained healthy throughout the experiment.

Crude protein content of experimental diets

The CP content of the experimental diets containing the various protein sources ranged from 20.8 % for the RSM and DPP diets to 21.4 % for the SPI diet (DM basis) (Table 4.2), of which approximately 75 % of the CP originated from the protein sources and the remaining 25 % from the supplemented SBM.

Table 4.2. Analysed chemical and amino acid composition of the experimental diets.

	Diets containing the various protein source[1]						
Item[2]	SBM	SPI	WG	RSM	DPP	BSF	Basal
Dry matter (% as-fed)	90.1	90.9	90.4	90.4	89.2	91.6	88.8
Ash	5.8	6.0	5.5	5.9	6.3	7.6	5.3
Crude protein	21.0	21.4	21.1	20.8	20.8	21.1	5.2
Crude fat	3.9	1.4	2.2	8.5	1.9	4.9	1.3
Starch	27.4	42.4	42.4	16.1	39.6	32.3	51.6
Sugars	17.7	15.0	15.8	19.2	15.5	14.9	15.1
Indispensable AA							
His	0.59	0.64	0.54	0.64	0.69	0.67	-
Ile	1.14	1.04	0.81	0.87	0.87	0.85	-
Leu	1.59	1.72	1.38	1.49	1.73	1.35	-
Lys	1.20	1.31	1.40	1.14	1.38	1.30	-
Met	0.51	0.30	0.41	0.40	0.47	0.60	-
Phe	1.03	1.12	0.89	0.86	1.01	0.90	-
Thr	0.82	0.99	0.77	0.91	1.04	0.88	-
Val	1.00	1.07	0.96	1.06	1.20	1.10	-
Dispensable AA							
Ala	0.91	0.95	0.60	0.92	1.03	1.20	-
Asx	2.40	2.57	1.09	1.75	1.97	1.91	-
Cys	0.28	0.27	0.37	0.41	0.63	0.18	-
Glx	3.72	4.06	5.98	3.52	3.39	2.39	-
Gly	0.88	0.91	0.69	1.02	0.91	0.93	-
Pro	1.19	1.26	2.02	1.34	1.38	1.18	-
Ser	1.07	1.14	0.97	0.96	1.09	0.83	-

[1] SBM= soybean meal; SPI= soy protein isolate; WG= wheat gluten; RSM= rapeseed meal; DPP= dried porcine plasma protein; BSF= black soldier fly larvae. [2] Values are expressed on a dry matter basis (DM) unless otherwise indicated.

Growth performance

The initial body weight of broilers did not differ among treatment (Table 4.3). The ADG, ADFI and FCR were affected by the diet ($P < 0.001$). Broilers fed the RSM diet showed the highest ADFI and broilers fed the SPI diet the lowest (181.7 and 135.3 g, respectively).

Digestion kinetics of dry matter and crude protein along the SI

The AD_{DM} and AD_{CP} increased along the SI for all diets ($P < 0.001$), mainly in between the duodenum and the distal jejunum (Table 4.4). The AD_{DM} along the SI was affected by the diet ($P < 0.001$). The SPI, WG and DPP diets showed a higher AD_{DM} than the SBM and RSM diets in both jejunum segments and the ileum ($P < 0.001$). The AD_{CP} along the SI was also affected by the diet ($P < 0.001$). In both jejunum segments, the WG diet showed the highest and the RSM diet the lowest AD_{CP}. The AD_{CP} in the ileum was higher for the SPI, WG and DPP diets than for the SBM, RSM and BSF diets ($P < 0.001$).

The retention time of the solid fraction (RT_{solid}) of digesta differed among the GIT segments ($P < 0.001$) (Table 4.5). The averaged RT_{solid} from all diets in the crop, gizzard, duodenum, proximal jejunum, distal jejunum and ileum was 15 ± 8, 27 ± 8, 1 ± 1, 45 ± 6, 50 ± 4, 21 ± 4 min, respectively, with an estimated mean RT_{solid} over the entire SI of 122 ± 16 min (mean ± standard deviation). The standard deviation of the RT_{solid} mainly originated from the variation among dietary treatments rather than the variation within dietary treatments. The SPI diet had a lower RT_{solid} in the crop than the SBM and RSM diets ($P < 0.01$). The RT_{solid} in the gizzard was higher for the WG and DPP diets than for the RSM and BSF diets ($P < 0.001$). The RT_{solid} in the proximal jejunum, distal jejunum and ileum did not differ among the diets ($P > 0.05$). The retention time of the liquid fraction (RT_{liquid}) of digesta also differed among the GIT segments ($P < 0.001$). The averaged RT_{liquid} from all diets in the crop, gizzard, proximal jejunum, distal jejunum and ileum was 17 ± 9, 16 ± 2, 42 ± 7, 44 ± 5, 23 ± 6 min, respectively, with an estimated mean RT over the entire SI of 107 ± 15 min. The duodenum digesta samples could not be analysed for Cr concentration due to the limited amount of digesta collected. The averaged RT_{liquid} in the duodenum, therefore, cannot be presented. Similar to the RT_{solid}, the standard deviation of the RT_{liquid} mainly originated from the variation among dietary treatments rather than the variation within dietary treatments. The RT_{liquid} in the crop was higher for the SBM and RSM diets than for the SPI and BSF diets ($P < 0.001$). The RT_{liquid} in the gizzard and distal jejunum did not differ among the diets ($P > 0.05$). The

RSM diet had a higher RT_{liquid} in the ileum than the WG diet ($P < 0.01$). Over all experimental diets, the solid fraction of digesta showed a longer RT in the gizzard than the liquid fraction of digesta (27 and 16 min, respectively; $P < 0.01$) and tended to show a longer RT in the SI (122 and 107 min, respectively; $P = 0.052$).

Large differences in DM and CP digestion rate constants were observed among diets (Table 4.6 and Figure 4.1). The DM digestion rate constant ranged from 0.026 min^{-1} for the RSM diet to 0.267 min^{-1} for the WG diet. The CP digestion rate constant ranged from 0.023 min^{-1} for the RSM diet to 0.365 min^{-1} for the SPI diet. The WG and SPI diets showed a high initial rate of protein digestion (65 and 67 g · min^{-1} per kg diet, respectively) followed by the DPP diet (17 g · min^{-1} per kg diet) and the SBM, RSM and BSF diets (7, 4 and 6 g · min^{-1} per kg diet , respectively).

Table 4.3. Growth performance[1] of broilers fed experimental diets containing different protein sources[2] (n = 6).

Parameter	Experimental diets						Pooled SEM	*P*-value
	SBM	SPI	WG	RSM	DPP	BSF		
Initial body weight (g)	1421	1436	1438	1399	1428	1441	20.24	0.707
Final body weight (g)	2343[a]	1974[b]	2390[a]	2414[a]	2418[a]	2502[a]	44.12	<0.001
ADG[3] (g/bird)	87.5[a]	57.9[b]	85.4[a]	86.5[a]	86.2[a]	92.6[a]	2.62	<0.001
ADFI[4] (g/bird)	167.2[ab]	135.3[c]	160.5[b]	181.7[a]	155.3[b]	167.5[ab]	3.44	<0.001
FCR[5] (g/g)	1.92[c]	2.34[a]	1.88[c]	2.10[b]	1.81[c]	1.81[c]	0.03	<0.001

[1] SBM= soybean meal; SPI= soy protein isolate; WG= wheat gluten; RSM= rapeseed meal; DPP= dried porcine plasma protein; BSF= black soldier fly larvae. [2] Performance results were measured between experimental day 13 and 20. [3] Average daily weight gain (g/bird). [4] Average daily feed intake (g/bird). [5] Feed conversion ratio (g/g). [a-c] Means within the row without a common superscript differ ($P < 0.05$).

Table 4.4. Apparent digestibility (AD) of dry matter (DM) and crude protein (CP) of experimental diets containing different protein sources[1] along the small intestine of broilers.

	Experimental diets												Pooled	*P*-value	
AD (%)	SBM		SPI		WG		RSM		DPP		BSF		SEM	Diet	Segment
Dry matter															<0.001
Duodenum	-17.8^{b}	(6)[2]	12.2^{a}	(6)	26.5^{a}	(6)	10.0^{a}	(6)	12.7^{a}	(6)	10.8^{a}	(6)	5.6	<0.001	
Proximal Jejunum	47.1^{c}	(6)	61.7^{b}	(6)	70.0^{a}	(6)	37.3^{d}	(6)	63.1^{ab}	(6)	58.4^{b}	(6)	1.8	<0.001	
Distal Jejunum	61.3^{d}	(6)	74.3^{bc}	(6)	79.9^{a}	(6)	54.8^{e}	(6)	77.1^{ab}	(6)	70.0^{c}	(6)	1.1	<0.001	
Ileum	62.3^{b}	(6)	76.0^{a}	(6)	76.8^{a}	(6)	53.6^{c}	(6)	79.2^{a}	(6)	66.7^{b}	(6)	2.0	<0.001	
Crude protein															<0.001
Duodenum	6.6^{ab}	(5)	26.8^{ab}	(6)	37.8^{a}	(6)	8.5^{ab}	(6)	-7.4^{b}	(5)	4.8^{ab}	(5)	8.3	0.008	
Proximal Jejunum	67.9^{c}	(6)	79.0^{b}	(6)	87.1^{a}	(6)	49.9^{d}	(6)	78.3^{b}	(6)	62.1^{c}	(6)	1.3	<0.001	
Distal Jejunum	83.8^{c}	(6)	88.2^{b}	(6)	93.7^{a}	(6)	72.1^{e}	(6)	87.5^{b}	(6)	75.5^{d}	(6)	0.7	<0.001	
Ileum	84.9^{b}	(6)	90.8^{a}	(6)	93.7^{a}	(6)	75.6^{c}	(6)	90.4^{a}	(6)	78.4^{c}	(6)	1.2	<0.001	

[1] SBM= soybean meal; SPI= soy protein isolate; WG= wheat gluten; RSM= rapeseed meal; DPP= dried porcine plasma protein; BSF= black soldier fly larvae.
[2] Values in brackets indicate the number of replicates of which samples could be collected and analysed. $^{a-e}$ Means within the row without a common superscript differ ($P < 0.05$).

Table 4.5. Retention time (RT, min) of the solid and liquid fraction of digesta along the gastrointestinal tract of broilers fed experimental diets containing different protein sources[1].

Fraction RT (min)	Experimental diets												Pooled SEM	*P*-value	
	SBM		SPI		WG		RSM		DPP		BSF			Diet	Segment
Solid															<0.001
Crop	23^{a}	(6)[2]	8^{b}	(6)	11^{ab}	(6)	27^{a}	(6)	10^{ab}	(5)	11^{ab}	(6)	3.6	0.003	
Gizzard	24^{bc}	(6)	29^{abc}	(6)	35^{ab}	(6)	17^{c}	(6)	39^{a}	(6)	20^{c}	(6)	3.2	<0.001	
Duodenum	1^{b}	(6)	2^{ab}	(6)	2^{ab}	(6)	2^{a}	(6)	1^{ab}	(6)	1^{ab}	(6)	0.5	0.011	
Proximal Jejunum	42	(6)	48	(6)	51	(6)	48	(6)	35	(6)	45	(6)	3.7	0.067	
Distal Jejunum	51	(6)	51	(6)	49	(6)	53	(6)	44	(6)	53	(6)	5.3	0.782	
Ileum	23	(6)	20	(6)	16	(6)	27	(6)	19	(6)	20	(6)	3.4	0.297	
Small intestine	117	(6)	122	(6)	117	(6)	130	(6)	98	(6)	120	(6)	9.8	0.357	
Liquid															<0.001
Crop	27^{a}	(6)	9^{b}	(5)	13^{ab}	(5)	29^{a}	(6)	14^{ab}	(4)	11^{b}	(6)	3.8	0.002	
Gizzard	17	(6)	16	(6)	16	(6)	15	(6)	17	(6)	12	(6)	1.7	0.353	
Proximal Jejunum	46^{a}	(6)	42^{ab}	(6)	45^{a}	(6)	49^{a}	(6)	31^{b}	(6)	38^{ab}	(6)	3.1	0.003	
Distal Jejunum	45	(6)	45	(6)	40	(6)	52	(6)	37	(6)	43	(6)	5.3	0.422	
Ileum	27^{ab}	(5)	19^{ab}	(6)	15^{b}	(5)	31^{a}	(6)	24^{ab}	(4)	19^{ab}	(6)	3.2	0.029	
Small intestine	113^{ab}	(6)	106^{ab}	(6)	98^{ab}	(6)	132^{a}	(6)	84^{b}	(6)	100^{ab}	(6)	10.1	0.046	

[1] SBM= soybean meal; SPI= soy protein isolate; WG= wheat gluten; RSM= rapeseed meal; DPP= dried porcine plasma protein; BSF= black soldier fly larvae. [2] Values in brackets indicate the number of replicates of which samples could be collected and analysed. [a-c] Means within the row without a common superscript differ ($P < 0.05$).

Table 4.6. Parameter estimates characterising digestion kinetics of dry matter and crude protein in the small intestine of broilers fed experimental diets containing different protein sources[1].

	Experimental diets											
Digestion	SBM		SPI		WG		RSM		DPP		BSF	
Dry Matter[2]												
D_{max} (g/kg diet)	609	(9)[4]	689	(22)	683	(13)	508	(39)	695	(33)	625	(19)
k (min^{-1})	0.027	(0.001)	0.038	(0.006)	0.267	(0.032)	0.026	(0.007)	0.069	(0.019)	0.047	(0.009)
$t_{1/2}$ (min)	25.3		18.3		2.6		26.4		10.0		14.6	
Initial rate ($g \cdot min^{-1}$/kg diet)[3]	17		26		182		13		48		30	
Crude Protein[2]												
D_{max} (g/kg diet)	182	(2)	184	(4)	193	(3)	164	(11)	184	(3)	166	(4)
k (min^{-1})	0.036	(0.004)	0.365	(0.053)	0.336	(0.034)	0.023	(0.005)	0.092	(0.011)	0.035	(0.004)
$t_{1/2}$ (min)	19.0		1.9		2.1		30.0		7.5		19.6	
Initial rate ($g \cdot min^{-1}$/kg diet)[3]	7		67		65		4		17		6	(19)

[1] SBM= soybean meal; SPI= soy protein isolate; WG= wheat gluten; RSM= rapeseed meal; DPP= dried porcine plasma protein; BSF= black soldier fly larvae. [2] D_{max} (g/kg diet) is the potentially digestible dry matter or crude protein per kg of diet (asymptote); k (min^{-1}) is the fractional rate constant; $t_{1/2}$ (min) is the time where half of D_{max} is reached. [3] Initial rate ($g \cdot min^{-1}$/kg diet) was calculated by multiplying D_{max} with k. [4] Values in brackets indicate the approximate standard error of the estimate.

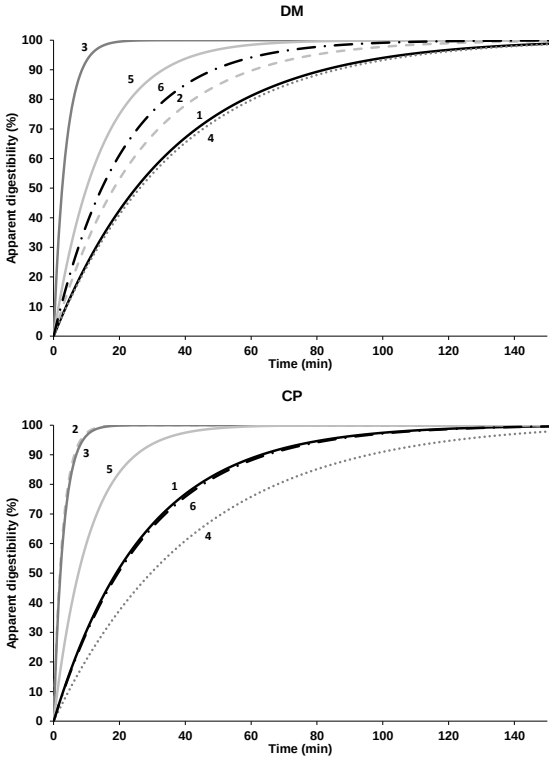
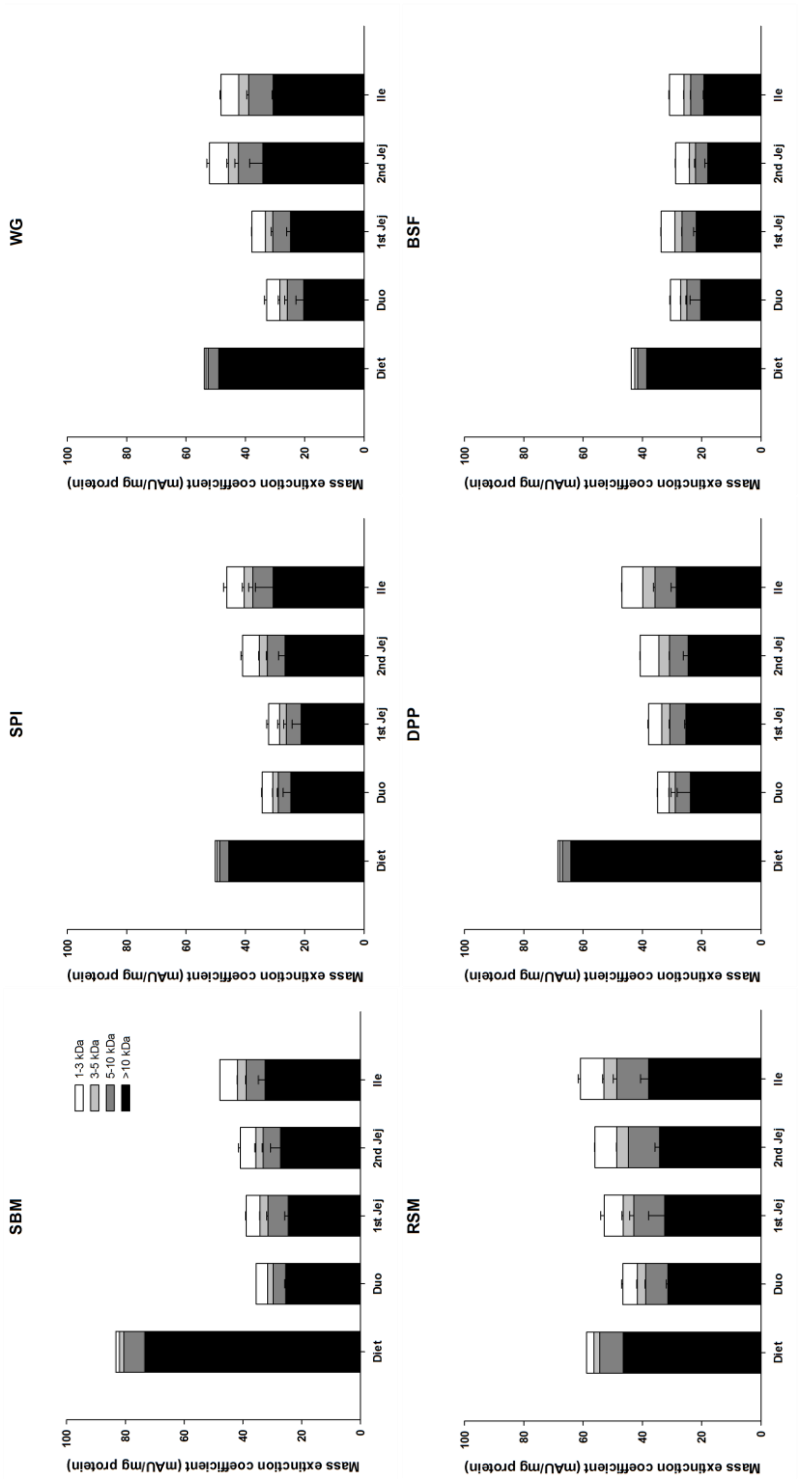


Figure 4.1. Rescaled digestion curves (setting D_{max} at 100 %) of dry matter (DM) and crude protein (CP) of experimental diets containing soybean mean (1), soy protein isolate (2), wheat gluten (3), rapeseed meal (4), dried porcine plasma protein (5) or black soldier fly larvae (6) as the main dietary protein source in the small intestine of broilers.

Molecular weight distribution of protein and peptides in digesta of the stomach and SI

Proteins and peptides in the diets predominantly had a molecular weight > 10 kDa (Figure 4.2). Fewer peptides with a molecular weight > 10 kDa were present in digesta of the SI than the diet. The molecular weight distribution of proteins and peptides in digesta over the SI was similar for each of the dietary treatments. The molecular weight fractions > 10 kDa accounted for the majority of proteins and peptides in digesta of the SI. Values for the mass extinction coefficients of peptides < 1 kDa were negative after the correction for the signals from the 2 % SDS and 100 mM dithiothreitol solution used for sample extraction.


SBM
SPI
WG
RSM
DPP
BSF
1-3 kDa
3-5 kDa
5-10 kDa
>10 kDa
Mass extinction coefficient (mAU/mg protein)
0
20
40
60
80
100
Diet
Duo
1st Jej
2nd Jej
Ile

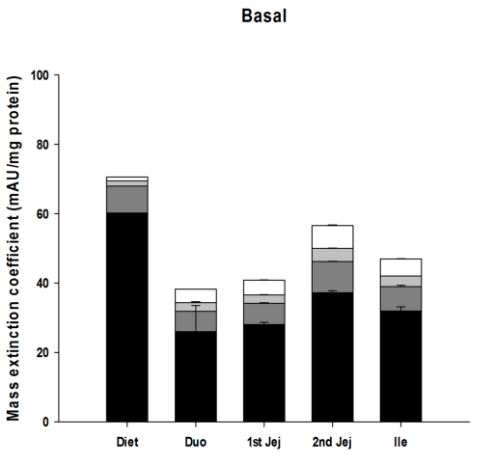


Figure 4.2. Molecular weight distribution (>10 kDa; 5-10 kDa; 3-5 kDa; 1-3 kDa; <1 kDa) of the experimental diets and digesta samples from the duodenum (Dou), proximal jejunum (1st Jej), distal jejunum (2nd Jej) and ileum (Ile) of broilers fed experimental diets contained soybean mean (SBM), soy protein isolate (SPI) wheat gluten (WG), rapeseed meal (RSM), dried porcine plasma protein (DPP) or black soldier fly larvae (BSF) as the main protein source. The basal diet contained 90 g/kg SBM as the protein source. Values are means ± SE calculated from two experimental units (pen with nine broilers; n = 2). Values for the mass extinction coefficients of peptides smaller than 1 kDa were negative after the correction for the signals from the SDS and dithiothreitol solution used for sample extraction and are, therefore, not presented.

DISCUSSSION

The aim of the present study was to determine the *in vivo* protein digestion kinetics of diets with different protein sources up to the end of the SI of broilers. The protein digestion kinetics was calculated from the digesta retention time and protein digestibility, which is the result of protein hydrolysis and absorption. At the start of the study, the tested protein sources were included as a single protein source in the respective experimental diets with supplemented free AAs to the diets to prevent severe deficiencies in the supply of essential AAs (total AA supply in free form among diets ranging from 0 to 17.5 g/kg diet). However, when feeding these diets, the birds on the SPI, WG and DPP diets showed a 20 % lower feed intake than the birds on the SBM and RSM diets over experimental day 7 to 12. To avoid large differences in feed intake among experimental diets that could influence both digesta passage rate and nutrient digestibility, all diets were supplemented with the same level of SBM (100 g SBM per kg diet, on top of) and fed subsequently. However, a lower ADFI was still observed in broilers fed the SPI diet. The differences in protein digestion kinetics of the experimental diets, therefore, can still be attributed to differences in protein sources included in the experimental diets, which provided approximately 75 % of the total amount of protein present in the diets. Among protein sources, SPI, WG and DPP had a higher CP content than the other protein sources. Since all experimental diets were formulated isoproteineous, the inclusion level of SPI, WG and DPP in the diets was lower than the other protein sources. Maize starch was used to fill up the gap of non-protein components present in SBM, RSM and BSF in the diets with SPI, WP and DPP, resulting in a higher starch content of these diets.

The AD_{CP} of the diets was determined in the duodenum, proximal jejunum, distal jejunum and the ileum. The AD_{CP} largely increased along the jejunum, with an average increase of 69 ± 12 % over all experimental diets. These results indicate that for broilers, dietary proteins from the evaluated protein sources were mainly hydrolysed and absorbed in the jejunum, especially the proximal jejunum. Values for the AD_{CP} measured in the ileum for the SBM and RSM diets (85 and 76 %, respectively) were comparable to published values (82 and 79 %, respectively) of apparent ileal protein digestibility of broilers in the literature (Ravindran et al., 2005). The SPI, WG and DPP diets showed a high AD_{CP} up to the end of the ileum (91, 94 and 90 %, respectively). SPI, WG and DPP, therefore, can be considered as highly digestible protein sources. In contrast, the RSM and BSF diets showed a relative low AD_{CP} up to the end of the ileum (76 and 78 %, respectively).

It is generally assumed that proteins present in highly digestible protein sources are more susceptible to hydrolysis by digestive enzymes, whereas proteins present in low digestible protein sources are more resistant to enzymatic hydrolysis. As a consequence, relatively more low and intermediate molecular weight peptides were expected to be present in the ileal digesta of broilers fed highly digestible protein sources. In contrast, relatively more high molecular weight proteins and peptides were expected to be present in ileal digesta of broilers fed low digestible protein sources. The molecular weight distributions of proteins and peptides in digesta of the ileum, however, were comparable between highly and low digestible protein sources. In addition, the molecular weight distributions remained rather similar throughout segments of the GIT for all diets. These results indicate that proteins from both highly and low digestible sources follow a "one-by-one" type of hydrolysis mechanism (Adler-Nissen, 1976), meaning intact proteins are hydrolysed to low molecular weight peptides and free AAs and absorbed by the intestinal mucosa in one sequence. As a result, proteins and peptides with a wide range of molecular weights were not observed in digesta of different segments of the GIT. The factor limiting the extent of digestion of protein sources in the SI of broilers then could be related to the initial rate of hydrolysis, calculated from the amount of potentially digestible protein in the diet (D_{max}) and the digestion rate constant (k) in the present study. Proteins present in highly digestible protein sources might show a higher initial hydrolysis rate than proteins present in low digestible protein sources. Nearly no peptides < 1 kDa were found in digesta of the SI after the correction for the signals from the extraction solution. This is different from our previous study in pigs, in which approximately 30 % of proteins and peptides in digesta of the SI were present in the molecular fraction < 1 kDa (Chapter 3). The presence of fewer peptides < 1 kDa in digesta of broilers might result from the occurrence of antiperistaltic contractions (i.e. digesta reflux) in the GIT of poultry (Duke, 1982). The reflux of digesta provides opportunity for further digestion and adsorption of nutrients (Basha and Duke, 1999).

The RT of the solid and liquid fraction of digesta along the GIT was measured using TiO_2 and Cr-EDTA, respectively. The liquid fraction of digesta showed a shorter RT in the gizzard than the solid phase of digesta, which agrees with the study of Vergara et al. (1989) who also measured the passage rate of digesta in the gizzard using both soluble and insoluble markers. Proteins, which are solubilised in the gizzard of broilers, therefore, are expected to be transported from the gizzard to the SI faster than proteins which are insoluble. As a result, they could be subjected to a more rapid

enzymatic hydrolysis and absorption in the SI, leading to a rapid postprandial appearance of AAs in the portal circulation. The solid fraction of digesta tended to show a longer RT in the SI than the liquid fraction of digesta (122 and 107 min, respectively; $P = 0.052$). Similar results were found in a study with pigs, in which the RT in the small intestine of the solid fraction of digesta was, on average, 30 min longer than that of the liquid fraction (Wilfart et al., 2007). The estimated mean RT_{solid} over the entire SI of broilers (122 ± 16 min) was lower than published data in the literature, which was between 140 to 180 min (Weurding et al., 2001; Liu et al., 2013). This is likely due to the use to semi-purified diets in the present study. Broilers fed semi-purified diets showed an 8 % lower RT of digesta along the GIT than cereal-based diets (Rochell et al., 2012). A relatively low RT_{solid} in the ileum for all experimental diets was observed. The AD_{DM} of all diets reached 95 % of its maximum value in the distal jejunum, indicating the digestion of nutrients in the diets is nearly completed at the end of the jejunum. This might explain the short RT_{solid} in the ileum.

Studies on digestion kinetics of nutrients commonly provide information on the potentially digestible fraction (D_{max}) and the digestion rate constant (k). The rate constant of the digestion of nutrients is independent of size of the potential digestible fraction. In quantitative nutrition studies, comparison of parameters accounting for both the rate constant and the size of the potentially digestible fraction is preferred over comparison of the rate constant only. Therefore, the initial rate ($g \cdot min^{-1}$ per kg diet), calculated by multiplying the potentially digestible fraction and the rate constant, was used to compare the digestion kinetics of the experimental diets. With respect to crude protein digestion, the SPI and WG diets showed the highest initial rate and the RSM diet the lowest. Although the DPP diet showed a similar AD_{CP} in the ileum to the SPI and WG diet, the initial rate was 3.8 times lower than that of the SPI and WG diet (17 vs. 67 and 65 $g \cdot min^{-1}$ per kg diet). These results indicate that protein sources with a similar ileal protein digestibility can differ in protein digestion kinetics up to the end of the SI in broilers. Over the six experimental diets, no correlation was found between the initial rate of protein digestion in the SI and the AD_{CP} up to the ileum ($P = 0.20$). This opposes the hypothesis that highly digestible protein sources are digested faster than less digestible sources in the SI of broilers.

Protein sources with a high CP content (i.e. SPI, WG and DPP), on average, showed a faster digestion kinetics than protein sources with a relatively low CP content (i.e. SBM, RSM and BSF). Similar results were found in pig studies when feeding soy protein concentrate and a mixture of untoasted and toasted SBM. Pigs fed soy protein

concentrate showed a more rapid postprandial portal appearance of AAs than pigs fed a mixture of untoasted and toasted soybean meal after meal ingestion (Jansman et al., 1997). This is likely due to the negative effects of non-protein constituents present in less purified protein sources on protein digestion. Soybean meal and RSM consist for 20-30 % non-starch polysaccharides (NSP) (CVB, 2016), which can be further divided into insoluble NSP (i.e. mainly cellulose) and soluble NSP. In broilers, an increase in soluble NSP content in the diet results in a decreased absorption of nutrients in the SI (Antoniou et al., 1981; Choct and Annison, 1990). This is likely due to the fact that soluble NSP can increase the viscosity of digesta (Bach Knudsen, 2001). Moreover, other anti-nutritional factors present in SBM (e.g. protease inhibitors, lectins) (Campbell and van der Poel, 1998) and RSM (e.g. sinapine, phytic acid, tannins) (Khajali and Slominski, 2012) could also hinder protein digestion in the SI of broilers.

In broiler diets, energy is mainly provided in the form of starch by cereal grains such as wheat, maize and barley, and proteins mainly by vegetable proteins such as SBM and RSM. In the SI of broilers, the average digestion rate constant of starch of these cereal grains was 0.045 ± 0.005 min^{-1} (Weurding et al., 2001). This value is 20 % higher than the digestion rate constant of protein from SBM and RSM as reported in the present study. This suggests that starch from cereal grains is digested faster than most dietary proteins in broiler diets, resulting in some degree of asynchrony in availability of AAs and glucose in the post-absorption metabolism in broilers. As a consequence, more AAs are likely to be oxidised to produce energy. This could explain why feeding slowly digestible starch or fast digestible proteins to broilers is beneficial for their growth performance (Weurding et al., 2003; Frikha et al., 2014).

Broilers have a shorter SI than pigs. The RT of the digesta is lower in the SI of broilers than of pigs (Weurding et al., 2001; Wilfart et al., 2007). Broilers, however, show a comparable ileal protein digestibility of most feed ingredients compared to pigs (Lemme et al., 2004). This could be due to a higher rate of hydrolysis and absorption of dietary proteins and AAs in the SI of broilers. In our previous study, the kinetics of protein digestion of the same protein sources as used in the present study were determined in the SI of pigs. Indeed, the fractional protein digestion rate of these protein sources was, on average, 2.7 times higher in broilers than in pigs (Chapter 3). Despite the differences in digestion between species, the ranking of the protein sources in terms of protein digestion kinetics were comparable in both species.

CONCLUSIONS

The kinetics of protein digestion differed among protein sources in the small intestine of broilers. Soy protein isolate, WG and DPP can be regarded as fast digestible while SBM, RSM and BSF as more slowly digestible protein sources. Purified protein sources (i.e. high in protein content) tend to be digested faster in the small intestine of broilers than less purified protein sources due to the negative effects on protein digestion of non-protein constituents such as fibre present in less purified protein sources.

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Chapter 5

Effect of synchronising the kinetics of protein and starch digestion in the small intestine on the growth performance and carcass characteristics in broilers

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ABSTRACT

The objective of the present study was to determine if synchronising the digestion kinetics of dietary starch and protein affects the growth performance and carcass characteristics in broilers. Four diets were evaluated in a 2 x 2 factorial design using pea (PS) and rice starch (RS) as a slow and fast digestible starch source, and soybean meal (SBM) and soy protein isolate (SPI) as a slow and fast digestible protein source, respectively. In addition, the experimental diets were also evaluated in a digestibility study with 300 birds in which the ileal digestibility of dry matter (DM), starch and crude protein (CP) and nitrogen-corrected apparent metabolisable energy (AME_n) were determined. In the growth performance trial, a total of 800 one-day-old male broilers were used to determine average daily feed intake (ADFI), average body weight gain (ADG), feed conversion ratio (FCR) and energy conversion ratio (ECR) over day 7 - 35. Broilers fed RS diets showed a 10 % higher AID_{Starch} than broilers fed PS diets, leading to a higher AME_n of RS diets ($P < 0.001$) compared to PS diets. The ADFI was affected by the dietary starch and protein source ($P < 0.001$), with values ranging from 98.0 g/d for birds on the PS-SBM diet to 81.4 g/d when fed on the RS-SPI diet. Over the entire experimental period, birds fed the PS-SBM (slow - slow) diet showed the highest ADG (62.6 g) while birds on the RS-SPI (fast - fast) diet the lowest ADG (48.6 g; $P < 0.001$). Broilers on SPI diets showed higher carcass, breast meat and abdominal fat yields (% of slaughter weight; $P < 0.001$) compared to birds on SBM diets ($P < 0.001$). In conclusion, broilers fed synchronised diets for starch and protein digestion kinetics did not show a higher growth performance and breast meat yield compared to birds fed asynchronized diets.

INTRODUCTION

Starch and protein are two major constituents in commercial broiler diets. Starch is mainly provided by cereal grains such as wheat, maize and barley, and protein mainly by vegetable proteins such as soybean meal (SBM) and rapeseed meal, apart from the protein fraction originating from cereal grains. The kinetics of starch and protein digestion could affect the appearance of glucose and amino acids (AAs) in blood after meal ingestion (Adeleye et al., 2016). In pigs, fast digestible starch sources, such as maize starch, showed a more pronounced but transient postprandial appearance of glucose and insulin in blood than slowly digestible starch sources, such as pea starch (van der Meulen et al., 1997). Similarly, fast digestible protein sources showed an earlier and higher postprandial appearance of AAs in blood than slowly digestible sources in pigs (Barbé et al., 2014). In chickens, starch hydrolysis and glucose absorption mainly take place in the duodenum and the proximal jejunum (Riesenfeld et al., 1980), whereas protein hydrolysis and AAs absorption mainly occur in the jejunum and ileum (Sklan and Hurwitz, 1980). This indicates that there might be asynchrony in the digestion of dietary starch and protein in the small intestine (SI) of broilers. Indeed, previous studies showed that the digestion rate of starch of cereal grains was on average approximately 20 % higher than the digestion rate of protein from SBM and rapeseed meal (Weurding et al., 2001; Chapter 4).

Digestible AAs provided via the diet are used by organs and tissues, either or not after transformation into other AAs, to synthesise proteins or as an energy source after deamination. An efficient utilisation of AAs for body protein deposition is economically important in modern-day broiler production. The simultaneous availability of AAs and glucose in organs and tissues increase protein deposition in growing animals (van den Borne et al., 2007). This indicates that a synchronised supply of glucose and AAs in organs and tissues is important for an efficient utilisation of dietary protein for body protein deposition. As starch might, on average, be digested faster than protein in broiler diets, glucose might show a faster postprandial appearance in blood than AAs. This might lead to an asynchrony in the supply of glucose and AAs in organs and tissues. It has been shown that feeding slowly digestible starch to broilers could increase body weight gain up to 5 % and decreased feed conversion ratio by approximately 2 % (Weurding et al., 2003). In addition, nitrogen retention was negatively correlated to the starch digestion rate in broilers fed sorghum-based diets, indicating slowly digestible starch benefited protein retention in broilers (Liu et al., 2013b). The former can probably

be explained by the release of glucose in the distal part of the SI from slowly digestible starch, thereby sparing AAs from being catabolised to produce energy in the intestinal mucosa of the distal SI, and increasing their quantitative appearance in the portal circulation (van der Meulen et al., 1997; Li et al., 2008).

The objective of the present study was to determine if synchronising the digestion kinetics of dietary starch and protein affects the growth performance and carcass characteristics in broilers. It was hypothesised that feeding diets synchronised for digestion rate of dietary starch and protein to broilers 1) improves the growth performance, and 2) increases carcass and breast meat yield and decrease abdominal fat yield.

MATERIALS AND METHODS

Experimental design

Two experiments were performed: a digestibility trial and a growth performance trial. The digestibility trial followed a randomized complete block design. Four experimental diets (2 × 2 factorial arrangement) were used. Cages were blocked on the location in the experimental room (five blocks per room, four cages per block) and pens within a block were randomly allocated to one of the four experimental diets. With respect to the growth performance trial, the experiment followed a randomized complete block design, in which pens were blocked on the location in the experimental room (ten blocks, four pens per block). Pens within a block were randomly allocated to one of the four experimental diets.

Protein sources and experimental diets

In a 2 × 2 factorial arrangement, diets including both starch and protein sources with different digestion rates were compared. Rice starch (RS) (obtained from Beneo-Remy NV, Leuven-Wijgmaal, Belgium) was used as a fast digestible starch source and pea starch (PS) (Cosucra Group Warcoing, Warcoing, Belgium) as a slowly digestible starch source. Soy protein isolate (SPI) (obtained from Archer Daniels Midland Company, Chicago, IL, USA) was used as a fast digestible protein source and soybean meal (SBM) (commodity batch obtained via Trouw Nutrition, Casarrubios del Monte, Spain) as a slowly digestible protein source. The digestion rates of starch and protein of the starch and protein sources derived from literature and Chapter 4 (Table 5.1).

Table 5.1. Estimated starch and protein digestion rates of starch and protein sources used in the present study.

Ingredient	Digestion rate (% · min^{-1})[1]	Species	Reference
Starch source			
Rice starch	0.65	Pig	van Kempen et al., 2010
Pea starch	0.34	Pig	
Protein source			
Soy protein isolate	32.3	Broiler chicken	Chapter 4
Soybean meal	3.1	Broiler chicken	

[1] Starch digestion rate was determined as the maximum rate of net portal glucose appearance using a modified Michaelis-Menten enzyme kinetics model.

All four diets were formulated to be equal in metabolisable energy (ME) and not limiting in digestible essential AAs (CVB, 2009). In a previous study, broilers fed a diet with SPI as a single protein source showed a substantially lower feed intake than broilers fed a diet with SBM as the only protein source. The difference in feed intake, however, was reduced when supplementing the SPI diet with 100 g SBM per kg diet (Chapter 4). To limit differences in feed intake between birds of the different experimental treatments in the present study, 100 g/kg SBM was included in all experimental diets. Soy fibre was included in the SPI diets (77 and 79 g/kg in the grower and finisher diets) to reconstitute the fibre fraction coming from the SBM in the SBM diets. In the diets used in the digestibility experiment, titanium dioxide (TiO_2) was included as an indigestible marker at 2.5 g/kg diet (as-fed basis). All diets were produced by Trouw Nutrition (Casarrubios del Monte, Spain). The ingredient, calculated and analysed nutrient composition of the experimental diets is presented in Table 5.2.

Table 5.2. Composition of the experimental diets for the growth performance trial (g/kg, as-fed basis).

Ingredients	Grower				Finisher			
	PS-SBM	PS-SPI	RS-SBM	RS-SPI	PS-SBM	PS-SPI	RS-SBM	RS-SPI
Pea starch (PS)	400.0	400.0	0.0	0.0	400.0	400.0	0.0	0.0
Rice starch (RS)	0.0	0.0	400.0	400.0	0.0	0.0	400.0	400.0
Soybean meal (SBM)	326.7	100.0	326.7	100.0	332.3	100.0	332.3	100.0
Soy protein isolates (SPI)	0.0	113.4	0.0	113.4	0.0	116.1	0.0	116.1
Soy Fibr	0.0	77.1	0.0	77.1	0.0	79.0	0.0	79.0
Oat hulls	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Soybean hulls	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Sucrose	45.0	63.1	45.0	63.1	45.0	63.6	45.0	63.6
Arbocel	0.0	18.1	0.0	18.1	0.0	18.6	0.0	18.6
Soybean oil	83.0	80.0	83.0	80.0	82.5	79.1	82.5	79.1
Premix[1]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Limestone	8.8	8.9	8.8	8.9	7.7	7.7	7.7	7.7
Salt	2.8	1.2	2.8	1.2	3.2	1.1	3.2	1.1
Potassium chloride	0.0	1.5	0.0	1.5	0.0	1.9	0.0	1.9
Monocalcium phosphate	12.7	13.4	12.7	13.4	10.9	11.6	10.9	11.6
Potassium carbonate	0.0	4.7	0.0	4.7	0.0	4.3	0.0	4.3
Sodium bicarbonate	1.1	0.0	1.1	0.0	0.6	0.0	0.6	0.0
Magnesium oxide	0.0	0.7	0.0	0.7	0.0	0.7	0.0	0.7
Diamol	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
L-Lysine HCl	2.2	1.5	2.2	1.5	1.6	0.9	1.6	0.9
DL-Methionine	3.8	3.8	3.8	3.8	3.5	3.5	3.5	3.5
L-Threonine	1.4	1.2	1.4	1.2	1.2	0.9	1.2	0.9
L-Arginine	0.4	0.0	0.4	0.0	0.0	0.0	0.0	0.0
L-Isoleucine	0.3	0.0	0.3	0.0	0.1	0.0	0.1	0.0
L-Valine	1.8	1.4	1.8	1.4	1.4	1.0	1.4	1.0

Calculated nutrient composition (%)								
ME, MJ/kg	13.2	13.2	13.2	13.2	13.2	13.2	13.2	13.2
Calcium	7.3	7.3	7.3	7.3	6.6	6.6	6.6	6.6
Available phosphorus	3.3	3.3	3.3	3.3	2.9	2.9	2.9	2.9
Sodium	1.5	2.0	1.5	2.0	1.5	2.0	1.5	2.0
Digestible Lys	12.2	12.5	12.2	12.5	11.9	12.2	11.9	12.2
Digestible Met + Cys	8.7	8.6	8.7	8.6	8.4	8.4	8.4	8.4
Digestible Thr	8.0	8.2	8.0	8.2	7.9	8.0	7.9	8.0
Digestible Trp	2.2	2.5	2.2	2.5	2.2	2.5	2.2	2.5
Analysed nutrient composition (%, DM basis)								
DM (% as-fed)	93.2	93.7	93.0	93.6	93.2	94.0	93.1	93.6
Ash	5.9	5.5	5.9	5.8	5.4	5.1	5.8	5.2
CP	18.7	20.8	20.7	21.4	18.8	20.4	21.7	21.5
Crude fat	10.4	6.8	9.5	6.0	9.4	8.0	9.3	7.7
Crude fibre	4.9	6.4	5.2	6.4	5.2	5.9	5.2	6.1

[1] The mineral and vitamin premix supplied per kilogram of diet: vitamin A, 12.000 IU; cholecalciferol, 0.6 mg; vitamin E, 50 mg; vitamin B2, 7.5 mg; vitamin B6, 3.5 mg; vitamin B1, 2.0 mg; vitamin K, 1.5 mg; vitamin B12, 20 µg; choline chloride, 460 mg; anti-oxidant, 125 mg; niacin, 35 mg; pantothenic acid, 12 mg; biotin, 0.2 mg; folic acid, 1 mg; Mn, 85 mg, as MnO; Fe, 80 mg, as $FeSO_4$; Zn, 60 mg, as $ZnSO_4$; Cu, 12 mg, as $CuSO_4$; I, 0.8 mg, as KI; Co, 0.4 mg, as CoSO4; Se, 0.15 mg, as Na_2SeO_3

Exp 1. Digestibility trial

Animals, housing and feeding

A total of 300 one-day-old male broilers (Ross 308, Aviagen Group, Newbridge, UK) were used. From experimental day 1 to 14, broilers were housed in four floor pens with 75 birds in each pen. Pens were randomly allocated to one of the four experimental diets. From day 15, 60 out 75 broilers were selected from each pen and housed in 40 digestibility cages with six broilers in each cage. During the first two days, lights were continuously on (24L : 0D). From day 3 onwards, the lighting schedule followed a four-cycle 2L : 4D regime. From day 1 to 7, broilers were fed a commercial diet and from day 7 onwards were fed only the experimental diets containing the indigestible marker. The experimental diets were provided in mash form and broilers had unlimited access to feed an water throughout the entire experiment.

Data collection and calculation

From day 21 to 23, excreta were collected twice a day, every morning and afternoon. Excreta samples obtained over three days of collection were pooled per cage and stored at -20 °C. Excreta samples from the same treatment in the same block of two experimental rooms were pooled to have sufficient sample for the planned chemical analyses. Samples were freeze-dried before dry matter (DM), gross energy, nitrogen, uric acid and Ti analysis to calculate total tract digestibility of DM and crude protein (CP), and nitrogen-corrected apparent metabolisable energy (AME_n). At day 24, broilers were euthanized by an intravenous T-61 injection. The broilers were placed on their posterior side and the body cavity was opened prior to careful removal of the GIT from the crop to the cloaca. The GIT was carefully located horizontally on the table without disturbing digesta. The jejunum and the ileum were separated by the Meckel's diverticulum. Digesta from the ileum was quantitatively collected by gentle stripping. Digesta samples from the same treatment in the same block of two experimental rooms were pooled before being freeze-dried for DM, starch, nitrogen, and Ti analysis to calculate digestibility of DM, starch and CP. Apparent digestibility (AD) of DM, starch, CP was calculated by equation 1:

$$\text{Apparent digestibility of nutrient (\%)} = \frac{(\text{Nutrient}_{diet}/\text{Ti}_{diet}) - (\text{Nutrient}_{digesta}/\text{Ti}_{digesta})}{(\text{Nutrient}_{diet}/\text{Ti}_{diet})} \times 100\ \% \quad (1)$$

where Nutrient_{diet} and $\text{Nutrient}_{digesta}$ (%) are the nutrient (DM, starch or CP) content in the experimental diets (as-fed basis) and in the freeze-dried digesta samples, respectively,

and Ti_{diet} and $Ti_{digesta}$ (%) are the Ti content in the sample experimental diets the digesta samples, respectively.

The AME_n value per g of each of the experimental diets (as-fed basis) were calculated according to the method described by Hill and Anderson, (1958) using a value of 34.39 kJ per g of retained nitrogen to correct to zero nitrogen-retention.

Exp 2. Growth performance trial

Design, animals, housing and feeding

A total of 800 one-day-old male broilers (Ross 308, Aviagen Group, Newbridge, UK) housed in 40 floor pens with 20 broilers in each pen were used. Wood shavings were used as bedding material throughout the experimental period and the ambient temperature was kept at 33 °C at experimental day 1 and gradually reduced to 21 °C. The relative humidity was at a minimal level of 60 % during the first three days and between 40 and 70 % from day 3 onwards. During the first two days, lights were continuously on (i.e. 24L : 0D) while from day 3 onwards, lighting schedule followed a four-cycle 2L : 4D regime. The experiment consisted of three stages: a starter (i.e. day 1 to 7), a grower (i.e. day 8 to 24) and a finisher (i.e. day 25 to 35) phase. During the starter phase, broilers were fed a commercial diet while the grower diets were fed from day 8 to 24 and the finisher diets from day 25 to 35. The experimental diets were provided in mash form and throughout the experiment, broilers had unrestricted access to feed and water.

Data collection and calculation

Total feed intake and body weight per pen were recorded at the end of each phase (i.e. day 7, 24 and 35). For each phase, feed conversion ratio (FCR) was calculated by dividing total feed intake by total body weight gain including dead birds. Average daily feed intake (ADFI) was calculated as the mean body weight gain (ADG) of live birds multiplied with the respective value for FCR. Energy conversion ratio (ECR) was calculated by dividing dietary ME intake, calculated using the results from the digestibility trial, by total body weight gain including dead birds. Average daily feed intake and ADG were expressed per bird and FCR and ECR per pen. At day 35, four broilers were randomly selected from each pen and slaughtered. Two out of these four broilers were used to determine carcass yield and breast meat yield (as % of live weight). The other two were used to determine abdominal fat pad (as % of live weight).

Chemical analysis

All chemical analyses were performed according to standard laboratory methods. Dry matter was determined gravimetrically after drying at 103 °C for 4 h (ISO 6496; ISO, 1999). Gross energy was determined using bomb calorimetry (IKA-C700, Janke & Kunkel, Heitersheim, Germany) (ISO 9831; ISO, 1998). Starch content was determined after enzymatically hydrolysis (method ISO 15914; ISO, 2004). Nitrogen was determined using Dumas method (method ISO 16634-1; ISO, 2008) using a Flash EA 1112 NC Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). Titanium was determined according to (Myers et al., 2004). Uric acid was analysed calorimetrically using a commercial test kit (Human GmbH, Wiesbaden, Germany).

Statistical analysis

All data were analysed by analysis of variance using the GLM procedure of SAS (version 9.3, SAS Institute Inc., Cary, NC) with starch source, protein source and their interaction as fixed effects and block as a random effect. Residuals of all dependent variables were tested for normality and non-normal distributed data were log transformed. Probability levels of less than 5 % were considered to be statistically significant, and levels between 5 to 10 % were considered a trend.

RESULTS

Apparent digestibility of experimental diets

Apparent ileal digestibility (AID) of DM was affected by starch source ($P < 0.001$) (Table 5.3). For the AID_{Starch} and AID_{CP}, there was an interaction between starch and protein source ($P = 0.008$ and $P < 0.001$, respectively). Broilers fed RS diets showed a higher AID_{DM} and AID_{Starch} than broilers fed PS diets. Apparent total tract digestibility (ATTD) of DM was affected by starch source ($P < 0.001$), with RS diets showing a higher $ATTD_{DM}$ than PS diets. For $ATTD_{CP}$, there was an interaction between starch and protein source ($P < 0.001$). Broilers fed the PS-SBM and RS-SPI diets had a higher $ATTD_{CP}$ (83.9 and 85.1 %, respectively) than broilers fed the PS-SPI and RS-SBM diets (82.5 and 82.4 %, respectively). Diets with RS showed a higher AME_n than diets with PS ($P < 0.001$).

Table 5.3. Growth performance (day 15-24) of broilers and apparent ileal digestibility (AID, %) and apparent total tract digestibility (ATTD, %) of dry matter (DM), crude protein (CP) and starch, and nitrogen-corrected metabolisable energy (AME_n, kJ) of experimental diets in the digestibility trial (n = 5).

	Experimental diets[1]				Pooled SEM	*P*-value		
	PS-SBM	PS-SPI	RS-SBM	RS-SPI		Starch	Protein	Interaction
Performance[2]								
ADG[3] (g)	49.3[a]	49.4[a]	46.5[a]	38.4[b]	1.51	<0.001	0.014	0.011
ADFI[4] (g)	67.5[a]	69.1[a]	64.3[a]	54.0[b]	1.64	<0.001	0.013	0.001
FCR[5] (g/g)	1.38	1.40	1.40	1.41	0.04	0.717	0.674	0.887
Digestibility								
AID_{DM}	68.1[b]	69.0[b]	73.5[a]	73.8[a]	0.80	<0.001	0.430	0.686
AID_{CP}	84.8[a]	82.7[ab]	81.9[b]	84.7[a]	0.55	0.428	0.512	<0.001
AID_{Starch}	87.1[c]	90.4[b]	99.0[a]	99.6[a]	0.46	<0.001	<0.001	0.008
$ATTD_{DM}$	71.3[b]	72.0[b]	76.0[a]	75.6[a]	0.38	<0.001	0.695	0.210
$ATTD_{CP}$	83.9[a]	82.5[b]	82.4[b]	85.1[a]	0.33	0.117	0.095	<0.001
AME_n	13.4[b]	13.3[b]	14.1[a]	13.9[a]	0.07	<0.001	0.031	0.332

[1] PS=pea starch; RS= rice starch; SBM= soybean meal; SPI= soy protein isolate. [2] Performance results were measured between experimental day 15 and 24. [3] Average daily weight gain (g). [4] Average daily feed intake (g). [5] Feed conversion ratio (g/g). [a-c] Means within the row without a common superscript differ ($P < 0.05$).

Growth performance

The initial body weight of broilers did not differ among treatments. During the grower stage (i.e. day 7-24), an interaction between starch and protein source was found for ADG, FCR and ERC. Broilers fed the RS-SPI diet showed the lowest ADG and ADFI (36.9 and 59.2 g, respectively), and highest FCR and ERC (1.61 g/g and 22.3 kJ/g, respectively) ($P < 0.001$) (Table 5.4). Broilers on the PS-SBM and PS-SPI diets showed a comparable growth performance. During the finisher stage (i.e. day 24-35), broilers on the PS-SBM diet showed the highest ADG and ADFI, and broilers on the RS-SPI diet the lowest, with broilers on the PS-SPI and RS-SBM diets being intermediate ($P < 0.001$). Broilers fed the RS-SPI diet showed a higher FCR and ECR (1.74 g/g and 24.1 kJ/g, respectively) than broilers fed the PS-SBM (1.66 g/g and 22.3 kJ/g, respectively; $P < 0.001$). Over the entire experimental period (i.e. day 7-35), an interaction between starch source and protein source was found for ADG, FCR and ERC ($P < 0.05$). Broilers on the PS-SBM diet showed the best performance and broilers on the RS-SPI diet the worst over the entire experiment ($P < 0.001$). No interaction between starch and protein source was found for ADFI for both the grower and the finisher stages.

Carcass characteristics

Carcass characteristics were affected by protein source ($P < 0.001$) (Table 5.5). Birds fed the PS-SBM diet showed the highest slaughter weight and birds fed the RS-SPI diet the lowest. Broilers on diets with SPI showed higher carcass, breast meat and abdominal fat yields ($P < 0.001$) and breast to carcass ratio than broilers on diets with SBM ($P < 0.001$).

Table 5.4. Growth performance of broilers fed experimental diets[1] during grower, finisher and overall period (day 7-35) in the growth performance trial (n = 10).

	Experimental diets				Pooled	*P*-value		
	PS-SBM	PS-SPI	RS-SBM	RS-SPI	SEM	Starch	Protein	Interaction
Initial body weight (g)	165.3	166.2	161.7	166.5	2.17	0.458	0.204	0.378
Final body weight (g)	1919[a]	1781[b]	1749[b]	1528[c]	19.90	<0.001	<0.001	0.045
Grower (day7-24)								
ADG[2] (g/bird)	47.1[a]	44.6[ab]	43.7[b]	36.9[c]	0.73	<0.001	<0.001	0.007
ADFI[3] (g/bird)	68.5[a]	65.4[ab]	64.6[a]	59.2[b]	1.14	<0.001	<0.001	0.315
FCR[4] (g/g)	1.46[b]	1.47[b]	1.48[b]	1.61[a]	0.01	<0.001	<0.001	<0.001
ERC[5] (kJ/g)	19.5[c]	19.5[c]	20.8[b]	22.3[a]	0.19	<0.001	<0.001	<0.001
Finisher (day 25-35)								
ADG[2] (g/bird)	86.5[a]	78.0[b]	76.7[b]	66.8[c]	1.09	<0.001	<0.001	0.535
ADFI[3] (g/bird)	143.5[a]	131.9[b]	128.8[b]	115.8[c]	1.40	<0.001	<0.001	0.604
FCR[4] (g/g)	1.66[b]	1.69[ab]	1.68[ab]	1.74[a]	0.02	0.047	0.008	0.464
ERC[5] (kJ/g)	22.3[b]	22.5[b]	23.7[ab]	24.1[a]	0.21	<0.001	0.135	0.811
Overall (day 7-35)								
ADG[2] (g/bird)	62.6[a]	57.7[b]	56.7[b]	48.6[c]	0.69	<0.001	<0.001	0.031
ADFI[3] (g/bird)	98.0[a]	91.5[b]	89.8[b]	81.4[c]	1.13	<0.001	<0.001	0.386
FCR[4] (g/g)	1.56[b]	1.59[b]	1.58[b]	1.68[a]	0.01	<0.001	<0.001	0.004
ERC[5] (kJ/g)	21.2[c]	21.5[c]	22.2[b]	23.2[a]	0.21	<0.001	0.031	0.015

[1] PS=pea starch; RS= rice starch; SBM= soybean meal; SPI= soy protein isolate. [2] Average daily weight gain (g/bird). [3] Average daily feed intake (g/bird). [4] Feed conversion ratio (g/g). [5] Energy conversion ratio (kJ/g). [a-c] Means within the row without a common superscript differ ($P < 0.05$).

Table 5.5. Slaughter weight (g), yield (% of slaughter weight) of carcass, breast meat and abdominal fat pad of broilers (day 35) fed the different experimental diets[1].

	Experimental diets				Pooled SEM	*P*-value		
	PS-SBM	PS-SPI	RS-SBM	RS-SPI		Starch	Protein	Interaction
Slaughter weight (g)	2009^{a}	1858^{b}	1866^{ab}	1633^{c}	39.45	<0.001	<0.001	0.295
Carcass (%)	64.6^{b}	65.0^{ab}	64.9^{ab}	66.0^{a}	0.37	0.077	0.048	0.341
Breast meat (%)	15.2^{b}	16.7^{a}	15.6^{ab}	16.5^{a}	0.32	0.718	<0.001	0.320
Abdominal fat pad (%)	2.3^{a}	2.0^{bc}	2.2^{ab}	1.8^{c}	0.08	0.095	<0.001	0.527
Breast to carcass (%)[2]	23.5^{b}	25.7^{a}	24.1^{ab}	25.0^{ab}	0.45	0.879	<0.001	0.167

[1] PS=pea starch; RS= rice starch; SBM= soybean meal; SPI= soy protein isolate. [2] Breast to carcass percentage was calculated as the weight of breast meet as a percentage of carcass weight.

DISCUSSION

The objective of the present study was to determine if synchronising the digestion kinetics of dietary starch and protein affects the growth performance and carcass characteristics in broilers. Broilers were fed *ad libitum* over the experiment. *Ad libitum* feeding is commonly considered as continuous feeding, resulting in an evenly distributed intake of feed during the period of light and a steady passage of feed and digesta through the gastrointestinal tract where nutrient hydrolysis and absorption take place. Therefore, *ad libitum* supply of feed could possibly diminish the extent of asynchrony of the supply of glucose and AAs caused by the differences in digestion kinetics of dietary starch and protein compared to meal feeding. *Ad libitum* feeding, however, is not necessarily equal to continuous feeding, especially in broilers that are subjected to an intermittent lighting schedule (Weaver and Siegel, 1968; Savory, 1976). In the present study, the daily lighting schedule followed a four-cycle of 2L : 4D regime. This intermittent lighting schedule might have induced a meal-like pattern of feed intake, potentially inducing an increase in asynchrony in the systemic supply of glucose and AAs.

The ADFI for all broilers in all treatments over the experimental period was 11-26 % lower than the tabulated ADFI values for male Ross 308 broilers (Aviagen Group, 2014). Studies have shown that broilers fed mash diets showed a lower feed intake compared to broilers fed pelleted diets (Engberg et al., 2002; Amerah et al., 2007). Pelleting, however, is a hydrothermal process, which could induce gelatinisation of starch in diets (Abdollahi et al., 2013; Lewis et al., 2015), thereby increasing the digestion rate of dietary starch (Liu et al., 2013a). Thus, in order to ensure adequate differences in starch digestion kinetics, experimental diets were provided as mash. Unexpectedly, the ADFI was affected by the dietary starch and protein sources in both the grower and the finisher stages. Broilers fed diets with RS showed a lower ADFI than broilers fed PS starch. This might be related to a higher AID_{starch} of the RS diets than the PS diets (99 vs. 89 %), leading to on average a 0.7 MJ/kg higher AME_n value of the RS diets than the PS diets. Broilers were shown to be capable of adjusting their feed intake in response to dietary energy level to maintain their BWG, by increasing intake of low-energy diets (Leeson et al., 1996). Broilers fed the SBM diets showed a higher ADFI than broilers fed the SPI diets. The AID_{CP} did not differ between the SBM and SPI diets. The difference in ADFI, therefore, could not be related to differences in the supply of digestible essential AAs. Soy protein isolate is a fast digestible protein source, which shows a fast release of AAs in the GIT of broilers (Chapter 4). A fast release of glucose and AAs from dietary

starch and protein could slow down the gastric emptying via both endocrine and neurocrine signal pathways, leading to an increased satiety and a decreased feed intake in poultry (Richards and Proszkowiec-Weglarz, 2007). This feedback mechanism could also contribute to the explanation why broilers fed RS diets showed a lower ADFI.

It was hypothesised that broilers fed synchronised diets (i.e. PS-SBM and RS-SPI) would have a higher growth performance and breast meat yield compared to broilers fed asynchronized diets (i.e. PS-SPI and RS-SBM). Over the experimental period (i.e. day 7-35), birds fed the synchronised PS-SBM diet showed the highest ADG among all treatments, with the concomitant highest ADFI compared to the other treatments. The FCR did not differ in broilers fed the PS-SBM and RS-SBM diets. This is not in agreement with a study of Weurding et al. (2003) showing that feeding slowly digestible starch to broilers improved the feed conversion ratio by approximately 2 %. Birds fed the RS-SPI diet showed the lowest growth performance and ADFI among all treatments. The differences in ADFI among treatment in the present study hindered a proper evaluation of the effect of synchronising the digestion kinetics of dietary starch and protein on the growth performance of broilers.

Studies have shown that the simultaneous availability of AAs and glucose in organs and tissues increase protein retention in both growing pigs and broilers (van den Borne et al., 2007; Liu et al., 2013b). This could be explained by sparing AAs from being catabolised to produce energy in organs and tissues when sufficient glucose as energy substrate is available. As a consequence, the AAs can be used primarily for protein synthesis. Moreover, the postprandial increase of glucose in blood is commonly accompanied by an increased postprandial plasma insulin concentration. Insulin stimulates protein deposition and inhibits protein breakdown in muscle tissues (Rooyackers and Nair, 1997; Bigot et al., 2003). These suggest that broilers fed protein and energy synchronised diets could show a higher protein deposition and breast meat yield than broilers fed non-synchronised diets. The breast meat yield of the broilers in the present study, however, was not affected by the interaction between dietary starch and protein source, suggesting no effect of nutrient asynchrony, but was only affected by protein source. Broilers fed the SPI diets increased breast meat yield by 8 % compared to broilers fed the SBM diets. This indicates, independent of the starch source, fast digestible protein sources might benefit muscle protein deposition in growing broilers. This is not in agreement with previous studies indicating that a more steady supply of AAs from slowly digestible protein sources, such as casein, could

increase the overall protein retention in healthy young human subjects (Boirie et al., 1997; Dangin et al., 2001).

Fast and slowly digestible starch and protein sources were selected based on their differences in digestion rates. The digestion rate of the same source of RS and PS as used in the present study were determined in a study with pigs, in which RS showed a higher maximal starch digestion rate than PS (0.65 vs. 0.34 % · min^{-1}) (van Kempen et al., 2010). With respect to protein sources, SPI had a 10-fold higher maximum protein digestion rate than SBM in broilers (32.3 vs. 3.1 % · min^{-1}) (Chapter 4). However, the methods to determine the digestion rate of starch and protein sources were different in these two studies. Moreover, the digestion rate of the starch sources was determined in pigs, and not in broilers. The differences in digestive physiology (i.e. size of the GIT relative to body weight, digesta passage rate, and secretion and concentration of digestive enzymes) in pigs and poultry could affect the digestion rate. As a consequence, the dietary starch and protein sources as used in the present study were not synchronised based on their actual digestion rates determined in broilers. Synchronisation of the digestion of dietary starch and protein based on actual digestion rates might be a better approach.

CONCLUSION

Broilers fed diets synchronised for the digestion rate of starch and protein (i.e. PS-SBM (slow-slow) and RS-SPI (fast-fast)) did not show a higher growth performance and breast meat yield compared to broilers fed the asynchronised diets (i.e. RS-SBM (fast-slow) and PS-SPI (slow-fast)). The evaluation of the effect of synchronising the supply of dietary starch and protein, however, was hindered by feed intake being affected by dietary protein and starch source, in favour of using SBM compared to SPI and PS compared to RS.

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Chapter 6

General discussion

Aim and main findings of the thesis

Increasing the protein efficiency is considered a main strategy for sustainable feeding of pigs and poultry. In practice, protein in pig and poultry diets originates from different ingredients, selected in diet formulation based on their nutritional value and costs of the ingredients. Currently, the nutritional value of protein sources in pig and poultry diets is based on the concentration of essential amino acids (AAs), and their digestibility up to the end of the ileum or the gastrointestinal tract (GIT) (NRC, 2012; CVB, 2016). The ileal and faecal digestibility of protein and AAs, however, only provide information on the quantity of protein and AAs apparently absorbed up to the end of the ileum or the GIT. They, however, do not provide information on the kinetics of protein digestion, which might affect the post-absorption metabolism of dietary AAs. The aim of this thesis, therefore, was to provide further insights into the digestion kinetics of dietary protein sources in the GIT of pigs and poultry, and the consequences of differences in digestion kinetics of dietary protein for the growth performance of broilers.

The main findings of this thesis are:

- Protein sources differ in digestion kinetics in growing pigs, with initial protein digestion rate ranging from 0.68 % · min^{-1} for a rapeseed meal based diet to 3.04 % · min^{-1} for a dried porcine plasma protein based diet.
- Protein sources differ in digestion kinetics in broilers, with initial protein digestion rate ranging from 1.76 % · min^{-1} for a rapeseed meal based diet to 30.7 % · min^{-1} for a wheat gluten based diet.
- The initial protein digestion rate is on average 2.7-fold higher in broilers than in pigs with the exception of wheat gluten, which is far higher in broilers than in pigs.
- A fast digestion kinetics of dietary protein results in a more rapid and pronounced postprandial appearance of AAs and peptides in systemic blood of pigs.
- Protein hydrolysis in the GIT of pigs and broilers follows a “one-by-one” mechanism to AAs.
- Approximately 30 % of peptides present in ileal digesta of pigs are < 10 kDa in dependent of protein source, whereas almost no peptides < 10 kDa are found in the ileal digesta of broilers.
- Synchronising the digestion kinetics of dietary starch and protein using rice starch (fast digestible), pea starch (slowly digestible), soy protein isolate (fast digestible), and soybean meal (slow digestible) does neither improve the growth performance

nor the breast muscle yield of *ad libitum* fed broilers kept under a 2 h L and 4 h D light regime.

Selection of protein sources

Soybean meal (SBM), wheat gluten (WG), rapeseed meal (RSM), dried porcine plasma protein (DPP), and black soldier fly larvae (BSF)) were the protein sources evaluated in the present thesis. Selection of these five protein sources was not only based on the differences in expected *in vitro* protein digestion kinetics (fast vs. slow) but also on their innate physicochemical characteristics. Soybean meal, WG and RSM are plant-derived sources, whereas DPP and BSF are protein sources of animal origin. Comparing the selected protein sources of plant origin, the protein fraction of SBM and RSM mainly consists of albumins and globulins while the protein fraction of WG mainly consists of prolamins and glutelins. Moreover, SBM, RSM and DPP are considered conventional sources, whereas BSF is a novel source. Although soy protein and rapeseed protein are also commercially available in isolated forms, SBM and RSM were selected because they are more widely used in commercial diets compared to soy and rapeseed protein isolates. Soy and rapeseed protein isolates are primarily used in weaner and starter diets for young animals. In addition, SBM and RSM contain a substantial fraction of non-protein constituents. The effect of the interaction between proteins and these constituents on protein digestion kinetics was of interest in the present thesis as well.

What determines the digestion kinetics of dietary protein?

The overall extent and the rate of dietary protein digestion are related to three aspects: 1) the passage of digesta along the GIT, 2) the hydrolysis of dietary protein, and 3) the absorption of AAs and di- and tri-peptides by the small intestinal mucosa. All three aspects can be affected by intrinsic characteristics of protein sources, the matrix of complete diets (i.e. the interactions with other ingredients) and the digestive physiology of animals (Figure 6.1).

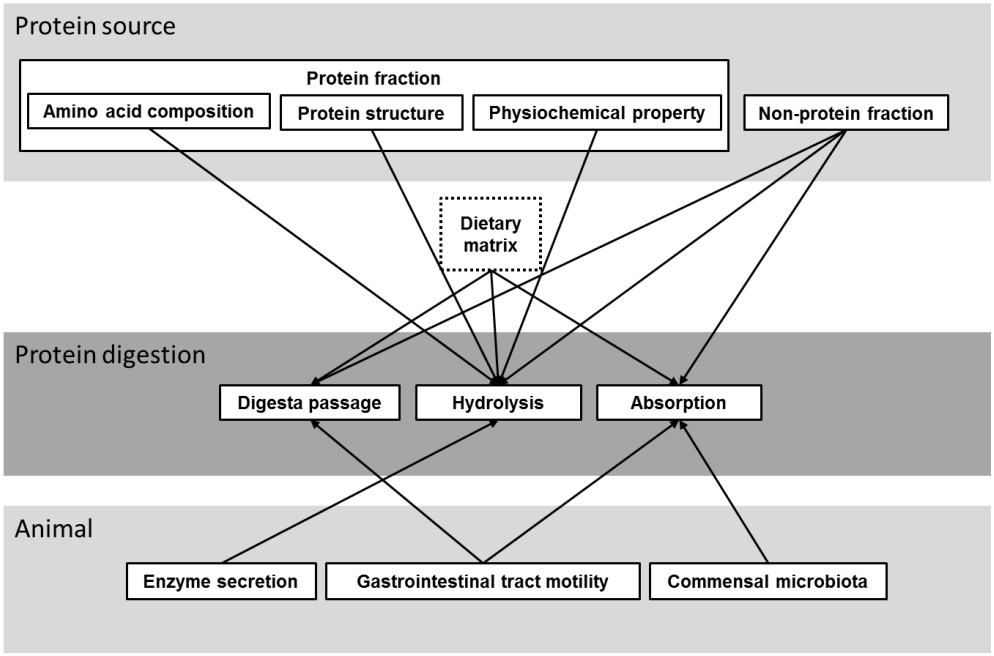

Figure 6.1. Overview of potential determinants of protein digestion kinetics in the gastrointestinal tract of pigs and poultry.

Intrinsic characteristics of protein sources

A protein source is composed of a protein fraction and can contain, depending on the protein source, a non-protein fraction including various levels of carbohydrates (i.e. sugars, starch and non-starch polysaccharides), fat and minerals. The protein content of the five sources evaluated in this thesis ranged from 36 % for RSM to 88 % for DPP. The protein fraction of most protein sources consists of different types proteins. The protein composition of SBM, RSM, WG and DPP is relatively well-defined (Table 6.1), whereas the protein composition of BSF remains largely unknown. The digestion of a protein source, therefore, is a combined result of the digestion of each protein present in the protein source.

Table 6.1. Protein content and composition of different protein sources.

Protein source	Protein content (%)	Protein type	Content (% of total protein)
Soybean meal	39-50	Glycinin	31
		β-Conglycinin	30-50
Rapeseed meal	30-40	Cruciferin	60
		Napin	15-45
Wheat gluten	73-84	Prolamin	34
		Gluenin	34
Porcine plasma	67-78	Albumins	55
		Globulins	37
		Fibrinogen	8

Reference: Delisle et al. (1984); Sikorski (2006); Jamroz et al. (2011); Kriger (2014).

In both pigs and poultry, WG and DPP can be regarded as fast digestible while SBM, RSM and BSF as more slowly digestible protein sources (Chapter 3 and 4). Intrinsic characteristics of protein sources can be related to their 1) AA composition, 2) structural conformation, 3) physicochemical properties and 4) interactions with the non-protein fraction of proteins present in protein sources. Digestion of dietary protein starts in the stomach by the action of pepsin and hydrochloric acid but mainly occurs in the small intestine by the action of pancreatic proteases (i.e. trypsin and chymotrypsin and carboxypeptidase A and B), followed by the hydrolysis by proteases and peptidases present at the intestinal brush border. Trypsin and chymotrypsin are endopeptidases, which cleaves the carboxyl end of basic AAs (arginine and lysine) and aromatic AAs (phenylalanine, tyrosine and tryptophan), respectively (Riviere and Tempst, 2001). These basic and aromatic AAs then can be released as free AAs by the action of carboxypeptidase A and B (Folk et al., 1960; Riviere and Tempst, 2001). Based on this, it could be assumed that basic and aromatic AAs would be released faster from dietary protein than the other AAs and therefore could be absorbed and enter the circulation faster. As a consequence, protein sources rich in basic and aromatic AAs might be digested faster. The portal appearance of these AAs, however, was not faster than that of the other AAs in pigs fed SBM and RSM diets (Jansman et al., 1994). In addition, DPP has a higher concentration of basic and aromatic AAs than the other protein sources. Dried porcine plasma protein, however, did not show a higher *in vitro* release rate of low molecular weight peptides (< 500 kDa) and free AAs than the other protein sources during the hydrolysis by the proteases in porcine pancreatin (Chapter 2). These results indicate that the effect of AA composition of protein sources on the kinetics of protein digestion is rather small.

The conformation of proteins is mainly determined by the AA sequence of the polypeptide chains. Studies have shown that *in vitro* protein digestibility can be affected by the secondary structure of protein (Carbonaro et al., 2012; Rubio et al., 2014; Yang et al., 2016). A negative linear correlation coefficient was found between the content of β-conformation and the degree of hydrolysis of soy protein isolate by the action of pepsin (Yang et al., 2016). In addition, the presence of intramolecular β-sheet structures negatively affected *in vitro* protein digestibility of both animal- and plant-origin food proteins being hydrolysed by porcine trypsin, chymotrypsin and peptidases (Carbonaro et al., 2012). The latter authors suggested that the decrease in *in vitro* protein digestibility with an increased proportion of intramolecular β-sheet can be explained by the higher hydrophobicity of β-sheet structures. Prolamin and gliadin from WG have a lower content of β-sheets and higher ratio between α-helix and β-sheet than glycinin and β-conglycinin from SBM and cruciferin and napin from RSM (Delisle et al., 1984; Sikorski, 2006). A higher ileal digestibility and faster protein digestion kinetics of WG than SBM and RSM, therefore, might be partly attributed to the secondary structure of proteins in wheat.

With respect to physicochemical properties, protein solubility plays a critical role in protein digestion. The *in vitro* hydrolysis rate of a water-soluble form of casein (i.e. Na^+-caseinate) was 2-fold higher than that of water-insoluble casein at pH 8.0 (Tonheim et al., 2007). Moreover, the soluble protein fraction of unprocessed RSM showed a 3-fold higher *in vitro* hydrolysis rate than the insoluble protein fraction (Salazar-Villanea et al., 2017). In Chapter 2, WG and DPP showed a higher nitrogen solubility under the simulated gastric (pH 3.5) and intestinal (pH 6.8) conditions of pigs, which might explain why WG and DPP showed a faster digestion kinetics in the GIT of pigs.

Soybean meal and RSM consist of 20-30 % of non-starch polysaccharides (NSP) (CVB, 2016), originating from plant cell wall structures. Non-starch polysaccharides can be further divided into insoluble NSP (i.e. mainly cellulose) and soluble NSP. An increase in the content of soluble NSP in pig and broiler diets results in a decreased absorption of AAs and other nutrients (King and Taverner, 1975; Choct and Annison, 1990). This is likely due to the fact that soluble NSP can increase the viscosity of digesta (Bach Knudsen, 2001), which could restrict the accessibility of proteases to dietary protein and absorption of AAs and di- and tri-peptides. Moreover, other anti-nutritional factors present in SBM (e.g. protease inhibitors, lectins, phytate) (Campbell and van der Poel,

1998; Selle et al., 2012) and RSM (e.g. sinapine, phytic acid, tannins) (Mangan, 1988; Khajali and Slominski, 2012) could also hinder protein digestion in the GIT of pigs and poultry by binding to either proteases or dietary protein.

Protein digestion in monogastric animals

Animals regulate protein digestion mainly via protease secretion and GIT motility. The secretion of endogenous proteases is regulated by both endocrine and neurocrine signal pathways. The secretion of pepsinogen, the precursor of pepsin, and hydrochloric acid in the stomach is initiated by stimulation of vagal nerve during feeding (Saladin and Miller, 1998). The secretion of pepsinogen and hydrochloric acid is further stimulated by the action of gastrin, which is released by the presence of peptides present in the stomach and the duodenum (Skak-Nielsen et al., 1988). The secretion of pancreatic juice including various proteases and bicarbonate is also initiated by stimulation of the vagal nerve during gastric digestion. The secretion of pancreatic juice is further stimulated by the action of cholecystokinin and secretin. Cholecystokinin is released by the presence free fatty acids and AAs in the lumen of the gut (Saladin and Miller, 1998). The presence of basic and aromatic AAs, the end product of trypsin and chymotrypsin digestion is proven to cause the release of pancreatic juice (Niederau et al., 1986), most likely via the action of cholecystokinin. The release of secretin is related to the decreased pH in the duodenum due to the hydrochloric acid emptied from the stomach. The amount of endogenous proteases being secreted is affected by the presence of peptides and AAs in the diet. The loss of AAs of endogenous origin was higher in rats fed a diet with a mixture of peptides and AAs than rats fed a protein-free diet (Moughan and Rutherfurd, 1990). Moreover, the loss of endogenous AAs at ileal level is positively correlated to the concentration of peptides in the diet in growing pigs (Hodgkinson et al., 2000). These results indicate that the presence of dietary peptides and AAs lead to a higher secretion of endogenous proteases. In addition, the activity of endogenous proteases also increases with a higher protein intake in weaning pigs (Makkink et al., 1994). The form in which the dietary AAs are provided can also regulate the amount of endogenous proteases secreted. In growing pigs, dietary AAs provided in the form of free AAs resulted in a lower endogenous ileal AA loss than AAs provided in the form of peptides (Butts et al., 1993). This can be related to peptides requiring further hydrolysis in the GIT prior to absorption in the form of peptides and free AA, whereas free AAs are absorbed directly by the intestinal mucosa without further hydrolysis.

Protein digestion requires a sufficient reaction time between proteolytic enzymes and dietary protein. A fast passage rate of digesta along the GIT, therefore, might hinder quantitative protein digestion. The passage rate is regulated by gut motility. The motility of the GIT is mainly regulated by the release of end products of digestion (i.e. glucose, AAs and fatty acids) via both endocrine and neurocrine signal pathways (Skak-Nielsen et al., 1988). The afferent vagal nerve acts as receptor which is activated by glucose, AAs and fatty acids along the small intestine. Once the vagal nerve detects the increased concentration of glucose, AAs and fatty acids, it slows down the GIT motility, leading to longer retention time of digesta in the GIT. With respect to endocrine pathways, gastric emptying is inhibited by the action of cholecystokinin, peptide YY and glucagon-like peptide (Moran and McHugh, 1982; Savage et al., 1987). As mentioned above, cholecystokinin is secreted in the duodenum, whereas peptide YY and glucagon-like peptide are secreted in the ileum. The release of peptide YY and glucagon-like peptide also reduce peristalsis of the small intestine, resulting in a longer retention time of digesta in the small intestine. In addition to GIT motility, the passage rate of digesta along the GIT also depends on the feeding pattern (i.e. feeding frequency and meal quantity) and the physicochemical properties (e.g. solubility, viscosity, water binding capacity) of digesta which are affected by dietary protein sources but also by characteristics of other ingredients in the diet. A large volume of a meal (Hunt and Stubbs, 1975), a high solubility of digesta (Low et al., 1978) and an increase in dietary fibre content (Wilfart et al., 2007) increase the passage rate of digesta along the GIT.

Dietary protein can also be fermented by the commensal microbiota in the GIT, which mainly occurs in the colon of pigs and the caeca of poultry. Dietary protein degraded in the hindgut by microbial fermentation does not significantly contribute to AA supply for animals and therefore does not directly affect the nutritional value of protein sources. The potentially toxic metabolites such as ammonia, amines, volatile phenols and indoles, produced during proteolytic fermentation can negatively affect gut health and animal performance (Williams et al., 2001). For example, ammonia produced by proteolytic fermentation can disturb the development of intestinal mucosa and reduce the villus height in chickens (Visek, 1984; Nousiainen, 1991), leading to a reduced nutrient absorption by the intestinal mucosa. Moreover, feeding poorly digestible protein sources and increasing the dietary protein concentration favoured the growth of pathogenic bacteria such as *Clostridium perfringens*, leading to an increased endogenous loss (Drew et al., 2004; Wilkie et al., 2005).

Identifying potential determinants of protein digestion, related to both intrinsic characteristics of protein sources and the digestive physiology of animals, could help to develop strategies to increase protein and AA digestibility of feed ingredients used in pig and poultry diets.

Possibilities to manipulate protein digestion

Several factors determining the extent and the rate of protein digestion, related to both intrinsic characteristics of protein sources and the digestive physiology of animals, have been discussed above. It still remains unclear whether these factors can be manipulated to increase either the extent or the rate of protein digestion or both, especially of protein sources with a rather low digestibility. An overview of current strategies to increase digestibility of protein and AA of feed ingredients used in pig and poultry diets is presented in Figure 6.2.

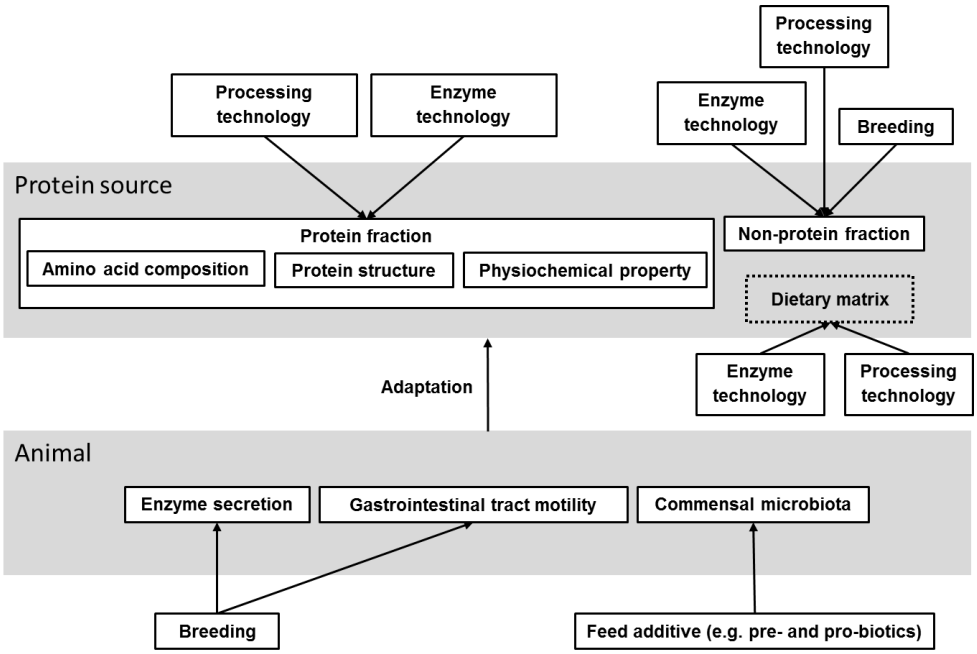

Figure 6.2. Overview of current strategies to increase protein and amino acid digestibility of feed ingredients used in pig and poultry diets.

With respect to the digestive physiology of monogastric animals, the secretion of digestive enzymes and the motility of the GIT are controlled by both endocrine and neurocrine systems, which involve several feedback mechanisms each. Possibilities to

manipulate enzyme secretion and GIT motility by interfering with these feedback mechanisms seem rather small. By using genetic variation within and between breeds with respect to nutrient digestibility in breeding programmes, some key features of the digestive system (e.g. digestive enzyme secretion and digesta passage rate) could be changed. However, breeding might be a time-consuming and costly approach. As a consequence, the possibilities to manipulate protein digestion via changing components of the digestive system of animals seem rather small. However, animals adapt their digestive system according to the composition of the diet, suggesting it is possible to modify digestive capacity of animals via different dietary interventions. (Moughan and Rutherfurd, 1990; Butts et al., 1993; Makkink et al., 1994; Hodgkinson et al., 2000), As mentioned above, colonization of pathogenic bacteria in the GIT negatively affects nutrient absorption by the intestinal mucosa. Inclusion of pre- and pro-biotics in the diet, therefore, could be an approach to inhibit the growth of pathogenic bacteria and maintain health and digestive functions of the gut (Patterson and Burkholder, 2003; de Lange et al., 2010)

Hydrothermal processing is commonly applied to SBM and RSM in order to remove the organic solvents used for oil extraction. It could cause both chemical and physical changes of the proteins present and therefore negatively affect nutritional value of SBM and RSM. During hydrothermal processing, the Maillard reaction, the reaction between reducing sugars and AAs (mainly lysine and arginine), might occur. The Maillard reaction decrease AA concentration, their digestibility and post-absorptive utilisation (Messerschmidt et al., 2012; Almeida et al., 2014; Hulshof et al., 2017). Moreover, hydrothermal processing can reduce protein solubility in SBM and RSM (Hulshof et al., 2016), likely resulting in a decreased digestion rate. Indeed, the protein digestion rate of untoasted RSM was 1.3- and 1.6-fold higher than RSM toasted for 60 and 120 min, respectively (Salazar-Villanea, 2017). However, the shear force applied during hydrothermally processing, such as when using extrusion, can reduce particle size of feed ingredients, leading to an increased surface area which allows a better accessibility of digestive enzymes and therefore a higher nutrient digestibility (Wondra et al., 1995; Fastinger and Mahan, 2003). Moreover, hydrothermal processing can inactivate the anti-nutritional factors, such as trypsin inhibitors in legume seeds, leading to a higher digestibility of protein and AAs (van der Poel, 1990). Thus, hydrothermal processing could both negatively and positively affect the extent and the rate of protein digestion of protein sources.

Supplementation of exogenous enzymes to pig and poultry diets is also applied to inactive antinutritional factors present in feed ingredients, which can increase the digestibility of AAs as well as other nutrient digestibility. For example, supplementation of dietary microbial phytase could increase ileal protein and AAs digestibility in pigs and poultry by releasing proteins bound to phytic acid (Ravindran et al., 1999; Traylor et al., 2001). In addition, exogenous proteases are used to inactive protease inhibitors present in legume seeds. However, the inclusion of exogenous proteases in a SBM-based broiler diet has inconclusive effects on ileal protein and AAs digestibility (Simbaya et al., 1996; Ghazi et al., 2002). This is likely due to the fact that the activity of protease inhibitors is largely reduced during the hydrothermal processing of SBM. The effect of proteases on the inactivation of protease inhibitors, therefore, is limited. Soluble NSP, originating from plant cell wall structures, can increase the viscosity of digesta (Bach Knudsen, 2001), which could restrict the accessibility of proteases to dietary protein and absorption of AAs and di- and tri-peptides. Supplementation of NSP-degrading enzymes, such as xylanases and β-glucanases, in pig and poultry diets have been shown to increase NSP degradation and reduce digesta viscosity, resulting in a higher ileal protein and AAs digestibility (Choct et al., 1999; Bedford, 2000; Nortey et al., 2008). Apart from processing technology and supplementation of dietary exogenous enzymes, plant breeding could also be an approach to reduce the content of antinutritional factors in feed ingredients. For example, low-phytic acid maize, barley and soybean meal have been successfully bred, with a 50-90 % reduction in phytic acid content compared to conventional ones (Raboy, 2002).

Current strategies to increase digestibility of protein and AA of feed ingredients mainly focus on the elimination of antinutritional factors present in feed ingredients. Hydrothermal processing could reduce protein solubility, resulting in an reduced protein digestion rate. Thus, the use of enzymes and plant breeding might be preferable methods to reduce the content of antinutritional factors. Also, prolonging the retention time of digesta in the GIT up to the large intestine, could increase the extent and rate of protein digestion. However, manipulating digesta retention time using dietary intervention, such as inclusion of guar gum and cellulose in the diet (Owusu-Asiedu et al., 2006), might also alter digesta viscosity and therefore affect protein hydrolysis and AA absorption. Future research, therefore, could focus on the alteration of digesta retention time without affecting digesta viscosity.

Digestion kinetics differs in pigs and poultry

Broilers showed, on average, a 1.1-fold higher ileal protein digestibility and a 2.7-fold higher small intestinal protein digestion rate than growing pigs, with the exception of WG, for which the protein digestion rate was 22-fold higher in broilers than in pigs. Moreover, a substantial proportion (30 %) of peptides present in ileal digesta of pigs are < 10 kDa, whereas almost no peptides < 10 kDa were present in the ileal digesta of broilers (Chapter 2 and 3). This is probably related to the differences in digestive physiology between pigs and broilers. Broilers have a greater size of the stomach and the small intestine relative to their body size than pigs (7.8 and 3.5 % of body weight, respectively) (Barea et al., 2011; Mabelebele et al., 2014). Chickens have a crop, a part of the oesophagus, whereas pigs do not. Digestion of dietary protein can already take place in the crop by microbial fermentation (Rehman et al., 2007) although its contribution to the overall extent of protein digestion is unknown. The stomach of broilers is separated into two parts: the proventriculus and the gizzard. The proventriculus functions as a glandular stomach similar to the stomach of pigs and the gizzard is regarded as a muscular stomach. The physical force exerted by the gizzard can reduce particle size of feed ingredients, leading to a higher nutrient digestibility (Wondra et al., 1995; Fastinger and Mahan, 2003). This is likely due to a reduction in particle size which increases the surface area and would allow a better accessibility of digestive enzymes. Moreover, plant proteins are commonly encapsulated by a fibrous cell wall structure. The physical force exerted by the gizzard can open the cell wall structure, which also increases the accessibility of digestive enzymes to proteins. The gastric pH of pigs and broilers is comparable, with an average pH of 4.4 and 4.6, respectively (Merchant et al., 2011; Mabelebele et al., 2014). Pepsin of chickens, however, shows a broader pH range for optimal activity than porcine pepsin (Crévieu-Gabriel et al., 1999). The physical force exerted by the gizzard together with a broader pH range for optimal activity of broiler pepsin might result in a better gastric protein digestion in broilers than in pigs. A longer digesta retention time in the GIT might accommodate a sufficient reaction time between digestive enzymes and dietary protein. In this thesis, pigs showed a longer digesta retention time in the stomach and the small intestine than broilers (Chapter 2 and 3), which is in agreement with published results in the literature (Weurding et al., 2001b; DeSesso and Williams, 2008). Although the digesta retention time is shorter in broilers, the occurrence of antiperistaltic contractions (i.e. digesta reflux) in the GIT of poultry (Duke, 1982) provides the

opportunity for an extended hydrolysis of nutrients in the gizzard and the small intestine (Basha and Duke, 1999).

Digestion is a result of both hydrolysis of dietary protein and absorption of released free AAs and di- and tri-peptides. The absorption rate of nutrients largely depends on the absorptive surface area of the intestinal mucosa. The absorptive surface not only relates to the luminal surface area of the small intestine but also to the characteristics (i.e. length, width, and density) of villi and microvilli. Chickens have a 1.2 fold larger total absorptive surface area per unit of body weight than pigs (43.3 vs. 36.7 cm^2 per g body weight) (Chivers and Hladik, 1980; Mitjans et al., 1997). This could also contribute to explaining why broilers showed a higher extent and rate of protein digestion than pigs.

Mechanism of protein digestion in the GIT of pigs and poultry

At the start of this PhD project, protein digestion was considered as a cascade process, in which intact proteins were hydrolysed into intermediate molecular weight peptides in the proximal part of the GIT (stomach and duodenum). The resulting intermediate peptides would subsequently be further hydrolysed into low molecular weight peptides as they further transit to the distal part of the small intestine. With this perception, a gradual shift of high molecular weight proteins and peptides to low molecular weight peptides and free amino acids along the GIT was expected. In addition, it was assumed that proteins present in highly digestible protein sources would be more susceptible to hydrolysis by digestive enzymes, whereas proteins present in low digestible protein sources would be more resistant to proteolytic hydrolysis. As a consequence, the shift of molecular weight distribution of proteins and peptides would occur more rapidly in highly digestible protein sources, resulting in relatively higher low molecular weight peptides being present in the ileal digesta of pigs and broilers. In contrast to expectation, the molecular weight distribution of proteins and peptides remained rather similar throughout the segments of the GIT of pigs and broilers (Chapter 3 and 4). These results indicate that protein hydrolysis in GIT of pigs and broilers likely follow a “one-by-one” type of hydrolysis mechanism (Adler-Nissen, 1976) (Figure 6.3), meaning intact proteins are hydrolysed in one sequence into di- and tri-peptides and AAs prior to absorption. Results also indicate that, despite the differences in intrinsic characteristics of protein sources and digestive physiology of pigs and poultry, the

mechanism of hydrolysis of proteins in protein sources and absorption of released AAs and di-and tri-peptides is rather similar in pigs and poultry independent of protein sources.

The rate limiting step in "one-by-one" type of hydrolysis mechanism is the denaturation of native proteins, in which native proteins lose their secondary and tertiary structures and are transformed into linear chains of AAs. Once the protein structure is opened and peptide bonds are exposed, proteases can rapidly hydrolyse proteins into end products. This suggests that protein denaturation might be a limiting factor in protein digestion in the GIT of pigs and poultry. Denaturation of dietary protein mainly occurs in the stomach by the action of hydrochloric acid. The degree of denaturation of proteins could be related to 1) the intrinsic characteristics of the protein sources, 2) the hydrothermal processing of feed ingredients and 3) the prevailing condition in the GIT of pigs and poultry. It can be speculated that proteins present in slowly and less digestible protein sources might show a lower degree of protein denaturation in the stomach. Indeed, food allergens, usually regarded as resistant proteins to enzymatic digestion, showed a higher stability towards the *in vitro* digestion by pepsin at pH 1.3 (Astwood et al., 1996). Moreover, increasing the pepsin concentration by 100-fold did not change the stability of food allergens to the hydrolysis by pepsin, suggesting pepsin concentration was not the limiting factor for the hydrolysis of food allergens. Therefore, the resistance of food allergens against pepsin digestion could be attributed to their rigid conformational structures, leading to a low degree of protein denaturation under the acidic condition of the stomach. Thus, to further increase protein digestibility of poorly digestible protein sources, increasing protein denaturation in the GIT of pigs and poultry could be a worthwhile approach.

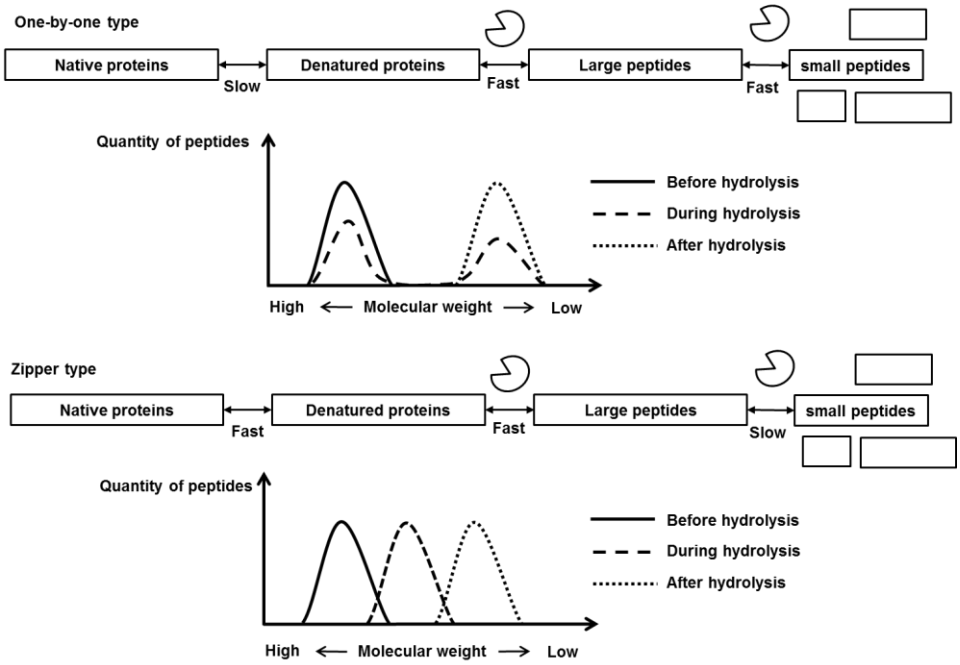

Figure 6.3. Schematic overview of the one-by-one type and zipper type mechanisms of protein hydrolysis in the digestive tract of pigs and poultry.

Classification of dietary protein using *in vitro* approaches

Dietary starch can be divided into different fractions: a rapidly digestible starch fraction (RDS), a slowly digestible starch fraction (SDS) and a resistant starch fraction (RS) using controlled *in vitro* enzymic hydrolysis (Englyst et al., 1992; Weurding et al., 2001a). The *in vivo* digestion kinetics of starch sources can be related to the proportional presence of these three fractions, of which fast digestible starch sources contain a higher RDS, whereas slow digestible starch sources contain a higher SDS (Weurding et al., 2001a). A similar classification might be applied to dietary protein in pig and poultry diets. In Chapter 2, the nitrogen present in feed ingredients after *in vitro* pepsin and pancreatin hydrolysis was separated into three fractions: a insoluble protein fraction (IPF), a soluble high molecular weight (> 500 Da) peptide fraction (HMW) and a soluble low molecular weight peptide fraction (LMW). The IPF can be compared with the resistant starch fraction, as it was assumed that nitrogen present in soluble form is absorbed in the GIT *in vivo*. Indeed, over the evaluated protein sources, the proportion of nitrogen present as soluble nitrogen was well-correlated to the *in vivo* apparent protein digestibility in

both pigs and poultry (Figure 6.4A). The LMW and the HMW factions after *in vitro* protein hydrolysis were assumed to be the rapidly digestible protein fraction and the slowly digestible protein fraction, respectively. With this assumption, protein sources with a higher LMW, therefore, would be digested faster *in vivo*. However, such correlation between the proportion of LMW and *in vivo* digestion rate was observed in broilers but not in pigs (Figure 6.4B). The reason of the poor correlation found in pigs is unclear. However, it should be noted that only five protein sources were evaluated in the studies presented in this thesis. Follow-up studies using multiple batches of a large number of protein-containing feed ingredients should be carried out to validate if the current fractionation method of dietary protein using the *in vitro* approach as presented in this thesis (Chapter 2) can be used to predict *in vivo* protein digestion kinetics.

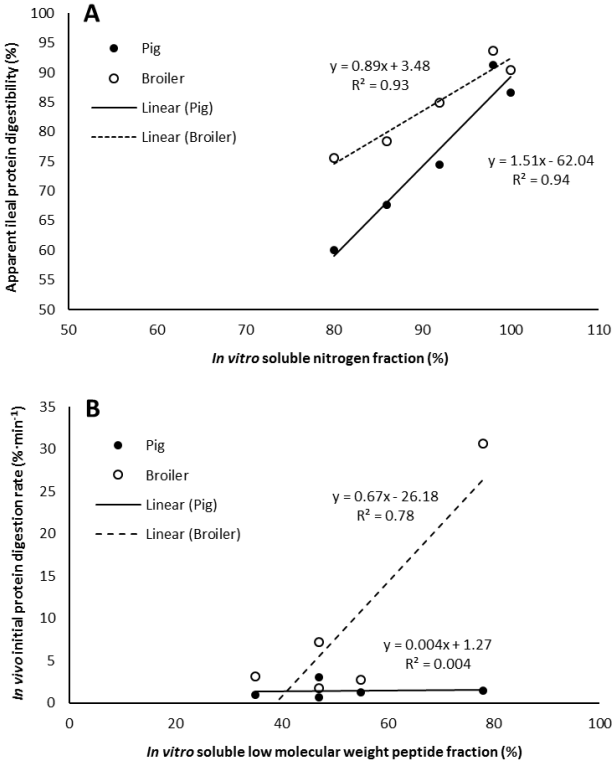

Figure 6.4. Relationship between soluble nitrogen fraction in protein sources after *in vitro* hydrolysis with pepsin and pancreatin (%) and *in vivo* apparent protein digestibility (%) of protein sources in pigs and poultry (A) and between soluble low molecular weight peptide fraction in protein sources after *in vitro* hydrolysis with pepsin and pancreatin (%) and *in vivo* initial protein digestion rate (% · min^{-1}) of protein sources in pigs and poultry (B).

Synchronization of the supply of nutrients towards post-absorptive metabolism

An efficient utilisation of dietary AAs for muscle protein synthesis is economically important in meat-producing animals particularly with forthcoming global protein scarcity. Amino acids are used by organs and tissues, either or not after transformation into other AAs, to synthesise proteins or as an energy source after deamination. The efficiency of protein utilisation depends on the balance between these two processes, in which a higher efficiency relates to a higher body protein deposition. The simultaneous availability of energy and AAs in tissues is prerequisite for protein synthesis (Geiger, 1950). Indeed, the simultaneous supply of energy and AAs, using the concept of nutrient synchronisation, increased nitrogen retention in growing pigs and broilers (van den Borne et al., 2007; Liu et al., 2013). In Chapter 5, synchronising the digestion kinetics of dietary starch and protein using both fast digestible sources (i.e. rice starch and soy protein isolate) or both slowly digestible source (i.e. pea starch and SBM) did not improve the performance nor the breast muscle yield of broilers. However, considerable differences in voluntary feed intake between experimental treatments, related to dietary starch and protein source, limited the capacity of the study to evaluate the concept of dietary energy and protein synchronization.

In addition to energy, a simultaneous availability of dietary AAs derived from either protein or from supplemented free AAs is also critical for maximizing body protein deposition. Protein sources are usually the most costly ingredients in pig and poultry diets. Commercially available free AAs (e.g. lysine, methionine, threonine, tryptophan, valine, isoleucine, arginine, glycine) allow to lower the crude protein content of pig and poultry diets by 2-3 % while maintaining performance (Ospina-Rojas et al., 2014; Molist et al., 2016). Low crude protein diets are not only of economic interest but also reduce nitrogen emission (Liu et al., 2017). However, a reduction in dietary crude protein content by 6 % reduced growth performance in pigs (He et al., 2016). This was related to the fast absorption of supplemented free AAs in the small intestine of pigs relative to the AAs derived from dietary protein, leading to an asynchrony in the availability of AAs in organs and tissues. Information on protein digestion kinetics as affected by protein sources presented in this thesis can be used to further develop concept of the synchronization of the supply of energy, protein-bound AAs and supplemented AAs to post-absorptive metabolism.

The asynchrony in the supply of glucose and AAs might be more substantial in meal-fed animals than in continuous-fed animals. Meal feeding might induce a more pronounced postprandial increase of plasma glucose and AAs. The fluctuation in plasma concentration of glucose and AAs is rather large over the day. In contrast, continuous feeding allows a steady flow of nutrients into the GIT, leading to smaller postprandial increase in plasma glucose and AA concentrations. Moreover, the plasma glucose and AAs concentrations remain more stable over the day (Figure 6.5). In practice, broilers are fed *ad libitum*. *Ad libitum* feeding, however, is not necessarily equal to continuous feeding, especially in broilers, for which the feeding pattern can be influenced by lighting schedule (Weaver and Siegel, 1968; Savory, 1976).

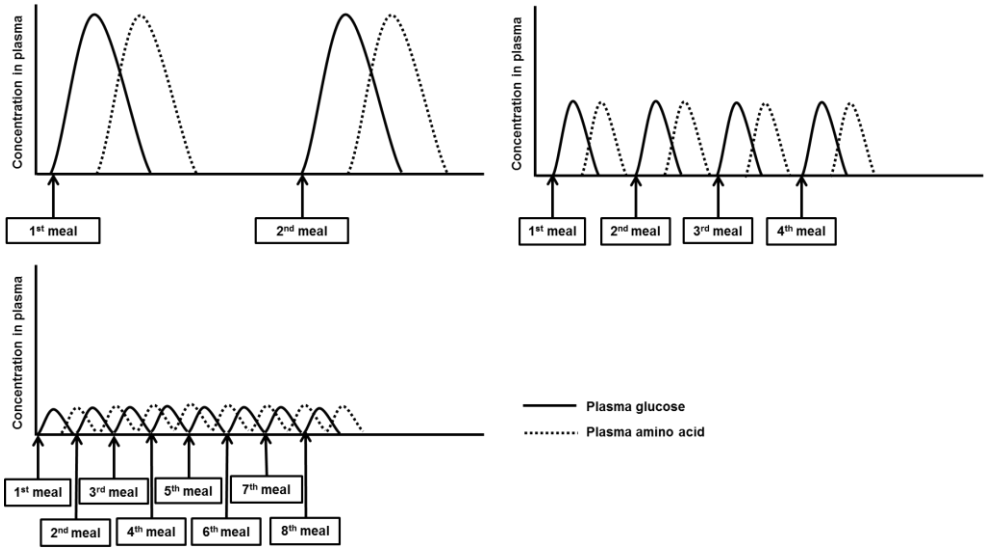

Figure 6.5. Schematic overview of postprandial increase in plasma concentrations of glucose and amino acids when feeding the same daily amount of amount feed in two, four or eight separate meals.

Overall conclusions and recommendations

- The kinetics of protein digestion in the GIT differed substantially among protein sources in both pigs and poultry. Wheat gluten and DPP can be regarded as fast digestible protein sources while SBM, RSM and BSF as more slowly digestible protein sources. The differences in protein digestion kinetics can be attributed to intrinsic characteristics of protein sources and their consequent effects on synthesis, secretion and activity of digestive enzymes in the gut and to effects on digesta retention time. Protein digestion kinetics, as determined in this thesis, is a combined result of the digestion kinetics of all AAs. Future studies could also focus on the digestion kinetics of individual AAs.

- A higher rate and extent of protein digestion were observed in broilers compared to pigs. This might be related to differences in digestive physiology of pigs and broilers. However, despite the differences in intrinsic characteristics of protein sources and digestive physiology of pigs and poultry, the *in vivo* mechanism of hydrolysis of proteins in protein sources seems to be similar between these species.

- Separation of the nitrogen present in feed ingredients after *in vitro* pepsin and pancreatin hydrolyses into an insoluble protein fraction, a soluble high molecular weight (> 500 Da) peptide fraction and a soluble low molecular weight peptide fraction, as presented in this thesis, could potentially be used to predict *in vivo* protein digestion kinetics of pigs and poultry. However, follow-up studies using multiple batches of a large number of protein-containing feed ingredients should be carried out to validate this *in vitro* approach before it can be applied in practice.

- Synchronising the digestion kinetics of dietary starch and protein using either fast or slow digestible sources did not improve the growth performance and the breast muscle yield of *ad libitum* fed broilers kept under an intermittent light regime. Rather than categorising starch and protein sources into fast and slowly digestible sources, synchronising the digestion of dietary starch and protein based on their actual digestion rates might be a better approach to evaluate the concept of dietary energy and protein synchronization.

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List of abbreviations

AA(s)	Amino acid(s)
ADFI	Average daily feed intake
ADG	Average daily gain
AID	Apparent ileal digestibility
Ala	Alanine
AME_n	Nitrogen-corrected apparent metabolisable energy
Arg	Arginine
Asx	Asparagine/Aspartic acid
ATTD	Apparent total tract digestibility
AUC	Area under the curve
BSF	Black soldier fly larvae
BW	Body weight
$BW^{0.75}$	Metabolic body weight
CP	Crude protein
Cr-EDTA	Chromium ethylenediamine tetraacetic acid
CVB	Centraal Veevoeder Bureau
Cys	Cysteine
Da	Dalton
DDGS	Distillers dried grains with solubles
DM	Dry matter
DPP	Dried porcine plasma protein
ERC	Energy conversion ratio
FAO	Food and Agricultural Organization of the United Nations
FCR	Feed conversion ratio
Glx	Glutamine/ Glutamic acid
GIT	Gastrointestinal tract
GLM	General linear model
Gly	Glycine
His	Histidine
HMW	High molecular weight peptides
Ile	Isoleucine
INRA	Institut National de la Recherche Agronomique (France)
IPF	Insoluble protein fraction
ISO	International Organization for Standardization
Leu	Leucine
LMW	Low molecular weight peptides

Lys	Lysine
Met	Methionine
MW	Yellow meal worm larvae
N	Nitrogen
NA	Not available
NE	Net energy
NRC	National research council
NSP	Non-starch polysaccharides
Phe	Phenylalanine
Pro	Proline
PS	Pea starch
RS	Rice starch
RSM	Rapeseed meal
RT	Retention time
SBM	Soybean meal
SD	Standard deviation
SEC	Size exclusion chromatography
SEM	Standard error of the mean
Ser	Serine
SI	Small intestine
SID	Standardized ileal digestibility / Standardized ileal digestible
SPF	Soluble protein fraction
SPI	Soy protein isolate
Thr	Threonine
TiO_2	Titanium dioxide
Trp	Tryptophan
Tyr	Tyrosine
UN	United nations
Val	Valine
VFA(s)	Volatile fatty acid(s)
WG	Wheat gluten
WP	Whey protein

Summary

Increasing the protein efficiency is considered a main strategy for sustainable feeding of pigs and poultry. In practice, protein in pig and poultry diets originates from different ingredients, selected in diet formulation based on their nutritional value and cost. Currently, the nutritional value of protein sources in pig and poultry diets is based on the concentration of essential amino acids (AAs), and their digestibility up to the end of the ileum or the gastrointestinal tract (GIT) (NRC, 2012; CVB, 2016). The ileal and faecal digestibility of protein and AAs, however, only provide information on the quantity of protein and AAs apparently absorbed up to the end of the ileum or over the entire GIT, respectively. They, however, do not provide information on the kinetics of protein digestion, which might affect the post-absorption metabolism of dietary AAs. The aim of this thesis, therefore, was to provide further insights into digestion kinetics of dietary protein sources in the GIT of pigs and poultry, and the consequences of differences in digestion kinetics of dietary protein for the growth performance of broilers.

Protein digestion kinetics in pigs and poultry

In Chapter 2, *in vitro* protein digestion kinetics of various protein sources (soybean meal (SBM), wheat gluten (WG), rapeseed meal (RSM), whey powder (WP), dried porcine plasma protein (DPP), yellow meal worm larvae (MW), and black soldier fly larvae (BSF)) were determined using a two-step method. Protein sources were incubated with pepsin at pH 3.5 for 0-90 min and subsequently with pancreatin at pH 6.8 for 0-210 min at 39 °C. Protein sources showed substantial differences in *in vitro* protein digestion kinetics as measured by the kinetics of N solubilisation and the release of low molecular weight peptides (< 500 Da). The N solubilisation rate ranged from 0.025 min^{-1} for BSF to 0.685 min^{-1} for WP during the incubation with pepsin, and from 0.027 min^{-1} for RSM to 0.343 min^{-1} for WP during the incubation with pancreatin. The rate of release of low molecular weight peptides ranged from 0.027 min^{-1} for WG to 0.093 min^{-1} for WP during the incubation with pepsin, and from 0.029 min^{-1} for SBM to 0.385 min^{-1} for WP. Over all protein sources evaluated, no correlation was found between the rate of N solubilisation and the rate of release of low molecular weight peptides.

Based on the *in vitro* results, SBM, RSM, WG, DPP and BSF were selected for further investigations into *in vivo* protein digestion kinetics in both pigs (Chapter 3) and broiler chickens (Chapter 4). Forty pigs were randomly allocated to one of the five experimental diets containing the respective protein sources as the only source of protein. Four pigs per experimental diet were fitted with an ear-vein catheter and blood samples were

collected before and after a morning meal. At dissection, digesta samples from the stomach and the small intestine, divided into four segments of equal length, were quantitatively collected. Apparent digestibility of crude protein (CP), and retention time (RT) of the solid fraction of digesta along the stomach and the SI were determined to calculate protein digestion kinetics. The initial protein digestion rate ranged from 0.68 % · min^{-1} for the RSM based diet to 3.04 % · min^{-1} for the DPP diet. A higher digestion kinetics of dietary protein resulted in a more rapid and pronounced postprandial appearance of AAs and peptides in systemic blood of pigs.

In the broiler trial, a total of 378 26-day-old male broilers with average body weight of 1430 ± 48 g were randomly allocated to 42 pens. Pens were randomly allocated to one of the seven diets (i.e. a basal diet and six experimental diets with SBM, soy protein isolate (SPI), WG, RSM, DPP or BSF as the main protein source). At dissection, digesta samples from the crop, gizzard, duodenum, proximal jejunum, distal jejunum, and ileum were quantitatively collected. The CP digestion kinetics of the experimental diets were calculated by relating the apparent CP digestibility coefficient at each segment of the small intestine to the sum of digesta retention up to that segment. The initial protein digestion rate ranged from 1.76 % · min^{-1} for the RSM based diet to 30.7 % · min^{-1} for the WG based diet.

Mechanism of protein hydrolysis in the GIT of pigs and poultry

It was hypothesised that proteins present in highly digestible protein sources (i.e. WG and DPP) are more susceptible to hydrolysis by digestive enzymes than slow digestible protein sources (i.e. SBM, RSM and BSF) and that enzymatic hydrolysis of protein progress stepwise in the small intestinal intestine, resulting in hydrolysis products (peptides) becoming smaller in size towards the end of the small intestine. As a consequence, relatively more low and intermediate molecular weight peptides were expected to be present in ileal digesta of pigs and broilers fed highly digestible protein sources, compared to sources with a lower digestibility. The molecular weight distribution of soluble proteins and peptides in digesta from the different segments of the GIT of pigs and broilers was analysed using size exclusion chromatography (Chapter 3 and 4). The molecular weight distribution of proteins and peptides in ileal digesta of pigs and broilers fed highly digestible protein sources was comparable to those of pigs and broilers fed low digestible protein sources. In addition, the molecular weight distributions were rather similar throughout segments of the GIT. These results indicate

that proteins from both highly and low digestible sources follow a "one-by-one" type of hydrolysis mechanism, meaning intact proteins are hydrolysed to low molecular weight peptides and free AAs and absorbed by the intestinal mucosa in one sequence. As a result, proteins and peptides with a wide range of molecular weights were not observed in digesta of different segments of the GIT. Approximately 30 % of peptides present in ileal digesta of pigs are < 10 kDa in dependent of protein source, whereas almost no peptides < 10 kDa were found in the ileal digesta of broilers.

Synchronisation the supply of dietary starch and protein

The effects of synchronising the supply of dietary protein and starch using information on their kinetics of digestion on the growth performance and carcass characteristics in broilers was investigated (Chapter 5). Two starch and two protein sources were used: pea starch (PS) and SBM as slowly digestible sources while rice starch (RS) and SPI as fast digestible sources. Broilers fed diets synchronised for digestion rate of starch and protein (i.e. PS-SBM (slow-slow) and RS-SPI (fast-fast)) did not show a higher growth performance and breast meat yield compared to broilers fed the asynchronised diets (i.e. RS-SBM (fast-slow) and PS-SPI (slow-fast)). The evaluation of the effect of synchronising the supply of dietary starch and protein, however, was hindered by feed intake being affected by dietary protein and starch source. Feed intake of birds was higher when fed diets with SBM compared to SPI and when PS was fed instead of RS.

Conclusions

The results of the present thesis indicate that the kinetics of protein digestion in the GIT of pigs and poultry differs substantially among protein sources. Wheat gluten and DPP can be regarded as fast digestible protein sources while SBM, RSM and BSF are more slowly digestible protein sources in both pigs and broilers. Broilers showed on average a 2.7-fold higher small intestinal protein digestion rate than pigs, excluding and with the exception of WG, for which the protein digestion rate was very high in broilers compared to pigs. However, despite differences in intrinsic characteristics (e.g. AA composition, protein conformation, physicochemical properties) of protein sources and in digestive physiology of pigs and poultry, the mechanism of hydrolysis of dietary proteins in the gut seems rather similar. Synchronising the digestion kinetics of dietary starch and protein using both fast digestible sources or both slowly digestible sources did not improve the performance nor the breast muscle yield of *ad libitum* fed broilers kept under an intermittent light regime.

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Hsuan/萱

About the author

Curriculum vitae

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Training and supervision plan

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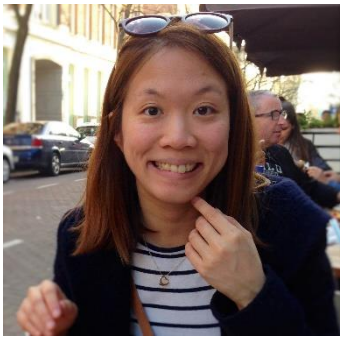

Hsuan Chen was born in Taipei, Taiwan on November 9, 1987. After graduating from Taipei Private Yan-Ping High School in 2006, Hsuan started her Bachelor study in the department of Animal Science and Technology at National Taiwan University. After obtaining her Bachelor degree in 2010, Hsuan continued her Master study in the Animal Science Department of Wageningen University. During her Master study, Hsuan did both her major and minor theses at the Animal Nutritional Group. For the major thesis, Hsuan investigated the effect of processing and enzyme technologies on fibre digestion in broiler chickens. For the minor thesis, Hsuan studied the effect of low dietary phosphorus on the gene expression of phosphorus transporters in the small intestine of pigs. In December 2012, Hsuan started working as a Ph.D. within the IPOP Customised Nutrition project at the Animal Nutritional Group of Wageningen University. During her Ph.D., Hsuan investigated protein digestion kinetics in pigs and poultry. The results of her PhD work are presented in this thesis. In May 2017, Hsuan joined Cagill as a poultry researcher in the Global Innovation Centre, Velddriel, the Netherlands.

List of publication

Peer reviewed scientific publications

Chen, H., P. A. Wierenga, W. H. Hendriks, and A. J. M. Jansman. *In vitro* protein digestion kinetics of protein sources for pigs. Revision submitted to Animal.

Chen, H., P. A. Wierenga, W. H. Hendriks, and A. J. M. Jansman. Protein sources differ in digestion kinetics in the small intestine of growing pigs and affect postprandial appearance of amino acids in blood. Submitted to Animal.

Chen, H., P. A. Wierenga, W. H. Hendriks, and A. J. M. Jansman. Protein digestion kinetics in the small intestine of broilers differs among protein sources. Submitted to Poultry Science.

Chen, H., J. de los Mozos Garcia, P. A. Wierenga, W. H. Hendriks, and A. J. M. Jansman. Effect of synchronising the kinetics of protein and starch digestion in the small intestine on the growth performance and carcass characteristics in broilers. To be submitted.

Conference and symposia proceedings

Chen, H., H. Gruppen, and A. J. M. Jansman. Dietary protein degradation: evaluating protein hydrolysis kinetics in the stomach and small intestine of pigs using a two-step *in vitro* method. Proceedings of the 3rd International Conference on Food Digestion, 11-13 March, 2014, Wageningen, the Netherland

Chen, H., P. A. Wierenga, H. Gruppen, and A. J. M. Jansman. Evaluation of the kinetics of protein hydrolysis in the stomach and small intestine of pigs using a two-step *in vitro* method. Proceedings of the 13th Digestive Physiology of Pigs, 19-21 May, 2015, Kliczków, Poland.

Chen, H., H. Gruppen, and A. J. M. Jansman. Evaluating protein digestion kinetics in the stomach and small intestine of pigs using a two-step *in vitro* method. Proceedings of the WIAS Science Day, 5 February, 2015, Wageningen, the Netherlands

Chen, H., P. A. Wierenga, and A. J. M. Jansman. Protein digestion kinetics of different protein sources in broilers. Proceedings of the 5th International Symposium on Energy and Protein Metabolism and Nutrition, 12-15 September, 2016, Krakow, Poland.

Chen, H., P. A. Wierenga, and A. J. M. Jansman. Protein digestion kinetics of different protein sources in pigs. Proceedings of the 5th International Symposium on Energy and Protein Metabolism and Nutrition, 12-15 September, 2016, Krakow, Poland.

Chen, H., P. A. Wierenga, and A. J. M. Jansman. Protein digestion kinetics of different protein sources in pigs. Proceedings of the 1st Protein for Life Conference, 23-26 October, 2016, Ede, the Netherlands,

Training and supervision plan[1]

The Basic Package (3 ECTS[2])

WIAS Introduction Course	2013
Course on philosophy of science and/or ethics	2013

Scientific Exposure (11 ECTS)

International conferences

3rd International Conference on Food Digestion, Wageningen, the Netherland	2014
13th Digestive Physiology of Pigs, Kliczkow, Poland	2015
5th EAAP International Symposium on Energy and Protein Metabolism and Nutrition, Krakow, Poland	2016
1st Protein for Life Conference, Ede, the Netherlands	2016

Seminars and workshops

WIAS Science Day	2013
Nutrient Requirements and Animal Health	2015
41th Animal Nutrition Research Forum	2016

Presentations

'Dietary protein degradation: evaluating protein hydrolysis kinetics in the stomach and small intestine of pigs using a two-step *in vitro* method', 3rd International Conference on Food Digestion, Wageningen, the Netherland, Poster presentation	2014
'Evaluation of the kinetics of protein hydrolysis in the stomach and small intestine of pigs using a two-step *in vitro* method', 13th Digestive Physiology of Pigs, Kliczkow, Poland, Poster presentation	2015
'Fast or slow: speed of protein digestion makes a difference', Quality of Protein in Animal Diets, Wageningen, the Netherland, Oral presentation	2015
'Protein digestion kinetics of different protein sources in broilers', 5th EAAP International Symposium on Energy and Protein Metabolism and Nutrition, Krakow, Poland, Oral presentation.	2016
'Protein digestion kinetics of different protein sources in pigs', 5th EAAP International Symposium on Energy and Protein Metabolism and Nutrition, Krakow, Poland, Poster presentation.	2016

'Protein digestion kinetics of different protein sources in pigs', 1st Protein for Life Conference, Ede, the Netherlands, Oral presentation.	2016

In-Depth Studies (7 ECTS)

Disciplinary and interdisciplinary courses

Industrial Food Proteins, Graduate School VLAG, Wageningen, the Netherlands	2013
Food Digestion and Human Nutrition, INFOGEST Cost Action, Budapest, Hungary	2014
Quality of Protein in Animal Diets, Wageningen Academy, Wageningen, the Netherlands	2015
Advances in Feed Evaluation Science, Wageningen Academy, Wageningen, the Netherlands	2015
Design of Experiment	2013
Statistics for life sciences	2014

Statutory Courses (3 ECTS)

Use of Laboratory Animals (mandatory when working with animals)	2014

Professional Skills Support Courses (3 ECTS)

Project and Time Management	2013
Course Supervising MSc thesis work	2013
Course Techniques for Scientific Writing	2014

Research Skills Training (6 ECTS)

Preparing own PhD research proposal	2012-2013

Didactic Skills Training (8 ECTS)

Supervising MSc major thesis (4x)	2013-2015

Education and training total	**41 ECTS**

[1]Completed in the fulfilment of the requirements for the education certificate of the Graduate School Wageningen Institute of Animal Sciences (WIAS).

[2]One ECTS equals a study load of 28 hours.

Colophon

The research described in this thesis is part of the IPOP Customized Nutrition project of Wageningen University & Research and was financially supported by Wageningen University & Research, the Dutch Ministry of Economic Affairs, Nutreco NV and Darling Ingredients International.

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