CHARACTERIZATION OF PROTEIN SOURCES AND THEIR EFFECTS ON BROILER PERFORMANCE, DIGESTIVE TRACT MORPHOLOGY AND CAECAL FERMENTATION METABOLITES

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By

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ABSTRACT

The goal of this thesis was to characterize protein sources and evaluate their effects on broiler performance, digestive tract morphology and caecal fermentation. Understanding the digestion kinetics of high protein ingredients is an important step in elucidating the impact protein sources have on poultry performance and health. An *in vitro* assay was used to characterize the digestion kinetics of feed ingredients fed to broilers. The assay predicted the rapidly, slowly and undigested protein fraction of a variety of ingredients, and their digestion rate and extent. Compared to corn distiller dried grain with solubles, soybean meal and fish meal crude protein (CP) was digested more rapidly and to a larger extent. The rate and extent of protein digestion in feed ingredients were also evaluated using broiler chickens, and further the ileal digesta CP was characterized. Protein sources varied in rate and extent of CP and amino acid (AA) digestibility, distal ileum digesta residual AA, and total and soluble CP content. Fish meal, corn gluten meal and soybean meal had similar CP and AA extent of digestion *in vivo*. Among the protein sources, fish meal had the highest digestion rate for most AA. The total and soluble CP in the distal ileal content of birds fed protein sources ranged from 54 to 1466 and 6 to 347 mg, respectively.

When broilers were vaccinated for coccidiosis and fed antibiotic free diets, dietary protein level (PL) and the ratio of indigestible protein (IDP) fraction affected broiler performance and meat yield. The disease status of the birds and the level of digestible AA in the diets influenced the bird's response to dietary IDP. The level of caecal metabolites depended on dietary protein source, disease status of the birds and PL. In conclusion, data generated on *in vitro* and *in vivo* protein digestion kinetics provide an opportunity to categorize feed ingredients and use this information to establish the impact of these characteristics on broiler nutrition. Dietary PL and IDP affected broiler performance and meat yield when birds were vaccinated for coccidiosis and this was related to the characteristics of the protein sources present in the diets.

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DEDICATION

I would like to dedicate this thesis to my wife. It is through her sacrifice, unconditional support, dedication to our family, and her encouragement over the years which motivated me to complete this thesis.

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LIST OF ABBREVIATIONS

AA	Amino Acid (s)
Acet	Acetic Acid
adr	Absolute Digestion Rate
Ag	Agmatine
AIPD	Apparent Ileal Protein Digestibility
ALA	Alanine
AME	Apparent Metabolizable Energy
AMEn	Apparent Metabolizable Energy With Nitrogen Correction
AMP	Adenosine Monophosphate
AOAC	Association of Official Analytical Chemists
ARG	Arginine
ASP	Aspartic Acid
Avg	Average
BAEE	Nα-Benzoyl-L-Arginine Ethyl Ester
BM	Blood Meal
BTEE	N-Benzoyl-L-Tyrosine Ethyl Ester
But	Butyric Acid
BW	Body Weight
BWG	Body Weight Gain
CA	Casein
Cap	Caproic Acid
Cd	Cadaverine
CDDGS	Corn Distillers Dried Grains With Solubles
cfu	Colony Forming Units
CGM	Corn Gluten Meal
CM	Canola Meal
cm	Centimeter
CP	Crude Protein
CV	Coefficient Of Variation
CYS	Cysteine
D	Dark
d	Day
Da	Daltons
DI	Distal Ileum
DIG	Digestion
DM	Dry Matter
DNA	Deoxyribonucleic Acid
FCR	Feed Conversion Ratio
FEM	Feather Meal

FG	Feed to Gain
FI	Feed Intake
FM	Fish Meal
g	Gram
GC	Gas Chromatography
GE	Gross Energy
GIT	Gastrointestinal Tract
GLU	Glutamic Acid
GLY	Glycine
h	Hour
H_2O	Water
HCl	Hydrochloric Acid
Hi	Histamine
HIP	High Indigestible Protein
HIS	Histidine
IDP	Indigestible Protein
ILE	Isoleucine
Isob	Isobutyric Acid
Isov	Isovalaric Acid
kd	Fractional Digestion Rate
kDa	Kilo-Daltons
kg	Kilograms
KOH	Sodium Hydroxide
L	Liter
LC	Liquid Chromatography
LEU	Leucine
LIP	Low Indigestible Protein
LYS	Lysine
m^2	Meter Square
MBM	Meat And Bone Meal
MET	Methionine
min	Minutes
mL	Milliliter
mm	Millimeter
mmol	Millimolar
mRNA	Messenger Ribonucleic Acid
MRT	Mean Retention Time
MS	Mass spectrometry
N	Normal
N_2	Nitrogen
NA	Not Analyzed

NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
ND	Non Detected
OD	Optical Density
OTUs	Operational Taxonomic Units
PCM	Porcine Meal
PD	Potential Digestibility
PDI	Protein Dispersibility Index
PER	Protein Efficiency Ratio
PHE	Phenylalanine
PI	Proximal Ileum
PL	Protein Level
PRO	Proline
Prop	Propionic Acid
Pu	Putrescine
rpm	Revolutions Per Minute
RNA	Ribonucleic Acid
s	Seconds
SBM	Soybean Meal
SCFA	Short Chain Fatty Acid/s
SD	Standard Deviation of the Mean
Sd	Spermidine
Se	Serotonin
SEM	Standard Error of the Mean
SER	Serine
SID	Standardized Ileal Digestible
Sm	Spermine
THR	Threonine
TiO_2	Titanium Oxide
Tr	Tryptamine
Ty	Tyramine
TYR	Tyrosine
UN	Undigested
V	Volume
Va	Valeric Acid
VAL	Valine
wt.	Weight
x *g*	Centrifugal force
µL	Micro Liter
µmol	Micro Mole

1.0 INTRODUCTION

Over the years broiler chickens have been selected for rapid growth which makes them very efficient at depositing body protein in a short period of time. This is important since the broiler sector is expected to contribute to the growing global demand for poultry meat. In light of this, the quality of proteins fed to poultry is becoming more important. Animal and plant ingredients are the main sources of protein used in poultry diets and they vary in digestibility and amino acid composition (Parsons et al., 1997; Adedokun et al., 2008; Kim et al., 2012).

The concept of protein nutrition is based on the sequential process through which proteins are digested, and the amino acids are absorbed and become available for metabolic processes. The nutritional quality of protein ingredients for poultry is based on their amino acid bioavailability. Animal proteins are composed of twenty-two amino acids (Bhagavan and Bhagavan, 1992). Ten of the twenty-two amino acids in poultry meat proteins cannot be synthesized in large enough quantity and, therefore, must be provided in the diet for proper growth and metabolic function (Ravindran and Bryden, 1999).

Digestibility is used in the practice as an estimator of the amino acid bioavailability in poultry diets (Lemme et al., 2004). Digestible protein is the proportion of protein which is digested and absorbed in the form of amino acids (Lemme et al., 2004). On the other hand, amino acid bioavailability is the proportion of an amino acid in a form that is suitable for protein synthesis after the protein has been digested and amino acids absorbed (Batterham, 1992). Since the 1990s, most poultry nutrition research used digestibility assays when evaluating protein feed ingredients instead of bioavailability (Ravindran and Bryden, 1999), because they do not require the free form of the amino acid during the evaluation (Batterham, 1992). The digestibility coefficient obtained can be used directly by nutritionist during ration formulation (Ravindran and Bryden, 1999).

Although *in vivo* digestibility assays for poultry are available, they are expensive and time consuming to conduct. *In vivo* digestibility assays are the optimum tool for characterizing protein sources to be used in commercial production, but it is not practical to conduct these assays in commercial setting. Commercial production therefore, relies on the use of other assays such as i*n vitro* assays to evaluate the quality of protein sources. The pros and cons of *in vitro* and *in vivo* assays are covered in the subsequent review. It was clear that there is a need for a poultry specific

in vitro protein digestibility assay for assessing protein sources commonly fed to poultry. The first objective of this research was to develop a poultry specific *in vitro* digestibility assay which could be used to characterise protein source base on their digestion kinetics.

Earlier research suggested that the rate at which amino acids were hydrolysed and released from dietary proteins was a key regulator of protein synthesis and deposition in tissue (Sklan and Hurwitz, 1980; Boirie et al., 1997). Understanding how this mechanism occurs in broiler chickens could provide tools and techniques which could be used to further maximize muscle protein deposition in broilers and increase overall meat production from available protein ingredients. The main obstacle in accomplishing this is related to the fact that, while the rate of starch digestion for poultry have been quantified *in vitro* and *in vivo*, no progress has been made for protein. The second objective of this research was to evaluate the *in vivo* rate and extent of digestion for some proteins sources using broiler chickens.

Data from the literature suggests that there is a link between protein nutrition and the modulation of pathogenic microorganisms such as *C. perfringens* in the poultry intestine (Drew et al 2004). Interest in this area is gaining more attention because of the current legislative changes on the use of sub-therapeutic antibiotics in some leading poultry producing countries. The limited supply of soybean meal and the growing demand for poultry products worldwide have seen an increase in the use of non-traditional protein sources in poultry diets in some countries. Limited supply of soybean meal and restrictions on the use of some antibiotics in poultry diets has increased concern about the interaction of protein source and pathogenic intestinal microorganism in poultry when fed traditional and non-traditional protein sources. Despite the existence of a relationship between dietary protein source and pathogenic intestinal microorganism in poultry, except for fish meal, there is limited research which explains this relationship for common protein source available to the poultry industry. Even for fish meal the mechanisms behind the response is still elusive. The third objective of this research was to evaluate the effects of individual proteins sources on the characteristics of the protein found at the distal ileum of broilers.

Dietary protein level has been known to influence broiler chicken performance (Temim et al., 2000) and it is often theorised that the undigested protein at the distal ileum can alter poultry performance (Qaisrani et al., 2015; Apajalahti and Vienola, 2016). The effects of undigested protein on broiler performance is yet to be tested using practical formulated diets in which birds were reared under commercial management practices. The fourth objective of this study was to

evaluate the effect of dietary protein levels and indigestible protein fractions on broilers chicken performance, digestive tract morphology and caecal fermentation metabolites.

This thesis had three main approaches to accomplish the objectives. The first approach was to develop and validate an *in vitro* assay which could be used to characterize protein sources based on their extent and rate of digestion (Chapter 3 and 4). The second approach was to modify an existing *in vivo* assay so that it could provide both extent and rate of digestion data (Chapter 5). The third approach was to use the *in vitro* data to formulate practical diets to test an hypothesis related to undigested protein and broiler chicken performance, and to elucidate mechanisms (Chapters 6 and 7).

2.0 LITERATURE REVIEW

The main focus of this review relates to the importance of protein nutrition in poultry production. Some important areas covered in this review include factors affecting protein quality for poultry diets, methods of assessing protein quality, protein digestion kinetics in poultry, the interaction of protein digestion kinetics with host microbiome and the potential impact of protein on poultry health.

2.1 Factors affecting protein quality in poultry diets

The quality of protein sources used in poultry diets maybe influenced by a number of factors. Some factors are inherent from the protein source, while others are due to the manufacturing processes associated with the production of that meal. Anti-nutritional factors are known to adversely affect the digestibility of protein in animal diets (Bones and Rossiter, 1996).

2.1.1 Anti nutritive agents

Anti-nutritional compounds are often secondary metabolites and structural components of plants that interfere with metabolic activities of animals when present in feed ingredients (Bones and Rossiter, 1996). These compounds provide structural support and some metabolites have evolved into defense chemicals to protect plants from insect damage (Chen and Andreasson, 2001). Some anti-nutritional compounds represent important storage minerals and intermediate molecules used in various pathways by the plant (Bones and Rossiter, 1996).

2.1.1.1 Glucosinolates

Glucosinolates are the plant's secondary defense metabolites used to ward off animals and microorganisms (Chen and Andreasson, 2001). All plants in the *Brassica* family contain some level of glucosinolates. The glucosinolates are converted by myrosinase hydrolysis to thiohydroximate-O-sulphonate during plant tissue damage (Bones and Rossiter, 1996). Thiohydroximate-O-sulphonate is then converted to thiocyanates, nitriles and isothiocyanates because it is very unstable (Bones and Rossiter, 1996; Chen and Andreasson, 2001). Rapeseed meal with high levels of glucosinolates is known to increase the incidence of haemorrhagic liver in broilers, resulting in reduced feed intake and weight gain (Campbell and Smith, 1979). Myrosinase is the key enzyme

involved in the conversion of glucosinolates to its toxic products and can be inactivated by heating during meal processing (Newkirk, 2002).

2.1.1.2 Sinapine and tannins

A phenolic compound found in many plant feed ingredient is sinapine, it is a choline ester derived from 3, 5-dimethoxy-4-hydroxyinnamic acid or tannins (Shahidi and Naczk, 1992). Growing plants use sinapine as their main source of sinapic acid and choline (Campbell and Smith, 1979). High levels of sinapic acid can react with other compounds to create a colour change and produce a bitter taste in plant feed ingredients (Kozlowska et al., 1990). During oxidation, phenolic acids may react with proteins to form indigestible complexes like quinines which binds to the functional group of lysine and methionine (Shahidi and Naczk, 1992).

Tannins are another set of water soluble polyphenolic compounds which may be found in protein meals of plant origin (Mangan, 1988). They are normally present in legume seeds, cereal grains and oil seeds (Shahidi and Naczk, 1992; Sarwar Gilani et al., 2012). Tannins are generally grouped into hydrolysable and condensed tannins. Hydrolysable tannins may have esters of gallic, m-digallic or hexahydroxydiphenic acids, which are easily hydrolyzed (Mangan, 1988). Condensed tannins resist hydrolysis and are polymers of flavan-2, 4-diol and flavan-3-ol or a mixture of both (Sarwar Gilani et al., 2012). Tannins precipitate protein out of solution through the formation of soluble and insoluble complexes (Shahidi and Naczk, 1992), and are known to reduce the digestibility of amino acids in poultry (Elkin et al., 1996). Tannins inhibit the absorption of protein from the digestive tract (Elkin et al., 1996; Sarwar Gilani et al., 2012). Low molecular weight tannins may be absorbed from the intestine and cause toxicity through the inhibition of key metabolic pathways (Elkin et al., 1996; Sarwar Gilani et al., 2012).

2.1.1.3 Protease inhibitors

Almost all plant protein sources available for use in animal production contain some type of protease inhibitor (Francis et al., 2001). Even commonly consumed foods such as legumes, cereal grains, and tomatoes contain protease inhibitors (Sarwar Gilani et al., 2012). Protease inhibitors block the activity of trypsin, chymotrypsin (Becker and Yu, 2013), elastase, and carboxypeptidase (Friedman and Brandon, 2001). Trypsin inhibitor can be found in field pea, peanut, wheat, soybean, rapeseed, lupin and sunflower seeds (Friedman and Brandon, 2001; Becker and Yu, 2013).

Of the plant protein sources used in poultry production, soybean is generally considered to have the highest trypsin inhibitor activity (Sarwar Gilani et al., 2012). The inhibitors bind to the active site of the enzyme hereby reducing their ability to lower the kinetic energy needed during proteolytic cleavage (Bhagavan and Bhagavan, 1992). The two main inhibitors found in soy bean are from the Kunitz and Bowman-Birk inhibitor families (Becker and Yu, 2013). Kunitz is about 21.4 kDa with high affinity for trypsin, while Bowman-Birk is about 8 kDa and has high affinity for both trypsin and chymotrypsin (Sarwar Gilani et al., 2012).

When birds were fed diets containing raw soybean, the granules of the pancreatic acini were totally depleted in 2 h after feeding and the size of the pancreas increased after 8 d (Applegarth et al., 1964). The pancreatic activity of the birds at 16 d was twice the activity before they were given the diet and the bird growth was reduced drastically. Protease inhibitor activities can be reduced through various heat processes, but complete elimination is often not possible in commercial soybean products (Parsons et al., 1991; Francis et al., 2001).

2.1.1.4 Phytate

Feed ingredients derived from plants contain some level of phosphorus stored as phytic acid or phytate which are also known as myo-inositol hexaphosphoric acid and myo-inositol hexaphosphate respectively (Nelson et al., 1968). Phytate is predominantly found in the seeds of plants, which makes animal feed derived from oil seeds and cereal grains a source of phytate (O'Dell et al., 1972). During germination, the inorganic phytate is hydrolyzed by enzymes to produce phosphate which the plant use for its growth (Urbano et al., 2000). Phytic acid has strong mineral binding capacity through its six phosphate groups, which actively bind zinc, iron, calcium, and magnesium (Urbano et al., 2000). Phytate's chelating ability results in complexes with nutrients such as proteins and minerals (Selle et al., 2000).

The anti-nutritional effects of phytic acid on protein digestion can occur via direct or indirect modes of action. During protein digestion, phytate may bind to metal cofactors needed for the activity of aminopeptidases and carboxypeptidases (Bhagavan and Bhagavan, 1992; Sarwar Gilani et al., 2012). Phytate may also bind with protein to form complexes in acidic and neutral pH conditions (Selle et al., 2000), which may inhibit the activities of digestive enzymes (Li et al., 1993). Intestinal phytase activity observed in poultry (Maenz and Classen, 1998) may depend on magnesium as a cofactor. In such a case intestinal phytase may not be able to hydrolyze a substantial amount of the dietary phytate if sufficient magnesium is not present.

Chickens cannot use all the phosphorous bound by phytate because inherent endogenous phytase is limiting (Cowieson et al., 2004). High excretion of phosphorous from poultry production has led to environmental concern (Nelson et al., 1968; Selle et al., 2000). In response to those concerns the poultry industry has incorporated exogenous phytase in poultry diets (Adeola and Cowieson, 2011). The exogenous phytase hydrolyzes the ester bond between the inositol ring and phosphate group, thereby releasing phosphorus.

Some research has shown improvements in protein and amino acid digestibility in poultry with exogenous dietary phytase supplementation (Ravindran et al., 1999; Selle et al., 2000). In a digestibility assay testing nine plant feed ingredients Ravindran et al. (1999) supplemented 1200 FTU/kg microbial phytase in diets. There were significant improvement in the protein and amino acids digestibility of the nine feed ingredients. Similar effects were also observed for amino acid digestibility when lower levels of phytase (1000 FTU/kg) were included in broiler chicken diets (Amerah et al., 2014). The use of a phytase from *Aspergillus oryzae* at 1000 U/kg in corn-soybean diets low in phosphorus resulted in a significant increase in ileal amino acid digestibility. The amino acids showing the largest increase in digestibility as a result of phytase supplementation were threonine, tyrosine, and histidine (Rutherfurd et al., 2012).

The specific response in digestibility of each amino acids to phytase will vary from ingredient to ingredient (Ravindran et al., 1999). The level of improvement gained in protein digestion with phytase supplementation in poultry diets varies and is still a controversial subject in the literature (Selle and Ravindran, 2007). The variability is likely due to differences in experimental parameters such as diet ingredient types, bird age, dietary Ca levels and the kind of phytase and digestibility marker used (Selle and Ravindran, 2007; Amerah et al., 2014).

The improvements seen in protein digestion as a result of phytase supplementation are thought to have occurred via two mechanisms (Ravindran et al., 1999; Cowieson et al., 2004; Selle and Ravindran, 2007). For the first mechanism, it is believed that phytase reduces the phytate-protein complex found in the feedstuff, which subsequently releases protein and soluble phytate during digestion. The lower level of phytate-protein complex also reduces phytate inhibition effects on protease enzymes (Selle et al., 2000). The second mechanism suggests that phytase reduce the amino acid endogenous loss associated with phytate. The reduced endogenous loss alleviates the depression in apparent ileal digestibility of amino acids (Ravindran et al., 1999; Cowieson et al., 2004; Liu and Ru, 2010).

2.1.2 Effects of processing

Proteins used in animal production are often by-products of other processing industries. The nutritional quality of these proteins is a function of the processes used in meal production. Plant based protein sources generally will contain some form of anti-nutrient, and thus require processing to reduce their effects when fed to animals. Proteins meals of animal origin are waste products from food processing facilities. As such the raw materials may contain higher levels of microbial contamination and require additional processing before it is fed to animals.

The major anti-nutritional compounds found in plant based protein sources can be reduced through some form of heat treatment. Unfortunately, amino acid digestibility in chickens may be compromised if the heat treatment used is excessive (Araba and Dale, 1990) or not enough (de Coca-Sinova et al., 2008). Autoclaving flaxseed at 120°C for 20, 40 and 60 min resulted in changes in the α-helix to β-sheet ratio of the protein fraction (Doiron et al., 2009). Rumen degradable protein is reduced with increased autoclaving time which suggested that the protein resisted digestion as a result of the change in α-helix to β-sheet ratio. This would be true if that same protein was fed to non-ruminants and the effects would be more severe.

During the commercial production of canola meal using the prepress-solvent extraction system, the meal is subjected to toasting during hexane removal (Newkirk, 2002). Amino acid digestibility and content of the meal are reduced after toasting. Newkirk (2002) suggested that elimination of the spurge steam during toasting could alleviate the loss of amino acids. Soybean meal production involves solvent extraction as well. Ideally the soybean is exposed to 105°C for half hour (Ljøkjel et al., 2000), but if the meal is heated to 121°C, the concentration and digestibility of amino acids, especially lysine, are reduced (Parsons et al., 1992). The loss of amino acids during the production of meals from the solvent extraction process may result in poor growth in chickens fed meals processed under such conditions (Lee and Garlich, 1992; Newkirk and Classen, 2002)

Amino acid loss during heating processing of protein meal may involve Maillard reactions, were a sugar-amine complex is formed from the reaction of sugars and ketones with amino acids, proteins and peptides in food (Mauron, 1981). Mauron (1981) suggested that Maillard reactions involve early, advanced and final stage reactions. Early Maillard reaction involves a reversible condensation of the carbonyl group of the sugar with the amino-group of the amino acid, peptide or protein to form a hydrolysable N-substituted glycosylamine and then 1-amino-1-deoxy-2-ketose. At the early stage, food does not have any browning or flavour, but its nutritive value is reduced.

During the advanced stage of the reaction, amines are released and are used as catalysts in reactions to form intermediate flavour products such as acetaldehyde and pyruvaldehyde (Hodge, 1953). The final reaction produces a dark brown nitrogen-containing pigment composed of decomposed amino acids, heterocyclic amines, melanoidin polymers and aldol condensation products (Mauron, 1981).

The stages of the Maillard reaction requires specific reaction conditions to be successful (Mauron, 1981). Temperature and moisture are the two important parameters which govern each stage of the Maillard reaction (Shallenberger, 1975; Mauron, 1981; Labuza and Ragnarsson, 1985). Experimental simulations of Maillard reaction generally takes place in solutions and the formation of melanoidin polymers is an exponential function of heating (Shallenberger, 1975). Reactions of D-xylose and glycine in aqueous solution at 22, 68 and 100°C produces a temperature dependent increase in aromaticity or high molecular weight melanoidin polymers (Benzing-Purdie et al., 1985). The rate of the Maillard reaction is defined as the function Q_{10} which is the increase in rate for every 10°C (Shallenberger, 1975). As the temperature increases from 22 to 100°C the quantity of high molecular weight melanoidin increase and the low soluble intermediate products of the Maillard reaction decrease (Benzing-Purdie et al., 1985).

Protein meals of animal origin do not contain the high levels of sugars found in meals of plant origin, so are less likely to undergo Maillard reaction when exposed to heat treatment. The natural soluble carbohydrate concentration of dried animal protein meals range from 0.3 to 1.3% (Schroeder et al., 1961), which is far less than what would normally be present in plant based meals (Lee and Garlich, 1992; Newkirk, 2002). The meals are prone to Millard reaction if they are exposed to soluble carbohydrate during autoclaving which has been shown to reduce meal digestibility (Schroeder et al., 1961).

Large amounts of meat and bone meal are produced by the rendering industry, but the quality of those meals can vary (Johnson and Coon, 1979). The variability in quality of meat and bone meal can limit its use in poultry production (Parsons et al., 1997). Oxidation and enzymatic denaturing may occur depending on location and source of the raw material used in the rendering process. Polyunsaturated fats are known to react with atmospheric oxygen which results in the production of peroxides and other auto-oxidation products (Labuza and Ragnarsson, 1985). If the meal is kept in warm conditions, this could increase the formation of peroxides and secondary oxidation products. The application of heat in the presence of oxygen and polyunsaturated fats is known to increase the production of peroxides and secondary oxidation products (Labuza and

Ragnarsson, 1985). This could be a factor during rendering if parameters such a temperature, time, and raw material polyunsaturated fat content are not controlled during meal production.

Peroxides and secondary auto-oxidation products are known to react with proteins and amino acids to form amino sulfone and indigestible polymers like lysinoalanine or to completely oxidize methionine and cysteine (Chang et al., 1985; Kanazawa et al., 1987; Piva et al., 2001). The bonding of secondary oxidation products to protein produces insoluble complexes whose concentration increases with the time that the secondary oxidation products interact with the protein (Kanazawa et al., 1987). Increases in amino sulfone and secondary oxidation products-protein complex reduce the *in vitro* and *in vivo* digestion rate of meat and bone meal samples (Chang et al., 1985; Kanazawa et al., 1987). These compounds could be playing a role in the variability in digestibility of meat and bone meal samples seen in poultry studies (Parsons et al., 1997). If an alkali treatment is used during the meal preparation, this could also reduce the digestibility of the samples via the production of lysinoalanine (Piva et al., 2001).

2.2 Methods for evaluating protein quality

Traditionally, protein quality is assessed by evaluating the extent to which amino acids are digested and absorbed from the ingredient. Estimation of protein digestibility is normally achieved by feeding the feed ingredient to the intended animal and assessing protein or amino acid digestibility. This technique is termed *in vivo*. Protein quality can also be evaluated using less expensive and time consuming *in vitro* chemical methods. These techniques are used to improve the level of precision while mimicking the physiological and chemical characteristics of the digestive system of the animal to which the ingredient will be feed.

2.2.1 In vivo methods

The most popular *in vivo* techniques used in assessing protein digestibility in poultry are (1) apparent amino acid digestibility and (2) standardized ileal amino acids digestibility. Most researchers agree that protein nutrition in poultry diets should be based on providing digestible amino acids (Lemme et al., 2004). The concept of true amino acid digestibility in poultry was established by Sibbald, (1979). The author demonstrated a linear relationship, not influenced by energy intake, between amino acid intake in feed and amino acid output in excreta of roosters.

The true amino acid digestibility procedure involved starving adult roosters for 24 h and then precision feeding with the ingredient being evaluated. Excreta samples were collected over a

24 h period from precision-fed and starved bird, and then analyzed for amino acids (Sibbald, 1979; Parsons, 1985). The data obtained by this method was criticized for not taking into consideration the effects of microbial fermentation, which can account for up to 25% of the amino acids in excreta and urine (Parsons et al., 1982). It is also well known that excreta samples from poultry can be contaminated by scales and feathers (Ivy et al., 1968).

If the excreta collection assay proposed by Sibbald (1979) and Parsons (1985) could account for the microbial influence, it would better predict amino acid digestibility (Parsons, 1985). Parsons (1985) investigated the effects of caecectomy on the digestibility of distillers' dried grains with solubles and found a strong relationship between amino acid digestibility and caecectomy in adult roosters. The digestibility coefficients of almost all amino acids were underestimated due to both microbial and endogenous amino acids in the excreta after collection from the intact birds. The true amino acid digestibility assay using caecectomized adult roosters gave a better prediction of amino acid digestibility for oil seeds and processed meals from animals (Ravindran and Bryden, 1999). The assay became a routine assay for assessing amino acid digestibility in poultry (Ravindran and Bryden, 1999).

Caecectomy was unable to address the presence of urinary and endogenous amino acids in excreta samples without starving or feeding a nitrogen free diet. The need for surgery, differences in digestibility due to bird age (Ravindran and Bryden, 1999), and animal welfare issues related to starving and presision-feeding birds led to the criticism of the precision-fed caecectomized rooster assay (Lemme et al., 2004). The cumulative effects of the issue surrounding the precision fed caecectomized assay suggested that there was a need for a new method. Other researchers have avoided surgical modifications, excreta, urine and hind gut microbial effects on amino acid digestibility by sampling digesta at the ileum of birds (Soaees and Kifer, 1971; Payne et al., 1986). With this method, birds of any age can be used to obtain digestibility estimates specific to each growth phase. Although the birds are killed, there are less welfare problems since birds are not force fed or starved. Instead, the birds are given a diet containing the test ingredient as the only source of amino acids or they are given two diets, a basal diet with no test ingredient and a test diet which is a mixture of test ingredient and basal diet (Lemme et al., 2004). After consuming the diet for a week the birds are sacrificed and the ileal content collected and used for analysis.

Collecting digesta from the ileum or from excreta of caecectomized birds assumes that the endogenous contributions of amino acids are insignificant. Digestibility data obtained with this

assumption are considered to be apparent because endogenous amino acid contributions are not independent of amino acid intake (McNab, 1995). Endogenous loss of amino acids can be classified as non-specific (basal loss) and specific in poultry (Lemme et al., 2004). Birds may consume ingredients with specific characteristics, which stimulate excessive release of endogenous secretions. This loss of amino acids in endogenous secretions should be applied to the ingredient amino acid digestibility coefficient (Ravindran and Bryden, 1999). This specific loss of amino acids from the animal would not have accrued normally, so it is a function of that ingredient. Basal loss of amino acids from the animal is independent of ingredient and diet composition (Lemme et al., 2004).

Ravindran and Bryden (1999) suggested that if the apparent ileal digestible amino acids values are not standardized, values would underestimate the digestible amino acids of the test ingredient. Additional amino acids from secretions and other endogenous sources found in the proximal ileum interfere with digestibility coefficient calculation (Lemme et al., 2004). Using a nitrogen free or a highly digestible protein source to assess endogenous amino acid flow at the ileum has been studied in humans (Moughan et al., 2005). Adedokun et al. (2008) also compared the use of a nitrogen free diet and a highly digestible casein diet to standardized ileal apparent values in broilers. The ileal amino acid digestibility of 21 d old broilers standardized with the highly digestible casein diet gave values which were higher than those standardized with the nitrogen free diet. Higher endogenous amino acid flow was also reported for the highly digestible protein source in humans (Moughan et al., 2005). Golian et al. (2008) compared three methods of standardizing apparent ileal values using the nitrogen free, highly digestible casein and the regression approach. There were no differences between the values of the regression and nitrogen free diets but there were differences between the nitrogen free and the highly digestible diets coefficients.

It is difficult to separate specific loss from basal loss when measuring endogenous loss so most techniques (protein-free diet, regression method and feeding hydrolyzed casein) correct for only basal loss when standardizing apparent values (Ravindran and Bryden, 1999; Lemme et al., 2004). The standardized ileal digestibility method described by Lemme et al. (2004) has become the preferred method for predicting amino acid digestibility in poultry nutrition research (Adedokun et al., 2008). The debate on which method is most appropriate for standardizing apparent amino acid values continues (Golian et al., 2008).

2.2.2 In vitro methods

To obtain useful information on the digestibility of nutrients without the use of *in vivo* assays, researchers often employ the use of *in vitro* assays. In theory, *in vitro* digestibility assays should closely simulate the digestive process of the intended animal (Boisen and Eggum, 1991). Depending on the nature of the research, it is expected that an intended *in vitro* assay should be reproducible, cheaper than available *in vivo* assays and simple to perform while giving fast results (Clunies and Leeson, 1984). Methods for evaluating nutrient digestibility *in vitro* for simple stomach animals has been reviewed by others (Boisen and Eggum, 1991; Fuller, 1991). Only those methods applicable to protein digestion will be discussed.

2.2.2.1 Chemical methods

Evaluating protein quality using chemical method provides less precision than *in vivo* techniques but can be used as a routine quality control measure. In the chemical engineering literature, it was known as early as the 1930s that an alkali solution could extract up to 95% of the protein from plant meal sources (Smith et al., 1938). In the late 1960s Rinehart was one of the first to employ the protein solubility technique as a measure of protein quality of soybean meal in the poultry industry (Araba and Dale, 1990). While working at Purina Mills Inc., Rinehart evaluated the suitability of protein from soybean meal derived from different processing systems using potassium hydroxide.

The ability to predict animal performance is one of the most important criteria of any chemical assay (Lee and Garlich, 1992). It was not until the 1950s that Lyman et al. (1953) established a relationship between bird performance and the solubility of protein feed ingredients used in poultry diets. The study evaluated the correlation between a chick growth assay and the use of a protein solubility technique using sodium hydroxide as the alkali solution. In the solubility technique, one gram of cottonseed meal with four glass beads was placed in an Erlenmeyer flask with 100 mL of 0.02 N sodium hydroxide solution. The flask was agitated continuously at 37º C for an h and then the mixture centrifuged for 5 min at 3000 rpm. After centrifuging, the solution was filtered and aliquots evaluated for protein concentration (Lyman et al., 1953).

The solubility index method was not adopted as a routine measure of protein quality in the poultry feed industry until the test was validated. Araba and Dale (1990) reported a study in which the protein solubility technique was used to evaluate soybean quality in poultry feed. This study provided the foundation for the evaluation of protein quality using the solubility technique. Lee

and Garlich (1992) revived the technique when they proposed the use of sodium tetraborate at 40ºC as a more sensitive test for detecting changes in protein quality due to over cooking of meals. By the end of the late 1990s, protein solubility using potassium hydroxide became a routine technique in research evaluating dietary protein (Parsons et al., 1991; Lee and Garlich, 1992; Fernandez et al., 1993; Batal et al., 2000). Newkirk (2002) used the protein solubility index to evaluate canola meal quality and found that the 0.5% sodium hydroxide assay did not accurately predict canola meal lysine digestibility in broiler chickens. This suggests that the relationship between protein solubility and amino acid digestibility is ingredient specific.

Protein dispersibility index (PDI) is another method used to evaluate the quality of protein ingredients. This technique involves high speed mixing of a protein sample in water followed by assessment of solubility (Batal et al., 2000). In the literature, PDI may be referred to as water dispersible protein or water-soluble protein (Johnson, 1970). In 1970, the PDI technique was published as two official and tentative methods of the American Oil Chemists Society (Johnson, 1970). Veltmann et al. (1986) evaluated the quality of soybean meal used in poultry diets employing the PDI method. The PDI method was able to distinguish between the normal processed meals and meal heat treated to escape rumen degradation. In that same study, a chick growth assay showed that there was no difference between the bioavailability of the protein from the two meals. This suggested that the PDI method did not correlate well to the bioavailability of protein from the ingredient tested.

In 1978, the American Oil Chemists Society published a revised PDI method which was corrected in 1979 as method Ba 10-65. In brief, 20 g of protein is mixed for 10 min at 7800 rpm with 300 mL of water. A portion of the mixture is centrifuged and the nitrogen content of the solid fraction and the original protein sample measured (Clarke and Wiseman, 2005). The percent dispersed protein is calculated as the protein loss from the original sample to the water. (Batal et al., 2000) compared the revived PDI method against the urease index and potassium hydroxide solubility test. Of the three tests, PDI method was more effective and more sensitive in detecting the minimum adequate heat processing conditions required for soybean meal fed to chickens.

Since the 1980s, PDI method has become a routine technique used worldwide by researchers (Veltmann et al., 1986; Batal et al., 2000; de Coca-Sinova et al., 2008; Pérez-Calvo et al., 2010; Serrano et al., 2012) to assess the quality of protein sources used in monogastric animal feeds. While chemical methods provide an overview of the protein quality of feed ingredients, they

do not give a good indication of how much of the nutrient will be absorbed by the animal. Protein solubility index and PDI methods are used as measures of ingredient quality in most poultry nutritional research evaluating high protein ingredients. The information gained from PDI method and protein solubility index does not provide useful information for diet formulation in a commercial setting, but they are often used in quality control programs.

2.2.2.2 pH-Stat/Drop method

As protein samples are hydrolyzed by digestive enzymes they release protons from the cleaved peptide bonds, which changes the pH of the reaction media (Maga et al., 1973). In the early 1970s, Maga, Lorenz and Onayemi evaluated the extent to which dietary protein undergoes proteolysis. They realized that there was a close relationship with the initial rate of hydrolysis of the proteins from 0 to 10 min and the digestibility of the protein samples. The rates of hydrolysis of the protein samples were evaluated as an indirect measure of the pH of the reaction mixture over time. In their system, the protein samples were incubated with trypsin at 37º C in a water bath for 10 min while evaluating the pH change. However, this method lacked precision in predicting bioavailability of protein (Vavak, 1975; Hsu et al., 1977).

To improve precision in predicting bioavailability with Maga et al. (1973) method, Rhinehart (1975) modified the above procedure in a master's thesis while working with distiller's grain protein concentrate. During the modification of the procedure, various enzyme combinations were tested in an effort to gain improvement in the correlation coefficients between pH drop and protein digestibility in rats. The trypsin-chymotrypsin combination gave superior correlation coefficients compared to the initial single trypsin proposed by Maga et al. (1973). Hsu et al. (1977) suggested that the methods presented by Maga et al. (1973) and Rhinehart (1975) were too time consuming and complicated for routine quality control.

A faster method was developed which could be completed in 1 h (Hsu et al., 1977). In this method the trypsin-chymotrypsin enzyme combination was replaced with a multi-enzyme mixture composed of trypsin, chymotrypsin and peptidase. The correlation coefficient with the apparent digestibility of protein from rats was 90% using this new multi-enzyme system after evaluating 23 food protein sources. The method was also able to detect the effects of trypsin inhibitor, chlorogenic acid and heat processing on the digestibility of the protein tested. The pH drop method was susceptible to the buffering capacity of the protein source, since high ash content affected the digestibility results (Hsu et al., 1977). Parsons, in (Fuller, 1991) used the pH drop method proposed

by Hsu et al. (1977) modified by Satterlee et al. (1982) to evaluate various high protein feed ingredients while correlating the results to true digestibility in caecectomized cockerels. There was good correlation with lysine digestibility in caecectomized cockerels and the pH drop test across the ingredients tested. The test however showed no relationship to lysine digestibility and protein efficiency ratio in various qualities of feather meal and meat meal samples.

To overcome the susceptibility of the pH drop test to the buffering capacity of protein samples, Pederson and Eggum (1983) revised the pH drop method proposed by Hsu et al. (1977). During revision, the consumption of alkali was used as an indirect measure of true protein digestibility values in rats. The pH of the reaction was held constant at 8 during titration with alkali over a 10 min period. The correlation coefficient was improved from 90 Hsu et al. (1977) to 96 with a residual error of 1.29 after evaluating 30 protein samples. Pedersen and Eggum (1983) suggested that the effects of ash content on the test results were due to differences in mineral content, which was mostly due to the influence of calcium. The authors proposed the use of two different regression equations to accurately predict digestibility of protein samples from plant and animal origins. Using a literature derived prediction equation for a specific kind of protein source was unreliable when using the pH stat method (Linder et al., 1997). To measure the degree of hydrolysis, the method requires knowledge of the average dissociation of the α–amino groups of the protein sample and the number of peptide bonds present in the territory structure of the main proteins present the ingredient (Pedersen and Eggum, 1983).

Due to the limitations mentioned, the pH stat test has been used mostly in food science research to predict the digestibility of highly digestible pure protein sources (Pedersen and Eggum, 1983; Linder et al., 1997; Wang et al., 2009). Such pure protein sources typically have data about the average dissociation of the α–amino groups and the number of peptide bonds present. Since the early 1990s, the pH stat method has been used to evaluate only aquatic animal feed ingredients (Dimes and Haard, 1994; Tibbetts et al., 2011). To address the limitations of the method, casein average dissociation constant and number of peptide bonds were used as the standards when calculating degree of hydrolysis (Tibbetts et al., 2011). So far, the data generated with the pH stat method has been consistent with *in vivo* digestibility assays, especially with the use of purified enzymes extracted from the species to which the ingredient has been fed (Dimes and Haard, 1994; Tibbetts et al., 2011). The pH stat method has become a valuable tool for aquatic nutritional research, but not for terrestrial animals. The good digestibility correlations seen with aquatic

species are probably due to the simple nature of their digestive tract and the use of highly digestible protein sources such as fish meal.

2.2.2.3 Closed enzymatic methods

These systems are used to evaluate the digestibility of nutrients with multiple or single enzymes while simulating part or all of the *in vivo* digestive process (Boisen and Eggum, 1991). The system is flexible, so the procedure and enzymes used may vary to meet the specific needs of the research objectives. Only those procedures used specifically to evaluate digestibility of protein samples will be reviewed. The digestibility of protein is tied to the amino acid content and to the specificity of digestive enzyme used to free them from complex peptides (Gauthier et al., 1982).

Pepsin

The pepsin digestibility assay is one of the most widely used assays to evaluate the quality of feed and protein ingredients. Gehrt et al. (1955) and Sheffner et al. (1956) were the first group of researchers to employ a single enzymatic method to evaluate the digestibility of protein using pepsin. In their procedure 1 g of protein was incubated with 25 mg of pepsin in 30 mL of 0.1 N sulfuric acid at 37º C for 24 h, during this time the samples were stirred intermittently (Gehrt et al., 1955). After incubation, the samples were placed in a boiling water bath for 10 min. Samples were cooled and the pH adjusted to 2 followed by the addition of one volume each of 10% sodium tungstate and 2/3 N sulfuric acid. The mixtures were filtered after standing for 10 min, and then the filtrate adjusted to pH 6.8 and analyzed for amino acids. When the digestibility data were regressed against the biological value of the protein samples for rats, there was a 0.998 correlation (Sheffner et al., 1956).

The pepsin digestibility assay was not accepted as a routine protein quality evaluation until 1959. The Association of Official Analytical Chemists (AOAC) adopted a revived version of the method proposed by Gehrt et al. (1955) and Sheffner et al. (1956). Hydrochloric acid was used instead of sulfuric acid and all the fat were extracted from the samples using ether before digestion. The sodium tungstate and pH steps were eliminated. In 1972 the procedure was revised to improve the filtration step and the pepsin concentration was defined as 0.2%.

Since the 1959 AOAC publication of the pepsin digestibility method, it has been used extensively to evaluate high protein feed ingredient quality of both plant and animal origin (Johnson and Coon, 1979; Parsons et al., 1991). Johnston and Coon (1979) were one of the first

group of researchers to use this method to evaluate poultry feed ingredients of animal origin. After evaluating 20 commercial animal by-product meals, they were able to get a 91% correlation with the net protein utilization and the protein efficiency ratio for chickens. The pepsin digestibility procedure proposed by Johnston and Coon (1979), adjusted the pepsin concentration to 0.002% while eliminating the preliminary grinding and defatting steps.

In another study, the same group of researchers evaluated various levels of pepsin in order to find a suitable level for use in the assay during routine evaluation of meat and bone meal samples fed to poultry (Johnson and Coon, 1979). Lower levels of pepsin (0.002%) were able to detect differences between the quality of the meat and bone meal samples which was contrary to that of the AOAC 0.2% pepsin. Parsons et al. (1997) did a comparative study on the ability of 0.2%, 0.002%, and 0.0002% pepsin to detect differences in quality among 14 meat and bone meal samples (Parsons et al., 1997). They confirmed the findings of Johnson and Coon (1979) that the 0.002% pepsin level gave the best correlation with lysine digestibility in chickens.

Pancreatin

Some testing systems involve the use of pancreatin as the only enzyme source to digest protein samples. Riesen et al. (1947) described a single enzymatic method that used pancreatin to evaluate the quality of soybean meal in poultry (Riesen et al., 1947). The samples were ground in a power-driven mortar, 100 mg or 300 mg of pancreatin was added to 2 g of the ground samples in 50 mL of 0.2 M disodium phosphate buffer at pH 8.3. One mL of toluene was added to the solution and the mixture incubated for 5 d or 12 h at 37º C. At the end of each digestion period, the samples were heated with steam for 15 min to facilitate enzyme deactivation. The pH of the mixture was adjusted with glacial acetic acid to precipitate the indigested proteins. This method was able to detect the difference between overheated and the normal heated meals, but not the difference between the normal and under heated soybean meals.

Ingram et al. (1949) modified the procedure by adding 1.2 g of pancreatin to 12 g of sample in 300 mL of buffer for 6 h. The pattern of amino acid released from the samples correlated with the growth of chickens fed the same samples of soybean meal (Ingram et al., 1949). In another study by Anwar (1962) the pancreatin *in vitro* test was used to evaluate the quality of cottonseed meal, groundnut meal, meat meal and fish meal (Anwar, 1962). The method was not reliable for fish meal and groundnut meal, but gave fair results for meat meal. The one step pancreatin method

has been used routinely by many food scientists to evaluate the digestibility of various protein foods, but not by poultry nutritionists (Altangerel et al., 2011).

Pancreatic digestion is controlled by substrate concentration. An increase in protein concentration will promote an increase in proteolytic enzyme secretion (Boisen and Eggum, 1991). In vitro digestibility methods using pancreatin as the only enzyme source keeps the enzyme concentration constant when evaluating a range of protein sources (Anwar, 1962). However, the method lacks precession when evaluating a variety of protein sources (Anwar, 1962). Other researchers have found no difference between *in vivo* chicken ileal digestibility and the pepsin or pancreatin assay when ranking feather meal digestibility (Bielorai et al., 1983).

Closed multi-enzymatic methods

A multi-enzyme method may use two or more enzymes while simulating one, two or all stages of the digestive process (Boisen and Eggum, 1991). Multi-enzyme methods are more comparable to *in vivo* conditions since many enzymes are involved in the digestion of proteins. The digestion of proteins starts in the stomach under the action of pepsin and hydrochloric acid. The partially digested protein enters the small intestine where they are hydrolyzed by trypsin, chymotrypsin, elastase and carboxypeptidases (Boisen and Eggum, 1991).

Akeson and Stahmann (1964) described a method using pepsin and pancreatin as enzyme sources. The method was developed to evaluate large numbers of food protein samples while reducing the labour load associated with the pepsin digestibility assay. The method involved incubating 100 mg of protein sample with 1.5 mg pepsin in 15 mL 0.1 N hydrochloric acid for 3 h at 37º C (Akeson and Stahmann, 1964). The reaction was neutralized with 7.5 mL of 0.2 N sodium hydroxide solution and then 4 mg pancreatin dissolved in 7.5 mL phosphate buffer with pH 8 was added. Fifty parts per million merthiolate was added to the mixture, which was incubated at 37º C for 24 h. Samples of the digestion mixture were precipitated with acid and centrifuged at 1,000 x *g* for 30 min after which the supernatant was analyzed for amino acids.

Saunders et al. (1973) described a two enzyme system using pepsin and trypsin. The test occurred in a closed system using centrifuge tubes containing 1 g of protein sample suspended in 20 mL of 0.1 N hydrochloric acid and then mixed with 50 mg pepsin dissolved in 1 mL 0.01 N hydrochloric acid (Saunders et al., 1973). The mixture was incubated at 37º C while gently shaken for 48 h, centrifuged at 20,000 x *g* for 5 min and the supernatant removed. The solid was suspended in 10 mL water and 10 mL of 0.1 M phosphate buffer with pH 8 and 5 mg of dissolved trypsin.

The mixture was incubated at 23ºC for another 12 h then centrifuged and the solids washed with 30 mL of water five times, with centrifuging and removal of the supernatant each time. The solid was filtered through a 1.2 µm Millipore filter, air dried and analyzed for amino acids.

Both the pepsin-pancreatin and pepsin-trypsin methods were able to give good correlation between the *in vivo* digestibility values for various food proteins using rats (Akeson and Stahmann, 1964; Saunders et al., 1973). The pepsin-pancreatin assay is known to give good amino acid digestibility correlation of 84% in cereal gains with true amino acid availability in chickens, but was less reliable for soybean meal and corn gluten meal (Cave, 1988). However, the pepsin-pancreatin test gave an excellent correlation of 91% between the *in vivo* ileal digestibility of protein of 15 feedstuffs in pigs (Boisen and Fernández, 1995). The test proposed by Saunders et al. (1973) has been used to some extent to evaluate protein digestibility in poultry (Saleh et al., 2003, 2004; Tahir et al., 2008).

Dialysis cell method is a non-static system in which products of digestion are removed from the substrate as they become available. When simulating *in vivo* protein digestion with *in vitro* techniques, the rate of hydrolysis maybe compromised by the accumulation of end product in the system (Robbins, 1978; Cave, 1988). The rate of hydrolysis can be improved if the digestion products are removed from the system as digestion occurs (Cave, 1988). To prevent the inhibition of proteolysis by the end products, dialysis has been proposed to remove digestion products (Mauron et al., 1955; Steinhart and Kirchgessner, 1973). They conducted their experiments in dialysis bags to facilitate the removal of the end products during incubation of the protein source with the enzymes.

Gauthier et al. (1982) adopted the dialysis principle of (Mauron et al., 1955; Steinhart and Kirchgessner, 1973) and presented a method in which the dialysis solution was continually replaced as the incubation proceeded. The content of the dialysis bag was stirred constantly during the digestion process. In brief, 400 mg nitrogen (6.25 x %N) of protein was suspended in a beaker with 100 mL of 0.1 N hydrochloric acid. The beaker was shaken and placed in a water bath at 37° C for 30 min. The pH of the solution was adjusted to 1.9 then 20 mL of solution containing 5 mg pepsin per mL of 0.1N hydrochloric acid added. The mixture was incubated for 30 min, the pH adjusted to 8 and transferred to a dialysis bag with a 1000 Da molecular weight cut off. The bag was placed in a U-shaped container with inlets from a peristaltic pump and outlets to a beaker. Twenty mL of solution containing 5 mg pancreatin per mL sodium phosphate buffer was added to the dialysis

bag, which was continuously washed with 37°C sodium phosphate buffer at a flow rate of 212 mL/h. Samples of dialysate were collected at different time intervals and analyzed. The method was able to detect effects of heat and alkali treatment on protein digestibility in foods.

The digestion unit size plus the use of handmade apparatus were limitations for its use in routine protein evaluation (Savoie and Gauthier, 1986). Savoie and Gauthier (1986) modified the design presented by (Gauthier et al., 1982). The improvements included the use of a magnetic stir bar and the construction of a cell with an inner compartment fitted with a dialysis membrane. The cell was 100 mm long in comparison to the 298 mm original unit. There was free access to the reaction chamber without disruption of the reaction. Each cell was designed to work as a single unit or part of the multi-unit system. The system developed was very flexible and could be used to measure the release of any product from enzymatic hydrolysis.

The dialysis cell method has been applied to study protein digestibility across a number of disciplines (Moyano and Savoie, 2001; Siddhuraju and Becker, 2005; Sáenz de Rodrigáñez et al., 2011). This method was able to identify difference in the rate of release of amino acids from different sea bream feed samples (Sáenz de Rodrigáñez et al., 2011). The system was flexible to accommodate the use of crude enzyme extract from sea bream as the digestive enzyme. A comparison between the pH stat and the dialysis cell method showed that the dialysis cell method was able to identify which products were released from the protein as well as the digestion kinetics of the protein samples (Moyano and Savoie, 2001). The effects of different processing method on the digestibility of legume proteins were identified with dialysis cell method (Siddhuraju and Becker, 2005). A detailed description of the availability of different amino acids and the rate at which they were released during digestion was obtained from different protein sources (Savoie et al., 1988; Siddhuraju and Becker, 2005). The main disadvantages of the dialysis cell method it its complexity and the number of samples which can be digested in a given run. This method uses custom made dialysis cell, peristalsis pumps and fraction collectors which can be expensive. Savoie and Gauthier (1986) recommend that no more than 6 cells should be used simultaneously due to the manual inputs needed. From a practical point of view an *in vitro* method must be simple and easy to implement for it to be adopted by poultry nutritionist.

2.3 Factors influencing *in vitro* digestibility systems

The digestibility data obtained by *in vitro* methods vary even within the same method for the same ingredient. This variation maybe due to a number of issues associated with *in vitro*

digestibility systems. Enzymes and their concentration seemed to be one of the most important factors influencing *in vitro* digestion (Vavak, 1975; Parsons et al., 1997). The specificity of enzymes and its ratio to substrate will determine the level of hydrolysis achieved (Boisen and Eggum, 1991).

2.3.1 Enzyme specificity

Table 2.1 shows a list of enzymes involved in protein digestion. The first enzyme responsible for the initiation of protein digestion in poultry is pepsin (Boisen and Eggum, 1991). This enzyme will only cleave the N-terminal of aromatic amino acids like tyrosine, tryptophan and phenylalanine (Bhagavan and Bhagavan, 1992) at low pH. Hydrolysis by pepsin results in smaller peptides which enter the duodenum for further hydrolysis by pancreatic protease (Boisen and Eggum, 1991). As suggested by Assoumani and Nguyen in (Fuller, 1991), trypsin will only break a lysyl or arginyl peptide bonds to expose lysine or arginine terminal residues at basic pH. Trypsin binds only to the positive side group of arginine and lysine where the peptide is cleaved at those amino acids (Bhagavan and Bhagavan, 1992).

The ability of enzymes to hydrolyze substrate may depend on the presence of other enzymes. The activation of chymotrypsin is dependent on the presence of trypsin (Bhagavan and Bhagavan, 1992). Chymotrypsin will act on proteins and peptides, but will also hydrolyze esters and amides (Appel, 1986). Chymotrypsin cleaves peptides over a wider range of sites than trypsin, both aromatic and hydrophobic side chains of amino acids residues (Bhagavan and Bhagavan, 1992). Peptide bonds involving tyrosine, tryptophan, phenylalanine and glutamyl, leucyl, asparaginyl residues are cleaved by chymotrypsin (Boisen and Eggum, 1991; Bhagavan and Bhagavan, 1992).

Lysine or arginine is released from small peptides by carboxypeptidase-B, which is specific for C-terminal basic groups (Boisen and Eggum, 1991). Animal protein meals may contain high levels of collagen due to the nature of the type of rendering material. Digestion of this meal *in vitro* may need additional collagenase enzyme during the pancreatic digestion stage (Straumfjord and Hummel, 1957). Bonds hydrolyzed in protein feed samples are enzyme specific, so *in vitro* digestion models should take this into account by using multiple enzymes (Boisen and Eggum, 1991).

Table 2.1. Enzyme and their specific bond cleavage

Enzymes	Bond cleave	Reference
Pepsin	N-terminal of aromatic amino acids phenylalanine, tryptophan and tyrosine	(Bhagavan and Bhagavan, 1992) and (Boisen and Eggum, 1991)
Trypsin	Lysyl or arginyl peptide bond to expose lysine or arginine	(Fuller, 1991)
Chymotrypsin	Aromatic or large hydrophobic amino acid residues such as tyrosine, phenylalanine, tryptophan, leucyl, methionyl, asparaginyl, and glutamyl	(Bhagavan and Bhagavan, 1992), (Appel, 1986) and (Boisen and Eggum, 1991)
Elastase	Glycine and alanine of elastin	(Boisen and Eggum, 1991; Bhagavan and Bhagavan, 1992)
Carboxypeptidase A	Peptide bond adjacent to the C- terminal end of a polypeptide chain,	(Boisen and Eggum, 1991; Bhagavan and Bhagavan, 1992)
Carboxypeptidase B	Basic amino acids from the C-terminal end of polypeptide chains	(Boisen and Eggum, 1991)
Collagenase	Alpha peptides and hydrogen bonds in the super helix of tropocollagen and collagen	(Straumfjord and Hummel, 1957)

2.3.2 Protein structure and forms

The structure of the protein samples and the food matrix in which the samples are presented will influence the protein *in vitro* digestibility (Savoie in Fuller, 1991). Protein feed ingredients may contain free amino acids, peptides of various length, secondary structure proteins (α-helix, β-pleated sheets, β-turns and super helix), tertiary structure proteins and quaternary structure proteins (Bhagavan and Bhagavan, 1992). Secondary structure proteins such as scleroproteins, which include collagen, elastin and keratin are poorly digested in simple stomach animals (Becker and Yu, 2013). Protein sources containing high levels of these proteins will have limited bioavailability. Higher protein structural configuration requires more time and higher enzyme concentration to achieve greater hydrolysis (Bhagavan and Bhagavan, 1992; Becker and Yu, 2013).

Secondary structure proteins resist digestion due to the nature of their individual structures. Feather meal for example contains high levels of keratin (Bielorai et al., 1983), which has highly cross-linked disulfide bonds along the pleated sheet configuration (Becker and Yu, 2013). This makes the protein almost insoluble in water and thereby reduces the action of pepsin and subsequent pancreatic actions (Bielorai et al., 1983). Samples of meat and bone meal may contain elastin and collagen after being produced from tendons, ligaments and bone scraps of animals. Elastin and collagen also contain cross-linking in their helix structures which may influence digestion (Becker and Yu, 2013).

The matrix in which the protein is presented in the protein source may limit the access of proteolytic enzymes. Plant proteins are often presented in a matrix with cell walls, lipids and complex sugars, and may also be organized into specialized storage vacuoles (Fuller, 1991; Becker and Yu, 2013). The ability of proteolytic enzymes to access those proteins may depend on the ability of other enzymes to free protein from the matrices (Boisen and Eggum, 1991). The digestion of protein from plant sources in mono gastric animals is closely linked to the protein associated with plant cell wall components (Theander et al., 1989). Non starch polysaccharides are known to protect proteins from enzymatic digestion in a variety of plant feed ingredients in poultry (Meng et al., 2005). Solubilisation of the cell wall components of plant source protein meals with various carbohydrase enzymes were able to improve the availability of the protein to chickens (Theander et al., 1989; Meng et al., 2005).

2.3.3 Enzyme activity

In vitro digestion maybe influenced by the activity of the enzymes used while enzyme activity is affected by factors such as pH, temperature, ratio of enzyme to substrate and incubation time (Boisen and Eggum, 1991). As proteins are hydrolyzed by enzyme *in vitro*, the pH of the mixture will be reduced by the release of protons from the cleaved peptide bonds (Maga et al., 1973). If the original pH of the reaction moisture is further away from the optimum pH of the enzyme, the rate of hydrolysis will be reduced drastically in a short period of time. In the pH stat method, pH is held constant in the optimal range for the enzyme via automated alkali titration (Pedersen and Eggum, 1983). To achieve optimal reaction conditions, most *in vitro* assays select appropriate starting pH for the enzyme used (Fuller, 1991). The pepsin digestibility assay requires an acidic condition (Sheffner et al., 1956), while the pancreatin assay requires a basic environment (Riesen et al., 1947).

Temperature may play a regulatory role as it relates to enzyme activity. Like all chemical reactions, temperature increases the amount of kinetic energy and increases the velocity at which molecules collide in an enzymatic reaction (Bhagavan and Bhagavan, 1992). *In vitro* digestibility assays using protease keep the temperature of their reaction between 37 and 45° C (Boisen and Eggum, 1991). Enzymes are proteins and all proteins can be denatured at high temperatures, therefore the optimal temperature for a given enzyme is always close to the body temperature of the organism from which the enzyme was derived (Bhagavan and Bhagavan, 1992). *In vitro* assays should reflect *in vivo* conditions so the temperature at which the reaction takes place is often that of the animal internal temperature (Boisen and Fernández, 1995; Tibbetts et al., 2011).

The ratio of enzyme to substrate and the incubation time varies across individual *in vitro* assays (Robbins, 1978; Boisen and Eggum, 1991; Parsons et al., 1997). Generally, the incubation time can range from 0.5 to 45 h depending on the kind of *in vitro* assay (Fuller, 1991). The enzyme to substrate ratio is often a function of the specific activity of the enzyme. The specific activity of an enzyme is often defined as the amount of product produced from a specific substrate over time while maintaining the reaction at a fixed pH and temperature range (Bhagavan and Bhagavan, 1992). Enzymes from different preparations with different specific activity are often used for the same *in vitro* assay (Savoie and Gauthier, 1986; Savoie et al., 1988). The ratio of pepsin used with 4 mg nitrogen of sample in the dialysis method ranged from 5 to 7 mg/mL pepsin (Gauthier et al., 1982; Savoie and Gauthier, 1986). Pepsin concentration used in the pepsin digestibility test ranged from 0.02 to 2.5 g/L and the sample size of the protein may be expressed as g of nitrogen per sample (Boisen and Eggum, 1991). To avoid confusion in the literature, an *in vitro* method should define the enzyme to substrate ratio and the specific activity of each enzyme in the assay (Gauthier et al., 1986).

2.4 Protein digestion kinetics in poultry

The process by which proteins are hydrolyzed and absorbed by chickens is well understood, but little is known about the rate at which such proteins are digested. Sklan and Hurwitz (1980) suggested that the solubilisation and sequential breakdown of protein into intermediate peptides are the rate limiting steps for the absorption of soybean meal protein in poultry diets. Protein digestion rate is known to modulate of postprandial protein accretion (Boirie et al., 1997; Dangin et al., 2001). It is often assumed that proteins with high digestibility are rapidly digested.

The digestion kinetic of any nutrient from a diet should be a function of the gut transit time of that diet. The mean retention time of digesta along the small intestine of broilers varies (Gutiérrez del Álamo et al., 2009a; b). Gut transit time is a function of the physical form of the diet and the nature of the ingredients in the diet (Bedford and Classen, 1993). There is evidence that digestion and absorption of nutrients, and possibly proteins, in broiler chickens is a function of the rate of passage of the diet (Gutiérrez del Álamo et al., 2009a). Digestion of protein in the small intestine of broiler chickens tends to show a linear increase from the proximal to the distal end of the tract (Gutiérrez del Álamo et al., 2009a). Liu et al. (2013) showed that 70% of the methionine present in diets composed of sorghum, soybean and canola meal, fed to broilers were digested in the proximal jejunum. Of that diet less than 50% of all other amino acids except lysine were digested in the proximal jejunum. The data show that different amino acids were digested to varying degrees across each section of the small intestine. This variation in digestion pattern across different sections of the small intestine was also seen for crude protein digestion in broilers (Gutiérrez del Álamo et al., 2009b). Sklan and Hurwitz (1980) suggested that different protein sources have different hydrolysis patterns as they move along the small intestine of broilers. They suggested that this could be related to each section of the intestine having a different distribution of peptides with different molecular weights in the soluble fraction of the digesta.

Understanding the kinetics of peptides and the rate of amino acid release from various protein ingredients as they move along the small intestine would give insight into where each amino acid gets digested and absorbed. Some of the above authors were not able to distinguish the quantity of protein or amino acids that were digested from the feed ingredient because various ingredients contributed to the protein pool of the diet. If they were able to quantify the amount of protein and amino acids digested from a specific ingredient source, they did not correct for the endogenous source of protein during digestibility calculation. Ignoring the endogenous protein contribution could result in negative rate of digestion in the proximal jejunum (Gutiérrez del Álamo et al., 2009b) and an underestimation of the protein digestibility (Lemme et al., 2004).

2.5 Protein absorption and its interaction with the gut

The digestion of proteins into free amino acids and peptides up to three amino acids long is followed by absorption across the intestinal epithelia. In order to facilitate the movement of these molecules, enterocytes use membrane bound protein transporters. The location of the transporters in the enterocytes membrane depends on the type of transporter and nutrients being transported

(Bröer, 2008). Many transporter families have been identified and cloned in rats, human and birds, but the molecular mechanism governing their regulation may vary from species to species, tissue to tissue or age to age within the same species (Humphrey et al., 2006; Frazier et al., 2008).

Amino acid transporters

Amino acid transporter types are defined by the chemical properties of the amino acids being transported and other molecules which aid in transporter function. The main amino acid transporters are classified as neutral transporters (B^oAT1, ASCT2), neutral and cationic transporter ($ATB^{o,+}$), L-like neutral transporter (4F2hc/LAT2), aromatic transporter (TAT1), glycoprotein independent L-type transporter (SLC43, SLC7), amino acid transporters of the SNAT family (SNAT2, SNAT4), cationic transporter (CAT1, rBAT/$b^{o,+}$AT, 4F2hc/y^+LAT1, 4F2hc/y^+LAT2), anionic transporter (EAAT3), imino acid and glycine transporter (PAT1, PAT2, IMINO), β-amino acid transporter (TauT), and peptide transporter (PepT1) (Bröer, 2008; Gilbert et al., 2010).

Evidence suggests that nutrient presence in the intestinal lumen regulates amino acid transporters (Bröer, 2008). Gilbert et al. (2010) reported a significant interaction between dietary protein and expression of cationic transporter rBAT/$b^{o,+}$AT and anionic transporter EAAT3 in the chicken small intestine. Chen et al. (2005) fed chickens three levels of crude protein 12, 18, and 24% from d 0 to 35 while evaluating the level of peptide transporter (cPepT1) mRNA abundance throughout the intestine. The chickens which were on the 18 and 24% CP diets feeds were restricted to the feed intake of the 12% CP diet. The abundance of intestinal cPepT1 mRNA in chickens fed 18 and 24% CP diets increased significantly over the experimental period. The duodenal and jejunal sections had the highest increase in cPepT1 mRNA abundance over the 35 d period (Chen et al., 2005). Humphrey et al. (2006) fed a lysine deficient and a lysine adequate diet to chickens and observed similar results in liver cationic amino acid transporter (CAT1-3) mRNA expression.

Chen et al. (2005) observed an interaction between the stage of development and the level of protein on intestinal cPepT1 mRNA abundance when chickens were given unrestricted access to a 24% CP diet. When the chickens were given the 24% diets from d 0 to 35, there was a reduction in the abundance of the intestinal cPepT1 mRNA until d 14 followed by a gradual increase up to d 35. Humphrey et al. (2006) observed an interaction between dietary lysine levels and tissue type on transporter (CAT1-3) mRNA expression in chickens. The developing lymphatic tissues in chickens had a higher expression of CAT1 than CAT3 mRNA expression when compared to other surrounding tissues of birds given a lysine deficient diet but not the lysine adequate diets. These

data suggest that dietary protein plays an important role in the regulation of amino acid and peptide transporters.

Tight junctions

The transportation of nutrients via the intercellular space adjacent to intestinal enterocytes is termed paracellular transport (Suzuki, 2013). The paracellular forces behind intestinal epithelial nutrient selectivity involve the use of tight junctions and nutrient transporters. Tight junctions are apical complexes composed of claudins, occludins and junctional adhesion molecules, which stretch from one enterocyte to neighbouring enterocytes via zonula occludens, which are anchored to each enterocyte by myosin and actin (Tsukita et al., 2001). The structural orientation and the roles of the proteins involved in tight junctions have been the subject of reviews (Tsukita et al., 2001; Van Itallie and Anderson, 2006; Suzuki, 2013). Intestinal tight junction permeability is known to be controlled by a number of factors including peptides, amino acids and SCFA (Suzuki, 2013).

Data from the literature suggests SCFA might be involved in the regulation of tight junctions, thereby affecting intestinal barrier function (Mariadason et al., 1997; Suzuki et al., 2008; Peng et al., 2009). The application of a mixture of acetate (80 mmol/L), propionate (40 mmol/L) and butyrate (20 mmol/L) swiftly increased the electrical resistance of caecal wall tissue mounted on Ussing chambers. Peng et al. (2009) evaluated the AMP activated kinase activity, electrical resistance and inulin permeability in a Caco-2 cell monolayer model after butyrate application in order to understand the molecular bases of increased electrical resistance following SCFA application to caecal tissues. The increase in electrical resistance was associated with the reorganization of tight junction proteins which accelerated the formation of tight junctions (Peng et al., 2009). The AMP activated kinase activity significantly increased during the process, which suggests that butyrate regulates intestinal barrier function via up regulation of AMP activated kinase activity (Peng et al., 2009). It has been shown that SCFA can restore intestinal barrier dysfunction by reorganizing zona occludens-1, occludin and filamentous-actin via AMP activated kinase activity in Caco-2 monolayers (Elamin et al., 2013)

Seth et al. (2004) investigated tight junction restoration potential of amino acids in a Caco-2 cell monolayer with tight junctions that were disrupted by acetaldehyde. L-glutamine was able to restore the transepithelial electrical resistance and the permeability of the monolayer to inulin and lipopolysaccharide. The acetaldehyde application dissociated the occludin and zona occludens-

1 of the tight junctions from the actin cytoskeleton. It appears that the amino acid was able to reorganize those proteins to their original location in the junction thus restoring the junction function. There is also evidence from animal research suggesting that glutamine provides gut barrier protection when animals develop intestinal barrier dysfunction caused by stress (Wijtten et al., 2011). Theories related to the mechanism by which glutamine helps to maintain intestinal barrier function has recently been reviewed by (Wang et al., 2014).

The dysfunction of the intestinal barrier caused by piglet weaning results from stress, which leads to intestinal atrophy and diarrhea (Wang et al., 2014). The supplementation of L-methionine in the diets of post-weaning piglets to achieve a total standardized ileal digestibility of 0.35% improves small-intestinal mucosa villi architecture and increases the transepithelial electrical resistance of the jejunal mucosa. There was greater abundance of occludin in the jejunum of L-methionine pigs (Chen et al., 2014). When the jejunum tissues were examined for apoptotic protein (active caspase-3), there was a significant reduction in the active caspase-3 level. This suggested that the L-methionine supplementation was able to maintain intestinal barrier integrity by increasing enterocyte lifespan via the active caspase-3 and the maintenance of paracellular transport through increased occluding production.

2.6 *In vivo* protein fermentation

2.6.1 Gastrointestinal microbial community composition

The gastrointestinal tract (GIT) of poultry host a wide range of microorganisms (Gong et al., 2007; Choct and Ao, 2009). The microbiota associated with the mucosa of older broilers is mainly composed of gram-positive bacteria (Gong et al., 2007). Gong et al. (2007) evaluated the mucosa-associated microbiota of the broiler chicken GIT using 16S rRNA gene-based analysis. Thirteen operational taxonomic units (OTUs) were detected in the crop, 11 OTUs in the gizzard, 14 OTUs in the duodenum, 12 OTUs in the jejunum, 9 OTUs in the ileum and 51 OTUs in the caeca. The upper GIT (crop, gizzard, duodenum, jejunum and ileum) microbiata was predominantly *Lactobacilli* (72%) while the other 20% consisted of *Clostridium, Escherichia, Eubacterium, Enterococcus, Faecalibaterium, Ruminococcus, Veillonella and Candidates arthromitus* (Gong et al., 2007). The caeca had the widest variation in microbes and sub species. Ninety six clones were isolated from the caeca which includes a*ctanaerbacterium, bilophila, bacteroides, clostridium, Escherichia, eubacterium, faesalibacterium, firmicutes, lactobacillus,*

peptococcus, psedobutyrvibrio, megamanas, ruminococcus, sporobacter, subdoligranulum and several species of uncultured bacteria (Gong et al., 2007).

2.6.2 Protein digestion and gut microbes

Choct and Ao (2009) defined gut health as a complex concept encompassing GIT macro and micro structural integrity, immune status and equilibrium of the microbial community. There are many factors which may influence gut health and are the subject of other reviews (Dahiya et al., 2006; Choct and Ao, 2009). This section will highlight some of the roles dietary protein play in the modulation of the microbial community in broilers.

One of the first detailed studies which linked dietary composition to bird health was conducted by Kaldhusdal and Skjerve (1996). They evaluated the incidence of necrotic enteritis in south-eastern Norwegian broiler chickens from 1969 to 1989. The univariate regression analysis indicated that incidence of necrotic enteritis changed with season and increased when animal protein was present in diets (Kaldhusdal and Skjerve, 1996). Drew et al. (2004) examined the relationship between dietary protein source and the concentration on *C. perfringens* in the intestine of broilers. Using fish meal and soybean concentrate as the two main protein sources, they observed a significant positive relationship between increasing levels of fish meal and the *C. perfringens* population in the ileum and caecum. Further investigation illustrated that high populations of *C. perfringens* were associated with the high concentration of dietary glycine from fish meal protein (Drew et al., 2004).

Wilkie et al. (2005) evaluated several dietary protein meals sources in broilers challenged with *C. perfringens* in order to identify correlations between specific amino acids and an increase in *C. perfringens* colonization of the intestine. A significant correlation was found between ileal digesta lysine content and the *C. perfringens* count in the ileum and caecum (Wilkie et al., 2005). The glycine content of the diet and the ileal digesta also correlated with the *C. perfringens* count in the ileum and caecum. The birds that were given feather meal, fish meal, potato protein concentrate and meat/bone meal had a significantly higher *C. perfringens* count in the ileum and caecum than the birds fed soybean meal, corn gluten meal or pea protein concentrate. Birds fed meat and bone meal had significantly less lactobacilli and other lactic acid producing bacteria in their ileum than those fed corn gluten meal (Wilkie et al., 2005).

Dietary methionine may play a role in the control of some pathogenic gut microbial population in broilers. The supplementation of broilers with diets containing 8% methionine

reduced the populations of *C. perfringens* streptococcus group D and coliforms in the ileum and caecum (Dahiya et al., 2007). There was a significant increase in the lactobacillus populations with 8% methionine supplementation. There was a trend for intestinal necrotic enteritis lesion scores to be lower with increased dietary methionine levels.

Dietary amino acids, especially glycine, appear to play an important role in the proliferation of *C. perfringens* in the small intestine of broilers (Drew et al., 2004; Wilkie et al., 2005). What is unclear from the literature is the influence of amino acid digestion kinetics on the count of *C. perfringens* in the small intestine. Could the rate at which various protein sources release amino acids and the host's ability to absorb those amino acids influence gut microbial growth? Research is required to identify relationships that may exist between protein digestion kinetics and the gut microbial community.

2.6.3 Gastrointestinal microbial metabolites

Diet is considered to be the most influential factor affecting the GIT microbial community and their metabolites regardless of animal species (Macfarlane and Macfarlane, 2007; Dahiya et al., 2007). There is evidence that some microbes in the GIT community have symbiotic relationships with the host animal, with the host supplying microbes with a suitable environment and nutrient supply while microbes helping to maintain a functional gut equilibrium (Scheppach, 1994; Lunn and Buttriss, 2007). In some cases the symbiotic microbes contribute to the host health through their metabolic activity (Williams et al., 2009). Some microbial species may produce SCFA, which are beneficial to the host (Elsden and Hilton, 1978).

The relationship between the host animal and its gut microbiota can become harmful due to the production of toxic levels of metabolites. Some microbes can produce compounds such as ammonia while others may produce biogenic amines (Barnes et al., 2001). These compounds can compromise host health if they are produced at high luminal concentrations. The kinds of microbial metabolites produced depends on the fermentation substrates available and the metabolic pathways of the bacteria involved in the process (Elsden and Hilton, 1978).

2.6.3.1 Short chain fatty acids

Apart from carbohydrates, protein of exogenous and endogenous origin is fermented by GIT microbiota (Macfarlane and Macfarlane, 2007). Short chain fatty acids are one type of by-products of intestinal microbiota protein fermentation and research related to SCFA has gained a

lot of attention over the years (Lunn and Buttriss, 2007; Williams et al., 2009). Fermentation of protein by microbes yields isobutyrate, valerate and isovalerate, which are branch chain amino acid origin (Elsden and Hilton, 1978), but other SCFA can be produced depending on the amino acids and bacteria present (Ramsay and Pullammanappallil, 2001). Clostridium species such as *C.difficile* and *C. lituseburense* present in poultry GIT (Gong et al., 2007) are known to produce branch chain fatty acids from threonine, L-valine, L-leucine, and L-isoleucine (Elsden and Hilton, 1978).

Sakata (1987) evaluated the effects of SCFA on intestinal epithelia cell proliferation in rats. All tested SCFA increased normal crypt cell proliferation, but butyrate gave the largest response (Sakata, 1987). Intracolonic infusion of SCFA is said to have stimulated intestinal mucosal growth. The infusion of 20 mM butyrate and a mixture of butyrate, propionate, and acetate in the colon of rats significantly increased mucosal weight, and total protein, RNA and DNA of the GIT (Kripke et al., 1989).

Studies have investigated the control of C*lostridium perfringens* through butyric acid dietary supplementation. At concentrations of 330 g/ton in the starter diets and 250 g/ton in the grower diets of chickens, butyrate was able to control *Clostridium perfringens* and reduce enteritis in chickens (Timbermont et al., 2010). Similar effects of butyrate were seen *in vitro* on the invasiveness of *Salmonella enteritis* using chicken intestinal epithelium (Immerseel et al., 2004a). By simply adding 25 mM butyric acid to the salmonella culture before inoculating the chicken caecal epithelial cells, the invasiveness of the bacteria was reduced by 10 fold (Immerseel et al., 2004a). Feeding microencapsulated SCFA to young chickens as a means to protecting the birds from early *salmonella* colonization have been studied. Butyric acid impregnated capsules significantly reduced the colonization of the caeca of the birds 2 d after the birds were challenged with 5.103 cfu *S. enteritidis* (Immerseel et al., 2004b).

2.6.3.2 Biogenic amines

Microbial fermentation of amino acids produces biologically active compounds called biogenic amines (Smith et al., 2000). Biogenic amines may result from microbial decarboxylation of amino acids (Barnes et al., 2001) or normal cell metabolism (Wang et al., 1991). Animal by-products used in feed production are considered to be a source of biogenic amines in the GIT (Smith et al., 2000) where they play various biological roles (Table 2.2).

Table 2.2. Biogenic amines their metabolic roles and amino acid precursor (Blum, 1985)

Biogenic amines	Metabolic roles	Amino acid precursor
γ-aminobutyric acid	An inhibitory amino acid	Synthesized from glutamic acid
Histamine	Local hormone	Synthesized from histidine
Tyramine	Neurotransmitter increases systolic blood pressure	Synthesized from tyrosine
Cadaverine	Unclear, found in urine and associated with bacterial vaginosis	Synthesized from lysine
Serotonin	Neurotransmitter	Synthesized from tryptophan
Dopamine Norepinephrine Epinephrine,	Neurotransmitters	Synthesized from tyrosine
Putrescine	Involved in cell metabolism (growth, tissue repair) Precursor of spermidine	Synthesized from ornithine and methionine
Spermidine	Plays multiple function in cells, precursor for spermine (crucial for cell survival)	Synthesized from putrescine
Spermine	Associated with nucleic acids, plays a key role in cell proliferation	Synthesized from spermidine

The presence of microbial biogenic amines in food or feed samples can be an indication of metabolic activity of spoilage microorganisms (Bardócz et al., 1993; Smith et al., 1996). High microbial activity leads to high levels of putrescine, tyramine, and cadaverine and as such they are used as indicators of spoilage level in refrigerated meat products (Lazaro et al., 2014). The presence of 0.2% histamine or a combination of 0.1% histamine and cadaverine in the broiler chicken diets have been suggested to increase the total number and severity of gizzard erosion and proventricular ulcers (Barnes et al., 2001). The presence of spermidine levels in excess of 0.2% in poultry diets can lead to reduced growth rate and feed consumption (Smith et al., 1996). Feeding 0.2, 0.4, and 0.6% putrescine in the diets of 60 week old Barred Rock hens resulted in an increased concentration of putrescine in eggs, and decreased feed consumption and egg mass (Chowdhury and Smith, 2001).

The presence of some polyamines in protein feed ingredients may not indicate spoilage since they are essential for growth of all living cells (Bardócz et al., 1993). The presence of luminal polyamines in rats is known to stimulate mucosal growth when rats were given an ornithine decarboxylase blocker in their diets (Wang et al., 1991). The ornithine decarboxylase blocker was

α-difluoromethylornithine, which decreases the activity of the enzyme thereby reducing the mucosal DNA, RNA, and protein content (Wang et al., 1991). It appears that some biogenic amines, especially polyamines, are related to cellular activities and when the GIT metabolic activity increases, the concentration of tissue polyamines also increases (Bardócz et al., 1993; Adeola et al., 2003). Polyamines such as putrescine when supplemented in coccidial challenge turkey diets at 0.3g/100g of diet were able to promote growth and help increase mucosal development of the small intestine. Putrescine aids in the recovery of birds from subclinical coccidiosis by restoring small intestinal morphology (Girdhar et al., 2006).

The potential benefits of feeding polyamines depends on the length of time that polyamines are fed, the kind and the final concentration in the diets. A study feeding 0 to 0.6% spermidine in the diets of broiler chickens found reduced feed consumption and growth at 0.2%. Birds fed 0.05% spermidine for one d had a higher growth rate than those fed no spermidine (Smith et al., 1996). Rats fed 0.5% spermidine also displayed reduced growth rate and feed consumption when compared to those fed 0.05%. It was suggested that concentrations less than 0.05% spermidine promote growth, but above 0.05% could lead to toxicity in rats (Jeevanandam et al., 1997). The reduced growth rate seen in rats given 0.1% spermidine was associated with increased concentrations of total amino acids and spermidine in the muscle and brain tissue accompanied by low levels in the plasma. This suggests that spermidine uses the same transporters as amino acids (Jeevanandam et al., 1997).

The source of dietary methionine and the ratio of arginine to lysine influence the concentration of polyamines in the proximal small intestine. Gonzalez-Esquerra and Leeson (2006) investigated the effects of amino acid ratio on the concentration of polyamines in selected chicken tissues grown at two different temperatures. There were significant negative correlations between pancreatic spermine concentration with body weight gain, feed intake, feed conversion ratio, efficacy for crude protein deposition and duodenal villus height in heat stressed birds. There was a significant positive correlation between duodenum spermidine concentration and feed intake. The effects of heat stress on poultry may be related to changes in the spermidine and spermine levels in the pancreas and proximal small intestine (Gonzalez-Esquerra and Leeson, 2006). Research related to dietary protein and polyamine (putrescine, spermidine and spermine) metabolism in poultry is lacking, but there is evidence suggesting dietary protein and biogenic amines are interrelated.

2.7 Summary

Protein quality assessment of feed ingredients is often achieved using *in vitro* or *in vivo* testing. *In vivo* technique estimates protein digestibility of feed ingredient by feeding the ingredient to the intended animal while assessing the nutrients absorbed from the ingredient. This method seemed to be the most suitable method for estimating the amino acid bio-availability of various feed ingredients high in protein. *In vivo* methods can be expensive and time consuming, which makes them more suitable to research environments. Despite the debate over the years on which *in vivo* method is most appropriate, evidence from the literature suggested that the standardized ileal digestible amino acid technique is the method of choice for assessing feed ingredient protein quality.

Protein quality can also be evaluated using less expensive and time consuming chemical methods, termed *in vitro*. These techniques are used to improve the user's efficiency when dealing with large sample numbers and some mimic the physiological and chemical characteristics of the animal digestive system to which the ingredient will be fed. The pepsin digestibility test is the *in vitro* method of choice for quick evaluation of protein sample during quality control and in most research settings. In 1993 the AOAC revised the pepsin digestibility index test and recommended that the test should not be used for evaluating samples that contain fats and carbohydrates. Even though the pepsin digestibility test uses enzymes to liberate the amino acids from the protein, it does not mimic normal *in vivo* digestive conditions. The results obtained with this method may be misleading if the samples tested contain fats or carbohydrates which they often do. To overcome the problem encountered when using the pepsin digestibility test, multi-enzyme tests have been proposed. These tests use a combination of enzymes in one or multiple steps customized to simulate the digestive process of the animal. Multi enzyme assays can predict animal digestibility but any inherited biological properties of the ingredients on the animal digestive tract are loss.

The process by which proteins are hydrolyzed and absorbed by chickens is well understood. Past research has focused on identifying the major sites of protein digestion and absorption in poultry. Standardized ileal digestible amino acid coefficients provide data on the extent to which protein sources are digested and absorbed, but data on the sequential break down of different kinds of protein meal are lacking. Little is known about the rate at which the vast majority of protein meals used in poultry rations are digested. The rate of protein digestion may have a significant impact on the ability of poultry to synchronize protein synthesis from dietary amino acids. This

can influence bird health via host microbial interaction and the animal's efficiency in using dietary proteins. Studying the rate of absorption of any nutrient in poultry *in vivo* has its limitations, which could be the reason for the lack of literature data. Due to the small size of the bird's digestive tract, it is often difficult to collect samples routinely over time from the same bird to measure the rate of nutrient disappearance. Some researchers have overcome this problem in other species by calculating the mean retention time of the food being digested in each section of the digestive tract. The retention time and the nutrient digestibility data could be used to calculate digestion rate in poultry based on the digestion kinetic model proposed by Ørskov and McDonald (1979). If an appropriate *in vitro* protein digestion model for poultry is developed, digestion kinetics for various protein meals used in poultry diets could be studied.

Data from the literature suggest that there is a link between protein nutrition and the modulation of pathogenic microorganisms such as *C. perfringens* microflora community in the bird's gut. The specific details of the relationship between poultry health and protein digestion are still elusive and theoretical. The nature of this relationship is yet to be defined for majority of the high protein feed ingredients available to the poultry industry. The main theory from the literature suggests that it is the undigested protein at the end of the distal ileum of poultry, which will have the most effects on bird's health via protein fermentation. The ability of that protein to be fermented depends on how soluble and how fine the particles are to get into the caeca. To date there is no research available for poultry which has characterized the distal ileum protein from high protein ingredients commonly fed to poultry.

2.8 General objective of research

It was hypothesized that protein sources will vary in digestion kinetics consistent with their unique digestibility characteristic and chemical composition, which will affect broiler performance, digestive tract morphology and caecal fermentation metabolites. Therefore, the main objective of this thesis was to identify the effects of protein digestion kinetics on broiler performance and digestive tract morphology. Understanding the digestion kinetics of high protein ingredients is the first step in elucidating the potential impact protein digestion kinetics could have on poultry performance. The first objective was to develop an *in vitro* assay which could be used to characterize the digestion kinetics of various high protein feed ingredients. The second objective of studies in this dissertation was to evaluate the extent and rate of digestion of different plant and animal protein meals by broiler chicken using an *in vivo* assay modified for collecting digestion

rate data. The third objective was to characterize the ileal digesta CP from chickens fed commonly available high protein feed ingredients. The fourth objective was to evaluate the effects of dietary protein level and indigestible protein fraction on the performance and meat yield of broiler vaccinated for coccidiosis and fed antibiotic free diets. The fifth objective was to evaluate the effects of dietary protein level and indigestible protein fraction on the digestive tract tissue characteristic and caecal fermentation products of broiler fed antibiotic free diets using coccidiosis vaccinated and non-vaccinated management protocol.

3.0 DEVELOPMENT OF AN *IN VITRO* PROTEIN DIGESTIBILITY ASSAY MIMICKING THE CHICKEN DIGESTIVE TRACT[1]

3.1 Abstract

It is difficult to obtain *in vivo* digestion kinetics data for high protein ingredients using chickens. Collecting kinetics data requires repeated sampling of digesta from the small intestine during the digestion process. The collection of digesta is not easily accomplished due to the anatomical structure of the chicken digestive tract. An *in vitro* technique is proposed for measuring the digestion kinetics of protein sources fed to chickens. The proposed method has a 30 min gastric and 3 h intestinal phase. Five hundred mg crude protein (CP) equivalent of each meal sample (CP = % N_2 x 6.25) was digested with (28,260 units) pepsin in 50 mL polyethylene centrifuge tubes for 30 min in a shaking water bath (150 strokes/min; 30 mm stroke length) at 41°C. Tube pH was then adjusted to 7±0.5 with 500 µL 4.9 N NaOH and 9.5 mL sodium acetate buffer (pH 12.5). Following the addition of 6.5 mL pancreatin and 3 glass marbles, the tubes were returned to the water bath. Aliquots (500 µL) were collected at 0, 15, 30, 45, 60, 90, 120, 150, 180, and 240 min of the intestinal phase. Digestion (DIG) was measured colourimetrically via a ninhydrin assay where aliquots were diluted (1:820) with 10 mL 0.1N HCl followed by 10 mL sodium acetate buffer (pH 6.5) then 100 µL of that mixture was added to 1900 µL H_2O. Samples are mixed with ninhydrin reagent (2:1) at 100°C ± 2 for 15 min and spectrometric readings are taken at 568 nm after 10 min cooling. To validate the assay, 5 replications of soybean (SBM), corn gluten (CGM), corn distiller dried grains with solubles (CDDGS), porcine meal (PCM), fish meal (FM), and casein (CA) were digested. The DIG data were modeled with PROC NLIN procedure, and the intra coefficient of variation (CV) assessed using PROC MEANS of SAS 9.4. The DIG values at 180 min were SBM 95 ± 4, FM 93 ± 3, PCM 68 ± 4, CGM 82 ± 3, and CDDGS 70 ± 2%. Intra CV for SBM, CGM,

[1] This chapter is a modification of the contents from the following paper: D.D.S.L. Bryan, D.A. Abbott and H.L. Classen, 2018. Development of an *in vitro* protein digestibility assay mimicking the chicken digestive tract. Animal Nutrition. https://doi.org/10.1016/j.aninu.2018.04.007

CDDGS, PCM and FM were 5, 5, 12, 10, and 2% respectively. The estimated fractional protein digestion rates for SBM, CGM, CDDGS, FM, and PCM were 0.023, 0.013, 0.009, 0.024, and 0.013 h^{-1}, respectively. In conclusion, the proposed *in vitro* technique estimated the rate and extent of protein digestion for the meals with low intra CV.

Key words: Digestion kinetics, soybean meal, corn gluten meal, corn dried distiller's grains with solubles, fish meal, porcine meal

3.2 Introduction

Broiler chickens have been extensively selected for rapid growth and as a consequence the ability of the birds to deposit body protein has increased dramatically (Zuidhof et al., 2014). Concurrently, the quality of protein in broiler diets has increased in importance, with quality being defined by amino acid digestibility and balance (Ravindran and Bryden, 1999). It is a general consensus among poultry nutritional researchers that the jejunum and proximal ileum are the major sites for amino acid absorption. However, little information can be found pertaining to how much protein from common ingredients gets digested in the proximal and distal portions of the small intestine.

In vivo assays are considered to be the gold standard for assessing ingredient nutritional quality in poultry (Fuller, 1991). *In vivo* estimation of protein quality of a feed ingredient is normally achieved by feeding the ingredient to the intended animal while assessing the extent to which nutrients are absorbed by the terminal intestine. Protein quality can also be evaluated using *in vitro* chemical methods (Boisen and Eggum, 1991). *In vitro* assays are less expensive, more ingredients can be evaluated, and they are less time consuming than *in vivo* assays. Historically the focal point of assessing protein quality for chickens has been based on the extent of digestion and as a result little data are available on the rate at which proteins are digested and absorbed.

The rate of digestion of proteins along the digestive tract has been known to have significant biological effects in other species (Ørskov and McDonald, 1979; Boirie et al., 1997) and the same could be true for poultry. *In vivo* protein nutritional research in humans suggested that the sequential breakdown of proteins having different digestion rates, modulate tissue protein synthesis and deposition (Boirie et al., 1997). The sequential breakdown of protein into intermediate peptides

is considered to be the rate limiting step for the digestion and absorption of soybean meal protein in poultry diets (Sklan and Hurwitz, 1980), which might also be the case for other protein sources commonly fed to poultry. Therefore, protein digestion kinetics and bioavailability of proteins are both important factors, which could be considered when trying to maximize yield in poultry production.

Extensive research is available on the extent of digestibility for various high protein ingredients estimated using *in vivo* and *in vitro* procedures. However, information on the protein digestion characteristics of feed ingredients (*in vivo* or *in vitro*) for poultry is scarce and this type of research is often limited to human research (Dangin et al., 2001; Koopman et al., 2009). Most *in vivo* techniques used to evaluate protein degradation in humans and other animal research require the use of expensive isotope labeling of pure proteins and tracers (Boirie et al., 1997). Less expensive and time consuming *in vitro* methods have been used to obtain protein digestion data in ruminant species (Boila et al., 1980) and may have value for poultry. Currently, there is no *in vitro* method, which estimates protein degradation kinetics for poultry.

The purpose of this research was to develop a poultry specific *in vitro* protein digestibility assay, which could predict the degradation kinetics of high protein feed ingredients commonly fed to poultry. A multi-enzymatic digestion technique using gastric and intestinal digestion phases was defined and validated. The digestive tract transit time in poultry has been reported to be between 2-3.5 h (Svihus et al., 2002; Hughes, 2008), so the optimum enzyme to substrate concentration that resulted in the most effective degree of digestion within 3 h was used as a criterion for the assay. The conditions for the colourimetric assay used to evaluate the degree of digestion were optimized. The effectiveness of the *in vitro* digestions technique on a variety of high protein ingredients was tested. This *in vitro* protein digestibility assay was developed to predict the rapidly, slowly and undigested protein fraction of ingredients, as well as the rate and extent of digestion of the proteins.

3.3 Material and methods

The following methods illustrate the stages which were involved in the development of the proposed *in vitro* assay. The first stage describes an appropriate colourimetry assay for identifying changes in a protein sample due to hydrolysis of peptide bonds. The chemical composition of the reagent, its shelf life and wavelength sensitivity during reactions were evaluated and optimized for the *in vitro* assay. The second stage involved the establishment of the conditions for the *in vitro* assay gastric and intestinal digestion phase. The composition of the buffers which were compatible

to the enzymes used in the gastric and intestinal phase were identified. The optimal units of pepsin for the gastric phase were elucidated using as dose response study over a 30 min digestion time frame. Selection of enzyme dose in the pancreatin for the intestinal phase was based on a dose response study building on the gastric phase results. The final stage of the research provides validation data for high protein ingredients using the colourimetry procedure and the two stage *in vitro* digestion assay.

Reagents and chemical used

The reagents used in this study were obtained from the following sources. Tin (II) chloride dehydrate (CAS 107-21-1), benzoic acid (CAS 65-85-0), glacial acetic acid (CAS 64-19-7), trichloroacetic acid solution (Sigma T0699), hemoglobin (Sigma H2625), pepsin (P7125-100g; CAS 9001-75-6), Nα-benzoyl-L-arginine ethyl ester (Sigma B4500), Trizma® Base (Sigma T1503), N-Benzoyl-L-Tyrosine Ethyl Ester (Sigma B6125), methanol (Sigma M1775), N-Succinyl-Ala-Ala-Ala-p-nitroanilide (Sigma S4760), and ninhydrin (CAS 485-47-2) were obtained from Sigma Chemical Co. (St. Louis, MO). Ethylene glycol (CAS 107-21-1), sodium hydroxide (CAS 1310-73-2), sodium acetate trihydrate (CAS 6131-90-4), calcium chloride (CAS 10035-04-8), guar gum (CAS 9000-30-0), and hydrochloric acid (7647-01-0) were obtained from Fisher Scientific (Pittsburgh, PA). The liquid bovine pancreatin (62,500 USP trypsin units/mL) was purchased from RENCO (10 London Street, Eltham 4322, New Zealand).

3.3.1 Colourimetry assay

3.3.1.1 Ninhydrin reagent composition

A 4 N sodium acetate buffer was prepared by dissolving 544 g of sodium acetate trihydrate in 100 mL of warm glacial acetic acid and then Millipore water was added to make a total volume of 1000 mL. Tin (II) chloride solution was prepared by adding 1.2 g tin (II) chloride to 12 mL of ethylene glycol and then vortexing to dissolve all the tin (II) chloride. To prepare the ninhydrin reagent, 9.75 g of ninhydrin were dissolved in 366 mL ethylene glycol, then 122 mL 4 N sodium acetate buffer were added. This solution was mixed for 5 min with a magnetic stir bar before the addition of 12 mL of tin (II) chloride solution and mixing for another 5 min.

3.3.1.2 Validation of ninhydrin reagent

The absorbance spectrum for SBM, CA (casein), CDDGS, and CGM were determined as follows. All samples were ground to pass through a 0.5 mm screen using a Retsch Ultra Centrifugal

Mill ZM 200 (Haan, Germany). The CP contents of all the meals were determined as nitrogen x 6.25, with N content determined using a Leco nitrogen analyzer. Samples (500 mg CP equivalent) were weighed and placed in individual 100 mL Pyrex® glass bottles (No.14395) after which 6 N hydrochloric acid was added at 4 mL per 100 mg of sample weight. The samples were gently mixed by swirling, and then capped and placed in an oven at 110°C for 24 h. After 24 h hydrolysis, samples were allowed to cool to room temperature and then filtered through Whatman Grade 601 filter paper. An aliquot of the sample was collected after filtering and the pH adjusted to 7±0.5 with sodium hydroxide. The filtered sample was diluted with Millipore water to give 0.36 mg CP per mL based on the initial 500 mg CP of the sample that was hydrolyzed.

Each sample (100 µL) was mixed with 1900 µL of Millipore water and 1000 µL of ninhydrin reagent in disposable glass culture tubes (Borosilicate glass 16 x100 mm, NO. 14-961-29). A blank sample with 2000 µL of Millipore water and 1000 µL of ninhydrin reagent was prepared. Glass marbles were placed on top of each tube and the tubes were placed in a boiling water bath for 10 min. The tubes were allowed to cool for 5 min before 200 µL of sample were pipetted in triplicate into a 96 well plate (Falcon 353910 U-Bottom well). The samples were read from 200 nm to 999 nm at 1 nm wavelength interval using a microplate reader (Epoch 2^{TM}, BioTeck) set at 22°C.

The concentration detection limits for the ninhydrin reagent with a lysine standard were identified as follows. The lysine standard was prepared and diluted over the range of 0.25 to 410 µg/mL. One mL of each dilution was mixed with 500 µL of ninhydrin reagent in disposable glass culture tubes. A blank tube was prepared by replacing the diluted sample with Millipore water. Marbles were placed on the tubes before being placed in a boiling water bath according to the process described previously. A 200 µL volume of sample was pipetted into a 96 well plate and read at maximum absorbance (OD) identified during the previous spectrum scan of the samples.

The shelf life of the ninhydrin reagent was evaluated over 304 d. A CA standard was prepared from the hydrolyzed CA sample. A fresh batch of ninhydrin reagent was prepared on the morning of d 1 at 8:00 h and placed in a dark glass bottle wrapped in aluminum foil. At 16:00 h the CA standard was reacted with the reagent as outlined in the absorbance spectrum test above, and then the ninhydrin reagent was placed on a shelf for storage at room temperature (22 ± 3°C). This test was repeated on d 10, 14, 120, and 304 after the first test was conducted.

3.3.2 In vitro digestion assay

The *in vitro* assay method had a 30 min gastric and 3 h intestinal phase mimicking digestion in chickens based on previous research (Svihus et al., 2002; Hughes, 2008). The optimum enzyme to substrate ratio was verified for the gastric phase and the intestinal phase using enzyme dose response assays with SBM as the model protein. Soybean was selected as the model protein because it is the most widely used protein source in poultry diets worldwide and its volume in production accounts for more than 69% of the world's total protein source for animal feed (USDA, 2016).

3.3.2.1 Buffer compositions

Multiple buffer compositions were evaluated in preleminary studies to test their interaction with the colourimetry reagent and their impact on the stability of enzymes. Sodium acetate buffers with pH 12.5 and 6.5 were the most suitable for maintaining enzyme activity of the glycerol based pancreatin and compatibility with the ninhydrin reagent used in this study. To prepare 1 L of a 10 mM HCl solution, 833 µL of concentrated HCl were mixed with 999.167 mL of Millipore water. A 0.1 M calcium chloride solution was prepared by dissolving 33.3 g of calcium chloride in 300 mL Millipore water. A benzoic acid solution was prepared by dissolving 5.8 g of benzoic acid into 2 L of Millipore water. For the sodium acetate buffer preparation, 27.2 g of sodium acetate trihydrate were dissolved in 500 mL benzoic acid solution. The pH was adjusted to 12.5 or 6.5 using saturated sodium hydroxide solution (50% wt/wt) and then the volume of the solution was made up to 2 L with Millipore water followed by the addition of 8 mL 0.1 M calcium chloride solution. All buffers were stored in the refrigerator until use.

3.3.2.2 Pepsin dose response assay

Pepsin activity was determined using the Sigma enzymatic assay for pepsin (3.4.23.1). One unit of pepsin was defined as a change in ΔA_{280} of 0.001 per min at pH 2.0 and 37°C measured as trichloroacetic acid soluble products using hemoglobin as the substrate. Pepsin was dissolved in 10 mM HCl solution to give 9,420, 14,130, 18,840, 28,260 or 32,970 units per mL of freshly prepared solution, which was used on the d of preparation. The pepsin dose response assay was carried out in 50 mL polyethylene screw cap centrifuge tubes (VWR 21008-178).

A 500 mg CP (N_2 x 6.12) equivalent of SBM sample was placed in centrifuge tubes with 50 mg of guar gum and 8.5 mL of 10 mM HCl solution. The tubes were vortexed to evenly mix and saturate the sample with the 10 mM HCl solution. After mixing, 1.5 mL pepsin solution with

either 14,130, 21,195, 28,260, 42,390 or 49,455 units of pepsin were added to 6 replicate tubes plus 3 blank tubes per enzyme concentration. All tubes were vortexed and 0.5 mL sample taken for electrophoresis, and another 0.5 mL was placed in 20 mL of sodium acetate buffers (pH 6.5) for colourimetric evaluation. The tubes were placed in a shaking water bath (150 strokes/min; 30 mm stroke length) at 41°C for 30 min. After the gastric phase digestion 0.5 mL sample was taken for electrophoresis. Another 0.5 mL of sample was placed in 20 mL of sodium acetate buffer for colourimetric evaluation.

The samples for electrophoresis were placed in a boiling water bath for 15 min immediately after collection to denature the pepsin and then samples were centrifuged at 2500 rpm. An aliquot was taken from the supernatant of all samples and used for electrophoresis. All samples were analyzed in a non-reducing condition using an Agilent 2100 Bioanalyzer System (Agilent Technologies, Lexington, USA) and the Protein 230 Chip Assay Kit following the manufacture's protocol.

The samples for colourimetric analysis were vortexed before centrifuging at 3000 rpm for 10 min. A 100 µL aliquot of the sample was diluted with 1900 µL of Millipore water in disposable glass culture tubes and then 1 mL of ninhydrin reagent was added. A marble was placed on top of the tubes before heat treatment as outlined previously in section 3.3.1.2. The cool reaction mixture (approximately 2 mL) was read in 4.5 mL disposable plastic cuvettes (Cat. No. 14955129 Fisherbrand®) using a Genesys 20 Spectrophotometer UV-Vis (Termo Fisher Scientific Inc., Waltham, USA).

3.3.2.3 Pancreatin dose response assay

The pancreatin used was a liquid bovine enzyme (65,000 trypsin unit/mL) from RENCO (New Zealand). The activity of trypsin (30,667 BAEE units/mL), chymotrypsin (2,157 BTEE units/ mL) and elastase (7 units/ mL) were determined using Sigma EC 3.4.21.4, EC 3.4.21.1, and EC 3.4.21.36 assays, respectively. Six pancreatin levels (1, 3, 5, 6.5, 7.5, and 9 mL) were evaluated in the intestial phase for the enzyme dose response assay. Six replicate tubes and 3 blank tubes per enzyme level were used during the pancreatin dose response assay.

All samples were digested for 30 min using the selected pepsin concentration identified in the pepsin dose response assay. A 500 µL volume of 4.9 N sodium hydroxide solution was added to each tube immediately after gastric digestion. Sodium acetate buffer (pH 12.5) was added to each tube and the pH was adjusted to 7.5. The selected volume of pancreatin solution was added

to the respective tubes to bring the final volume of the tubes up to 26.5 mL. All tubes were vortexed and 0.5 mL sample was taken for electrophoresis. Another 0.5 mL was taken from the tubes for colourimetric evaluation and placed in 10 mL 10 mM HCl solution, the mixture was then vortexed followed by the addition of 10 mL of sodium acetate buffers (pH 6.5). Three marbles were placed in each tube and the tubes were placed in a shaking water bath (150 strokes/min; 30 mm stroke length) at 41°C for 180 min. During the intestinal digestion phase, a 0.5 mL aliquot was taken for colourimetry assay evaluation at 15, 30, 40, 60, 90, 120, 150, and 180 min. At 180 min of digestion, a 0.5 mL aliquot was taken from each tube for electrophoresis.

3.3.2.4 In vitro assay validation

The assay intra-variability was evaluated using high protein feed ingredients. The ingredients selected for the validation study were SBM, CGM, CDDGS, PCM, and FM; CA was used as a control because it represents a pure protein source. All samples were ground to pass through a 0.5 mm screen as outlined in section 3.3.1.2 before proximate analysis. The moisture content of all meal samples were determined using (AOAC, 2006; Method 990.03). Protein sources were analyzed for N using a Leco nitrogen analyzer (Model 601–500–100, Serial # 3211, Leco Corporation, St. Joseph, MA, USA) according to the combustion method (AOAC, 2006) Method 990.03 using 6.25 as the conversion factor to calculate CP. Soybean meal was analyzed for trypsin inhibitor activity following AOAC Method 22-40. All meals were also analyzed for protein dispersibility index (PDI) as outlined by Johnson (1970) and protein solubility in 0.2% potassium hydroxide solution using the method of Araba and Dale (1990). The calcium and magnesium content of all meal samples were analyzed using inductively coupled plasma optical emission spectrometry (ICP-OES) after total acid digestion with HCl.

A subset of each meal sample was hydrolyzed with 6 N HCl as outlined in the validation of ninhydrin reagent section. The protein content of the samples was calculated as N_2 x 6.25, then 500 mg CP equivalent of each ground sample were placed in 5 replicate tubes. The samples were digested using the optimum pepsin and pancreatin concentrations identified during the two dose response assays. The 0.5 mL aliquots for the colourimetry assay were only collected during the intestinal digestion phase at 0, 15, 30, 40, 60, 90, 120, 150 and 180 min.

3.3.3 Calculations and statistics

The digestible protein of the samples was calculated using the OD of the digested sample and the OD of the totally hydrolyzed sample as follows:

$$\text{Digestible protein } (\%) = \frac{\text{OD of digested sample}}{\text{OD of 6N HCl hydrolyzed sample}} \times 100 \quad \text{.....(3.3.3.1)}$$

Where OD = the absorbance at 568 nm.

The absolute percentage of CP digested per min was calculated using the following rate formula:

$$\text{Protein digested per min} = \frac{\text{digestible CP @ time (x) - CP @ time (y)}}{\text{time (x)-time (y)}} \quad \text{.....(3.3.3.2)}$$

Where x and y represents different time point during the 180 min digestion period.

All the digestible protein data were fitted to the following modified two tail compartmental statistical model proposed by Ørskov & McDonald (1979) using the PROC NLIN procedure of SAS 9.4:

$$P = A + B\,(1 - e^{-kd*t}) \quad \text{.....(3.3.3.3)}$$

Where P = CP digested at a specific time point, A = rapidly digested CP fraction, B = slowly digested CP fraction, kd = the rate at which B is digested over time (fractional rate). This constant was set to negative since the data represented increasing protein digestion over time, and t = Time. The undigested fraction of the proteins UD was calculated as 100-(A+B) and the potential digestible of the protein PD was calculated as (A+B).

The spectrum scan data were analyzed for the maximum inflection point using the Proc REG procedure. Correlation analysis was performed between calculated digestible CP and the models predicted digestible CP using Proc Corr, and the means of the kinetic constants were compared using the Proc Mixed procedure of SAS 9.4 with probability of $P \leq 0.05$ considered significant. If significant differences were found between means, LSD means statement was used to separate treatment means.

3.4 Results

3.4.1 Validation of ninhydrin reagent

Two major peaks were identified after a full spectrum scan of the reactions between the Ninhydrin reagent and the samples as illustrated in Figure 3.1. The first peak span was from 300

nm to 450 nm while the second peak was from 500 to 650 nm. The reaction was monitored for 30 min during which time there was no change in the OD reading of the second peak, however the first peak OD decreased with time and by 30 min it was no longer present. Evaluation of the second peak data from 500 nm to 630 nm (Figure 3.2) using the Pro REG function of SAS 9.4 revealed that the inflection point for all the samples was 568.

The relationship of the ninhydrin reaction with the lysine standard was used to determine the detection limits of the reaction (Figure 3.3). Below 2 µg/mL lysine, the absorbance values did not show a linear trend, and above 400 µg/mL, the detector of the spectrophotometer was saturated. The R^2 value of the point between the lysine standard concentrations and OD values obtained at each concentration was 0.97 for the range of 2 to 400 µg/mL of lysine. This inferred that the OD reading of the sample was a good predictor of the amount of free amino and carboxyl group present in the reaction.

Aging the ninhydrin reagent in dark bottles shielded from light, kept the reagent relatively stable up to 120 d (Figure 3.4). It took 14 d for the reagent to stabilize, during which time there was a 0.119 OD reduction in the absorbance reading.

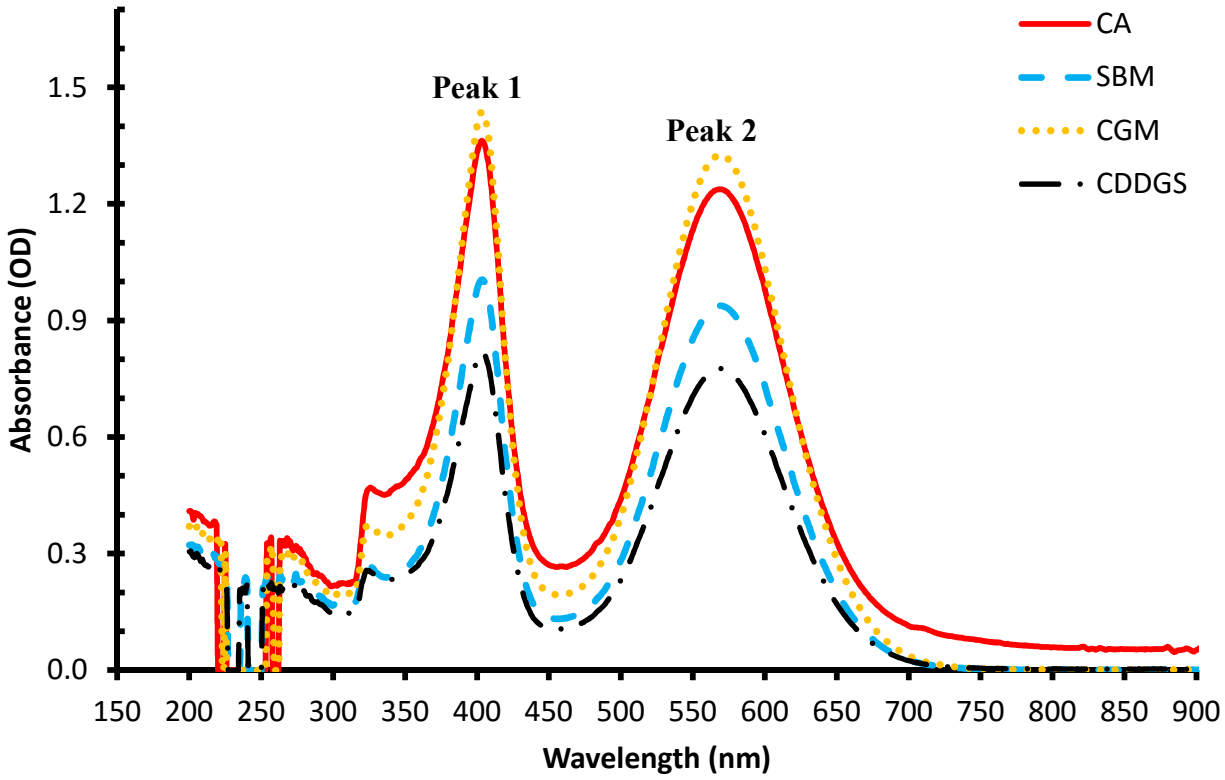

Figure 3.1. Absorbance spectrum from 150 to 950 nm for ninhydrin reagent reaction with casein (CA), soybean meal (SBM), corn gluten meal (CGM), and corn distillers' grain with solubles (CDDGS) hydrolysed with 6 N HCl at 100°C for 24 h.

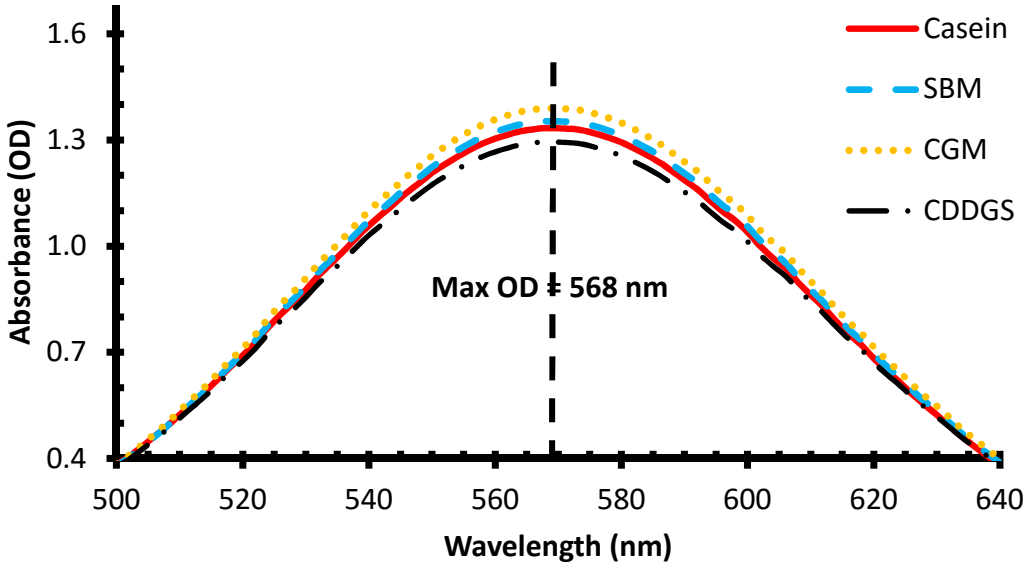

Figure 3.2. Absorbance spectrum from 150 to 950 nm for ninhydrin reagent reaction with casein, soybean meal (SBM), corn gluten meal (CGM), and corn distillers' grain with solubles (CDDGS) hydrolyzed with 6 N HCl at 100°C for 24 h.

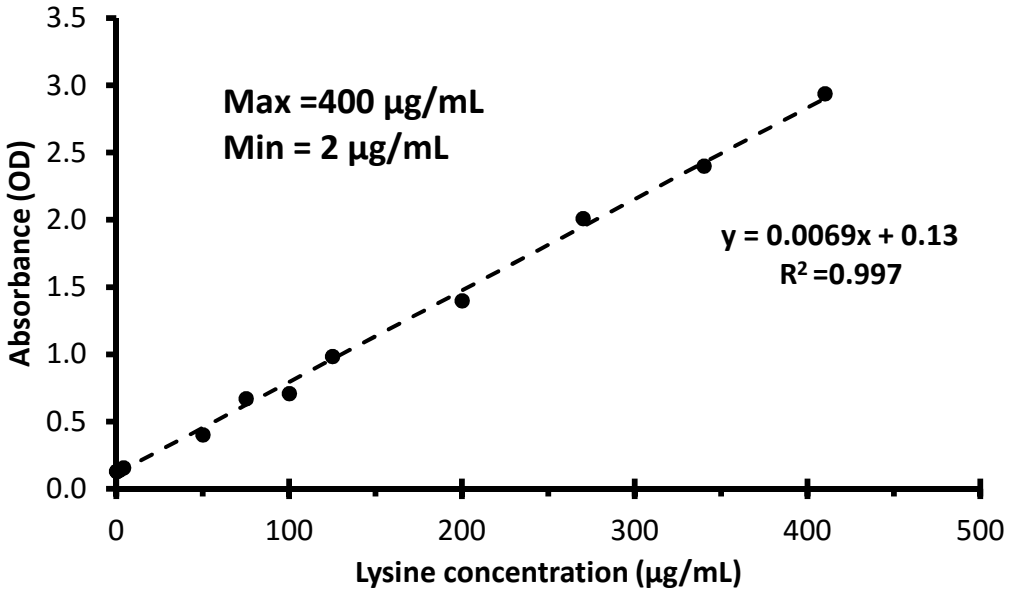

Figure 3.3. Relationship between concentrations of lysine standard and absorbance values when reacted with ninhydrin reagent.

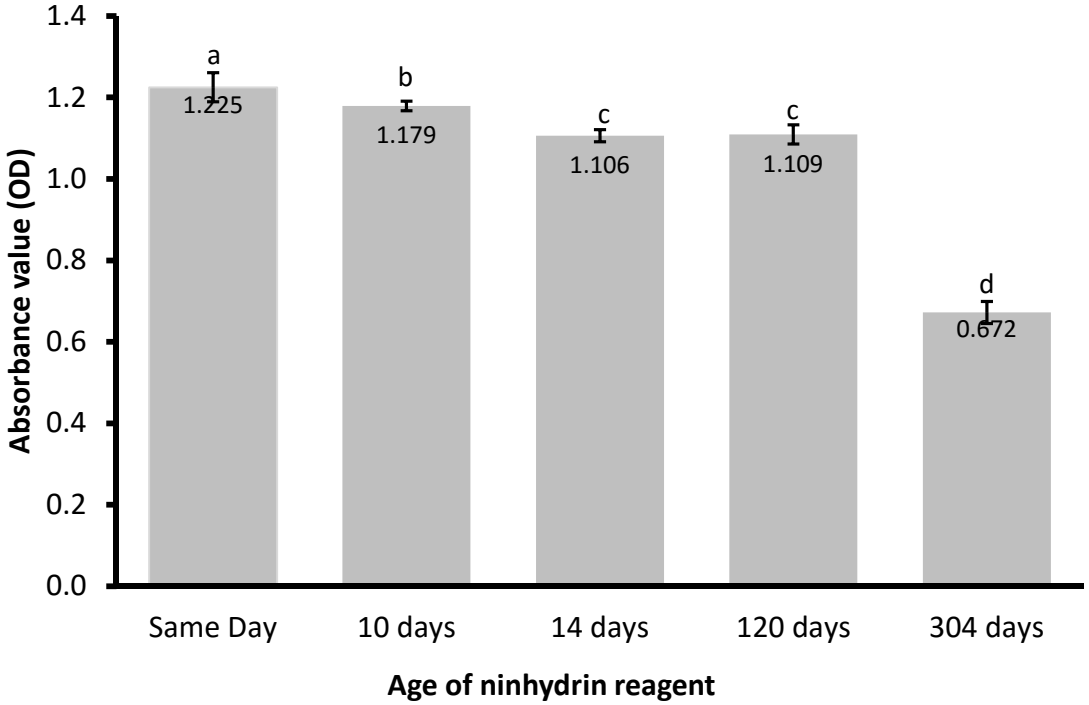

Figure 3.4. Effects of ninhydrin reagent storage time on the absorbance reading of hydrolyzed casein. [a-d]Means ± standard deviation with different letters are significantly different (P<0.05).

3.4.2 Enzyme dose response assay

Increasing the concentration of pepsin from 14,130 to 49,455 units reduced the abundance of polypeptides between 46-28, 63-46 and 95-63 kDa (Figure 3.5). This reduction resulted in an increase in the concentration of peptides between 12-7 kDa and it confirmed increased hydrolytic activity with enzyme dose. The colourimetry assay data presented in Figure 3.6 had a similar trend to what was observed for the peptide concentration between 7 to 12 kDa. By dividing the units of pepsin used in the assay by the percentage CP hydrolyzed (Figure 3.6) the CP hydrolyzed per unit of enzyme can be calculated. This resulted in 0.188, 0.189, 0.163, 0.116, and 0.114% hydrolyzed CP per unit of enzyme for the five enzyme concentrations, respectively.

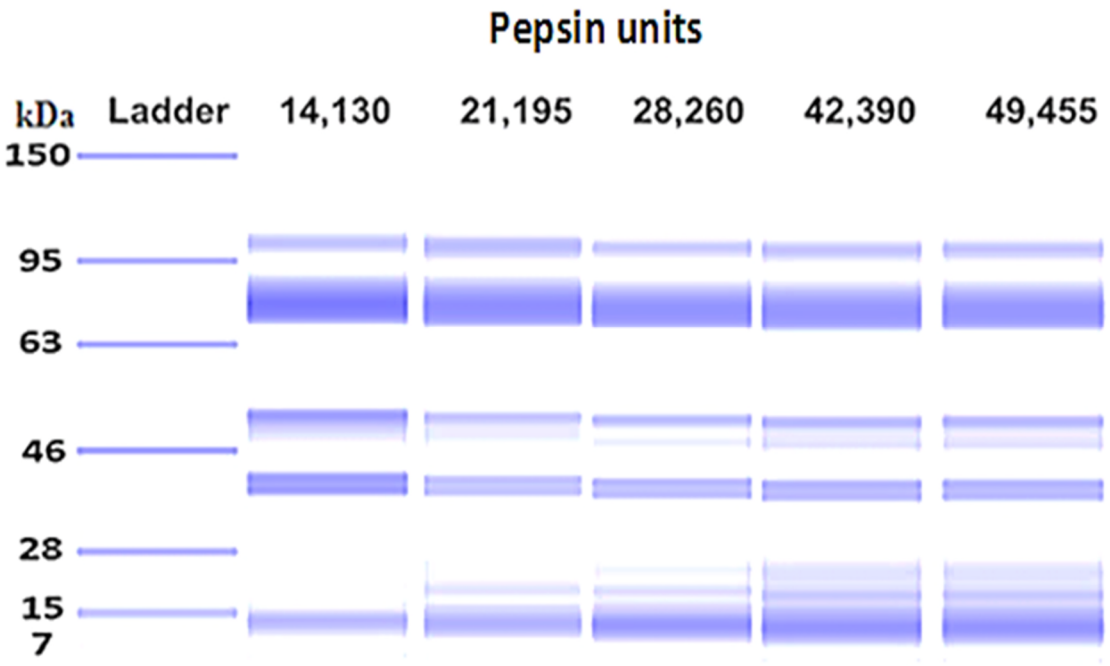

Figure 3.5. Effects of pepsin concentration (units) on the molecular weight distribution of peptide from soybean meal digested for 30 min at 41°C. Ladder represents protein and peptide fragments with molecule weights measured in kilo-Daltons.

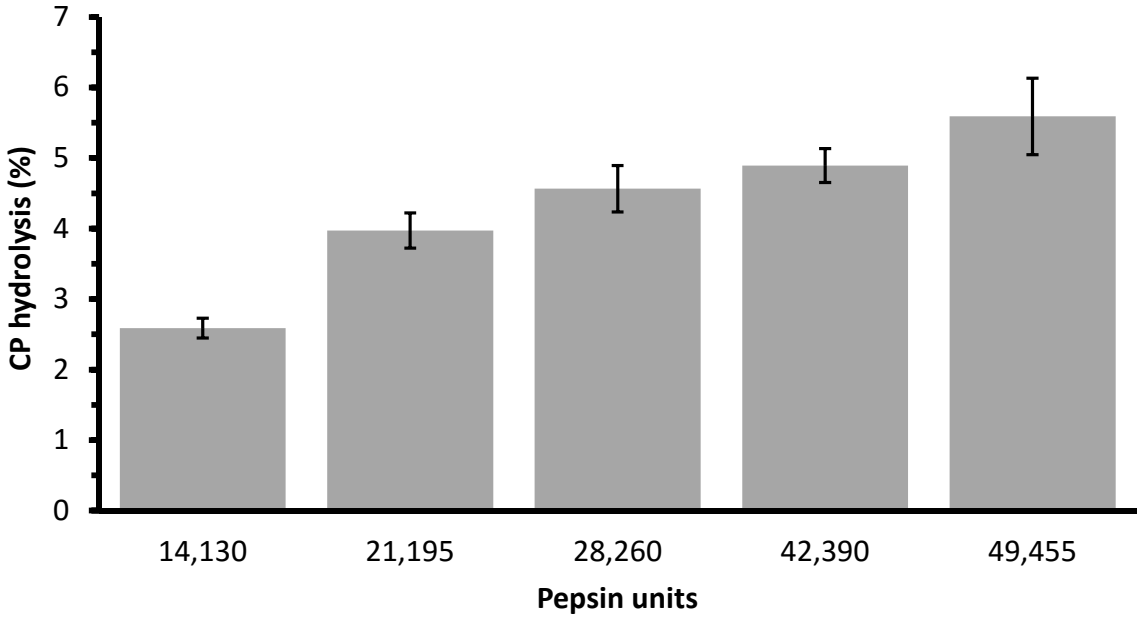

Figure 3.6. Effects of pepsin concentration (units) on CP hydrolysis (%) from soybean meal digested for 30 min at 41°C.

The pepsin concentration of an *in vitro* assay can be selected base on a number for criteria. In this study taking the cost of the pepsin into consideration, having a minimum of 4% CP hydrolysis in the gastric phase and the ability of the selected pepsin concentration to produce a typical digestion curve (Figure 3.7) in the intestinal phase were the basic criteria for this assay. The 28,260 unit of pepsin was selected because the units of pepsin below that level did not achieve 4% CP hydrolysis during the 30 min of the assay. Concentrations above 28,260 units gave a substantial reduction in the percentage of CP hydrolysis per unit of enzyme, which indicated that these levels of enzyme might not be economical since the concentrations were almost doubled. In a preliminary study the four lowest pepsin concentrations were used to digest a sample of SBM, then a standard 7 mL volume of pancreatin was used for the intestinal phase of the digestion. The digestion of the samples was monitored over a 3 h period. The 28,260 units of pepsin gave a time dependent digestion curve which was gradual, which we assumed to be the case *in vivo*, while the 42,390 units curve was very steep (data not shown). Therefore the 28,260 units of pepsin was selected as the standard pepsin concentration because the pepsin efficiency measured as percentage CP hydrolyzed per unit of enzyme was drastically reduced after 28,260 unit of pepsin, the shape of the digestion curved from the preliminary data gave a gradual digestion with time and the extent of hydrolysis for the SBM by 28,260 units were above 4%.

The criteria for the selection of the pancreatin was based on the extent of hydrolysis which mimicked that of *in vivo* SBM CP digestion by poultry. The 7.5 and 9 mL pancreatin gave the highest degree of hydrolysis which was above 90% at the end of the intestinal incubation time (180 min). Both the 7.5 and 9 mL pancreatin treatments also had the steepest digestion curve over time. The 1, 3 and 5 mL volumes were only able to hydrolyze less than 60% of the CP in the SBM samples after 180 min incubation. The digestion curve from the 6.5 mL volume of pancreatin was more gradual over time. Approximately 81% of the CP in the SBM sample was hydrolyzed by the 6.5 mL volume of pancreatin at the end of the 180 min intestinal digestion phase.

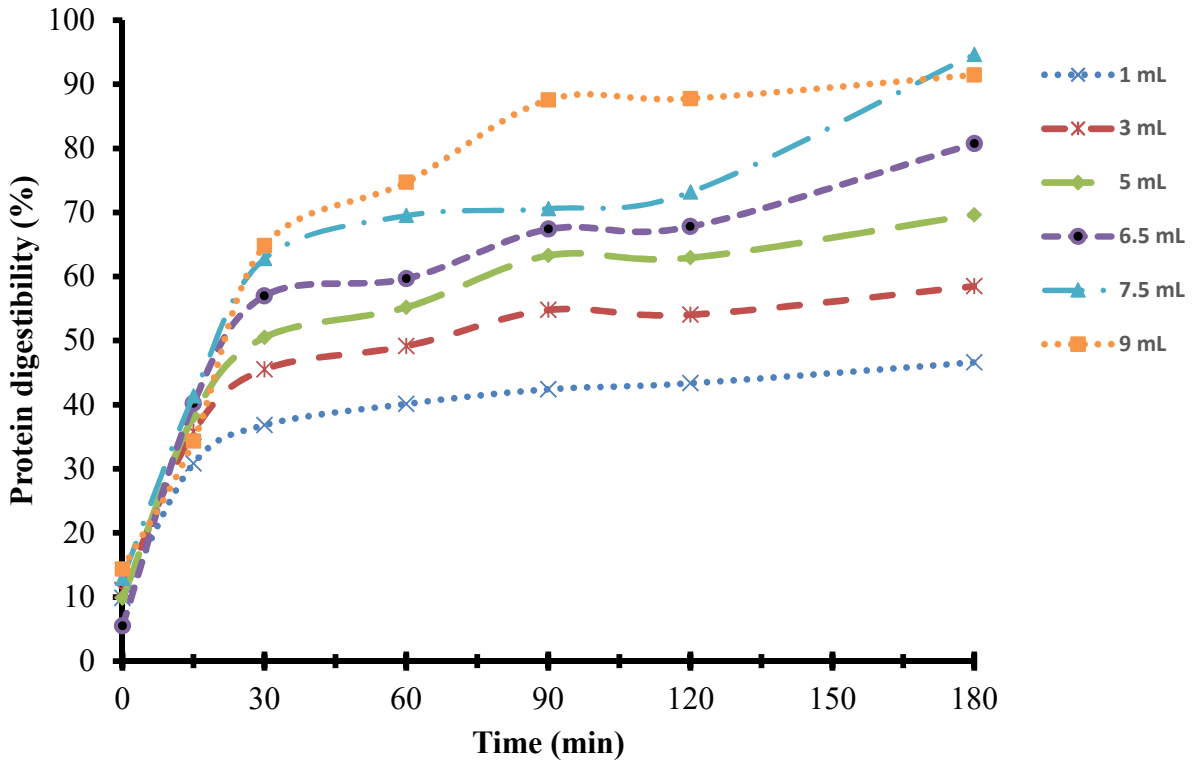

Figure 3.7. Effects of pancreatin concentrations (1 mL= 30,667 BAEE units of trypsin; 2,157 BTEE units of chymotrypsin, and 7 units of elastase) on the digestible CP of soybean meal over 180 min of the intestinal phase at 41°C and after predigesting with 28,260 unit of pepsin.

A preliminary literature search suggested that SBM samples from 4 different countries had an average *in vivo* CP digestibility of 82% (Ravindran et al., 2014). The percentage of CP hydrolyze by the 6.5 mL of pancreatin was similar to the 82%, plus the digestion curve was more gradual over time, which is assumed to be the case for protein digestion *in vivo*. The shape of the curve also provided the opportunity to obtained relevant digestion kinetic data from the assay. Based on the criteria listed above the 6.5 mL of pancreatin (199,335.5 BAEE units of trypsin; 14,020.5 BTEE units chymotrypsin, and 445.5 units elastase) was selected as the optimum enzyme dosage for the intestinal phase of the assay.

3.4.3 In vitro assay validation

The composition and chemical properties of the feed ingredients used in this assay are shown in Table 3.1. These data are presented in order to give the reader a clearer over view of the status of the ingredients that were used. Ingredient composition (mineral, CP, and DM content)

was similar to values previously reported for samples used as poultry feed ingredients (National Research Council, 1994).

Table 3.1. Feed ingredients composition and chemical properties.

	Meals					
Item	CA	FM	PCM	SBM	CGM	CDDGS
Dry mater (%)	98.0	89.2	95.4	89.2	90.6	97.7
Crude Protein (%)	90.2	67.2	62.0	45.3	62.1	28.3
Calcium (%)	ND	3.54	4.32	0.50	0.10	0.06
Magnesium (%)	ND	0.33	0.22	0.29	0.05	0.34
Trypsin inhibitor (TIU/g)	ND	ND	ND	4335	ND	ND
Protein disperseability index (%)	ND	32	25	15	15	2
Protein solubility (%)	ND	45	39	78	24	28

CA= casein, FM = fish meal, PCM = porcine meal, SBM = soybean meal, CGM = corn gluten meal, CDDGS = corn distillers' grain with solubles, ND= not determined

The rapidly digested CP fraction (A) was higher ($P \leq 0.05$) for FM and PCM than SBM and CDDGS, while other protein fractions were intermediate (Table 3.2). The coefficient of variation for fraction (A) of the samples were numerically higher for CDDGS, CGM, and SBM then FM, PCM and CA. Fraction (B) which represents the proportion of the proteins which were digested over time was higher ($P \leq 0.05$) for CA, FM, SBM, and CGM when compared to PCM, while CDDGS was similar to all samples evaluated. The coefficient of variation for fraction (B) was higher for CDDGS when compared to the other ingredients.

The SBM and FM samples had the highest ($P \leq 0.05$) fractional digestion rate (rate at which faction (B) was digested over time; kd) compared to all other samples. The CDDGS had a higher ($P \leq 0.05$) fractional digestion rate than CA, but all other samples were intermediate. The coefficient of variation for the fractional digestion rate was lowest for CDDGS and SBM. The trend observed for the absolute digestion rate (adr), which was calculated by dividing the extent of digestion by the total digestion time, was different from that of the fractional digestion rate. SBM had a higher ($P \leq 0.05$) absolute digestion rate than all other samples. The absolute digestion rate for PCM and CDDGS were similar, but lower ($P \leq 0.05$) than that of all the other ingredients. The coefficient of variation for the absolute digestion rate of the samples were similar.

The undigested protein fraction was calculated as the difference between the total protein content of the sample and the total protein digested. There was more ($P \leq 0.05$) undigested protein in the PCM sample than all other samples except of the CDDGS which was intermediate. Numerically, lower coefficients of variation were seen for the undigested protein of FM and SBM compared the other samples.

The potential digestible (PD) CP of samples equals the sum of fraction (A and B) and values were higher ($P \leq 0.05$) for Ca, FM, SBM, and CGM than that for PCM. The value for CDDGS was intermediate and not different from any of the protein sources tested. The coefficients of variation for the potential digestible CP of the samples were generally low, but PCM and CDDGS values were twice that of the other values. The *in vitro* digestible CP values calculated using the OD values of the samples at 180 min of intestinal digestion expressed as a percentage of the OD values after 24 h acid hydrolysis of the samples ranged from 68 to 90%. After modeling the data, the predicted digestible CP of the samples ranged from 60 to 84%. The correlation (R^2) value between meal *in vitro* digestible CP and the model's predicted digestible CP were above 0.9 for all the meals evaluated (Table 3.3).

Table 3.2. Digestion kinetic constant of meals generated with the *in vitro* digestion data fitted to Ørskov and McDonald (1979) model A+B ($1-e^{-kd*t}$).

	Degradation Kinetics Constants											
	A (%)		B (%)		kd (h^{-1})		Adr (%/min)		UD (%)		PD (%)	
Meals	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV
CA	16.9^{a}	16	71.9^{a}	5	0.018^{b}	18	0.443^{b}	3.5	13.0^{b}	27	87.0^{a}	5
FM	13.2^{ab}	9	70.6^{a}	4	0.024^{a}	20	0.463^{b}	4.0	16.1^{b}	9	83.9^{a}	2
PCM	13.9^{ab}	11	55.2^{b}	7	0.013^{bc}	20	0.340^{c}	3.8	30.9^{a}	23	69.1^{b}	10
SBM	6.5^{c}	30	78.8^{a}	5	0.023^{a}	13	0.507^{a}	5.0	14.6^{b}	15	85.4^{a}	5
CGM	10.3^{bc}	20	72.7^{a}	7	0.013^{bc}	20	0.433^{b}	5.0	17.1^{b}	27	82.9^{a}	5
CDDGS	8.1^{c}	24	66.8^{ab}	12	0.009^{c}	12	0.346^{c}	4.1	25.1^{ab}	31	74.9^{ab}	12
SEM	1.2		3.6		0.001		0.008		3.5		3.5	
ANOVA P-Value	<.0001		0.0023		<.0001		<.0001		0.0039		0.0039	

[a-c] Means (n = 6 cages) within a column with different superscripts are significantly different ($P<0.05$).
CV = coefficient of variation. CA= casein, FM = fish meal, PCM = porcine meal, SBM = soybean meal, CGM = corn gluten meal, CDDGS = corn distillers' grain with solubles; A = rapidly digested CP fraction; B = slowly digested CP fraction; kd = the rate at which the B fraction is digested over time; UD= undigested fraction calculate as 100-(A+B); PD = potential digestible fraction calculated as A+B; adr = absolute digestion rate (percentage of protein digested per min from 0 to 180 min).

Table 3.3. *In vitro* and predicted digestibly coefficient of meals over 180 min and their correlation coefficients and p-Values.

	In vitro digestible CP means						Predicted digestible CP means[1]					
Min	CA	SBM	FM	CDDGS	CGM	PCM	CA	SBM	FM	CDDGS	CGM	PCM
0	12 ± 3.1[2]	5 ± 2.9	9 ± 3.1	7 ± 1.3	4 ± 1.8	7 ± 2.5	0	0	0	0	0	0
15	27 ± 6.5	31 ± 6.1	30 ± 5.1	15 ± 6.4	27 ± 2.5	28 ± 2.6	19	25	25	9	15	12
30	50 ± 2.7	52 ± 1.6	54 ± 3.0	29 ± 1.2	38 ± 3.0	34 ± 3.3	33	43	43	18	27	22
45	56 ± 4.0	59 ± 2.3	58 ± 3.1	31 ± 1.9	43 ± 5.0	38 ± 2.8	45	55	55	25	37	31
60	67 ± 2.4	67 ± 2.5	64 ± 8.6	37 ± 6.8	49 ± 6.9	45 ± 3.0	54	64	64	31	45	37
90	69 ± 7.1	67 ± 3.9	66 ± 3.7	38 ± 6.8	50 ± 3.3	45 ± 2.2	66	75	74	42	57	48
120	69 ± 2.1	71 ± 1.3	71 ± 1.8	49 ± 3.0	62 ± 1.8	48 ± 2.9	74	80	79	49	66	55
180	93 ± 3.2	95 ± 3.9	93 ± 3.2	70 ± 1.8	82 ± 3.0	68 ± 3.7	82	84	83	60	75	62

Pearson correlation coefficients between model predicted and *in vitro* digestible CP of meals over 180 min of digestion

		In vitro digestible CP					
	Meals	CA	SBM	FM	CDDGS	CGM	PM
Model predicted digestible CP	CA	0.97 <.01[3]					
	SBM		0.97 <.01				
	FM			0.97 <.01			
	CDDGS				0.97 <.01		
	CGM					0.97 <.01	
	PM						0.95 <.01

[1]Model = A+B (1-e^{-kd*t}) where A, B and kd are CA = 16.93, 71.9 and 0.018; SBM = 6.5, 78.8 and 0.023; FM = 13.2, 70.6 and 0.024; CDDGS = 8.1, 66.8 and 0.009; CGM = 10.3, 72.7 and 0.013 and PM = 13.9, 55.2 and 0.013, respectively; [2]Means ± SD (n= 6 cages); [3]P value
CA = casein, FM = fish meal, PCM = porcine meal, SBM = soybean meal, CGM = corn gluten meal, CDDGS = corn distiller's grain with solubles.

3.5 Discussion

3.5.1 Colourimetry assay

The oxidative deamination of an amino acid to form Ruhemann's purple is a complex reaction with a wide absorbance spectrum (Bottom et al., 1978). The nitrogen from the amino acids is incorporated in the Bluish-Violet pigment after reacting with ninhydrin in the presence of Tin (II) chloride dehydrate as a reducing agent (Bottom et al., 1978). The full spectrum scan of this reaction reveals that all the samples tested had maximum OD reading at 568 so this OD was chosen as the OD for the colourimetry assay. Identifying this OD provides an opportunity to increase the assay to increase its sensitivity and precision in detecting the amino and carboxyl end of peptide bonds as they are broken during hydrolysis. It is possible that the first peak identified during the spectrum scan was as a result of intermediate products of the reaction.

An ethylene glycol sodium acetate base was chosen for the ninhydrin reagent because it provided a stable reagent and it is easy to make. The reagent does not require a nitrogen atmosphere and similarly it is not required for storage, unlike dimethyl sulfoxide base reagents (Moore, 1968). The ninhydrin reagent is susceptible to light during storage, and in this study, it took up to 14 d for the reagent to stabilize and provide a constant OD reading. If the reagent is stored in a dark sealed bottle, it can be stored up to 120 d and still give stable OD readings. Even though there was a reduction in the OD readings of the reagent over time, this would only be of significance if OD values from different digestion runs were being compared directly. The reagent is very sensitive in detecting α amino acids, and ammonia (Moore, 1968), so proper precaution must be taken to prevent amino acid or ammonia contamination of solutions used to make the reagent and buffers.

Due to the sensitivity of the reagent, the relationship between the concentrations of free α amino and carboxyl group in solution with the OD reading was linear from 2 µg to 400 µg. The maximum concentration from that range was at the upper limit of the detector in the spectrophotometer that was used in the study. This close relationship makes it possible to track changes in the hydrolysis of the CP samples over time as more free α amino and carboxyl groups are exposed. In theory, the OD intensity is directly proportional to the degree of hydrolysis, which has occurred as seen in Figure 3. If the OD from the total hydrolysis of an ingredient is known, the degree of hydrolysis can be calculated using the OD values. The very low detection limit of the reagent means any small change in the concentration of amino acid or available amino and

carboxyl side group will induce a large change in OD reading. This can produce large variation in the reading of a sample if pipetting is not accurate; therefore, it is advisable to read samples in triplicate when using the reagent as outlined in this colourimetry assay. Proper controls and blank samples should be run with every batch of samples that goes into the water bath in order to generate a correction factor for any change in temperature of the water bath during the assay.

3.5.2 Enzyme dose response assay

One of the most important elements of an enzymatic *in vitro* assay is the enzyme to substrate ratio at known enzyme activity (Boisen and Eggum, 1991). The pepsin dose response assay suggested that the greatest change in the degree of hydrolysis over the 30 min was between 14,130 and 28,260 units of pepsin to 500 mg of CP. When the pepsin concentration increased above 28,260 units the equivalent change in the degree of hydrolysis per unit of enzyme addition was reduced. Using a pepsin concentration which maximizes the hydrolysis acheived per unit of enzyme can help to develop the most economical assay.

Electrophoresis data of the gastric phase sample presented in Figure 3.5 suggests that the two tertiary structures normally seen in proteins extracted from soybean seed were subdivided into five major peptide and many smaller groups in the meal. The major shift in these peptides of the meal due to pepsin concentration was seen at the lower molecular weight 11S globulins 38-39 kDa compared to the 7S fractions 62-90 kDa. Similar results were observed by Yang et al. (2016) after peptic digestion of SBM isolated CP. The authors suggested that 11S glycinin is more susceptible to pepsin digestion because of its lower surface hydrophobicity and less β-sheet secondary structures (Yang et al., 2016). Never-the-less, electrophoresis and the colourimetry assay both show that hydrolysis had taken place after pepsin digestion of the SBM.

The digestion kinetic data obtained is dependent to a large extent on the shape of the digestion curve during the intestinal phase. The pancreatin concentration which produced a curve which fits the model proposed by Ørskov and McDonald (1979) and gave a value approximating *in vivo* CP digestion for SBM at the end of the digestion period were considered key criteria for the pancreatin concentration selection. Colourimetric testing of samples from the 7.5 mL and 9 mL pancreatin after 180 min gave OD values which were higher than the OD values of SBM sample total hydrolyzed (data not shown). This suggested that at those higher concentrations of pancreatin, there might have been auto-hydrolysis of enzyme after 180 min of digestion. Even after

240 min of intestinal digestion the 6.5 mL pancreatin did not produce OD values which were higher than those of the total hydrolysis sample. The 1, 3, and 5 mL enzyme concentration gave final digestible CP values below the 82% average *in vivo* digestibility for SBM (Ravindran et al., 2014), The 6.5 mL pancreatin was selected as the enzyme concentration for the intestinal phase, based on the shape of its digestion curve, extent of digestion mimicking SBM *in vivo* digestion and the stability of the enzyme after 180 min during the intestinal digestion phase,

3.5.3 In vitro assay validation

Based on fractional digestion rates (kd) values, SBM and FM can be classified as rapidly digested protein sources and CDDGS slowly digested. The kd value represented the rate at which faction (B) of the proteins were digested over time assuming that the process followed the first order of kinetics. The absolute digestion rate represents as (adr) is a different kind of measurement which assumed that the rate of digestion is linear. The data presented in Figure 3.6 suggested that the rate at which the proteins were digested followed the first order of kinetics which is typical of most biological reactions and therefore is a true representation of that process.

The animal based protein ingredients tend to have higher fraction (A). It is possible that this difference relates to a higher proportion of peptides or free amino acids in animal than plant based ingredients. Another reason for the difference between fraction (A) of plant and animal ingredients might relate to the nature of the proteins in these meals. Plants tend to store protein in vacuoles in cells which are often surrounded by a fibre matrix (Staswick, 1994), while animal proteins do not have a fibre matrix associated with the protein and there are also free amino acids and peptides present in extracellular space of animal tissue. These factors could have made the animal based proteins more susceptible to enzymatic hydrolysis than the plant proteins. Predicting fraction (A) produced higher variability in the plant based compared to animal based ingredients, but the reason for this is still to be determined.

The potential digestible CP was quite similar for all the ingredients except for PCM, which was lower than all the other samples. It is possible that the PCM meal has a higher elastin and collagen content, which would require more elastase to hydrolyze this meal than the 445.5 units present in pancreatin that was used. Porcine meal also tends to have high levels of arginine (Wang and Parsons, 1998), which could mean that more carboxypeptidase B is needed to break arginine bonds present in small peptides. Most likely, the processing conditions during the rendering

process could have damaged the PCM proteins, which makes them more resistant to digestion (Wang and Parsons, 1998).

The CDDGS potential digestible CP values were the second lowest of all ingredients evaluated, but they were in the range for *in vivo* values previously reported for CDDGS in broilers (Adedokun et al., 2015). Corn products like CDDGS are known to contain zein which is a prolamine that is insoluble in water and resistant to most proteolytic enzymes except alcalase (Shukla and Cheryan, 2001). The level of zein present in the protein fraction could reduce the digestible protein of CDDGS during gastric and pancreatic digestion. Apart from the zein content of the CDDGS, the drying process used during the postharvest of corn have been shown to reduce its protein digestibility (Barrier-Guillot et al., 1993).

3.5.4 Assay advantages and disadvantages

The *in vitro* assay presented in this work for measuring digestible CP is not the first of its kind. Other two stage *in vitro* methods have been previously described for measuring digestible CP in poultry (Clunies and Leeson, 1984; Ravindran and Bryden, 1999). The main problems with these assays lies in the length of the 4 h gastric digestion period, which is not representative of poultry *in vivo* digestion, the use of just a single enzyme and the lack of information pertaining to the activity of the major enzymes in the pancreatin used. All in vitro assays will suffer from various degrees of uncertainty due to the complexity of simulating the mechanism involved in the digestion of proteins. However, enzymatic digestion *in vitro* assays can provide meaningful characterization of feed ingredients (Ravindran and Bryden, 1999).

One of the major disadvantages of the current assay is that it requires a minimum of three people to collect the samples during the intestinal phase. The sequential timing of sample collection is affected by the length of time required for sample collection and processing. For example, the lowest sample interval that was achieved in the assay was 15 min with four people conducting the assay with 30 digestion tubes. Due to the sensitivity of the ninhydrin reagent, proper pipetting skills are needed, and all buffers and solution used in the assay must be free of ammonia, peptide, proteins and ammo acids. During the colour development stage of the assay, the water bath should always be at boiling to obtain consistent sample colour development.

Most i*n vitro* digestion methods suffer from some degrees of imprecision. The assay presented in this study has the following advantages, many samples can be analyzed in a short time

frame, it is relatively inexpensive and easy to perform in a basic animal nutrition lab, and no special training is needed to use required equipment. The assay can be easily transferred to an automated platform for running the entire assay. This would significantly reduce the number of personnel needed to collect the kinetic data. The level of precision between sample collection time intervals would be increased, and the timing interval could also be reduced below 15 min.

The digestibility assay was able to generate kinetic data for all the ingredients tested because their digestion curve over time all followed the first order kinetics plot. The model developed from the digestion constant was able to predict the *in vitro* digestible CP of the meals over time with a high degree of accuracy. However, it should be noted that the digestion constants generated for each meal only represent that specific sample and may not predict the response of other samples of the same ingredient. Based on the correlation coefficients in Table 2, it is safe to say that the constants generated from the model reflected the digestion characteristic of those samples tested.

3.5.5 Implication on future research

This study provides a basic assay, which can be used to generate kinetic data for high protein poultry feed ingredients in a short time frame. It is well known that protein digestion rate modulates tissue protein synthesis and deposition, but this process is still unknown in poultry due to the lack of kinetic data for high protein ingredients. Data from this assay can be used to develop diets for studying the metabolic response of poultry to specific ingredient digestion characteristics. More research is needed to test the assay inter-variability and to develop more precise digestion constants for each high protein ingredient, which would be representative of the ingredient and not the sample.

Conclusion

A multi-enzymatic *in vitro* protein digestion technique mimicking the chicken digestive tract was defined and validated. The effectiveness of the *in vitro* digestion technique was tested on a variety of high protein ingredients. The *in vitro* digestible protein assay predicted the rapidly, slowly and undigested protein fraction of ingredients, as well as the rate and extent of digestion of the proteins. The *in vitro* assay described in this study can be used to study the digestion kinetic of high protein ingredients fed to poultry.

Transition statement

The work completed in Chapter 3 outlined a poultry specific *in vitro* digestion assay, but only five high protein feed ingredients were evaluated in the validation study. Using the assay outlined in Chapter 3, nine high protein feed ingredients that were purchase in large quantities for this thesis *in vivo* research were evaluated in Chapter 4.

4.0 DIGESTION KINETICS OF PROTEIN SOURCES DETERMINED USING AN *IN VITRO* CHICKEN MODEL[2]

4.1 Abstract

In vivo assays are the main techniques used to evaluate high protein ingredients before diet formulation, but they are time consuming, expensive and do not generate digestion kinetics data. This paper presents digestion kinetics data for commonly available high protein feed ingredients using an *in vitro* model that mimics the gastric and intestinal phases of chickens. Soybean meal (SBM), corn gluten meal (CGM), corn distiller dried grains with solubles (CDDGS), porcine meal (PM), fish meal (FM), canola meal (CM), meat, and bone meal (MBM), feather meal (FEM), and blood meal (BM) were digested in 6 replicate tubes. Meal sample equivalent to 500 mg crude protein (CP) = (% N_2 x 6.25) was digested with 28,260 units of pepsin in 50 mL polyethylene centrifuge tubes for 30 min in a shaking water bath at 41°C. After gastric digestion, tube pH was adjusted to 7 ± 0.5 using NaOH. A 9.5 mL volume of sodium acetate buffer (pH 12.5) and 6.5 mL pancreatin, and 3 glass marbles were placed in the tube and then incubated for 180 min at 41°C in a water bath. Tubes were sampled at 0, 15, 30, 45, 60, 90, 120, 150, and 180 min of the intestinal phase and digestible (DIG) CP determined calorimetrically with ninhydrin reagent per time point. The DIG data were fitted to the Ørskov & McDonald (1979) model using PROC NLIN procedure of SAS 9.4 and all derived constants were analyzed using the PROC Mix procedure. The predicted DIG values (%) of the meals were SBM 87, FM 88, PM 79, CGM 73, MBM 56, CM 82, BM 47, FEM 48 and CDDGS 58. Estimated fractional protein digestion rate (kd) values for BM and FEM were 0.062 and 0.054, respectively while MBM, CGM, FM, PM, CM, SBM, and CDDGS were 0.046, 0.041, 0.040, 0.038, 0.035, 0.027, and 0.017, respectively. In conclusion, protein digestion

[2]This chapter is a modification of the contents from the following paper: D.D.L.S. Bryan, D.A. Abbott and H.L. Classen, 2018. Digestion kinetics of protein sources determined using an *in vitro* chicken model. Submitted to Animal Feed Science and Technology.

of high protein meals was determined with an *in vitro* technique, which provided the opportunity to categorize the ingredients based on their digestion kinetics as well as extent of digestion.

Keywords: protein digestibility, digestion rate, soybean meal, fish meal, canola meal

4.2 Introduction

Protein quality is important in poultry diets because its effects animal growth performance and potentially impacts bird health (Drew et al., 2004). The quality of a protein ingredient in poultry diets is often based on its amino acid balance and the general process by which that protein is hydrolyzed and absorbed in the gastrointestinal tract of poultry. These criteria have been studied extensively *in vivo* and reliable data exists on the extent of digestion for a number of ingredients. Similarly, a number of techniques have been used to assess the extent of protein (amino acid) digestion *in vivo,* with the standardized ileal digestibility assay widely used (Ravindran et al., 2017).

Because of the time and cost associated with conducting *in vivo* assays to estimate digestibility, moderately successful *in vitro* procedures have been developed (Fuller, 1991). These chemical methods or *in vitro* techniques, are less expensive and time consuming, but any metabolic response to the ingredient will be absent (Boisen and Eggum, 1991; Fuller, 1991). *In vitro* protein digestibility assays currently available for other monogastric animals take h to perform and digestion conditions are not specific to chickens (Fuller, 1991). Therefore, little is known about the *in vitro* digestion kinetics of high protein feed ingredient with poultry digestive specifications.

Current *in vivo* and *in vitro* assays, which simulate digestion of commercially relevant monogastric animals have not focused on the location and kinetics of protein digestion, and how that varies with protein source and samples. However, the *in vivo* rate of protein digestion is not well studied in chickens for a number of reasons. *In vivo* evaluation of protein digestion kinetics in poultry has limitations and challenges due to the small size of the bird's digestive tract, plus difficulty in repeated digesta collection along the digestive tract. *In vivo* techniques often require that birds are killed for sample collection (Wilson and Leibholz, 1981), and they require time for animal growth and development. *In vivo* assays require more resources, and require animal

research ethics approval, which can be difficult to obtain if the experimental protocol contains invasive techniques.

The location of protein digestion can be important for poultry because of its significant impact on the bird's ability to synchronize the provision of dietary amino acids for protein synthesis (Sklan and Hurwitz, 1980). For example, in meal fed chickens the rate at which dietary amino acids are digested, absorbed and become available in the blood determine if they undergo decarboxylation or if they are used directly for protein synthesis (Nonis and Gous, 2006). The location where dietary amino acids and peptides are released as the feed moves along the digestive tract can also influence their availability to the animal. Some microorganisms residing in the gastro-intestinal tract of poultry have similar amino acid requirements to chickens and may compete for digested amino acids and peptides (Apajalahti and Vienola, 2016). The amount of protein entering the distal ileum can influence the hindgut fermentation metabolites, some of which are thought to negatively impact bird health (Barnes et al., 2001)

Little is known about the rate at which high protein feed ingredients used in poultry rations are digested. Data from human studies suggest that protein digestion rate modulates tissue protein synthesis and deposition, where rapidly digested protein stimulated protein synthesis by 68% and slowly digested protein inhibited body protein breakdown by 34% (Boirie et al., 1997). Protein digestion rate is also a regulator of postprandial protein retention, where diets with slowly digested protein seem to have better postprandial utilization than rapidly digested proteins (Dangin et al., 2001). Before any benefits of protein digestion rate of ingredients can be applied to poultry nutrition, avaliable feed ingredients must first be characterized based on their digestion kinetics.

An *in vitro* poultry digestion model that estimates digestibility as well as protein digestion kinetics has been developed in previous research (Chapter 3). The objective of this study was to apply this procedure to an array of protein sources used in poultry feeding. It was hypothesized each ingredient will have unique digestion kinetics consistent with their known digestibility characteristics and chemical properties which can be used to formulation feed to better understand the impact of these protein sources in poultry feeding.

4.3 Material and methods

4.3.1 *Test meals*

The meals used in this study were soybean meal (SBM), meat and bone meal (MBM), fish meal (FM), porcine meal (PM), blood meal (BM), feather meal (FEM), canola meal (CM), corn gluten meal (CGM), and corn distiller dry grains with solubles (CDDGS). All meal samples were analyzed in duplicate except for analysis of amines. Minerals were analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES) after total acid digestion with HCl. Protein sources were analyzed for N_2 using a Leco nitrogen analyzer (Model 601–500–100, Serial # 3211, Leco Corporation, St. Joseph, MA, USA) according to the combustion method (AOAC, 2006; Method 990.03); 6.25 was used to convert N_2 to CP. Meals were also analyzed for protein dispersibility index (PDI; Johnson, 1970), protein solubility in 0.2% potassium hydroxide solution (Araba and Dale, 1990) and reactive lysine by AOAC Method 975.44. Soybean meal was analyzed for trypsin inhibitor activity using AOAC Method 22-40.

Polyamine and biogenic amines were measured in BM, CM, CDDGS, CGM, FEM, FM, MBM, PM, and SBM by LC/MS/MS at the Analytical Facility for Bioactive Molecules (The Hospital for Sick Children, Toronto, Canada). One gram from each samples was extracted with perchloric acid then neutralized with NaOH. For polyamine analysis the neutralized samples were extracted with methanol and pH adjusted with sodium carbonate. Samples were derivatized with isobutyl chloroformate then suspended in ethyl ether and the supernatants were dried under N_2 at 35°C. Samples and standards were reconstituted in 1 mL (80:20, water: acetonitrile +0.1% formic acid) and separated on a Agilent 1290 LC system coupled to a Sciex Q-Trap 5500 mass spectrometer using a Kinetex XB-C18 2.6u 100A 50 x 3.0 mm column (Phenomenex). Samples were eluted using a gradient of solution A (water +0.1% formic acid) and solution B (acetonitrile +0.1% formic acid) over the following gradient: 0 min = 15% B, 2 min = 15% B, 3.5 min = 90% B, 4.5 min = 90% B, 4.6 min = 15% B and 6 min = 15% B.

Biogenic amines were extracted from neutralized samples with acetonitrile/methanol (90:10), then taken to dryness under a gentle stream of nitrogen. Samples were reconstituted into 1 mL of solution containing acetonitrile/methanol (90:10 + 0.1% formic acid). The samples were then separated with an Agilent 1200 LC system coupled to a Sciex Q-Trap 5500 mass spectrometer using a Kinetex HILIC 2.6u 100A 50 x 4.6 mm column (Phenomenex). Samples were eluted via

gradient separation over 11 min using solution A composed of (90/10 water/acetonitrile 5 mmol ammonium formate pH 3.2) and solution B composed of (10/90 water/acetonitrile 5 mmol ammonium formate pH 3.2). The following gradient was used for sample elution: 0 min = 100% B, 1 min = 100% B, 3 min = 5% B, 3.5 min = 5% B, 3.6 min = 100% B and 11 min = 100% B.

4.3.2 *Reagents and chemicals used*

The reagents used in this study and their sources were Tin (II) chloride dehydrate (CAS 107-21-1), benzoic acid (CAS 65-85-0), glacial acetic acid (CAS 64-19-7), trichloroacetic acid solution (Sigma T0699), hemoglobin (Sigma H2625), pepsin (P7125-100g; CAS 9001-75-6 647-008-00-6), Nα-benzoyl-L-arginine ethyl ester (Sigma B4500), Trizma® Base (Sigma T1503), N-benzoyl-L-tyrosine ethyl ester (Sigma B6125), methanol (Sigma M1775), N-succinyl-ala-ala-ala-p-nitroanilide (Sigma S4760), and ninhydrin (CAS 485-47-2) were obtained from Sigma Chemical Co. (St. Louis, MO). Ethylene glycol (CAS 107-21-1), sodium hydroxide (CAS 1310-73-2), sodium acetate trihydrate (CAS 6131-90-4), calcium chloride (CAS 10035-04-8), guar gum (CAS 9000-30-0), and hydrochloric acid (7647-01-0) were obtained from Fisher Scientific (Pittsburgh, PA). Liquid bovine pancreatin (62,500 USP trypsin units/mL) was purchased from RENCO (10 London Street, Eltham 4322, New Zealand).

4.3.3 *Ninhydrin reagent and buffer composition*

The 4 N sodium acetate buffer was prepared by dissolving 544 g of sodium acetate trihydrate in 100 mL of warm glacial acetic acid and then Millipore water was added to make a total volume of 1000 mL. The tin (II) chloride solution was prepared by adding 1.2 g to 12 mL of ethylene glycol followed by vortexing to dissolve all solids. To prepare the ninhydrin reagent, 9.75 g of ninhydrin was dissolved in 366 mL ethylene glycol, then 122 mL 4 N sodium acetate buffer was added. This solution was mixed for 5 min with a magnetic stir bar and then 12 mL of tin (II) chloride solution was added before mixing for another 5 min. One liter of a 10 mM HCl solution was prepared by mixing 833 µL of concentrated HCl with 999,167 µL of Millipore water. A 0.1 M calcium chloride solution was prepared by dissolving 33.3 g of calcium chloride in 300 mL of Millipore water. Benzoic acid solution was prepared by dissolving 5.8 g of benzoic acid into 2 L of Millipore water. For the preparation of sodium acetate buffers, 27.2 g of sodium acetate trihydrate was dissolved in 500 mL benzoic acid solution. The pH was adjusted to 12.5 or 6.5 using saturated sodium hydroxide solution (50% wt/wt), then the volume of the solution made up to 2 L

with Millipore water followed by the addition of 8 mL 0.1 M calcium chloride solution. All buffers were stored in the refrigerator until use.

4.3.4 *In vitro digestion*

The *in vitro* assay was performed according to the procedure described in detail in Chapter 3. Ingredient samples were ground using a Retsch Ultra Centrifugal Mill ZM 200 (Haan, Germany) to pass through a 0.5 mm screen. To establish assay values representing 100% hydrolysis, a 500 mg CP equivalent of the samples was weighed and placed in individual 100 mL Pyrex® glass bottles (No.14395); 6 N HCl was then added at 4 mL per 100 mg of sample. The samples were gently mixed by swirling, capped and placed in an oven at 110°C for 24 h. After 24 h of hydrolysis, samples were allowed to cool to room temperature and then filtered through a Whatman Grade 601 filter paper. An aliquot of the sample was collected after filtering and the pH adjusted to 7 ± 0.5 with sodium hydroxide. The filtered sample was diluted with Millipore water to give 18.8 mg CP per mL using the initial 500 mg CP of the sample that was hydrolyzed.

Enzymatic digestion was conducted using 500 mg CP (N_2 x 6.25) equivalent of each meal sample was placed in 50 mL centrifuge tubes with 50 mg of guar gum and 8.5 mL of 10 mM HCl solution. The tubes were vortexed to saturate and evenly mix the sample with the 10 mM HCl solution. After mixing, 1.5 mL pepsin solution containing 28,260 units of pepsin was added to 6 replicate tubes per sample and 3 blank tubes. All tubes were vortexed and placed in a shaking water bath (150 strokes/min; 30 mm stroke length) at 41°C for 30 min. At the end of 30 min, 500 µL volume of 4.9 N sodium hydroxide solution was added to each tube immediately after removal from the water bath. A 9.5 mL volume of sodium acetate buffer (pH 12.5) was placed in each tube, and the pH was adjusted to pH 7.5 with 6 N HCl or 4.9 N sodium hydroxide solution. A 6.5 mL volume of pancreatin solution from RENCO (Eltham, New Zealand) which contained 30,667 BAEE units/mL trypsin, 2,157 BTEE units/ mL chymotrypsin and 7 units/ mL elastase was added to the respective tubes to make the final volume of 26.5 mL. Three marbles (16 mm diameter) were placed in each digestion tube and then the tubes were placed on their sides in a shaking water bath (150 strokes/min; 30 mm stroke length) at 41°C for 180 min. All tubes were vortexed and a 0.5 mL sample was taken from the tubes at 0, 15, 30, 45, 60, 90, 120, 150, and 180 min. The 0.5 mL sample was placed in tubes containing 10 mL 10 mM HCl solution and then vortexed. After 2-3 min, 10 mL of sodium acetate buffer (pH 6.5) was added to the mixture; then samples were later used in the colourimetric assay.

After adding the 10 mL 10 mM HCl and 10 mL of sodium acetate buffers (pH 6.5) to each 0.5 mL of collected sample, the tubes were centrifuged at 3000 rpm for 10 min. A subsample of 100 µL was taken from each tube after centrifugation. A 100 µL subsample was also taken from the samples previously hydrolyzed to 100% with HCl at 110°C for 24 h. Each 100 µL subsample was mixed with 1900 µL of Millipore water and 1000 µL of ninhydrin reagent in disposable glass culture tubes (Borosilicate glass 16 x100 mm, NO. 14-961-29). A blank sample with 2000 µL of Millipore water and 1000 µL of ninhydrin reagent was prepared. Glass marbles were placed on top of each tube then the tubes were placed in a boiling water bath for 15 min. The tubes were allowed to cool for 10 min then read at 568 nm on a Genesys 20 Spectrophotometer UV-Vis (Thermo Fisher Scientific Inc., Massachusetts, USA) using 4.5 mL disposable polystyrene standard cuvettes.

4.3.5 Calculations and statistics

The digestible protein of the samples was calculated using the OD of the digested sample and the OD of the totally hydrolyzed sample as follow:

$$\text{Digestible protein} = \frac{\text{OD of digested sample}}{\text{OD of 6N HCl hydrolyzed sample}} \times 100 \quad \text{……..……...……(4.3.5.1)}$$

where OD= the absorbance at 568 nm.

The absolute CP digested per min (adr) was calculated using the following formula:

$$\text{adr} = \frac{\text{digestible CP @ time(x)} - \text{digestible CP @ time(y)}}{time(x) - time(y)} \quad \text{…….……..…(4.3.5.2)}$$

Where x and y represent different time points during the 180 min digestion period.

Using the PROC NLIN procedure of SAS 9.4 all the digestible protein at each sampling interval over 180 min of digestion and the sampling interval times were fitted to the following statistical model proposed by Ørskov & McDonald (1979): $P=A+B\ (1-e^{-kd*t})$……....(4.3.5.3)

Where P = digestible CP at a specific time point during the 180 min of digestion, A = rapidly digestible CP fraction, B = CP fraction that was digestible over time, kd = the rate at which B fraction was digested over time (fractional digestion rate). The kd constant was set to negative since the data represented increasing protein digestion over time, and t = Time. The undigested fraction of the protein (UD) was calculated as 100-(A+B), and the potential digestible protein (PD) was calculated as A+B. The each component of the kinetic data set were analyzed as the response variable in a completely randomized design where each meal represented a treatment using the

Proc Mixed procedure of SAS 9.4 with probability of $P \leq 0.05$ considered significant. If significant differences were found between means, LSD means statement was used to separate treatment means.

4.4 Results

The nutrient composition, physiochemical properties and amine composition of the meals are presented in Table 4.1. These analyses were completed to provide a general overview of the nature of the samples used in this assay. Comparing CP content of the samples to National Research Council (1994) values, suggested that they were representative samples of those ingredients. The amine data suggested that the histamine levels of the FM was unusually higher than what was reported previously for fish meal, however all other ingredients amine levels were in the normal range (Brinker et al., 2003).

Table 4.1. Nutrient and amine composition, and physiochemical properties of high protein feed ingredients.

	Meals								
Nutrient composition	BM	CM	CDDGS	CGM	FM	FEM	MBM	PM	SBM
Moisture (%)	3	10	12	9	11	8	6	5	11
CP (%)	96	38	30	64	71	80	51	61	48
Ca (%)	0.10	0.70	0.06	0.10	3.54	0.79	5.66	4.32	0.50
P (%)	0.22	1.04	0.79	0.35	2.14	0.50	2.96	2.62	0.66
Na (%)	0.32	0.03	0.18	0.08	1.34	0.19	1.04	0.55	0.04
K (%)	0.19	1.29	1.11	0.15	1.23	0.25	0.71	0.87	2.22
Mg (%)	0.02	0.58	0.34	0.05	0.33	0.04	0.14	0.22	0.29
Zn (ppm)	35	55	56	187	94	159	125	215	93
Mn (ppm)	ND	69	15	20	16	5	14	45	85
Cu (ppm)	8	6	3	16	7	9	8	32	22
Fe (ppm)	2476	165	68	122	594	194	519	635	132
Amine content (ng/mg)									
Histamine	0.6	0.14	1.70	63.13	2040	5	28	87	0.18
Serotonin	0.04	0.07	0.45	0.05	ND	ND	ND	0.33	0.09
Tryptamine	1.2	0.12	0.99	4.42	4	0.13	3	4	0.16
Tyramine	3.4	0.21	2.03	196	292	4.7	54	89	0.35
Agmatine	0.29	0.73	0.48	0.57	214	0.63	5	13	15.21
Putrescine	46	3.02	79.77	12.53	380	29	116	374	12.74
Cadaverine	21	ND	3.570	3.88	880	28	195	643	16.38
Spermidine	2	51	42	6	42	10	11	23	144
Spermine	4	11.02	15.82	7.35	20	7	14	21	24.14
Physiochemical properties									
PDI (%)	0.4	11	15	4	32	9	11	25	15
KOH (%)	5	38	26	28	45	15	20	39	78
Reactive lysine (%)	7.27	1.78	0.75	0.97	4.63	1.59	2.62	2.98	2.72

BM = blood meal; CM = canola meal; CDDGS = corn distiller dry grains with solubles; CGM = corn gluten meal, FM = fish meal; FEM = feather meal; MBM = meat and bone meal; PM = porcine meal; SBM = soybean meal; PDI = protein dispersibility index; KOH solubility in 0.02% potassium hydroxide; ND = non detected;

The *in vitro* digestion kinetics data for protein meals are presented in Table 4.2. As indicated by fraction A, a higher proportion of MBM, FM, and BM proteins were digested earlier during the digestion process when compared to CDDGS, CGM, and FEM, while CM, PM, and SBM values were intermediate. As digestion continued over time, which is represented by fraction B, FM, SBM, CM, and PM had the greatest proportion of CP digested over time when compared to CDDGS, MBM, FM, and BM, with CGM being intermediate and not different from CM and PM. At 88 and 89% respectively, SBM and FM potential digestible CP values (which represent the sum of fractions A and B) were higher than all the other meals. Canola meal and PM potential digestible CP values were similar and both were higher than CGM. CDDGS, MBM, FM and BM. The values for potential digested CP for MBM and CDDGS were not different, but were higher than FEM and BM. Blood meal had the lowest potentially digested CP, which was lower than FEM. At 180 min, the *in vitro* digestible CP of the meals showed the same trends as seen in the potential digestible values, but values were slightly higher than those generated from modeling the data. The values ranged from a low of 49% for BM to a high of 91% for SBM and FM.

Fraction UD represents the undigested fraction of the CP, which is calculated as the difference between 100 and the potential digested fraction. Feather meal and BM had larger portions of CP that were not digested at the end of the assay when compared to the other meals. Fish meal and SBM had the lowest undigested CP fraction when compared to the other meals. Following FM and SBM, CM and PM undigested fractions were the same but lower than CDDGS and MBM whose undigested fractions were similar. The undigested fraction of CGM was intermediate between that of PM and MBM, but statistically different.

Table 4.2. *In vitro* digestion kinetics of nine high protein feed ingredients.

	Meals										
Constants	BM	CM	CDDGS	CGM	FM	FEM	MBM	PM	SBM	SEM	ANOVA *P* value
A (%)	14.9^{ab}	8.1^{bc}	6.1^{c}	5.2^{c}	10.2^{abc}	7.3^{c}	16.6^{a}	8.2^{bc}	9.0^{bc}	2.4	<.01
B (%)	26.6^{e}	74.3^{ab}	54.6^{c}	68.1^{b}	78.2^{a}	40.4^{d}	39.8^{d}	70.7^{ab}	78.5^{a}	2.7	<.01
PD (%)	41.1^{f}	82.6^{b}	60.7^{d}	73.4^{c}	89.2^{a}	49.0^{e}	59.7^{d}	79.2^{b}	88.1^{a}	1.4	<.01
UD (%)	58.9^{a}	17.4^{d}	39.3^{b}	26.6^{c}	10.7^{e}	51.0^{a}	41.8^{b}	20.7^{d}	11.8^{e}	2.0	<.01
Kd (h^{-1})	0.062^{a}	0.035^{d}	0.017^{f}	0.041^{cd}	0.040^{cd}	0.054^{ab}	0.046^{bc}	0.038^{cd}	0.027^{e}	0.004	<.01
Adr (%/min)	0.179^{e}	0.453^{ab}	0.330^{c}	0.432^{ab}	0.459^{ab}	0.253^{d}	0.204^{e}	0.426^{b}	0.478^{a}	0.015	<.01
					In vitro digestible CP (%)						
Time	BM	CM	CDDGS	CGM	FM	FEM	MBM	PM	SBM		
0	14 ± 3.6	4 ± 2.1	1.7 ± 0.6	3 ± 1.5	8 ±5.3	10 ± 5.9	16 ± 6.3	6 ± 3.2	8 ± 1.0		
15	30 ± 3.0	48 ± 2.0	24 ± 1.0	43 ± 3.1	54 ± 2.1	34 ± 1.1	43 ± 3.8	47 ± 2.0	46 ± 4.0		
30	36 ± 4.2	56 ± 4.7	30 ± 1.7	52 ± 4.9	64 ± 3.2	40 ± 3.1	46 ± 4.1	53 ± 3.0	53 ± 6.8		
45	38 ± 5.0	67 ± 1.0	37 ± 1.0	62 ± 3.0	71 ± 1.2	43 ± 2.2	49 ± 5.0	62 ± 2.3	64 ± 2.1		
60	38 ± 0.7	67 ± 3.1	39 ± 3.1	65 ± 2.5	81 ± 5.3	46 ± 5.5	55 ± 12	74 ± 7.5	66 ± 2.5		
90	39 ± 4.1	74 ± 4.7	45 ± 4.9	67 ± 3.5	82 ± 2.1	48 ± 2.7	57 ± 3.0	75 ± 3.8	77 ± 3.2		
120	47 ± 6.7	80 ± 0.6	52 ± 2.6	68 ± 1.5	85 ± 3.8	52 ± 3.3	59 ± 3.9	75 ± 0.5	84 ± 4.5		
150	48 ± 5.3	84 ± 3.6	56 ± 4.0	72 ± 0.5	90 ± 2.1	54 ± 2.6	61 ± 3.1	79 ± 0.5	84 ± 0.6		
180	49 ± 5.5	86 ± 3.6	61 ± 2.7	80 ± 4.9	91 ± 3.0	55 ± 2.9	56 ± 5.1	82 ± 1.5	91 ± 1.0		
					Predicted digestible CP (%)						
180	43	82	58	73	88	48	56	79	87		

[a-e] Means (n = 6 tubes) within a row with common superscripts are not significantly different at $\alpha \leq 0.05$.
SEM= pooled standard error of means.
BM = blood meal; CM = canola meal; CDDGS = corn distillers' dry grains with solubles; CGM = corn gluten meal, FM = fish meal; FEM = feather meal; MBM = meat and bone meal; PM = porcine meal; SBM = soybean meal.
A = rapidly digested CP fraction; B = slowly digested CP fraction; PD = potentially digested CP fraction (A+B); UD = undigested CP fraction; kd = the rate at which B is digested over time; adr = absolute percentage of CP digested per min.

The fractional digestion rate (kd) represents the rate at which fraction B was digested over time. Blood meal and FEM had similar fractional digestion rates, which were higher than PM, SBM, FM, CGM, CDDGS and CM. Feather meal and MBM fractional digestion rates were not different. Meat and bone meal, PM, FM, and CGM had similar fractional digestion rates which were higher than CDDGS and SBM. The CDGGS sample had the lowest fractional digestion rates of all the meals evaluated. The absolute percentage of CP which was digested over time (adr) was calculated by dividing the difference between the *in vitro* digestible CP at 180 min and 0 min by the 180 min value. The adr values ranked the meals in a similar way as their potential digestibility. Figure 4.1 shows the *in vitro* digestible CP of the nine meals during the 180 min *in vitro* digestion time. At 15 min of digestion FM, SBM, CM, PM and CGM separated from the other meals to form a group, which had higher digestible CP as digestion progressed over time.

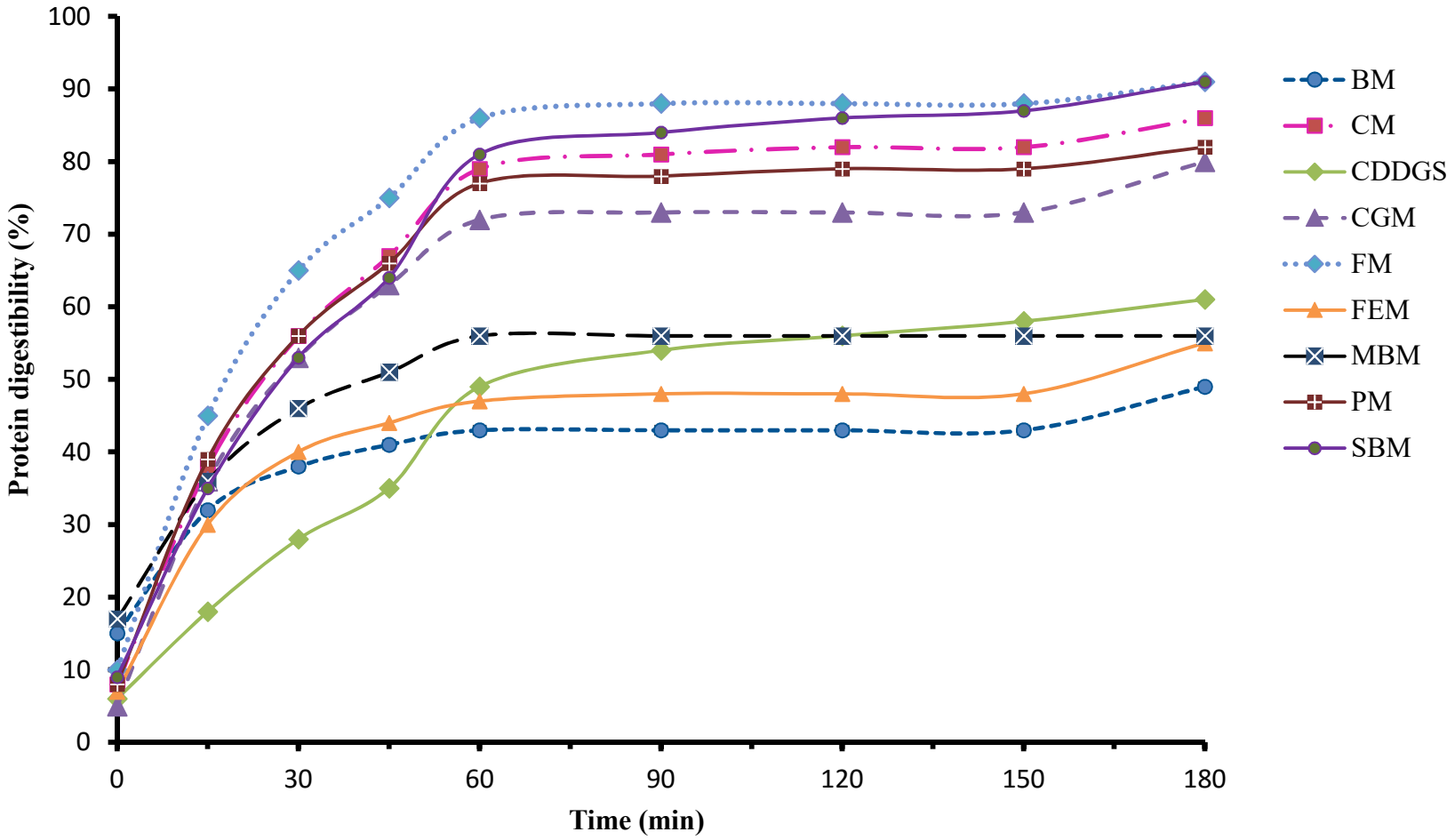

Figure 4.1 *In vitro* digestible protein of high protein meals (n = 6 reps per meal) during 180 min of digestion in the intestinal phase of a chicken *in vitro* model. BM = blood meal; CM = canola meal; CDDGS = corn distillers' dry grains with solubles; CGM = corn gluten meal, FM = fish meal; FEM = feather meal; MBM = meat and bone meal; PM = porcine meal; SBM = soybean meal.

4.5 Discussion

The nutrient composition and physiochemical properties of protein sources were measured to provide some data on their nutritional value and to indicate if the samples are representative of the ingredient. The CP values for all the meals were within 10% of values shown in National Research Council (994). The mineral contents were more variable but in general followed ingredients trends. Ingredients properties such as typsin inhibitors, physiochemical properties and mineral content can influence protein source hydrolysis *in vitro* (Boisen and Eggum, 1991).

Trypsin inhibitors are well known to impede CP digestibility (Clarke and Wiseman, 2005). However, the level of trypsin inhibitors in the SBM sample used in this work (4335 TIU/g) was relatively low and within the range expected for SBM produced in North America (Loeffler et al., 2013; Ravindran et al., 2014). It is also possible that trypsin inhibitor effects might be less importance for *in vitro* digestion models such as the current work, which tend to use relatively high levels of digestion enzymes (McGinnis and Menzies, 1946).

Protein dispersibility index and CP solubility in potassium hydroxide both assess the solubility of proteins, which can influence the ability of enzymes to access the proteins during *in vitro* digestion. Physiochemical properties such as PDI and CP solubility have been used extensively as assessment tools to evaluate the quality of meals (Batal et al. 2000), but they can be biased towards a specific ingredient and therefore are more applicable for evaluating multiple samples of the same ingredient. Despite compelling data from the literature that these assays may not be good indicators of protein bioavailability for some ingredients (Parsons et al., 1991; Batal et al., 2000; Newkirk, 2002) the feed industry continues to use them as broad spectrum protein quality assessment tools.

Protein dispersibility index and solubility values can give information about the condition under which meal samples were prepared. A notably low solubility was found for BM using PDI or solubility in potassium hydroxide. Blood meal solubility values and its DM suggest that the meal was drum dried instead of spray dried. The drying process could have altered the tertiary structure of the native proteins present in the sample, and thereby reduced protein solubility (Doiron et al., 2009). Alteration of the protein structure could lead to reduced hydrolysis and therefore the low *in vitro* digestible CP of that meal.

Reactive lysine is an estimation of how much lysine is available to the animal from a protein source based on the amount of ε-NH2 groups of lysine present in the sample. The reactive

lysine for blood meal was very high when compared to the other meals. This may be due to the fact that BM contains twice as much lysine as the other meals and as such the assay was biased towards BM. Another example of how assays based on physiochemical properties can be biased is the CP solubility assay using potassium hydroxide. This assay was developed specifically for SBM, so it is no surprise that SBM had the highest solubility values. Despite SBM high solubility, both SBM and FM had comparable *in vitro* digestible CP values, but FM solubility was almost one-half that of SBM. It is difficult to use physiochemical properties to predict the *in vitro* digestible CP outcome when evaluating different kinds of ingredients.

Fish meal, MBM, and PM calcium levels were numerically higher than the other meals. The mineral content of meals especially calcium and magnesium can introduce high buffering capacity during *in vitro* digestion (Boisen and Eggum, 1991). This effect was not observed in this assay due to the low level of FM, MBM and PM used compared to the high volume of buffer used for the hydrolysis process during the intestinal phase of the assay. This was confirmed to be the case based on the pH readings taken during the gastric and intestinal phase of the assay.

The amine content of meals can be very important from a meal quality perspective, but are of lesser importance during *in vitro* digestion. High levels of putrescine, tyramine, and cadaverine can be used as an indicator of protein putrefaction by microbes (Lazaro et al., 2014). Microbial growth is usually eliminated during the rendering process when animal by products are produced. The presence of amines in the meals after rendering could be related to the quality of the raw materials used or the length of time the ingredients were in storage after inadequate rendering. The presence of 1000 to 2000 mg/kg dietary histamine or cadaverine in poultry diet has been shown to increase the incidence of proventricular ulcers (Barnes et al., 2001). The highest levels of histamine 1620 mg/kg, putrescine 1340 mg/kg and cadaverine 1350 mg/kg detected in North American and Australian rendered animal by-products were considered too low to be of commercial significance (Bermudez and Firman, 1998; Brinker et al., 2003).

Of all the animal by-products, FM had the highest levels of histamine 2040, tyramine 292, agmatine 214, putrescine 380 and cadaverine 880 mg/kg, but only histamine levels were higher than values previously reported for FM in North America and Australia (Bermudez and Firman, 1998; Brinker et al., 2003). The purchasing history of the FM sample used in this study indicated that the meal was imported and not produced in North America. High levels of amines in the meal samples could lead to false positives during the colourimetry assay since amines will actively bind

to ninhydrin (Bottom et al., 1978). Because of concern about possible interference of amines from the FM sample with the colourimetry assay, the OD values for a digested FM sample known to have low total amine was compared to the OD values of the FM sample used in this study. The OD values were similar for both FM samples after they were digested *in vitro*. One advantage of this *in vitro* assay is the small amount of sample needed for the colourimetry assay and the original sample gets diluted more than 100 fold before it is used in the colourimetry assay. This low sample volume and high dilution of the original sample makes it possible to dilute any compound present in the ingredients which could have interfered with the colourimetry assay.

The SBM and FM evaluated in this study were highly digestible, in agreement with *in vivo* AA digestibility studies in chickens (Lemme et al., 2004). Slightly lower digestibility was found for CM, PM, and CGM. Corn distiller dry grains with solubles, BM, FEM, and MBM meals had the lowest extent of digestion and these meals tend to have low *in vivo* digestibility (Lemme et al., 2004; Adedokun et al., 2015). Figure 4.1 shows the visual separation and classification of the meals into two distinctive groups (quickly and slowly digested) based on their time dependent digestion. The mean predicted potential digestible CP (180 min) of the meals were a few points lower than the mean determined digestible CP of the meals except for MBM meal, but all values were within the range of the standard deviation of the determined values. Except for CGM, the *in vitro* assay ranking of the meals based on their potential digestible CP had a similar trend to those found *in vivo* (Lemme et al., 2004; Adedokun et al., 2015).

Corn gluten meal had unusual behaviour during the gastric phase of the assay in which solubility of the meal was very low and this persisted to some extent in the intestinal phase of digestion. The initially low solubility of the meals led to a low digestible CP of CGM which might have needed extended intestinal digestion time beyond 180 min. Therefore, CGM may not have given reliable *in vitro* results on the extent of digestion at 180 min, which means that the assay might need some modification when assessing GCM samples.

Fraction A, which represents the fraction of protein that was rapidly hydrolyzed, was high for BM, FM, and MBM. This suggests BM, FM, and MBM had a portion of their protein which can be easily accessed by proteolytic enzymes during the first few min of the digestion process, particularly when compared to CGM and CDDGS. Cells of animal tissues are enclosed by a simple lipid bilayer which can easily be disrupted by the action of processing and temperature during the manufacturing of animal by-products into meals. Animal cells tend to have gaps between them,

which are filled with extracellular fluid containing free amino acids and peptides of various lengths. Plant tissues on the other hand have no extracellular fluid instead, the cell membrane containing the cytoplasm are encapsulated in a cell wall made of pectin, cellulose and hemicellulose. The proteins in plants tissue are stored inside vacuoles, which are located in the cell cytoplasm. Since BM, FM, and MBM are of animal origin, they might contain more protein in the form of small peptides which can quickly become accessible to enzyme hydrolysis during the first few min of the digestion process.

It is also possible that solubility may have contributed to FM having a large fraction A since it had a high PDI, however the low PDI for BM and MBM are not in agreement with this possibility. Alternatively, the high fraction A values might be related to how each meal responded to pepsin hydrolysis during the gastric phase of the assay. Time zero of the intestinal phase presented in Table 4.2 shows that of the nine meals, MBM and BM had the highest pepsin digestibility.

The *in vitro* fractional digestion rate (kd) represents the theoretical nonlinear rate at which fraction B was digested over time. Even though BM, FEM, and MBM meal had very low total digestion, the available proteins were digested at a faster rate compared to the other meals. Information about the kd values of the high protein meals used in this study is virtually non-existent in the literature for poultry or other monogastric animals *in vivo* or *in vitro*. However, it has been reported previously that BM and MBM had higher kd values compared to FM and CGM using an *in vitro* ruminant model, but the FEM kd value was lower than FM and CGM (Hernández et al., 2002). There is not enough data in the literature comparing the digestion rate of BM, MBM, FM and FEM to conclude that the *in vitro* rate seen for those meals in the current study were ingredient specific. More research is need to evaluate more samples of ingredients before a conclusion can be made about the digestion rate characteristic of specific ingredients.

It has been previously shown that FEM digestibility varies depending on the processing condition during rendering (Latshaw et al., 1994). The FEM sample used in the present study had undergone steam and pressure hydrolysis during the rendering process, which may have been different for the meals used by Hernández et al. (2002). Steam and pressure hydrolysis have been shown to increase pepsin digestibility of feather meal (Latshaw et al., 1994). This processing method could have contributed to a larger amount of smaller peptides within the digestible CP fraction of the FEM which were quickly hydrolyzed during the digestion process. The absolute

digestion rate (adr) of the meals ranked the meals in a different way from that of the kd values, and this was directly related to the extent of digestion of the meals. The adr assumed that the time dependent digestion of the meals was linear, but Figure 4.1 shows nonlinear digestion curves. As such the adr values might not give a true representation of the meal digestion rates.

The digestion constants presented in this study represent the unique digestion characteristics of the meals samples which were analyzed using a chicken *in vitro* model. At this stage, it is not possible to conclude that the digestion constants for each ingredient presented in this study are representative of each ingredient. For example, not all SBM will have a PD fraction of 88.1%. Larger sample numbers per meal should be evaluated before a stronger conclusion can be made about the general kinetic properties of a single ingredient. However, the data of this study does confirm that ingredients vary in their digestion kinetics. Further, ingredients could be classified based on their digestion kinetic.

Formulating diets using the kinetic data of the protein sources could modulate biological response in poultry such as protein synthesis (Boirie et al., 1997; Dangin et al., 2001). It has been known that the rate of digestion of other macro nutrients such as carbohydrates can influence the growth performance of boilers chicken (Gutiérrez del Álamo et al., 2009b). The kinetic data from the present study highlighted the unique properties of the meals, but the *in vivo* biological consequence of those properties are yet to be determined.

Conclusion

The data presented in this study provides a basic foundation for building knowledge about the digestion kinetics of high protein feed ingredients for poultry. The *in vitro* model serves as a tool which can be used to evaluate rate and extent of digestion of high protein ingredients rate and extent of digestion quickly and economically. The kinetics data can be used as a tool to manipulate ration formulation to explore the potential metabolic and physiological response of poultry to specific kinetic parameters of ingredients predicted by the *in vitro* model.

Transition statement

Chapters 3 and 4 evaluated the *in vitro* digestion characteristic of nine high protein source in an attempt to understand the *in vivo* digestion behaviour of those ingredients. The work outlined in Chapter 5 evaluated similar digestion characteristic as Chapters 3 and 4 using and *in vivo* approach and the same samples as those evaluated in Chapter 4.

5.0 *IN VIVO* DIGESTION KINETICS OF PROTEIN SOURCES FED TO POULTRY

5.1 Abstract

The rate and extent of protein digestion are relevant to broiler performance and health, but information is lacking on the rate of digestion and the characteristics of the undigested fraction for common high protein feed ingredients. Therefore, this study evaluated the digestion kinetics and the distal ileum (DI) digesta protein characteristics of protein meals fed to broiler chickens. Using a completely randomized design, 360 male broilers at 14 d of age were assigned to 60 battery cages and fed semi-purified diets composed of wheat starch (N-free) or wheat starch with either corn distillers dried grains with solubles (CDDGS), corn gluten meal (CGM), meat and bone meal (MBM), soybean meal (SBM), fish meal (FM), porcine meal (PM), canola meal (CM), blood meal (BM) or feather meal (FEM). At d 21, the protein digestion kinetics, and total and soluble protein of the DI content were determined. Differences were considered significant when $P \leq 0.05$. Protein source affected the extent of amino acid (AA) and CP digestibility at the DI. The CP and average AA digestibility of the meals ranged from 39 to 85% and 46 to 85%, respectively. The average AA digestibility ranked CGM, SBM, and FM to have similar overall AA extent of digestion. At the DI, CGM CP digestibility was greater than all protein sources followed by SBM and FM, while FEM and BM were the least digested. The results demonstrated differences in the rate of digestion of AA and CP among protein sources. For example, FM had the highest digestion rate for most of the AA evaluated among the protein sources, while CDDGS had the lowest. In turn, the total and soluble CP in the distal ileal contents ranged from 54 to 1466 and 6 to 347 mg, respectively. In conclusion, dietary protein source influences the amount and solubility of the undigested protein in the distal ileum and the digestion kinetics of AA and CP along the small intestine of broilers. These parameters of protein source could modulate muscle deposition and could influence the impact protein sources have on gut health through protein fermentation.

Keywords: protein digestion rate, broilers, soybean meal, fish meal

5.2 Introduction

Animal and plant protein meals supply most of the amino acids (AA) found in poultry diets, but they vary in their digestibility and AA composition (Parsons et al., 1997; Adedokun et al., 2008; Kim et al., 2012). Good knowledge about the digestibility and AA content of protein sources can be found in the literature, although variation can occur between samples within a source (Parsons et al., 1997; Ravindran and Bryden, 1999; Lemme et al., 2004; Adedokun et al., 2008). The variability in meal digestibility can lead to incorrect diet formulation when using database digestibility coefficients values. The routine evaluation of ingredients digestibility adds to the database and helps nutritionists refine feed formulation. Both apparent AA digestibility and standardized AA digestibility techniques have been used to assess protein digestibility in poultry (Lemme et al., 2004; Ravindran et al., 2017). The latter technique has been viewed as the gold standard for estimating protein digestibility despite the debate on how digestible values are standardized (Lemme et al., 2004). Nevertheless, both *in vivo* digestibility techniques are routinely used to evaluate the extent of protein digestion in poultry and the data obtained are essential for the delivery of poultry AA requirements.

Despite the knowledge of AA digestibility, other characteristics of protein sources have been less well studied in avian species. One such area relates to the rate of digestion of protein sources. There is little to no information available on the rate of digestion of high protein feed ingredients because available *in vivo* assays do not readily provide such data due to the difficulty in estimating this characteristic *in vivo*. Due to the small size of the bird's digestive tract, it is often difficult to collect samples routinely over time from the same bird to measure the rate of nutrient disappearance in the small intestine. Some researchers have overcome the problem associated with routine digesta sampling over time by calculating the mean retention time of the digesta in each section of the small intestine of pigs (Wilson and Leibholz, 1981). This procedure requires sacrificing the animal and collecting samples from each section of the digestive tract for nutrient disappearance calculation. The calculated retention time and the nutrient digestibility data obtained can be used to calculate digestion rate based on the digestion kinetic model proposed by Ørskov and McDonald (1979). This approach could provide an alternative way to obtain *in vivo* protein

digestion kinetic data from currently available digestibility assays used in poultry nutritional research.

In addition to the extent of AA digestion, the rate at which proteins are hydrolyzed, and AA released and absorbed has also been suggested to play an important role in animal and human nutrition. Protein digestion rate has been suggested to be a key regulator of protein synthesis and deposition in tissue (Sklan and Hurwitz, 1980; Boirie et al., 1997) of birds and humans. Studies with poultry have shown that the synchronization of AA supply for protein synthesis differs between ad libitum and meal fed birds (Nonis and Gous, 2006). In this research, high levels of crystalline AA reduced performance in broiler breeders fed less frequently because these AA were absorbed more quickly than amino acids derived from protein and as a consequence there was a lack of synchronization of AA at the location of protein synthesis. If proteins vary in rate of digestion, this principle could also be true for intact protein and therefore influence the choice of protein source given to the birds based on specific feeding strategies. It has been shown in humans that protein digestion rate has the ability to influence plasma cholecystokinin, glucagon-like peptide-1 and insulin, which were linked to the satiety response of different protein sources (Hall et al., 2003; Pennings et al., 2011). The rate at which individual AA are digested from a protein will determine where along the digestive tract they are released and could, therefore, influence their availability to the host or its microbiota. The microbiota population increases in the distal gut (Gong et al., 2007), which means that there will be greater competition between the host and its microbiota for AA released in this location.

It has been suggested that the undigested protein at the distal ileum can influence poultry gut health (Apajalahti and Vienola, 2016). The mechanism is thought to involve bacterial dysbiosis due to an increase in the putrefactive microbial population, which in turn predisposes broilers to enteric disease and exposes the digestive tract to undesirable fermentation metabolites (Rinttila and Apajalahti, 2013; Apajalahti and Vienola, 2016). Before protein present in the distal ileum can be fermented, it must first be in a form which is available to the microbiota. Little is known about the characteristics of distal ileum protein derived from commonly fed protein sources and their fermentation capacity in the lower digestive tract of poultry. Further, the protein influence on digestive tract microbiota may relate to specific AA as the presence of ileal AA such as glycine correlate with the number of *C. perfringens* in the ileum and caecum of broilers (Wilkie et al., 2005). The majority of studies evaluating the digestible AA in common feedstuffs did not report

available data on ileal AA content. Such information could have value in understanding the link between dietary proteins and enteric disease in poultry.

The objective of this research was to evaluate the rate and extent of digestion of different plant and animal protein meals by broiler chickens and characterize the ileal digesta CP from the birds fed those protein sources. It was hypothesized that protein sources would vary in protein digestion kinetics and ileal digesta CP characteristics.

5.3 Material and methods

This work was approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use (Canadian Council on Animal Care, 2009).

5.3.1 Experimental design

The experimental design was completely random with ten dietary treatments including nine protein sources plus a nitrogen-free (N-Free) diet. Each dietary treatment was replicated six times using cage as the experimental unit and each cage had six birds.

5.3.2 Bird management

A total of 384 Ross x Ross 308 male broilers were obtained from a local commercial hatchery (Sofina Foods Inc., Wynyard, Saskatchewan, Canada) and randomly housed in a double tier battery cage system at the University of Saskatchewan Poultry Centre. Each cage (L 51 cm x W 51 cm x H 46 cm) had a wire mesh floor (2.54 cm x 2.54 cm) that was covered by a removable floor (1.27 cm x 1.27 cm) for the first 7 d. Feed and water were provided by a front mounted feed trough (L 51 cm x W 12 cm x H 10 cm) and 2 height adjustable nipple drinkers per cage, respectively. Supplemental water in ice cube trays and supplemental feed in 50 cm long plastic chick feeders were used for the first 5 d after placement. Room temperature was set at 34°C for the first d and then gradually reduced by 0.43°C every per d for the rest of the experiment. The light intensity was set to 20 lux, and the lighting program was 23 h light, 1 h dark (23L:1D) for the first 7 d, followed by 22L:2D for the rest of the experiment; a 15 min dawn and 15 min dusk were included in the light period.

All birds were given a standard mash starter diet for the first 13 d post-hatch. On d 14, birds with similar body weights were selected and randomly distributed to 60 cages with 6 birds per

cage and 6 cage replicates per dietary treatment. Birds were given the test diets ad libitum from 14 to 21 d of age. Feed intake was recorded from d 14 to 21 and also for the last 24 h (d 21) just before bird sampling.

5.3.3 Diet formulation and assay diets

Diets were manufactured at the Canadian Feed Research Centre (North Battleford, Saskatchewan, Canada). The starter diet was based on wheat and soybean meal (Table 5.1), and met or exceeded Ross 308 starter nutrient specifications (Aviagen, 2014). The protein sources evaluated were blood meal (BM), corn gluten meal (CGM), canola meal (CM), feather meal (FEM), fish meal (FM), meat and bone meal (MBM), porcine meal (PM) and soybean meal (SBM). The FEM was obtained from Saskatoon Processors (Saskatoon, Saskatchewan, Canada), the BM, FM, MBM and PM were from West Cost Reduction LTD (Vancouver, British Columbia, Canada) the CM, CDDGS, CGM and SBM were from Cargill (North Battleford, Saskatchewan, Canada). The test diets were semi-purified and formulated to contain approximately 20% CP solely derived from the test protein source. Diets contained a minimum of 2800 kcal/kg AME and used wheat starch (WHETSTAR-4TM, ADM, Montreal, Canada) as a digestible carbohydrate source. The N-Free diet was composed of wheat starch, solkafloc, canola oil, vitamins, and minerals. All test diets contained 0.3% titanium oxide and were micro-pelleted using a roller die with a 2 mm diameter hole after exposure to a conditioning temperature of 65°C for 35 s.

5.3.4 Sample collection and processing

On d 21, all birds within a cage were killed with an intravenous injection of T61 solution with embutramide, mebezonium iodide and tetracaine hydrochloride as active agents (Merck animal health, Kikland, Quebec, Canada). The digestive tract was carefully removed after clamping 1 cm proximal to the ileocaecal junction and 1 cm distal to the end of the pancreas. The portion of the small intestine from the Meckel's diverticulum to 1 cm of the ileocaecal junction was considered to be the ileum and the rest of the small intestine the jejunum. Both the jejunum and ileum were cut into proximal and distal halves. The digesta was removed from each section by flushing with distilled water into a drum vial and the flushed samples were pooled per intestinal section per cage. Gut contents were placed on dry ice immediately after collection and then stored at -20°C. The frozen samples were freeze-dried, weighed, and ground with a mortar and pestle.

5.3.5 Chemical analyses

All ingredients were analyzed for minerals by SGS Agrofood laboratories (Guelph, Ontario, Canada) using AOAC methods 985.01 (AOAC International, 2006) before diet formulation. Diets, feed ingredients, and distal ileal digesta samples were analyzed for AA by Experiment Station Chemical Laboratories (University of Missouri, Columbia) using AOAC method 982.30 E (a, b). The CP content and DM of all diets, test meals, and digesta samples were analyzed using AOAC 990.03 (Leco combustion) and AOAC 934.01 methods, respectively. The titanium in diets and digesta samples were analyzed using the method of (Myers et al., 2004). The soluble CP in the distal ileum dried content was extracted using normal saline solution. In brief, 250 mg of dried content was placed in a 13 x 100 mm glass culture tube followed by the addition of 3 ml of 0.9% NaCl solution. The tubes were vortexed, placed in a test tube rack and capped with silicone covers. The test tube rack was placed in an orbital shaker at a 45 degree offset from the horizontal base of the shaker and shaken at 300 rpm for 1 h. The tubes were centrifuged in a Beckman centrifuge (Model GS-6, Beckman Instruments, Palo Alto, California, USA) at 3000 rpm for 10 min, then the supernatant discarded and solid dried at 130°C for 2 h before CP analysis.

5.3.6 Calculations and statistics

The soluble CP was calculated as the difference between the total extracted CP content and the total CP content of the unextracted sample. The basal ileal endogenous AA and CP flow per kg of DM intake for birds fed the N-Free diet were calculated as follows (Moughan et al., 1992).

Basal ileal AA or CP flow (mg/kg DM intake) = [AA or CP in ileal digesta (mg/kg)] x [diet marker (mg/kg) ÷ ileal marker (mg/kg)]…….……(5.3.6.1)

The apparent ileal digestibility and standardized ileal digestibility for AA and CP were determined using the following formulas as described by Lemme et al., (2004).

Apparent ileal digestibility = 100-{[maker in diet (mg/kg) x AA or CP (mg/kg) in ileal digesta] ÷ [marker in ileal digesta (mg/kg) x AA or CP (mg/kg) in diet] x 100}…….(5.3.6.2)

Standardized ileal digestibility = Apparent ileal digestibility (%) + {[Basal endogenous AA or CP losses (g/kg DM intake)] ÷ [AA or CP content of ingredients (g/kg DM)]} x 100…...(5.3.6.3)

Digesta retention times (MRT) per gut section were calculated using the following formulas proposed by Wilson and Leibholz (1981).

MRT=[1440 x marker in digesta(mg/g) x gut digesta dried weight(g)] ÷ [maker intake over 24h (feed consumed x maker in feed)]……..…..…(5.3.6.4)

Where 1440 represents total min in 24 h. Mean retention time (MRT) in the duodenum was assumed to be 5 min based on previous work (Shires et al., 1987; Weurding et al., 2001) and it was assumed that no absorption of peptides or AA occurred prior to the duodenum.

The digestibility data along the digestive tract and the digesta mean retention times per gut sections were fitted to the following Ørskov and McDonald (1979) digestion model using the PROC LIN procedure of SAS (9.4) as described by Weurding et al. (2001) to determine the digestion rate of CP and AA. Digestion kinetics parameters were calculated as follows:

$P = a (1 - e^{-kdt})$……..…….……(5.3.6.6)

where P = digestiblility after time 't', a = the potentially digestible protein or AA which will be digested over time, kd = the rate constant for the digestibility of 'a.'

The rate and extent data were appropriately transformed when necessary to meet the statistical analysis assumptions of normal distribution and homogeneity of variance then subjected to a one-way ANOVA using the PROC mixed procedures of SAS (9.4). Each meal was a treatment with 6 birds per cage and six replicate cage per treatment. If significant effects ($P \leq 0.05$) were found among the treatments, the least squares means were separated using the PDIFF procedure in SAS (9.4). Correlation analyses were conducted using the Proc Corr procedures of SAS (9.4).

5.4 Results

The analyzed AA and CP levels of the test ingredients and diets are shown in Tables 5.2 and 5.3, respectively. All diets had similar CP levels, which approximated the calculated values in Table 5.1. Body weight and feed intake data are presented in Table 5.4. Average treatment body weights were not different at the beginning of the trial, but growth between 14 and 21 d of age was markedly affected by dietary treatment. The range in 21 d body weight was 366 to 821 g per bird and the ranking from lowest to highest was N-Free, CGM, BM, FEM, CDDGS, MBM, PM, SBM, and CM. Over the 7 d, experimental period, the range in feed intake was 285 to 707 g per bird and the treatment ranking from lowest to highest was CGM, BM, N-Free, FEM, CDDGS, MBM, PM, FM, SBM and CM.

Table 5.1. Composition of starter and test diets fed during the digestibility experiment.

	Starter	Test diets									
Ingredients (%)		BM	CDDGS	CGM	CM	FEM	FM	MBM	N-Free	PM	SBM
Wheat starch	0.0	69.38	22.75	58.68	37.99	65.58	65.93	52.65	84.79	62.94	47.76
Wheat	58.92	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
BM	0.0	20.84	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
CDDGS	0.0	0.0	69.05	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
CGM	0.0	0.0	0.0	31.48	0.0	0.0	0.0	0.0	0.0	0.0	0.0
CM	0.0	0.0	0.0	0.0	54.27	0.0	0.0	0.0	0.0	0.0	0.0
FEM	0.0	0.0	0.0	0.0	0.0	25.34	0.0	0.0	0.0	0.0	0.0
FM	0.0	0.0	0.0	0.0	0.0	0.0	28.92	0.0	0.0	0.0	0.0
MBM	0.0	0.0	0.0	0.0	0.0	0.0	0.0	40.41	0.0	0.0	0.0
PM	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	31.63	0.0
SBM	32.00	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	43.34
Solfa Floc	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.00	0.0	0.0
Canola oil	4.97	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Mono di-calcium phosphate	1.20	2.20	0.89	2.21	1.37	1.81	0.00	1.52	2.43	0.00	1.96
Limestone	1.48	1.62	2.23	1.64	1.15	1.31	0.00	0.00	1.57	0.00	1.49
Ameri-Bond 2X[1]	0.0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Vit/min premix[2]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Potassium carbonate	0.0	0.45	0.00	0.38	0.00	0.42	0.08	0.02	0.52	0.23	0.00
Magnesium oxide	0.0	0.32	0.00	0.31	0.00	0.31	0.17	0.24	0.33	0.22	0.12
Titanium oxide	0.0	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Sodium chloride	0.43	0.29	0.18	0.40	0.32	0.33	0.00	0.26	0.46	0.08	0.43
Choline chloride	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.1	0.10
DL-Methionine	0.29	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
L-Threonine	0.10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Econase[3]	0.01	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

BM = blood meal; CDDGS = corn distillers' dry grains with solubles; CGM = corn gluten meal; CM = canola meal (solvent extracted); FEM (steam and pressure hydrolyzed) = feather meal; FM = fish meal; MBM = meat and bone meal; N-Free = nitrogen free; PM = porcine meal; SBM = soybean meal. [1]Pellet binder (LignoTech, Rothschild, Wisconsin, USA). [2]Vitamin-mineral premix provided the following per kg of complete diet: vitamin B12, 4 mg ; vitamin D, 440,000 IU; vitamin A, 2,200,000 IU; vitamin E, 6000 IU; menadione, 400 mg; thiamine, 300 mg; riboflavin, 1200 mg; pyridoxine, 800 mg; niacin, 12,000 mg; pantothenic acid, 2000 mg; folic acid, 120 mg; biotin 30 mg; copper, 2000 mg; iron, 16,000 mg; manganese 16,000 mg; iodine, 160 mg; zinc, 16,000 mg; selenium, 60 mg; calcium carbonate 100,000 mg; Ethoxyquin 125 mg; wheat middlings 754,546 mg. [3]Econase XT (ABVista, Wiltshire, UK), β 1-4 endo-xylanase enzyme, xylanase activity –160,000 BXU/g.

Table 5.2. Determined amino acid and protein composition (%) of meals samples on a dry matter basis.

Amino acid	BM	CDDGS	CGM	CM	FEM	FM	MBM	PM	SBM
Indispensable amino acids									
Arginine	4.39	1.45	2.12	2.30	6.03	3.90	3.60	3.81	3.60
Cysteine	1.08	0.66	1.15	0.88	4.33	0.57	0.47	0.52	0.66
Isoleucine	2.00	1.31	2.97	1.70	4.22	3.29	1.89	2.25	2.47
Leucine	12.24	4.04	11.35	2.85	6.97	5.45	3.67	4.17	3.95
Lysine	8.48	1.17	1.20	2.34	2.44	5.71	3.47	3.80	3.26
Methionine	1.08	0.66	1.60	0.75	0.73	1.83	0.88	0.94	0.64
Threonine	4.45	1.33	2.10	1.60	3.98	2.79	1.91	1.93	1.80
Valine	8.00	1.81	3.33	2.09	6.55	3.90	2.60	3.01	2.68
Dispensable amino acids									
Alanine	7.30	2.34	5.93	1.76	4.03	4.44	3.84	4.37	2.17
Aspartic Acid	9.32	2.14	3.94	2.74	5.91	6.24	4.26	4.54	5.48
Histidine	6.30	1.05	1.37	1.07	0.77	2.16	1.24	1.38	1.31
Glutamic Acid	9.22	4.53	14.74	6.73	9.37	9.20	6.86	7.51	9.09
Glycine	4.27	1.30	1.98	2.05	6.79	4.28	6.04	6.52	2.19
Phenylalanine	6.64	1.61	4.26	1.61	4.17	2.92	2.03	2.23	2.54
Proline	3.71	2.66	6.51	2.59	8.05	3.07	3.62	4.40	2.79
Serine	4.34	1.50	2.66	1.31	8.42	2.11	1.96	1.78	1.93
Tyrosine	3.05	1.27	3.23	1.00	2.08	2.13	1.61	1.61	1.70
Total	96.02	31.26	70.92	36.43	87.31	66.37	52.72	58.16	49.00
Protein (N_2 x 6.25)	99.19	32.72	68.59	40.55	82.73	75.74	55.17	64.75	49.37

BM = blood meal; CDDGS = corn distillers' dry grains with solubles; CGM = corn gluten meal; CM =canola meal (solvent extracted); FEM (steam and pressure hydrolyzed) = feather meal; FM = fish meal; MBM = meat and bone meal; PM = porcine meal; SBM = soybean meal.

Table 5.3. Determined amino acid and protein composition (%) of test diets on a dry matter basis.

Amino acid	BM	CDDGS	CGM	CM	FEM	FM	MBM	PM	SBM
Indispensable amino acids									
Arginine	0.92	1.42	0.68	1.22	1.42	1.07	1.28	1.28	1.52
Cysteine	0.26	0.99	0.37	0.49	0.99	0.17	0.19	0.19	0.29
Isoleucine	0.47	1.06	0.94	0.91	1.06	0.89	0.79	0.78	1.03
Leucine	2.72	1.76	3.61	1.60	1.76	1.51	1.48	1.47	1.69
Lysine	1.88	0.73	0.45	1.21	0.73	1.55	1.39	1.37	1.41
Methionine	0.24	0.22	0.51	0.42	0.22	0.54	0.39	0.39	0.29
Threonine	0.97	0.97	0.74	0.92	0.97	0.81	0.74	0.73	0.84
Valine	1.75	1.61	1.06	1.16	1.61	1.07	1.06	1.06	1.09
Dispensable amino acids									
Alanine	1.61	1.06	1.91	0.99	1.06	1.23	1.42	1.45	0.93
Aspartic Acid	2.06	1.53	1.30	1.50	1.53	1.73	1.66	1.63	2.40
Histidine	1.32	0.22	0.44	0.57	0.22	0.55	0.48	0.47	0.57
Glutamic Acid	2.12	2.51	4.83	3.69	2.51	2.64	2.85	2.82	3.95
Glycine	0.95	1.72	0.64	1.10	1.72	1.20	2.03	2.09	0.92
Phenylalanine	1.44	1.04	1.39	0.90	1.04	0.83	0.83	0.82	1.11
Proline	0.82	2.01	1.99	1.33	2.01	0.77	1.32	1.35	1.00
Serine	0.94	1.97	1.02	0.83	1.97	0.67	0.72	0.71	0.97
Tyrosine	0.56	0.52	0.92	0.53	0.52	0.53	0.59	0.57	0.68
Total	21.10	22.25	22.87	19.95	22.26	18.50	20.38	20.40	20.92
Protein (N_2 x 6.25)	21.67	21.99	22.52	22.01	21.84	20.69	21.76	21.53	20.94

BM = blood meal; CDDGS = distillers' dry grains with solubles; CGM = corn gluten meal; CM =canola meal (solvent extracted); FEM (steam and pressure hydrolyzed) = feather meal; FM = fish meal; MBM = meat and bone meal; PM = porcine meal; SBM = soybean meal.

Table 5.4. Growth performance of broilers fed diets containing different protein meals.

Item	BM	CDDGS	CGM	CM	FEM	FM	MBM	N-Free	PM	SBM	SEM	P-value
						Performance (g/bird)						
Body weight d 14	416	397	420	396	419	402	397	399	397	422	11.4	0.48
Body weight d 21	470^{e}	622^{c}	468^{e}	797^{ab}	527^{d}	821^{a}	742^{b}	366^{f}	744^{b}	788^{ab}	17.4	<0.01
Feed intake d 14-21	310^{ef}	538^{d}	285^{f}	707^{a}	355^{e}	612^{bc}	574^{cd}	313^{ef}	602^{bc}	638^{b}	20.9	<0.01

$^{a-f}$ Means (n =6 cages) within a column sharing a common superscript are not significantly different at $\alpha \leq 0.05$.
BM = blood meal; CDDGS = corn distillers' dry grains with solubles; CGM = corn gluten meal; CM =canola meal (solvent extracted); FEM (steam and pressure hydrolyzed) = feather meal; FM = fish meal; MBM = meat and bone meal; PM = porcine meal; SBM = soybean meal.

Standardized and apparent ileal digestibilities of CP and AA for protein sources are shown in Table 5.5. All AA and total protein digestibilities were affected by protein source. The standardized ileal digestible (SID) CP values ranged from 39 to 85 %. The CGM had the highest SID CP which was only similar to that of SBM. The SID CP of SBM was similar to that of CDDGS, FM, PM and MBM, but higher than BM and, FEM which had the lowest SID CP. The apparent ileal digestible CP for CGM, SBM, and FM were all similar but higher than BM and FEM. However, the apparent CP digestibility of SBM, FM, CDDGS, PM, and MBM were not different.

The average of the AA SID evaluated values ranged from 46 to 85%, which ranked CGM, SBM and FM to have similar overall digestibility values where as CGM was higher than the other ingredients. Nonetheless, SBM, CDDGS, CM, FM, PM, and MBM average AA SID were not different from each other, while BM and FEM values were lower than all other meals. The SID of the indispensable AA of the ingredients ranged 31 to 94%, which was dependent on the AA and the ingredient been evaluated. For example, cysteine SID was the lowest value for the range reported for SBM, CM, FM, PM, MBM, FEM, and BM. Methionine SID was at the lower end of the range of values reported for CDDGS and lysine for CGM. Blood meal had the lowest SID for most of the AA evaluated except in the case of FEM which had lower SID cysteine, and aspartic acid. In most cases, CGM, SBM, FM, and CDDGS had high SID AA values when compared to FEM and BM, while CM, PM, and MBM tended to be intermediate.

Table 5.5. The standardized and apparent distal ileal amino acid and CP digestibility of high protein meals in 21 d old male broiler chickens.

Item	BM	CDDGS	CGM	CM	FEM	FM	MBM	PM	SBM	SEM	P-value
Indispensable amino acid standardized ileal digestibility (%)											
ARG	45^{d}	89^{a}	84^{ab}	76^{b}	59^{c}	84^{ab}	77^{b}	79^{b}	85^{ab}	2.2	<.01
CYS	35de	87^{a}	76^{ab}	52^{cd}	31^{e}	51^{cd}	53^{cd}	44de	67^{bc}	3.5	<.01
ILE	44^{d}	81^{a}	86^{a}	69^{bc}	65^{c}	81^{a}	77^{ab}	71^{bc}	79^{ab}	1.8	<.01
LEU	46^{e}	71^{c}	94^{a}	72^{bc}	57^{d}	81^{b}	78^{bc}	72^{c}	78^{bc}	1.8	<.01
LYS	53^{d}	71^{b}	75^{ab}	69^{bc}	58^{cd}	84^{a}	79^{ab}	77^{ab}	83^{a}	2	<.01
MET	48^{e}	65^{cd}	91^{a}	80^{b}	61^{d}	82^{ab}	81^{b}	74^{bc}	81^{b}	2.2	<.01
THR	46^{d}	71^{abc}	77^{a}	59^{cd}	46^{d}	75^{ab}	70^{abc}	65^{bc}	74^{ab}	2.5	<.01
VAL	46^{d}	81^{a}	81^{a}	64^{bc}	57^{cd}	74^{ab}	74^{ab}	69^{b}	75^{ab}	2.2	<.01
Dispensable amino acid standardized ileal digestibility (%)											
ALA	47^{e}	70^{c}	92^{a}	70^{c}	58^{d}	81^{b}	75^{bc}	75^{bc}	77^{bc}	2	<.01
ASP	43^{d}	66^{bc}	80^{a}	60^{cd}	24^{e}	66^{bc}	64^{bc}	56^{cd}	78^{ab}	3.1	<.01
GLU	47^{e}	70^{d}	92^{a}	78^{bcd}	52^{e}	81^{bc}	75^{bcd}	72^{cd}	82^{b}	2.1	<.01
GLY	46^{d}	85^{a}	74^{b}	64^{bc}	60^{c}	75^{ab}	65^{bc}	74^{b}	74^{b}	2.2	<.01
HIS	47^{cd}	36^{d}	86^{a}	75^{ab}	53^{c}	81^{ab}	76^{ab}	70^{b}	82^{a}	2.4	<.01
PHE	46^{d}	79^{b}	91^{a}	73^{b}	59^{c}	77^{b}	78^{b}	72^{b}	79^{b}	2.2	<.01
PRO	35^{e}	79^{ab}	89^{a}	62^{cd}	42^{e}	74^{bc}	59^{d}	67^{bcd}	75^{bc}	2.9	<.01
SER	46^{e}	88^{a}	84^{a}	60^{cde}	53de	72^{bc}	64^{c}	63^{cd}	80^{ab}	2.6	<.01
TYR	46^{c}	72^{b}	90^{a}	69^{b}	52^{c}	77^{b}	75^{b}	72^{b}	81^{b}	2.2	<.01
Avg	46^{c}	74^{b}	85^{a}	68^{b}	52^{c}	76^{ab}	72^{b}	69^{b}	78^{ab}	2.1	<.01
CP	39^{d}	71^{bc}	85^{a}	64^{c}	50^{d}	75^{bc}	71^{bc}	68^{bc}	77^{ab}	2.3	<.01
Indispensable amino acid apparent[1] ileal digestibility (%)											
ARG	44^{c}	86^{a}	83^{a}	75^{a}	58^{b}	83^{a}	76^{a}	77^{a}	83^{a}	2.2	<.01
CYS	29^{ef}	82^{a}	70^{ab}	51^{bcd}	26^{f}	48^{cde}	46de	41^{def}	64^{bc}	4	<.01
ILE	42^{d}	78^{ab}	85^{a}	68^{bc}	63^{c}	79^{ab}	75^{b}	69^{bc}	76^{ab}	2.1	<.01
LEU	45^{e}	69^{c}	93^{a}	71^{c}	55^{d}	81^{b}	76^{bc}	71^{c}	76^{bc}	2	<.01
LYS	51^{e}	65^{c}	74^{abc}	66^{cd}	56de	83^{a}	77^{ab}	72^{bc}	80^{ab}	2.1	<.01
MET	46^{d}	63^{c}	90^{a}	78^{b}	59^{c}	81^{b}	80^{b}	74^{b}	79^{b}	2.1	<.01
THR	43^{d}	67^{abc}	75^{a}	57^{c}	43^{d}	74^{ab}	67^{abc}	62^{bc}	70^{abc}	2.6	<.01
VAL	44^{e}	77^{ab}	80^{a}	63^{cd}	54de	73^{abc}	71^{abc}	67^{bc}	72^{abc}	2.5	<.01
Dispensable amino acid apparent ileal digestibility (%)											
ALA	46^{d}	68^{c}	91^{a}	69^{c}	56^{d}	80^{b}	74^{bc}	74^{bc}	75^{bc}	2.0	<.01
ASP	42^{d}	62^{c}	79^{a}	58^{cd}	23^{e}	65^{bc}	62^{bc}	54^{cb}	75^{ab}	3.1	<.01
GLU	46^{e}	68^{d}	91^{a}	77^{bcd}	51^{e}	80^{bc}	73^{bcd}	71^{cd}	81^{b}	2.1	<.01
GLY	45^{d}	80^{a}	73^{ab}	63^{bc}	57^{c}	74^{ab}	64^{bc}	71^{ab}	72^{ab}	2.4	<.01
HIS	45^{d}	34^{e}	85^{a}	72^{bc}	52^{d}	80^{ab}	74^{bc}	69^{c}	80^{ab}	2.4	<.01
PHE	44^{d}	77^{b}	90^{a}	72^{b}	58^{c}	77^{b}	76^{b}	71^{b}	77^{b}	2.1	<.01
PRO	34^{e}	77^{ab}	88^{a}	61^{cd}	40^{e}	73^{bc}	58^{d}	66^{bcd}	73^{bc}	2.9	<.01
SER	43^{e}	83^{a}	81^{ab}	59^{cd}	49de	71^{bc}	61^{c}	60^{cd}	76^{ab}	2.6	<.01
TYR	44^{c}	69^{b}	89^{a}	67^{b}	50^{c}	76^{b}	73^{b}	71^{b}	77^{b}	2.2	<.01
Avg	43 ^{c}	71^{b}	84^{a}	66^{b}	50^{c}	75^{ab}	70^{b}	67^{b}	75^{ab}	2.2	<.01
CP	38^{c}	68^{b}	83^{a}	64^{b}	48^{c}	73^{ab}	68^{b}	67$_{b}$	75^{ab}	2.3	<.01

[a-f] Means (n = 6 cages) within a row per subsection sharing a common superscript are not significantly different at $\alpha \leq 0.05$.[1]Apparent values were standardized using a nitrogen-free diet fed to birds in the same room as the treatment birds during the assay. BM = blood meal; CDDGS = corn distillers' dry grains with solubles; CGM = corn gluten meal; CM =canola meal (solvent extracted); FEM (steam and pressure hydrolyzed) = feather meal; FM = fish meal; MBM = meat and bone meal; PM = porcine meal; SBM = soybean meal.

Using treatment standardized digestibility coefficients and feed intake, it is possible to estimate the AA content of the terminal ileum content (g) per 100 g of feed intake (Table 5.6). The values derived for all AA and were affected by dietary treatment. The total AA content which is a summary of measured AA ranged from 10.36 (N-Free diet) to 108.12 (BM) g per 100 g of feed consumed. In the distal ileum content, the BM, and FEM diets gave more total AA than all other diets. Not surprisingly, the N-Free diet gave the lowest level of total AA when compared to the other diets. Following the N-Free diet were CM, CDDGS, and SBM which had similar values, then PM, MBM, FM, and CGM preceded those values. In general, BM, and FEM tended to produce the highest level of indispensable and dispensable AA in the ileum content when compared to the other diets.

Mean retention time for the proximal and distal sections of the jejunum and ileum, as well as for the small intestine are shown in Table 5.7. For all sections, dietary treatment affected MRT with ranges of 5 to 19, 11 to 37, 18 to 41, and 14 to 50 min for the proximal jejunum, distal jejunum, proximal ileum and distal ileum, respectively. As a consequence, total small intestine MRT was also affected by treatment with a range of 46 (FM) to 142 min (CDDGS).

In vivo digestion rates of AA and CP in protein sources were estimated by modeling the extent of digestion and MRT of digesta along the small intestine for each ingredient. For detailed assessment of the effects of protein source on the digestion rate for AA see Table 5.8. The CP digestion rate was affected by protein source and PM CP was digested and absorbed more rapidly than all the other meals evaluated. Numerically, CDDGS had the lowest CP digestion rate, which was statistically similar to CGM, SBM, CM, FEM, and BM, but lower than MBM. The digestion rate for all AA was similarly affected by protein source and statistical examination of these effects are shown in Table 5.8. The average digestion rate for all AA analyzed showed that FM had the highest fractional digestion rate, which was similar to PM. Porcine meal average AA digestion rate was not different from that of FEM, while CDDGS and CM had the lowest digestion rate with CGM, SBM, MBM and BM having intermediate values.

Table 5.6. Amino acid in distal ileal content of 21 d old male broiler chickens fed plant and animal by-product meals.

Item	BM	CDDGS	CGM	CM	FEM	FM	MBM	N-Free	PM	SBM	SEM	P-value
Indispensable amino acid g per 100 g feed consumed												
ARG	4.82^{a}	0.60^{e}	1.67^{b}	0.71^{de}	5.04^{a}	1.33^{bc}	1.28^{bc}	0.47^{e}	1.35^{bc}	0.93^{cd}	0.178	<.01
CYS	1.73^{b}	0.53^{cd}	1.59^{b}	0.56^{cd}	6.20^{a}	0.65^{c}	0.44^{de}	0.31^{f}	0.50^{cde}	0.39^{ef}	0.112	<.01
ILE	2.52^{ab}	0.70^{ef}	2.12^{b}	0.69^{ef}	3.35^{a}	1.38^{c}	0.84^{de}	0.53^{f}	1.09^{cd}	0.89^{de}	0.114	<.01
LEU	13.21^{a}	1.57^{de}	4.09^{c}	1.09^{fg}	6.68^{b}	2.08^{d}	1.50^{ef}	0.80^{g}	1.93^{de}	1.50^{ef}	0.314	<.01
LYS	8.61^{a}	0.74^{g}	1.69^{cd}	0.98^{efg}	2.71^{b}	1.95^{c}	1.36^{de}	0.77^{eg}	1.74^{cd}	1.02^{ef}	0.195	<.01
MET	1.21^{a}	0.24^{d}	0.71^{b}	0.22^{d}	0.77^{b}	0.75^{b}	0.34^{cd}	0.18^{d}	0.46^{c}	0.22^{d}	0.04	<.01
THR	5.23^{a}	0.95^{de}	2.48^{b}	0.93^{de}	4.77^{a}	1.58^{c}	1.05^{de}	0.78^{e}	1.26^{cd}	0.93^{de}	0.129	<.01
VAL	9.18^{a}	1.10^{f}	2.83^{c}	1.02^{fg}	6.29^{b}	2.11^{d}	1.29^{ef}	0.81^{g}	1.57^{e}	1.11^{f}	0.223	<.01
Dispensable amino acid g per 100 g feed consumed												
ALA	7.60a	0.99^{e}	2.47^{c}	0.71^{fg}	3.99^{b}	1.77^{d}	1.59^{d}	0.52^{g}	1.67^{d}	0.85^{ef}	0.187	<.01
ASP	11.27^{a}	1.74^{def}	4.07^{b}	1.48^{ef}	10.06^{a}	4.45^{b}	2.67^{cd}	1.10^{f}	3.43^{bc}	2.22^{de}	0.317	<.01
GLU	10.79^{a}	2.39^{cd}	6.46^{b}	2.03^{cd}	10.57^{a}	3.87^{c}	3.23^{c}	1.48^{d}	3.70^{c}	2.75^{cd}	0.331	<.01
GLY	4.85^{a}	0.99^{c}	2.57^{b}	0.97^{c}	6.24^{a}	2.37^{b}	3.04^{b}	0.61^{d}	2.70^{b}	0.94^{c}	0.191	<.01
HIS	6.75^{a}	0.43^{ef}	0.98^{b}	0.37^{f}	0.90^{b}	0.79^{bc}	0.53^{de}	0.24^{g}	0.66^{cd}	0.42^{ef}	0.151	<.01
PHE	7.49^{a}	0.72^{fg}	2.15^{c}	0.59^{gh}	3.71^{b}	1.42^{d}	0.83^{efg}	0.45^{h}	1.07^{de}	0.94^{ef}	0.186	<.01
PRO	5.03^{b}	1.36^{f}	3.61^{c}	1.22^{fg}	10.08^{a}	1.54^{ef}	2.34^{d}	0.62^{h}	2.08^{de}	0.98^{g}	0.268	<.01
SER	5.07^{b}	0.96^{ef}	2.41^{c}	0.80^{ef}	8.46^{a}	1.47^{d}	1.18^{de}	0.62^{f}	1.27^{de}	0.87^{ef}	0.192	<.01
TYR	2.91^{a}	0.47^{ef}	1.53^{b}	0.41^{ef}	2.20^{a}	0.94^{c}	0.67^{cd}	0.37^{f}	0.75^{cd}	0.56^{de}	0.099	<.01
Total	108.12^{a}	16.45^{d}	41.69^{b}	14.75^{d}	91.97^{a}	30.42^{c}	24.16^{c}	10.36^{e}	27.18^{c}	17.50^{d}	2.834	<.01

$^{a-f}$ Means (n = 6 cages) within a column sharing a common superscript are not significantly different at $\alpha \leq 0.05$.
BM = blood meal; CDDGS = corn distillers' dry grains with solubles; CGM = corn gluten meal; CM =canola meal (solvent extracted); FEM (steam and pressure hydrolyzed) = feather meal; FM = fish meal; MBM = meat and bone meal; PM = porcine meal; SBM = soybean meal.

Table 5.7. Digesta mean retention time of broiler chickens fed diets containing different protein meals.

Item	BM	CDDGS	CGM	CM	FEM	FM	MBM	N-Free	PM	SBM	SEM	P-value
						Mean retention time (min)						
Proximal jejunum	12^{ab}	19^{a}	10^{ab}	14^{a}	7^{bc}	5^{c}	7^{bc}	7^{bc}	7^{bc}	11^{ab}	2.3	<0.01
Distal jejunum	37^{a}	31^{abc}	18^{cd}	23^{abcd}	32^{ab}	11^{d}	21^{bcd}	18^{cd}	19^{bcd}	19^{bcd}	4.8	0.01
Proximal ileum	40^{ab}	41^{ab}	30^{bc}	25^{cd}	43^{a}	18^{d}	34^{abc}	25^{cd}	25^{cd}	26^{cd}	3.9	<0.03
Distal ileum	36^{b}	50^{a}	19^{cd}	39^{ab}	36^{b}	14^{d}	40^{ab}	15^{d}	28^{bc}	35^{b}	4.7	<0.01
Jejunum + ileum	115^{bc}	142^{a}	77^{de}	75^{de}	119^{ab}	46^{f}	98^{bcd}	55^{ef}	75^{de}	91^{cd}	8.9	<0.01

[a-f] Means (n = 6 cages) within a column sharing a common superscript are not significantly different at $\alpha \leq 0.05$.
BM = blood meal; CDDGS = corn distillers' dry grains with solubles; CGM = corn gluten meal; CM =canola meal (solvent extracted); FEM (steam and pressure hydrolyzed) = feather meal; FM = fish meal; MBM = meat and bone meal; PM = porcine meal; SBM = soybean meal.

Table 5.8. The *in vivo* digestion rate of amino acid and CP of high proteins meals by 21 d old male broiler chickens.

Items	BM	CDDGS	CGM	CM	FEM	FM	MBM	PM	SBM	SEM	P-value
Indispensable amino acids fractional digestion rate (kd)											
ARG	0.186^{bcd}	0.155^{bcd}	0.150^{bcd}	0.083^{d}	0.194^{bcd}	0.792^{a}	0.330^{b}	0.995^{a}	0.266^{bc}	0.044	<.01
CYS	0.260^{abc}	0.148^{bc}	0.271^{abc}	0.071^{c}	0.408^{ab}	0.308^{ab}	0.199^{bc}	0.609^{a}	0.206^{bc}	0.048	<.01
ILE	0.111^{cd}	0.097^{d}	0.084^{d}	0.082^{d}	0.325^{abc}	0.390^{ab}	0.210^{bcd}	0.562^{a}	0.219^{bcd}	0.038	<.01
LEU	0.152^{bc}	0.056^{c}	0.099^{bc}	0.083^{bc}	0.233^{bc}	0.696^{a}	0.253^{b}	0.851^{a}	0.233^{bc}	0.044	<.01
LYS	0.283^{c}	0.036^{d}	0.716^{b}	0.084^{d}	0.703^{b}	1.841^{a}	0.252^{c}	0.666^{b}	0.209^{c}	0.038	<.01
MET	0.153^{d}	0.016^{e}	0.119^{d}	0.109^{d}	0.582^{c}	1.764^{a}	0.416^{c}	1.021^{b}	0.212^{d}	0.038	<.01
THR	0.181^{b}	0.143^{b}	0.134^{b}	0.116^{b}	0.474^{ab}	0.283^{ab}	0.190^{b}	0.580^{a}	0.204^{b}	0.055	<.01
VAL	0.140^{b}	0.131^{b}	0.159^{b}	0.077^{b}	0.197^{b}	0.635^{a}	0.241^{b}	0.737^{a}	0.216^{b}	0.043	<.01
Dispensable amino acids fractional digestion rate (kd)											
ALA	0.257^{cd}	0.046^{f}	0.090^{def}	0.082^{ef}	0.198^{cde}	1.543^{a}	0.368^{c}	0.990^{b}	0.202^{cde}	0.039	<.01
ASP	0.170^{cde}	0.044^{e}	0.081^{de}	0.102^{de}	0.232^{cd}	1.128^{a}	0.247^{c}	0.548^{b}	0.260^{c}	0.052	<.01
GLU	0.140^{cd}	0.032^{e}	0.091^{de}	0.131^{d}	0.241^{bcd}	1.351^{a}	0.321^{bc}	1.025^{a}	0.334^{b}	0.038	<.01
GLY	0.219^{bc}	0.122^{c}	0.148^{bc}	0.072^{c}	0.255^{bc}	0.989^{a}	0.429^{b}	1.314^{a}	0.229^{bc}	0.058	<.01
HIS	0.174^{cde}	0.078^{e}	0.117^{de}	0.091^{e}	0.316^{c}	1.543^{a}	0.286^{cd}	0.847^{b}	0.236^{cd}	0.038	<.01
PHE	0.224^{bc}	0.083^{c}	0.137^{c}	0.073^{c}	0.254^{bc}	0.462^{ab}	0.251^{bc}	0.685^{a}	0.262^{bc}	0.042	<.01
PRO	0.247^{bc}	0.101^{c}	0.081^{c}	0.124^{c}	0.183^{bc}	0.202^{bc}	0.337^{b}	1.132^{a}	0.247^{bc}	0.039	<.01
SER	0.123^{bc}	0.135^{bc}	0.095^{c}	0.133^{bc}	0.110^{c}	0.085^{c}	0.181^{bc}	0.387^{a}	0.216^{b}	0.039	<.01
TYR	0.161^{bcd}	0.051^{e}	0.066^{de}	0.088^{cde}	0.197^{bc}	0.158^{bcd}	0.228^{b}	0.759^{a}	0.238^{b}	0.023	<.01
Avg	0.282^{c}	0.094^{d}	0.105^{cd}	0.081^{d}	0.616^{b}	1.281^{a}	0.280^{c}	0.895^{ab}	0.236^{cd}	0.05	<.01
CP	0.075^{cd}	0.031^{d}	0.076^{cd}	0.133^{bcd}	0.096^{cd}	0.180^{bc}	0.210^{b}	0.604^{a}	0.126^{bcd}	0.05	<.01

[1]The rate constant values were calculated as kd from the digestibility of each amino acid along the small intestine and the digesta mean retention time (MRT) per section of the small intestine using the following equation; Amino acid or CP digestibility = A (1-EXP$^{(-kd \times MRT)}$).

[a-f] Means (n =6 cages) within a column sharing a common superscript are not significantly different at $\alpha \leq 0.05$.

BM = blood meal; CDDGS = corn distillers' dry grains with solubles; CGM = corn gluten meal; CM =canola meal (solvent extracted); FEM (steam and pressure hydrolyzed) = feather meal; FM = fish meal; MBM = meat and bone meal; PM = porcine meal; SBM = soybean meal.

Individual AA digestion rate varied based on protein source and the AA in question. Within the indispensable AA for example, lysine, methionine, arginine, leucine and valine digestion rate were higher in FM and PM than all other meals. In the case of lysine, methionine, isoleucine, and leucine it was CDDGS which had the lowest digestion rate which was often similar to that of CM. The digestion rate of threonine and isoleucine were greater for FM, PM, and FEM. However, it was FM, PM, FEM and BM which had the highest cysteine digestion rate. Within the dispensable AA the effects of protien source also varied depending on the AA been evalauted. For example FM and PM had higher glycine and glutamic acid digestion rate compared to the other meals. For glycine, CM and CDDGS had the lowest digestion rate, while the CDDGS value was lowest for glutamic acid.

The total dry matter content of the terminal ileum digesta as well as the proportional and actual content of total and soluble CP are shown in Table 5.9. As a proportion of the DM content, total CP ranged from 19 to 57% and soluble CP from 1 to 9% and in both cases, protein source affected levels. The total DM content of the terminal ileum was also affected by protein source and varied considerably (range 200 to 6768 mg) with a portion of the variability associated with feed intake (R^2 =0.55). Multiplication of the proportion of total and soluble CP by total dry matter content yields the actual content in mg of these components in the digesta. Total CP ranged from 54 (CGM) to 1466 (CM) mg while the range for soluble CP was 6 (FEM) to 347 (CM) mg.

Table 5.9. Soluble protein in the distal ileum digesta content of 21 d old broiler chickens fed plant and animal protein sources semi-purified diets.

	Distal ileum				
	CP (%) in DM		Content (mg)		
Meals	Total	Soluble	Total DM	Total CP	Soluble CP
CDDGS	19^{f}	5^{bc}	6230^{a}	1148^{ab}	304^{a}
CGM	24^{e}	3^{cd}	200^{e}	54^{f}	8.6^{c}
CM	22^{ef}	5^{bc}	6768^{a}	1466^{a}	347^{a}
FEM	57^{a}	1^{d}	1000^{d}	565^{d}	6^{c}
FM	41^{b}	9^{a}	715^{d}	289^{e}	64^{c}
MBM	28^{d}	6^{abc}	3960^{b}	1089^{abc}	231^{b}
PM	33^{c}	7^{ab}	2198^{c}	726^{cd}	168^{b}
SBM	21^{f}	6^{abc}	3717^{b}	786^{bcd}	230^{b}
SEM	0.9	1.1	432	108	26
ANOVA P-value	<.01	<.01	<.01	<.01	<.01

$^{a-f}$ Means (n = 6 cages) within a column with the same superscript letters are not significantly different at $\alpha \leq 0.05$. CDDGS = corn distillers' dry grains with solubles; CGM = corn gluten meal; CM = canola meal (solvent extracted); FEM (steam and pressure hydrolyzed) = feather meal; FM = fish meal; MBM = meat and bone meal; PM = porcine meal; SBM = soybean meal.

5.5 Discussion

There is a growing concern globally about how the poultry sector will meet regulatory requirements on the use of sub-therapeutic antibiotics while satisfying the increasing demand for poultry meat. A reduction in the use of antibiotics in poultry diets has led the poultry industry to consider new approaches to deal with issues such as bird health and reduced animal performance.

Various nutritional strategies have been developed to reduce subsequent disease and performance loss associated with a reduction or absence of in feed antibiotics. One approach to help reduce production losses is the use of highly digestible diets. Diets with high nutrient bioavailability are thought to result in animals expending less energy and other nutrients required for digestion. As a result, more nutrients will be available to the animal during stressful periods such as illness or extreme environmental conditions. A second approach is to formulate diets and use additives to positively modulate the gastrointestinal microbiota (Choct and Ao, 2009).

Dietary protein has been one of the nutrients of most interest because the amount and source of protein are often associated with increased susceptibility to diseases such as necrotic enteritis (Apajalahti and Vienola, 2016). In terms of protein, feeding highly bioavailable protein sources is possible, but not without economic penalty as more poorly digested protein sources can be relatively inexpensive. In addition, as human demand increases for high quality protein sources, less may be available for animal production, therefore increasing the use of less well digested ingredients for feed formulation. In regards to modulation of gastrointestinal tract microbiota, information on the impact of protein sources is limited, although evidence for a negative impact does exist for animal in comparison to plant based protein sources (Wilkie et al., 2005; Rodgers et al., 2015). Further the mechanism (s) whereby protein sources affect microbial populations and enteric disease are poorly researched, particularly in poultry species. Potential mechanisms include protein fermentation and microbiota dysbiosis due to the AA content of the distal ileum (Wilkie et al., 2005; Rinttila and Apajalahti, 2013).

The potential impact of protein nutrition on bird health and ultimately performance could be related to protein fermentation in the hindgut of poultry (Rinttila and Apajalahti, 2013; Qaisrani et al., 2015). In theory, if a large amount of protein is available for fermentation in the distal digestive tract and the fermentable carbohydrate is limited, bacteria capable of fermenting protein will proliferate. This change in the microbiome establishes a favorable microbial community for *C. perfringens*, which is known to cause necrotic enteritis in poultry (Al-Sheikhly and Truscott, 1977). The fermentation of protein can also lead to the formation of undesirable compounds such as ammonia, amines and indoles which are capable of negatively impacting bird health. It is essential that effects of protein sources on gut health are understood and taken into account when formulating diets for poultry. Individual AA, for example, glycine have been shown to increase *C. perfringens* numbers *in vitro* and was correlated with higher *C. perfringens* count in the lower digestive tract of poultry (Wilkie et al., 2005). The data from the current study provides some insight into the digestion characteristics of some protein sources used in poultry production.

The chicken's ability to digest proteins in a digestibility study is dependent on ingredient quality, bird age, assay methodology and variation due to endogenous loss associated with specific ingredients (Lemme et al., 2004; Ravindran et al., 2017). The AA concentrations of the meals used in the current study approximated other published values (Bandegan et al., 2010; Kim et al., 2012; Rochell et al., 2012; Adedokun et al., 2015). The extent of digestion of the protein sources are in

agreement with other published values (Ravindran et al., 2005; Adedokun et al., 2008); however, direct comparison cannot be made due to differences in assay methodology among studies. Knowing the extent of digestion of the ingredients makes it possible to formulate diets on a digestible AA basis (Dari et al., 2005) and it also provides indirect information on how much protein is available for hindgut fermentation. The latter of the two was considered to be of lesser importance, but recently, due to the worldwide push to remove sub-therapeutic antibiotics usage from poultry diets, it has gained more attention (Rinttila and Apajalahti, 2013; Apajalahti and Vienola, 2016).

The characterization of protein in the distal digestive tract includes understanding the remaining AA content. These data are relevant as previous research has demonstrated that levels of specific AA relate to the nature of microbial populations and are associated with enteric disease. High distal ileum glycine levels are positively correlated with *C. perfringens* proliferation and necrotic enteritis lesion scores (Dahiya et al., 2005; Wilkie et al., 2005). All of the meals evaluated in this study had unique AA profiles (Table 5.2), which translated into variable distal ileum AA content. The BM and FEM had higher ileum levels of AA than all the other samples. This was expected since those meals had the lowest AA digestibility. More glycine was seen in the ileum content of birds fed the animal based meals compared to those fed meals of plant origin. Even though broiler distal ileum glycine have been implicated as a reason for the difference in *C. perfringens* count between plant and animal protein (Dahiya et al., 2005; Wilkie et al., 2005), other research suggest ingredient specific effects might also exist (Palliyeguru et al., 2010; Fernando et al., 2011; Annett-Christianson, 2012). The level of each AA present in the distal ileum content presented in Table 5.6. might be bias due to feed intake differences between the diets when each AA is express per 100 g of feed intake. More research is needed to fully elucidate the role distal ileum AA from different protein source might have on the proliferation of *C. perfringens* in the distal small intestine of poultry.

The chicken's ability to use AA from a protein source for growth has been shown to be influenced by the rate at which AA were liberated from the protein during digestion (Sklan and Hurwitz, 1980) and there is evidence suggesting that rate of protein digestion modulates postprandial protein accretion in humans (Boirie et al., 1997; Dangin et al., 2001). To our knowledge, this is the first study to have evaluated the digestion rate of AA and CP from various protein sources by broiler chickens. The data from this study provide a starting point for further

research into the concept of how protein digestion rate influences postprandial muscle protein synthesis in poultry and the potential effects specific protein sources could have on poultry gut health. Understanding this mechanism could be beneficial to the poultry industry by maximizing broiler muscle protein deposition from available protein sources or managing the delivery of AA to the portal blood of broiler breeders.

The current study suggested that using the CP digestion rate to characterize different kinds of protein meals may not provide the best ranking of meals based on their overall AA digestion rates, due to inherent properties of each meal. For example the CP digestion rate ranked only CDDGS to have the lowest digestion rate of all the meals, but the average of all the AA digestion rate per meal ranks both CDDGS and CM to have a low digestion rate. The CP digestion rate of the meals evaluated in this study indicated that PM had the highest digestion rate followed by MBM. However, the average of the digestion rates of all AA differed from that of the CP digestion rate when ranking the meals in terms of their rate of digestion. This difference in ranking of the meals between the two forms of measurement could be related to the concentration of individual AA in each ingredient or other inherent properties of the feedstuff. The digestion rate averages of all AA seem to be a more precise indicator of the overall digestion rate of the AA from individual protein sources. However, using the average AA digestion rate might not be comparable across different ingredients if the digestion rate of individual AA is influenced by meal AA content. Nonetheless, using the average of the AA indicates that the AA from FM were digested most rapidly, followed by PM, and then FEM.

Another confounding factor which could be influencing the digestion of CP and AA is the level of CP in each meal sample. If the CP digestion rates of ingredients are influenced by their CP level, using a fixed CP level in the test diets should eliminate or reduce potential bias when comparing CP digestion rate across ingredients. However, it is possible that higher meal inclusion levels also increase the level of anti-nutritional compounds in diets. Despite the potential inherent effects of ingredient composition on digestion rate, overall, FM and PM AA were rapidly digested, while CDDGS and CM AA were at the opposite end of the spectrum.

From a practical point of view, it is easier to use the CP digestion rate or the average of all AA as an estimator of AA digestion rate, but this will depend on the level of correlation between the two forms of measurements. Of the 17 AA evaluated only proline, serine, and tyrosine digestion rate did not correlate with the average AA digestion rate of the meals, while histidine,

glutamic acid, aspartic acid, alanine, methionine and lysine digestion rate did not correlate with CP digestion rate.

The level of correlation between the CP or average AA digestion rate with that of individual AA might been influenced by the assay technique used to evaluate the meal samples. The technique used in this assay relied on the feed intake of the diets over 24 h and the MRT of digesta in the small intestine. Correlations between feed intake and digestion rate data (individual AA digestion rate, average AA digestion rate and meal CP) were not significant, suggesting that the current method of estimating digestion rate was valid despite large differences in feed intake. Furthermore, the MRT was calculated in order to have a time component to use in the digestion kinetic model, therefore it is expected that it should be influenced by the characteristics of the ingredient. There were differences in the MRT between the diets possibly due to the physical and chemical characteristic of the feed ingredients. Diet structure can affect passage rate (MRT) and as such all diets were pelleted to reduce this confounding effect and to encourage higher feed intake. The technique used to evaluate the AA digestion rate of the samples is novel and therefore requires future research to confirm these finding using multiple samples per ingredients.

The protein entering the ileocaecal junction can have a significant impact on poultry health based on its fermentation capacity (Choct and Ao, 2009; Rinttila and Apajalahti, 2013). The chicken's ability to ferment protein (particularly in the caeca) might depend on the solubility and to a lesser extent the size of the particles of the proteins in the distal ileum. To the author's knowledge, this is the first study to have determined the total soluble CP present in the distal ileum content of birds fed semi-purified diets containing 9 different protein sources. Each diet had a single meal as the only protein source, as such, any protein effects seen on the soluble CP is not confounded by feeding a diet with multiple protein sources. The soluble CP could not be determined for the BM and N-free diets, and therefore no data were provided for these meals.

The concentration of CP in the distal ileum digesta of broilers from the current study was related to the digestibility of the meals and their inherent characteristics. Broilers fed the FEM diet, which had low CP digestibility, translated into a high concentration of CP in the distal ileum digesta when compared to the other meals, and the same was true for those fed the PM diet. This confirmed that the CP present in the ileum was influenced more by the individual protein sources than the level of CP in the diets. The dry matter of the distal ileum content from birds fed FEM contained 57% total and 1% soluble protein, which suggests that 98% of the protein may not have

access to the caeca, and therefore not be fermented if access to the caeca is based on solubility. In comparison, the total and soluble content (dry matter basis) of distal ileum digesta of birds fed FM was 41 and 9%, respectively. The SBM, PM, and MBM had similar soluble CP levels as FM, which suggests that they could have the same fermentation capacity in the caeca if that capacity depends only on the soluble CP present at the distal ileum. The proportion of individual AA in the soluble fraction of the CP of the distal ileum content was not determined in this study, but it is expected that these proportions would be influenced by individual protein sources.

The diets produced different levels of DM in the distal ileum, which could be related to feed intake, digest passage rate, diet fibre level and diet digestibility. Correlation analysis revealed that feed intake (R^2 =0.55) attributed to a portion of the dry matter content of the DI. The amount of DM present in the distal ileum could influence the level of CP and soluble CP present for fermentation. The lowest DM content and subsequently the lowest mg of soluble CP were found for FEM, CGM, and the FM diets, while CDDGS and CM had the highest DM content which corresponded with the highest mg of soluble CP. It is possible that the high DM content was related to the fibre level of the CM and CGGDS diets. Even though specific protein sources, such as high levels of FM or animal by-product meals in poultry diets, are often associated with increased susceptibility to diseases like necrotic enteritis (Kaldhusdal and Skjerve, 1996; Drew et al., 2004; Gholamiandehkordi et al., 2007), it is yet to be proven whether the distal ileum concentration of soluble CP or the volume of CP influence protein fermentation in the caeca of poultry.

Conclusion

In conclusion, the extent and rate of CP and AA digestion of protein meals were determined *in vivo* for poultry. Protein sources were shown to vary in rate and extent of CP digestibility, as well as residual AA, and total and soluble protein content in the digesta of the distal ileum. This could have been related to differences in CP and AA intake. These data are a first attempt to characterize proteins sources beyond AA digestibility and thereby facilitate research on the importance of these characteristics in bird performance and health.

Transition statement

The *in vivo* and *in vitro* digestion characteristic of nine high protein sources were evaluated in the previous chapters. It is still unknown if the digestion characteristics identified in the previous chapters can have any impact on broiler growth performance and meat yield proteins when birds are grown under industry management standards. In Chapter 6 the level of undigested protein, which was one of the digestion characteristic identified from the protein sources evaluated in Chapter 4, was used as a formulation criteria to produce practical diets. The diets where fed to broilers grown with an antibiotic free management protocol, which involved the use of live coccidiosis vaccination.

6.0 THE INFLUENCE OF INDIGESTIBLE PROTEIN ON THE PERFORMANCE AND MEAT QUALITY OF BROILERS VACCINATED FOR COCCIDIOSIS

6.1 Abstract

High dietary protein and the use of poorly digested protein sources have been suggested to negatively impact broiler health, possibly because of protein fermentation in the distal intestinal tract. The effect of dietary protein levels with low or high indigestible protein fractions (LIP or HIP) on male and female broiler performance were evaluated. The trial was completely randomized with a 2x3x2 factorial arrangement where gender, dietary protein levels (24, 26, and 28%) and dietary indigestible protein fractions were the main factors. Ross 308 male (1944) and female (2232) were allocated to 72 pens with 54 males or 62 females per pen. Six grower diets 24-LIP, 24-HIP, 26-LIP, 26-HIP, 28-LIP, and 28-HIP were fed from 0-32 d of age. Birds were vaccinated with Coccivac-B52 on d 5, and feed intake and body weight were recorded at 0, 12, 22, and 32 d. On d 32, 24 birds per treatment were processed for meat yield. Males were heavier than females at all post-hatch ages and the LIP birds were heavier than their HIP counterparts at 32 d. At 22 d, birds fed 24 and 26% CP were heavier than those fed 28% CP. Birds fed the 28-LIP diet consumed less total feed than their 24 and 26-LIP equivalents. Birds fed 24% CP had the highest total feed to gain ratio, while LIP fed birds had a lower total feed to gain than those fed HIP diets. LIP diets resulted in higher total mortality than the HIP diets. Carcass yield was higher for females than males, increased with CP level and was lower in HIP than LIP birds. An interaction between CP level and dietary indigestible protein resulted in the 28 and 26-LIP having higher breast yield than all other diets. In conclusion, broiler growth performance and meat yeild were affected by dietary indigestible protein alone or in combination with gender and dietary CP level.

Key words: Indigestible protein, coccidiosis vaccination, antibiotic free diet, carcass yield, dietary protein level

6.2 Introduction

There are concerns regarding the prophylactic use of medication in poultry feed. Regulation concerning the use of medication in poultry diets varies depending on the geographical location. Some countries have none whatsoever, while others have legislation governing the amount and nature of sub-therapeutic medications used in poultry diets. Recently more efforts have been placed on reducing and in some cases like antibiotic-free production, eliminating the use of sub-therapeutic antibiotics in poultry production.

To compensate for less or no use of antibiotics and other medication, the poultry industry is using a multifaceted approach including changing the nature of poultry diets. A number of feed additives such as enzymes, essential oils, organic acids, and vaccines are available to the poultry industry to help manage poultry performance and health in the absence or removal of antibiotics from poultry diets. A second approach is to formulate diets which enhance the bird's ability to withstand disease. Regardless of the approach used, the main objective is to reduce the potential for disease without loss of performance.

One aspect of diet formulation changes is the amount and nature of the dietary protein. As such, recommendations for antibiotic-free production includes a reduction in dietary protein and the reduced use of poorly digested protein sources. The main reason for these recommendation is to reduce the amount of undigested, and potentially fermentable protein, in the distal small intestine, caeca, and colon. Protein fermentation in the distal small intestine can lead the formation of ammonia, polyamine, indoles, and skatole, which are thought to be toxic (Barnes et al., 2001; Qaisrani et al., 2015; Apajalahti and Vienola, 2016).

The undigested protein may also increase the levels of specific amino acids in the distal gastrointestinal tract, which may affect the microbiota and change the host animal susceptibility to intestinal disease. There is evidence suggesting that individual dietary protein sources have the potential to influence the population of pathogenic bacteria in the gastrointestinal tract of poultry (Drew et al., 2004; Wilkie et al., 2005; Dahiya et al., 2007). Some of these dietary protein sources can negatively shift microbiota in the presence of coccidiosis, which subsequently leads to necrotic enteritis (Rodgers et al., 2015). A high intestinal microbial load can lead to stimulation of the immune system, which will utilize nutrients intended for growth. It has, therefore, been suggested that poorly digested dietary proteins (high indigestible fraction) can lead to poor health and reduced performance (Qaisrani et al., 2015; Apajalahti and Vienola, 2016).

Coccidiosis is estimated to cost the poultry industry millions of dollars worldwide due to poor performance (Williams, 1999). The disease is also a predisposing factor for necrotic enteritis in commercial production, so it is often controlled by the use of anticoccidial agents or vaccination (Timbermont et al., 2011). Anticoccidials are considered medication and due to complications with legislative description between antibiotics and coccidiostats in some countries, vaccination is becoming more prevalent in commercial broiler production. The use of live oocyte vaccines has been shown to reduce growth performance of broilers due to the initial challenge of the immune system (Williams, 2002). Despite the reduced performance, coccidiosis vaccination is part of many antibiotic-free feeding programs. To be industrially relevant in antibiotic free production, Research on the effects of the nature and amount of dietary protein needs to be completed with animals grown under simulated commercial management conditions.

The objective of this study was to evaluate the effects of dietary protein level (PL) and dietary indigestible protein fraction (IDP) on the performance and meat yield of broilers vaccinated for coccidiosis and fed antibiotic-free diets. It was hypothesized that diets with high IDP will compromise broiler production performance and meat yield and that increasing the PL will decrease broiler production performance but maximize meat yield portions.

6.3 Material and methods

All animals used in this study were cared for using the Canadian Council on Animal Care guidelines On the Care and Use of Farm Animals in Research, Teaching, and Testing (Canadian Council on Animal Care, 2009). The experimental procedures of this study were approved by the University of Saskatchewan Animal Research Ethics Board.

6.3.1 Experimental design and bird management

This study evaluated the effects of three dietary protein levels (24, 26, and 28%) with low or high indigestible protein (LIP, HIP) on broiler performance. The trial was completely randomized, with a 3 x 2 x 2 factorial arrangement, where dietary protein levels, indigestible dietary protein, and gender were the main factors. The pens were the experimental units with rooms as a blocking factor.

A total of 2,232 female and 1,944 male Ross 308 broilers chicks were obtained from a commercial hatchery (Sofina Foods Inc., Wynyard, Saskatchewan, Canada). On the d of arrival, the birds were sorted into groups of 62 females or 54 males, and then randomly distributed to 72

floor pens in 6 rooms. The stocking density of the pens was set at 23.2 kg/m^2 based on the estimated trial end weight and the incidence of mortality (Aviagen, 2014a). Each room had 12 floor pens, each 2.3 m wide by 2 m long. The floor of each pen was covered with wheat straw to a thickness of 10 cm. Each pen was equipped with a tube feeder (diameter = 36 cm from 0-21 d or 43 cm from 21-32 d), and a drinker which had 6 Lubing-4087 nipples. A cardboard egg filler flat (30 x 30 cm, 30 eggs) and a polypropylene ice cube tray provided supplemental feed and water for the first 5 d of the trial. The temperature in each room was set at 33°C on the d of bird placement and subsequently temperatures were reduced by 0.75°C for each of the first 7 d and then by 0.42°C per d until 21°C where it remained for the rest of the trial. The lighting program and light intensity was 23 h light 1 h dark (23L:1D) at 20 lux for 0-2 d, 22L:2D at 18 lux from 3-4 d, 21L:3D at 16 lux from 5-6 d, 20L:4D at 14 lux for 7-8 d, 19L:5D at 12 lux from 9-10 d, 18L:6D at 10 lux from 10-11 d, and 17L:7D at 10 lux for the remainder of the trial (12-32 d).

Three days before bird placement, the relative humidity in experimental rooms was set at 60%, regulated using a humidifier (AIRCARE® Evaporative Humidifier Model SS390DWHT, Little Rock, Arkansas, USA) and confirmed by a Mason hygrometer (Cole-Parmer 7109, Montreal, Quebec, Canada). The wet/dry bulb temperature in each room was checked at least two times per d until d 14 of the trial. Over the 3 d prior to the bird's arrival litter in each room was sprayed with water using a backpack garden sprayer (ECHO® Model MS40BP, London, Ontario, Canada) several times daily until the humidity in each room stabilized around 55-60%. The humidity was maintained in the rooms until 14 d after initial bird placement, by periodically spraying the walkways with water and adjusting the humidifiers. The humidifiers were removed 15 d after bird placement.

Five d after bird placement, a new cardboard egg filler flat was placed in each pen and feed was put evenly in each cell; clean water was also added to pen ice cube trays. Feeders were raised in each pen so that the birds only had access to feed in the filler flats and lowered at the end of the d. The egg flats and ice cube trays were placed approximately 25 cm from the drinker line. A 30 cm wide piece of Kraft brown paper (Model S-8511S, ULINE Canada, Milton, Ontario, Canada) was placed under the full length of the water line to promote coprophagy and recycling of coccidial oocysts; the paper was removed at 14 d of age. One bottle of Coccivac-B52 (1,000 doses) was diluted with 500 ml distilled water and placed in a one-gallon Chapin Lawn, and Garden Ploy Sprayer (Model 20000, Batavia, New York, USA) set to light spraying with a wide spray pattern

and used for one room (696 chickens). The vaccine was sprayed over the feed and water in the cardboard egg trays and ice cube tray using 3 even passes while agitating the sprayer bottle.

6.3.2 Diet formulation

The birds were fed the six diets using a one phase feeding program, and they had free access to water and feed throughout the experiment. The non-medicated diets were formulated to meet or exceed the as hatched 2.0 to 2.5 kg Ross 308 broiler grower nutrient requirements (Aviagen, 2014b), and all contained the same level of digestible methionine and cysteine. The diets were formulated on a digestible amino acid basis, and the digestibility coefficients were based on Evonik Degussa AMINODat 4.0 (Evonik, 2010) and Ajinomoto Heartland Inc. digestible amino acid databases (Ajinomoto Heartland LLC, 2009).

All ingredients which contributed protein to the diets were analyzed for amino acids (Table 6.1) by the Agriculture Experiment Station Chemical Laboratories (University of Missouri, Columbia) using AOAC method 982.30 E (a, b, c) (AOAC, 2006) before diet formulation. All protein meals and the wheat sample used to make the diets were subjected to *in vitro* digestion using the assay developed in Chapter 4 to obtain the level of IDP in the ingredients. The *in vitro* model mimics the gastric and intestinal phases in chickens and all ingredients were digested in 6 replicate tubes. Meal sample equivalent to 500 mg CP (% N_2 x 6.25) was digested with 28,260 units of pepsin in 50 mL polyethylene centrifuge tubes for 30 min in a shaking water bath at 41°C. The pH of the tube contents was adjusted to 7±0.5 using NaOH after gastric digestion, then 9.5 mL of sodium acetate buffer (pH 12.5) was added to each tube. A 6.5 mL pancreatin solution and 3 glass marbles were placed in the tubes, which were incubated for 180 min at 41°C in a water bath. Tubes were sampled at the end of the intestinal phase and the degree of hydrolysis determined calorimetrically with ninhydrin reagent. The digestibility of the samples was calculated using the degree of hydrolysis of the samples as a percentage of that sample totally hydrolyzed with 6N HCl. All synthetic amino acids were considered to have 0% IDP. The diets were formulated to have different levels of IDP at each protein level. Using this formulation criteria, the following 6 diets (Table 6.2) were formulated: 24-LIP (24% CP with low IDP), 24-HIP (24% CP with high IDP), 26-LIP (26% CP with low IDP), 26-HIP (26% CP with high IDP), 28-LIP (28% CP with low IDP), and 28-HIP (28% CP with high IDP). All diets were analyzed for minerals and CP by SGS Agrofood laboratories (Guelph, Ontario, Canada) using AOAC (2006) methods 990.03 and 985.01, respectively.

Table 6.1. Amino acid and protein composition (%) of the ingredient as is.

Amino acid	Wheat	CGM	SBM	CDDGS	FM	PCM
Indispensable amino acids						
Arginine	0.58	1.92	3.21	1.27	3.48	3.63
Cysteine	0.27	1.04	0.59	0.58	0.51	0.5
Isoleucine	0.48	2.69	2.2	1.15	2.93	2.15
Leucine	0.87	10.28	3.52	3.54	4.86	3.98
Lysine	0.39	1.09	2.91	1.03	5.09	3.62
Methionine	0.2	0.67	0.57	0.58	1.63	0.9
Threonine	0.34	1.9	1.61	1.17	2.49	1.84
Tryptophan	0.17	0.34	0.58	0.23	0.72	0.46
Valine	0.59	3.02	2.39	1.59	3.48	2.87
Dispensable amino acids						
Alanine	0.45	5.37	1.94	2.05	3.96	4.17
Aspartic Acid	0.61	3.57	4.89	1.88	5.56	4.33
Glutamic	3.9	13.36	8.11	3.97	8.2	7.16
Acid						
Glycine	0.55	1.79	1.95	1.14	3.82	6.22
Histidine	0.3	1.24	1.17	0.92	1.93	1.32
Phenylalanine	0.6	3.86	2.27	1.41	2.6	2.13
Proline	1.35	5.9	2.49	2.33	2.74	4.2
Serine	0.46	2.41	1.72	1.32	1.88	1.7
Tyrosine	0.28	2.93	1.52	1.11	1.9	1.54
Total	12.39	63.38	43.64	27.27	57.78	52.72
CP	13.27	62.09	45.28	28.29	67.23	61.92

CGM=Corn gluten meal; SBM=Soybean meal; CDDGS=Corn distiller's dry grain with solubles; FM=Fish meal; PCM=Porcine meal; CP=crude protein.

Table 6.2. Experimental diet composition with calculated and analyzed nutrient levels.

Ingredients	24-LIP[1]	24-HIP[2]	26-LIP	26-HIP	28-LIP	28-HIP
Wheat	60.11	50.48	55.36	44.74	49.34	42.71
Soybean	26.94	28.32	30.02	25.59	34.88	24.03
Fish meal	5.00	0.00	7.00	0.00	8.00	0.00
CDDGS[3]	0.00	10.50	0.00	15.00	0.00	15.00
Corn gluten meal	0.00	0.00	0.00	4.00	0.00	6.00
Porcine meal	0.00	1.50	0.00	2.00	0.00	5.00
Canola oil	4.20	4.84	4.45	4.60	4.94	4.17
Vit/min broiler[4]	0.50	0.50	0.50	0.50	0.50	0.50
Mono di-calcium phosphate	0.60	0.87	0.33	0.73	0.19	0.19
Limestone	1.39	1.50	1.30	1.49	1.24	1.24
Sodium chloride	0.24	0.36	0.17	0.32	0.13	0.29
Choline chloride	0.10	0.10	0.10	0.10	0.10	0.10
L-Lysine HCl	0.03	0.15	0.00	0.16	0.00	0.09
DL-Methionine	0.26	0.26	0.21	0.18	0.17	0.14
L-Threonine	0.12	0.12	0.05	0.07	0.00	0.03
Ameri-Bond 2x[5]	0.50	0.50	0.50	0.50	0.50	0.50
Econase[6]	0.01	0.01	0.01	0.01	0.01	0.01
Calculated analysis (%)						
AME (kcal/g)	3.1	3.1	3.1	3.1	3.1	3.1
DM	89	89	89	89	89	89
CP	24	24	26	26	28	28
Fat	6.1	7.3	6.4	7.5	6.9	7.3
Calcium	0.9	0.9	0.9	0.9	0.9	0.9
Non-phytate phosphorus	0.45	0.45	0.45	0.45	0.45	0.45
Total phosphorus	0.66	0.67	0.67	0.67	0.68	0.66
Potassium	0.85	0.89	0.91	0.88	0.98	0.85
Indigestible CP	4.18	5.15	4.50	6.29	4.80	7.02
Dig. Arginine	1.2427	1.2352	1.3684	1.2478	1.5127	1.3113
Dig. Isoleucine	0.8870	0.8776	0.9786	0.9457	1.0753	1.0012
Dig. Leucine	1.5187	1.6378	1.6606	2.0305	1.8070	2.2448
Dig. Lysine	1.1500	1.1500	1.2814	1.1500	1.4321	1.1500
Dig. Met+Cys	0.8700	0.8700	0.8700	0.8700	0.8700	0.8700
Dig. Methionine	0.5729	0.5584	0.5630	0.5332	0.5512	0.5255
Dig. Threonine	0.7700	0.7700	0.7700	0.7700	0.7851	0.7700
Dig. Tryptophan	0.2583	0.2467	0.2783	0.2424	0.3002	0.2452
Dig. Valine	1.0289	1.0295	1.1261	1.1131	1.2252	1.1847
Analysed values (%)						
DM	89	90	89	90	90	90
CP	24	24	26	26	28	28
Fat	6.0	5.0	7.0	7.1	5.3	6.9
Calcium	0.77	0.94	0.85	0.95	0.80	0.84
Total phosphorus	0.64	0.67	0.63	0.67	0.68	0.65
Potassium	0.99	1.05	1.01	0.94	1.04	1.04

[1]Low indigestible CP; [2]High indigestible CP. [3]Corn distillers' dried grains with solubles. [4]Vitamin-mineral premix provided the following per kilogram of diet: 4 mg vitamin B12; vitamin D, 440,000 IU; vitamin A, 2,200,000 IU; vitamin E, 6000 IU; menadione, 400 mg; thiamine, 300 mg; riboflavin, 1200 mg; pyridoxine, 800 mg; niacin, 12,000 mg; pantothenic acid, 2000 mg; folic acid, 120 mg; biotin 30 mg; copper, 2000 mg; iron, 16,000 mg; manganese 16,000 mg; iodine, 160 mg; zinc, 16,000 mg; selenium, 60 mg; calcium carbonate 100,000 mg; Ethoxyquin 125 mg; wheat middlings 754,546 mg. [5]Pellet binder (LignoTech, Wisconsin, USA). [6]Econase XT (ABVista, Wiltshire, UK), β 1-4 endo-xylanase enzyme, xylanase activity (160,000 BXU/g).

6.3.3 Data collection

Body weight (BW) was measured per pen at 0, 12, 22, and 32 d of age. Feed intake (FI) was measured on d 12, 22, and 32. Body weight gain (BWG) was calculated for 0 to 12, 12 to 22, 22 to 32, and 0 to 32 d. Feed to gain ratio (FG) was determined based on BWG and FI for each data collection period and for the overall experiment. The FG calculation was corrected for mortality by adding the BW of dead and cull birds to the end-day pen weights. Death loss and culling were recorded daily, and dead birds were weighed, wing banded and sent to the Prairie Diagnostic Services lab (Saskatoon, Saskatchewan, Canada) for necropsy.

At trial end, 24 birds per treatment (6 birds per pen) were randomly selected and double wing banded for meat yield determination. Feed and water were withdrawn for 2 and 0.5 h, respectively, before birds were individually weighed prior to transport to a commercial slaughtering plant (Sofina Foods Inc, Wynyard, SK, Canada). The birds were slaughtered no later than 10 h after feed withdrawal. After slaughter, the birds were chilled, packed in ice and shipped to the Meat Science lab at the University of Saskatchewan for meat yield evaluation. Each carcass was weighed and then separated into a whole right drumstick, whole right thigh, left drum and thigh separated into skin, meat and bone portions, breast (skin, pectoralis major, pectoralis minor), wings (2), abdominal fat pad, and the remaining carcass (back/rack). All the component weights were expressed as a percentage of live body weight.

6.3.4 Statistical analysis

All statistical assumptions (normality and heterogeneity of variance) were checked prior to analysis using the PROC Univariate test of SAS 9.4. The production performance and the meat yield data were subjected to analysis of variance using PROC MIXED (SAS 9.4) with a 2 x 2 x 3 factorial arrangement of treatments with the room as a blocking factor. Statistical significance was determined at $P \leq 0.05$. Tukey's Studentized Range Test was used for mean separation of significant main effects or their interaction, and pdmix800 macro (Saxton, 1998) was used to provide letter grouping for differences. Initial analysis of the data indicated that the blocking factor was not significant for any of the variables tested and the F ratio was close to 1. Therefore, the data were reanalyzed without block to maximize the usage of the degrees of freedom.

6.4 Results

6.4.1 Growth performance

Males weighed more than females at all ages including d 0 (Table 6.3). At 12 d of age, IDP, PL and their interaction affected BW which was as a result of a significant reduction in BW of the HIP compared to the LIP treatment at the 26% CP level. In contrast, IDP did not affect BW at the 24 and 28% CP levels and birds fed 24% CP or 26% CP with LIP had higher BW than birds fed 26-HIP, 28-HIP and 28-LIP. At 22 d of age, IDP did not affect BW, but birds fed the 28% PL were smaller than those fed either the 24 or 26% CP treatments. Body weights on d 32 were influenced independently by PL and IDP where high levels of IDP lowered BW, while birds on the 26% CP diets had higher BW compared to those on the 28% CP with broilers fed the 24% CP diets being intermediate.

Males grew faster than females for all periods of the trial (Table 6.3). Body weight gain from 0-12 d was affected by a 3-way the interactions between gender, IDP, and PL in which BWG decreased in both males and females as PL increased. There was a concurrent lower BWG in the HIP diets at each PL with the exception of females fed 28% CP where it was reversed with LIP being lower than the HIP diet. From 12 to 22 d, BWG was influenced by the interaction between PL and IDP, where birds fed 24% CP with LIP and those fed 26% CP with HIP had higher gains than those fed 28% CP with LIP; other treatments were intermediate and not different than the low and high values. Day 22 to 32 BWG was affected by the combined effects of gender and IDP in which females gained less than males, but males fed LIP diets gained more than those on the HIP diets. Birds on the LIP diets had higher total gain than those on HIP diets, and birds given the 26% CP diets had higher gain than those given 28% CP, while those given 24% CP were intermediate. The interaction between gender and IDP approached significance (P=0.0936), with the HIP treatment having a negative effect on male growth in comparison to the LIP treatment, but having no effect on females.

Table 6.3. Effects of diet levels of total and indigestible protein on the body weight and body weight gain of Ross 308 broiler chickens.

Day	0	12	22	32	0-12	12-22	22-32	Total
	Body weight (g/bird)				Body weight gain (g/bird)			
Gender (G)								
Male	46[a]	407[a]	1095[a]	2164[a]	362	685[a]	1073	2119[a]
Female	45[b]	387[b]	1018[b]	1929[b]	344	631[b]	908	1884[b]
SEM	0.1	1.1	4.9	9.1	0.8	3.9	5.9	9.1
Indigestible protein (IDP)[1]								
LIP	45	400	1056	2060[a]	355	653	1007	2015[a]
HIP	45	394	1057	2033[b]	350	664	974	1988[b]
SEM	0.1	1.2	4.8	9.2	0.8	3.6	5.7	9.2
Protein levels (PL)								
24% CP	45	403	1066[a]	2057[ab]	358	666	992	2012[ab]
26% CP	45	396	1063[a]	2063[a]	352	660	1000	2017[a]
28% CP	45	392	1040[b]	2020[b]	349	649	980	1975[b]
SEM	0.1	1.4	5.9	11.2	0.9	4.5	7.6	11.1
IDP *G								
LIP * Male	46	412	1094	2189	367	677	1099[a]	2143
HIP * Male	45	404	1056	2140	358	693	1047[b]	2094
LIP * Female	45	389	1017	1932	344	628	914[c]	1887
HIP * Female	45	387	1019	1927	343	634	901[c]	1882
SEM	0.1	1.3	6.8	12.5	1.2	5.4	8.3	12.5
PL* IDP								
24% CP * LIP	45	405[a]	1071	2080	360	672[a]	1009	2035
24% CP * HIP	45	401[a]	1061	2035	356	660[ab]	974	1989
26% CP * LIP	45	402[a]	1067	2080	358	649[ab]	1019	2034
26% CP * HIP	45	389[b]	1060	2046	345	671[a]	981	2000
28% CP * LIP	46	393[b]	1029	2021	348	637[b]	992	1975
28% CP * HIP	45	391[b]	1052	2020	350	661[ab]	968	1975
SEM	0.2	1.6	8.8	15.1	1.3	6.7	9.4	15.7
PL*IDP*G								
24% CP * LIP * Male	46	415	1107	2211	370[a]	704	1104	2165
24% CP * HIP * Male	45	412	1100	2132	367[a]	688	1031	2086
26% CP * LIP * Male	46	415	1103	2197	369[a]	660	1106	2152
26% CP * HIP * Male	46	398	1103	2157	353[bc]	705	1063	2111
28% CP * LIP * Male	46	404	1071	2157	361[ab]	667	1086	2112
28% CP * HIP * Male	45	400	1084	2131	355[bc]	687	1047	2086
24% CP * LIP * Female	45	395	1034	1949	350[c]	640	915	1904
24% CP * HIP * Female	45	391	1022	1938	346[cd]	631	916	1893
26% CP * LIP * Female	45	392	1031	1962	348[c]	639	931	1917
26% CP * HIP * Female	45	383	1016	1934	337[de]	636	898	1889
28% CP * LIP * Female	45	381	987	1884	336[e]	606	897	1839
28% CP * HIP * Female	45	387	1019	1909	345[cde]	634	890	1864
SEM	0.2	2.2	11.9	22.2	2.1	9.9	14.6	22.2
ANOVA	*P*-Value							
G	0.0002	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
IDP	0.4576	0.0003	0.7912	0.0412	<.0001	0.0426	0.0002	0.0414
PL	0.6546	<.0001	0.0062	0.0188	<.0001	0.0327	0.1548	0.0184
IDP*G	0.4503	0.2567	0.9556	0.0930	0.0036	0.3207	0.0203	0.0936
PL*G	0.5217	0.7937	0.9515	0.7771	0.6167	0.4654	0.5074	0.7695
PL*IDP	0.2268	0.0132	0.1150	0.3534	<.0001	0.0111	0.7259	0.3439
PL*IDP*G	0.5033	0.3344	0.6042	0.6747	0.018	0.0654	0.2686	0.6686

[a-e] Means (n = 12 cages) within a column with no common superscript per main effects or interactions are significantly different ($P \leq 0.05$). [1]LIP-low indigestible protein; HIP-high indigestible protein.

Feed intake for all periods of the trial was influenced by gender (Table 6.4) with males consuming more than females. Indigestible CP alone did not affect FI. The combined effects of PL and IDP impacted FI in all periods except for 22-32 d. For 0-12 d birds fed the 24-LIP and HIP diet had similar but higher FI than those fed the other diets, while birds fed 28-LIP had the lowest FI with all other diets being intermediate. Over the 12 to 22 d period, birds on the 24-LIP diet consumed more feed than all the other diets except those on the 26-LIP, which were statistically similar, while those fed 28-LIP diets consumed the least feed with the rest of diets being intermediate. The effect of PL was significant for d 22-32 FI, as feed consumption decreased with increasing dietary CP levels. For total FI there was a shift in feed consumption relative to IDP that is PL dependent. Birds fed the HIP treatment had decreased FI at 24% CP, but increased FI at 28% CP; IDP had no effects when birds were fed 26% CP.

The FG over the first 12 d (Table 6.4) was influenced by the interaction between PL and IDP, where birds fed 26-LIP and 28-LIP had lower FG than their corresponding HIP diets, but the effect of LIP was not seen when birds were given the 24% CP diets. All three main effects independently influenced the FG from d 12 to 22; females were more feed efficient than males, and birds fed the HIP diets had a lower FG than birds given the LIP diet, and FG decreased in a linear fashion with increasing PL. Similarly, FG decreased with PL from 22 to 32 d of age. The interaction between gender and IDP was also significant for this period, where feeding a HIP diet increased FG for males but not the females. For the total FG of the trial, birds fed the HIP diet were less efficient than those fed the LIP diet and FG decreased with increasing PL.

The protein efficiency ratio was affected by the levels of indigestible and total protein for all time periods (Table 6.5). General trends show higher PER values for LIP diets and for diets with less total protein. An exception is a higher value for the HIP than LIP treatment from 12-22 d of age. Male PER values were higher than females for the 12-22 and 22-32 time periods. However, interactions among main effects were also noted, particularly in young birds. From 0 to 12 d, an IDP by PL interaction showed that HIP diets resulted in lower PER values than their protein equivalent counterparts, except for the 24% protein level where IDP level did not result in a change. A gender by PL interaction for the same time period demonstrated that PER values decreased with increasing PL for both males and females, but the pattern of change was slightly different. For 12 to 22 d, the interaction between IDP and PL was significant again and in this case, the values were higher for the HIP than LIP treatments for the 24 and 26% PL, but not for the 28%

level. A gender by IDP interaction was found for the 22-32 time period, where all treatment had similar PER except for males fed the LIP diets which had the highest value. No interactions were found for the total trial.

Table 6.4. Effects of diet levels of total and indigestible protein on feed consumption and feed to gain mortality corrected of Ross 308 broiler chickens.

Day	0-12	12-22	22-32	Total	0-12	12-22	22-32	Total
	Feed consumption (g/bird)				Feed to gain (kg/kg)			
Gender (G)								
Male	425[a]	1031	1705[a]	3164[a]	1.163	1.481[a]	1.606	1.482
Female	404[b]	915	1490[b]	2807[b]	1.170	1.442[b]	1.635	1.483
SEM	1.67	5.38	7.52	11.69	0.003	0.008	0.009	0.005
Indigestible protein (IDP)[1]								
LIP	413	976	1592	2977	1.151	1.475[a]	1.589	1.470[b]
HIP	416	970	1603	2995	1.182	1.448[b]	1.652	1.495[a]
SEM	1.67	5.59	7.40	12.19	0.003	0.008	0.009	0.005
Protein levels (PL)								
24% CP	429	992	1620[a]	3037	1.188	1.483[a]	1.645[a]	1.506[a]
26% CP	413	982	1601[ab]	2999	1.166	1.459[ab]	1.610[ab]	1.477[b]
28% CP	402	944	1572[b]	2921	1.146	1.442[b]	1.606[b]	1.464[b]
SEM	2.01	6.54	10.20	14.12	0.004	0.010	0.016	0.006
IDP *G								
LIP * Male	424	1036	1703	3156	1.147	1.503	1.558[b]	1.465
HIP * Male	426	1025	1708	3173	1.180	1.459	1.653[a]	1.499
LIP * Female	403	916	1482	2798	1.156	1.447	1.620[a]	1.475
HIP * Female	406	915	1499	2817	1.184	1.437	1.650[a]	1.491
SEM	2.32	7.37	10.47	16.19	0.005	0.011	0.012	0.008
PL*G								
24% CP * Male	442	1045[a]	1720	3212	1.185	1.497	1.627	1.504
26% CP * Male	419	1030[a]	1706	3161	1.156	1.472	1.599	1.473
28% CP * Male	414	1016[a]	1690	6120	1.148	1.475	1.591	1.467
24% CP * Female	417	940[b]	1520	2863	1.192	1.469	1.664	1.507
26% CP * Female	406	934[b]	1496	2836	1.175	1.447	1.621	1.481
28% CP * Female	390	872[c]	1454	2723	1.143	1.410	1.622	1.460
SEM	2.98	9.47	12.51	20.80	0.006	0.014	0.015	0.009
PL*IDP								
24% CP * LIP	433[a]	1013[a]	1622	3049[a]	1.195[a]	1.506	1.615	1.503
24% CP * HIP	426[a]	972[b]	1619	3025[ab]	1.182[ab]	1.460	1.676	1.509
26% CP * LIP	412[b]	994[ab]	1598	2998[ab]	1.133[c]	1.479	1.581	1.464
26% CP * HIP	414[b]	969[b]	1605	3000[ab]	1.198[a]	1.439	1.639	1.491
28% CP * LIP	395[c]	921[c]	1558	2883[c]	1.125[c]	1.440	1.572	1.441
28% CP * HIP	409[b]	968[b]	1586	2960[bc]	1.166[b]	1.444	1.640	1.485
SEM	2.84	9.47	13.02	19.83	0.006	0.014	0.015	0.009
ANOVA	P-Value							
G	<.0001	<.0001	<.0001	<.0001	0.1602	0.0012	0.0204	0.8853
IDP	0.2752	0.4156	0.3013	0.2755	<.0001	0.0201	<.0001	0.0015
PL	<.0001	<.0001	0.0014	<.0001	<.0001	0.0182	0.0232	<.0001
IDP * G	0.8347	0.4772	0.5718	0.9063	0.6658	0.1484	0.0121	0.2284
PL* G	0.0767	0.0321	0.3468	0.1977	0.1256	0.2929	0.8862	0.7368
PL*IDP	0.0024	<.0001	0.4551	0.0423	<.0001	0.1615	0.9394	0.1613
PL* IDP * G	0.0764	0.6029	0.0569	0.0709	0.8599	0.5743	0.3661	0.4247

[a-c] Means (n = 12 cages) within a column with no common superscript per main effects or interactions are significantly different ($P \leq 0.05$). [1]LIP-low indigestible protein; HIP-high indigestible protein.

Table 6.5. Effects of diet levels of total and indigestible protein on protein efficiency ratio of Ross 308 broiler chickens from 0 to 32 d of age.

Day	0-12	12-22	22-32	0-32
		PER[1] (kg/kg)		
Gender (G)				
Male	3.28	2.65[a]	2.41[a]	2.58
Female	3.28	2.56[b]	2.36[b]	2.59
SEM	0.013	0.018	0.014	0.011
Indigestible protein (IDP)[2]				
LIP	3.31	2.57[b]	2.43[a]	2.60[a]
HIP	3.24	2.65[a]	2.34[b]	2.56[b]
SEM	0.013	0.018	0.013	0.011
Protein level (PL)				
24% CP	3.48	2.77[a]	2.54[a]	2.75[a]
26% CP	3.27	2.61[b]	2.39[b]	2.58[b]
28% CP	3.09	2.45[c]	2.22[c]	2.42[c]
SEM	0.016	0.022	0.017	0.013
PL*G				
24% CP * Male	3.47[a]	2.72	2.57	2.74
26% CP * Male	3.30[b]	2.59	2.41	2.58
28% CP * Male	3.07[c]	2.38	2.26	2.40
24% CP * Female	3.48[a]	2.82	2.51	2.75
26% CP * Female	3.23[b]	2.63	2.38	2.58
28% CP * Female	3.12[c]	2.52	2.20	2.43
SEM	0.022	0.031	0.023	0.018
PL*IDP				
24% CP * LIP	3.46[a]	2.71	2.59	2.75
24% CP * HIP	3.49[a]	2.83	2.49	2.74
26% CP * LIP	3.33[b]	2.55	2.43	2.60
26% CP * HIP	3.20[c]	2.66	2.35	2.57
28% CP * LIP	3.20[c]	2.45	2.27	2.45
28% CP * HIP	3.05[d]	2.44	2.18	2.39
SEM	0.022	0.032	0.023	0.018
ANOVA				
G	0.8175	0.0008	0.0113	0.4378
IDP	0.0004	0.0071	<.0001	0.0208
PL	<.0001	<.0001	<.0001	<.0001
IDP * G	0.3322	0.2420	0.0133	0.2261
PL* G	0.0427	0.3146	0.7207	0.7758
PL*IDP	0.0015	0.1033	0.9723	0.3739
PL* IDP * G	0.6397	0.3089	0.4890	0.6107

[a-b] Means (n = 12 cages) within a column with no common superscript per main effects or interactions are significantly different ($P \leq 0.05$). [1]Protein efficiency ratio. [2]LIP-low indigestible protein; HIP-high indigestible protein.

The total mortality (death loss and culls) in this trial was 10.76% of birds placed. There were no effects of PL on total morality or causes of mortality in the trial. Total mortality was

influenced separately by gender and IDP over all the production periods except for IDP which had no effect between 22-32 d of the trial (Figures 6.1 and 6.2). In both cases, more males died than females, and more birds died when fed the LIP diets compared to the HIP diets. Mortality was separated into infectious (coccidiosis, necrotic enteritis, arthritis, cellulitis, hepatitis, endocarditis, osteomyelitis, pericarditis, polyserositis, and yolk sac infection), metabolic (sudden death syndrome and ascites), skeletal (rickets, valgus varus deformities, rotated tibia, spondylolisthesis ,and tibial dyschondroplasia), other (pendulous crop, autolysis, starve out, and dehydration) and unknown (birds whose cause of death was undetermined) causes. In the case of gender (Figure 6.3), more males died from infectious and metabolic causes, while for indigestible protein (Figure 6.4), more birds died from infectious and unknown causes of mortality when they were given the LIP diets compared to the HIP diets.

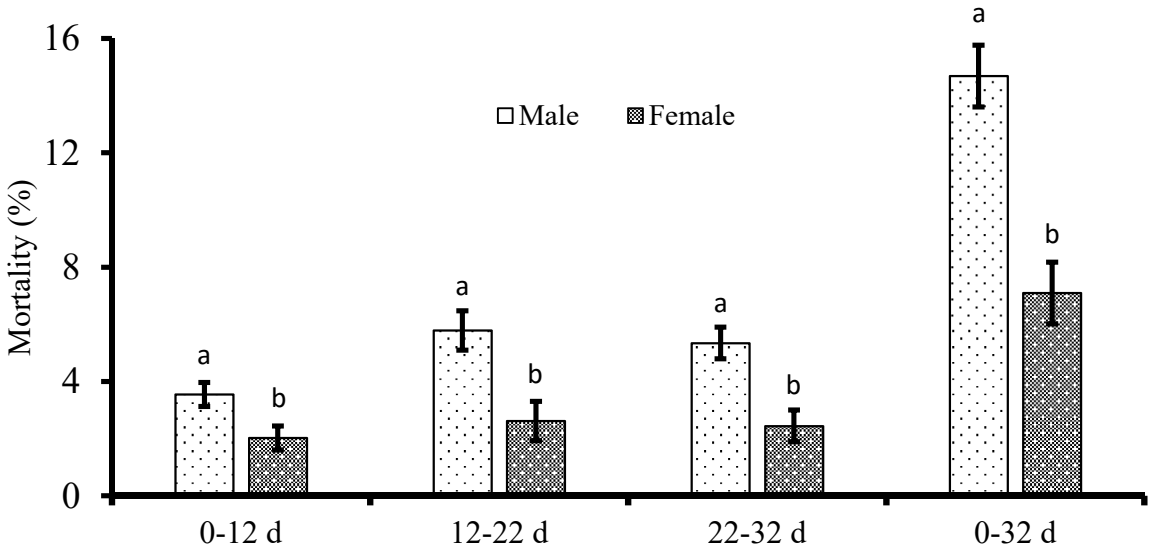

Figure 6.1. Total mortality (mean ± pooled standard error of means) of Ross 308 broiler chickens as influenced by gender per production period express in d (d). Means (n = 12 pens) within a production period with a common superscript are not significantly different at ($P \leq 0.05$).

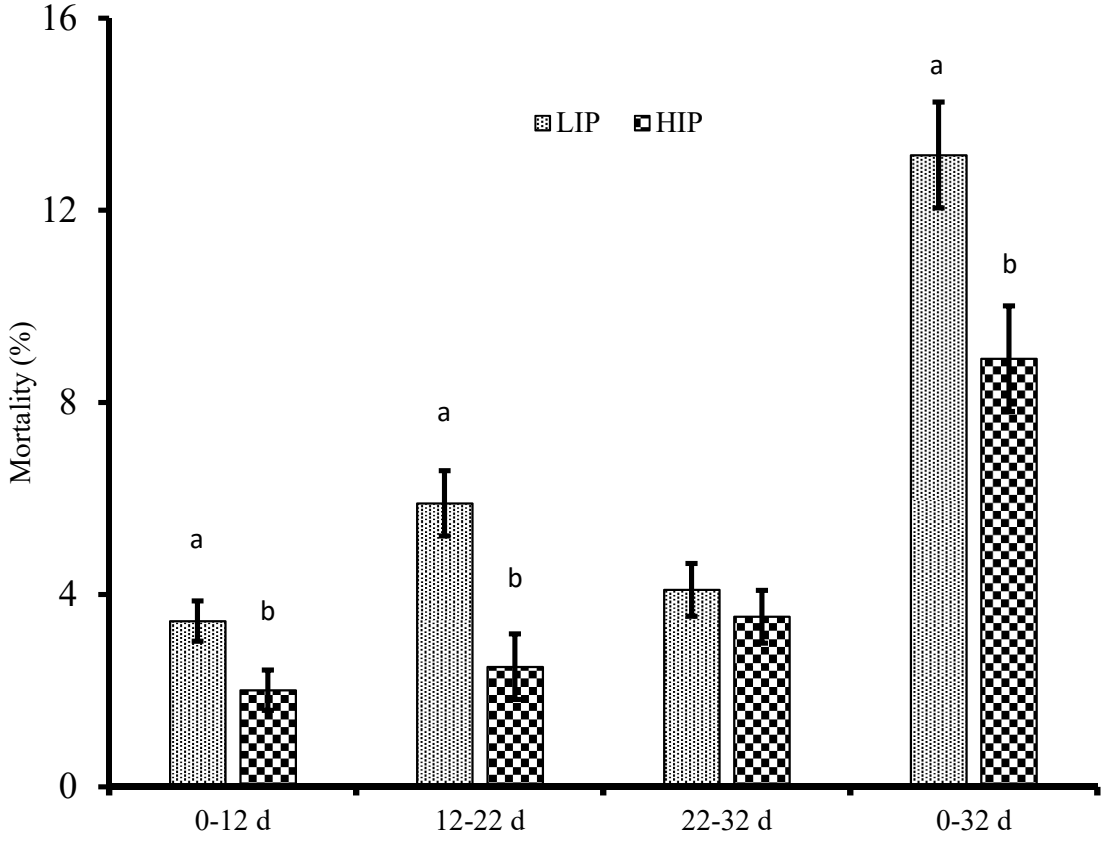

Figure 6.2. Total mortality (mean ± pooled standard error of means) of Ross 308 broiler chickens as influenced by dietary indigestible protein (LIP=low indigestible protein, HIP=high indigestible protein) per production period express in d (d). Means (n = 12 pens) within a production period with a common superscript are not significantly different ($P \leq 0.05$).

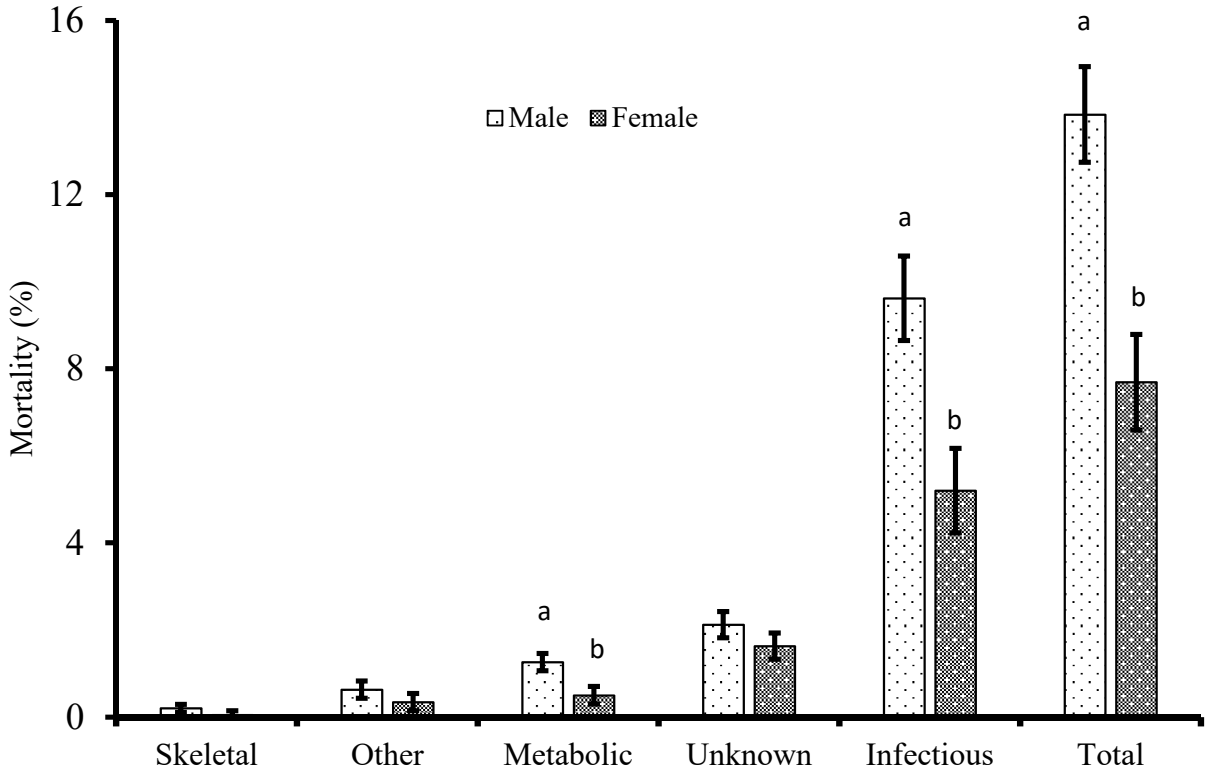

Figure 6.3. Total mortality by cause (mean ± pooled standard error of means) for Ross 308 broiler chicken as influenced by gender. Where infectious includes: coccidiosis, necrotic enteritis, arthritis, cellulitis, hepatitis, endocarditis, osteomyelitis, pericarditis, polyserositis, and yolk sac infection; metabolic: includes sudden death syndrome and ascites; skeletal: includes rickets, valgus varus deformities, rotated tibia, spondylolisthesis, and tibial dyschondroplasia; other: includes pendulous crop, autolysis, starve out, and dehydration while unknown includes birds who's cause of death was undetermined. On each means (n = 12 pens) within a mortality category different superscript are significantly different ($P \leq 0.05$).

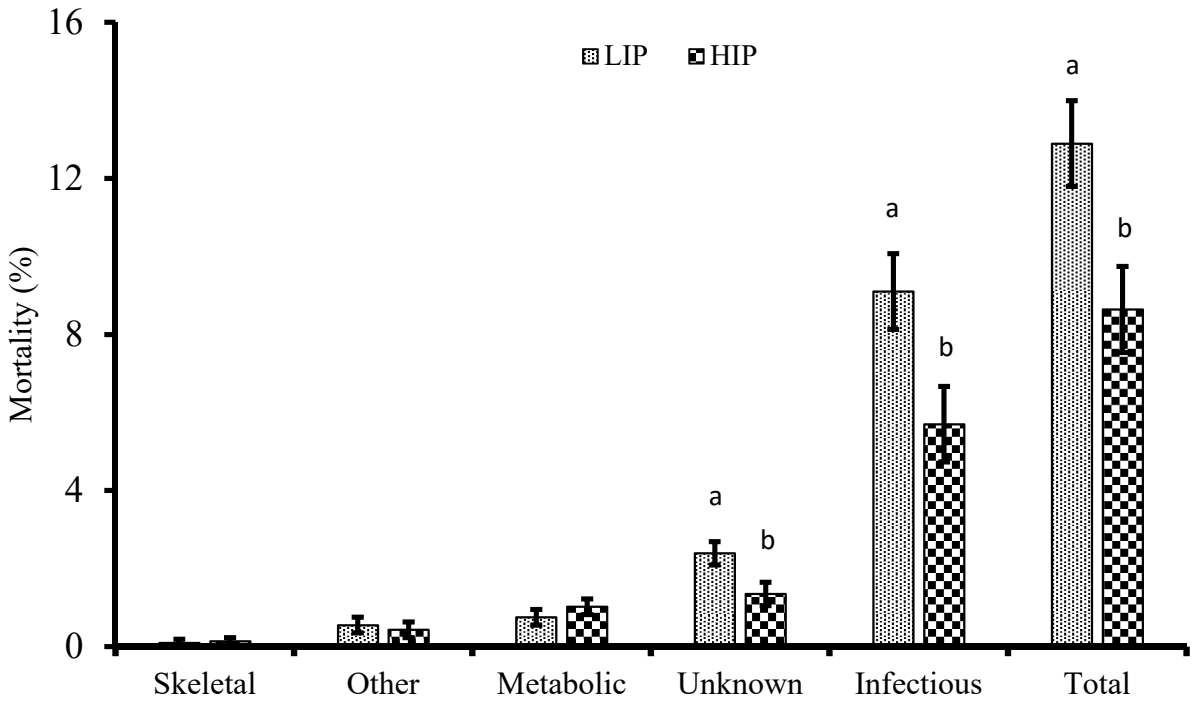

Figure 6.4. Total mortality by cause (mean ± pooled standard error of means) for Ross 308 broiler chickens as influenced by dietary indigestible protein (LIP=low indigestible protein, HIP=high indigestible protein). Where infectious includes: coccidiosis, necrotic enteritis, arthritis, cellulitis, hepatitis, endocarditis, osteomyelitis, pericarditis, polyserositis, and yolk sac infection; metabolic: includes sudden death syndrome and ascites; skeletal: includes rickets, valgus varus deformities, rotated tibia, spondylolisthesis, and tibial dyschondroplasia; other: includes pendulous crop, autolysis, starve out and dehydration while unknown includes birds who's cause of death was undetermined. On each means (n = 12 pens) within a mortality category different superscript are significantly different ($P \leq 0.05$).

6.4.2 Meat yield

The live weights of the birds sampled for meat yield were affected by the interaction of all three main effects (Figure 6.5). In general, males were heavier than females across all PL in which males live weight decreased with increasing protein levels, while female weights were unchanged. Meals fed the lowest PL with high IDP had a slight reduction in live weight, while the opposite was seen in females fed the highest PL.

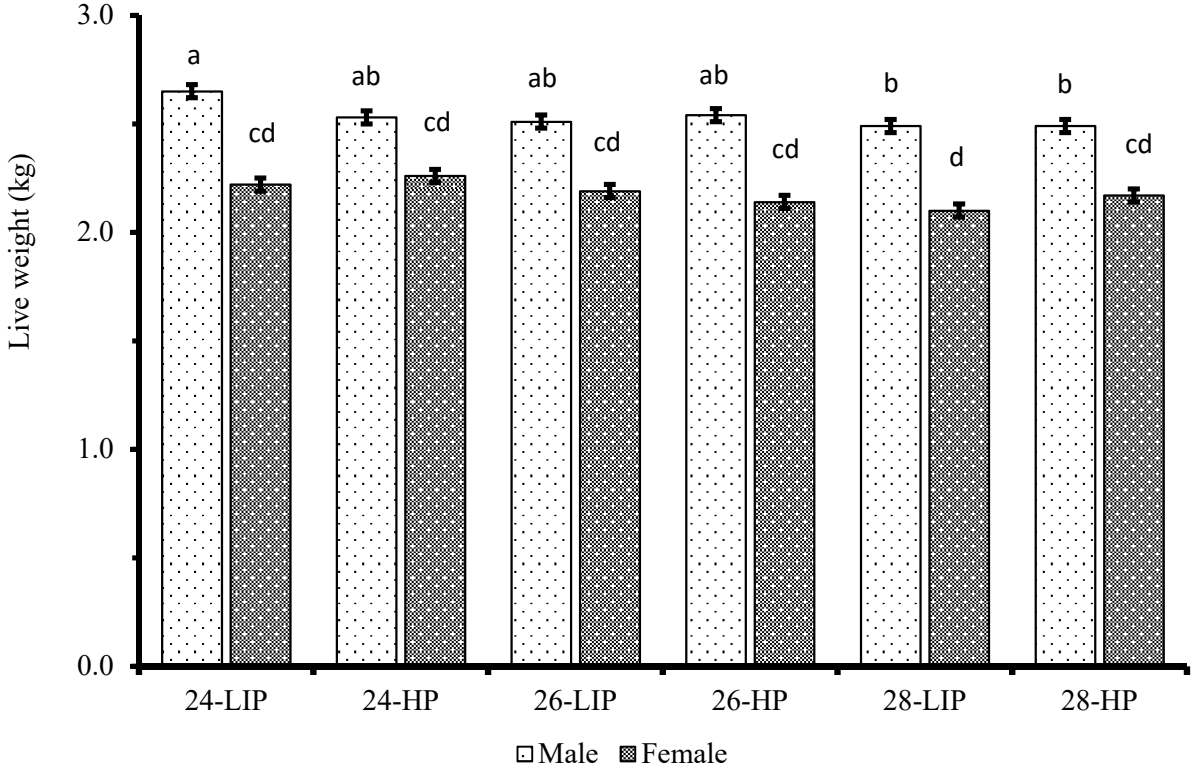

Figure 6.5. Live weight of Ross 308 (mean ± pooled standard error of means) as influenced by the 3-way interaction of gender, indigestible protein (LIP-low indigestible protein, 28-HIP-high indigestible protein) and dietary protein level (24, 26, and 28%). Means (n = 12 pens) with a common superscript are not significantly different at ($P \leq 0.05$).

Carcass weight and breast yield expressed as a percentage of live weight are presented in Table 6.6. Males had heavier carcasses than females and the higher levels of IDP lowered carcass weights. Females had a higher proportion of the whole breast, pectoralis minor, and skin than males, while pectoralis major values were unaffected. Pectoralis minor values were higher for the LIP than HIP treatments and increased with increasing protein levels. Proportional skin weights decreased with increasing dietary protein level. Protein level by IDP interactions for total breast and pectoralis major yield revealed that birds fed the 26-LIP and 28-LIP diets had a higher yield than those on the corresponding HIP diets, but a similar increase was absent in birds fed 24% CP.

Drum and thigh proportional meat yield characteristics are presented in Table 6.7. Males had larger drum and thigh bones, and more thigh meat than females, but less thigh skin. Birds fed LIP diets had larger whole drums and less drum skin than those fed HIP diets. Protein level did

not affect drum and thigh characteristics. Drum meat and whole thigh yield were affected by the interaction between the three main effects (Table 6.7). For the drum interaction, meat yield increased in a quadratic manner in the males and linearly in females as PL increased and when they were fed the LIP diets. Drum meat from both males and females fed LIP diets responded in a quadratic fashion as protein level increased, but at 26% CP females had the largest yield while males had the lowest. For the whole thigh interaction, yield was lowest in males fed the 24-LIP diet and highest in males fed the 26-LIP and 28-HIP diets, while all other diets were intermediate.

Carcass, wing, back-rack, and abdominal fat as a percentage of live weights are presented in Table 6.8. There were no effects of dietary treatments on wing yield. Back-rack yield was only affected by PL where birds fed 24% CP diets had the lowest yield. Both carcass and abdominal fat yield were influenced by 3-way interactions between gender, PL, and IDP in which IDP reduced carcass yield for both sexes, and females had a higher yield than males. When fed the LIP diets, male and female carcass yield increased from low to high PL and females had a similar response when given the HIP diets, but male values were unchanged. During sampling, it was observed that the abdominal fat remaining on the carcass was variable as a result of the processing equipment at the processing plant. Therefore, the abdominal fat pad was only reported for data completeness.

Table 6.6. Effects of diet levels of total and indigestible protein on carcass weights and total breast meat yield per bird as a percentage of live weight.

	Weights (kg)		Breast (%)			
	Live	Carcass	Whole	Major	Minor	Skin
Gender(G)						
Male	2.53	1.78[a]	21.90[b]	18.4	3.49[b]	1.65[b]
Female	2.18	1.54[b]	22.39[a]	18.6	3.83[a]	1.81[a]
SEM	0.13	0.18	0.12	0.11	0.03	1.02
Indigestible protein (IDP)[1]						
LIP	2.36	1.68[a]	22.64	18.93	3.71[a]	1.71
HIP	2.35	1.64[b]	21.65	18.04	3.61[b]	1.75
SEM	0.01	0.18	0.12	0.11	0.03	1.02
Protein level (PL)						
24% CP	2.41	1.68	21.66	18.10	3.60[b]	1.79[a]
26% CP	2.34	1.66	22.34	18.69	3.65[ab]	1.73[ab]
28% CP	2.31	1.64	22.43	18.69	3.74[a]	1.67[b]
SEM	0.02	0.20	0.14	0.13	0.04	1.02
PL*IDP						
24% CP * LIP	2.43	1.70	21.81[b]	18.21[b]	3.60	1.78
24% CP * HIP	2.39	1.65	21.51[b]	17.92[b]	3.60	1.80
26% CP * LIP	2.35	1.68	23.04[a]	19.34[a]	3.70	1.67
26% CP * HIP	2.34	1.64	21.64[b]	18.05[b]	3.60	1.78
28% CP * LIP	2.29	1.65	23.08[a]	19.25[a]	3.84	1.68
28% CP * HIP	2.33	1.64	21.78[b]	18.14[b]	3.64	1.66
SEM	0.22	0.23	0.21	0.18	0.05	1.03
ANOVA			**P-Values**			
G	<.0001	<.0001	0.0035	0.3476	<.0001	<.0001
IDP	0.8589	0.0081	<.0001	<.0001	0.0142	0.3712
PL	<.0001	0.1489	0.0003	0.0005	0.0213	0.0409
IDP * G	0.2168	0.5534	0.9303	0.9834	0.7819	0.8147
PL* G	0.9720	0.9628	0.8879	0.9999	0.1398	0.1562
PL * IDP	0.2112	0.4269	0.0122	0.0165	0.1592	0.3791
PL * IDP * G	0.0347	0.4661	0.1550	0.1031	0.3736	0.9038

[a-b] Means (n = 12 cages) within a column with no common superscript per main effects or interactions are significantly different ($P \leq 0.05$). [1]LIP-Low indigestible protein; HIP-High indigestible protein.

Table 6.7. Effects of diet levels of total and indigestible protein on total drum and thigh per bird expressed as a percentage of live weight.

	Drum				Thigh			
	Whole	Meat	Bone	Skin	Whole	Meat	Bone	Skin
Gender (G)								
Male	4.78	3.13	1.13[a]	0.43	6.03	4.59[a]	0.73[a]	0.75[b]
Female	4.72	3.05	1.09[b]	0.44	6.02	4.47[b]	0.71[b]	0.90[a]
SEM	0.03	0.06	0.01	0.01	0.04	0.04	0.01	0.03
Indigestible protein (IDP)[1]								
LIP[1]	4.79[a]	3.12	1.11	0.43[b]	6.02	4.55	0.73	0.81
HIP[2]	4.71[b]	3.07	1.11	0.45[a]	6.03	4.51	0.72	0.83
SEM	0.03	0.06	0.01	0.01	0.04	0.04	0.01	0.03
Protein level (PL)								
24% CP	4.72	3.04	1.10	0.43	6.00	4.52	0.70	0.84
26% CP	4.75	3.12	1.11	0.44	6.01	4.56	0.72	0.81
28% CP	4.78	3.11	1.13	0.44	6.07	4.51	0.74	0.81
SEM	0.03	0.07	0.02	0.01	0.05	0.05	0.02	0.3
PL*IDP*G								
24% CP * LIP * Male	4.72	3.02[abc]	1.13	0.42	5.76[b]	4.50	0.72	0.64
24% CP * HIP * Male	4.80	3.16[abc]	1.14	0.43	6.03[ab]	4.59	0.72	0.75
26% CP * LIP * Male	4.79	3.23[a]	1.10	0.43	6.25[a]	4.76	0.72	0.72
26% CP * HIP * Male	4.73	3.07[abc]	1.14	0.44	5.94[ab]	4.57	0.74	0.72
28% CP * LIP * Male	4.83	3.14[abc]	1.17	0.40	5.92[ab]	4.50	0.76	0.64
28% CP * HIP * Male	4.78	3.17[ab]	1.12	0.48	6.26[a]	4.61	0.75	0.81
24% CP * LIP * Female	4.78	3.06[abc]	1.09	0.43	6.07[ab]	4.50	0.70	1.01
24% CP * HIP * Female	4.56	2.93[c]	1.03	0.46	6.10[ab]	4.51	0.67	0.90
26% CP * LIP * Female	4.78	3.10[abc]	1.06	0.44	5.98[ab]	4.50	0.72	0.88
26% CP * HIP * Female	4.71	3.06[abc]	1.14	0.46	5.88[ab]	4.40	0.72	0.87
28% CP * LIP * Female	4.81	3.14[abc]	1.11	0.44	6.14[ab]	4.56	0.73	0.85
28% CP * HIP * Female	4.70	3.00[bc]	1.11	0.43	5.96[ab]	4.40	0.70	0.87
SEM	0.04	0.14	0.03	0.02	0.10	0.09	0.03	0.06
ANOVA	**P-Values**							
G	0.1267	0.0030	0.0337	0.3903	0.9168	0.0235	0.0343	<.0001
IDP	0.0345	0.0797	0.9887	0.0237	0.8528	0.4580	0.4695	0.4331
PL	0.2655	0.0606	0.3613	0.7987	0.5157	0.7262	0.0973	0.7079
IDP * G	0.0640	0.0886	0.8566	0.2102	0.1143	0.3257	0.4503	0.0627
PL* G	0.6612	0.9541	0.3958	0.5087	0.0420	0.3728	0.6447	0.2946
PL * IDP	0.9740	0.3464	0.0825	0.8295	0.0284	0.2647	0.6010	0.4230
PL * IDP * G	0.1706	0.0170	0.3344	0.0938	0.0268	0.3083	0.9923	0.4571

[a-b] Means (n = 12 cages) within a column with no common superscript per main effects or interactions are significantly different ($P \leq 0.05$). [1]LIP-low indigestible protein; HIP-high indigestible protein.

Table 6.8. Effects of diet levels of total and indigestible protein on carcass, wing, back-rack and abdominal fat yield per bird expressed as a percentage of live weight.

	Carcass	Wing	Back-rack	Abdominal fat
Gender (G)				
Male	69.76	7.41	15.90	0.5770
Female	70.55	7.40	16.00	0.4939
SEM	0.18	0.04	0.10	0.0002
Indigestible protein (IDP)[1]				
LIP[1]	70.77	7.39	15.96	0.5616
HIP[2]	69.54	7.42	15.92	0.5084
SEM	0.18	0.04	0.10	0.0002
Protein level (PL)				
24% CP	69.14	7.36	15.64^{b}	0.5615
26% CP	70.58	7.39	16.09^{a}	0.5344
28% CP	70.75	7.46	16.09^{a}	0.5088
SEM	0.23	0.04	0.12	0.0003
PL*IDP*G				
24% CP * LIP * Male	68.316^{cd}	7.24	15.79	0.5544^{ab}
24% CP * HIP * Male	69.39^{bcd}	7.46	15.82	0.5064^{ab}
26% CP * LIP * Male	71.04^{ab}	7.47	15.90	0.5062^{ab}
26% CP * HIP * Male	69.25^{bcd}	7.26	15.89	0.4540^{b}
28% CP * LIP * Male	71.09^{ab}	7.53	16.20	0.5031^{ab}
28% CP * HIP * Male	69.49^{bcd}	7.47	15.80	0.4432^{b}
24% CP * LIP * Female	70.68^{ab}	7.37	15.69	0.7234^{a}
24% CP * HIP * Female	68.18^{d}	7.39	15.25	0.4765^{ab}
26% CP * LIP * Female	71.61^{a}	7.36	16.16	0.6042^{ab}
26% CP * HIP * Female	70.41^{ab}	7.47	16.42	0.5799^{ab}
28% CP * LIP * Female	71.91^{a}	7.37	16.02	0.4942^{ab}
28% CP * HIP * Female	70.52^{ab}	7.46	16.33	0.6008^{ab}
SEM	0.45	0.09	0.24	0.0011
ANOVA		**P-Values**		
G	0.0027	0.9517	0.5687	0.0040
IDP	<.0001	0.5552	0.7638	0.0643
PL	<.0001	0.3139	0.0115	0.3290
IDP * G	0.0777	0.3688	0.5502	0.9279
PL * G	0.8424	0.5392	0.0970	0.7578
PL * IDP	0.3746	0.4022	0.6407	0.0687
PL * IDP * G	0.0018	0.1044	0.2317	0.0411

[a-d] Means (n = 12 cages) within a column with no common superscript per main effects or interactions are significantly different ($P \leq 0.05$). [1]LIP-low indigestible protein; HIP-high indigestible protein.

6.5 Discussion

The main goal of this study was to evaluate the effects of dietary protein level, and indigestible protein fraction on the performance and meat yield of broiler vaccinated for coccidiosis and fed antibiotic-free diets. One limitation in designing experiments to evaluate the effects of indigestible CP in practical nutritionally balanced diets is the use of different types of ingredients to create the protein pool of the diets, which in turn affects other components of the diets. This makes it impossible to separate ingredient specific effects from treatment effects.

With the recognition of the confounding approach, other experimental practice aimed to reduce this effect. The nutrient content of major ingredients was evaluated before the diets were formulated and amino acid digestibility coefficients utilized in the diet formulation were based on two well-recognized databases (Ajinomoto Heartland LLC, 2009; Evonik, 2010). The indigestible CP fraction of major protein contributing ingredients was assessed using an *in vitro* approach (Chapter 5) and these values were used for diet formulation. Finally, the single batches of soybean meal, wheat, fish meal, porcine meal, corn gluten meal and corn distiller's dried grains with solubles were used for all experimental diets. The digestible amino acid content of the diets were evaluated elsewhere (Chapter 7). The data from Tables 7.2 and 7.11 in Chapter 7 indicate that digestible methionine plus cysteine levels (first limiting) were relatively similar across diets and that differences in digestibility of protein between the LIP and HIP diets were as expected.

Dietary carbohydrate and protein are interrelated at the gut level in poultry nutrition (Choct and Ao, 2009). The nature of the carbohydrate and amount present in the lower digestive tract can influence the fate of the protein present (Apajalahti and Vienola, 2016). If the carbohydrate is fermentable and in a large amount relative to the protein, this will stimulate microorganisms to use the protein for cell growth rather than energy. In such a situation, bacteria will convert the excess fermentable carbohydrate to energy and other by-products such as short chain fatty acids, which can be beneficial to the host animal. The opposite effect is true if the proportion of protein is greater than that of the fermentable carbohydrate. Higher protein fermentation in the lower digestive tract of poultry could lead to bacterial dysbiosis (Apajalahti and Vienola, 2016). If the microbial population is out of balance it will predistpose the birds to other problems like enteric disease and reduced feed efficiency. The delivery of nutrients to the portal blood supply can also be compromised, which in turn, will affect growth and meat yield.

6.5.1 Growth performance

In the current study, HIP diets reduced BWG for 0-12 and 22-32 d production periods, but for the 12-22 d period the LIP fed birds had the reduced BWG. The differential response may be due to the disease status of birds. The birds were vaccinated on d five and it is likely that the impact of vaccination during the first 12 d of the trial was small. Similarly from 22-32 d the birds had likely recovered from the vaccination challenge. Indigestible dietary CP has been reported to reduce broiler average daily gain and BWG in other research (Widyaratne and Drew, 2011; Qaisrani et al., 2014) and during the periods 0-12 and 22-32 d, the current research supports that concept. During the 12-22 d production period the birds were in a subclinical disease state, which was different from the previous studies, and this might be the factor which caused the LIP diets to produce the negative effect on BWG. It is possible that the LIP diets promoted an increased disease state in comparison to the HIP diets and therefore growth (and FG) were poorer. This is supported by the increased infectious mortality seen for the LIP treatments.

The reason for increased infectious mortality of birds fed the LIP diets is not clear, since the LIP diets should have provided more amino acids to aid the animals recovery compared to the HIP diets. It should be noted that a one phase feeding program was use in the current study and all the diets were formulated to the Ross 308 grower nutrient specifications where all diets had the same content of digestible methionine plus cysteine. In line with the diet formulation, all the BW and BWG of the birds in this study were higher than Aviagen performance objectives (Aviagen, 2014a) and were maximized when 24 or 26% CP was fed. However there seem to be a negative biological response when the highest level of dietary CP was fed which was not link to the disease state of the birds.

Vaccination of broiler chicks with live oocysts reduced BW (Lee et al., 2011; Arczewska-Włosek et al., 2017) and it is possible that this occurred in the present study, but it cannot be deduced from the experiment because all the birds were vaccinated on d five. Birds vaccinated on the first d of age tend to see the most negative effects on performance associated with the vaccination during the starter period (Lee et al., 2011; Arczewska-Włosek et al., 2017). Other researchers have suggested that providing additional protein during the starter period can reduce the adverse effects of vaccination on performance (Lee et al., 2011). The effects of dietary CP on ameliorating the negative effects of live oocyst vaccination was reported to have occurred d 1 to 21 and 22 to 42 (Lee et al., 2011; Arczewska-Włosek et al., 2017). The effects of PL was

significant for most of the performance variables evaluated in our study, but increasing the PL reduced BW, BWG, FI, and FG. Even though FI went down and FG was improved with increasing PL, the PER demonstrate that it was more protein efficient to feed the lower protein diets. The PL in our experiment ranged from 24 to 28% which was higher than the 20 to 24% CP reported by Lee et al. (2001) and Arczewska-Wlosek et al. (2017). It is possible that increasing the PL from 24 to 28% was sufficiently high so that positive effects gained from PL were counterbalanced by the negative metabolic effect of birds needing to eliminate excess dietary nitrogen.

The feed consumption from 0-32 d in this study was approximately 250 g more for males and 200 g more for females than Aviagen performance objectives (Aviagen, 2014a). The effects of IDP alone did not influence the total FI of the birds in our study and the same was true for the starter and grower periods evaluated by Widyaratne and Drew (2011). However, the interaction between PL and IDP was significant for total FI, where both the LIP and HIP diets had reduced FI as the PL increased, but only the LIP reduction was statistically significant. The reduction in FI could, therefore, be related to the dietary protein level, which was also observed by Cheng et al. (1997). The effect of PL on feed intake translated into higher feed efficiency in the birds fed the higher levels of CP. The high indigestible dietary CP reduced feed efficiency because the birds on the LIP diets gained more weight than those on the HIP diet while the feed consumption remained the same for the LIP and HIP diets except during d 12-22. Qaisrani et al. (2014) observed a reduction in feed efficiency due to higher levels of dietary indigestible protein, which was related to changes in both the feed consumption and weight gain, suggesting possible anti-nutrient effects.

Unlike the feed efficiency, the PER suggested that birds on the higher protein diets were less efficient in converting the protein they consumed to equivalent gain in body mass, which is in agreement with Cheng et al. (1997). Except for the 12-22 d period, the LIP diets were more protein efficient than the HIP diets which was also observed by Widyaratne and Drew (2011). The switch which occurred in PER between the LIP and HIP fed birds at 12-22 d coincides with the period when the oocysts from the vaccination would start to recycle in the small intestine of the birds. During the 12-22 d period, the LIP and HIP fed birds consumed 253 and 252 g of protein per bird, respectively, but BWG for the LIP birds was lower indicating an inability of the birds to use the protein for gain, possibly because they were sicker. The PER values were in the range of previously reported values (Cheng et al., 1997; Widyaratne and Drew, 2011). It has been reported that lower

PL and diets with less IDP tend to promote higher PER in broilers (Widyaratne and Drew, 2011), which was confirmed in this study.

The total mortality in this trial was high compared to that of industry standard. The primary cause of death was due to infections, which is not surprising since the birds were vaccinated with live oocysts and environmental conditions were designed to increase enteric disease. Coccidiosis infections result in epithelial cell damage, which predisposes birds to secondary infection from a wide range of gastrointestinal tract bacteria (Wu et al., 2010; Timbermont et al., 2011). Secondary bacterial infections can occur when birds are vaccinated with live oocysts due to cell damage in the intestinal epithelium, which permits bacterial translocation.

The general assumption relating to IDP and the incidence of diseases suggests that birds fed diets with high levels of IDP are more likely to have enteric disease. Similarly, the general recommendation to reduce the risk of enteric disease in poultry operations with reduced or no antibiotic usages is to decrease the level of dietary protein. The results from the current study which used over four thousand birds did not find an effect of PL on the incidence of mortality in any of the production periods evaluated. What was surprising, however, was that more birds died when fed the LIP relative to the HIP diets.

Based on the general assumption about PL and the incidence of enteric disease in poultry, it would be expected that increasing the PL from 24 to 28% should have resulted in more undigested protein reaching the distal small intestine. In turn, excess protein should have predisposed the birds to more enteric disease and resulting death loss; however, this was not observed in the current study. It is likely that the underlying mechanism might be more protein ingredient dependent rather than PL, which might also explain the effect of LIP diets on mortality. The LIP diets were composed of soybean meal and fish meal as the major dietary protein sources. Elevated dietary levels of fish meal have been shown to interact with the presence of coccidiosis to result in necrotic enteritis (Wu et al., 2010; Rodgers et al., 2015), but the mechanisms are unclear. Most of the differences in mortality between birds fed the LIP and HIP diets were due to infectious causes, which included coccidiosis, necrotic enteritis, arthritis, cellulitis, hepatitis, endocarditis, osteomyelitis, pericarditis, polyserositis and yolk sac infection.

There are two possibilities which could explain the effects of the LIP diet on mortality. The first possibility is that the negative effects were due to the fish meal in the diets since fish meal has been shown to be associated with necrotic enteritis infection (Wu et al., 2010; Rodgers et al., 2015).

The maximum level of fish meal used in the diets of this study was only 8% which was much lower than the 25 and 50% use to induce necrotic enteritis in broiler chickens (Wu et al., 2010; Rodgers et al., 2015). However the histamine levels of the fish meal used in the current study was 2040 ng/mg (Chapter 4).

The second possibility is that the protein sources in the HIP diets were aiding the recovery of the birds during the vaccination. The HIP diets protein were composed of soybean supplemented with porcine meal, corn gluten meal and corn distillers' dried grains with solubles. The maximum amount of CDDGS used in the diets was 15% and CGM was only included at a maximum of 6%. Corn distillers' dried grains with solubles is a by-product of the fermentation of corn by *S. cerevisiae* and it may contain up to 6% yeast biomass (Alizadeh et al., 2016). Yeast cell-derived products have been shown to help maintain the performance of birds when challenged with *C. perfringens* by stimulating the innate immune response, which allows birds to have a stronger and faster immune response (Alizadeh et al., 2016). Apart from the immuno-modulating effect of yeast biomass, some yeast cell components, such as mannanoligosaccharides, have been shown to decrease caecal *E. coli*, while increasing lactobacilli and bifidobacteria (Baurhoo et al., 2007). Research in our lab has shown that CGM hydrolysate had the potential to reduce the proliferation of *C. perfringens* (Annett-Christianson, 2012).

Male broilers are often reported to have a higher incidence of mortality and culling than females, particularly associated with metabolic causes like sudden death syndrome, ascites and skeletal disorders. This is in agreement with findings from the current study. However, the finding that males had a higher level of death loss than females due to infectious causes is of interest. It appears that males of many species including mammals and birds are more susceptible than females to infections caused by bacteria, parasites, viruses, and fungi (Klein, 2000). The reasons for the gender difference are not fully understood, but there is evidence suggesting the effect is due to immuno-modulation by sex steroids and genes involved in disease resistance (Klein, 2000; Furman et al., 2014).

6.5.2 Meat yield

Proportional carcass weight is strongly influenced by balanced digestible protein level (Lily et al., 2011), but the current research attempted to minimize this effect by formulating diets to have the same first limiting amino acids (MET+CYS) which was confirmed to be the case in Chapter 7. Table 7.2. While methionine plus cysteine where the limiting amino acids the digestible

lysine increased in the diets with increasing CP level. The LIP diets had more digestible lysine than the HIP diets and correspondingly LIP birds had more carcass and breast yield, which suggests that the increased digestible lysine was used to synthesize muscle. The live weight of the birds was sensitive to all the treatments applied in the present study and as such their combined effects also affected carcass yield. Increased dietary amino acid levels has been shown to increase proportional carcass yield of broilers, and this was confirmed in the present study (Lilly et al., 2011). The carcass yield differences between the LIP and HIP diets might not be related to the level of IDP in the diet per se, but instead might be due to the availability of excess amino acids.

The birds fed the LIP diets with 26 and 28% CP produced more breast meat than those fed the 26 and 28% CP HIP diets. This response was not seen when the diets contained 24% CP. The diets were formulated to that of the Ross 308 broiler grower nutrition specifications for as-hatched broilers - target live weight of 1.70 - 2.40 kg and they were fed from the d of hatch to 32 d of age. This means that the 24% CP diets, as depicted in Table 6.2 digestible amino acids were below that which was needed for growth during the first 10 d of life (starter phase) of the birds. Broiler chicken AA deficiency early in life have been shown to reduce breast meat yield later in life due to reduced satellite cell proliferation which caused lower muscle fibre development as the animal developed (Halevy et al., 2000).

The differences in breast meat yield between the LIP and HIP diets was mainly due to changes in the pectoralis major component of the whole breast. The effects of PL on the breast meat yield were more definitive in the pectoralis minor, and the values were similar to those previously reported (Dozier et al., 2007; Widyaratne and Drew, 2011). Only the total sulfur containing amino acid and threonine levels remained constant across all diets as the level of dietary CP increased. The response of breast meat yield to IDP and PL might be related to changes in lysine level (Kerr et al., 1999) or the density of other amino acids in the diets (Dozier et al., 2007). It has been shown in other studies that when the level of methionine plus cysteine was kept constant at 0.75% and increasing level of lysine was provided, breast meat yield also increased in response to lysine level (Rezaei et al., 2004). This response of breast meat yield to increasing digestible lysine might be limited to the level of digestible methionine plus cysteine in the diet.

Even though the drum meat was influenced by the interaction of all three main factors in the study, the only differences seen were due to males fed the 26-LIP diet producing more drum meat than females on the 24-HIP or 28-HIP diets. However, only the main effects of gender were

significant, which suggests that gender might be a more important factor when evaluating drum meat yield. Thigh meat and bone were only influenced by gender, which was in line with what is expected.

Conclusion

The objective of this study was to evaluate the effects of dietary protein level and indigestible protein fraction on the performance and meat yield of broiler vaccinated for coccidiosis and fed antibiotic-free diets. A re-evaluation of the initial study hypothesis leads to the conclusion that dietary protein level and the ratio of indigestible protein affected broiler performance and meat yield when birds were vaccinated for coccidiosis. The unexpected effects of less indigestible CP increasing infectious mortality may be due to a protein source effect on mortality when birds were challenges with coccidiosis. Whether the effects seen on performance and meat yield were due to changes in the digestibility of the diets or fermentation of indigestible protein needs further investigation and will be the subject of Chapter 7.

Transition statement

The broilers given the diets with low levels of indigested proteins had statistically better performance, but there was a period (12-22 d) when those given diets with high levels of indigestible protein had the best performance. The data from Chapter 6 suggested that more birds died when they were fed a diet which had low level of undigested protein. It was unclear as to why the low indigestible diets gave such response. Chapter 7 confirmed the digestibility of the test diets using a traditional digestibility assay as well as from birds grown under the management protocol used in Chapter 6. Digestive tract morphological evaluation was done in Chapter 7 to try and understand the reason for the high mortality associated the diets which had low indigestible protein.

7.0 THE INFLUENCE OF INDIGESTIBLE PROTEIN ON BROILER DIGESTIVE TRACT MORPHOLOGY AND CAECAL PROTEIN FERMENTATION METABOLITES

7.1 Abstract

Indigestible dietary protein fermentation products have been suggested to negatively influence broiler performance due to their impact on health and digestive tract morphology. This study evaluated the digestive tract morphology and caecal protein fermentation metabolites of broiler fed 3 dietary protein levels (24, 26, and 28%) with low or high indigestible protein (LIP, HIP). Two completely randomized 3 x 2 factorial trials were conducted with protein level and indigestible protein as the main factors. Ross 308 males (1,200 birds) were randomly assigned to 24 litter floor pens for 32 d (trial 1) or 216 birds were assigned to 36 battery cages for 21 d (trial 2). In both trials, birds received 6 diets (24-LIP, 24-HIP, 26-LIP, 26-HIP, 28-LIP, 28 HIP) formulated to broiler grower nutrient specifications and containing no medication. On d 5, trial 1 birds were vaccinated in feed and water with Coccivac-B52. Tissue, caecal and distal ileum samples were collected from 16 birds per treatment on both d 14 and 28 in trial 1. Caecal and distal ileum samples were collected from 32 birds per treatment on d 21 in trial 2. All caecal contents were analyzed for protein fermentation metabolites. Differences were considered significant when $P \leq 0.05$. At d 14 LIP diets had greater histamine, agmatine and cadaverine levels, while HIP diets resulted in increased serotonin, tryptamine, and spermidine levels. Indigestible protein (IDP) level did not affect histamine, serotonin, and tryptamine at d 28. There were no effects of treatment on ammonia at d 14 or 28. HIP fed birds had lower total short-chain fatty acids, higher caecal pH, and heavier pancreas, proventriculus, gizzard, jejunum, and ileum weights at 14 d. As in trial 1, the same effects of IDP were observed for histamine, agmatine, cadaverine, serotonin, tryptamine, and spermidine at d 21 for the cage non-vaccinated birds. Protein level (PL) affected tryptamine (26>24 with 28 intermediate) and cadaverine (28>26=24). Interactions between PL and IDP influenced tyramine, spermidine (28-LIP>24-LIP) and spermine with values increasing with PL for LIP diets and remaining constant for HIP diets. The interaction between PL and IDP affected ammonia

levels which was similar to interactions for biogenic amines. There were no effects of treatments on short chain fatty acids, but caeca pH was affected by the interaction of PL and IDP. In conclusion, dietary PL and IDP influence broiler caecal protein fermentation metabolites and those effects varied with coccidiosis vaccination and rearing environment.

Keywords: Short chain fatty acids, antibiotic-free, polyamine, ammonia, chicken

7.2 Introduction

The public has raised concerns about the use of medication in animal feed and how this practice might have influenced the development of antibiotic resistant bacteria. The animal agriculture industry has been working to reduce and in some cases eliminate its dependency on some medications. Some sectors of the poultry industry have been able to remove the use of sub-therapeutic antibiotics from their production practices, but it requires that more effort is placed on managing the health of the animals. To be able to feed poultry without medication, more information is needed about how commonly used ingredients and nutrient levels affect enteric disorders in birds, which often arise under reduced antibiotic management practices. One area that may affect health is microbial fermentation of protein and carbohydrate.

The gastrointestinal tract (GIT) of chickens hosts a wide range of microorganisms (Gong et al., 2007; Choct and Ao, 2009) and diet is considered to be the most influential factor capable of changing the GIT microbial community (Dahiya et al., 2007; Macfarlane and Macfarlane, 2007). Fermentation of nutrients in the digestive tract of animals leads to a variety of effects on the digesta environment and host due to the metabolites which are produced. Among the most important are short-chain fatty acids (SCFA) derived from the fermentation of carbohydrates and protein (Elsden and Hilton, 1978) with the nature and degree of fermentation dependent on substrate and microbial ecology. Carbohydrate fermentation in the avian caeca generally leads to the formation of lactate, acetate, propionate, and butyrate (Józefiak et al., 2004). Protein fermentation by *Clostridium* species such as *C. difficile* and *C. lituseburense* present in poultry GIT (Gong et al., 2007) can produce a wide range of SCFA such as acetate, propionate and butyrate and SCFA such as isobutyrate, valerate, and isovalerate, which are derived from branch chain AA (Elsden and Hilton, 1978).

The production of SCFA in the digestive tract can reduce digesta pH. Such changes in the digesta pH can alter the enteric microbiota and decrease the proliferation of some bacteria as the content of undissociated SCFA increases (Immerseel et al., 2004b). It is, therefore, assumed that the production of SCFA in the lumen of the small intestine of poultry reduces the risk of developing intestinal dysbiosis. It has been reported that SCFA have the potential to stimulate epithelial cell proliferation (Immerseel et al., 2004a), which can be beneficial in promoting a healthy intestinal epithelium. Short chain fatty acids are a source of energy for enterocytes and this may have a sparing effect on glucose needed by the intestine. It is through these and other actions that SCFA are thought to be beneficial when poultry are suffering from enteric disease.

The level of undigested fermentable carbohydrate present in the distal ileum will have a direct impact on the fate of the protein present (Rinttila and Apajalahti, 2013). If there is a large supply of undigested carbohydrate in the form of slowly digested or resistant starch, or non-starch polysaccharides, bacteria will use the carbohydrate as an energy source and the protein as a source of AA for growth. On the other hand, when carbohydrate is limited or absent, the bacteria will ferment the peptides and AA for energy, and produce undesirable metabolites.

Protein fermentation by microbes may produce ammonia, polyamines, indoles, and skatoles, which are suggested to compromise host health if they are produced at high luminal concentrations (Barnes et al., 2001; Qaisrani et al., 2015; Apajalahti and Vienola, 2016). Microbial fermentation of proteins most often leads to the formation of biogenic amines (Smith et al., 2000) and ammonia. These compounds can be produced from diets high in protein content or diets which contain poorly digested proteins, provided that the protein is in a form that gains access to the caeca, the primary site of fermentation (Rinttila and Apajalahti, 2013).

Ammonia in the small intestine of poultry can be a source of nitrogen for some bacteria, but systemically it is considered to be toxic. Whenever ammonia is produced from metabolic activities in chicken cells, it is quickly converted to uric acid for transportation and excretion. Intravenous injection of ammonium acetate up to 2 μ-moles/kg of body weight in adult male chickens has been shown to have no effects on mortality, but 3.8 μ-moles/kg resulted in 100% mortality (Wilson et al., 1968). Under certain bacterial infection conditions *in vitro* and *in vivo*, ammonia has been shown to induce apoptosis in gastric epithelium cells of mammals (Tsujii et al., 1993; Igarashi et al., 2001). This suggests that the accumulation of a large amount of luminal ammonia can potentially compromise animal health.

Amines are formed from the decarboxylation of AA by microorganisms or by decarboxylase enzymes in animal cells (Wang et al., 1991). Amines that are produced by protein fermentation are biologically active and the nature of their effects is dependent on the kind of amine and their concentration. This is not surprising since amines are also normal cell metabolites in animals (Wang et al., 1991). Increased growth rate was reported when spermidine and putrescine were fed to chickens at 0.05 and 0.3%, respectively (Smith et al., 1996; Girdhar et al., 2006). However, spermidine and histamine dietary levels higher than 0.05% were shown to reduce the growth of chickens (Smith et al., 1996; Barnes et al., 2001), while feeding putrescine at 0.2% or greater tends to reduce feed intake in laying hens (Chowdhury and Smith, 2001). Histamine and cadaverine at 0.1% acting alone or in combination are known to cause mild proventriculus and gizzard lesions (Barnes et al., 2001).

Studies in humans have shown that high levels of soluble protein in the colon result in correspondingly high levels of protein fermentation products (Macfarlane et al., 1986). Therefore, the solubility of proteins may be one important criteria for protein fermentation. If this is the case, it would be particularly important for chickens, where access to the most important fermentation site, the caeca, is restricted to soluble or small particle size material (Svihus, 2014). Little information exists on the impact of dietary protein level and source on the characteristics of proteins found in the distal ileum and in turn fermentation in caeca. Further, information on the luminal concentration of naturally produced ammonia and biogenic amines and their effects on the GIT morphology of poultry is lacking. Therefore, the objective of this study was to evaluate the effects of three dietary protein levels (24, 26 and 28%) with low (LIP) or high (HIP) indigestible protein (IDP) on digestive tract tissue characteristics and caecal protein fermentation in coccidiosis vaccinated and non-vaccinated of broilers. It was hypothesized that high dietary protein levels (LP) and IDP will increase protein fermentation, which in turn will produce high levels of protein fermentation products in the caeca.

7.3 Material and methods

All animals used in this study were cared for using the Canadian Council on Animal Care guidelines On the Care and Use of Farm Animals in Research, Teaching, and Testing (Canadian Council on Animal Care, 2009). The experimental procedures of this study were approved by the University of Saskatchewan Animal Research Ethics Board.

7.3.1 Experimental design and bird management

Experiment 1

The effects of three dietary protein levels (24, 26 and 28%) with low or high indigestible protein (LIP, HIP) on broiler digestive tract morphology and caecal protein fermentation metabolites were evaluated in this study. The trial was completely randomized, with a 2 x 3 factorial arrangement, where indigestible dietary protein and dietary protein levels were the main factors. Pens were considered to be the experimental units for this study. This trial was conducted concurrently with the production trial presented in Chapter 6.

Three d prior to chick arrival humidifiers (AIRCARE® Evaporative Humidifier Model SS390DWHT) set to a minimum of 60% relative humidity were placed in each room used in the experiment. Humidity levels were maintained for 14 d after initial bird placement, and humidity and temperature levels were measured at least two times daily. Prior to chick placement, litter in each pen was sprayed with water several times daily to achieve and maintain desired humidity levels. Thereafter, humidifiers were adjusted daily and walkways periodically sprayed with water to accomplish the same goal; humidifiers were removed 15 d after chick placement.

One thousand two hundred and ninety-six male Ross 308 broilers chicks were provided by a local hatchery. On the d the birds arrived, they were allocated to 24 groups (54 birds per group) and randomly distributed to 24 pens located in two rooms. The stocking density of the pens was estimated to be 23.2 kg/m^2 at marketing weight. Each floor pen measured 2.3 m wide by 2 m long and was covered with wheat straw to a thickness of 10 cm. Each pen had one tube feeder (diameter = 36 cm from 0-21 d or 43 cm from 21-32 d) and a drinker with 6 Lubing-4087 nipples. On the d of chick placement, a 30 cm wide piece of brown paper spanning the length of the drinkers was placed under the drinkers to promote excreta consumption and oocyst cycling. One polypropylene ice cube tray (16 cell, W 20 cm x L 28.6 cm x H 3 cm) and one cardboard egg tray were added to each pen. The birds had ad libitum access to water and the same 6 diets from 0 to 28 d.

Room temperature was initially set at 33°C on the d of chick placement and then gradually reduced by 0.75°C every d for 7 d and by 0.42°C until 21°C was reached. The lighting program and light intensity settings were 23 h light and 1 h dark (23L:1D) at 20 lux (age 0-2 d), 22L:2D) at 18 lux (age 3-4 d), 21L:3D at 16 lux (age 5-6 d), (20L:4D) at 14 lux (age 7-8 d), 19L:5D at 12 lux (age 9-10 d), 18L:6D at 10 lux (age 10-11d) and 17L:7D at 10 lux (age 12-32 d).

A clean cardboard egg tray was placed in each pen 5 d after chick placement and feed was evenly distributed over all cells. Ice cube trays were filled with clean water and placed about 25 cm from the drinker along with the cardboard egg trays. One bottle of Coccivac-B52 (Merck Animal Health, Omaha, USA) containing 1000 doses in 500 ml distilled water was placed in a garden sprayer set to a light spraying with a wide spray pattern. The vaccine was sprayed over the feed and water in the cardboard egg trays and ice cube trays using 3 even passes while simultaneously agitating the sprayer bottle. Each pen received approximately 83 doses of the vaccine during the vaccination. The feeders were raised in each pen so that the birds only had access to the vaccinated feed until the feeders were lowered at the end of the d.

Experiment 2

This study evaluated the amino acid and CP digestibility of six diets having three dietary protein levels (24, 26 and 28%) with low or high indigestible protein (LIP, HIP). Diet effects on caecal protein fermentation metabolites of broilers were also assessed. The trial was completely randomized, with a 2 x 3 factorial arrangement, where indigestible dietary protein and dietary protein levels, were the main factors and cages were the experimental units.

A total of 250 Ross 308 male chicks were provided by a local hatchery and placed in a 16 cage per tier (L 51 cm x W 46 cm X H 46 cm) double tier battery cage system. The cages were housed in an environmentally controlled room. Each cage had a feed trough (L 53.3 cm x W 12 cm x H 10 cm) mounted on the front of the cage and 2 height adjustable nipple drinkers. The cage system had a wire mesh floor with a 2.54 cm x 2.54 cm mesh gap, but the chicks were provided with a removable mesh floor with a 1.27 cm x 1.27 cm mesh gap from 0 to 7 d of life. The room temperature was initially set at 34°C on the d of chick placement and then gradually reduced by 0.43°C each d until the end of the trial. The light intensity was set to 20 lux, and the lighting program was 23L:1D 0 to 7 d then 22L:2D for the rest of the experiment. Gradual increases (dawn) and decreases (dusk) in light intensity (15 min each) were included in the light portion of the d.

All birds were given a mash starter diet (Table 7.1) for the first 13 d of life. During the first five d in the cages, the chicks were provided supplemental water in ice cube trays and additional feed in 50 cm long plastic chick feeders. Fourteen d after chick placement birds with body weights which were within 494 ± 8 g were selected and randomly distributed to 36 cages with 6 birds per cage and 6 replicate cages per dietary treatment.

Table 7.1. Ingredient composition of test diets and nutrient levels for experiment 1 and 2.

Ingredients	Starter	24-LIP[1]	24-HIP[2]	26-LIP	26-HIP	28-LIP	28-HIP
Wheat	58.92	59.81	50.18	55.06	44.44	49.04	42.41
Soybean	32.00	26.94	28.32	30.02	25.59	34.88	24.03
Fish meal	0.00	5.00	0.00	7.00	0.00	8.00	0.00
CDDGS[3]	0.00	0.00	10.50	0.00	15.00	0.00	15.00
Corn gluten meal	0.00	0.00	0.00	0.00	4.00	0.00	6.00
Porcine meal	0.00	0.00	1.50	0.00	2.00	0.00	5.00
Canola oil	4.97	4.20	4.84	4.45	4.60	4.94	4.17
Vit/min broiler[4]	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Limestone	1.48	1.39	1.50	1.30	1.49	1.24	1.24
Choline chloride	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Sodium chloride	0.43	0.24	0.36	0.17	0.32	0.13	0.29
Mono di-calcium phosphate	1.20	0.60	0.87	0.33	0.73	0.19	0.19
L-Lysine HCl	0.00	0.03	0.15	0.00	0.16	0.00	0.09
DL-Methionine	0.29	0.26	0.26	0.21	0.18	0.17	0.14
L-Threonine	0.10	0.12	0.12	0.05	0.07	0.00	0.03
Ameri-Bond 2x[5]	0.00	0.50	0.50	0.50	0.50	0.50	0.50
Econase[6]	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Titanium dioxide	0.00	0.03	0.03	0.03	0.03	0.03	0.03
Calculated analysis (%)							
AME (kcal/g)	3.1	3.1	3.1	3.1	3.1	3.1	3.1
DM	89.5	89	89	89	89	89	89
CP	23	24	24	26	26	28	28
Fat	6.4	6.1	7.3	6.4	7.5	6.9	7.3
Calcium	0.87	0.9	0.9	0.9	0.9	0.9	0.9
Non-phytate phosphorus[7]	0.44	0.45	0.45	0.45	0.45	0.45	0.45
Indigestible CP[8]	0.00	4.18	5.15	4.50	6.29	4.80	7.02
Dig. Arginine	1.37	1.24	1.24	1.37	1.25	1.51	1.31
Dig. Isoleucine	0.84	0.89	0.88	0.98	0.95	1.08	1.00
Dig. Leucine	1.48	1.52	1.64	1.66	2.03	1.81	2.25
Dig. Lysine	1.15	1.15	1.15	1.28	1.15	1.43	1.15
Dig. Met+Cys	0.87	0.87	0.87	0.87	0.87	0.87	0.87
Dig. Methionine	0.55	0.57	0.56	0.56	0.53	0.55	0.53
Dig. Threonine	0.77	0.77	0.77	0.77	0.77	0.79	0.77
Dig. Tryptophan	0.26	0.26	0.25	0.28	0.24	0.30	0.25
Dig. Valine	0.88	1.03	1.03	1.13	1.11	1.23	1.19
Amines (g/kg)							
Agmatine	NA[9]	0.015	0.005	0.02	0.005	0.023	0.005
Cadaverine	NA	0.048	0.018	0.067	0.023	0.076	0.042
Histamine	NA	0.102	0.002	0.143	0.005	0.163	0.009
Putrescine	NA	0.027	0.021	0.034	0.026	0.038	0.037
Serotonin	NA	0.000	0.000	0.000	0.000	0.000	0.000
Spermidine	NA	0.058	0.06	0.062	0.057	0.068	0.054
Spermine	NA	0.012	0.012	0.013	0.012	0.014	0.013
Tryptamine	NA	0.000	0.000	0.000	0.000	0.000	0.001
Tyramine	NA	0.015	0.002	0.021	0.01	0.024	0.017
Total amine	NA	0.278	0.120	0.359	0.138	0.406	0.177
Determined analysis (% DM)							
Soluble fibre[10]	NA	5.24	5.48	3.32	3.56	3.75	3.87
Insoluble fibre[10]	NA	19.95	23.46	19.04	19.24	20.73	20.06

[1]Low indigestible CP; [2]high indigestible CP; [3]corn distillers dried grains with solubles. [4]Vitamin-mineral premix provided the following per kilogram of complete diet: 4 mg vitamin B12; vitamin D, 440,000 IU; vitamin A, 2,200,000 IU; vitamin E, 6000 IU; menadione, 400 mg; thiamine, 300 mg; riboflavin, 1200 mg; pyridoxine, 800 mg; niacin, 12,000 mg; pantothenic acid, 2000 mg; folic acid, 120 mg; biotin 30 mg; copper, 2000 mg; iron, 16,000 mg; manganese 16,000 mg; iodine, 160 mg; zinc, 16,000 mg; selenium, 60 mg; calcium carbonate 100,000 mg; Ethoxyquin 125 mg; wheat middlings 754,546 mg [5]pellet binder (LignoTech, Wisconsin, USA); [6]Econase XT (ABVista, Wiltshire, UK), β 1-4 endo-xylanase enzyme (160,000 BXU/g.); [7]phosphorus not derived from the hydrolysis activity of phytase; [8]protein which is not digested after *in vitro* digestion with pepsin and pancreatin, [9]Not analyzed, [10]Determined with Megazyme dietary fibre assay kit (Megazyme International Ireland Ltd., Wicklow, Ireland).

7.3.2 Diet formulation experiments 1 and 2

The test diets fed in both trials (Table 7.1) were formulated based on the same criteria and manufactured at the same time using the same batch of ingredients as those fed in the production trial (Chapter 6), except 0.03% titanium dioxide was included as an indigestible maker at the expense of wheat.

7.3.3 Data collection

Experiment 1

On d 14 and 28 of the trial, 16 birds per treatment were selected and individually weighed. The birds were killed with an intravenous injection of T61 euthanasia solution (Intervet Canada Corp., Kirkland, Quebec, Canada) prior to tissue (heart, pancreas, liver and digestive tract) removal. The digestive tract was separated into the crop, proventriculus, gizzard, jejunum, ileum, and caeca. The weights of the organs and empty digestive tract sections were taken along with the length of each section of the small intestine and caeca. The pH of the ileum and caecal content was measured *in situ* using a benchtop pH meter (Knick model 766, Elektronische Messgeräte GmbH & Co. KG, Berlin) equipped with one SE 106 N probe. Caecal samples for SCFA analysis were collected into pre-weighed 25 ml centrifuge tube by gently squeezing immediately after an incision was made at the end of the caecal pouch. The remaining caecal contents were pooled per pen into a 25 ml centrifuge tube. Subsequently, 0.5 g of the pooled sample was placed into 2 mL of 0.6 N HCl solution for ammonia analysis and the remaining content was used for amine analysis. The distal ileum content per pen was pooled in a drum vial for ileal soluble CP analysis and amino acid digestibility. All gut content samples were immediately placed on dry ice then stored at –20°C. The frozen ileum samples were freeze-dried, weighed and then ground with a mortar and pestle.

Experiment 2

On d 21 all birds within a cage were killed with an intravenous injection of T61 and then the digestive tract was carefully removed from 36 birds per treatment after clamping 1 cm proximal to the ileocaecal junction and at 1 cm distal from the end of the pancreas after the duodenum loop. The portion of the small intestine from the Meckel's diverticulum to 1 cm of the ileocaecal junction was considered to be the ileum and the rest of the small intestine the jejunum. Both sections of the

small intestine were cut into proximal and distal halves. The digesta was removed from the distal ileum by gently squeezing into a drum vial and ileal digesta was pooled per cage. Caecal samples for SCFA, ammonia, and amine analysis were collected, stored and ground as described in Experiment 1.

7.3.4 Chemical analyses

The diets, feed ingredients and dried ileal digesta samples were analyzed for AA by Experiment Station Chemical Laboratories, University of Missouri–Columbia using (AOAC, 2006) method 982.30 E (a, b, c). The CP content and DM of all diets, test meals, and ileal digesta samples were analyzed using AOAC (2006) methods 990.03 and 934.01, respectively. The titanium in diets and ileal digesta samples were analyzed using the method of Myers et al. (2004). Total and soluble dietary fibre of diets were determined using the Megazyme fibre assay kit (Megazyme International Ireland Ltd., Wicklow, Ireland) following the manufacturer instructions.

The soluble CP in the dried distal ileum content was extracted using normal saline solution. Dried ileal content (250 mg) was placed in a 13 x 100 mm glass culture tubes followed by 3 ml of 0.9% NaCl solution. The tubes were vortexed, capped with silicone covers and placed in a test tube rack. The test tube rack was placed in an orbital shaker at a 45 degree offset from the horizontal base of the shaker and shook at 300 rpm for 1 h. The tubes were then centrifuged, and the supernatant poured off, and the solid dried at 130°C for 2 h before analyzing for CP.

The ammonia content of the caecal samples was determined using a modification of the phenol-hypochlorite assay described by Broderick and Kang (1980). All caecal samples were pre-weighed and collected in 6 N HCl solution (0.5 g/3 ml) on the d of sampling. The tubes were allowed to thaw at 4°C, vortexed for 30 s and then centrifuged at 3000 rpm for 10 min. The supernatant was filtered through a 0.45 micron syringe filter into a 1.5 mL microcentrifuge vial. A 25 µL volume of sample was mixed with 2.5 mL of phenol reagent, and then 2 mL of hypochlorite reagent was added prior to tube vortexing. The tubes were placed in a water 95°C bath for 5 min, allowed to cool and the mixture was read at 630 nm on a spectrophotometer.

Samples were analyzed for biogenic and polyamines by LC/MS/MS at the Analytical Facility for Bioactive Molecules (The Hospital for Sick Children, Toronto, Canada) using the following procedure. Meal and caecal samples were weighed to 1 g in 15 mL polypropylene tubes. To this, 2 mL 0.4 N perchloric acid was added. Samples were incubated on ice for 10 min before being homogenized by Polytron homogenizer for approximately 15 s. Samples were then

centrifuged at 600 x g for 15 min and 1.5 mL supernatant was transferred into a 2 mL polypropylene tube for further centrifugation at 20,000 x g for 15 min at 4°C. A 1 mL volume of this supernatant was then transferred to a clean 1.5 mL polypropylene tube and the samples were neutralized with approximately 125 µL 2N NaOH. Samples were stored at -80°C until analysis.

For polyamines and agmatine sample extraction, 20 µL of internal standard (50 ng/ml cadaverine-d4, agmatine-d8, putrescine-d8, spermine-d8, and spermidine-d8), 820 µL of methanol, and 10 µL extracted samples were placed in a 1.5 mL polypropylene tube. Tubes were vortexed for 1 min and centrifuged at 20,000 x g for 10 min at 4°C. Supernatants were mixed with 500 µL water and 75 µL 1M sodium carbonate pH 9.0 in 15 mL conical polypropylene tubes. Samples were derivatized with 20uL isobutyl chloroformate at 37°C for 25 min. Following derivatization, 2 mL ethyl ether was added and the tubes were vortexed 1 min and centrifuged at 500 x g for 10 min. The organic supernatant was transferred to a clean polypropylene tube. A further 2 mL ethyl ether was added to the first tube, the process was repeated and the supernatants combined. Supernatants were then taken to dryness under nitrogen at 35°C. Samples and standards were reconstituted in 1 mL (80:20, water: acetonitrile +0.1% formic acid). Sample (200 µL) was transferred to plastic inserts in autosampler vials for agmatine and spermine analysis. For the remainder of the polyamines, 5 µL were diluted in 495 µL of the reconstitution solution in a plastic autosampler vial. Samples were stored at -20°C until analysis by LC/MS/MS.

For biogenic amine sample extraction, 100 µL caecal or 10 µL meal extracted supernatant were added to 10 µL internal standards and 1 mL of acetonitrile/methanol (90:10) solution in a 1.5 mL polypropylene. Tubes were vortexed and centrifuged at 20,000 g. Supernatants were transferred to a conical tube containing 2 mL acetonitrile/methanol (90:10) solution and taken to dryness under a gentle stream of nitrogen. Samples were reconstituted into 1 mL acetonitrile/methanol solution (90:10 + 0.1% formic acid) before analysis by LC/MS/MS.

Polyamines were quantified by LC/MS/MS by injecting samples onto Kinetex XB-C18 2.6u 100A 50 x 3.0 mm column (Phenomenex, Torrance, Californian, USA) on an Agilent 1290 LC system coupled to a Sciex Q-Trap 5500 mass spectrometer. Samples were eluted using a gradient of solution A (water +0.1% formic acid) and solution B (acetonitrile + 0.1% formic acid) over the following gradient: 0 min = 15% B, 2 min = 15% B, 3.5 min = 90% B, 4.5 min = 90% B, 4.6 min = 15% B and 6 min = 15% B. Separate injections were made for Agmatine (15 µL), Spermine (3 µL), and the remaining polyamines (2 µL).

Biogenic amine samples were injected onto Kinetex HILIC 2.6u 100A 50 x 4.6 mm column (Phenomenex) on an Agilent 1200 LC system coupled to a Sciex Q-Trap 5500 mass spectrometer. Samples were eluted using a gradient of solution A (90/10 water/acetonitrile 5 mmol ammonium formate pH 3.2) and solution B (10/90 water/acetonitrile 5 mmol ammonium formate pH 3.2) over 11 min. The following gradient was used for sample elution: 0 min = 100% B, 1 min = 100% B, 3 min = 5% B, 3.5 min = 5% B, 3.6 min = 100% B and 11 min = 100% B. Biogenic and polyamine data were collected and analysed using Sciex Analyst v1.6.3.

SCFA analysis of caecal contents was determined by gas chromatography (GC) using the method of Zhao et al. (2006) with minor modifications. Caecal samples were extracted with 25% (v/v) phosphoric acid to obtain a solution which had 17% (w/w) sample. The samples were allowed to sit for 10 min at room temperature during which time they received occasional shaking and then they were centrifuged for 10 min. The supernatants were collected and filtered through a 0.45 micron filter; then 1 mL of supernatant was mixed with 1 mL of internal standard (0.5 g 3-methyl-n-valeric acid in 1 L of 0.15 mol/L oxalic acid) and 3 ml distilled water. The samples were placed in GC autosampler vials then the vials were placed on a Thermofisher Al 1310 autosampler connected to a Thermofisher Trace 1310 GC system equipped with a flame ionization detector. A 0.2 µL sample volume was injected onto a Zebron^{TM} ZB-FFAP column (length=30 meters, internal diameter=0.25 mm and film thickness =0.25 µm, Penomenex®, Torrance, Californian, USA) with a sample run time of 17.7 min. Helium was the carrier gas at 2.5 mL/min, the oven temperature was initially set at 120°C for 0.1 min, then raised to 180°C at 8 min and held at 250°C. The flame ionization detector temperature was set to 350°C and the air, hydrogen, and mix gas flow rates were 350, 40 and 35 mL/min, respectively.

7.3.5 Calculations and statistics

The soluble protein in the distal ileum content was calculated as the difference between the crude protein content of the dried content before and after extraction. The apparent ileal digestibility coefficients for diets CP and amino acids were calculated using the following formula:

Digestibility = [1-((titanium in diets ÷ titanium in ileal digesta) x (amino acid or CP in diet ÷ amino acid or CP in ileal digesta))] x 100……(7.3.5.1)

All statistical assumptions were checked prior to analysis using the PROC Univariate test of SAS 9.4 for both experiments. All data were subjected to analysis of variance using PROC

MIXED Procedure (SAS 9.4) with a 2 × 3 factorial arrangement of treatments with protein digestibility and dietary protein level as the main factors. Statistical significance was determined at $P \leq 0.05$ and trends $P \leq 0.1$ but ≥ 0.05. Significant main effects or interaction, mean were separation with Tukey's Studentized Range Test and pdmix800 macro (Saxton, 1998) provided letter grouping for differences. Contrast statements were used to check the relationship of some variables to dietary protein level.

7.4 Results

Experiment 1

The CP and amino acid content and digestibility of the diets are presented in the Table 7.2. The level of indigestible CP in the diets were 5.55, 6.29, 5.97, 6.77, 6.41 and 7.50 for 24-LIP, 24-HIP, 26-LIP, 26-HIP, 28-LIP and 28-HIP respectively which were higher than the calculated values (Table 7.1). The diets were also formulated to have 0.87% digestible methionine plus cysteine (Met+Cys) which were the first limiting AA in the diet. However, the determined digestible Met+Cys values were 0.76, 0.75, 0.75, 0.82, 0.77, and 0.78 for 24-LIP, 24-HIP, 26-LIP, 26-HIP, 28-LIP, and 28-HIP, respectively.

Tables 7.3 to 7.5 show the organ and empty intestinal tissue data at 14 and 28 d. Dietary treatments influenced heart, pancreas, proventriculus, gizzard, jejunum and ileum weights at d 14. The heart and pancreas were affected by the interaction of IDP and PL where birds fed the 28-HIP diets had heavier hearts than those fed the 24-HIP diets while all the other diets had intermediate heart weights. Birds fed the 26-HIP had larger pancreas weights than all other diets. The IDP influenced proventriculus, gizzard, jejunum and ileum weights in which the LIP diets gave lower weights for those tissues compared to the HIP diets. Duodenal length was affected by IDP (LIP>HIP) and PL (highest value for 26% CP) at 14 d.

The pancreas, crop, proventriculus, gizzard and ileum weights were the only components influenced by dietary treatments at 28 d. The interaction between IDP and PL affected crop and ileum weights. Crop weights were lowest for the 24-LIP treatment in comparison to the 24-HIP, 26-LIP and 28-LIP treatments; other values were intermediate. For the ileum, the 28-LIP value was lower than 24-HIP, 26-LIP and 28-HIP diets, and again other values were intermediate and not statistically different from the extreme values. The pancreas, proventriculus and gizzard weights were affected by IDP in a similar manner as 14 d of age, where birds fed the LIP diets had

lower weights compared to those fed the HIP diets. The ileum and caeca lengths were influenced by the interaction between IDP and PL. Ileum lengths peaked at 26% CP and had a quadratic response as PL increased in the LIP diets. However, the HIP diets had the opposite quadratic response as PL increased with the lowest ileum length at 26% CP. A similar response as seen for the ileum length of birds fed HIP diets, was seen for caeca lengths in birds fed LIP diets, but there is a tendency for shorter caeca as PL increased in the HIP fed birds.

Table 7.2. Test diets analyzed crude protein and amino acid composition and digestibility in experiment 1 (n = 36 birds per treatment).

	24-LIP[1]		24-HIP[2]		26-LIP		26-HIP		28-LIP		28-HIP	
Item (%)	Content	Dig.[3]	Content	Dig.	Content	Dig.	Content	Dig.	Content	Dig.	Content	Dig.
CP	24.13	77	24.20	74	25.96	77	26.02	74	27.89	77	27.76	73
Alanine	1.05	74	1.13	74	1.14	78	1.40	78	1.26	76	1.58	75
Arginine	1.49	83	1.52	83	1.62	86	1.52	84	1.77	84	1.62	81
Aspartic Acid	2.13	73	2.17	72	2.35	74	2.22	72	2.57	73	2.37	69
Cysteine	0.40	69	0.42	67	0.41	70	0.46	70	0.43	67	0.48	64
Glutamic Acid	5.19	87	5.18	85	5.42	87	5.38	85	5.65	86	5.69	82
Glycine	1.08	74	1.12	72	1.17	76	1.16	73	1.25	74	1.36	70
Histidine	0.65	79	0.68	79	0.71	82	0.70	79	0.77	80	0.75	75
Isoleucine	1.09	77	1.11	77	1.17	80	1.18	78	1.28	78	1.27	74
Leucine	1.79	79	1.96	78	1.94	81	2.41	81	2.12	79	2.65	78
Lysine	1.33	79	1.40	79	1.45	82	1.32	80	1.60	80	1.37	75
Methionine	0.58	86	0.56	86	0.54	88	0.58	87	0.57	86	0.59	82
Met+Cys[4]	0.98	78	0.98	77	0.95	79	1.04	79	1.00	77	1.07	73
Phenylalanine	1.20	79	1.25	79	1.29	81	1.38	81	1.39	79	1.49	78
Proline	1.60	83	1.70	81	1.67	84	1.91	82	1.74	82	2.09	78
Serine	1.01	77	1.08	77	1.10	79	1.15	77	1.16	77	1.22	75
Threonine	0.92	74	0.95	73	0.97	75	1.00	74	1.01	73	1.05	69
Tyrosine	0.74	80	0.78	80	0.80	82	0.91	82	0.87	81	0.98	79
Valine	1.22	74	1.25	74	1.31	78	1.35	76	1.42	75	1.44	72

[1]LIP-low indigestible protein; [2]HIP-high indigestible protein; [3]Dig.-digestibility; [4] Met+Cys-methionine + cysteine.

Table 7.3. Effects of diet levels of total and indigestible protein on organ and empty intestinal tissue as a percentage of bird weight at 14 d of age in experiment 1.

Item	Heart	Liver	Pancreas	Crop	Proventriculus	Gizzard	Duodenum	Jejunum	Ileum	Caeca
Indigestible Protein (IDP)[1]										
LIP	0.62	3.88	0.35	0.44	0.60^{b}	1.63^{b}	0.94	1.92^{b}	1.34^{b}	0.42
HIP	0.62	3.91	0.39	0.46	0.67^{a}	1.80^{a}	0.97	2.08^{a}	1.48^{a}	0.43
SEM	0.01	0.08	0.01	0.01	0.02	0.03	0.02	0.03	0.03	0.01
Protein level (PL)										
24	0.60	3.87	0.35	0.43	0.62	1.71	0.93	1.97	1.38	0.43
26	0.61	3.86	0.40	0.47	0.65	1.70	1.00	2.04	1.43	0.44
28	0.63	3.95	0.35	0.45	0.64	1.74	0.93	1.99	1.42	0.41
SEM	0.01	0.10	0.01	0.02	0.02	0.04	0.03	0.04	0.04	0.02
PL*IDP										
24*LIP	0.62^{ab}	3.91	0.35^{b}	0.42	0.55	1.59	0.90	1.91	1.33	0.42
24*HIP	0.59^{b}	3.82	0.35^{b}	0.44	0.69	1.84	0.97	2.03	1.43	0.44
26*LIP	0.62^{ab}	3.66	0.35^{b}	0.46	0.63	1.66	0.98	1.95	1.34	0.44
26*HIP	0.60^{ab}	4.07	0.45^{a}	0.48	0.66	1.74	1.01	2.13	1.52	0.44
28*LIP	0.60^{ab}	4.06	0.34^{b}	0.45	0.63	1.65	0.93	1.90	1.36	0.41
28*HIP	0.66^{a}	3.84	0.37^{b}	0.46	0.66	1.82	0.92	2.07	1.49	0.41
SEM	0.02	0.15	0.02	0.02	0.03	0.06	0.04	0.06	0.05	0.02
ANOVA					*P*-Values					
IDP	0.85	0.79	0.01	0.33	<0.01	<0.01	0.32	<0.01	<0.01	0.81
PL	0.15	0.77	0.01	0.14	0.59	0.82	0.15	0.43	0.59	0.36
PL*IDP	<0.01	0.07	0.02	0.95	0.13	0.32	0.56	0.84	0.73	0.88

[a-b] Means (n = 16 birds per treatment) within a column with no common superscript per main effect or interaction are significantly different ($P \leq 0.05$).
[1]LIP-low indigestible protein; HIP-high indigestible.

Table 7.4. Effects of diet levels of total and indigestible protein on organ and empty intestinal tissue as a percentage of bird weight at 28 d of age in experiment 1.

Item	Heart	Liver	Pancreas	Crop	Proventriculus	Gizzard	Duodenum	Jejunum	Ileum	Caeca
Indigestible Protein (IDP)[1]										
LIP	0.51	3.12	0.23[b]	0.36	0.42[b]	1.09[b]	0.80	1.55	1.08	0.34
HIP	0.54	3.19	0.25[a]	0.37	0.47[a]	1.19[a]	0.80	1.57	1.11	0.35
SEM	0.01	0.07	0.01	0.01	0.01	0.04	0.02	0.04	0.03	0.01
Protein level (PL)										
24	0.54	3.21	0.23	0.35	0.44	1.12	0.80	1.54	1.12	0.35
26	0.56	3.08	0.24	0.37	0.46	1.12	0.79	1.58	1.10	0.35
28	0.52	3.18	0.25	0.37	0.43	1.17	0.83	1.56	1.07	0.33
SEM	0.02	0.09	0.01	0.01	0.02	0.05	0.03	0.05	0.03	0.01
PL*IDP										
24 * LIP	0.53	3.23	0.22	0.31[b]	0.44	1.13	0.80	1.47	1.10[ab]	0.34
24 * HIP	0.55	3.20	0.24	0.38[a]	0.44	1.12	0.79	1.62	1.20[a]	0.36
26 * LIP	0.55	3.04	0.24	0.38[a]	0.44	1.07	0.82	1.64	1.14[a]	0.33
26 * HIP	0.56	3.12	0.24	0.36[ab]	0.47	1.16	0.76	1.52	1.10[ab]	0.36
28 * LIP	0.52	3.12	0.23	0.38[a]	0.39	1.06	0.81	1.54	1.00[b]	0.33
28 * HIP	0.52	3.25	0.28	0.37[ab]	0.48	1.28	0.85	1.58	1.14[a]	0.32
SEM	0.02	0.12	0.01	0.02	0.03	0.064	0.04	0.07	0.05	0.02
ANOVA						*P*-Values				
IDP	0.72	0.53	0.01	0.30	0.02	0.04	0.76	0.63	0.36	0.33
PL	0.33	0.52	0.13	0.21	0.60	0.68	0.49	0.85	0.48	0.34
PL*IDP	0.82	0.80	0.15	<0.01	0.13	0.20	0.37	0.11	0.05	0.41

[a-b] Means (n = 16 birds per treatment) within a column with no common superscript per main effects or interactions are significantly different ($P \leq 0.05$).
[1]LIP-low indigestible protein; HIP-high indigestible protein.

Table 7.5. Effects of diet levels of total and indigestible protein on intestinal tissue length as a percentage whole intestine[1] of broiler at 14 and 28 d of age in experiment 1.

	D 14				D 28			
Item	Duodenum	Jejunum	Ileum	Caeca	Duodenum	Jejunum	Ileum	Caeca
Indigestible Protein (IDP)[2]								
LIP	15.9[a]	35.7	34.9	13.2	15.4	34.9	35.2	14.5
HIP	15.2[b]	35.8	35.5	13.4	15.2	35.0	35.6	14.3
SEM	0.2	0.2	0.3	0.2	0.2	0.3	0.3	0.2
Protein level (PL)								
24	15.3[b]	36.0	35.2	13.3	15.6	34.3	35.3	14.8
26	16.1[a]	35.2	35.0	13.7	15.1	35.4	35.4	14.2
28	15.2[b]	36.1	35.5	13.0	15.2	35.1	35.5	14.2
SEM	0.2	0.3	0.4	0.3	0.2	0.3	0.3	0.3
PL*IDP								
24 * LIP	15.7	36.0	34.6	13.3	16.1	34.3	34.7[b]	14.8[a]
24 * HIP	15.0	35.9	35.8	13.3	15.2	34.3	35.8[ab]	14.7[ab]
26 * LIP	16.2	35.1	34.8	13.9	14.9	35.4	35.9[ab]	13.8[b]
26 * HIP	16.0	35.4	35.2	13.4	15.3	35.3	34.8[b]	14.6[ab]
28 * LIP	15.8	36.0	35.5	12.4	15.4	34.9	34.9[b]	14.8[a]
28 * HIP	14.7	36.2	35.6	13.6	15.1	35.3	36.1[a]	13.7[b]
SEM	0.3	0.4	0.5	0.4	0.3	0.5	0.5	0.4
ANOVA				*P*-Values				
IDP	0.01	0.69	0.17	0.51	0.31	0.82	0.26	0.70
PL	<0.01	0.08	0.56	0.22	0.29	0.08	0.89	0.22
PL*IDP	0.37	0.88	0.55	0.09	0.16	0.87	0.01	0.02

[a-b] Means (n = 16 birds per treatment) within a column with no common superscript per main effects or interactions are significantly different ($P \leq 0.05$).
[1]Whole intestine- (duodenum + jejunum + ileum +caeca); [2]LIP-low indigestible protein; HIP-high indigestible protein.

Caecal SCFA for d 14 and expressed as µmol/g of wet caecal content SCFA are presented in Table 7.6. Total SCFA, acetic, isobutyric and isovaleric acid concentrations were influenced by dietary treatments. Both total SCFA and acetic acid concentrations were influenced by the interaction between IDP and PL. For both, levels decreased in caecal content as PL increased when birds were fed the HIP diets, but levels went up as PL increased in the LIP diets. Dietary IDP affected isobutyric acid level with birds fed the LIP diets having higher levels than those fed the HIP diets. Isovaleric acid content increased with increasing PL.

As a percentage of total SCFA, only isovaleric and caproic acid were not affected by dietary treatment on d 14, while all other SCFA were affected by the interaction between IDP and PL (Table 7.6). The proportion of acetic acid increased with increasing PL for LIP fed birds, while values decreased with PL for the HIP treatment birds. Propionic acid and butyric acids, proportional levels decreased with increasing PL for LIP birds and increased for HIP birds. The isobutyric acid percentage of total SCFA in birds fed the 28-HIP diets was higher than those fed 24- or 26-HIP diets. However, isobutyric acids levels for LIP diets were similar regardless of dietary PL. Valeric acid levels was unchanged regardless of PL in the LIP diets but increased with increasing PL in a linear fashion in the HIP diets.

At 28 d of age, the ANOVA analysis indicated that the caecal content concentration of SCFA in µmoles/g of wet caecal content were not affected by any dietary treatment (Table 7.7). However, the orthogonal contrast suggested that there was a positive linear effect of PL on isovaleric concentration in µmoles/g of wet caecal content. When SCFA were expressed as a proportion of total SCFA, only isovaleric acid was affected by the interaction between IDP and PL. The interaction indicates that isovaleric acid levels increased as PL increased in the HIP diets, but no effect of PL was seen when birds were fed the LIP diets.

Table 7.6. Effects of diet levels of total and indigestible protein on caecal SCFA of 14 d old male broiler chicken vaccinated for coccidiosis in experiment 1.

	SCFA[1] µmol/g of wet caecal content								Percentage of total SCFA						
Item	Total	Acet	Prop	Isob	But	Va	Isov	Cap	Acet	Prop	Isob	But	Va	Isov	Cap
Indigestible protein (IDP)[2]															
LIP	206	119	36	1.15^{a}	41	0.77	1.54	0.88	59	18	0.54	21	0.74	0.37	0.47
HIP	177	97	32	0.70^{b}	37	0.60	1.80	0.73	56	19	0.41	23	1.09	0.33	0.45
SEM	8.9	9.7	2.2	0.11	2.5	0.14	0.16	0.11	2.7	1.2	0.05	1.6	0.08	0.07	0.06
Protein level (PL)															
24	223	129	35	0.86	41	0.56	1.38^{b}	0.78	59	17	0.41	22	0.62	0.25	0.39
26	165	91	32	0.78	37	0.67	1.52^{ab}	0.79	55	20	0.42	23	0.93	0.40	0.51
28	189	106	34	1.14	39	0.82	2.12^{a}	0.85	57	19	0.60	22	1.18	0.43	0.48
SEM	11.0	11.9	2.6	0.14	2.9	0.17	0.20	0.13	3.3	1.5	0.07	1.9	0.09	0.08	0.07
IDP*PL															
24 * LIP	176^{cd}	89^{b}	35	1.00	44	0.55	1.26	0.84	52^{ab}	20^{ab}	0.56^{ab}	26^{ab}	0.71^{b}	0.31	0.52
24 * HIP	282^{a}	187^{a}	35	0.73	38	0.58	1.49	0.72	67^{a}	13^{b}	0.26^{b}	19^{ab}	0.53^{b}	0.20	0.27
26 * LIP	198^{bc}	111^{ab}	38	1.22	42	1.01	1.53	0.95	57^{ab}	20^{ab}	0.60^{ab}	21^{ab}	0.78^{b}	0.51	0.54
26 * HIP	138^{d}	75^{b}	27	0.34	32	0.44	1.50	0.65	54^{ab}	20^{ab}	0.24^{b}	24^{ab}	1.09^{ab}	0.32	0.49
28 * LIP	249^{ab}	172^{a}	34	1.24	37	0.81	1.84	0.85	69^{a}	14^{ab}	0.48^{ab}	15^{b}	0.73^{b}	0.33	0.36
28 * HIP	144^{d}	65^{b}	33	1.04	40	0.83	2.40	0.84	45^{b}	23^{a}	0.72^{a}	28^{a}	1.63^{a}	0.57	0.60
SEM	15.5	16.8	3.7	0.19	4.1	0.24	0.28	0.19	4.7	2.1	0.09	2.7	0.13	0.12	0.10
ANOVA								*P*-Values							
IDP	0.01	0.04	0.19	0.01	0.22	0.27	0.27	0.24	0.36	0.67	0.08	0.24	<0.01	0.62	0.83
PL	<0.01	0.03	0.68	0.16	0.56	0.41	0.03	0.88	0.70	0.35	0.08	0.93	<0.01	0.11	0.46
IDP*PL	<0.01	<0.01	0.22	0.18	0.23	0.21	0.56	0.61	<0.01	<0.01	<0.01	<0.01	<0.01	0.12	0.07
CP Contrast															
Linear	0.02	0.11	0.64	0.16	0.54	0.18	0.05	0.64	0.65	0.38	0.04	0.77	<0.05	0.05	0.38
Quadratic	<0.01	0.02	0.46	0.19	0.36	0.94	0.34	0.85	0.48	0.25	0.30	0.81	0.79	0.38	0.37

[a-b] Means (n = 16 birds per treatment) within a column with no common superscript per main effects or interactions are significantly different ($P \leq 0.05$).
Acet=acetic acid, Prop=propionic acid, Isob=isobutyric acid, But=butyric acid, Va=valeric acid, Isov=isovaleric acid, Cap=caproic acid.
[1]Short chain fatty acids; [2]LIP-low indigestible protein; HIP-high indigestible protein.

Table 7.7. Effects of diet levels of total and indigestible protein on caecal SCFA of 28 d old male broiler chicken vaccinated for coccidiosis in experiment 1.

	SCFA[1] µmol/g of wet caecal content								Percentage of total SCFA						
Item	Total	Acet	Prop	Isob	But	Va	Isov	Cap	Acet	Prop	Isob	But	Va	Isov	Cap
Indigestible protein (IDP)[2]															
LIP	287	201	33	1.84	46	1.45	3.01	0.76	69	12	0.65	15	1.07	0.52	0.27
HIP	276	195	33	1.92	40	1.56	3.07	0.72	70	12	0.73	14	1.10	0.64	0.28
SEM	20.7	18.4	1.5	0.24	3.9	0.22	0.20	0.04	2.8	0.7	0.07	1.5	0.07	0.08	0.04
Protein level (PL)															
24	280	195	33	1.83	44	1.47	2.56	0.71	69	12	0.64	15	0.97	0.51	0.25
26	297	211	34	1.66	46	1.23	3.19	0.76	70	12	0.59	15	1.11	0.43	0.26
28	268	188	33	2.15	38	1.89	3.37	0.75	70	13	0.84	14	1.18	0.84	0.31
SEM	25.3	22.5	1.8	0.29	4.9	0.26	0.24	0.05	2.2	1.0	0.11	1.8	0.09	0.10	0.04
IDP*PL															
24 * LIP	277	196	31	1.92	43	1.81	2.38	0.72	70	11	0.71	14	0.88	0.66[ab]	0.26
24 * HIP	282	194	34	1.73	46	1.20	2.73	0.70	68	12	0.58	16	1.05	0.43[b]	0.25
26 * LIP	310	218	35	1.26	50	0.98	3.39	0.82	68	12	0.43	16	1.15	0.33[b]	0.28
26 * HIP	284	203	33	2.07	41	1.54	3.00	0.70	71	12	0.75	14	1.06	0.54[ab]	0.25
28 * LIP	276	190	34	2.34	44	1.72	3.26	0.74	69	13	0.83	15	1.18	0.64[ab]	0.27
28 * HIP	261	187	32	1.97	33	2.07	3.48	0.76	70	13	0.86	13	1.19	1.12[a]	0.27
SEM	35.8	31.8	2.6	0.41	6.8	0.37	0.34	0.07	3.1	1.2	0.15	2.6	0.11	0.13	0.06
ANOVA								*P*-Values							
IDP	0.69	0.80	0.88	0.80	0.32	0.67	0.83	0.43	0.99	0.85	0.53	0.62	0.22	0.18	0.79
PL	0.72	0.77	0.92	0.49	0.51	0.15	0.08	0.75	0.62	0.57	0.23	0.75	0.76	<0.01	0.53
IDP*PL	0.90	0.97	0.55	0.32	0.54	0.14	0.54	0.54	0.66	0.79	0.34	0.62	0.54	0.02	0.49
CP Contrast															
Linear	0.75	0.83	0.83	0.44	0.38	0.25	0.03	0.58	0.91	0.35	0.20	0.51	0.09	0.02	0.30
Quadratic	0.46	0.50	0.74	0.37	0.45	0.11	0.45	0.63	0.95	0.61	0.24	0.71	0.73	<0.01	0.70

[a-b] Means (n = 16 birds per treatment) within a column with no common superscript per main effects or interactions are significantly different ($P \leq 0.05$).
Acet=acetic acid, Prop=propionic acid, Isob=isobutyric acid, But=butyric acid, Va=valeric acid, Isov=isovaleric acid, Cap=caproic acid.
[1]Short chain fatty acids; [2]LIP-low indigestible protein; HIP-high indigestible protein.

Caecal ammonia, biogenic and polyamine data for 14 d are presented in Table 7.8. Histamine, agmatine, cadaverine and total amine levels were lower in the caecal contents of birds fed the HIP diets compared to those fed the LIP diets. There was less serotonin, tryptamine, and spermidine in the caecal content of birds fed the LIP diets than those fed the HIP diets. There were no effects of dietary treatments on tyramine, putrescine, spermine, and ammonia, but statistical trends for ammonia and tyramine suggest levels increased with increasing PL. Protein level had a linear effect on ammonia and a trend (P=0.06) for a linear effect on tyramine.

Of the amines evaluated in the caecal content at 28 d (Table 7.9), only the biogenic ammines histamine, serotonin and tryptamine were affected by dietary treatments. Indigestible protein influenced histamine and serotonin levels, while both IDP and PL independently affected tryptamine. Birds fed the LIP diets had higher histamine, but lower serotonin and tryptamine compared to those fed the LIP diets. Caecal tryptamine level increased with increasing PL. Ammonia level showed a trend towards significance for PL with the response quadratic in nature where the minimal ammonia level was found for the 26% PL.

Day 14 and 28 d crop, gizzard, ileum, and caeca pH values are presented in Table 7.10. Only the crop, ileum, and caeca pH values were affected by the dietary treatments at 14 and 28 d. At 14 d birds fed the LIP diets had higher crop pH and lower caeca pH values compared to those fed the HIP diets. The PL affected d 14 ileum pH and birds fed the 26% protein diets had higher pH than those fed the 24 or 28% protein diets. The interaction between IDP and PL influenced d 28 crop, ileum, and caeca pH values. Birds fed the 28-LIP diets had higher crop pH values than those fed the 26-LIP or 26- and 28-HIP diets. Ileum pH tends to increase with PL in the LIP diets, while no change was seen for the HIP diets. The caeca pH values at 28 d were lowest for birds on the 26-HIP diets compared to those on the 26-LIP diets, while other diets were intermediate.

Table 7.8. Effects of diet levels of total and indigestible protein on amine and ammonia content in wet caecal content of 14 d old male broiler chicken vaccinated for coccidiosis in experiment 1.

	Biogenic amine (ng/mg)				Polyamine (ng/mg)					ng/mg	µg/mg
Item	Hi	Se	Tr	Ty	Ag	Cd	Pu	Sd	Sm	Total amine	NH_3
Indigestible protein (IDP)[1]											
LIP	32.1[a]	0.09[b]	0.11[b]	5.0	0.076[a]	12.4[a]	11.0	58.5[b]	1.7	126.3[a]	0.81
HIP	8.2[b]	0.18[a]	0.24[a]	5.1	0.016[b]	5.3[b]	7.7	71.2[a]	1.6	99.7[b]	0.76
SEM	2.6	0.01	0.03	1.0	0.004	0.9	1.8	4.1	0.1	5.7	0.03
Protein level (PL)											
24	20.7	0.13	0.17	4.2	0.036	7.7	8.1	60.9	1.8	103.7	0.74
26	21.1	0.13	0.17	3.7	0.047	9.7	11.3	65.4	1.6	121.2	0.75
28	18.7	0.14	0.16	7.5	0.038	9.1	8.7	68.3	1.5	114.1	0.85
SEM	3.3	0.02	0.03	1.2	0.005	1.1	2.1	5.3	0.2	7.0	0.04
IDP*PL											
24 * LIP	31.8	0.09	0.08	3.3	0.062	10.9	9.7	56.1	1.6	113.6	0.77
24 * HIP	9.6	0.17	0.25	5.2	0.017	4.4	6.5	65.7	2.0	93.9	0.72
26 * LIP	33.2	0.10	0.12	4.0	0.094	13.1	13.1	57.1	2.0	139.0	0.77
26 * HIP	8.9	0.19	0.21	3.4	0.017	6.4	9.4	73.5	1.3	103.4	0.74
28 * LIP	31.3	0.07	0.11	7.6	0.075	13.1	10.1	62.3	1.7	123.3	0.89
28 * HIP	6.1	0.19	0.25	7.2	0.014	5.1	7.2	74.3	1.4	101.9	0.81
SEM	5.0	0.02	0.04	1.7	0.007	1.6	2.9	8.0	0.2	9.9	0.05
ANOVA							P-Values				
IDP	<0.01	<0.01	<0.01	0.82	<0.01	<0.01	0.19	0.04	0.46	<0.01	0.19
PL	0.84	0.84	0.66	0.07	0.18	0.43	0.52	0.56	0.55	0.26	0.08
IDP*PL	0.93	0.59	0.30	0.71	0.16	0.87	0.98	0.89	0.06	0.73	0.88
CP Contrast											
Linear	0.71	0.90	0.50	0.06	0.70	0.37	0.85	0.29	0.55	0.31	0.04
Quadratic	0.26	0.58	0.55	0.15	0.07	0.35	0.26	0.90	0.46	0.19	0.35

[a-c] Means (n = 16 birds per treatment) within a column with no common superscript per main effects or interactions are significantly different ($P \leq 0.05$).
Hi=histamine, Se=serotonin, Tr=tryptamine, Ty=tyramine, Ag=agmatine, Cd=cadaverine, Pu=putrescine, Sd=spermidine, Sm=spermine.
[1]LIP-low indigestible protein; HIP-high indigestible protein.

Table 7.9. Effects of diet levels of total and indigestible protein on amine and ammonia content in wet caecal content of 28 d old male broiler chicken vaccinated for coccidiosis in experiment 1.

	Biogenic amine (ng/mg)				Polyamine (ng/mg)					ng/mg	µg/mg
Item	Hi	Se	Tr	Ty	Ag	Cd	Pu	Sd	Sm	Total amine	NH_3
Indigestible protein (IDP)[1]											
LIP	32.1[a]	0.12[b]	0.06[b]	5.1	0.318	23.4	7.4	78.8	2.0	154.3	0.88
HIP	17.3[b]	0.30[a]	0.30[a]	5.9	0.437	25.9	8.5	91.0	2.3	156.7	0.84
SEM	4.9	0.04	0.03	0.81	0.205	5.5	2.6	4.4	0.2	13.8	0.06
Protein level (PL)											
24	23.7	0.14	0.08[b]	5.4	0.581	22.1	6.6	85.9	2.4	149.9	0.85
26	22.4	0.20	0.16[a]	4.9	0.316	28.0	8.3	85.5	2.0	159.0	0.75
28	27.9	0.28	0.19[a]	6.2	0.219	23.8	9.0	83.2	2.1	156.7	0.99
SEM	6.0	0.05	0.04	1.0	0.258	6.9	3.2	5.4	0.3	11.0	0.08
IDP*PL											
24 * LIP	31.2	0.13	0.03	6.4	0.393	16.9	4.8	75.3	2.5	138.7	0.93
24 * HIP	16.3	0.16	0.23	4.4	0.770	27.2	9.1	96.4	2.3	161.1	0.79
26 * LIP	32.7	0.10	0.09	3.4	0.350	29.6	13.1	84.5	2.1	176.8	0.73
26 * HIP	12.2	0.30	0.30	6.5	0.088	26.5	6.2	86.6	2.0	141.2	0.76
28 * LIP	32.4	0.13	0.09	5.7	0.180	23.6	6.4	76.4	1.5	147.5	1.00
28 * HIP	23.4	0.43	0.40	6.6	0.453	24.0	10.7	90.0	2.6	165.9	0.97
SEM	8.5	0.07	0.05	1.4	0.389	10.5	4.5	7.7	0.4	18.1	0.11
ANOVA							P-Values				
IDP	0.04	<0.01	<0.01	0.54	0.65	0.74	0.65	0.06	0.37	0.91	0.62
PL	0.79	0.13	<0.01	0.68	0.56	0.81	0.68	0.92	0.60	0.88	0.08
IDP*PL	0.79	0.12	0.23	0.21	0.63	0.75	0.13	0.47	0.17	0.27	0.69
CP Contrast											
Linear	0.62	0.04	<0.01	0.58	0.44	0.85	0.54	0.73	0.41	0.72	0.23
Quadratic	0.65	0.87	0.21	0.50	0.46	0.54	0.54	0.87	0.56	0.73	0.05

[a-c] Means (n = 16 birds per treatment) within a column with no common superscript per main effects or interactions are significantly different ($P \leq 0.05$).
Hi=histamine, Se=serotonin, Tr=tryptamine, Ty=tyramine, Ag=agmatine, Cd=cadaverine, Pu=putrescine, Sd=spermidine, Sm=spermine.
[1]LIP-low indigestible protein; HIP-high indigestible protein.

Table 7.10. Effects of diet levels of total and indigestible protein on digestive tract pH and total and soluble crude protein in ileum content of male broiler chicken vaccinated for coccidiosis in experiment 1.

	D 14 pH				D 28 pH				D 28 CP in ileum DM (%)	
Item	Crop	Gizzard	Ileum	Caeca	Crop	Gizzard	Ileum	Caeca	Total	Soluble
Indigestible Protein (IDP)[1]										
LIP	5.15[a]	3.27	6.59	5.64[b]	5.09	3.63	6.19	6.05	24.15	8.16
HIP	4.99[b]	3.09	6.57	5.82[a]	4.82	3.57	6.42	5.92	24.41	8.64
SEM	0.05	0.07	1.06	0.05	0.07	0.08	1.01	0.07	0.52	0.31
Protein level (PL)										
24	5.10	3.20	6.49[b]	5.66	4.98	3.61	5.98	5.88	23.27[b]	7.78[b]
26	5.01	3.12	6.78[a]	5.71	4.83	3.62	6.46	5.98	23.38[b]	8.12[ab]
28	5.10	3.23	6.46[b]	5.82	5.05	3.57	6.48	6.09	26.20[a]	9.30[a]
SEM	0.07	0.09	1.17	0.07	0.08	0.09	1.02	0.09	0.64	0.38
PL*IDP										
24 * LIP	5.09	3.28	6.54	5.59	5.05[ab]	3.57	5.58[b]	5.79[ab]	23.48	7.27
24 * HIP	5.11	3.11	6.44	5.72	4.90[ab]	3.66	6.42[a]	5.96[ab]	23.06	8.29
26 * LIP	5.15	3.14	6.79	5.69	4.86[b]	3.78	6.46[a]	6.25[a]	24.00	8.10
26 * HIP	4.87	3.09	6.76	5.73	4.81[b]	3.47	6.45[a]	5.72[b]	22.76	8.14
28 * LIP	5.22	3.39	6.42	5.65	5.37[a]	3.56	6.59[a]	6.09[ab]	24.97	9.10
28 * HIP	4.99	3.07	6.51	6.01	4.73[b]	3.58	6.36[ab]	6.07[ab]	27.42	9.51
SEM	0.09	0.13	1.41	0.09	0.12	0.13	1.03	0.12	0.91	0.54
ANOVA						P-Values				
IDP	0.03	0.08	0.89	0.02	<0.01	0.54	0.18	0.22	0.72	0.28
PL	0.52	0.66	0.05	0.17	0.15	0.90	0.02	0.23	<0.01	0.02
PL*IDP	0.21	0.56	0.80	0.21	0.02	0.28	0.02	0.01	0.13	0.66
PL Contrast										
Linear	0.94	0.81	0.87	0.07	0.52	0.73	0.02	0.09	<0.01	0.01
Quadratic	0.26	0.38	0.02	0.66	0.07	0.76	0.20	0.99	0.10	0.37

[a-b] Means (n = 16 birds per treatment) within a column with no common superscript per main effects or interactions are significantly different ($P \leq 0.05$).
[1]LIP-low indigestible protein; HIP-high indigestible protein.

The total percentage crude protein in the dried distal ileum content of the birds at d 28 and the portion of the protein which was soluble was affected by the level of protein in the diets (Table 7.10). At 24 and 26% dietary protein, the total percentage of protein in the dry matter was the same but lower than for birds fed the 28% protein diets. There was a linear increase in the soluble crude protein content of the distal ileum dry matter content as the level of dietary protein went up, where birds on the 24% protein diet had lower values compared to those on the 28% protein with 26% being intermediate.

Experiment 2

The analyzed crude protein and amino acid composition and digestibility of experimental diets are shown in Table 7.11. Protein levels closely approximated calculated values and with some exceptions, amino acid levels increased with level of dietary protein. Numerically, the determined digestible amino acids values were marginally lower than the calculated values in Table 7.1. After calculating the digestible methionine plus cysteine content of the diets from the analyzed content and digestibility coefficient, numerically diet methionine plus cysteine values were similar except for 24-LIP which was lower than the other diets.

The levels of SCFA in the caecal content of birds are presented in Table 7.12. Dietary treatment had no effect on the concentration of SCFA in μmole/g of wet caecal content. However, an interaction trend between IDP and PL was seen for butyric acid, plus the orthogonal contrast of PL suggested a linear decrease in butyric acid with increasing PL. When each SCFA was expressed as a proportion of total SCFA, acetic acid, butyric and valeric acids were affected by the interaction between IDP and PL. Birds fed the 26-LIP diet had a lower amount of acetic acid as a percentage of total SCFA when compared to those fed the 28-LIP diets, while other treatments were intermediate and not different from the extreme values. Butyric acid levels were lower in birds fed the 28-LIP diets than those fed the 24-, 26-LIP, and 28-HIP diets; other diets were intermediate. Valeric acid increased as PL increased in the HIP diets and there was more valeric acid in the caecal content of birds fed the 26 LIP diets than the other two LIP diets.

Table 7.11. Test diets analyzed crude protein and amino acid composition and digestibility in experiment 2.

	24-LIP[1]		24-HIP[2]		26-LIP		26-HIP		28-LIP		28-HIP	
Item (%)	Content	Dig.[3]	Content	Dig.	Content	Dig.	Content	Dig.	Content	Dig.	Content	Dig.
CP	24.13	69	24.20	73	25.96	77	26.02	70	27.89	74	27.76	70
Alanine	1.05	67	1.13	76	1.14	79	1.40	74	1.26	78	1.58	75
Arginine	1.49	77	1.52	83	1.62	85	1.52	82	1.77	84	1.62	80
Aspartic Acid	2.13	61	2.17	72	2.35	76	2.22	70	2.57	73	2.37	67
Cysteine	0.40	60	0.42	70	0.41	73	0.46	68	0.43	70	0.48	66
Glutamic Acid	5.19	82	5.18	86	5.42	87	5.38	82	5.65	86	5.69	82
Glycine	1.08	64	1.12	74	1.17	78	1.16	71	1.25	75	1.36	69
Histidine	0.65	73	0.68	80	0.71	83	0.70	77	0.77	81	0.75	76
Isoleucine	1.09	72	1.11	79	1.17	82	1.18	76	1.28	80	1.27	76
Leucine	1.79	72	1.96	79	1.94	81	2.41	76	2.12	80	2.65	78
Lysine	1.33	73	1.40	81	1.45	84	1.32	78	1.60	82	1.37	76
Methionine	0.58	83	0.56	88	0.54	87	0.58	84	0.57	86	0.59	83
Met+Cys[4]	0.98	72	0.98	79	0.95	80	1.04	76	1.00	78	1.07	75
Phenylalanine	1.20	72	1.25	80	1.29	81	1.38	77	1.39	80	1.49	77
Proline	1.60	78	1.70	82	1.67	84	1.91	78	1.74	84	2.09	78
Serine	1.01	69	1.08	77	1.10	80	1.15	74	1.16	78	1.22	73
Threonine	0.92	64	0.95	73	0.97	77	1.00	70	1.01	74	1.05	69
Tyrosine	0.74	73	0.78	81	0.80	83	0.91	79	0.87	82	0.98	79
Valine	1.22	67	1.25	76	1.31	79	1.35	73	1.42	77	1.44	73

[1]LIP-low indigestible protein; [2]HIP-high indigestible protein; [3]Dig.-digestibility; [4] Met+cys -methionine + cysteine
(n = 36 birds per treatment).

Table 7.12. Effects of diet levels of total and indigestible protein on caecal SCFA of 21 d old male broiler chicken in experiment 2.

	SCFA[1] µmol/g of wet caecal content								Percentage of total SCFA						
Item	Total	Acet	Prop	Isob	But	Va	Isov	Cap	Acet	Prop	Isob	But	Va	Isov	Cap
Indigestible protein (IDP)[2]															
LIP	174	115	24	0.64	27	1.83	1.02	0.41	67	14	0.28	16	1.04	0.65	0.25
HIP	161	106	23	0.49	28	1.87	0.73	0.41	65	14	0.21	17	1.17	0.46	0.26
SEM	7.1	6.0	0.7	0.09	1.3	0.17	0.13	0.01	1.3	0.6	0.06	0.8	0.08	0.09	0.02
Protein level (PL)															
24	168	112	24	0.47	30	1.67	0.78	0.41	66	14	0.21	17	0.99	0.47	0.26
26	172	110	24	0.62	29	2.04	0.98	0.43	64	15	0.32	18	1.16	0.67	0.28
28	162	109	22	0.62	25	1.84	0.86	0.40	69	14	0.22	15	1.17	0.54	0.23
SEM	8.6	7.3	0.8	0.11	1.7	0.21	0.16	0.02	1.6	0.7	0.08	1.1	0.10	0.11	0.02
IDP*PL															
24 * LIP	175	115	24	0.43	32	1.82	0.70	0.40	66[ab]	14	0.16	18[a]	1.05[b]	0.40	0.25
24 * HIP	162	108	23	0.51	27	1.51	0.86	0.41	67[ab]	13	0.26	17[ab]	0.93[b]	0.54	0.26
26 * LIP	176	108	25	0.78	28	2.18	1.23	0.42	62[b]	15	0.45	18[a]	1.19[ab]	0.90	0.28
26 * HIP	168	111	24	0.45	30	1.91	0.73	0.44	65[ab]	15	0.19	17[ab]	1.13[ab]	0.43	0.27
28 * LIP	171	120	22	0.72	22	1.49	1.12	0.42	73[a]	13	0.25	12[b]	0.88[b]	0.66	0.21
28 * HIP	152	99	22	0.52	28	2.20	0.60	0.37	64[ab]	15	0.20	18[a]	1.46[a]	0.41	0.25
SEM	12.2	10.4	1.1	0.16	2.2	0.30	0.22	0.2	2.2	1.0	0.11	1.5	0.15	0.15	0.3
ANOVA								P-Values							
IDP	0.19	0.32	0.34	0.25	0.13	0.85	0.66	0.65	0.35	0.67	0.44	0.17	0.26	0.12	0.60
PL	0.70	0.96	0.15	0.55	0.56	0.46	0.12	0.38	0.08	0.52	0.52	0.10	0.40	0.42	0.22
IDP*PL	0.90	0.51	0.92	0.43	0.06	0.16	0.22	0.26	0.02	0.55	0.24	0.01	0.03	0.13	0.70
CP Contrast															
Linear	0.60	0.81	0.19	0.35	0.05	0.55	0.68	0.71	0.27	0.77	0.89	0.06	0.23	0.66	0.37
Quadratic	0.52	0.92	0.14	0.59	0.46	0.27	0.42	0.18	0.04	0.27	0.26	0.24	0.55	0.21	0.14

[-b] Means (n = 36 per treatment) within a column with no common superscript per main effects or interactions are significantly different ($P \leq 0.05$).
Acet=acetic acid, Prop=propionic acid, Isob=isobutyric acid, But=butyric acid, Va=valeric acid, Isov=isovalaric acid, Cap=caproic acid.
[1]Short chain fatty acids; [2]LIP-low indigestible protein; HIP-high indigestible protein.

The effects of treatment on biogenic amines, polyamines, and ammonia levels in the caecal content are shown in Table 7.13. Low indigestible protein diets resulted in higher levels of caecal histamine, agmatine, and cadaverine and lower levels of serotonin and tryptamine than birds fed the HIP diets. The orthogonal contract of PL suggested that levels of caecal tryptamine, tyramine, cadaverine, spermine, total amine, and ammonia increased with PL. Interactions between IDP and PL were found for tyramine, spermidine, and spermine. In all three cases, values tended to increase with PL for LIP diets with no effects of PL seen in the HIP diets. The same interaction was seen for caecal ammonia levels.

The soluble and total crude protein in the distal ileum content and the pH of the ileum and caeca are presented in Table 7.14. Dietary protein level affected the total and soluble protein (% of content DM) which increased in the distal ileum content with increasing dietary PL. Caecal pH was influenced by the interaction of IDP and PL, where birds on 28-LIP diets had higher pH values than those on the 24- and 28-HIP diets, with all other diets being intermediate. There were no effects of treatment on ileum pH.

Table 7.13. Effects of diet levels of total and indigestible protein on wet caecal amine and ammonia content of 21 d old male broiler chickens in experiment 2.

	Biogenic amine (ng/mg)				Polyamine (ng/mg)					ng/mg	µg/mg
Item	Hi	Se	Tr	Ty	Ag	Cd	Pu	Sd	Sm	Total amine	NH_3
Indigestible protein (IDP)[1]											
LIP	17.9[a]	0.15[b]	0.12[b]	3.0	0.109[a]	3.6[a]	2.6	78.4	2.4	107.2	0.87
HIP	5.6[b]	0.26[a]	0.32[a]	3.4	0.047[b]	2.5[b]	3.1	78.5	1.8	95.3	0.76
SEM	1.5	0.02	0.03	0.9	0.008	0.3	0.3	3.0	0.2	3.5	0.03
Protein level (PL)											
24	10.9	0.20	0.12[c]	0.8	0.078	2.0[b]	2.5	75.3	1.8	94.9	0.70
26	10.2	0.21	0.30[a]	1.2	0.071	2.7[b]	2.8	77.3	2.0	96.9	0.83
28	14.1	0.19	0.24[b]	1.6	0.086	4.4[a]	3.3	82.7	2.6	112.0	0.91
SEM	1.8	0.03	0.04	1.1	0.010	0.32	0.4	3.7	0.3	4.3	0.04
IDP*PL											
24 * LIP	16.1	0.18	0.04	1.4[b]	0.100	2.2	1.9	65.3[b]	1.7[b]	89.3[b]	0.64[c]
24 * HIP	5.8	0.23	0.20	3.4[ab]	0.055	1.8	3.2	85.3[ab]	1.8[b]	100.5[b]	0.76[bc]
26 * LIP	16.9	0.14	0.16	3.1[ab]	0.103	3.7	3.0	79.3[ab]	2.1[ab]	103.7[ab]	0.95[ab]
26 * HIP	3.5	0.29	0.44	3.5[ab]	0.038	1.7	2.6	75.4[ab]	1.9[b]	90.1[b]	0.72[bc]
28 * LIP	20.8	0.14	0.17	6.6[a]	0.123	4.9	3.1	90.5[a]	3.5[a]	128.5[a]	1.02[a]
28 * HIP	7.4	0.24	0.31	3.3[ab]	0.048	3.9	3.5	74.8[ab]	1.7[b]	95.5[b]	0.79[abc]
SEM	2.8	0.04	0.05	1.5	0.013	0.5	0.5	5.4	0.4	6.7	0.06
ANOVA							P-Values				
IDP	<0.01	<0.01	<0.01	0.60	<0.01	<0.01	0.23	0.97	0.04	0.02	<0.01
PL	0.28	0.84	<0.01	0.03	0.52	<0.01	0.22	0.33	0.07	0.02	0.02
IDP*PL	0.79	0.49	0.33	0.02	0.51	0.19	0.10	<0.01	0.01	<0.01	<0.01
CP Contrast											
Linear	0.22	0.68	0.03	0.01	0.53	<0.01	0.08	0.15	0.02	0.01	<0.01
Quadratic	0.31	0.68	0.01	0.91	0.34	0.21	0.87	0.70	0.60	0.23	0.58

[a-c] Means (n = 36 per treatment) within a column with no common superscript per main effects or interactions are significantly different ($P \leq 0.05$).
Hi=histamine, Se=serotonin, Tr=tryptamine, Ty=tyramine, Ag=agmatine, Cd=cadaverine, Pu=putrescine, Sd=spermidine, Sm=spermine.
[1]Low indigestible protein; HIP-high indigestible protein.

Table 7.14. Effects of diet levels of total and indigestible protein on ileal and caecal pH, and total and soluble crude protein in ileum content of 21 d old male broilers in experiment 2.

	CP in DM (%)		pH	
Item	Total	Soluble	Ileum	Caeca
Indigestible protein (IDP)[1]				
LIP	21.31	6.74	7.06	5.58
HIP	21.28	7.00	7.24	5.47
SEM	0.35	0.18	0.09	0.07
Protein level (PL)				
24	19.07[c]	5.16[c]	7.03	5.50
26	21.65[b]	6.87[b]	7.25	5.52
28	23.17[a]	8.81[a]	7.17	5.55
SEM	0.42	0.22	0.11	0.06
IDP*PL				
24 * LIP	18.80	5.90	6.94	5.59[ab]
24 * HIP	19.33	5.44	7.11	5.41[b]
26 * LIP	21.80	6.64	7.22	5.44[ab]
26 * HIP	21.50	7.11	7.28	5.60[ab]
28 * LIP	23.33	8.99	7.01	5.69[a]
28 * HIP	23.00	8.63	7.32	5.41[b]
SEM	0.58	0.30	0.16	0.09
ANOVA		P-Values		
IDP	0.94	0.16	0.17	0.16
PL	<0.01	<0.01	0.39	0.85
IDP*PL	0.71	0.09	0.75	0.04
PL Contrast				
Linear	<0.01	<0.01	0.39	0.58
Quadratic	0.31	0.97	0.28	0.94

[a-c] Means (n = 36 per treatment) within a column with no common superscript per main effects or interactions are significantly different ($P \leq 0.05$).
[1]LIP-low indigestible protein; HIP-high indigestible protein.

7.5 Discussion

It is commonly stated that diets with high levels of indigestible protein promote hindgut protein fermentation and the formation of undesirable fermentation metabolites that can compromise animal health (Qaisrani et al., 2015; Apajalahti and Vienola, 2016). The objective of this study was to evaluate this theory by examining the effects of three dietary PL (24, 26, and 28%) with low or high IDP on the digestive tract morphology and caecal protein fermentation metabolites in coccidiosis vaccinated and non-vaccinated broilers.

Performance data from a comparison of the same diets (Chapter 6) in vaccinated broilers showed that birds fed LIP diets had better performance in terms of body weight gain, feed to gain ratio and carcass yield than broilers fed HIP diets, but mortality in these treatments was higher due to enteric and systemic infection. The mortality results were opposite to current literature theories related to IDP. However, changes in the digestive tract morphology due to vaccination (Lee et al., 2011) or the production of some protein fermentation metabolites (Barnes et al., 2001; Igarashi et al., 2001) could have promoted higher systemic infection when the birds were fed the LIP diet. The battery cage experiment provided an environment free of coccidiosis infection and was used to evaluate the digestibility of the diets, while the floor experiment was conducted concurrently with the production experiment described in Chapter 6 and the 14 and 28 collections represented periods of active response and recovery to coccidiosis vaccination, respectively.

7.5.1 Diet digestibility

The diets used in Chapter 6 were formulated to have different levels of IDP at each level of dietary protein inclusion (Table 7.1). By using the analyzed CP and determined digestibility coefficients, the digestible protein contents of the diets were confirmed to be 16.7, 17.7, 20.0, 18.2, 20.6, and 19.4%, for 24-LIP, 24-HIP, 26-LIP, 26-HIP, 28-LIP, and 28-HIP diets, respectively in the non-vaccinated birds (Table 7.11). The *in vivo* digestibility of the diets in the non-vaccinated birds suggested that the 24-LIP diet had more of its protein which was not digested when compared to the 24-HIP diets, the opposite of what was anticipated. However the 28 d digestible CP data from the vaccinated birds were 18.58, 17.91, 19.99, 19.25, 21.48, and 20.26 for 24-LIP, 24-HIP, 26-LIP, 26-HIP, 28-LIP, and 28-HIP diets, respectively. These values were in general agreement with formulated values.

The digestibility data from both the vaccinated (Table 7.2) and non-vaccinated birds (Table 7.11) suggest that the *in vitro* prediction of the indigestible CP in the diets (Table 7.1) underestimated the IDP in all the diets. The *in vitro* extent of digestion that was predicted may have overestimated both soybean and fish meal extent of digestion which was 88 and 89% *in vitro* (Table 4.2, Chapter 4) compared to 77 and 75% respectively *in vivo* (Table 5.4 Chapter 5). Regardless of the overestimation of SBM and fish meal CP digestibility *in vitro*, all diets met the formulation requirements of low and high IDP.

The LIP diet protein sources were fish and soybean meal. The fish meal had unusually high levels of amines relative to levels normally found in fish meal samples in North America (Barnes et al., 2001). The total amine levels in the diets LIP (Table 7.1) were higher than those of the corresponding HIP diets, which could have influenced the digestibility of the diets *in vivo* through an effect on the digestive tract, a physiological response that would not have been predicted by the *in vitro* model. A common disadvantage with *in vitro* digestion models is the lack of host feedback mechanisms on the digestive process and overestimation of digestibility (Boisen and Eggum, 1991; Fuller, 1991). Regardless of the *in vitro* predictions, the digestibility data confirmed that there were actual differences between the IDP fractions of the diets.

The work of Macfarlane et al. (1986) suggested that there was a link between soluble protein and the production of protein fermentation products in the large intestine of humans. In both trials of the current study, the total and soluble CP in the ileum of the birds increased as the protein level went up in the diets. This suggested that more protein should be available for fermentation as the digesta moves towards the colon. In theory the dietary PL should, therefore, have an impact on the level of protein fermentation products found in the caeca of the birds, however, the response seen might also be influenced by the carbohydrate fraction of the digesta. Unfortunately, the level of carbohydrate in the ileal digesta from the current study was not evaluated.

7.5.2 SCFA and pH

A part of the general theory about protein fermentation in poultry suggests that higher levels of SCFA lower the digesta pH, and thereby reduce the population of acid-sensitive pathogenic bacteria. The total SCFA μmol/g present in the caecal content of non-vaccinated birds fed the LIP (174 μmol/g) and HIP (161 μmol/g) diets were statistically the same (Table 7.12). A similar pattern was seen between the LIP (287 μmol/g) and HIP (276 μmol/g) diets for the total

SCFA at 28 d in the vaccinated birds. In neither case, the numeric deviations in the concentration of total SCFA between the LIP and HIP fed birds were not enough to cause a statistical difference in pH values. However, at 14 d of age, the vaccinated birds had significantly higher levels of total SCFA in the birds fed the LIP (206 µmol/g) diet compared to the HIP (177 µmol/g). This difference translated into statistically lower pH values in the caeca of birds fed the LIP diet, however it should be noted that at 14 d of age the birds were actively responding to a coccidiosis vaccination.

Vaccination of boilers have been shown to lower the pH of the caecal content of broiler chickens (Arczewska-Włosek et al., 2017), but the reason for this effect is yet to be elucidated. The difference seen at 14 d between the LIP and HIP fed birds caecal pH could be related to the disease status of those birds. The LIP fed birds had higher mortality (Chapter 6) an indication of increased disease, which was accompanied by more tissue damage and nutrient in the digestive tract for fermentation. At 28 d this effect was absent, instead, the interaction between PL and IDP influenced the caecal pH, which was also seen at 21 d in the non-vaccinated birds. At 28 d the change in caecal pH was in part due to changes in the PL. Numerically pH values were higher at 14 d relative to those at 28 d in the vaccinated and 21 d in the non-vaccinated birds, but it is unlikely that the increased systemic infection in birds fed the LIP diets (Chapter 6) was as a result of higher levels of pathogenic bacteria due to increased pH level caused by the low SCFA production in the LIP fed birds.

The data from the current study indicate the production of SCFA in caeca of the birds depended on vaccination and dietary treatments. Acetic acid was the primary SCFA and would, therefore, have the largest effect on the total SCFA in the caeca. At 14 d there was an interaction between IDP and PL for total acetic acid in the vaccinated birds, where levels increased with PL in the LIP diets while it decreased with PL in the HIP diets. The response of caecal acetic acid at 14 d seems to be a diet and PL response. It is not clear why acetic acid content went down with increasing PL in the HIP diets. Interestingly, except for isovaleric and isobutyric acid, no other SCFA evaluated at d 14 or 28 d responded to dietary treatment. As expected isovaleric acid content increased with PL which might indicate that its production was related to protein fermentation. Isobutyric acid only responded to IDP where the LIP fed birds had higher levels than HIP fed birds, which were unexpected. The reason for the unusual response of isobutyric acid to IDP is unknown, but this response might indicate that the kind of SCFA produced in the caeca reflect the nature of the dietary components.

The proportion (% of total SCFA) of propionate, isobutyric acid, butyric acids, and valeric acid production at 14 d increased with increasing PL in the HIP fed birds, while except for butyric acid, which declined with PL in the LIP diets, the other SCFA remain unchanged. Only isovaleric acid was affected by dietary treatment at 28 d in the vaccinated birds while acetic acid, butyric acid, and valeric acid were affected in the non-vaccinated birds. The interaction of IDP and PL did not produce any noticeable trend for acetic acid in the non-vaccinated birds, but butyric acid and valeric acid tend to increase with increasing PL in the HIP fed birds. Since acetic acid had the largest proportion, there is some uncertainty that the response of the SCFA in the proportional data might only be a reflection of changes in the acetic acid level for at least the d 14 data.

The response of SCFA to dietary treatment might be in part related changes in the digestive tract environment in terms of the substrate available for fermentation. In both experiments the total protein available for fermentation in the distal ileum increased with protein level, however, the source from which that was derived varied. Short chain fatty acids can be produced from anaerobic fermentation of proteins and the quantity of acetic acid produced varies based on the kind of proteins available for fermentation (Macfarlane et al., 1986) and the species of bacteria present (Ramsay and Pullammanappallil, 2001). The nature of the proteins present in the digesta of the birds at 14 d is unknown. Due to the vaccination, it is possible that the digesta would include epithelial cell cytoplasmic protein along with digestive enzymes, mucin, and an increased dietary protein fraction, all of which would contribute to the fermentable protein pool.

Ramsay and Pullammanappallil (2001) summarized the literature regarding the fermentation of amino acids. The data predict that alanine, arginine, aspartic acid, cysteine, glycine, and serine predominantly produce acetate via the Stickland reaction. However, there is no amino acid digestibility available for the current trial at 14 d in the vaccinated birds to confirm the availability of amino acids for fermentation. It is possible that the ileal digesta composition of the LIP and HIP fed birds differed in alanine, arginine, aspartic acid, cysteine, glycine or serine level in the distal small intestine. The trends seen for proportional butyric acid and valeric acid when the non-vaccinated birds were fed the HIP diets would suggest that the ileal digesta from those birds should have more arginine, histidine, lysine, proline, and threonine as substrate (Ramsay and Pullammanappallil, 2001). Numerically, the AA digestibility data in Table 7.2 and 7.11 suggested more undigested histidine, lysine, proline, and threonine in the ileal digesta of birds fed HIP diets.

Dietary protein affects SCFA production in the caeca of broilers. The effects might be more related to the source and or digestibility than the level of protein fed in the diets. The disease status of the birds played a role in inducing more protein related effects on SCFA production. In relation to the nature of the proteins in the diets, the SCFA produced in the caecal content of the healthy birds or those recovering from the coccidiosis vaccination had no impact on the caecal content pH. In contrast, 10 d post vaccination, the reduced protein digestibility of diets, as well as damage due to enteric disease, could have changed the caecal SCFA, which in turn affected the pH of the caecal contents.

7.5.3 Amine production

The effects of IDP on the amine level in the caecal content of the birds were fairly consistent across experiments. It seems that vaccination (disease status) may have influenced the response of some amines to dietary IDP. As it relates to the IDP in the diets, the total amine content increased from 95 in the non-vaccinated birds to 99.7 and 154 ng/mg in the vaccinated birds at 9 and 24 d respectively post vaccination when fed the HIP diets. In the case of the birds fed the LIP diets the values were higher; increasing from 107 (non-vaccinated) to 126 and 156 ng/mg (9 and 24 d post vaccination, respectively). To our knowledge, this is the first experiment to have such a comprehensive list of amines analyzed from the caecal content of both coccidiosis vaccinated and non-vaccinated birds fed diets which varied in IDP.

The vaccine used in this study contained *E. acervulina. E. maxima, E. maxima MFP, E. mivati,* and *E. tenella*. Under normal situations, *E. acervulina* reside mostly in the upper part of the small intestine (mainly the duodenum), while *Eimeria mivati* can be found duodenum and the upper part of the jejunum (Sharma, 1964). *Eimeria maxima* tend to be located in the middle part of the intestine and *tenella* resides mostly in the caeca (Sharma, 1964). The species present in the Coccivac-B52 vaccine gave a complete coverage of the intestine. Apart from that, the environment in which the birds were housed was manipulated to encourage cycling of the *Eimeria* species. Each species has a slightly different cycling period (Williams, 2002), which could have resulted in a constant supply of substrate for protein fermentation up to 24 d post infection due to intestinal cells damage from the cycling of the different *Eimeria*.

The effect of IDP on the caecal amine content of the vaccinated birds over time might be explained by shifts in microbiota composition, alterations in the absorption and metabolism of amines by the intestinal epithelium possibly due to the *Eimeria* infection. The data from

experiments 1 and 2 consistently showed higher levels of histamine, agmatine, and cadaverine in the caecal content of birds fed the LIP diets. The birds fed the LIP diets were the same group which had higher death loss due to systemic infection (Chapter 6). The histamine, agmatine, and cadaverine ranges for LIP diets were 0.0102 to 0.0163%, 0.0015 to 0.0023% and 0.0048 to 0.0076%, respectively. Diets with 0.2% histamine and 0.1% cadaverine have been shown to cause gizzard erosion and proventricular ulcers in chickens (Barnes et al., 2001), which could lead to more pathogenic bacteria entering the blood. None of those pathological changes were observed in the present study, probably due to the potentially low levels of histamine and cadaverine in the diets. This suggests that the higher death loss due to systemic infection in the LIP diets might not be related to dietary amine levels.

In the absence of vaccination and during the early and late stage following vaccination the level of histamine in the caecal content of the LIP fed birds was significantly higher than there HIP counterparts. It is unlikely that the caeca selectively accumulates dietary histamine so the other alternative for the higher caecal histamine relates to microbial fermentation in response to the diets fed. This alternative implied that the caecal residential microbes were able to ferment the undigested feed from the LIP diets to produce more amine than those fed the HIP diets.

It may be that the caecal histamine played a role in the mortality associated with the LIP fed birds. Other research has shown that the administration of 10 μmoles of histamine in whole blood culture exposed to bacteria lipopolysaccharides has been shown to reduce T-helper type1 cells (Fleisher et al., 1998), which are responsible for promoting an immune response against intercellular parasites such as bacteria. Alongside this, cadaverine and agmatine can enhance the ability of rodent intestinal cells to absorb and retain histamine by inhibiting histamine-N-methyltransferase and diamine oxidase enzymes which catabolize histamine (Taylor and Lieber, 1979; Lyons et al., 1983). Even though the diets may have had low levels of amine, theoretically, the combined effects of dietary cadaverine and agmatine plus caecal microbial histamine production could have increased the systemic blood concentration of histamine over time. If the blood had high levels of histamine this could reduce the bird's ability to fight off bacterial infections, because histamine tends to shift the immune system towards greater T-helper type 2 cell response (Fleisher et al., 1998).

The HIP fed birds were associated with higher levels of tryptamine and serotonin in their caecal content, so there is a possibility that they were providing beneficial effects to the birds

during the cycling of the vaccine. Tryptamine and serotonin are derived from tryptophan, while tryptamine can act as a serotonin receptor agonist, which preferentially stimulates the release of serotonin (Barnes et al., 2001; Blough et al., 2014). Serotonin has a variety of functions and is known to enhance wound healing through its stimulatory effects on fibroblast proliferation (Boucek and Alvarez, 1970). The serotonin could have reduced intestinal lesions via enhanced wound healing in the vaccinated birds fed the HIP diets. Serotonin is also a potent activator of macrophages which is a key component of the innate immune response (Polanski et al., 1995). It is likely that serotonin was involved in macrophage activation that lowers the number of bacteria translocated to the blood from the intestinal lumen due to damage of epithelial cells by the *Eimeria* parasites.

The levels of amines in the caecal content were consistent across experiments and treatments. The primary differences between treatments might be due to protein source and digestibility. The effects of PL were only seen in healthy birds at 21 d with some amine responding to the interacting effects of IDP and PL. Except for tryptamine at d 28, PL effects were not seen for the amine data in the vaccinated birds. In those birds the PL effect might have been covered up by the increased protein loss to the intestinal lumen from enteric disease.

7.5.4 Ammonia production

Ammonia can accelerate apoptosis of digestive tissue cells during bacterial infection (Igarashi et al., 2001) in humans and this can alter the intestinal barrier. While there were statistically higher levels of ammonia from the non-vaccinated birds fed the LIP diets compared to those fed the HIP diets, it was not the case for the vaccinated birds at either 14 or 28 d. However, it should be noted that the ANOVA values from the vaccinated birds indicate trends for significance for PL (*P=0.08*) at both ages. The ammonia data from the healthy birds seem to be opposite to what was expected for diets with low levels of IDP, but Qaisrani et al. (2014) also observed higher ammonia levels in the caecal content of broilers fed diets with low levels of IDP. Those authors did not provide an explanation for their finding. The data from the current study may suggest that the effects of PL on caecal ammonia are in part related to the total or soluble CP present in the distal ileum digesta of the healthy birds. However, the interacting effects of IDP and PL on the caecal ammonia in the healthy birds indicate that dietary protein source might also be involved.

Caecal ammonia increased with PL, but only in the healthy birds fed LIP diets; ammonia level did not increase with increasing PL birds in fed the HIP fed diets. It is possible that ammonia contributed to the higher level of systemic infection found when broilers were fed the LIP diets, but more detailed evaluation is required.

7.5.5 Morphology and tissue weights

The high feed intake and growth rate of broiler chickens can potentially make the digestive tract susceptible to impaired functionality (Svihus, 2014). The nature of the diet can lead to digestive tract dysfunction so it is relevant to evaluate the morphology of this organ when a nutritional assessment is done. The tissue and organ data from Tables 7.3 and 7.4 were similar to values previously reported (Qaisrani et al., 2014). Indigestible protein in the diets consistently influenced the proventriculus, gizzard, pancreas, and ileum weights of broilers at younger and older ages, but dietary protein seemed to have little to no effect. In general, all of those tissues were lighter in the birds fed the LIP diets and the effects of IDP on the jejunum seemed to be dependent on age or disease status of the birds.

Gut segments have the ability to change in response to diet in an effort to optimize nutrient retention (Svihus, 2011). In theory, if dietary nutrients are poorly digested, it might be expected that the digestive tract and associated tissues would adapt, including an increase in size, in an attempt to increase nutrient digestibility. Proportional increases in the size of the pancreas, proventriculus and gizzard at both 14 and 28 for the HIP in contrast to LIP diets suggest that this may be the case. Further, the proportional weights of the jejunum and ileum for HIP birds were also heavier than LIP birds at 14 d. However, since the diets contained different ingredients other components than protein digestibility may be responsible for these changes. A possible candidate is the diets insoluble fibre since the HIP diets had 1% more insoluble fibre than the LIP diets. Other possibilities may include difference in diet particle size after adding moisture to the feed in the gizzard. Large feed particle size entering the gizzard has been shown to stimulate gizzard and proventriculus size (Hetland and Svihus, 2001; Svihus, 2011). Increase in gizzard activity due to larger diet particle size can simulate gizzard development and increase pancreatic secretions via cholecystokinin (Svihus, 2011). Continuous stimulation of the pancreas could lead to increased size. The HIP and LIP diets could have had different particle size due to CDDGS addition. Previous work by Qaisrani et al. (2014) has shown that the size of the gizzard was larger in birds fed diets

with larger particle size. The biological changes that stimulated increased proventriculus, gizzard, and pancreas weights of birds fed the HIP diets in the current study are not clear.

Conclusion

In conclusion, indigestible protein increased the size of some digestive tract tissue in young birds. Indigestible protein modified the caecal fermentation metabolites of broilers, but this effect varied depending on vaccination status of the birds and the metabolites been evaluated. Some caecal metabolites produced by broilers chicken might be related to the amount of protein present in the distal ileum digesta, however, the source of dietary proteins and the cocivac-B52 vaccine were the two most important factors.

8.0 GENERAL DISCUSSION

8.1 *In vitro* and *in vivo* digestion kinetics research

The poultry meat sector is expected to fill the growing global demand for poultry meat, as such, the quality of proteins fed to poultry has become more important. The major sources of protein available to the poultry industry are of animal and plant origin, which can be variable in digestibility and amino acid composition (Lemme et al., 2004). Consumers of animal derived food are becoming more conscious about how it is produced. In some countries, customers are demanding food products from poultry fed all vegetable diets, which limits the number of protein ingredients that can be used by the feed industry. There have been some research efforts to find alternative protein sources such as algae and insect meals (Veldkamp et al., 2012; Gatrell et al., 2014; Allegretti et al., 2017), but these sources are still some time from broad application. Until new sources of proteins become available to the poultry sector, the industry will have to find new ways to increase the efficient utilization of currently available protein sources.

The proportion of amino acids from a protein source that is used for protein synthesis determines the ingredient's protein bioavailability (Batterham, 1992). Determining the bioavailability of protein sources for poultry feeding is rarely done, as such, *in vivo* digestibility assays are often used as an estimate of bioavailability (Lemme et al., 2004). *In vivo* digestibility assays provide data on ingredients that can be applied directly to the feed industry when used by a nutritionist in ration formulation (Ravindran and Bryden, 1999). The major concerns about *in vivo* digestible assays are their cost and the time it takes to complete them; regardless, these assays continue to be the optimum tool for assessing protein ingredients. However, other rapid assessment tools are also needed to judge ingredient digestibility and quality. In practice, commercial feed manufacturers rely on book digestibility values and the quality of feed ingredients is often evaluated using a combination of *in vitro* assays. This practice is unlikely to change in the near future so more precise *in vitro* models are needed for evaluating poultry protein feedstuffs.

Apart from digestibility of protein, there might be other unexplored biological mechanisms present in avian species, which could aid in meeting the future global demand for poultry meat. Earlier work by Sklan and Hurwitz (1980) indicated that protein digestion rate could be a key regulator of protein synthesis and deposition in tissue of chickens. This phenomenon was

confirmed to be the case in humans (Boirie et al., 1997), but additional data on this subject are absent for avian species. The mechanisms by which protein digestion rate regulates protein deposition in poultry is an under researched area. The main obstacle in studying the effects of protein digestion rate on poultry performance is related to the fact that no progress has been made on quantifying protein digestion rate *in vivo* or *in vitro* for poultry. Despite the lack of progress in this area of research, an AA digestibility study with meal-fed broiler breeders suggested that the release of amino acids into the portal blood after digestion had to be gradual and synchronized before the birds could utilize them for protein synthesis (Nonis and Gous, 2006).

The main focus of Chapters 3 and 4 of this thesis was to address the need for more precise *in vitro* models for assessing the rate and extent of digestion of protein feedstuffs for poultry. One major challenge often encountered when developing *in vitro* models to evaluate protein digestion is the ability of a single model to effectively assay multiple kinds of feed ingredients. Due to this challenge, multiple quality control assays such as those based on the physiochemical properties of ingredients have been developed to help the feed industry. The *in vitro* model developed in Chapter 3 evaluated nine different protein sources which are known to have variable digestibility and physiochemical properties. Correlation analysis between the PDI and KOH solubility of the ingredients and *in vitro* extent of digestion were all significant, with correlation coefficients (r) of 0.64 and 0.84, respectively. There was no correlation between the *in vitro* CP digestibility and the reactive lysine assay, which might be an indication that the assay was not useful when evaluating multiple kinds of ingredients.

Chapter 5 on the other, provided some progress towards the development of an *in vivo* assessment tool for evaluating protein feed ingredient digestion rate along with the routine extent of digestion. The same samples were evaluated in Chapters 4 and 5 in order to study the relationship between the *in vitro* and *in vivo* data. Correlation analysis between the *in vivo* extent of digestion and the PDI, KOH solubility and reactive lysine were all significant. The PDI (0.38) and KOH solubility (0.56) correlations were positive while reactive lysine (-0.62) was negatively correlated. Correlation analyses were performed between the *in vivo* and *in vitro* CP digestion data sets. The *in vitro* and *in vivo* CP digestibility of the ingredients were positively correlated with a correlation coefficient of 0.75 (Figure 8.1).

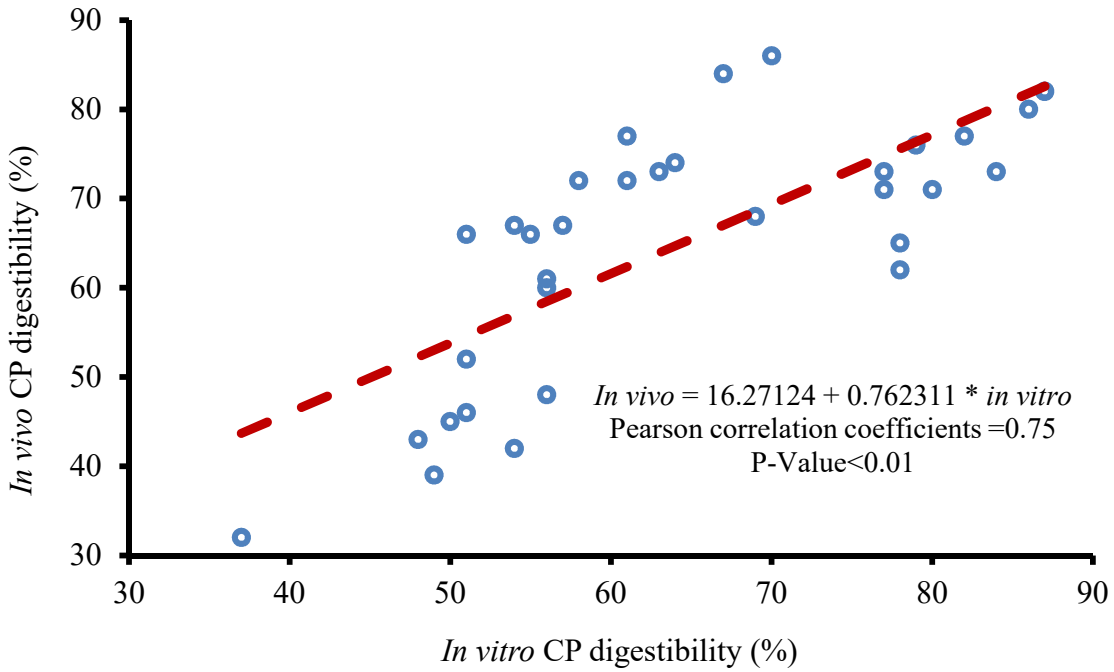

Figure 8.1. Plot of correlation between *in vivo* and *in vitro* CP digestible of nine high protein feed ingredients.

A one sample T-Test was performed comparing the difference between the *in vitro* and *in vivo* CP digestibility data to a mean of 0 to see if there were differences between the two methods of assessing digestibility. This comparison suggests that there is no difference between *in vivo* and *in vitro* CP extent of digestion for the meals evaluated. The Bland Altman plot of the data presented in Figure 8.2 shows that there was no proportional bias between *in vitro* and *in vivo* CP digestibility data for any of the nine meals evaluated and all the data points collected during the assay fell in the 95% confidence limit. This indicates that the *in vivo* and the *in vitro* CP digestible data were in agreement for the digestibility of nine meals. Based on the correlation and the Bland Altman plot results, the *in vitro* assay was able to predict the *in vivo* CP digestibility of the ingredients. The *in vitro* assay could, therefore, serve as a tool for assaying CP digestible of meals for broiler chickens.

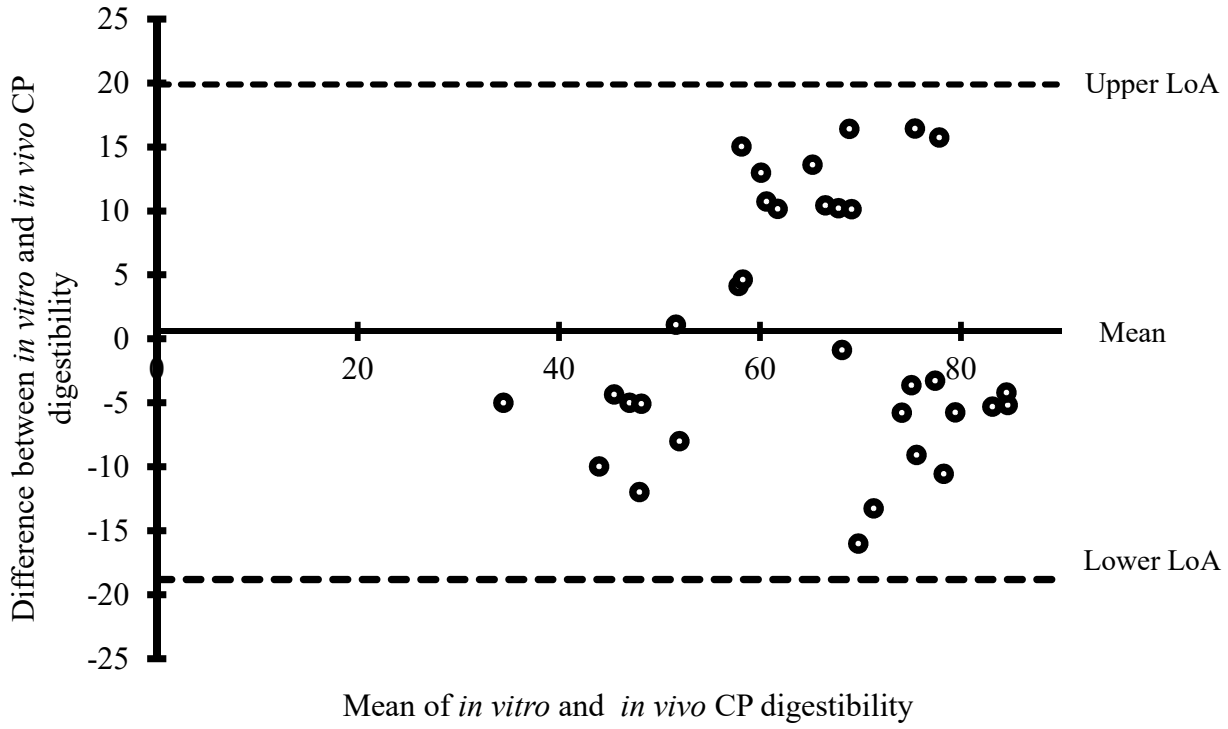

Figure 8.2. Bland Altman Plot of the difference between *in vivo* and *in vitro* CP digestible of nine high protein feed ingredients.

In order to know if *in vitro* CP digestibility data are representative of the *in vivo* AA digestibility, regression and correlation analysis (Table 8.1) were performed between the two sets of data. The *in vitro* CP digestibility was positively correlated with all amino acids except for CYS, which had a regression estimate *P* value of 0.1. The correlation coefficients ranged from 0.43 to 0.71 except for CYS which was 0.30. The *in vitro* model was developed using SBM as the model protein source which has both pros and cons. Using SBM might have put the other ingredients at a slight disadvantage since the method optimized SBM digestibility for each stage of digestion and not the other meals. This could have accounted for some of the variation seen in the correlation coefficients of the AA with the *in vitro* CP digestibility. Based on the data presented in Table 8.1 the *in vitro* CP digestibility can be used as a predictor of *in vivo* AA digestibility, however, the correlation coefficients varied among AA so more samples need to be tested to form stronger prediction equations.

Table 8.1. Simple linear regression and Pearson correlation of *in vitro* digestible CP and *in vivo* standardized ileal amino acids digestibility of the nine meal samples.

	Regression coefficients		ANOVA		*In vitro* digestible CP	
Item	Intercept	*In vitro* digestible CP	R^2	MSE	Correlation coefficients	P-Value
ASP	3.27	0.83	0.35	252.55	0.59	<0.01
Estimate SE	13.68	0.21	—	—	—	—
Estimate P-Value	0.81	<0.01	—	—	—	—
THR	27.35	0.55	0.35	115.73	0.59	<0.01
Estimate SE	9.25	0.14	—	—	—	—
Estimate P-Value	<0.01	<0.01	—	—	—	—
SER	38.29	0.43	0.18	168.08	0.43	0.02
Estimate SE	11.15	0.17	—	—	—	—
Estimate P-Value	<0.01	0.02	—	—	—	—
GLU	22.19	0.75	0.50	112.11	0.71	<0.01
Estimate SE	9.11	0.14	—	—	—	—
Estimate P-Value	0.02	<0.01	—	—	—	—
PRO	13.72	0.75	0.35	206.60	0.59	<0.01
Estimate SE	12.36	0.19	—	—	—	—
Estimate P-Value	0.28	<0.01	—	—	—	—
GLY	41.58	0.40	0.25	94.69	0.50	<0.01
Estimate SE	8.37	0.13	—	—	—	—
Estimate P-Value	<0.01	<0.01	—	—	—	—
ALA	35.01	0.56	0.39	95.38	0.63	<0.01
Estimate SE	8.40	0.13	—	—	—	—
Estimate P-Value	35.01	0.56	—	—	—	—
CYS	26.18	0.41	0.09	326.99	0.30	0.09
Estimate SE	15.55	0.23	—	—	—	—
Estimate P-Value	0.10	0.09	—	—	—	—
VAL	42.74	0.40	0.21	113.32	0.46	<0.01
Estimate SE	9.16	0.14	—	—	—	—
Estimate P-Value	<0.01	<0.01	—	—	—	—
MET	32.256	0.63	0.45	97.07	0.67	<0.01
Estimate SE	8.47	0.13	—	—	—	—
Estimate P-Value	<0.01	<0.01	—	—	—	—
ILE	43.74	0.44	0.26	110.68	0.51	<0.01
Estimate SE	9.05	0.14	—	—	—	—
Estimate P-Value	<0.01	<0.01	—	—	—	—
LEU	35.38	0.56	0.35	113.76	0.59	<0.01
Estimate SE	9.17	0.14	—	—	—	—
Estimate P-Value	<0.01	<0.01	—	—	—	—
TYR	28.97	0.63	0.39	121.17	0.62	<0.01
Estimate SE	9.47	0.14	—	—	—	—
Estimate P-Value	<0.01	<0.01	—	—	—	—
PHE	39.97	0.5	0.29	120.73	0.54	<0.01
Estimate SE	9.45	0.14	—	—	—	—
Estimate P-Value	<0.01	<0.01	—	—	—	—
LYS	34.44	0.57	0.50	62.50	0.71	<0.01
Estimate SE	6.80	0.10	—	—	—	—
Estimate P-Value	<0.01	<0.01	—	—	—	—
HIS	12.17	0.84	0.48	150.04	0.70	<0.01
Estimate SE	10.54	0.16	—	—	—	—
Estimate P-Value	0.26	<0.01	—	—	—	—
ARG	33.27	0.63	0.40	119.31	0.63	<0.01
Estimate SE	9.39	0.14	—	—	—	—
Estimate P-Value	<0.01	<0.01	—	—	—	—

There was no correlation between in vitro and in vivo CP digestion rate which could be due to a number of factors. The in vivo model used to evaluate the digestion kinetics of the protein samples depended to a large extent on the dietary feed intake. There were large differences in feed intake for broilers fed the test diets, which is likely due to the nature of the diets. All the diets were semi-purified, were not balanced, plus, they may have had other ingredient specific differences affecting feed intake. The in vivo model could be refined with the introduction of the substitution method, which could provide the opportunity to test the ingredients in diets which are more balanced in nutrients. This type of dietary approach would encourage higher feed intake and the ingredients could be evaluated at dietary levels which was commonly used in practical diet formulation. However, the level of each test ingredient in the diets would vary if similar CP levels were to be fed, plus it would have to be assumed that the digestion and absorption of nutrients from the ingredients in the test diets are additive.

The rate at which CP is digested *in vivo* is sensitive to feedback mechanisms of the animal during the digestion process and this is lacking in the *in vitro* model. If the animal responds in this way, there are many factors that might be involved in the process such as anti-nutrient agents. Compounds such as phytate, protease inhibitors, sinapine, glucosinolate, amines, and tannins might have a greater impact on the rate of digestion than one would expect in a traditional digestibility assay. The goal of the developed *in vivo* model was to provide a starting point to stimulate future development in this area of research. This *in vivo* model needs to be refined with special emphasis placed on key factors which might be influencing the feedback mechanism of the chicken on the digestion process of proteins. The *in vivo* and *in vitro* digestion rate data does provide some level of distinction between ingredients evaluated. Whether or not these differences can be used to stimulate a biological response in poultry needs to be evaluated. Diets can be formulated to have different rate of AA digestion which could be used to test their effects on body protein synthesis and accretion.

8.2 Impacts of protein digestion kinetics on poultry performance

Data from the literature suggest that there is a link between protein nutrition and the modulation of pathogenic microorganisms such as *C. perfringens* in the poultry digestive tract (Drew et al 204). Interest in this area is gaining more attention because of the legislative change in antibiotics use as well as voluntary removal of antibiotics to meet customer demand in some leading poultry producing countries. As demand grows for poultry products, pressure will be placed

on the supply of protein sources including SBM. As a result there has and will continue to be increased use of non-traditional sources of protein. The increased pressure on SBM supply and restrictions on the use of some antibiotics in poultry diets have increased concerns about the effects protein digestion may have on intestinal pathogenic microorganisms in poultry when fed traditional and non-traditional protein sources. There are also indications that necrotic enteritis, a serious poultry disease worldwide, is more prevalent in production systems where antibiotic usage is limited or absent (Van Immerseel et al., 2009).

Despite much discussion about the relationship between dietary protein source and pathogenic intestinal microorganisms in poultry, there is limited published research which explains or confirms this relationship for common protein sources available to the poultry industry. An exception is FM, which has been used in necrotic enteritis models (Rodgers et al., 2015), but even for FM the mechanism behind the response is still elusive. Despite the scarcity of information, the majority of recommendations for managing gut health include feeding low protein diets with highly digestible CP and limited or more often no CP from animal sources. This recommendation is based on the theory that undigested proteins at the terminal ileum will become available to the resident microbiota and this will shift the microbial ecology towards pathogenic microorganisms. In theory, the characteristics of the protein present in the distal ileum content of poultry should affect its availability for fermentation and in turn the resulting negative effects of protein fermentation products. An unanswered question is whether the nature of the protein present in the distal ileum can affect its fermentative capacity.

Chapter 5 examines characteristics of the protein present in the distal ileum of birds fed semi-purified diets where specific ingredients were the only source of protein in the diets. Similar data presented in Chapter 7, was taken from birds given balanced diets, which were formulated to have differences in the level of IDP. Chapter 5 data demonstrate that the amount and solubility of the CP in the ileal digesta are ingredient specific. In the semi-purified diets, which were formulated to have the same CP levels, the amount of CP present in the distal ileum content was based on the extent to which the ingredients were digested. In contrast, the amount of soluble CP which was based on the individual characteristics of the ingredients and not as much on their digestibility.

In the case where birds were fed diets designed to have high and low IDP, the soluble CP in the distal ileum was not affected by coccidiosis vaccination or diet IDP. However, increasing the dietary CP resulted in a proportional increase in soluble CP of the distal ileum content. The

reason of this could be related to specific ingredients ability to change endogenous protein or not all the digestible protein from the diets was removed before the digesta reaches the distal ileum. It is possible that the soluble CP faction of the LIP and HIP diets might have contained a large proportion of endogenous proteins whose level were altered by the individual characteristic of the protein sources. By increasing the level of the protein sources in the LIP and HIP diets, the endogenous portion of the soluble protein also increased. Another possibility is that the animals were less efficient at utilizing all the digestible CP as the diets CP level increased. Those two reasons could explain the proportional change in soluble CP due to increasing dietary CP levels that was seen in both the vaccinated and non-vaccinated birds.

Diet protein level has been shown to influence broiler chicken performance (Temim et al., 2000) and it is often theorized that the undigested protein at the distal ileum can alter poultry performance due to changes in microbiota and caecal fermentation metabolites (Qaisrani et al., 2015; Apajalahti and Vienola, 2016). The effects of undigested protein on broiler performance were evaluated in Chapters 6 and 7 using practical diets and commercial rearing management practices. The diets were formulated based on the *in vitro* IDP content of the ingredients and by increasing the protein level. It was hypothesized that changes in IDP and CP levels should amplify the effects of IDP.

The level of diet IDP affected broiler performance with the specific effect apparently related to the disease status of the birds. Over the 32 d of production, HIP diets had negative effects on feed to gain and body weight gain of the birds except for d 12-22 period where the HIP fed birds gave better feed efficiency and body weight gain. The HIP diets tended to reduce carcass and most meat yield parameters measured while increasing the dietary CP levels of the LIP diets benefited meat yield and feed to gain. Some of the effects seen in the performance and meat yield data attibuted to the IDP might be related to the AA digestibility of the diets. There were numerical differences in digestibility of AA between the HIP and LIP diets from the cage study and the 28 d data from the vaccinated birds, so it is expected that during the vaccination cycling period in the vaccinated birds, the digestibility of AA should be lower (Adedokun et al., 2016). Surprisingly, the performance of the birds was above Aviagen objectives despite the challenge from the coccidiosis vaccination and manipulation of the environmental humidity. The diets had three protein levels 24, 26 and 28% and all met the as hatched 2.0 to 2.5 kg Ross 308 broiler grower nutrient requirements and contained no medication. Based on the criteria used to formulate the diets, there might have

been excess of some individual AA. The excess of some AA (Lys) in the diets altered some of the meat yield parameters measured in this study.

Mortality in vaccinated broiler chickens was not affected by protein level, which is contrary to conventional thinking. Similarly, feeding the high IDP reduced systemic infection death loss and overall mortality, which does not match the negative expectation. There were no major gross morphological changes in the digestive tract tissue, which could have provided any indication as to why the LIP diets increased infectious mortality. However, the pancreas size increased when high IDP was fed to the birds.

Of interest is the finding that the levels of fermentation metabolites, histamine, agmatine cadaverine, and total amines in the caecal content were higher in the birds fed diets with lower IDP. Similarly, caecal ammonia levels were higher in the LIP fed birds even when they were not exposed to vaccination. Both the amine and ammonia data suggest higher CP fermentation in the caecal content of birds fed the LIP diets compared to the HIP diets despite the HIP diets being formulated to have a larger proportion of undigested (and potentially fermentable) protein. The protein pool of the LIP diets was derived from wheat, SBM, and FM. Why was there more CP fermentation seen in the LIP diets compared the HIP diets in both the vaccinated and non-vaccinated birds? One possibility is that the caecal amine levels are a reflection of diet amine levels. One way to assess this possibility is to express each amine as a percentage of total amine in the diet and caecal content, respectively, and then compare the ratios. When this was done, the ratios in the diet and caecal contents did not align, suggesting that diet did not directly influence caecal levels. This finding plus the difference in ammonia between the LIP and HIP caecal content support the role of the microbial activity in producing these fermentation products. There were no differences between the total or soluble CP in the distal ileum of LIP and HIP fed (vaccinated or non-vaccinated birds), which would explain the difference seen in protein fermentation.

It should be noted that the technique used to extract the soluble CP from the ileal digesta was less aggressive when compared to urea and dimethyl sulfoxide extractions. Any protein which required aggressive extraction would not have been extracted using the normal saline solution. The CP fermentation capabilities of the caeca might be dependent on the size of digesta particles entering the caeca instead of the solubility of the CP in the ileal digesta.

Is it possible that the difference in infectious death loss between the LIP and HIP diets seen in this study was due to a mechanism other than CP fermentation? It is proposed that increased

infectious mortality associated with the LIP diets was due to the effects of a compound(s), such as an amine, indoles or skatoles in the LIP diet, which reduce the host ability to respond to the disease or enhanced the invasive ability of the coccidiosis oocysts. In either case more damage to the intestinal epithelium would increase the probability of death from coccidiosis and necrotic enteritis, as well as systemic infection as a result of bacterial translocation.

8.3 Implication and future research

To the author's knowledge, this thesis is one of first to have evaluated and developed both *in vitro* and *in vivo* techniques for assessing the rate of CP digestion for poultry. There are many metabolic systems which are affected by the rate of digestion of proteins in *Homo sapiens* (Boirie et al., 1997; Hall et al., 2003), which presents questions about how and which metabolic systems might be influenced by protein digestion rate in avian species. Future work could use molecular technology and bioinformatics to study the metabolic effects of protein digestion rate on avian species. This thesis provides a set of tools which can be used to explore the effects protein digestion rate may have on the health and production efficiency of commercial poultry flocks.

It is a general consensus that the poultry industry will have to change the way animals are fed and managed. These changes are due to concerns about the development of highly resistant strains of pathogenic microorganism in the human health sector and the growing demand for poultry products worldwide. This thesis provides a small insight into some of the nutritional strategies and challenges the broiler industry might face when birds are grown under antibiotic-free management strategies. Apart from that, this thesis presents initial data pertaining to the ability of broilers to fermentation dietary indigestible protein when reared without antibiotic. The data from this thesis suggest that dietary proteins sources available to the poultry industry are not just sources of AA. These protein sources possess both functional and biological properties which require further investigation. Future research should focus on the functional and biological properties of available protein sources in an effort to identify health effects they might have in antibiotic-free production systems.

8.4 Conclusion

In conclusion, an *in vitro* protein digestibility assay was developed to predict the rapidly, slowly and undigested protein fraction of ingredients, as well as the rate and extent of digestion of protein sources. The proposed *in vitro* technique estimated the rate and extent of protein digestion

for high protein ingredients with low assay variability. The extent and rate of CP and AA digestion of plant and animal protein meals were also determined *in vivo* for poultry and variation was seen for CP and AA both between and within protein sources. The *in vitro* and *in vivo* protein digestion kinetics data generated were in agreement, and therefore provide an opportunity to categorize feed ingredients based on their digestion kinetics as well as the extent of digestion. The amount of soluble CP present in the distal ileum content of broiler fed semi-purified diets was influenced by dietary protein source, and this could potentially affect CP fermentation in the caeca of broiler chickens.

This thesis evaluated the effects of dietary protein level and indigestible protein fraction on the performance and meat yield of broiler vaccinated for coccidiosis and fed antibiotic-free diets. The dietary protein level and the level of indigestible protein affected broiler performance and meat yield when birds were vaccinated for coccidiosis. The negative effects seen on performance and meat yield due to indigestible protein could be related to differences in the digestibility of the diets AA. The unexpected mortality seen when birds were fed diets with lower indigestible protein required further investigation since the caecal metabolites evaluated, might in part, be related to the source of the dietary proteins.

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