INVESTIGATING THE IMPACT OF EXOGENOUS ENZYMES AND PHOSPHORUS-INDUCED APPETITE REGULATION IN BROILER CHICKENS

by

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Dedicated to God, my wife, my lovely kids, my parents.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

SYMBOL	DESCRIPTION
AA	Amino acid
AD	Apparent digestibility
AI	Anterior ileum
AID	Apparent ileal digestibility
AJ	Anterior jejunum
AGRP	Agouti-related peptide
AME	Apparent metabolizable energy
AMEn	Nitrogen-corrected metabolizable energy
AMPK	Adenosine monophosphate activated protein kinase
ANF	Antinutritional factor
ANOVA	Analysis of variance
AOAC	Association of official analyst chemist
ARC	Arcuate nucleus
ASCT-2	Neutral amino acid transporter
BW	Body weight
CART	Cocaine- and amphetamine-regulated transcript
CCK	Cholecystokinin
CCKAR	Cholecystokinin receptor
CD	Crypt depth
CM	Crude mucin
CP	Crude protein
CRH	Corticotropin-releasing hormone
DDGS	Distillers' dried grains with solubles
DE	Digestible energy
DEB	Dietary electrolyte balance
DF	Dietary fiber
DM	Dry matter
DMD	Dry matter digestibility

DMI	Dry matter intake
DMN	Dorsomedial nucleus
EAA	Endogenous amino acid
EXT	Excreta
FYT	Phytase unit
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GE	Gross energy
G: F	Gain to feed
GIT	Gastrointestinal tract
GLM	General linear model
GLP-1	glucagon-like peptide 1
GLUT	Glucose transporter
IDE	Ileal digestible energy
IP	Inositol phosphate
IL	Interleukin
KNU	Amylase unit
LHA	Lateral hypothalamic area
LNC-2	Lipocalin-2
MCR	Melanocortin receptor
MSH	Melanocyte-stimulating hormone
MUC-2	Mucin
N	Nitrogen
NaPi-IIb	Sodium-phosphate cotransporter
NFD	Nitrogen free diet
NSP	Non starch polysaccharide
NPP	Non phytate phosphorus
NPY	Neuropeptide Y
OXM	Oxyntomodulin
PF	Pair-feeding
PI	Posterior ileum
PJ	Posterior jejunum

POMC	Pro-opiomelanocortin
PROT	Protease unit
PTI	Purified trypsin inhibitor
PVN	Paraventricular nucleus
PYY	Peptide YY
qPCR	Quantitative real-time polymerase chain reaction
RANKL	Receptor activator of nuclear factor-κB ligand
SA	Sialic acid
SBM	Soybean meal
SGLT-1	Sodium glucose transporter
SLC7A-2	Cationic amino acid transporter
TI	Trypsin inhibitor
TIU	Trypsin inhibitor unit
TTR	Total tract retention
VH	Villus height
VMN	Ventricular nucleus

ABSTRACT

For this dissertation, four experiments were conducted to evaluate the effect of dietary addition of exogenous protease and amylase enzymes on growth performance and nutrient utilization in broiler chickens. An additional fifth experiment was designed to determine the role of central and peripheral appetite regulators in birds fed diets deficient in dietary phosphorus (P). This arose from consistent reports in literature of a direct effect of dietary P concentration on feeding response in broiler chickens.

Experiment 1 examined the growth performance and protein utilization responses of broiler chickens to purified trypsin inhibitors (TI) and exogenous protease additions. Experimental diets were arranged as a 2 × 2 factorial with factors being dietary TI (1,033 or 10,033 TIU/g) and exogenous protease (0 or 15,000 PROT/kg). Protease supplementation improved BW gain ($P < 0.01$) and gain to feed ratio ($P < 0.05$) of birds. The relative weight of pancreas increased ($P < 0.05$) with added TI on d 14 and 21 but was reduced ($P < 0.001$) with protease supplementation. Apparent ileal digestibility (AID) of all amino acids (AA), except methionine, decreased ($P < 0.001$) with added TI, but increased ($P < 0.05$) with protease supplementation. Duodenal trypsin and chymotrypsin activities were reduced ($P < 0.05$) with added TI but increased ($P < 0.01$) with protease supplementation. It was concluded that dietary addition of purified TI negatively affects nutrient utilization by broiler chickens and that the efficacy of the exogenous protease might be independent of dietary TI concentration. A follow-up experiment was conducted (Experiment 2) to evaluate the impact of TI and exogenous protease supplementation on endogenous AA loss in broiler chickens. Four diets were arranged as a 2 × 2 factorial with factors being dietary TI (0 or 8,000 TIU/g) and exogenous protease (0 or 15,000 PROT/kg). There was no effect of TI, exogenous protease, or their interaction on growth performance of birds. Endogenous nitrogen (N) loss and all AA (except Cys) increased ($P < 0.05$) due to added dietary TI. Exogenous protease had no effect on endogenous loss of N and all AA. The AID of Ca, Fe, Mg, Mn, and Cu was reduced ($P < 0.05$) by added dietary TI. Protease supplementation improved the AID of Cu ($P < 0.01$) and K ($P < 0.05$). Secretion of crude mucin and sialic acid (g/kg DM intake) increased ($P < 0.05$) with increased dietary TI and was not recovered by protease supplementation. It was concluded from this study that TI increases the endogenous loss of AA, reduces the digestibility

of minerals in broiler chickens, and that exogenous protease had no effect on endogenous AA flow, irrespective of added dietary TI.

In Experiment 3, the responses of broiler chickens fed corn-soybean meal-based diets to dietary α-amylase supplementation during 4 growth phases were evaluated. Birds were assigned to 8 treatment diet in a 2 × 4 factorial arrangement of 2 dietary levels of α-amylase supplementation (0 or 80 kilo-Novo alpha amylase units (KNU) per kg diet) and 4 post hatching growth phases (d 0 to 11, d 11 to 21, d 21 to 42, or d 42 to 56). Body weight gain and feed efficiency of birds improved ($P < 0.01$) with α-amylase supplementation. There were main effects of α-amylase, growth phase and interaction ($P < 0.01$) on AID of starch. The total tract retention (TTR) of starch increased ($P < 0.05$) with amylase supplementation but was not different across growth phases. Amylase supplementation improved ($P < 0.05$) gross energy utilization in birds, and specifically, during d 11 to 21 post hatching, the viscosity of jejunal digesta and pancreatic amylase activity increased ($P < 0.01$) with amylase supplementation. The conclusion from the study was that the growth phase of birds may affect the response to exogenous amylase. Following the result of this study, Experiment 4 was conducted to evaluate the effect of amylase supplementation on starch and energy digestibility at various intestinal sites in broiler chickens. Experimental diets comprised 3 concentrations of α-amylase supplementation (0, 80, or 160 KNU/kg diet) and sampling was done on 4 intestinal sites: anterior jejunum (AJ), posterior jejunum (PJ), anterior ileum (AI) and posterior ileum (PI). There were linear and quadratic ($P < 0.01$) responses of increasing α- amylase supplementation on starch and energy digestibility at the PJ and AI, with only linear effects on TTR of starch ($P < 0.05$). A linear increase in starch disappearance and digestible energy (kcal/kg) was observed ($P < 0.01$) with digesta flow from AJ to PJ with increasing amylase supplementation, which may be related to the observed decrease in the viscosity of the jejunal digesta ($P < 0.05$). Results from this experiment demonstrate the efficacy of exogenous amylase to improve starch, and energy digestibility in broiler chickens, with the highest impact observed in the posterior jejunum.

A final study (Experiment 5) was conducted to evaluate the impact of dietary phosphorus (P) concentration on hypothalamic molecular regulation of appetite by broiler chickens. Birds were randomly assigned to 3 experimental diets which contained 1.2 (P-deficient), 2.8 (P-marginal) or 4.4 (P-adequate) g/kg non-phytate P (nPP). A decrease in feed intake and BW gain was observed ($P < 0.001$) in birds fed the P-deficient diet. There was upregulation ($P < 0.05$) in the mRNA

expression of Sodium-phosphate cotransporter (NaPi-IIb), anorexia-related hypothalamic cholecystokinin receptor (CCKAR) and melanocortin receptors (MC3R and MC4R) in birds fed P-deficient diets, whereas cholecystokinin (CCK) mRNA was downregulated ($P < 0.01$). It may be concluded that a deficiency in dietary P decreases feed intake in broiler chickens by altering the expression of anorexigenic genes in the gut and hypothalamus.

CHAPTER 1. LITERATURE REVIEW

1.1 Introduction

Over the last century, there has been an explosion in global human population, and a recent estimate by the UN projects that this may reach 9.7 billion by 2050 (Roberts, 2011). This growth causes significant dietary changes within the population, the most notable is increase in the consumption of animal protein. Additionally, rising incomes in developing countries has resulted in food demand spike, estimated between 59% to 98% by 2050 (Valin et al., 2014). These factors increase the pressure on the animal food sector to maximize production and reduce waste, especially in the context where key resources, such as land and water, are likely to be scarcer and where climate change impact will intensify.

The global animal feed production estimate was 1.13 billion metric tons in 2020. Proportionally, the broiler industry represents about 28% of this gross estimate (Alltech, 2020). Because feed represent about 70% of total poultry production cost, there have been a gradual shift by producers towards cheaper alternative feed ingredients that can also supply essential nutrients to the animals. Another promising solution is the use of exogenous feed enzymes that can be used as individual additions or in combination, with the dual purpose of improving animal productivity and profit margin.

Furthermore, animals maintain their body weight by adjusting feed intake and as a result, energy expenditure. However, feed consumption is controlled by a complex network of central and peripheral appetite signaling (Kuenzel et al., 1999; Richards, 2003) which can be influenced by dietary nutrient composition (Wang et al., 2015; McConn et al., 2018). Curiously, several previous reports show changes in feeding response of broiler chickens to dietary phosphorus (P) concentration, however, the molecular basis for this response remains unclear.

For proper context to this dissertation, which may be necessary to clarify the research gap in current monogastric nutrition, this chapter reviews factors affecting nutrient utilization in broiler chickens, the impact and mode of action of exogenous enzymes and molecular regulation of appetite in broiler chickens.

1.2 Factors Affecting Nutrient Utilization in Broiler Chickens

There are several factors that may affect nutrient utilization in broiler chickens. These include, but not limited to, the nutritional quality of feed ingredients, form and processing of the feed, housing and environmental condition, age, and overall health status of the birds. Some of these factors are discussed below.

1.2.1 Anti-Nutritional Factors in Common Feed Ingredients

Plants synthesize a range of secondary metabolites generally for protection from feeding by wild animals or against adverse weather conditions. These compounds also called anti-nutritional factors (ANF) are predominantly found in legume grains (Ramadoss and Shunmugam, 2014) and include oligosaccharides, trypsin inhibitors (TI), lectins, and phytate. The ANF stimulate varying effect on animals when consumed (Khokhar and Apenten 2003) by binding to dietary nutrients, inhibiting digestive enzyme activity, increasing intestinal digesta viscosity, reduction in protein solubility, and a consequent increase in loss of endogenous nutrients (Cowieson and Ravindran, 2007; Ao, 2011).

1.2.1.1 Trypsin Inhibitor

Although soybean is an excellent source of dietary energy and protein (Waldroup, 1982), it contains significant amounts of ANF, the most problematic being TI. The TI affects the digestibility of protein and amino acid (AA) by inhibiting and reducing the activities of proteolytic enzymes via formation of indigestible enzyme-TI complexes (Liener, 1994; Cabrera-Orozco et al., 2013). There are at least five TI reported in soybeans, but only two, Kunitz and Bowman-Birk, have been purified and extensively studied (Kunitz, 1947; Birk et al., 1963; Balloun, 1980). The Kunitz inhibitor is a single polypeptide chain with a molecular mass of about 20,000 and two disulfide bridges and has 1:1 specificity against trypsin. The other subtype, the Bowman-Birk inhibitor (Birk, 1985), has a molecular mass of about 8,000, is rich in disulfide bonds and has specificity for both chymotrypsin and trypsin using independent binding pockets (DiPietro and Liener, 1989; Francis et al., 2001; Winiarska-Mieczan, 2007). Due to the inhibition of proteolytic enzyme activation by TI, there is feedback enzyme secretion by the pancreas resulting in pancreatic hypertrophy (Leeson and Summers, 2001). In broiler chickens, increased TI intake leads to a linear

increase in pancreas size suggesting a dose-dependent response (Clark and Wiseman, 2005; Erdaw et al., 2017). Increased TI intake may also increase endogenous protein loss from the body, with accompanying implications on nitrogen (N) balance (Clark and Wiseman, 2005) and disease susceptibility (Herkelman et al., 1991).

1.2.1.1.1 Method For Inactivation of Soybean Trypsin Inhibitor

Soybean TI is resistant to enzymatic degradation in the gastrointestinal tract (Astwood et al., 1996). However, TI is heat labile and about 90% of residual TI activity is routinely destroyed during processing steps in preparing soybean meal (SBM) by denaturing the native protein structure of TI (Rackis, 1972). A report by Van Eys et al. (2004) showed the average TI concentration in raw soybean to be around 45-60 mg/g CP and when to processed, reduces to 4-8 mg/g CP. However, over-heating can reduce protein digestibility in SBM, therefore optimal temperatures and heating times must be maintained. Other methods for TI deactivation include physical, chemical, and enzymatic treatments (Friedman and Brandon, 2001; Van Der Ven et al., 2005; Chen, 2015), with varying efficacy and limitations.

1.2.1.2 Phytate

Phytic acid [inositol hexaphosphate, myoinositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate)] is found in almost all plant materials (Graf, 1983) and when ingested by animals can form indigestible complexes with dietary nutrients in the gut (Selle et al., 2000; Kies et al., 2001). Although, nonruminant animals can readily degrade phytate with endogenous phytases and phosphatases (Birge and Avioli, 1981; Maenz and Classen, 1998), the efficacy is limited by the poor phytate solubility in the small intestine (Schlemmer et al., 2001). Amino acids such as Gly, Thr, Ser, and Pro and minerals such as Ca, Zn, Fe, Mg, and Na are the nutrients most detrimentally affected by the ingestion of phytate (Cowieson and Ravindran, 2007; Peter et al., 2009; Schlegel et al., 2009). Phytate ingestion also results in increased excretion of crude mucin and endogenous AA from the gastrointestinal tract of chickens (Ravindran et al., 1999; Selle et al., 2000; Cowieson et al., 2004; Onyango et al., 2009).

1.2.1.3 Fiber

Many feed ingredients such as rye, wheat and wheat middlings, barley, and distillers dried grains and solubles (DDGS) have high proportion of dietary fiber (DF) comprising of non-starch polysaccharides (NSP) which are indigestible by nonruminants (Thebaudin et al., 1997). The antinutritive properties of fiber include the encapsulation of nutrients within the plant cell walls. Increased DF intake may also result in increased digesta viscosity which depresses nutrient digestibility and utilization in the animal (Choct and Annison, 1992; Bederska-Łojewska et al., 2017). Broiler chickens lack the appropriate endogenous enzymes required to breakdown these fiber fractions, and the intake level and nature of the DF have marked implications on endogenous AA economy (Montagne et al., 2004; Kluth and Rodehutscord, 2009). It is expedient to note, however, that DF is not totally undesirable and that several types of fibers may possess intestinal immune modulatory benefits by its prebiotic action on the proliferation of beneficial gut microbiota (Mateos et al., 2012; Sánchez et al., 2017).

1.2.2 Age

Nutrient utilization is also age-of-bird dependent. Previous reports in boiler chickens reveal a rapid growth rate of the digestive intestinal sections compared to the entire body weight until 6 or 7 d of age (Nitsan et al., 1991; Obst and Diamond, 1992). This results in increased secretion of digestive enzymes (Nitsan et al., 1991), and villus height increases between 25 to 100% in all intestinal segments until d 10 (Uni et al., 1995). This suggests age-related differential capacity of birds to digest and utilize feed. Increasing age results in increased starch intake, and broiler chickens increase their starch digestion capacity by increasing the secretion of pancreatic amylase (Krogdahl and Sell, 1989). However, Noy and Sklan (1995) compared birds at d 14 and d 42 and observed a leveling off of pancreatic amylase production as starch intake continues to rise, suggesting a limitation to the bird's endogenous capacity. This phenomenon may be ascribed to the increasing disproportionality in digestive capacity relative to the metabolic needs of birds as they grow older (Croom et al. 1999). Therefore, there is strong evidence that changes in the level of maturity and absorptive capacity of the bird's GIT with advancing age will result in differences in nutrient utilization of the birds.

1.2.3 Diet Form and Particle Size

The physical form of feed and particle size has substantial impact on feed intake and nutrient utilization by broiler chickens (Dozier et al., 2010). It is widely reported that broilers fed pelleted feed showed improved performance and nutrient utilization compared to mash diets (McKinney and Teeter, 2004; Chewning et al., 2012). This is attributed to decreased feed wastage, increased nutrient density, and improved palatability. On the other hand, diets with smaller particle size may result in higher digestibility indices and is attributed to greater interaction of the feed material with digestive enzymes in the gut (Preston et al., 2000). However, large particle size may also have beneficial effect on gizzard functions, which would have marked implications on overall nutrient utilization metrics (Choct, 2009; Svihus, 2011).

1.3 Historical Perspective for Enzyme Supplementation in Broiler Chickens

The first report of enzyme use in animal feed was in 1925 (Hervey, 1925). In this pioneering work, female leghorns were fed diet containing a 'fungal enzymatic material' added at 50 g/kg for a 20-week period, which caused a 22% increase in final BW of the birds. However, because the diet comprised of mainly cereal and its by-products, the improvement in BW may have been due to the separate nutrients provided by the supplemental enzyme like limiting AA, vitamins, and minerals rather than an 'enzyme' activity *per se*. Later in the 1950s, scientists added amylases and proteases to animal diets and observed additional benefits in productivity (Adeola and Cowieson, 2011). Since then, there has been tremendous growth in the use of exogenous enzyme in the animal industry, and in 2015, the global animal feed enzyme market was worth more than $ 1.16 billion. Global adoption of exogenous enzymes provides numerous economic and environmental benefits to animal agriculture (Zakaria et al., 2010). However, despite more than 50 years of research, the exact mode of action of most exogenous enzymes is not fully understood (except for phytases) and has produced poor correlation with expected responses. This is likely because commercial enzymes generally have differing properties such as thermostability, specific activity, and complementarity with the gut environment (Menezes-Blackburn and Greiner, 2014). This necessitates a thorough assessment of the structural properties of substrates for specific enzymes, which will be important in developing the next generation of enzymes.

1.3.1 Digestive Enzymes and Mode of Action

Enzymes help to breakdown large compounds, such as starch, protein and fiber into simpler monomers that can be absorbed for utilization by the animal for maintenance and production purposes. For enzymes to work, two conditions must be met. First is the need for a suitable match between the enzyme and its substrate, and secondly, enzymes need a proper environment to function in terms of acidity and alkalinity of the gut. The latter is critical because enzymes secreted in the stomach or upper GIT work best under low pH while those secreted in the small intestine or lower gut work best in higher pH. A wide range of endogenous enzymes are naturally secreted by the pancreas, stomach, and small intestine of all animals as part of the process of feed nutrient digestion. However, the production level of endogenous enzymes may be insufficient for optimal nutrient digestion, especially as the animal grows older (Noy and Sklan, 1995; Croom et al., 1999). More importantly is the presence of ANF in feed which may not be entirely degraded or at all in the gut. This provides an opportunity for exogenous enzyme addition to either augment endogenous hydrolytic capacity or provide novel degradative function. Exogenous enzymes are also natural proteins, produced by controlled microbial fermentation, and like endogenous secretions, they also require a suitable substrate and intestinal pH to exert their full effect.

1.3.1.1 Protease

The principal function of protease is to breakdown storage proteins in plant materials into small peptides and AA needed by animals. Endogenous proteases are chiefly produced by the pancreas in an inactive form or zymogens (Kraut, 1977). These zymogens are secreted into the duodenum, where trypsinogen is activated into the active trypsin by the serine protease, enteropeptidase. This activation begins an activation cascade in which trypsin activates all other proteolytic enzymes (Williams, 2004). Exogenous proteases are routinely added in poultry diets either as stand-alone additive (Castanon and Marquardt, 1989; Guenter et al., 1995) or as enzyme mixtures containing other enzymes such as phytase and carbohydrase (Cowieson and Adeola, 2005; Cowieson and Ravindran, 2008). However, the effectiveness of supplemental protease *in vivo* is highly variable and depend on the source and inherent characteristics or declared activity of the protease. Previous studies (Ghazi et al., 1997; Rooke et al.,1998) showed that an acid fungal rather than an alkaline bacterial protease was more effective in enhancing growth performance in both

poultry and swine. High variations observed in exogenous protease efficacy could also be due to issues relating to cooperativity with endogenous proteases or substrate interactions (Blazek, 2008). Nevertheless, several previous reports suggest an overall benefit of exogenous protease application in poultry nutrition (Mahagna et al., 1995; Odetallah et al., 2003; Cowieson and Roos, 2014).

1.3.1.1.1 Effects on AA Digestibility

A direct benefit of exogenous protease supplementation is improvements in apparent ileal AA digestibility, which has been consistent in most of recent literature. Results from a meta-analysis (Cowieson and Roos, 2014), showed a mean improvement of +3.7% in AA digestibility, which ranged from +2.7% for Glu to +5.6% for Thr (Fig. 1 and 2). An interesting observation is that the efficacy of exogenous protease may depend on the digestibility of AA in the control diet. Generally, the efficacy of protease to improve AA digestibility increased (about 10%) when the digestibility of AA in the control diet was less than 70%. On the other hand, when AA digestibility in the control diet was more than 90%, protease effect was approximately 2% (Cowieson and Roos (2014). This brings to fore the relevance of the "starting material", which may account for a significant portion in the variability observed in the evaluation of exogenous protease value.

1.3.1.1.2 Extra-Proteinaceous Impact of Protease

Improvement in production metrics by protease supplementation (Simbaya et al., 1996; Ghazi et al., 2002; Odetallah et al., 2003; Cowieson and Roos, 2014) may not be totally unrelated to improvement in AA digestibility. Angel et al. (2010) showed a dose-dependent increase in BW gain in broiler chickens with dietary protease addition, similar to another report by Freitas et al. (2011). Furthermore, the use of supplemental protease indirectly reduces N excretion, which has direct implications on environmental health and climate. By cleaving storage proteins from starch-protein complexes, exogenous proteases can release bound starch for absorption by the animal (Yu et al., 2007). Protease addition to a corn-SBM-based has been reported to increase ileal digestible energy (3,077 to 3,154 kcal/kg) and apparent metabolizable energy (3,130 to 3,261 kcal/kg) in broiler chickens (Cowieson et al., 2015). Additionally, exogenous proteases may directly cleave proteinaceous ANF such as TI which improves dietary protein utilization (Huo et al., 1993; Marsman et al., 1997; Cowieson et al., 2015). However, the degradative capacity of ANF has been

attributed more to an acid-protease and not an alkaline-protease (Thorpe and Beal, 2001). Other non-proteinaceous effects of supplemental proteases include improvements in enteric resilience, immune competence, litter quality and carcass yield (Mynott et al., 1991; Peek et al., 2009; Zuo et al., 2015, Cowieson et al., 2015).

1.3.1.2 Carbohydrase

Carbohydrase is a collective name for a group of enzymes including xylanase, cellulase, glucanase, α-galactosidase, α-amylase, pectinase and β-mannanase. The use of carbohydrase has received increased interests because of increasing use of fibrous feed stuff such as wheat, barley, sunflower meal, or canola meal in animal diets. However, these feed ingredients contain large concentrations of overlapping layers of different structural carbohydrates called NSP that includes beta-glucans, pentosans, oligosaccharides, cellulose, and lignin. For digestive enzymes access to trapped nutrients, these layers must be unfolded (Petry and Patience, 2020). However, birds lack the appropriate endogenous enzymes needed to digest the beta linkages that make up the NSP resulting in inefficient use of these feedstuff and affects nutrient utilization and growth performance. Soluble NSP increases the digesta viscosity in the gut, which affects nutrient utilization by limiting digestive enzyme access to substrates. Therefore, certain carbohydrases like xylanase, β-mannanase and glucanase are exogenously supplemented as feed additives to specifically target NSP hydrolysis. This reduces the digesta viscosity by decreasing the polymerization of feed and liberating the carbohydrate oligomers (Kalmendal and Tauson, 2012; Guo et al., 2014). Other carbohydrases act by hydrolyzing insoluble NSP contained in the cell wall into low molecular weight oligomers or convert them into soluble forms (Pettersson and Aman, 1989). Generally, carbohydrases improve nutrient utilization by decreasing digesta viscosity or disrupting the nutrient caging tendency of NSP.

1.3.1.2.1 Amylase

Starch is the main energy storage in plants and non-ruminant animals like pigs and poultry generally secrete sufficient pancreatic amylase to cater for digestion of dietary starch. The kinetics of starch digestion has been related to intestinal efficiency or the proportion of total amylose in feedstuff (Moran, 1982; Weurding et al., 2001). However, several factors not intrinsic to starch

itself may affect the level of starch utilization (Weurding et al., 2001). Some portions of dietary starch can escape enzymatic digestion in the gut (called resistant starch) and may only be fermented in the hindgut by microbes (Tiwari et al., 2019). However, fermentation in the distal gut is a much less efficient usage than breaking it down in the more proximal regions of the gut. These factors have led to increased interests in exogenous amylase supplementation for poultry. Particularly in young birds, in which exogenous amylases may augment the function of endogenous amylase that may have limited production by the maturation of the gastrointestinal tract (Croom et al., 1999). However, the efficacy of exogenous amylases in non-ruminant nutrition have been inconsistent and is largely attributed to the relatively high innate starch digestion capacity of the animals. Therefore, some studies have suggested that a better applicability of amylase supplementation may be as part of a carbohydrase mix containing xylanases or glucanases, which results in improved animal performance (Kocher et al., 2003; Olukosi and Adeola, 2008).

1.3.1.3 Phytase

The storage form of P in plant is phytate (Graf, 1983) which is not readily usable by non-ruminant animals. When ingested, phytate can form indigestible complexes with proteins and other nutrients in the gut (Selle et al., 2000; Kies et al., 2001). A previous solution to insufficient P availability from phytate intake is the supplementation of diet with inorganic P to satisfy nutritional requirements of the birds. A more recent approach is exogenous phytase use, which helps to dephosphorylate the insoluble phytic acid into inositol phosphate esters and orthophosphate. Although some endogenous phytase activity is present in the gut of most non-ruminant animals (Maenz and Classen, 1998), it is limited by substrate solubility at different luminal pH (Schlemmer et al., 2001). There are two broad categories of phytases: 3- and 6-phytase, depending on phosphate cleaving action at the 3-carbon atom (3-phytase) or 6-carbon atom (6-phytase) of the inositol ring (Dvoráková, 1998). Alternatively, phytases can be categorized as either acidic, neutral, or alkaline. This depends on the optimum pH of the phytase product (Greiner and Konietzny, 2011). However, most microbial phytases used in monogastric nutrition are acidic with specificity in the upper section of the GIT such as the crop, proventriculus, and gizzard where the pH is between 2-3. Phytate hydrolysis in most instances is incomplete and usually results in a mixture of inositol-phosphate (IP) esters, while complete phytate hydrolysis to myoinsitol and orthophosphate is rare *in vivo*.

Several authors have reported on the efficiency of exogenous microbial phytase to improve animal performance and phytate P utilization (Simons et al., 1990; Adeola et al., 2006; Augspurger et al., 2006). In addition, extraphosphoric benefits of phytase supplementation have been widely reported. This includes improvement in AA availability because of alterations in protein structure, solubility, and digestibility (Selle et al., 2003; Dilger et al., 2004; Onyango et al., 2005). From an environmental viewpoint, an additional benefit of microbial phytase is in reducing phosphate excretion from animal waste, enabling compliance of the animal industry with environmental regulations. However, the effect of microbial phytase on P release in non-ruminant animals depends on several factors. These include Ca-P relationship in the diet and gut, length of feeding, age, and species of animal (Menezes-Blackburn and Greiner, 2014; Babatunde et al., 2019).

1.4 Starch Structure and Classification

Starch comprises of two discrete populations: amylopectin and amylose. Amylopectin consists of glucose chains linked with α-1–4 bonds with frequent α-1–6 branching whereas amylose consists of only α-1–4 glucose chains (Hizukuri et al., 1997). On average, α-1–6 branching in amylopectin occur in every 20 to 25 glucose molecules. The molecular weight of amylose and amylopectin is approximately 100 kDa and 106 kDa, respectively (Buléon et al., 1998). Starches in feed grains can be classified based on the proportion of amylose (Ring et al., 1988); as waxy (< 15% amylose), normal (20 to 35% amylose) or high (> 40% amylose). The native starch granule is a very complex organized amylose-amylopectin structure with multiple concentric semi crystalline and amorphous shell (Gallant et al., 1992; Tester et al., 2006). Using X-ray diffraction technique, starch can be further classified into A-type, B-type, or C-type, based on variances in amylopectin chain lengths, compactness, and crystal lattices of the granule (Oates, 1997; Tester et al., 2004). Starches with the A-pattern are found in cereals, whereas tubers and legumes exhibit the B- and C-pattern, respectively (Gallant et al. 1997). In addition, other non-starch components that are associated with the starch granule such as proteins, lipids, and minerals in the feed grains may affect the physical state and amylolysis of starch in animals (Cornell et al., 1994; Baldwin, 2001).

1.4.1 Starch Digestion in Broiler Chickens

A major proportion of poultry diet is cereals, such as corn and wheat, which serve as the primary source of energy for the birds. Therefore, poultry diets may contain up to 500 g/kg of starch on DM basis. However, rate and extent of digestion and absorption of dietary starches vary between different feed grains which is attributable to differences in the physico-chemical properties of the starch granule. Additionally, the organization of the granule or crystallinity (Björck et al., 2000; Zhang et al., 2006), influences the rate of starch digestibility. Generally, digestion of the starch granule starts at the surface pores, which creates internal pockets that allows the amylase to digest the granule from within (Zhang et al., 2006). Chickens have no salivary amylase; therefore, starch digestion is mainly by pancreatic amylase, which is an endo-acting α-1,4–glucan hydrolase. In the intestinal lumen, amylose is hydrolyzed to maltose and maltotriose or maltose, maltotriose and α-limit dextrins when amylopectin is the substrate. These di- and oligosaccharides are further hydrolyzed to glucose by the brush border enzymes before absorption by the enterocytes (Hasjim et al., 2010; Shirazi-Beechey et al., 2011) and transport to the portal vein (Knudsen et al., 2006).

1.4.2 Factors Affecting Starch Digestibility

Although several previous experiments report high (> 90%) starch digestive capacity for broiler chickens (Svihus, 2001; Hetland et al., 2003; Zelenka and Ceresnakova, 2005), values below 90% starch digestion are not uncommon (Wiseman et al., 2000; Maisonnier et al., 2001; Weurding et al., 2001; Zimonia and Svihus, 2009). The main reason for the variations in starch digestibility is largely unknown. However, it may be due to several intrinsic factors relating to the cereal type, such as surface area and structure of the starch granule, the degree of crystallinity, or interactions with other dietary components like fiber and protein or animal-related factors. Some of these factors are discussed in the following section.

1.4.2.1 Granule Morphology and Architecture

Variations in the morphology of starch granule affect the rate and extent of starch digestion. Expectedly, and attributable to increased surface area for enzyme adsorption, several studies (Zhang et al., 2006; Kasemwong et al., 2008; Naguleswaran et al., 2012) suggests that smaller

starch granules (such as in rice or oats) are more efficiently digested when compared to larger granules (such as in corn or wheat) (Bednar et al., 2001; Capriles et al., 2008). Furthermore, enzymatic hydrolysis of the native starch granule can be affected by its shape and surface characteristics. High amylose starch is less digestible due to the presence of extensive hydrogen bonding in its numerous glucose chains, compared to amylopectin which is more susceptible to enzymatic hydrolysis due to numerous branching and a larger surface area (Brewer et al., 2012). Starch granule surface can either be smooth as in tubers and legume starches or contain several pores like corn starch (Fannon et al., 1992, 1993). The surface pores enable easy access of enzymes to cause the "inside out" digestion of the granule, whereas smooth granules are largely impermeable to digestive enzymes which results in an "outside in" approach (Fig. 3). The digestion potential of starch granule is also related to the level of crystallinity within the granule, which is defined mainly by the branch structure of the amylopectin (Witt et al., 2012). Generally, the A type- crystal structure of cereal starch is highly digestible due to the open structure, compared to B-type pattern found in tubers that are closely packed and resistant to enzymatic hydrolysis (Blazek and Gilbert, 2010).

1.4.2.2 Protein-Starch Matrices

In the endosperm, starch granules typically exist in close interaction with protein bodies (Berg et al., 2012). This contiguous layer of protein around individual starch granule limits access to amylase and slows down digestion of starch. This is due to the extensive disulphide linkages of the storage proteins forming a close knit with the starch granule (Zhang and Hamaker, 1998; Dona et al., 2010). Choi et al. (2008) observed an increase in starch digestibility following removal of proteins in sorghum by proteolytic enzymes or reducing agents. Similarly, the mechanical disruption of both protein networks and cell wall integrity during grain milling has been reported greatly improve starch digestibility in sorghum and barley. Although it is not clear if this observation was simply due to a decreased particle size which increased the surface area for enzyme adsorption rather than a disruption in the protein matrix *per se*. Interactions of starch with other feed components such as beta-glucans, arabinoxylans or ANF can potentially influence starch digestion in livestock, for instance, by increasing the digesta viscosity (Vasanthan and Bhatty, 1996).

1.4.2.3 Bird-Related Factors

Regardless of the complex processes described above, the extent of starch digestion is also dependent on innate bird characteristics. Studies show that newly hatched chicks rapidly adapt to starch digestion as indicated by increased disaccharidase (Mahagna and Nir, 1996) and α-amylase activities in the gut (Lehrner and Malacinski, 1975; Sklan and Noy, 2000). However, as birds grow older, starch digestibility decreases linearly. This is especially true for fast-growing genotypes like the broiler chickens (Mahagna and Nir, 1996; Sklan and Noy, 2000). Compared to wild jungle fowl, Kadhim et al. (2011) observed that the duodenal and jejunal amylase activitites were thrice as much. Osman (1982) concluded that the proximal gut was the main site of starch digestion in the chicken based on higher amylase activity in the jejunum compared to the ileum. Therefore, starch that escape digestion in the proximal gut, or resistant starch, is less utilizable by the birds. Starch digestibility in chickens is also limited by the relatively short residence time of feed in gut which limit adequate interaction with the amylase. Additionally, starch digestibility is increased when birds are fed mash compared to pelleted diet (Svihus and Hetland, 2001), which indicate inverse correlation of feed intake level with starch digestibility.

1.5 Appetite Regulation in Broiler Chickens

For all animal species, the act of feeding is a basic and critical process necessary for survival, growth, and reproduction. Therefore, appetite, or the desire to eat is strongly regulated and controlled by complex mechanisms. Several external factors control appetite and include feed availability and dietary nutrient composition, temperature and humidity and internal signals like immune status, hormones, and nutrient levels in blood and tissues (Richards and Proszkowiec-Weglarz, 2007). In poultry species, as in mammals, regulation of feed intake is by unique signaling pathways between central nervous system (CNS) and peripheral tissues (Denbow, 1994; Kuenzel, 1994; Kuenzel et al., 1999; Richards, 2003). These pathways comprise of an interconnected network of orexigenic and anorexigenic signals that regulate appetite in animals (Kalra and Kalra, 1996; Kalra, 1997). The hypothalamus is the central processor of appetite and expresses genes that encodes for specific neuropeptides and their respective receptors (Blevins et al., 2002; Berthoud, 2002). Peripheral signals originate from the gastrointestinal tract, liver, adipose tissue, and pancreas, and integrates with regulatory centers in the CNS (e.g., via vagal afferent nerves) to

cause changes in appetite. However, peripheral signals, unlike those originating in the brain, are short-lived and are not believed to cause sustained impact on animal feeding response. Additionally, the impact of specific dietary nutrients and biomolecules on appetite have been reported. For instance, free fatty acids cause an anorexigenic effect in animals and is observed to be mediated through K_{ATP} channels (Obici et al., 2002).

1.5.1 Regulatory Sites

The CNS and GIT are the main appetite regulatory sites in animals (McMinn et al., 2000; Blevins et al., 2002). Specific neural centers and network in the brainstem and hypothalamus relays signaling molecules such as neuropeptides, hormones, and nutrients from the peripheral tissues and vice-versa (Woods et al., 1998). The intricate coordination between these two regions ultimately dictates feed intake and energy status of the animal.

1.5.1.1 Hypothalamic Regulation

Within the hypothalamus are several interconnecting nuclei. These includes arcuate nucleus (ARC), lateral hypothalamic area (LHA), paraventricular nucleus (PVN), dorsomedial nucleus (DMN) and ventromedial nucleus (VMN) (Yu, et al., 2012). Of these nuclei, the ARC is the most sensitive to peripheral metabolic signals and comprise of two distinct neuronal populations: the orexigenic Neuropeptide Y (NPY) and Agouti-related peptide (AgRP) or anorexigenic Cocaine- and amphetamine-regulated transcript (CART) and proopiomelanocortin (POMC). Appetite is controlled via the energy sensing regulation of adenosine monophosphate-activated protein kinase (AMPK) (Dridi, 2017). Central administration of NPY in chickens shows that it is a potent orexigenic agent (Kuenzel and McMurtry, 1988). On the other hand, secretion of α-melanocortin-stimulating hormone (α-MSH) from POMC neurons strongly inhibits feed intake in chickens (Kawakami et al., 2000). Like NPY, AGRP stimulate appetite through direct inhibition of anorexigenic neurons or by intercepting α-MSH secretion and inactivating the melanocortin receptor (MC4-R) (Dridi, 2017).

1.5.1.2 Peripheral Regulation

In addition to digestive and absorptive functions, the GIT plays a role in short-term appetite regulation. Orexigenic signals is achieved through hormones such as ghrelin while CCK and bombesin inhibit feed intake (Jensen, 2001; Blevins et al., 2002). However, several studies have shown an inhibitory effect of ghrelin on feed intake in chickens (Furuse et al., 2001; Saito et al., 2002), contrary to observations in mammals ((Wren et al., 2000). The CCK is secreted in the duodenum by I-type enteroendocrine cells (Kuenzel, 1994; Jensen, 2001), and transfers satiety signals through the vagus nerve terminal to the hypothalamus (Horvath, 1992). One mode of action of CCK is by interaction with the melanocortin system to slow down intestinal motility (Denbow, 1999). Furthermore, the adipokine leptin may also have direct or indirect effect on the melanocortin system to regulate feed intake in chickens (Dridi et al., 2005). Other appetite regulating hormones originating from the GIT include pancreatic polypeptide (PP), peptide tyrosine-tyrosine (PYY), Oxyntomodulin (OXM) and glucagon-like peptide 1 (GLP-1).

1.5.2 Macronutrient Regulation

Specific dietary components such as AA, minerals, fatty acids, and carbohydrates can also influence feeding response (Anderson, 1979; White et al., 2003; McConn et al., 2018). In mammals, high protein diets induce satiety (Bensaid et al., 2003) while low protein diets increase appetite and feed intake (Anderson 1979). The induced satiety due to intake of high protein diets is related to changes in palatability or protein to energy ratio of the diet or both (Bensaid et al., 2003). In a separate study, rats fed a low- protein diet had increased feed consumption compared to control-fed animals (White et al., 2003) and increased intake of simple sugars promotes hyperphagia and obesity in animals (Sclafani, 1987). Furthermore, there was decreased feed intake in rats fed a high-fat diet (White et al., 2003). This was related to a downregulation of the orexigenic NPY gene in the hypothalamus (Giraudo et al., 1994). On the contrary, chickens fed a high fat (Nelson et al., 2015) or high protein diet (Wang et al., 2015) had increased feed intake. It is typical for animals to alter their feed intake level to satisfy appetite for specific nutrient deficiency, as in the case of Ca for broiler chickens (Wilkinson et al., 2013). However, several studies (Adeola, 2010; Rousseau et al., 2016; Imari et al., 2020) show suppressed feeding in broiler

chickens fed diets with deficient P concentration. The underlying molecular mechanisms responsible for this observation are unknown (Bensaid et al., 2003) and warrants investigation.

1.6 Objective

The overall aim of the studies summarized in this dissertation was to determine the impact of exogenous administration of protease and amylase on growth performance and nutrient utilization in broiler chickens. Additionally, the nutritional impact of varying soybean TI intake in broiler chickens, with or without exogenous protease supplementation, was investigated. Furthermore, the effect of alpha-amylase supplementation on starch digestibility in broiler chickens at different age group, and in distinct intestinal sites was investigated. A final objective was to examine transcriptional regulation of appetite in broiler chickens in response to dietary P deficiency.

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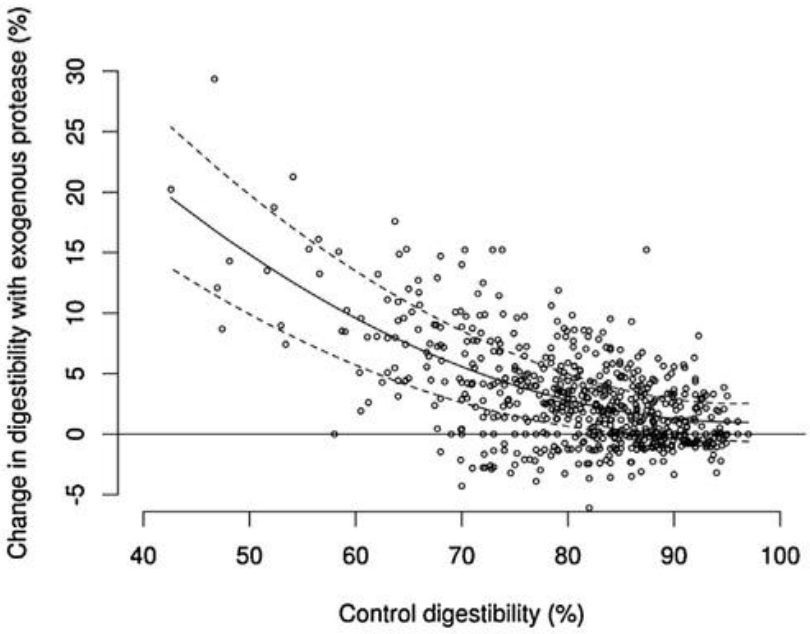

Figure 1.1 Correlation between control diet AA digestibility (%) and exogenous protease effect (% change relative to control diet). The solid quadratic line represents the best fit model while dotted lines represent the 95% confidence intervals. (Figure referenced from Cowieson and Roos, 2014).

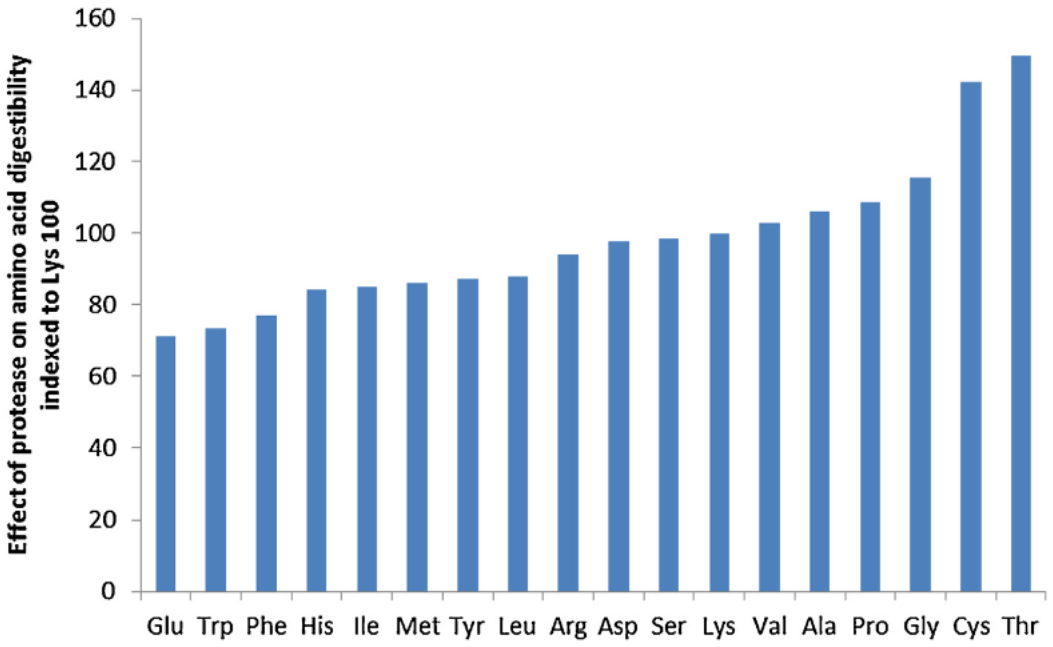

Figure 1.2 The effect of exogenous protease on ileal amino acid digestibility in pigs and poultry (Figure referenced from Cowieson and Roos, 2016).

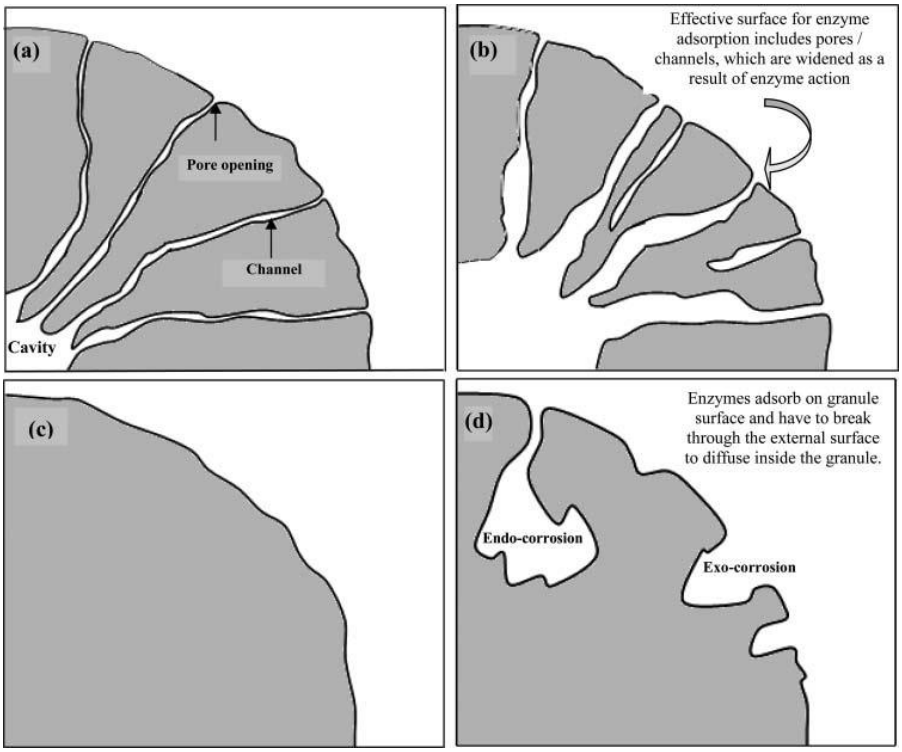

Figure 1.3 A model showing the catalytic patterns of amylase in corn and potato starches (Adapted from Dhital et al., 2010): (a) Corn starch showing pore channels, and cavity, (b) Inside-out amylase adsorption of corn starch due to amylase action on pore channels and cavity (c) Smooth potato starch surface lacking pores, channels, and cavity, (d) Outside-in endo- and exo-corrosion of potato starch by amylase action.

CHAPTER 2. GROWTH PERFORMANCE AND AMINO ACID DIGESTIBILITY RESPONSES OF BROILER CHICKENS FED DIETS CONTAINING PURIFIED SOYBEAN TRYPSIN INHIBITOR AND SUPPLEMENTED WITH A MONO-COMPONENT PROTEASE

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2.1 Abstract

Trypsin inhibitors (TI) present in soybeans affects protein utilization. While heat treatment influences residual TI, it simultaneously affects the structure and solubility of the soybean proteins and confounds any response to exogenous proteases. Using purified TI, the effect of exogenous protease to TI can be dissociated from changes in the soybean protein. Thus, the current study was designed to evaluate the growth performance and protein utilization responses of broiler chickens to purified TI and exogenous protease. Soybean meal (SBM) was pre-analyzed for basal TI (2,996 TIU/g SBM), formulated into nutritionally adequate experimental diets to contain 1,033 TIU/g diet, and purified TI was added at 9,000 TIU/g diet. A total of 320 Cobb-500 broiler chicks were allocated to 4 diets, each with 8 replicate cages and 10 birds per replicate. The experimental diets were arranged as a 2 × 2 factorial with factors being dietary TI (1,033 or 10,033 TIU/g) and exogenous protease (0 or 15,000 PROT/kg). On d 7, 14 and 21 post hatching, protease supplementation improved the BW gain ($P < 0.01$) and gain to feed ratio ($P < 0.05$) of birds. On d 14 and 21 post hatching, the relative weight of pancreas increased ($P < 0.05$) with added TI but was reduced ($P < 0.001$) with protease supplementation. Apparent ileal digestibility of all amino acids (**AA**), except methionine, decreased ($P < 0.001$) with added TI, but increased ($P < 0.05$) with protease supplementation. Jejunal MUC-2 was downregulated ($P < 0.01$) and SLC7A-2 was upregulated ($P < 0.05$) by protease supplementation. Duodenal trypsin and chymotrypsin activities reduced ($P < 0.05$) with added TI but increased ($P < 0.01$) with protease supplementation. Exogenous protease produced longer villi ($P < 0.05$) and deeper crypts ($P < 0.01$) in the jejunal tissue. In conclusion, dietary addition of purified TI negatively affects nutrient utilization by

broiler chickens. Furthermore, the study showed that the efficacy of the exogenous protease might be independent of dietary TI concentration.

Key words: broiler, gene expression, protease, soybean meal, trypsin inhibitor.

2.2 Introduction

Although soybean meal (SBM) is an excellent protein feed ingredient for non-ruminants, it contains several anti-nutritional factors (ANF), which may contribute to variance in its nutritional value. Among these are the protease inhibitors, specifically of the Bowman-Birk and Kunitz types. Both inhibitors have considerable anti-nutritive effects which impedes the activation of gastrointestinal proteolytic enzymes, thereby affecting dietary protein digestion. In soybeans, the Kunitz-type inhibitor is bigger (> 20 kDA), found in much larger concentrations and acts by forming stable stoichiometric complexes with the digestive enzyme trypsin and chymotrypsin (Liener, 1994). The resulting non-covalent complex renders the proteases inactive and significantly reduces the digestibility and utilization of proteins and amino acids (AA) by non-ruminants (Rawlings et al., 2004; Erdaw et al., 2017). Although the inhibitors are heat labile and can be deactivated by heat treatment, excessive processing can negatively influence the nutritional quality of SBM, necessitating care in the management of processing conditions (Newkirk, 2010).

Supplementing poultry diets with exogenous proteases may be a complementary strategy to improve the digestibility of SBM for non-ruminant animals (Ghazi et al., 2002, 2003; Clarke and Wiseman, 2005; Costa et al., 2008; Erdaw et al 2017)). One potential mode of action of microbial protease is by outcompeting the trypsin inhibitor (TI) for active sites, thereby improving the overall protein and AA utilization. Alternatively, the microbial protease could also destroy or inactive the TI. Huo et al. (1993) found that fungal and bacterial protease enzymes could inactivate TI in raw soybean and low-temperature extruded soybean *in vivo*. Rooke et al. (1998) incubated soybean meal using 0.1% acid protease for 3 h at 50℃ and pH 4.5 and reported fewer antigenic proteins compared to the non-protease treatments. In another study, dietary addition of mono-component protease reduced the SBM-specific antibodies in serum of broiler chicks fed a corn-SBM-based diet (Ghazi et al., 2002)

Previous work to explore the potential for microbial protease to reduce the antinutritional effect of TI in SBM have been conducted using variably heat-treated SBM. Thus, the effect of the exogenous protease on utilization of the soybean protein may be confounded by the level and or

method of processing. Hence, by the dietary addition of a purified source of soybean TI, it becomes possible to clearly delineate any direct effects of the exogenous protease on growth performance and nutrient utilization of the birds.

To our knowledge, there has not been any previous reports in literature that assess the efficacy of exogenous protease on nutrient utilization of birds fed diets containing purified TI. Therefore, the hypothesis of the current study was that purified soybean TI and exogenous protease will not affect the nutrient utilization and performance of broiler chickens. To test this hypothesis, specific objectives were set out to: 1) determine the effect of dietary addition of purified soybean TI on growth performance, nutrient utilization, intestinal morphology, and enzyme secretion of broiler chickens from d 0 to 21 post hatching; 2) evaluate the effect of exogenous protease administration in diets containing purified TI on growth performance, nutrient utilization, intestinal morphology, and enzyme secretion of broiler chickens from d 0 to 21 post hatching.

2.3 Materials and Methods

Protocols of animal experiments were reviewed and approved by the Purdue University Animal Care and Use Committee (#: 1112000389).

2.3.1 Diets and Experimental Birds

A batch of SBM obtained from a local supplier was set aside, sub-sampled and subsequently analyzed (Eurofins Scientific, IA) for quality indices (Table 2.1). Given the basal concentration of TI in the SBM, basal diets were supplemented with 0 or 9,000 TIU/g of a commercially available purified form of soybean TI (Sigma Aldrich, EC 2329069). This produced either a low TI diet or a high TI diet, which is 10 times as concentrated in dietary TI as the low TI diet.

A total of 320 male 0-d-old broiler chicks (Cobb 500, Siloam Springs, AR) were purchased from a commercial hatchery. Birds were individually tagged, weighed, and raised in heated battery brooders (model SB 4 T; Alternative Design Manufacturing, Siloam Springs, AR) with temperature and lighting maintained as previously described by Park et al. (2017). Using a 2 × 2 factorial arrangement, with 2 concentrations of dietary TI (1,033 or 10,033 TIU/g) and exogenous protease (0 or 15,000 PROT/kg; Ronozyme ProAct, DSM Nutritional Products, Kaiseraugst,

Switzerland) the chicks were allotted to 4 experimental diets (Table 2) in a randomized complete block design, each diet with 8 replicates cages and 10 birds per replicate cage. All diets were corn-SBM based, formulated to meet breeder nutrient specifications, and fed as mash. All diets were formulated to be nutritionally equivalent in terms of energy, protein, calcium, phosphorus, and dietary electrolyte balance. All diets contained phytase (RONOZYME HiPhos, DSM Nutritional Products, Switzerland) at 1,000 FYT/kg and titanium dioxide was included as an indigestible marker.

2.3.2 Sampling Procedures

For growth performance assessment, feed and water was available *ad libitum* during the 21-d experimental period. Body weights and feed intake was recorded weekly on d 7, 14, and 21 post hatching, and mortality records was taken daily. Gain to feed ratio was calculated and corrected for the body weight of any bird that died or was culled during the experimental period. On d 7 and 14 post hatching, two birds (heaviest and lightest) from each cage were selected and euthanized by CO_2 asphyxiation. The pancreas, liver and duodenal loop were excised for respective weight and length measurements. On d 21 post hatching, the six birds remaining in each cage were euthanized by CO_2 asphyxiation and exsanguination. Ileal digesta was collected from the distal two-thirds of the ileum (i.e. from the Meckel's diverticulum to approximately 2 cm cranial to the ileocecal junction), by flushing with distilled water into plastic containers and stored at –20°C prior to nutrient analyses.

2.3.3 Intestinal Morphological Analysis

On d 21 post hatching, mid-jejunal segments were collected from 1 bird per replicate with median BW, flushed with ice-cold 10% phosphate-buffered saline (VWR International, Radnor, PA) and fixed in 10% neutral buffered formalin (VWR International, Radnor, PA) for approximately 30 d. Samples were subsequently dehydrated with ethanol (VWR International, Radnor, PA), cleared with Sub-X® (Polysciences, Inc., Warrington, PA) and placed in paraffin (Polyfin paraffin, Sigma Polysciences, St. Louis, MO). The segments (5 μm) were stained with hematoxylin and eosin at the Purdue Histology and Phenotyping Laboratory (Purdue University, West Lafayette, IN USA). Villus height and crypt depth were measured from 4 complete, vertically

oriented villi per slide and villus height to crypt depth ratio was calculated. Villus length is defined as the length from the villus tip to the valley between each villus while crypt depth is defined as the length between the crypt opening and base. All measurements were performed under a binocular light microscope (National Optical and Scientific Instruments, Inc., Schertz, TX).

2.3.4 Digestive Enzyme Assay

Duodenal digesta and the pancreas was collected from 1 bird per replicate with median BW d 21 post hatching, frozen in liquid nitrogen and stored at –80°C until required for assay. Enzyme activities were determined using a commercially available assay kit (Sigma Chemical Co, St. Louis, MO). The absorbance of the colorimetric final product was measured in a UV/visible spectrophotometer, and the concentration of the respective enzymes was calculated accordingly. For duodenal digesta, the samples were centrifuged at 13,000 rpm at 4°C for 10 min, and aliquots of the supernatant was used for enzyme assay. The activity of the pancreatic enzymes was determined after the whole organ was homogenized in appropriate buffers and centrifuged at 13,000 rpm at 4°C for 10 min, to get a clear supernatant. Trypsin activity (EC 3.4.21.4) was determined with benzoyl-DL-arginine-p-nitroanalide as substrate (Sigma Aldrich, CN MAK290). The product of p-nitroaniline was measured at an absorbance of 405 nm. One activity unit of trypsin was expressed as nanomoles of p-nitroaniline released per minute per milligram of protein. Chymotrypsin activity (EC 3.4.4.5) was determined with N-Benzoyl-L-tyrosine ethyl ester as the enzyme substrate and absorbance was measured at 405 nm. Amylase activity (EC 3.2.1.1) was determined using a coupled enzyme assay and absorbance of ethylidene-pNP-G7 cleaved by the amylase was measured at 405 nm. One unit is the amount of amylase that cleaves ethylidene-pNP-G7 to generate 1.0 μmol of *p*-nitrophenol per minute at 25 °C. Lipase activity (EC 3.1.1.3) was determined at an absorbance of 570 nm using a coupled enzyme reaction. One unit of lipase is the amount of enzyme that will generate 1.0 μmol of glycerol from triglycerides per minute at 37°C.

2.3.5 Total RNA Extraction and Reverse Transcription

On d 21 post hatching, a section of the jejunum from 1 bird per replicate with median BW was removed and flushed with ice-cold PBS (VWR International, Radnor, PA), cut longitudinally in half exposing the lumen, and mucosal contents were scraped with a metal spatula. Mucosal

contents were immediately placed in 2 mL of Trizol reagent (Invitrogen, Grand Island, NY) and stored at -80°C until RNA isolation. Total RNA was extracted from the tissues using Trizol reagent (Invitrogen) following the manufacturer's protocol. RNA concentrations were determined by NanoDrop 1000 (Thermo Scientific), and RNA integrity was verified by 1% agarose gel electrophoresis. To prevent the contamination of the DNA, extracted RNA was purified with DNA-*free* DNase Treatment and Removal Kit (Ambion). Afterwards, 2 mg of total RNA from each sample were reverse transcribed into cDNA using the MMLV reverse transcription system (Promega). The cDNA was then diluted 1:10 with nuclease-free water (Ambion) and stored at -20°C until use.

2.3.5.1 Quantitative Real-time PCR Analysis

Real-time PCR was performed with Bio-Rad iCycler with the Faststart SYBR green-based mix (Life Technologies). PCR programs for all genes were designed as follows: 10 min at 95°C; 40 cycles of 95°C for 30 s, primer-specific annealing temperature for 30s, and 72°C for 30s; followed by melting curve analysis. The primer sequences used in the current study are listed in Table 2.3. Primer specificity and efficiency were verified, subsequently the samples were analyzed in duplicate, and a difference lesser than or equal to 5% was acceptable. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with normalization against glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as the housekeeping gene (Tan et al., 2014).

2.3.6 Chemical Analyses

Ileal digesta samples were freeze-dried for 96 h and subsequently ground to pass through a 0.5-mm screen (Retsch ZM 100, GmbH, Haan, Germany). For DM analysis, diets and ileal digesta samples were analyzed by drying overnight at 105°C (Precision Scientific Co., Chicago, IL; method 934.01; AOAC, 2006) and the nitrogen content of the samples was subsequently determined by combustion using a LECO FP-428 nitrogen analyzer (LECO Corp., St. Joseph, MI) with EDTA as a calibration standard. Samples for AA analysis were prepared using a 24-h hydrolysis in 6 *N* hydrochloric acid at 110°C under an atmosphere of nitrogen. Samples were oxidized in performic acid prior to acid hydrolysis for methionine and cysteine analyses. Samples

for tryptophan analysis were hydrolyzed using barium hydroxide. Amino acids in hydrolysates were determined by cation-exchange chromatography coupled with post-column derivatization (AOAC, 2000; method 982.30 E [a, b, c]). Titanium concentration was measured on a UV spectrophotometer following the method of Short et al. (1996).

The index method was used to calculate the apparent ileal digestibility (AID) of N and AA, according to the following equation:

$$AID, \% = 100- [(Ti_I/Ti_O) \times (P_O/P_I) \times 100]$$

where Ti_I is Titanium concentration in diets; Ti_O is Titanium concentration in output (ileal digesta); P_I is N, or AA concentration in diets; and P_O is nitrogen, or AA output in ileal digesta.

2.3.7 Statistical Analyses

Data were analyzed as a randomized complete block design using the GLM procedures of SAS (SAS Inst. Inc., Cary, NC). Initial body weight was used as the blocking criterion. The main effects of dietary TI and protease concentrations and the interaction were tested accordingly and an α level of 0.05 was considered significant. Where interactions exist, Tukey's mean separation test was used to make pairwise comparisons.

2.4 Results

The analyzed nutrient composition and quality characteristics of the soybean meal are presented in Table 2.1 and the ingredient composition and nutrient provision of the experimental diets are presented in Table 2.2. The effect of exogenous protease and dietary TI concentration on growth performance of the birds are presented in Table 2.4. There were no interactions between protease and TI on growth performance. Supplementation of the diets with exogenous protease resulted in an increase in BW gain ($P < 0.01$) and gain to feed ratio ($P < 0.05$) in all experimental phases. Increased dietary TI resulted in a reduction in BW gain ($P < 0.05$) in all experimental phases but had no effect on the gain to feed ratio. There was no effect of any of the experimental treatments on feed intake.

Table 2.5 shows the effect of exogenous protease and TI supplementation of diets on pancreas weight, liver weight and length of the duodenal loop. On an absolute basis, addition of TI to diets reduced ($P < 0.01$) the liver weight of birds only on d 7 post hatching. Notably, exogenous protease supplementation reduced ($P < 0.01$) the relative weight of the liver on d 21 post hatching. On the other hand, absolute pancreas weight on d 14 and 21 increased ($P < 0.01$) in response to dietary TI increase. Relative to BW, the increase in dietary TI led to 8 and 29% increases in pancreas weight on d 14 ($P < 0.05$) and d 21 ($P < 0.01$) post hatching, respectively. Addition of exogenous protease to the diet resulted in a reduction ($P < 0.01$) in both absolute and relative pancreas weight on d 21, to a greater extent in the diet that contained supplemental TI compared with the diet with low TI, resulting in a protease by TI interaction ($P < 0.01$).

Added dietary TI reduced ($P < 0.01$) the AID of all AA (Table 6). There were no interactions between dietary TI concentration and exogenous protease on AA digestibility. Protease supplementation increased the AID of N ($P < 0.01$) and all AA ($P < 0.05$), with the exception of Met. The increase in Cys digestibility by exogenous protease supplementation was only marginal ($P = 0.057$). Overall, the magnitude of the relative protease effect was numerically greater in the low TI diet for most of the AA than the high TI diet (Figure 2.1). For example, the protease effect on Lys digestibility was +1.6% in the low TI diet and +1.2% in the high TI diet.

The effects of exogenous protease and dietary TI concentration on histology of jejunal tissue and mRNA expression of markers of inflammation, integrity of the intestinal wall, and nutrient transport in the mucosa of the chicken jejunum are presented in Table 7. There were no interactions between dietary TI concentration and exogenous protease. The expression of interleukin (IL)-1β, -8, -10 and neutral amino acid transporter-1 (ASCT-1) were not affected by dietary TI or exogenous protease supplementation. However, increased dietary TI downregulated ($P < 0.05$) the expression of cationic amino acid transporter-2 (**SLC7A-2)**. Protease supplementation downregulated ($P < 0.01$) the expression of mucin-2 (MUC-2) and upregulated ($P < 0.05$) the expression of SLC7A-2 in the jejunum. Exogenous protease increased the villus height ($P < 0.05$) and crypt depth ($P < 0.01$) in the jejunal tissue of birds.

Table 8 shows the effect of exogenous protease and dietary TI concentration on digestive enzyme activities. In the pancreas, there were no effects (or interactions) of exogenous protease and dietary TI concentration on digestive enzyme activities. Despite this, there was a reduction in trypsin activity ($P < 0.05$) in the duodenal digesta in response to increased dietary TI. However,

duodenal trypsin activity increased ($P < 0.01$) with exogenous protease supplementation. Similarly, chymotrypsin activity was reduced ($P < 0.01$) by increased dietary TI concentration but increased ($P < 0.01$) with exogenous protease supplementation.

2.5 Discussion

There is a substantial body of work on the nutritional implications of TI in broiler chickens. To the authors' knowledge, the data obtained from the current study are the first to test the efficacy of a mono-component protease on a purified source of trypsin inhibitor in broiler chickens. This approach becomes valuable for optimization and indeed, validation of the protease enzyme as a useful additive for improvements in the nutrient utilization of broiler chickens.

In the current study, the effect of increased dietary TI on the final BW of birds diminished with age, although there were improvements due to protease supplementation. This was evident with marked improvements in BW gain and feed efficiency over the entire feeding period, and are similar to previous reports (Wang et al., 2008; Barekatain et al., 2013). However, there have also been reports of a lack of positive effect of exogenous protease on BW gain, even though the feed conversion significantly improved (Ghazi et al., 2002; Freitas et al., 2011). The diminishing effect of increased dietary TI on live BW agrees with previous authors (Erdaw et al., 2017) at 24-d or 35-d post hatching. However, Ruiz and De Belalcázar (2005) previously reported that the impact of dietary TI may simply be intake related and as age approaches 21-d and older, excess TI intake could cause moderate to severe rapid feed passage syndrome and a consequent drop in overall growth performance of the birds. In the current study, there were no differences in feed intake between birds fed the low or high TI diets at 21-d post hatching which could open up an avenue for exogenous protease in complementing the functions of the endogenous proteases (Noy and Sklan, 1995).

Addition of purified TI to the diets increased both absolute and relative weight of the pancreas. This was especially true for birds at d 14 and 21 post hatching and is indicative of pancreatic hypertrophy or hyperplasia (Embaby, 2010). Due to an intrinsic negative feedback control, enzyme secretion by the pancreas is inversely related to the enzyme activity in the gut. The ingested TI acts by forming an irreversible complex with trypsin in the small intestine, which limits the concentration and function of trypsin and leads to overproduction of digestive enzymes and thus, enlargement of the pancreas (Cabrera-Orozco et al., 2013). It is surprising that increased

dietary TI had no effect on the relative pancreas weight at d 7 post hatching, even though the growth performance data suggests that birds were more susceptible to TI at this age. One possible explanation is that although dietary TI was high, low feed intake in the first week of the bird's life could limit total TI intake, which might not be enough to elicit major morphological changes in the pancreas. This is corroborated by a previous report by Clarke and Wiseman (2007) who showed a strong dose-dependent response of relative pancreas weight to dietary TI intake. However, Erdaw et al (2017) reported that the pancreas of younger birds was more sensitive to dietary TI concentrations, but it is pertinent to state that responses in that study was for 10-d-old birds. Interestingly, the magnitude of change in the relative weight of the pancreas to dietary TI tends to increase with age and this might be partly due to increased dietary intake of TI.

These results contradict findings by Erdaw et al (2017), that the relative weight of the chicken pancreas fed extruded full fat SBM compared with the control, decreased with age. This suggests that purified TI exerts a more critical effect on the pancreas than the native TI in full fat SBM. In the current trial, birds were fed diets containing high quality SBM with highly digestible AA and this might increase the negative effects of the added purified TI. On the other hand, previous experiments that fed raw SBM, full-fat SBM or under-processed SBM used SBM with inherently low AA digestibility with a lot of residual TI. Therefore, it is possible that the TI as an antinutrient, tends to exert different influences depending on the inherent quality of the diet that it is associated with. Although both lectin and TI contained in raw or full fat soybean may have complementary effects on pancreatic function (Grant, 1989), TI seems a more relevant antinutritional factor in young broilers (Douglas et al., 1999). Although lectin can increase pancreas weight (Liener, 1994) by stimulating the accumulation of polyamines (Pusztai et al., 1995), Fasina et al. (2004) reported no trophic changes in pancreas weights of turkey poults fed diets containing increasing concentrations of purified soybean lectin. Nevertheless, the current study showed that the exogenous protease was effective in ameliorating the negative effect of increased dietary TI on pancreas weight.

In the current study, increased dietary TI as a result of the addition of purified TI decreased the apparent ileal digestibility (AID) of nitrogen (N) and all of the reported amino acids (AA). This was at least a 4% drop in AID of Thr and Trp and up to 8% decrease for Cys. This result is similar to reports by Schulze (1994), who observed a significant decrease in N digestibility, and increased ileal endogenous N flow when growing pigs were fed diets containing increasing

concentrations of purified TI. However, relative to the control, protease supplementation improved the overall N digestibility by 2.5%. This is similar to observations of Cowieson and Ravindran (2008) when a multienzyme complex, containing protease, was used. Angel et al. (2011) also reported an increased N digestibility with increasing dietary protease concentration. Bertechini et al. (2009) reported increased true AA digestibility for soybean meal and Carvalho et al. (2009) reported increased true AA digestibility for corn, in the presence of a mono-component protease. This indicates that variable quantities of N that could escape digestion and absorption was partially captured by the protease and made available to the birds. The relative effect of protease on control AA digestibility were consistent with those in the review of 25 independent experiments published by Cowieson and Roos (2014).

These improvements in growth performance responses can also be attributed to the observed improvements in the digestibility of AA. This is because birds that more readily reach their target intake of digestible amino acids have improved feed efficiency, compared to the corresponding controls. In the current study, we observed a lack of effect of exogenous protease on methionine digestibility, which is also consistent with previous reports (Ravindran et al., 2005; Angel et al., 2011; Cowieson and Roos, 2014). This may be related to the typically high AID of methionine (90-95%) in a corn-SBM based diet. However, there are some reports of significant improvements in AID of methionine when protease was supplemented at dietary concentrations of 400 mg/kg or higher (Angel et al., 2011). Although relatively low, but similar to a previous report (Cowieson, 2010), the AID of Cys in birds was improved by protease supplementation. These data on AA digestibility indicate a reliance by the exogenous protease on the inherent digestibility of dietary AA or 'starting material' (Cowieson and Roos, 2014). However, in diets with high TI concentration, the protease effect was not large enough to counteract the drastic drop in digestibility of AA in the control diet. This suggests that the exogenous protease may not directly act on the TI and any improvements in AA digestibility, attributable to the exogenous protease, is largely independent of dietary TI concentration. The reason for this observation is not clear but might be ascribed, in part, to a lack of an effective enzyme-substrate specificity, interference with other dietary components, or interference of endogenous proteolytic activity by the exogenous protease effect (Yuan et al., 2015).

Exogenous protease downregulated the expression of MUC-2 in the jejunum of birds fed diets with increased dietary TI concentration. This agrees with reports (Cowieson and Roos, 2014;

Cowieson et al., 2017) that noted a beneficial effect of exogenous protease on mucin secretion in the intestine of the chicken. This is usually associated with a reduction in mucin secretion or an increase in the autolytic recovery of mucin or both. It is possible that the protease acts by reducing the metabolic demand for mucoprotein by reducing the erosion of the mucosal layer by the incoming feed matrix. Hence, there is usually a marked reduction in the mucin layer thickness, goblet cells and endogenous protein losses. For example, birds fed diets supplemented with protease show significant reductions in the digesta sialic acid concentration (a component of mucin) and goblet cell numbers (Peek et al., 2009; Cowieson et al., 2017). It is also possible that a portion of the beneficial effect of protease on AA digestibility is conferred through a reduction in the loss of mucoprotein from the intestine, with possible implications for gut health.

In addition, mucin being a family of mucus glycoproteins have some essential AA in the core structure (e.g., Trp and Thr) and it is possible that an increase in the digestibility of these AA, conferred by the exogenous protease, would correlate with improvements in the integrity of mucus layer. Protease supplementation also upregulated (almost doubling) the expression of the Na-independent cationic AA transporter, SLC7A-2. Consistent with the reports of Cowieson et al. (2017), this suggests an effect of the exogenous protease in modulating protein absorption, which may also be due to the availability of more amino acids released in the gut. However, there was no protease effect on the expression of Na-dependent neutral AA transporter, ASCT-1 and the reason for this observation is not totally clear. The exogenous protease also increased the villus height and crypt depth in the jejunal tissue of the birds. This suggests a better absorptive capacity of dietary nutrients and may corroborate the observed improvements in growth performance and nutrient utilization responses previously noted. This observation is similar to reports by Wang et al. (2008) in broilers and Zuo et al (2015) in piglets.

In the current study, increased dietary TI and exogenous protease supplementation had no effect on pancreatic enzyme activities. Although, birds fed high TI diet showed signs of pancreatic hypertrophy, it is unclear why the pancreatic enzyme activity was not different from birds fed the low TI diet. This is in dissonance with reports by Erdaw et al (2017), who observed reduced activities of pancreatic trypsin and chymotrypsin of 24 d-old birds fed diets with increasing levels of raw soybean meal. However, in the duodenum, the activities of trypsin and chymotrypsin decreased as a result of increased dietary TI but increased with exogenous protease administration. The reduction in enzymatic activity might be because TI has inhibitory specificity to trypsin, but

will also affect the activity of chymotrypsin, which is activated by trypsin. On the other hand, the improvements in intestinal trypsin and chymotrypsin activity due to protease supplementation suggests a complementarity or additivity between the exogenous and endogenous proteases. This is because the enzyme activity in the small intestine is composed of both exogenous and endogenous components.

Given the foregoing, the current study shows that increased dietary trypsin inhibitor affected the growth performance and digestibility of amino acids, and that exogenous protease administration improves these responses in the birds. Furthermore, responses to protease administration seem more likely to have arisen from a general improvement in protein digestion. Pronounced pancreatic hypertrophy was identified in birds fed diets with added purified trypsin inhibitor, which was ameliorated by exogenous protease administration. The data from the current study suggest that the efficacy of dietary exogenous protease might be independent of dietary trypsin inhibitor concentration.

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Table 2.1 Analyzed values of nutrient composition and quality measuring parameters of soybean meal used in the study

Items	
Composition	
Dry matter, g/kg	866.3
Crude protein, g/kg (N × 6.25)	480.6
Ether extract, g/kg	6.1
Gross energy, kcal/kg	4,113
Quality measuring parameters	
Protein solubility in KOH, g/kg	781.8
Trypsin inhibitor, TIU/g	2,996
Urease activity, ΔpH	0.02
Amino acid, g/kg	
Arg	34.9
His	12.5
Ile	22.7
Leu	36.5
Lys	30.2
Met	6.2
Cys	6.6
Phe	24.2
Tyr	17.5
Thr	18.1
Trp	6.1
Val	23.4

Table 2.2 Ingredient and calculated nutrient composition of experimental diets, as-fed basis.

Purified trypsin inhibitor, TIU/g:	0		9,000	
Protease, PROT/kg:	0	15,000	0	15,000
Ingredients, g/kg				
Corn	562.6	552.6	512.6	502.6
Soybean meal	345.0	345.0	345.0	345.0
Soybean oil	18.0	18.0	18.0	18.0
Monocalcium phosphate[1]	11.0	11.0	11.0	11.0
Limestone[2]	13.5	13.5	13.5	13.5
Salt	2.8	2.8	2.8	2.8
Vitamin-mineral premix[3]	3.0	3.0	3.0	3.0
$_{DL}$-Methionine	2.1	2.1	2.1	2.1
$_{L}$-Lysine HCl	2.2	2.2	2.2	2.2
Threonine	1.5	1.5	1.5	1.5
Tryptophan	0.3	0.3	0.3	0.3
$NaHCO_3$	3.0	3.0	3.0	3.0
Trypsin inhibitor premix[4]	0.0	0.0	50.0	50.0
Ronozyme ProAct premix[5]	0.0	10.0	0.0	10.0
Ronozyme HiPhos premix[6]	10.0	10.0	10.0	10.0
Titanium dioxide premix[7]	25.0	25.0	25.0	25.0
Total	1,000	1,000	1,000	1,000
Calculated composition				
Crude protein, g/kg	222.34	222.34	222.34	222.34
ME, kcal/kg	3,000	3,000	3,000	3,000
Ca, g/kg	7.95	7.95	7.95	7.95
P, g/kg	6.11	6.11	6.11	6.11
Non-phytate P, g/kg	3.54	3.54	3.54	3.54
Ca:total P	1.30	1.30	1.30	1.30
Na, g/kg	2.34	2.34	2.34	2.34
K, g/kg	9.66	9.66	9.66	9.66
Cl, g/kg	3.72	3.72	3.72	3.72
Dietary electrolyte balance, mEq/kg	244.49	244.49	244.49	244.49
Digestible amino acids, g/kg				
Arg	13.23	13.23	13.23	13.23
His	5.20	5.20	5.20	5.20
Ile	8.34	8.34	8.34	8.34
Leu	17.38	17.38	17.38	17.38

Table 2.2 continued

Lys	12.24	12.24	12.24	12.24
Met	5.19	5.19	5.19	5.19
Cys	3.81	3.81	3.81	3.81
Phe	9.49	9.49	9.49	9.49
Tyr	7.36	7.36	7.36	7.36
Thr	8.61	8.61	8.61	8.61
Trp	2.89	2.89	2.89	2.89
Val	9.04	9.04	9.04	9.04
TSAA	9.00	9.00	9.00	9.00
Phe + Tyr	16.85	16.85	16.85	16.85
Analyzed composition				
Crude protein, g/kg	232.1	233.0	237.8	238.6
Crude fiber, g/kg	24.7	25.2	25.2	25.0
Ether extract, g/kg	36.5	35.7	36.2	34.1
Protease, PROT/kg[8]	LOD	16,760	LOD	17,010
Trypsin inhibitor, TIU/g	1,181	1,218	8,882	8,833

[1] 16% Ca, 21% P.

[2] 38% Ca.

[3] Supplied the following per kg diet: vitamin A, 5,484 IU; vitamin D3, 2,643 ICU; vitamin E, 11 IU; menadione sodium bisulfite,4.38 mg; riboflavin, 5.49 mg; pantothenic acid, 11 mg; niacin, 44.1 mg; choline chloride, 771 mg; vitamin B12, 13.2 ug; biotin, 55.2 ug; thiamine mononitrate, 2.2 mg; folic acid, 990 ug; pyridoxine hydrochloride, 3.3 mg; I, 1.11 mg; Mn, 66.06 mg; Cu, 4.44 mg; Fe, 44.1 mg; Zn, 44.1 mg; Se, 300 ug.

[4] Purified trypsin inhibitor (PTI) from soybeans product contains 9,000,000 TIU/g. 1 g of PTI added to 49 g of corn supplied 180,000 TIU/ g of premix. 50 g premix delivered 9,000,000 TIU/kg feed.

[5] Product contained 75,000 PROT/g. 1 g Protease added to 49 g ground corn supplied 1,500 PROT/ g premix. 10 g premix delivered 15,000 PROT/ kg feed.

[6] Phytase product contained 5000 units/g. 1 g of phytase added to 49 g of ground corn supplied 100 units/g of premix. 10 g premix delivered 1,000 units/kg feed. 1,000 units/kg supplied 1.5 g P/kg and 1.7 g of Ca/kg.

[7] 1 g of Titanium dioxide added to 4 g of corn.

[8] LOD = limit of detection

Table 2.3 Primers used in real –time quantitative PCR

Genes	Primer sequence (5'to 3')	Gene Bank ID	Reference
Housekeeping gene			
GAPDH	F: TCCTAGGATACACAGAGGACCA R: CGGTTGCTATATCCAAACTCA	ENSGALG00000014442*	Grenier et al., 2015
Markers of inflammation			
IL-1β	F: GCATCAAGGGCTACAAGCTC R: CAGGCGGTAGAAGATGAAGC	NM_204524	Adedokun et al., 2012
IL-8	F: GCGGCCCCCACTGCAAGAAT R: TCACAGTGGTGCATCAGAATTGAGC	ENSGALG00000011670*	Grenier et al., 2015
IL-10	F: GCTGAGGGTGAAGTTTGAGG R: AGACTGGCAGCCAAAGGTC	ENSGALG00000000892*	Grenier et al., 2015
Marker of gut integrity			
MUC-2	F: GCTACAGGATCTGCCTTTGC R: AATGGGCCCTCTGAGTTTTT	XM_421035	Adedokun et al., 2012
Markers of nutrient transport			
ASCT-1	F: TTGGCCGGGAAGGAGAAG R: AGACCATAGTTGCCTCATTGAATG	XM_001232899.4	Paris and Wong (2013)
SLC7A-2	F: TGCTCGCGTTCCCAAGA R: GGCCCACAGTTCACCAACAG	NM_001199102.1	Gilbert et al. (2007).

F, forward primer; R, reverse primer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; MUC2, mucin 2; ASCT-1, Neutral amino acid transporter-1; SLC7A-2, cationic amino acid transporter-2. *Sequence obtained from Ensembl chicken genome data resources.

Table 2.4 Growth performance of broiler chickens fed diets supplemented with protease (PROT/kg) and purified trypsin inhibitor (TIU/g), from d 0 to 21 post hatching

	TI											
	0		9,000									
	Protease		Protease			Protease		TI		*P*-value		
Item[1]	0	15,000	0	15,000	SEM	0	15,000	0	9,000	Protease	TI	P × TI
BW, kg												
d 0	36.3	36.3	36.3	36.3	0.01	36.3	36.3	36.3	36.3	0.238	0.728	0.728
d 7	141	148	132	145	2.29	137	146	144	138	< 0.001	0.017	0.159
d 14	415	432	394	429	6.36	405	430	423	411	0.001	0.074	0.172
d 21	899	943	870	936	11.63	884	940	921	903	< 0.001	0.144	0.356
d 0 to 7												
BW gain, g	105	111	96	108	2.29	100	110	108	102	< 0.001	0.017	0.159
Feed intake, g	134	137	132	132	2.35	133	134	136	132	0.634	0.126	0.594
G:F, g/kg	783	813	727	824	20.20	755	819	798	776	0.005	0.285	0.114
d 0 to 14												
BW gain, g	379	395	358	392	6.36	368	394	387	375	0.001	0.044	0.169
Feed intake, g	466	465	465	463	10.66	466	464	466	464	0.873	0.879	0.957
G:F, g/kg	813	851	773	854	23.74	793	853	832	813	0.019	0.429	0.379
d 0 to 21												
BW gain, g	862	906	834	876	15.32	848	891	884	855	0.011	0.046	0.946
Feed intake, g	967	983	970	971	10.29	969	977	975	971	0.452	0.692	0.431
G:F, g/kg	892	922	860	902	14.46	876	912	907	881	0.019	0.087	0.656
N	8	8	8	8		16	16	16	16			

[1] G:F, gain to feed ratio; P, protease; TI, trypsin inhibitor; SEM, standard error of the mean

Table 2.5 Organ response of broiler chickens at d 21, fed diets supplemented with protease (PROT/kg) and purified trypsin inhibitor (TIU/g)

	TI											
	0		9,000			Protease		TI		*P*-value		
	Protease		Protease									
Item[1]	0	15,000	0	15,000	SEM	0	15,000	0	9,000	Protease	TI	P × TI
Day 7, Absolute												
Pancreas, g	0.64	0.59	0.68	0.62	0.029	0.66	0.61	0.62	0.65	0.074	0.266	0.838
Liver, g	6.22	6.11	5.63	5.45	0.200	5.92	5.78	6.17	5.54	0.477	0.005	0.854
Duodenal loop, cm	14.37	14.31	14.29	14.99	0.389	14.33	14.65	14.34	14.64	0.415	0.448	0.338
Day 7, Relative												
Pancreas, g/kg BW	4.75	4.24	4.99	4.63	0.233	4.88	4.44	4.50	4.81	0.072	0.189	0.736
Liver, g/kg BW	45.39	42.36	41.62	41.35	1.872	43.50	41.85	43.87	41.48	0.388	0.216	0.469
Duodenal loop, cm/kg BW	110.1	103.50	109.1	114.9	3.89	109.6	109.2	106.8	112.0	0.919	0.195	0.127
Day 14, Absolute												
Pancreas, g	1.58^{b}	1.55^{b}	1.92^{a}	1.58^{b}	0.063	1.75	1.57	1.56	1.75	0.009	0.008	0.027
Liver, g	13.67^{b}	14.94^{ab}	15.13^{a}	13.70^{b}	0.455	14.40	14.32	14.30	14.42	0.861	0.799	0.007
Duodenal loop, cm	19.22^{b}	20.73^{a}	19.62^{ab}	19.09^{b}	0.454	19.42	19.91	19.98	19.36	0.289	0.188	0.036
Day 14, Relative												
Pancreas, g/kg BW	4.20	3.73	4.75	3.82	0.149	4.47	3.78	3.97	4.28	< 0.001	0.046	0.137
Liver, g/kg BW	36.67	35.80	37.38	33.59	1.583	37.03	34.70	36.23	35.49	0.156	0.642	0.367
Duodenal loop, cm/kg BW	52.44	50.64	49.59	46.68	1.796	51.02	48.66	51.54	48.14	0.204	0.072	0.759
Day 21, Absolute												
Pancreas, g	2.74^{b}	2.78^{b}	4.31^{a}	2.86^{b}	0.113	3.53	2.82	2.76	3.59	< 0.001	< 0.001	< 0.001
Liver, g	27.00	25.62	28.10	25.86	0.694	27.55	25.74	26.31	26.98	0.016	0.346	0.545
Duodenal loop, cm	21.56	21.31	21.57	21.41	0.451	21.56	21.36	21.43	21.49	0.662	0.902	0.924
Day 21, Relative												
Pancreas, g/kg BW	3.10^{b}	3.04^{b}	4.78^{a}	3.17^{b}	0.128	3.94	3.11	3.07	3.97	< 0.001	< 0.001	< 0.001
Liver, g/kg BW	30.44	27.96	31.06	28.46	0.781	30.75	28.21	29.20	29.76	0.004	0.485	0.942
Duodenal loop, cm/kg BW	24.37	23.49	23.93	23.91	0.699	24.15	23.70	23.93	23.92	0.530	0.990	0.521
N	8	8	8	8		16	16	16	16			

^{a,b} means within the same row with different superscripts are significantly different ($P < 0.05$) [1] P, protease; TI, trypsin inhibitor; SEM, standard error of the mean

Table 2.6 Effect of exogenous protease (PROT/kg) and purified trypsin inhibitor (TIU/g) concentration on apparent ileal digestibility (%) of nitrogen and amino acids in broiler chickens, at d 21 post hatching

	TI											
	0		9,000									
	Protease		Protease			Protease		TI		*P*-value		
Item[1]	0	15,000	0	15,000	SEM	0	15,000	0	9,000	Protease	TI	P × TI
Nitrogen	84.8	86.3	80.9	83.6	0.43	82.8	84.9	85.5	82.3	< 0.001	< 0.001	0.152
Indispensable AA												
Arg	89.6	90.7	87.5	88.1	0.38	88.5	89.4	90.1	87.8	0.034	< 0.001	0.541
His	86.6	87.9	83.8	84.7	0.43	85.2	86.3	87.2	84.3	0.019	< 0.001	0.624
Ile	84.5	86.0	81.0	82.0	0.47	82.8	84.0	85.2	81.5	0.017	< 0.001	0.573
Leu	85.9	87.2	82.7	84.0	0.42	84.3	85.6	86.5	83.4	0.006	< 0.001	0.904
Lys	87.7	89.1	85.2	86.2	0.44	86.5	87.7	88.4	85.7	0.012	< 0.001	0.706
Met	92.9	93.3	90.8	90.7	0.29	91.8	92.0	93.1	90.8	0.609	< 0.001	0.315
Phe	85.2	86.6	82.3	83.6	0.46	83.8	85.1	85.9	83.0	0.009	< 0.001	0.915
Thr	80.3	82.3	76.0	77.2	0.51	78.2	79.8	81.3	76.6	0.005	< 0.001	0.442
Trp	86.9	88.3	82.8	84.4	0.43	84.8	86.3	87.6	83.6	0.002	< 0.001	0.763
Val	82.8	84.4	78.8	80.2	0.48	80.8	82.3	83.6	79.5	0.004	< 0.001	0.819
Dispensable AA												
Ala	84.8	86.2	81.4	82.5	0.45	83.1	84.3	85.5	82.0	0.013	< 0.001	0.825
Asp	82.6	84.2	78.6	79.7	0.50	80.6	81.9	83.4	79.2	0.015	< 0.001	0.606
Cys	73.7	75.7	65.5	66.8	0.82	69.6	71.3	74.7	66.2	0.057	< 0.001	0.675
Glu	89.0	90.0	86.9	87.5	0.36	87.9	88.8	89.5	87.2	0.028	< 0.001	0.528
Gly	80.1	82.2	75.9	76.7	0.55	78.0	79.5	81.1	76.3	0.014	< 0.001	0.277
Pro	85.0	86.5	82.0	83.3	0.41	83.5	84.9	85.7	82.6	0.002	< 0.001	0.799
Ser	82.6	84.3	78.5	79.9	0.46	80.6	82.1	83.5	79.2	0.003	< 0.001	0.745
Tyr	84.8	86.3	80.9	83.6	0.43	82.8	84.9	85.5	82.3	< 0.001	< 0.001	0.152
N	8	8	8	8		16	16	16	16			

[1] AA, amino acid; P, protease; TI, trypsin inhibitor; SEM, standard error of the mean

Table 2.7 Relative gene expression† of cytokines, mucosa, amino acid transporter proteins in jejunal mucosa and histology of jejunal tissue of broiler chickens fed diets containing exogenous protease (PROT/kg) and purified trypsin inhibitor (TIU/g), at d 21 post hatching.

	TI											
	0		9,000									
	Protease		Protease			Protease		TI		*P*-value		
Item[1]	0	15,000	0	15,000	SEM	0	15,000	0	9,000	Protease	TI	P × TI
Genes												
IL-1β	1.18	0.96	0.96	0.95	0.246	1.07	0.95	1.07	0.95	0.638	0.651	0.683
IL-8	1.23	1.51	1.02	1.14	0.258	1.12	1.33	1.37	1.08	0.435	0.278	0.763
IL-10	1.14	0.95	0.92	0.69	0.164	1.03	0.82	1.05	0.81	0.217	0.154	0.913
MUC-2	1.31	1.02	1.25	0.57	0.130	1.28	0.79	1.16	0.91	0.001	0.062	0.155
ASCT-1	1.11	1.18	0.79	1.06	0.206	0.95	1.12	1.14	0.92	0.407	0.301	0.629
SLC7A-2	1.01	1.41	0.52	0.93	0.177	0.77	1.17	1.21	0.73	0.035	0.014	0.945
Histology												
Villus height, μm	1057.5	1273.8	849.4	1084.0	88.88	953.5	1178.9	1165.7	966.7	0.019	0.036	0.919
Crypt depth, μm	100.6	130.5	96.7	112.8	6.75	98.7	121.7	115.6	104.8	0.003	0.125	0.322
VH:CD ratio	10.7	9.8	8.8	9.7	0.78	9.8	9.7	10.2	9.3	0.968	0.216	0.244
N	8	8	8	8		16	16	16	16			

†Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated with GAPDH as the endogenous control

[1] P, protease; TI, trypsin inhibitor; SEM, standard error of the mean; IL, interleukin; MUC2, mucin 2; ASCT-1, neutral amino acid transporter-1; SLC7A-2, cationic amino acid transporter-2; VH, villus height; CD, crypt depth

Table 2.8 Effect of exogenous protease (PROT/kg) and purified trypsin inhibitor (TIU/g) concentration on enzyme activity in the duodenal digesta (units/mL) and Pancreas (units/mg), at d 21 post hatching

	TI										
	0		9,000								
	Protease		Protease			Protease		TI		*P*-value		
Item[1]	0	15,000	0	15,000	SEM	0	15,000	0	9,000	Protease	TI	P × TI
Duodenal digesta												
Trypsin	10.13	11.69	7.76	10.40	0.699	8.95	11.04	10.91	9.08	0.007	0.016	0.446
Chymotrypsin	5.49	6.77	3.79	5.41	0.123	4.64	6.09	6.13	4.60	< 0.001	< 0.001	0.178
Amylase	121.49	115.10	117.99	119.44	3.284	119.74	117.27	118.30	118.71	0.460	0.900	0.247
Lipase	1.30	1.32	1.41	1.35	0.047	1.35	1.33	1.31	1.38	0.682	0.140	0.393
Pancreas												
Trypsin	5.81	5.67	6.02	5.40	0.333	5.92	5.53	5.74	5.71	0.263	0.915	0.473
Chymotrypsin	4.28	4.31	4.12	4.21	0.076	4.20	4.26	4.29	4.16	0.433	0.102	0.708
Amylase	34.91	33.59	35.66	35.13	1.331	35.28	34.36	34.25	35.39	0.495	0.399	0.770
Lipase	0.95	0.98	1.07	1.00	0.045	1.01	0.99	0.97	1.03	0.670	0.143	0.325
N	8	8	8	8		16	16	16	16			

[1] P, protease; TI, trypsin inhibitor; SEM, standard error of the mean

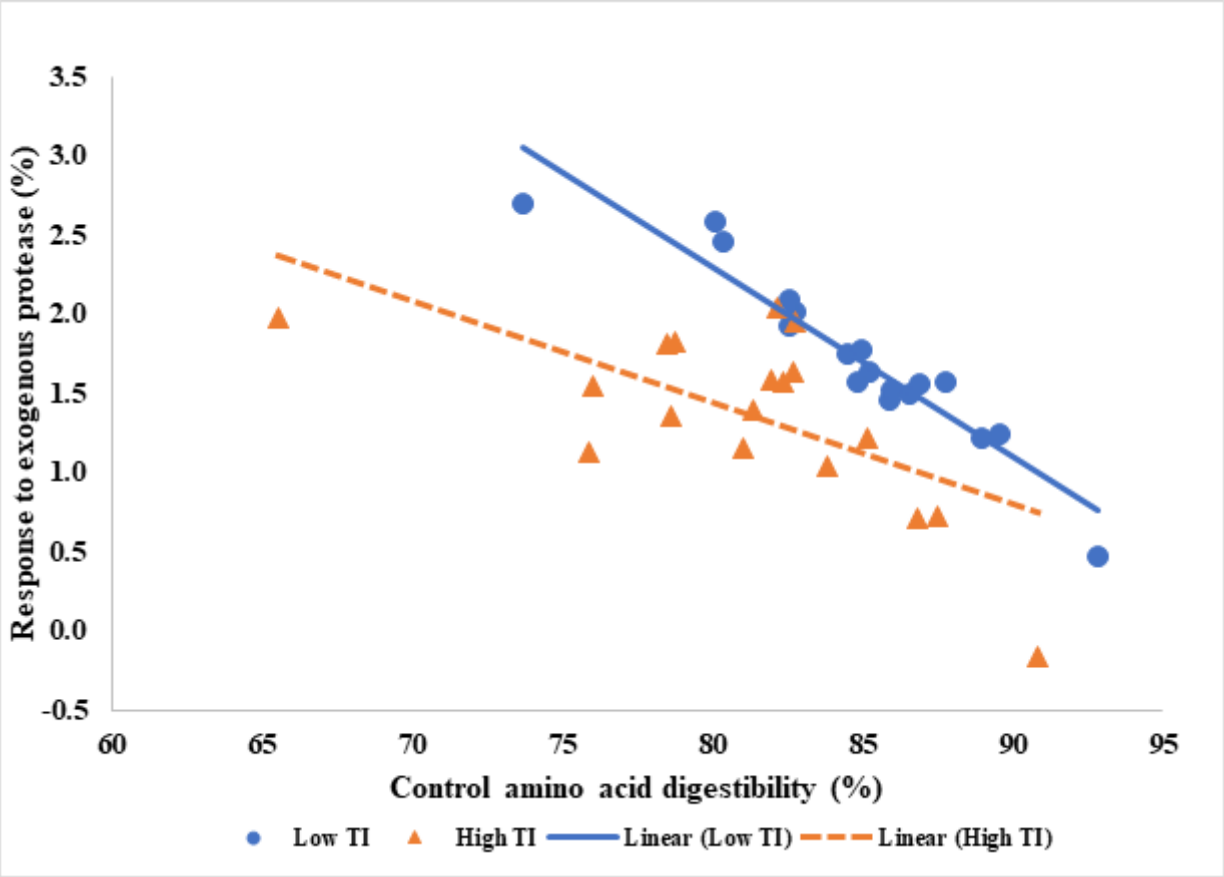

Figure 2.1 Correlation between inherent amino acid digestibility in the control diet (%) and response to exogenous protease (% change relative to the control diet without added protease). Solid blue circles represent data points from the Low TI diet group and solid orange triangles represent data points from the High TI diet group. Solid and dashed linear lines indicates the respective best fit model for the Low and High TI groups.

CHAPTER 3. CONTRIBUTION OF PURIFIED SOYBEAN TRYPSIN INHIBITOR AND EXOGENOUS PROTEASE TO ENDOGENOUS AMINO ACID LOSSES AND MINERAL DIGESTIBILITY

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3.1 Abstract

The primary objective of this experiment was to evaluate the impact of trypsin inhibitor (TI) and exogenous protease supplementation on endogenous amino acids (AA) loss in broiler chickens. A total of 384 Cobb-500 broiler chicks were allocated to 4 nitrogen-free diets, each with 8 replicate cages and 12 birds per replicate. The diets were arranged as a 2 × 2 factorial with factors being dietary TI (0 or 8,000 TIU/g) and exogenous protease (0 or 15,000 PROT/kg). Desired dietary TI concentration was achieved by addition of commercially available, purified soybean TI. There was no effect of TI, exogenous protease, or their interaction on growth performance of birds. However, endogenous loss of nitrogen (N) and all AA increased ($P < 0.05$) due to dietary TI concentration except for Cys. The increase in endogenous AA due to TI ranged from 17% for Thr to 52.2% for Trp. Exogenous protease had no effect on endogenous loss of N and all AA. There was no effect of TI, exogenous protease, or their interaction on AID of P, however AID of Ca, Fe, Mg, Mn, and Cu was reduced ($P < 0.05$) by dietary TI. The AID of Cu ($P < 0.01$) and K ($P < 0.05$) improved with exogenous protease supplementation. Significant interactions ($P < 0.05$) between exogenous protease and TI existed for Zn, Mg, Cu and Na. The concentration (g/kg DM intake) of crude mucin and sialic acid increased ($P < 0.05$) with increased dietary TI. Decreased trypsin ($P < 0.001$) and increased chymotrypsin ($P < 0.001$) activity in the pancreas were observed as a result of exogenous protease supplementation. In conclusion, our results indicate that TI increases endogenous AA loss and reduces mineral digestibility in broiler chickens. Furthermore, exogenous protease did not affect endogenous AA flow, irrespective of added purified dietary TI.

Key words: amino acid, broiler chicken, endogenous loss, mineral, protease, trypsin inhibitor

3.2 Introduction

During the digestive process in the gastrointestinal tract, endogenous proteins from digestive secretions (saliva, bile, gastric, and pancreatic secretions as well as intestinal secretions), mucoproteins, sloughed intestinal epithelial cells, serum albumin, and amides (Nyachoti et al., 1997; Ravindran and Bryden, 1999) are continuously secreted into the lumen of the intestine. A previous estimate (Nasset and Ju, 1961) reveals that endogenous protein secretion may be five times as abundant as those of dietary origin and, about 60 to 79% of this gross endogenous secretion may be reabsorbed (Krawielitzki et al, 1990; Souffrant et al., 1993). However, the degree of reabsorption will vary depending on the relative ratio of individual endogenous components and their point of entry into the gut (Souffrant et al., 1991). Regardless of the intestinal site, net endogenous losses are the result of total secretion, reabsorption, and reutilization of reabsorbed endogenous protein. The unabsorbed portion is inevitably lost to the animal and referred to as basal (or diet-independent) endogenous losses, representing the amino acids that are lost irrespective of whether the animal is fed protein.

The level of endogenous amino acid (EAA) flow may also depend on the presence of antinutritive factors in the gut (Sauer et al., 1977; Barth et al., 1993; Schulze, 1994). Schulze (1994) studied the effect of various inducing agents such as trypsin inhibitors (**TI**), lectins, and fiber, on EAA flow at the terminal ileum in pigs and found that endogenous nitrogen (**N**) secretions increased by 7.3, 5.2, and 0.04 g per g lectins, TI, and fiber respectively. For TI and fiber, there were linear increases in EAA flow with increasing amounts of the inducing agent (Schulze, 1994). While soybean meal **(SBM)** provides an excellent source of protein for non-ruminants, it contains variable amounts of TI depending on processing conditions (Newkirk, 2010). This may lead to increased TI intake which affects dietary protein digestion and amino acid utilization in non-ruminants by inhibiting the activation of the proteolytic enzymes in the gut (Rawlings et al., 2004; Erdaw et al., 2017). On the other hand, exogenous protease supplementation is a widely accepted strategy to improve dietary protein utilization in non-ruminants (Clarke and Wiseman, 2005; Erdaw et al 2017; Aderibigbe et al., 2020). Additionally, exogenous protease has been shown to improve EAA recovery in birds (Mateos et al., 2004). Likewise, an *in vitro* trial (Nielsen et al., 2013) showed that exogenous protease can directly degrade TI, with a degradation efficiency of up to 96%.

There are limited number of studies that report the direct impact of TI on EAA flow, and to the best of our knowledge, these have not been reported for broiler chickens. Therefore, the hypothesis of the current study was that TI and exogenous protease will affect the loss of endogenous protein in broiler chickens. To test this hypothesis, specific objectives were to: 1) determine the contribution of purified soybean TI and exogenous protease to EAA flow and mineral digestibility in broiler chickens fed nitrogen-free diet (NFD); 2) evaluate the effect of exogenous protease in diets containing purified soybean TI on EAA flow and mineral digestibility in broiler chickens fed NFD.

3.3 Materials and Methods

Protocols of animal experiments were reviewed and approved by the Purdue University Animal Care and Use Committee (#: 1112000389).

3.3.1 Diets and Experimental Birds

A total of 384 male Cobb 500 broiler chicks (Cobb-Vantress, Siloam Springs, AR) were procured from a commercial hatchery on the day of hatch. Birds were individually tagged, weighed, raised in heated battery brooders (model SB 4 T; Alternative Design Manufacturing, Siloam Springs, AR) and fed a nutritionally adequate corn-SBM based diet (210 g/kg CP; 3,100 kcal/kg ME) until d 20 post hatching. Temperature and lighting were maintained as previously described by Aderibigbe et al. (2020). Twelve hours prior to experimental diet allocation, birds were fasted to empty the gut of residual dietary N. Subsequently on d 21 post hatching, chicks were allotted to a 2 × 2 factorial arrangement, with 2 concentrations of dietary TI (0 or 8,000 TIU/g) (Sigma Aldrich, EC 2329069) and 2 concentrations of exogenous protease (0 or 15,000 PROT/kg; Ronozyme ProAct, DSM Nutritional Products, Kaiseraugst, Switzerland) (Table 3.1) in a randomized complete block design, with BW as a blocking factor, resulting in 8 blocks per treatment. Each diet comprised of 8 replicates cages and 12 birds per cage. Titanium dioxide was included in all diets as an indigestible marker.

3.3.2 Sampling Procedures

Birds were fed experimental diets on an *ad libitum* basis for a 3-d period, and mortality records was taken daily. On d 24 post hatching, feed intake was measured per cage and birds were individually weighed. Gain to feed ratio was calculated and corrected for the body weight of any bird that died or was culled during the experimental period. Subsequently, all birds were euthanized by CO_2 asphyxiation and exsanguination and eviscerated for sample collection. Ileal digesta was collected from the distal two-thirds of the ileum from the Meckel's diverticulum to approximately 2 cm cranial to the ileocecal junction. Ileal digesta samples were pooled within cage and stored at –20°C for subsequent nutrient analyses.

3.3.3 Digestive Enzyme, Crude Mucin, and Sialic Acid Assays

The pancreas was collected from 2 median bird per cage, frozen in liquid nitrogen and stored at –80°C until required for enzyme assay. Trypsin and Chymotrypsin activities were determined as previously described by Aderibigbe et al. (2020) using a commercially available assay kit (Sigma Chemical Co, St. Louis, MO). Crude mucin and sialic acid concentration were determined based on methods previously described by Horn et al. (2009). Briefly, excreta samples were freeze-dried for 96 h and subsequently ground to pass through a 0.5-mm screen (Retsch ZM 100, GmbH, Haan, Germany). Subsequently, 3 g of lyophilized excreta was placed in a 50-mL plastic centrifuge tube. Twenty milliliters of chilled NaCl solution (0.15 *M* NaCl, 0.02 NaN_3, kept at 4°C) was added to the excreta sample and homogenized for 30 s (T25 Basic, IKA Corp., Staufen, Germany). The mixture was then centrifuged at 12,000 × *g* for 20 min at 4°C, and the soluble supernatant was decanted into a 50-mL pre-weighed tube. For extraction of mucin proteins, 15 mL of chilled (4°C) absolute ethanol was added to the supernatant and allowed to sit overnight at −20°C. The mixture was then centrifuged at 1,400 × *g* for 10 min at 4°C and the mucin pellet was retained. The mucin pellet was washed with a mixture of 10 mL of chilled NaCl solution (0.15 *M* NaCl, 0.02 NaN_3, kept at 4°C) and 15 mL of chilled absolute ethanol and the sample was extracted overnight at −20°C. Subsequently, the mixture was then centrifuged at 1,400 × *g* for 10 min at 4°C and the wash-extraction cycle was repeated until a clear supernatant was obtained. Water was removed from the mucin pellet by suction and the pellet was then weighed to obtain crude mucin yield. The pellet was then dissolved in 2 mL of distilled water and immediately frozen at −40°C.

Sialic acid concentration was determined from the purified crude mucin samples. Briefly, 100 μL of crude mucin solution was diluted with 100 μL of distilled water in a 1.5-mL microcentrifuge tube and 200 μL of Bial reagent (Ward's Science, Rochester, NY) was added to the samples and heated for 15 min at 100°C in a water bath (Precision, GCA Corp., College Park, MD). The samples were cooled in room temperature water for 5 min. One ml of isoamyl alcohol (Sigma Chemical Co, St. Louis, MO) was added to the sample, vigorously vortexed, and chilled on ice for 5 min. The samples were then centrifuged at 1,200 × *g* for 1 min and 200 μL of the upper color phase was gently transferred to a 96-well plate. Absorbance was measured at 560 nm on a UV spectrophotometer (Spark 10M, TECAN, Baldwin Park, CA). Sialic acid concentration was determined from regression of the standard (*N*-acetylneuraminic acid; Sigma Chemical Co, St. Louis, MO) versus absorbance.

3.3.4 Chemical Analyses and Calculations

Ileal digesta were freeze-dried for 96 h and subsequently ground to pass through a 0.5-mm screen (Retsch ZM 100, GmbH, Haan, Germany). The dry matter (DM) content of diets and digesta were analyzed by drying overnight at 105°C (Precision Scientific Co., Chicago, IL; method 934.01; AOAC, 2006). Nitrogen content was determined by combustion using a LECO FP-428 nitrogen analyzer (LECO Corp., St. Joseph, MI) with EDTA as a calibration standard. Samples for amino acid (**AA**) analysis were prepared using a 24-h hydrolysis in 6 *N* hydrochloric acid at 110°C under an atmosphere of N. Samples were oxidized in performic acid prior to acid hydrolysis for methionine and cysteine analyses. Samples for tryptophan analysis were hydrolyzed using barium hydroxide. Amino acids in hydrolysates were determined by cation-exchange chromatography coupled with post-column derivatization (AOAC, 2000; method 982.30 E [a, b, c]). Following wet digestion using a mixture of nitric and perchloric acid, concentrations of Ca, Fe, Zn, Mg, Mn, Cu, Na, and K in diets and lyophilized ileal digesta samples was determined with appropriate standards using an atomic absorption spectrometer (AAnalyst 300; Perkin Elmer, Norwalk, CT; method 985.01; AOAC, 2006). Concentration of P was determined by spectrophotometry at 620 nm (Spectra Count, model AS1000, Packard, Meriden, CT; AOAC, 2006). Titanium concentration was measured on a UV spectrophotometer (Spark 10M, TECAN, Baldwin Park, CA) following the method of Short et al. (1996).

The index method was used to calculate endogenous flow of N and AA in ileal digesta, crude mucin and sialic acid in the excreta and apparent ileal digestibility (AID) of macro and trace minerals, according to the following equations:

$$\text{Endogenous loss, g/kg DM intake} = P_o \times (Ti_i/Ti_o);$$
$$\text{AID, \%} = 100 - [(Ti_i/Ti_o) \times (Q_o/Q_i) \times 100]$$

where Ti_i and Ti_o are the respective titanium concentrations (g/kg DM) in experimental diets and output (ileal digesta or excreta); P_o is the concentration of N, AA, crude mucin, or sialic acid (g/kg DM) in the ileal digesta or excreta; Q_i and Q_o are the respective concentrations (g/kg DM) of macro and trace minerals in experimental diets and excreta.

Ileal endogenous energy flow (IEEF) was calculated according to the following equations:

$$\text{IEEF, kcal/kg DM intake} = (F_{aa} \times G_{aa})/1000$$

Where F_{aa} is the endogenous flow of AA (g/kg DM intake) in the ileal digesta and G_{aa} is the gross energy of individual AA (kcal/kg) adapted from Boisen and Verstegen (2000).

3.3.5 Statistical Analyses

Data were analyzed as a randomized complete block design using the GLM procedures of SAS (SAS Inst. Inc., Cary, NC). Initial body weight was used as the blocking criterion. Block was treated as a random factor. The main effects of TI and exogenous protease and the interaction were tested accordingly and an α level of 0.05 was considered significant. Where interactions exist, Tukey's mean separation test was used to make pairwise comparisons.

3.4 Results

Overall, there were 11 mortalities during the 3-d trial period, and on-site postmortem examinations did not reveal any direct link to specific dietary treatment. Although the mortality was relatively high, the exact cause was unclear, and is more likely related to the non-physiological nature of the NFD. The effect of purified TI and exogenous protease on growth performance of

broiler chickens fed NFD are shown in Table 3.2. There were no effects of TI, exogenous protease, or their interaction on growth performance of birds. As shown in Table 3.3, there was no interaction between TI and exogenous protease on EAA loss in birds. Exogenous protease had no effect on endogenous loss of N and all AA. The calculated energetic losses associated with endogenous protein flows are presented in Table 3.4. Similar to AA, the ingestion of TI increased ($P < 0.05$) energy loss associated with EAA loss, except Cys and exogenous protease addition did not reduce energy loss in EAA. There were no effects of TI or exogenous protease or their interaction on AID of P (Table 3.5). However, the AID of Ca, Fe, Mg, Mn, and Cu were reduced ($P < 0.05$) by dietary TI. Exogenous protease improved AID of Cu ($P < 0.01$) and K ($P < 0.05$). Furthermore, significant interactions ($P < 0.05$) between exogenous protease and TI existed for Zn, Mg, Cu and Na. Added TI reduced ($P < 0.05$) the AID of Zn reduced by 43.7% in birds fed diets containing protease. Exogenous protease increased ($P < 0.05$) the AID of Mg irrespective of dietary TI concentration. The AID of Na increased ($P < 0.05$) with added TI, similar to the effect of exogenous protease. However, exogenous protease did not affect the AID of Na in diets containing added TI.

Dietary TI concentration significantly increased ($P < 0.05$) the excretion (g/kg DM intake) of crude mucin and sialic acid (Table 3.6). Pancreas weight was unaffected by TI or exogenous protease (Table 3.7). Exogenous protease resulted in a reduction in trypsin activity ($P < 0.001$) but increased pancreatic chymotrypsin activity ($P < 0.001$).

3.5 Discussion

The primary objective of this study was to evaluate the direct contribution of TI to EAA losses in broiler chickens, and whether this is affected by exogenous protease supplementation. The impact of soybean TI on growth performance and nutrient digestibility in broiler chickens is widely reported (Clarke and Wiseman,2005; Erdaw et al., 2017; Aderibigbe et al., 2020). Currently, there are no published data on the effect of TI on endogenous secretion in birds. Because NFD is a commonly used assay for estimating EAA flow in broiler nutrition, we also evaluated the digestibility of macro and trace minerals in an experimental NFD and whether or not this is affected by TI concentration. Our data showed that growth performance of birds was unaffected by dietary TI concentration or exogenous protease supplementation. This is not surprising because all the birds were fed NFD, which is not expected to support tissue accretion. Compared to conventional

diets, feeding NFD, irrespective of protease or TI addition, resulted in losses in BW and feed efficiency over the 3-d trial period. The BW losses observed for birds in the current trial may be attributed to increased body protein degradation because of the sustained dietary protein deficiency, which becomes critical for sustenance of biological functions under NFD feeding. As previously noted, the primary purpose of the current study was not to evaluate the growth performance of birds, as the sensitivity of the performance variables may be affected by the protein deficiency. An important observation, however, is the lack of effect of TI on feed intake response of birds. This is especially crucial because variances in feed intake will influence endogenous nutrient losses between dietary treatments (Boisen and Moughan, 1996, Adedokun et al., 2011) and potentially confounding any TI effect.

Endogenous AA secretion in the current study falls within the range of previously published data typical of the NFD assay (Ravindran, 2021) and was strongly affected by added dietary TI. This is similar to a previous report by Barth et al. (1993) who observed a 7.8-fold increase in endogenous protein secretion when pigs were fed casein meal containing 3,000 mg of purified TI per test meal. This observation suggests that the influence of TI on metabolic amino acid economy may be quantitatively more affected by a loss of endogenous protein. However, it is difficult to ascertain the specific contribution of the sources of secreted endogenous protein, because digesta collected at the terminal ileum represents the net result of the digestive dynamics of endogenous sources along the entire digestive tract (Hee et al., 1988; Nyachoti et al., 1997; Ravindran and Bryden, 1999). Indeed, Souffrant et al. (1993) estimated that about 79% of the gross endogenous secretion is reabsorbed, but the degree of reabsorption will vary depending on the relative ratio of individual endogenous components and their point of entry into the gut. For instance, when digestive enzymes dominate the endogenous flow, the proteins pass through the duodenum and jejunum where there is greater opportunity for digestion and absorption. However, opportunity for digestion is lower and relatively higher endogenous losses will be inevitable at the ileal level if mucus secretion or desquamation is significant, which particularly occurs more distal to the duodenum.

In the current study, and similar to previous observations (Chung and Baker, 1992; Adedokun et al., 2007), four AA (Glu, Asp, Ser and Thr) were predominantly identified in endogenous AA, which could represent up to 34% of total AA flow in the broiler fed an NFD (Adedokun et al., 2007). In the current study, the average increase in EAA loss attributable to TI

was 32.3% and was more apparent in Trp (52.2%), Arg (45.9%) and Lys (42.4%). Generally, the impact of TI on EAA secretion were higher for indispensable AA, which may have great nutritional consequences in birds. Interestingly, pancreatic enzyme secretion or pancreas weight were not affected by TI, indicative of no TI-induced hypertrophy. This is partly expected due to a lack of dietary protein needed for constant stimulation of the pancreas through the inhibitory actions of TI on trypsin. This also suggests that the pancreatic juice may not be a significant source for TI-induced EAA flow, however, this inference only applies to NFD conditions. Notwithstanding, digestive enzymes are degraded during the digestion process, absorbed into blood, reaccumulated by the pancreas, and reutilized (Rothman et al., 2002). Indeed, it is argued that a large fraction of the intact digestive enzymes secreted by the pancreas are absorbed and recycled in an entero-pancreatic circulation, instead of being reduced to their constituent amino acids in the intestines (Rothman et al., 2002). Perhaps TI disrupts the digestive enzyme recycling process that may result in a surge in EAA flow, however this needs to be investigated.

Supplemental protease had no impact on the reduction or recovery of EAA secretion irrespective of dietary TI concentration, and contrary to a previous report (Huo et al., 1993) did not appear to be involved in inactivation or degradation of the TI. However, *in vitro* trials such as the report by Huo et al. (1993) may not be easily replicated *in vivo*. Perhaps fluctuations in the *in vivo* environment, especially in birds fed a protein-free non-physiological diet like the NFD, interferes with the exogenous protease ability to recognize, and degrade the TI. However, these postulations have to be experimentally verified. Even more importantly, most commercially available proteases are chymotrypsin-like semi-alkaline endopeptidases (Glitso et al., 2012) and compared with an acid protease, may have limited capacity to degrade TI and other antigenic proteins (Hessing et al., 1996; Rooke et al., 1998). Although alkaline, the protease used in the current study may likely hydrolyze TI, but this reaction would more likely occur further down the gut (pH optima of Ronozyme ProAct is > 6; Cowieson and Roos, 2016). However, this might be too late to prevent TI-induced EAA flow, especially if most of the secretions originated from the upper gastrointestinal tract (e.g., HCl, pepsin). Asides from lack of substrate with the use of NFD, the exogenous protease action may be limited by the low total EAA flow compared with a conventional diet. Furthermore, using data from Boisen and Verstegen (2000), which reported that the gross energy (**GE**) of amino acids ranges from 2,890 kcal/kg for Asp to 6, 740 kcal/kg for Phe, we calculated the GE of the lost proteins based on their amino acid composition and gross flow.

Similar to phytic acid (Cowieson et al., 2008), endogenous protein loss from the terminal ileum of broilers as a result of TI ingestion had considerable energetic consequences for the host. This is without considering the substantial amount of energy required in the synthesis of the endogenous proteins in the first place.

A major source of EAA secretion is the intestinal mucus glycoprotein. In the current study, and similar to phytic acid (Cowieson et al., 2004), TI increased the excretion of crude mucin and its metabolic marker, sialic acid (Jourdian et al., 1971) indicative of continuous sloughing of the mucus layer. However, contrary to previous reports (Peek et al., 2009; Cowieson and Roos, 2014) exogenous protease did not alleviate loss of mucoproteins from the gut. Cowieson and Roos (2014) showed a significant correlation between the AA profile of intestinal mucin and the effect of exogenous protease on AA digestibility and submits that part of the beneficial effect of exogenous protease may be mediated via a reduction in the loss of mucoprotein from the intestine. Although the mechanism by which this may occur is not clear, it was suggested (Cowieson and Roos, 2014) that exogenous protease may likely reduce the secretion of HCl and pepsin in the gastric phase of digestion, reducing the need for mucin as a protective agent in the intestine. However, as previously discussed, the observed lack of muco-protective capacity by protease may be related to the non-physiological nature of the NFD or the absence of protein in the feed or both. Intestinal mucoprotein is highly resistant to enzymatic hydrolysis (Montagne et al., 2004), compared to pancreatic secretions, which hinders AA recycling and is predominated by Thr, Pro, Ser and Glu in the ileal flow (Lien et al., 2001; Ravindran, 2021). This disruption in thickness and fluidity of the mucosal layer by TI would also disrupt nutrient digestion, absorption and intestinal barrier function (Smirnov et al., 2004). Interestingly, a previous study (Sambeth, 1967) identified TI as a stimulant of gall bladder contraction in birds. The resultant increase in bile production will increase EAA flow, especially for taurine (data not included), which almost exclusively conjugates with bile acid in birds (Bremmer, 1958; Ravindran, 2021).

The current study also provides novel data on mineral digestibility in broiler chickens fed an experimental NFD and offers valuable insight into possible interactions of macro and trace minerals with TI. Feeding birds with NFD resulted in negative digestibility values for Fe, Mg, Cu and K indicating increased endogenous flow at the terminal ileum, which was further exacerbated by added TI. Increased endogenous mineral excretion may be explained by the interactions of various cations with the mucus components, where divalent ions have a greater affinity than

monovalent ions for the mucins (Powell et al., 1999). Similarly, Cowieson et al. (2004) and Woyengo et al. (2009) previously reported increased endogenous excretion of Fe, Mg, Na and S in broiler chickens and pigs due to phytic acid ingestion. Phytic acid is a potent chelator of mineral ions (Maenz et al., 1999) and the observation in the current study suggests similar propensity of TI to form insoluble complexes with minerals, which is eventually excreted. Exogenous protease improved the digestibility of Cu, irrespective of dietary TI concentration in the current study. This is an interesting observation because aside from the primary function in enzyme systems within cells, Cu intake is the main determinant of bone strength (Roughead and Lukaski 2003). This is ascribed to a Cu-containing enzyme, lysyl oxidase, which is responsible for enhancing bone strength (Ilich and Kerstetter 2000). In fact, Cu and Fe deficiency inhibits bone growth and decreases bone strength even when Ca and P levels are adequate (Medeiros et al., 1997). With few exceptions, the negative digestibility values observed in the current study suggest that the impact of protease may be more related to reductions in endogenous flow rather than improved dietary retention of the minerals. This may be a more appropriate description of the exogenous protease effect because of the consistent weight loss of birds as a result of being fed a NFD. It is unclear why addition of exogenous protease affected the digestibility of Zn and Mg in birds fed NFD containing TI. It is pertinent to state that a careful interpretation of these data may be required because mineral intake through the water source was unaccounted for in the current study. However, drinking water may not be a major source of minerals for broilers and due to high variability of individual daily water consumption, it is impossible to calculate mineral intakes from drinking water (Underwood, 1999). More importantly, complex interactions among minerals exist in the gut, which is a major cause of variation in availability and utilization or endogenous secretion. For instance, Zn absorption is impaired by Fe (Solomons and Jacob 1981), while excess Zn interferes with Fe incorporation into ferritin (Settlemire and Matrone 1967).

It can be concluded that the ingestion of TI by broilers increased the excretion of EAA, minerals, crude mucin, and sialic acid. Although supplementing with protease differentially affected proteolytic enzyme secretion by the pancreas, it did not reduce EAA losses, irrespective of added purified dietary TI. However, there were significant interactions between exogenous protease and TI for the AID of Zn, Mg, Cu and Na. In particular, the AID of Cu and Mg increased with protease supplementation, but protease reduced or caused no change in other mineral utilization in diets containing added TI. It should be noted that the effects observed in the current

study were obtained in birds fed NFD, and it is unclear how these will be mediated in birds fed on conventional diets, containing SBM with variable TI concentrations. However, these results emphasize the impact of trypsin inhibitor on endogenous nutrient loss and hence should be considered when formulating poultry diets.

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Table 3.1 Ingredient and calculated nutrient composition of experimental diets, as-fed basis.

Protease, PROT/kg:	0		15,000	
Purified trypsin inhibitor, TIU/g:	0	8,000	0	8,000
Ingredients, g/kg				
Dextrose	615.5	565.5	605.5	555.5
Cornstarch	200.5	200.5	200.5	200.5
Soybean oil	50.0	50.0	50.0	50.0
Solka floc	50.0	50.0	50.0	50.0
Ground limestone[1]	15.5	15.5	15.5	15.5
Monocalcium phosphate[2]	21.0	21.0	21.0	21.0
Potassium carbonate	2.6	2.6	2.6	2.6
Magnesium oxide	2.0	2.0	2.0	2.0
Sodium bicarbonate	7.5	7.5	7.5	7.5
Choline chloride	2.5	2.5	2.5	2.5
Potassium chloride	2.9	2.9	2.9	2.9
Vitamin-mineral premix[3]	5.0	5.0	5.0	5.0
Trypsin inhibitor premix[4]	0.0	50.0	0.0	50.0
Ronozyme ProAct premix[5]	0.0	0.0	10.0	10.0
Titanium dioxide premix[6]	25.0	25.0	25.0	25.0
Total	1,000	1,000	1,000	1,000
Calculated composition				
MEn, kcal/kg	3,606	3,606	3,606	3,606
Crude protein, g/kg	0.60	0.60	0.60	0.60
Ether extract, g/kg	48.50	48.50	48.50	48.50
Ca, g/kg	9.10	9.10	9.10	9.10
Available P, g/kg	4.52	4.52	4.52	4.52
DEB, mEq/kg diet[7]	90.98	90.98	90.98	90.98
Analyzed composition				
Crude protein, g/kg	1.16	3.15	1.24	2.58
Trypsin inhibitor, TIU/g[8]	LOD	6,244	LOD	5,921

[1] 38% Ca.

[2] 16% Ca, 21% P.

[3] Supplied the following per kg diet: vitamin A, 8,575 IU; vitamin D_3, 4,300 IU; vitamin E, 28.58 IU; menadione, 7.30 mg; riboflavin, 9.15 mg; $_D$-pantothenic acid, 18.33 mg; niacin, 73.5 mg; choline chloride, 1,285 mg; vitamin B_{12}, 0.02 mg; biotin, 0.09 mg; thiamine mononitrate, 3.67; folic acid, 1.65 mg; pyridoxine hydrochloride, 5.50 mg; I, 1.85 mg; Mn, 178.5 mg; Cu, 7.40 mg; Fe, 73.5 mg; Zn, 178.5 mg.

[4] Purified trypsin inhibitor (PTI) from soybeans product contains 9,000,000 TIU/g. 1g of STI added to 55.25 g of corn supplies 160,000 TIU/ g of premix. 50g/kg premix delivers 8,000,000 TIU/kg in feed.

[5] Product contained 75,000 PROT/g. 1 g Protease added to 49 g ground corn supplied 1,500 PROT/ g premix. 10 g premix delivered 15,000 PROT/ kg feed.

[6] 1 g of Titanium dioxide added to 4 g of dextrose.

[7] Dietary electrolyte balance (mEq/kg diet) was calculated as Na+K-Cl.

[8] LOD = limit of detection.

Table 3.2 Growth performance of broiler chickens fed nitrogen-free diets supplemented with protease (PROT/kg) and purified trypsin inhibitor (TIU/g), from d 21 to 24 post hatching.

	Protease											
	0		15,000									
	TI		TI			Protease		TI		*P*-value		
Item[1]	0	8,000	0	8,000	SEM	0	15,000	0	8,000	Protease	TI	P × TI
D 21 BW, g	631.6	631.2	631.7	631.6	0.44	631.4	631.7	631.7	631.4	0.614	0.614	0.664
D 24 BW, g	550.1	550.8	551.1	552.7	4.09	550.4	551.9	550.6	551.8	0.723	0.776	0.903
BW gain, g	-81.2	-81.3	-80.6	-79.2	4.07	-81.2	-79.9	-80.9	-80.3	0.751	0.872	0.863
Feed intake, g	204.5	240.4	231.1	248.9	18.1	222.4	239.9	217.8	244.6	0.342	0.151	0.622
G: F g/kg	-424.7	-347.9	-365.7	-318.3	44.4	-386.4	-341.9	-395.2	-333.1	0.329	0.177	0.744
N	8	8	8	8		16	16	16	16			

[1] G: F, gain to feed ratio; P, protease; TI, trypsin inhibitor; SEM, standard error of the mean.

Table 3.3 Ileal endogenous flow (mg/kg DMI) of nitrogen and amino acid in broiler chickens fed nitrogen-free diets supplemented with exogenous protease (PROT/kg) and purified trypsin inhibitor (TIU/g), at d 24 post hatching

	Protease											
	0		15,000									
	TI		TI			Protease		TI		*P*-value		
Item[1]	0	8,000	0	8,000	SEM	0	15,000	0	8,000	Protease	TI	P × TI
Nitrogen	1,975	2,215	1,725	2,341	157.5	2,095	2,033	1,850	2,278	0.699	0.013	0.244
Indispensable AA												
Arg	434	575	376	607	44.5	505	492	405	591	0.773	< 0.001	0.325
His	208	262	189	280	17.6	235	235	199	271	0.993	< 0.001	0.310
Ile	523	622	452	669	43.2	573	561	487	646	0.787	0.001	0.185
Leu	791	960	681	1,019	66.6	876	849	736	989	0.701	0.001	0.217
Lys	414	552	388	591	47.9	483	490	401	571	0.890	0.002	0.499
Met	146	169	119	190	14.8	158	155	133	179	0.851	0.004	0.120
Phe	461	569	399	596	40.7	515	497	429	583	0.667	0.001	0.290
Thr	860	908	747	973	64.2	884	860	804	941	0.713	0.045	0.179
Trp	74	102	65	108	10.4	88	87	69	105	0.885	0.003	0.491
Val	887	982	743	1044	73.2	934	893	815	1,013	0.585	0.013	0.174
Dispensable AA												
Ala	500	582	429	634	42.1	541	531	465	608	0.823	0.003	0.156
Asp	1,008	1,203	883	1,294	85.1	1,105	1,088	945	1,248	0.844	0.002	0.218
Cys	474	493	381	518	41.9	483	449	427	506	0.436	0.076	0.176
Glu	1,142	1,359	981	1,477	103.0	1,251	1,229	1,062	1,418	0.838	0.002	0.193
Gly	625	712	531	773	52.6	668	652	578	742	0.759	0.005	0.155
Pro	689	748	572	814	55.6	719	693	631	781	0.648	0.013	0.114
Ser	703	798	599	873	63.9	751	736	651	835	0.819	0.009	0.178
Tyr	368	434	324	469	30.9	401	397	346	452	0.893	0.003	0.215
N	8	8	8	8		16	16	16	16			

[1] AA, amino acid; P, protease; TI, trypsin inhibitor; SEM, standard error of the mean.

Table 3.4 Calculated ileal endogenous energy flow (kcal/kg DMI)[1] associated with endogenous amino acids in broiler chickens fed nitrogen-free diets supplemented with exogenous protease (PROT/kg) and purified trypsin inhibitor (TIU/g), at d 24 post hatching

	Protease											
	0		15,000									
	TI		TI			Protease		TI		*P*-value		
Item[2]	0	8,000	0	8,000	SEM	0	15,000	0	8,000	Protease	TI	P × TI
Indispensable AA												
Arg	2.22	2.94	1.93	3.11	0.227	2.58	2.52	2.07	3.02	0.773	< 0.001	0.325
His	1.08	1.36	0.98	1.45	0.091	1.22	1.22	1.03	1.40	0.993	0.001	0.310
Ile	3.45	4.11	2.98	4.42	0.285	3.78	3.70	3.21	4.26	0.787	0.001	0.185
Leu	5.22	6.33	4.49	6.72	0.439	5.78	5.61	4.86	6.53	0.701	0.001	0.217
Lys	2.33	3.10	2.18	3.32	0.269	2.71	2.75	2.25	3.21	0.890	0.002	0.499
Met	0.65	0.75	0.53	0.85	0.065	0.70	0.69	0.59	0.80	0.851	0.004	0.120
Phe	3.11	3.84	2.69	4.02	0.275	3.47	3.35	2.90	3.93	0.667	0.001	0.290
Thr	3.54	3.73	3.07	4.00	0.264	3.63	3.54	3.30	3.87	0.713	0.045	0.179
Trp	0.49	0.67	0.43	0.71	0.068	0.58	0.57	0.46	0.69	0.885	0.003	0.491
Val	5.29	5.87	4.44	6.24	0.437	5.58	5.34	4.87	6.05	0.585	0.013	0.174
Dispensable AA												
Ala	2.18	2.53	1.87	2.76	0.183	2.35	2.31	2.02	2.64	0.823	0.003	0.156
Asp	2.91	3.48	2.55	3.74	0.246	3.20	3.15	2.73	3.61	0.844	0.002	0.218
Cys	2.08	2.17	1.68	2.28	0.185	2.13	1.98	1.88	2.22	0.436	0.076	0.176
Glu	4.17	4.97	3.59	5.40	0.377	4.57	4.50	3.88	5.19	0.838	0.003	0.193
Gly	1.93	2.19	1.64	2.38	0.162	2.06	2.01	1.78	2.29	0.759	0.005	0.155
Pro	3.91	4.23	3.24	4.61	0.315	4.07	3.92	3.57	4.42	0.648	0.014	0.114
Ser	2.32	2.63	1.98	2.88	0.211	2.48	2.43	2.15	2.76	0.819	0.009	0.178
Tyr	2.19	2.58	1.93	2.79	0.184	2.39	2.36	2.06	2.69	0.893	0.003	0.215
N	8	8	8	8		16	16	16	16			

[1] Calculated using the flow of each amino acid in Table 3 and the gross energy of amino acids from Boisen and Verstegen, (2000). Gross energy estimates (kcal/kg) for individual AA from Boisen and Verstegen (2000) are as follows: Arg, 5115; His, 5186; Ile, 6597; Leu, 6597; Lys, 5617; Met, 4446; Phe, 6740; Thr, 4111; Trp, 6568; Val; 5975; Ala, 4350; Asp, 2890; Cys, 4398; Glu, 3657; Gly, 3083; Pro, 5664; Ser, 3298; Tyr, 5951. [2] AA, amino acid; P, protease; TI, trypsin inhibitor; SEM, standard error of the mean.

Table 3.5 Apparent ileal digestibility (%) of minerals in broiler chickens fed nitrogen-free diets supplemented with exogenous protease (PROT/kg) and purified trypsin inhibitor (TIU/g), at d 24 post hatching

	Protease											
	0		15,000									
	TI		TI			Protease		TI		*P*-value		
Item[1]	0	8,000	0	8,000	SEM	0	15,000	0	8,000	Protease	TI	P × TI
P	54.9	62.6	63.8	57.5	3.59	58.8	60.7	59.4	60.1	0.612	0.839	0.065
Ca	59.2	53.3	70.5	49.4	5.95	56.2	59.9	64.9	51.3	0.541	0.034	0.215
Fe	-25.7	-82.9	1.72	-84.5	22.09	-54.3	-41.4	-11.9	-83.7	0.965	0.004	0.519
Zn	48.9^{ab}	56.6^{a}	60.2^{a}	33.9^{b}	5.48	52.8	47.1	54.6	45.2	0.313	0.104	0.006
Mg	-82.6^{b}	-80.1^{b}	-21.3^{a}	-90.6^{b}	14.74	-81.3	-55.9	-51.9	-85.3	0.101	0.034	0.024
Mn	90.4	86.4	89.6	85.4	1.39	88.4	87.5	90.0	85.9	0.522	0.008	0.933
Cu	-31.3^{bc}	-63.8^{c}	14.9^{a}	-10.1^{ab}	10.92	-47.5	2.4	-8.2	-37.0	< 0.001	0.015	0.039
Na	35.2^{b}	62.7^{a}	65.2^{a}	51.8^{ab}	6.15	48.9	58.5	57.2	50.2	0.136	0.267	0.003
K	-9.62	-23.9	18.1	9.59	13.0	-16.8	13.8	4.2	-7.2	0.029	0.391	0.825
N	8	8	8	8		16	16	16	16			

[1] P, protease; TI, trypsin inhibitor; SEM, standard error of the mean.

Table 3.6 Effect of exogenous protease (PROT/kg) and purified trypsin inhibitor (TIU/g) on crude mucin and sialic acid excretion in broiler chickens, at d 24 post hatching

	Protease										
	0		15,000								
	TI		TI			Protease		TI		*P*-value	
Item[1]	0	8,000	0	8,000	SEM	0	15,000	0	8,000	Protease	TI	P × TI
CM, g/kg excreta	317.2	342.3	312.2	353.1	25.56	329.8	332.7	314.7	347.7	0.911	0.211	0.759
CM, g/kg DMI	70.2	71.8	57.5	77.4	4.97	71.0	67.4	63.8	74.6	0.479	0.042	0.081
SA, mg/kg excreta	187.8	270.0	189.1	241.3	23.73	228.9	215.2	188.4	255.7	0.568	0.010	0.534
SA, mg/kg DMI	40.2	50.7	34.1	48.5	5.56	45.3	41.3	37.1	49.6	0.477	0.035	0.739
N	8	8	8	8		16	16	16	16			

[1] P, protease; TI, trypsin inhibitor; SEM, standard error of the mean; CM, crude mucin; SA, sialic acid.

Table 3.7 Effect of exogenous protease (PROT/kg) and purified trypsin inhibitor (TIU/g) on pancreas weight and proteolytic enzyme secretion in broiler chickens, at d 24 post hatching

	Protease											
	0		15,000									
	TI		TI			Protease		TI		*P*-value		
Item[1]	0	8,000	0	8,000	SEM	0	15,000	0	8,000	Protease	TI	P × TI
Pancreas wt., g	1.39	1.47	1.35	1.32	0.075	1.42	1.33	1.36	1.39	0.231	0.698	0.504
Pancreas wt., g/kg BW	2.55	2.66	2.57	2.50	0.143	2.60	2.54	2.56	2.58	0.652	0.909	0.532
Trypsin, U/mg	5.78	5.89	5.11	4.62	0.247	5.83	4.86	5.44	5.26	< 0.001	0.438	0.252
Chymotrypsin, U/mg	4.53	4.04	5.25	5.34	0.240	4.28	5.30	4.88	4.69	< 0.001	0.466	0.240
N	8	8	8	8		16	16	16	16			

[1] P, protease; TI, trypsin inhibitor; SEM, standard error of the mean.

CHAPTER 4. GROWTH PHASE AND DIETARY ALPHA-AMYLASE SUPPLEMENTATION EFFECTS ON NUTRIENT DIGESTIBILITY AND FEEDBACK ENZYME SECRETION IN BROILER CHICKENS

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4.1 Abstract

Growth performance, nutrient digestibility, intestinal health, and endogenous enzyme secretion responses to dietary α-amylase supplementation during 4 growth phases of broiler chickens fed corn-soybean meal-based diets were evaluated in the current study. A total of 1,136 male broiler chicks were assigned at d 0 post hatching to 8 treatments in a 2 × 4 factorial arrangement. There were 2 dietary levels of α-amylase supplementation of 0 or 80 kilo-Novo alpha amylase units (KNU) per kg diet and 4 post hatching growth phases of d 0 to 11, d 11 to 21, d 21 to 42, or d 42 to 56 in a randomized complete block design. Each treatment comprised 8 replicate pens, with either 25 (d 0 to 11), 20 (d 11 to 21), 16 (d 21 to 42) or 10 (d 42 to 56) birds per pen. Body weight gain and feed efficiency of birds improved ($P < 0.01$) with α-amylase supplementation. There were main effects of α-amylase, growth phase and interaction ($P < 0.01$) on apparent ileal digestibility (AID) of starch. This ranged from 0.8% during d 11 to 21 to 2.8% during d 0 to 11 post hatching. The total tract retention of starch increased ($P < 0.05$) with amylase supplementation but was not different across growth phases. Amylase supplementation increased ($P < 0.05$) AID of gross energy, AME (kcal/kg) and AMEn (kcal/kg). Villus height in the jejunal tissue was increased ($P < 0.01$) by α-amylase supplementation. During d 11 to 21 post hatching, the viscosity of jejunal digesta and pancreatic amylase activity increased ($P < 0.01$) with amylase supplementation. In conclusion, dietary amylase supplementation improved growth performance, apparent nutrient digestibility and digestive enzyme activity of broiler chickens fed a corn-soybean diet. The study indicates that the growth phase of birds may affect response to exogenous amylase.
Key words: amylase, broiler, digestibility, enzyme, starch

4.2 Introduction

The energy derived from the components of plants feedstuffs by broiler chickens is affected by enzyme access to substrates such as starch or protein (Theander et al., 1989; Slominski et al., 1993). Among the nutrients in poultry diets, starch is quantitatively the most important energy-yielding source. For instance, corn contains about 69% starch (Knudsen, 1997), which leads to its high content in corn-based diets. Although starch degradability is relatively high in broiler chickens, some proportion of the dietary starch may escape digestion in the small intestine (Englyst et al., 1982; Svihus, 2014). This varies among feed ingredients and in a complete diet, can significantly influence the metabolizable energy content for the birds (Tester et al., 2004). Therefore, there have been increased interests in the use of supplemental enzymes to improve the utilization of substrates that release energy for poultry.

Exogenous carbohydrases such as xylanases, amylases, and glucanases have been shown to improve energy utilization and the performance of broiler chickens (Olukosi and Adeola, 2008). In conventional diets formulated with corn and soybean meal (SBM), an estimated 450 kcal/kg of energy is available for utilization via exogenous enzymes, which may include up to 37% from undigested starch (Cowieson et al., 2010). One mode of action is by improving the access of endogenous enzymes to cell contents (Kocher et al., 2003; Meng et al., 2005). Another is by augmenting endogenous enzyme secretions (Gracia et al., 2003). Similarly, previous studies showed that α-amylase, supplemented alone, increased starch and energy digestibility in broiler chickens (Cowieson et al., 2019; Stefanello et al., 2019; Woyengo et al., 2019) fed corn-SBM based diets.

However, there are variations in nutrient utilization by birds and age is one of the explanatory variables (Noy and Sklan, 1995; Uni et al. 1995). It has been suggested that the immaturity of the digestive system of younger birds may result in the relatively poor utilization of dietary nutrients (Jin et al., 1998), and nutrient digestion rather than the ability to absorb nutrients may be a primary limiting factor (Parsons, 2004). This has led to findings that poultry develop an increased capacity to digest starch as the intestinal tract matures, and there is elevated pancreatic amylase production in older birds compared with their juvenile counterparts (Krogdahl and Sell, 1989). Therefore, the effect of animal age on nutrient digestibility may be relevant and the interaction between age and exogenous enzymes needs to be explored.

There are few reports in literature that evaluated the effect of dietary α-amylase supplementation in broiler chickens, as in most instances, amylase is added as part of a cocktail of carbohydrases. There are yet fewer data on the effect of α-amylase supplementation across different growth phases of broiler chickens. Therefore, the hypothesis for the current study is that responses to α-amylase supplementation would be affected by bird age. The current study was designed to evaluate the effects of *α*-amylase supplementation on growth performance, nutrient digestibility, and feedback enzyme secretion in broiler chickens fed a corn-SBM diet during 4 growth phases of d 0 to 11, 11 to 21, 21 to 42 or 42 to 56 post hatching.

4.3 Materials and Methods

Protocols of animal experiments were reviewed and approved by the Purdue University Animal Care and Use Committee (#: 1112000389).

4.3.1 Diets and Experimental birds

A total of 1,136 male 0-d-old broiler chicks (Cobb 500, Siloam Springs, AR) were purchased from a commercial hatchery. Birds were individually tagged, weighed, and raised in floor pens with temperature and lighting maintained as previously described by Park et al. (2017). The birds were assigned to 8 dietary treatments in a 2 × 4 factorial arrangement. There were 2 dietary levels of α-amylase (Ronozyme HiStarch, DSM Nutritional Products, Kaiseraugst, Switzerland); 0 or 80 kilo-Novo alpha amylase units (KNU) per kg of diet and 4 post hatching growth phases of d 0 to 11, d 11 to 21, d 21 to 42, or d 42 to 56 in a randomized complete block design. Each dietary treatment comprised 8 replicate pens, with either 25 (d 0 to 11), 20 (d 11 to 21), 16 (d 21 to 42) or 10 (d 42 to 56) birds per replicate. All diets were corn-SBM based, formulated to meet breeder nutrient specifications, and fed as mash (Table 4.1). The α-amylase was a granulated enzyme preparation produced by submerged fermentation of *Bacillus amyloliquefaciens* and contained 600 KNU/g. Birds on d 0 to 11 growth phase were fed experimental diets throughout. Birds on d 11 to 21 growth phase were fed the standard broiler starter diet until d 11, but the experimental diets from d 11 to 21. Birds on d 21 to 42 growth phase were fed the standard broiler starter diet until d 21 but the experimental diets from d 21 to 42 post hatching. Birds on d 42 to 56 growth phase were fed the standard broiler starter until d 21 and

grower diets until d 42 post hatching but the experimental diets from d 42 to 56 post hatching. All diets contained phytase (Ronozyme HiPhos: DSM Nutritional Products, Kaiseraugst, Switzerland) at 1,000 phytase units (FYT)/kg and titanium dioxide was added at 5 g/kg as an indigestible marker.

4.3.2 Sampling Procedures

Feed and water were available *ad libitum* during the entire experimental period. Initial and final BW and average feed intake per pen were recorded within each growth phase. Mortality records was taken daily and was used to correct the calculated gain to feed ratio during the experimental period. Two days prior to the end of each growth phase, birds were randomly selected and transferred to metabolic cages for a 2-d excreta collection. Specifically, 5 birds per pen for during d 0 to 11, d 11 to 21; 3 birds per pen during d 21 to 42 and 2 birds per pen during d 42 to 56 growth phases respectively. At the end of the trial for each of the growth phase, which corresponds to d 11, 21, 42 or 56 post hatching, the remaining birds in each pen were euthanized by $C0_2$ asphyxiation and exsanguination. The pancreas was excised and weighed and digesta was collected from the distal two-thirds of the ileum (i.e., from the Meckel's diverticulum to approximately 2 cm cranial to the ileocecal junction), by flushing with distilled water into plastic containers and stored at –20°C prior to nutrient analyses. For viscosity measurement, the jejunal content was gently squeezed into plastic tubes and stored at –20°C prior to analysis.

4.3.3 Intestinal Morphological Analysis

On d 11, 21, 42 and 56 post hatching, mid-jejunal segments were collected from 1 bird per replicate with median BW, flushed with ice-cold 10% phosphate-buffered saline (VWR International, Radnor, PA) and fixed in 10% neutral buffered formalin (VWR International, Radnor, PA) for approximately 30 d. Fixed samples were subsequently dehydrated with ethanol (VWR International, Radnor, PA), cleared with Sub-X® (Polysciences, Inc., Warrington, PA) and placed in paraffin (Polyfin paraffin, Sigma Polysciences, St. Louis, MO). Segments (5 μm) were stained with hematoxylin and eosin at the Purdue Histology and Phenotyping Laboratory (Purdue University, West Lafayette, IN). Villus height and crypt depth were measured from 4 complete, vertically oriented villi per slide and subsequently, the villus height to crypt depth ratio was calculated. Villus length is defined as the length from the villus tip to the valley between each

villus while crypt depth is defined as the length between the crypt opening and base. The histological sections were evaluated using a binocular light microscope (National Optical and Scientific Instruments, Inc., Schertz, TX). Quantitative measurements were performed with a computerized image analyzer software (AmScope version 3.7, Irvine, CA).

4.3.4 Viscosity Measurements

Approximately 10 g of jejunal digesta sample were placed in a 50 mL plastic centrifuge tube, vortexed for 10 s and centrifuged at 10,000 × *g* for 10 min at 4°C. The supernatant was transferred into a 2-mL sample cup. The cup containing the supernatant was placed in a water bath (Precision, GCA Corp., College Park, MD) that had been preheated to 40°C until the temperature of the sample equilibrated with that of the water in the water bath (approximately 15 min). The viscosity, in centipoise (cP), of these samples was determined using a viscometer (Vibro viscometer, model SV-1A, A&D Instruments Ltd, Oxfordshire, United Kingdom).

4.3.5 Digestive Enzyme Assay

Duodenal digesta and the pancreas was collected from 1 bird per replicate with median BW, except for group d 0-11 where 2 birds per pen with median BW was selected to obtain sufficient samples for analysis. The digesta and pancreas were frozen in liquid nitrogen and stored at –80°C until required for assay. Enzyme activities were determined using a commercially available assay kit (Sigma Chemical Co, St. Louis, MO). The absorbance of the colorimetric final product was measured in a UV/visible spectrophotometer, and the concentration of the respective enzymes was calculated accordingly. For duodenal digesta, the samples were centrifuged at 13,000 rpm at 4°C for 10 min, and aliquots of the supernatant was used for enzyme assay. The activity of the pancreatic enzymes was determined after the whole organ was homogenized in appropriate buffers and centrifuged at 13,000 rpm at 4°C for 10 min, to get a clear supernatant. Amylase activity (EC 3.2.1.1) was determined using a coupled enzyme assay and absorbance of ethylidene-pNP-G7 cleaved by the amylase was measured at 405 nm. One unit is the amount of amylase that cleaves ethylidene-pNP-G7 to generate 1.0 μmol of *p*-nitrophenol per minute at 25 °C.

4.3.6 Total RNA Extraction, Reverse Transcription and Real-Time PCR Analysis

A section of the jejunum was removed from 1 bird per replicate with median BW and flushed with ice-cold PBS (VWR International, Radnor, PA), cut longitudinally in half exposing the lumen, and mucosal contents were scraped with a metal spatula. Mucosal contents were immediately placed in 2 mL of Trizol reagent (Invitrogen, Grand Island, NY) and stored at -80°C prior to RNA isolation. Total RNA was extracted from the tissues using Trizol reagent (Invitrogen) following the manufacturer's protocol. RNA concentrations were determined by NanoDrop 1000 (Thermo Scientific), and RNA integrity was verified by 1% agarose gel electrophoresis. Extracted RNA was purified with DNA-*free* DNase Treatment and Removal Kit (Ambion). Afterwards, 2 mg of total RNA from each sample were reverse transcribed into cDNA product using the MMLV reverse transcription system (Promega). The cDNA was then diluted 1:10 with nuclease-free water (Ambion) and stored at -20°C until use. Real-time PCR was performed with Bio-Rad iCycler with the Faststart SYBR green-based mix (Life Technologies). PCR programs for all genes were designed as follows: 10 min at 95°C; 40 cycles of 95°C for 30 s, primer-specific annealing temperature for 30s, and 72°C for 30s; followed by melting curve analysis. The primer sequences used in the current study are listed in Table 4.2. Primer specificity and efficiency were verified, subsequently the samples were analyzed in duplicate, and a difference lesser than or equal to 5% was considered acceptable. Relative gene expression was subsequently calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with normalization against glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as the housekeeping gene (Tan et al., 2014).

4.3.7 Chemical Analyses and Calculations

The ileal digesta and excreta samples were freeze-dried for 96 h and subsequently ground to pass through a 0.5-mm screen (Retsch ZM 100, GmbH, Haan, Germany). A portion of the samples were analyzed for DM by drying overnight at 105°C (Precision Scientific Co., Chicago, IL; method 934.01; AOAC, 2006) and the nitrogen content of the samples was subsequently determined by combustion method (Leco model TruMac N analyzer, Leco Corp., St. Joseph, MI; AOAC, 2000; Method 990.03) with EDTA as a calibration standard. Starch was determined using a Megazyme total starch determination kit (Method 996.11; AOAC, 2000). The absorbance of the colorimetric final product was measured in a UV/visible spectrophotometer at 510 nm and

converted to the amount of glucose released by comparison with a standard curve. Gross energy (GE) concentration in diets, ileal digesta and excreta samples was determined by isoperibol bomb calorimeter (Parr 1261; Parr 105 Instrument Co., Moline, IL). Titanium concentration was measured on a UV spectrophotometer following the method of Short et al. (1996).

The index method was used to calculate the apparent ileal digestibility (AID) or total tract retention (TTR) of nutrients, according to the following equation:

$$\text{AID or TTR, \%} = 100 - [(Ti_I/Ti_O) \times (P_O/P_I) \times 100]$$

where Ti_I is Titanium concentration in diets; Ti_O is Titanium concentration in output (ileal digesta or excreta); P_I is nutrient concentration in diets; and P_O is nutrient concentration in output (ileal digesta or excreta).

The ileal digestible energy (IDE; kcal/kg DM) and AME (kcal/kg DM) of the diet was calculated as the product of the coefficient and GE concentrations (kcal/kg DM) in the diet. The AMEn was calculated by correcting for zero N retention using a factor of 8.22 kcal/g (Hill and Anderson, 1958):

$$\text{AMEn (kcal/kg)} = \text{AME} - (8.22 \times \text{Nret})$$

where N_{ret} is N retention in g/kg of DM intake. The Nret was calculated as follows:

$$N_{ret}\,(\text{g/kg DM}) = N_i - (N_o \times T_i/T_o)$$

where N_i and N_o are the N concentrations (g/kg DM) in the diet and excreta, respectively

4.3.8 Statistical Analyses

The data obtained were analyzed as a randomized complete block design using the GLM procedures of SAS (SAS Inst. Inc., Cary, NC). Initial body weight was used as the blocking factor. The pen of birds was used as the experimental unit for all analyses. The main effects of dietary α-amylase supplementation and growth phase, and the interaction were tested accordingly. Statistical significance was declared at $P \leq 0.05$, with $0.05 < P \leq 0.10$ considered as a tendency.

4.4 Results

There were few recorded mortalities throughout the trial and were not directly related to the dietary treatments. Overall, there were 4, 4, 6, and 2 mortalities during d 0 to 11, d 11 to 21, d 21 to 42 and d 42 to 56 post hatching, respectively. The performance parameters of the broiler chickens in response to α-amylase supplementation is shown in Table 4.3. There was no interaction between α-amylase supplementation and growth phase for any of the growth performance indices. However, the final BW and BW gain increased ($P < 0.01$) with α-amylase supplementation and growth phase, while G:F increased ($P < 0.01$) with α-amylase supplementation but decreased ($P < 0.01$) as birds grew older. Numerical improvements in BW gain were lower during d 0 to 11 (0.8%), but relatively higher during d 42-56 (5.7%) resulting in a tendency ($P = 0.08$) for an interaction between dietary α-amylase supplementation and growth phase.

Amylase supplementation improved ($P < 0.01$) the AID of DM, starch, and GE (Table 4.4). There was an interaction ($P < 0.01$) between α-amylase supplementation and growth phase on AID of starch. Amylase supplementation improved ($P < 0.01$) the AID of starch in all growth phases and ranged from 0.8% during d 11 to 21 to 2.8% during d 0 to 11 post hatching. Furthermore, amylase supplementation improved ($P < 0.01$) the TTR of DM, starch, and GE (Table 4.4). There was no interaction between α-amylase supplementation and growth phase on TTR ($P < 0.05$) of starch. There were no interactions between α-amylase supplementation and growth phase on IDE, AME and AMEn (kcal/kg DM).

The effect of amylase supplementation on ileal digestible starch (IDS) intake is presented in Figure 4.1. The mean improvement in IDS intake due to amylase supplementation are 6.1, 41.4, 21.0, and 81.0 g/d during d 0 to 11, 11 to 21, 21 to 42 and 42 to 56 growth phases, respectively. As shown in Table 4.5, there were increases in villus height ($P < 0.01$), and crypt depth ($P < 0.05$) of the jejunal tissue due to dietary α-amylase supplementation. However, there was a tendency for an interaction ($P = 0.058$) between α-amylase and growth phase for villus height. The improvements in villus height due to α-amylase supplementation were 2.4% (d 0-11), 7.9% (d 11-21), 38.8% (d 21-42) and 23.1% (d 42-56).

Although affected by growth phase ($P < 0.01$), the absolute and relative pancreas weight was not affected by α-amylase supplementation. There was an effect of α-amylase supplementation and growth phase and an interaction ($P < 0.01$) on viscosity of jejunal digesta. Amylase supplementation reduced the viscosity ($P < 0.01$) of the jejunal digesta during d 0 to 11, d 21 to

42, and d 42 to 56 post hatching. However, during d 11 to 21 post hatching, α-amylase supplementation increased ($P < 0.01$) the viscosity of jejunal digesta.

The amylase activities in the duodenal digesta and pancreas and gene expression of glucose transporters of broiler chickens in response to α-amylase supplementation is shown in Table 4.6. There were effects of α-amylase supplementation and growth phase and an interaction ($P < 0.01$) on amylase activities in the duodenal digesta and pancreas. In all growth phases, duodenal amylase activity increased ($P < 0.01$) with amylase supplementation. Amylase supplementation decreased ($P < 0.01$) the pancreatic amylase activity in all phases, except during d 11 to 21 post hatching. There was no effect of α-amylase supplementation or growth phase on the mRNA expression of markers of glucose transport.

4.5 Discussion

The current study showed that exogenous amylase supplementation of diets improved the growth performance response of broiler chickens. This observation is similar to previous reports (Onderci et al., 2006; Vieira et al., 2015; Stefanello et al., 2019) for broilers fed amylase-supplemented, corn-SBM based diets. Likewise, Ritz et al. (1995) showed 3% improvements in BW gain for 21-d-old poults fed a corn-SBM diet supplemented with an enzyme complex containing predominantly amylase. Although improvements were observed relative to the control, the current study showed that the effect of the exogenous amylase on BW gain and feed efficiency was not different across the four growth phases. This might be due to the lack of change in feed intake response of the birds because of the enzyme supplementation. Although birds eat more as they grow older, it is possible that this lack of effect of amylase supplementation on feed intake could be a limiting factor to substrate availability for the enzyme. This might partly explain the observed similarity in amylase effect on bird performance responses across the four growth phases. However, Svihus and Hetland (2001) previously indicated that increases in feed intake in birds reduces the digesta transit time and is inversely correlated with starch digestibility. There are other previous reports that show this lack of effect of exogenous amylase on feed intake (Kaczmarek et al. 2014; Stefanello et al., 2019), however Gracia et al. (2003) reported increased feed intake due to exogenous amylase with increasing age of birds. Similarly, Jiang et al. (2008) showed a linear increase in feed intake and BW gain but observed no effect on feed efficiency with birds fed diets supplemented with amylase. These inconsistencies in the effect of amylase supplementation on

growth performance could be due to discrepancies in the source, composition and concentration of the enzyme preparation or age of the birds used in the various studies.

Furthermore, the current study showed significant improvements in the AID and TTR of starch and GE because of dietary amylase supplementation. This observation is similar to a previous report by Stefanello et al. (2019), who observed an increase in energy utilization in broiler chickens fed corn-SBM diet supplemented with amylase. Zanella et al. (1999) found that the respective AID and TTR of starch in 37-d-old broilers increased from 91.2 to 93.0% and from 98.2 to 98.5% when fed a corn-SBM diet supplemented with an enzyme complex containing amylase. It is presumed that while chickens readily adapt well to starch-based diets (Svihus, 2011), the very high feed intake of the modern fast-growing broiler chickens may present some physiological limitations for starch digestion and absorption. These limitations include factors such as the nature of the starch crystals, inadequacies in endogenous amylases and issues around extraction of glucose from the intestinal lumen via Na-dependent transport systems. This could leave significant portions of the dietary starch undigested and available to react with the supplemental amylase. Furthermore, the improvements in apparent ileal starch and energy digestibility were also observed across all four growth phases, with the greatest impact during d 0 to 11 post-hatching and suggests an efficacy of the enzyme irrespective of the stage of digestive system development of broilers.

Starch is an extremely heterogeneous structure (Tester et al., 2004), and inherent properties such as its crystallinity (Bjorck et al., 2000) and the ratio between the amylose and the waxier amylopectin fractions would play a major role in its rate of digestion by digestive amylases (Zhang et al., 2006). Compared to other species, the increased capacity to digest native starch by chickens may be due to the high pancreatic secretion of amylolytic juice (Lehrner and Malacinski, 1975).

However, previous work by Croom et al (1999) noted that as birds grow older, the intestinal mass and pancreatic tissue become an increasingly diminished proportion of the metabolic weight of the bird which may limit the overall effectiveness of the enzyme secreted. This has led to the assumption that birds may be responsive to exogenous amylases due to a limiting supply of endogenous amylase to cater for the changes in body weight and physiological needs. Conversely, Gracia et al. (2003) observed a significant increase in starch and energy digestibility when exogenous amylase was added to corn-based diets, thus indicating that α-amylase secretion may be a limiting factor. In the current study, the improvements in starch digestibility in older birds could also be due to an amylase-induced increase in the digestible starch intake. It is therefore

possible that the newly hatched chicks require assistance to augment pancreatic amylase production due to their relatively immature gut while the older birds would require exogenous amylase to augment pancreatic output only at a time of very high starch intake.

An elevation of duodenal amylase activity in all growth phases, especially during d 0 to 11 post hatching, with an associated feed-back inhibition of pancreatic amylase secretion was seen in the current study, which is similar to observations by Gracia et al. (2003) and Onderci et al. (2006). However, during d 11 to 21 post hatching, an increase in duodenal amylase activity because of the amylase supplementation did not result in sparing of pancreatic amylase secretion. Instead, there was an increase and the reason for this observation is not clear but may be related to the degree of homology between exogenous and endogenous amylases. Additionally, it may be that compared to other growth phases, there was a relatively low change in duodenal amylase activity due to the exogenous amylase and could suggest a compensatory action by the pancreas. In previous work and largely consistent with the current study, Cowieson et al. (2019) suggested that birds may have 2 windows of exogenous amylase sensitivity, which is immediately post-hatch, and in the grower-finisher phase. Furthermore, there were inconsistencies in the intestinal and pancreatic amylase activity and this difference in response, also observed in previous data in literature, may be due to age of birds. For example, Zhu et al. (2014) reported inconsistent pancreatic amylase activities on d 7, 14 and 21 post hatching in birds fed diets supplemented with an enzyme cocktail containing 800 U/g of amylase. Yuan et al. (2008) reported increased amylase activities in both pancreas and duodenal digesta, due to an enzyme cocktail supplementation containing predominantly amylase. Inborr (1990) and Ritz et al. (1995) opined that the inconsistencies in literature may also be due to differences that exists between the chemical characteristics of endogenous amylase and that of bacterial or plant origin which may not always result in feedback inhibition of pancreatic amylase production.

In the current study, exogenous amylase altered the morphology of the gut. This was observed as increases in the length of the villi and crypt depth within the jejunal tissue, which may have enhanced nutrient absorption (Caspary, 1992). This improvement by the exogenous amylase increases with the age of bird. This is like a previous report by Onderci et al. (2006) who observed increased villi length in broilers fed diets supplemented with two strains of amylase producing bacteria. Therefore, it is possible that the observed improvements in growth performance of the birds may not only be due to increased release of simple sugars from starch digestion but rather to

the changes in the morphology of the small intestine which would have favored nutrient absorption. Similarly, Ritz et al. (1995) reported that α-amylase supplementation increases the length of the villi within the jejunal, and ileal sections of 21-d-old turkey poults fed corn-soybean meal diets. Although there were changes in gut morphology and increases in starch degradability, it is pertinent to note that exogenous amylase did not affect the expression of glucose transporters in the jejunum in any of the growth phases. While this observation is not clear, it was reported that the rate of digestion of starch differs along the length of the chicken intestine (Weurding et al., 2001). This would lead to variation in the amount of glucose available for absorption at each different intestinal site and could have resulted in the lack of change in the glucose transporter expressions. In the current study, only the mid-jejunal section was assayed for glucose transporters.

The viscosity of the jejunal digesta was significantly reduced by amylase supplementation in all phases, except during d 11 to 21. This reduction in viscosity is however in dissonance to previous reports (Zanella et al., 1999; Garcia et al., 2003) for corn-SBM based diets. Corn and soybeans, compared to barley or wheat, are relatively low in non-starch polysaccharides and therefore should not present problems of viscosity. Given they make the bulk of the experimental diets for chickens, it is curious that amylase supplementation alone, and not as part of a carbohydrase cocktail, affected the viscosity of the digesta. However, due to the interfering effects of the branched amylopectin α-1,6 bonds on crystal formation, waxy starches with a high proportion of amylopectin relative to amylose tend to be more amorphous and soluble. This could create viscous gels in the intestine of the birds and interfere in the digestion and absorption of nutrients (Gohl and Gohl, 1977; Van der klis and Van Voorst, 1993). Hence, the improvements observed in nutrient digestibility by exogenous amylase may also have been partially due to a reduction in the viscosity of the digesta and a greater access to digestive enzymes. Again, it is not clear why the viscosity of the jejunal digesta was increased by amylase supplementation during d 11 to 21compared to other growth phases.

Anatomically, the relative pancreas weight decreased with age of birds and are consistent with reports by Nitsan et al. (1991a, b). However, there was no effect of α-amylase supplementation on the relative pancreas weight, for all growth phases. This response is similar to previous report by Onderci et al. (2006). However, it is in dissonance to Garcia et al. (2003) that reported a reduction in relative pancreas weight at d 7 and d 28 post hatching due to amylase supplementation. The pancreas produces and secretes digestive enzymes which are consequently

affected by the concentration of enzymes and substrates or products of their hydrolysis in the lumen of the small intestine (Moran, 1985). Therefore, a reduction in pancreas weight has been related to less secretion of endogenous enzymes, which is partly due to the presence of exogenous enzyme in the intestine.

In conclusion, the data showed that exogenous amylase improves growth performance and apparent nutrient digestibility of broiler chickens fed diets containing mostly corn and SBM. Additionally, the study showed that the apparent ileal digestibility of starch, viscosity of the jejunal digesta and intestinal amylase activity is age-of-bird dependent. However, there were marked deviations in the overall responses of birds during d 11 to 21 post hatching compared to other growth phases and this observation warrants further investigations.

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Table 4.1 Ingredient and calculated nutrient composition of experimental diets, as-fed basis.

Growth phase	d 0 to 11		d 11 to 21		d 21 to 42		d 42 to 56	
d post hatching:								
Amylase, KNU/kg:	0	80	0	80	0	80	0	80
Ingredients, g/kg								
Corn	576.2	556.2	623.8	603.8	638.6	618.6	663.3	643.3
Soybean meal	340.0	340.0	291.0	291.0	271.0	271.0	245.0	245.0
Soybean oil	6.5	6.5	9.5	9.5	18.5	18.5	18.5	18.5
Monocalcium phosphate[1]	10.2	10.2	9.0	9.0	8.0	8.0	8.5	8.5
Limestone[2]	12.2	12.2	11.5	11.5	10.5	10.5	11.0	11.0
Salt	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Vitamin-mineral premix[3]	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
$_{DL}$-Methionine	2.0	2.0	1.7	1.7	1.5	1.5	1.5	1.5
$_{L}$-Lysine HCl	1.9	1.9	2.0	2.0	0.9	0.9	1.2	1.2
$_{L}$-Threonine	0.0	0.0	0.5	0.5	0.0	0.0	0.0	0.0
Amylase premix[4]	0.0	20.0	0.0	20.0	0.0	20.0	0.0	20.0
Titanium dioxide premix[5]	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0
Phytase premix[6]	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Total	1,000.0	1,000.0	1,000.0	1,000.0	1,000.0	1,000.0	1,000.0	1,000.0
Calculated composition								
Crude protein, g/kg	220.2	220.2	200.8	200.8	190.8	190.8	180.6	180.6
ME, kcal/kg	3,036.6	3,036.6	3,108.2	3,108.2	3,180.1	3,180.1	3,203.5	3,203.5
Ca, g/kg	7.3	7.3	6.7	6.7	6.1	6.1	6.4	6.4
P, g/kg	6.0	6.0	5.6	5.6	5.3	5.3	5.3	5.3
Non-phytate P, g/kg	3.4	3.4	3.1	3.1	2.8	2.8	2.9	2.9
Ca: total P	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
Ca: non-phytate P	2.2	2.2	2.2	2.2	2.2	2.2	2.2	2.2
Starch, g/kg	452.8	452.8	483.0	483.0	492.1	492.1	507.7	507.7

Table 4.1 continued

Total amino acids, g/kg								
Arg	14.2	14.2	12.6	12.6	12.0	12.0	11.2	11.2
His	5.8	5.8	5.3	5.3	5.0	5.0	4.8	4.8
Ile	9.0	9.0	8.1	8.1	7.7	7.7	7.2	7.2
Leu	18.9	18.9	17.5	17.5	16.9	16.9	16.2	16.2
Lys	13.1	13.1	11.9	11.9	10.5	10.5	10.0	10.0
Met	5.4	5.4	4.8	4.8	4.5	4.5	4.4	4.4
Cys	3.6	3.6	3.3	3.3	3.2	3.2	3.0	3.0
Phe	10.3	10.3	9.3	9.3	8.9	8.9	8.4	8.4
Tyr	8.5	8.5	7.7	7.7	7.3	7.3	6.9	6.9
Thr	8.1	8.1	7.9	7.9	7.0	7.0	6.6	6.6
Trp	2.9	2.9	2.6	2.6	2.4	2.4	2.2	2.2
Val	10.0	10.0	9.1	9.1	8.7	8.7	8.3	8.3
Met + Cys	8.9	8.9	8.1	8.1	7.7	7.7	7.4	7.4
Phe + Tyr	18.8	18.8	17.0	17.0	16.2	16.2	15.3	15.3
Analyzed composition								
Amylase (KNU/kg)[7]	LOD	84	LOD	89	LOD	81	LOD	83

[1] Contained 16% Ca, 21% P.

[2] Contained 38% Ca.

[3] Supplied the following per kg diet: vitamin A, 5,484 IU; vitamin D3, 2,643 ICU; vitamin E, 11 IU; menadione sodium bisulfite,4.38 mg; riboflavin, 5.49 mg; pantothenic acid, 11 mg; niacin, 44.1 mg; choline chloride, 771 mg; vitamin B12, 13.2 ug; biotin, 55.2 ug; thiamine mononitrate, 2.2 mg; folic acid, 990 ug; pyridoxine hydrochloride, 3.3 mg; I, 1.11 mg; Mn, 66.06 mg; Cu, 4.44 mg; Fe, 44.1 mg; Zn, 44.1 mg; Se, 300 ug.

[4] Provided 80 kilo-Novo alpha amylase units (KNU) per kg of diet (Ronozyme HiStarch; DSM Nutritional Products, Kaiseraugst, Switzerland)

[5] 1 g of Titanium dioxide added to 4 g corn.

[6] Provided 1,000 FYT /kg of diet (Ronozyme HiStarch; DSM Nutritional Products, Kaiseraugst, Switzerland)

[7] LOD = limit of detection

Table 4.2 Primers used in real-time quantitative PCR

Genes	Primer sequence (5' to 3')	Gene Bank ID	Reference
Housekeeping gene			
GAPDH	F: TCCTAGGATACACAGAGGACCA R: CGGTTGCTATATCCAAACTCA	ENSGALG00000014442*	Grenier et al., 2015
Markers of glucose transport			
SGLT-1	F: GATGTGCGGATACCTGAAGC R: AGGGATGCCAACATGACTG	AJ236903	Hu et al., 2009
GLUT-1	F: GGCTTTGTCCTTTGAGATGC R: CGCTTTGTTCTCCTCATTGC	L07300	Humphrey et al. (2004)
GLUT-2	F: TGTTCAGCTCCTCCAAGTACC R: ACAACGAACACATACGGTCC	Z22932	Humphrey et al. (2004)

F, forward primer; R, reverse primer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase, SGLT-1, sodium-dependent glucose co-transporter 1, GLUT , glucose transporter. *Sequence obtained from Ensembl chicken genome data resources.

Table 4.3 Effect of amylase supplementation on growth performance of broiler chickens in different growth phases[1]

Growth phase d post hatching:	d 0 to 11		d 11 to 21		d 21 to 42		d 42 to 56			*P*-value		
Amylase, KNU/kg:	0	80	0	80	0	80	0	80	SEM	Amylase	Phase	A × P
Initial BW, g	52	52	403	403	1,145	1,145	3,113	3,112	0.6	0.689	< 0.001	0.907
Final BW, g	295	298	1,072	1,089	3,254	3,313	4,601	4,685	17.5	0.003	< 0.001	0.105
BW gain, g/bird	243	245	668	686	2,109	2,167	1,488	1,573	17.0	0.002	< 0.001	0.081
Feed intake, g/bird	369	365	1,112	1,146	3,934	3,870	4,540	4,527	46.8	0.726	< 0.001	0.774
G: F, g/kg	658	672	601	598	536	560	328	348	6.0	0.003	< 0.001	0.147

[1] Data are least square means of 8 replicates cages; A, amylase; P, phase.

Table 4.4 Effect of amylase supplementation and growth phase on nutrient digestibility and retention responses of broiler chickens[1]

Growth phase, d post hatching:	d 0 to 11		d 11 to 21		d 21 to 42		d 42 to 56			*P*-value		
Amylase, KNU/kg:	0	80	0	80	0	80	0	80	SEM	Amylase	Phase	A × P
Ileal digestibility												
DM, %	73.6	76.4	72.6	75.4	71.5	73.4	70.6	75.7	0.75	< 0.001	0.014	0.213
Starch, %	95.5	98.2	96.5	97.3	96.0	98.2	96.6	98.7	0.24	< 0.001	0.007	0.003
Energy, %	70.6	75.1	73.5	75.9	71.8	74.3	71.3	76.5	0.75	< 0.001	0.015	0.286
IDE, kcal/kg DM	3,184	3,289	3,397	3,432	3,266	3,411	3,321	3,478	34.1	< 0.001	0.289	0.715
Total tract retention												
DM, %	74.7	79.0	73.5	77.3	71.7	75.0	74.8	76.4	0.46	< 0.001	< 0.001	0.041
Starch, %	98.0	98.2	97.7	98.1	97.6	98.3	98.1	98.3	0.15	0.010	0.087	0.576
AME, %	76.9	80.3	76.3	78.9	75.6	78.1	76.0	79.5	0.50	< 0.001	0.022	0.661
AME, kcal/kg DM	3,466	3,514	3,523	3,566	3,512	3,586	3,539	3,612	23.2	0.001	0.008	0.867
Nitrogen	71.7	76.0	71.1	74.7	70.4	72.8	72.3	73.8	0.61	< 0.001	0.009	0.120
AMEn, %	72.1	75.0	71.4	73.5	70.9	73.1	71.0	74.2	0.48	< 0.001	0.024	0.650
AMEn, kcal/kg DM	3,252	3,284	3,297	3,324	3,291	3,357	3,310	3,375	22.1	0.005	0.016	0.722

[1] Data are least square means of 8 replicates cages; A, amylase; P, phase; IDE, ileal digestible energy.

Table 4.5 Effect of amylase supplementation and growth phase on pancreas weight, gut morphology, and viscosity of jejunal digesta of broiler chickens[1]

Growth phase d post hatching:	d 0 to 11		d 11 to 21		d 21 to 42		d 42 to 56			*P*-value		
Amylase, KNU/kg:	0	80	0	80	0	80	0	80	SEM	Amylase	Phase	A × P
Villus height, μm	959.8	982.6	1,154.7	1,246.6	1,067.3	1,481.7	1,427.8	1,757.3	79.13	0.001	< 0.001	0.058
Crypt depth, μm	124.0	147.2	124.3	141.4	164.5	180.9	148.5	173.1	13.06	0.036	0.012	0.985
Villus: crypt ratio	7.9	7.0	9.3	9.1	6.6	8.3	10.7	10.3	0.65	0.937	< 0.001	0.217
Pancreas, g	1.11	1.06	2.36	2.35	3.94	4.09	4.73	4.54	0.079	0.685	< 0.001	0.224
Pancreas, g/kg BW	3.14	3.06	2.03	2.02	1.14	1.19	0.97	0.92	0.061	0.609	< 0.001	0.774
Viscosity, mPas	3.30	3.04	2.78	2.82	2.82	1.94	3.04	2.98	0.066	< 0.001	< 0.001	< 0.001

[1] Data are least square means of 8 replicates cages; A, amylase; P, phase.

Table 4.6 Effect of amylase supplementation and growth phase on amylase activity and mRNA expression of glucose transporters in the jejunal tissue of broiler chickens[1]

Growth phase d post hatching:	d 0 to 11		d 11 to 21		d 21 to 42		d 42 to 56			*P*-value		
Amylase, KNU/kg:	0	80	0	80	0	80	0	80	SEM	Amylase	Phase	A × P
Amylase activity												
Duodenum, u/ml	174.20	258.80	126.30	131.50	91.90	122.80	143.90	178.60	5.081	< 0.001	< 0.001	< 0.001
Pancreas, u/mg	33.60	17.54	17.80	19.18	28.60	16.43	17.70	11.30	1.821	< 0.001	< 0.001	< 0.001
Glucose markers												
GLUT-1	0.83	0.72	0.60	1.20	1.35	1.07	0.99	1.01	0.157	0.623	0.096	0.058
GLUT-2	1.13	1.35	1.12	0.87	1.06	0.67	1.27	0.93	0.269	0.336	0.555	0.713
SGLT-1	0.64	1.08	1.14	1.02	0.80	0.86	0.98	0.88	0.325	0.768	0.873	0.772

[1] Data are least square means of 8 replicates cages; GLUT, glucose transporter; SGLT-1, sodium-dependent glucose co-transporter 1

124

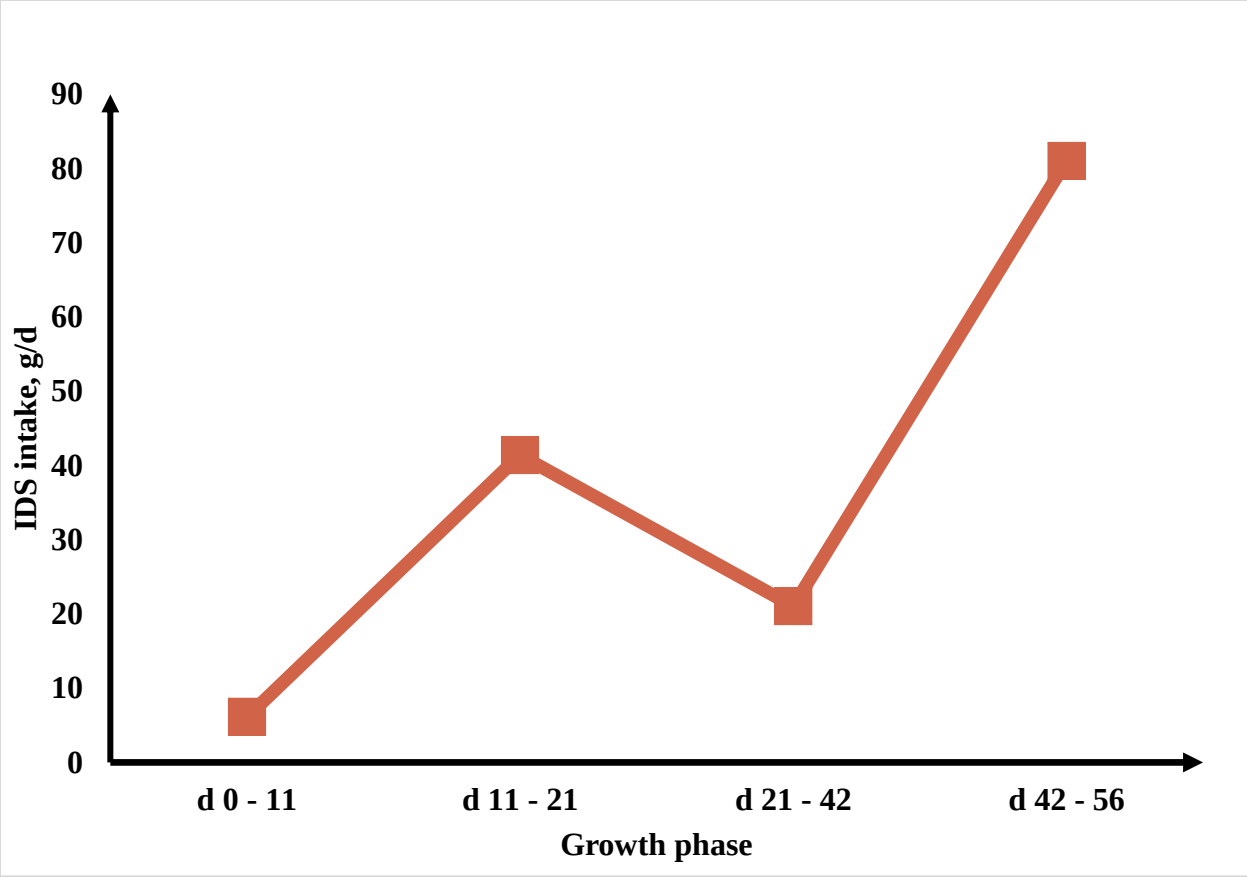

Figure 4.1 Changes in ileal digestible starch (IDS) intake of broiler chickens in the four growth phases as a result of α-amylase supplementation. Square data points represent the mean α-amylase effect on IDS intake, relative to the control diet, in the four growth phases.

CHAPTER 5. INTESTINAL STARCH AND ENERGY DIGESTIBILITY IN BROILER CHICKENS FED DIETS SUPPLEMENTED WITH ALPHA-AMYLASE

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5.1 Abstract

Dietary starch is the major energy source for broiler chickens, therefore relevant information on its intestinal utilization is important. The current study was designed to evaluate intestinal starch and energy digestibility of broiler chickens fed diets supplemented with *α*-amylase. A total of 240 d-0 male broiler chicks were randomly assigned to 3 nutritionally adequate corn-soybean based experimental diets comprising 3 levels of α-amylase supplementation (0, 80, or 160 KNU/kg diet). Each treatment comprised 8 replicate cages of 10 birds each. At d 21 post hatching, digesta was collected from 4 intestinal sites: anterior jejunum (AJ), posterior jejunum (PJ), anterior ileum (AI) and posterior ileum (PI). Increasing α- amylase supplementation linearly improved ($P < 0.01$) overall BW gain and feed efficiency of the birds. There were linear and quadratic ($P < 0.01$) responses of increasing α- amylase supplementation on starch and energy digestibility at the PJ and AI. Total tract digestibility of starch increased ($P < 0.05$) with increasing α- amylase supplementation. Starch disappearance and digestible energy (kcal/kg) linearly increased ($P < 0.01$) with digesta flow from AJ to PJ as dietary α- amylase supplementation increased. There were linear ($P < 0.01$) and quadratic ($P < 0.05$) effects of increasing α- amylase supplementation on villus height in the jejunum. The viscosity of the jejunal digesta decreased ($P < 0.05$) with increasing dietary α- amylase supplementation. The results from this study showed the efficacy of exogenous amylase in improving growth performance, starch, and energy digestibility in broiler chickens. Furthermore, the digestibility of starch and energy and the impact of the exogenous amylase was higher at the posterior jejunum compared with other intestinal sites.

Key words: broiler chickens, digestibility, energy, enzyme, starch

5.2 Introduction

Among the nutrients in poultry feed ingredients and diets, starch is quantitatively the most important source of energy. However, starch degradability is affected by the proportion of amylose (Moran, 1982) and its variability in corn and other cereal grains can significantly influence the AMEn content of feedstuffs to livestock (Wiseman et al., 2000; Tester et al., 2004). Additionally, there are physical barriers in cell walls of feed ingredients that restricts enzyme access to substrates (Ravindran, 2013). Therefore, the use of exogenous carbohydrases such as xylanases, amylases, and glucanases as feed additives, have been reported to improve energy utilization and the performance of non-ruminant animals (Gracia et al., 2003; Kocher et al., 2003; Olukosi and Adeola, 2008). However, some reports have not found effects in response to these enzyme combinations (Hong at al., 2002; Olukosi et al., 2007). Factors not directly related to starch itself may also affect its digestibility, and the dynamics of starch digestion relative to bird intestinal efficiency may have considerable nutritional consequences. Previously, Weurding et al. (2001) showed that site, rate, and extent of starch digestion in the small intestine of broiler chickens may differ considerably between a wide range of feedstuffs and concluded that rapid starch digestion may lead to the same extent of starch digestion as gradual starch digestion, but the amount of starch digested at specific sites of the intestine would differ. The differences that exist in the site of starch digestion may therefore have metabolic consequences that affect feed utilization in broiler chickens. There are few reports on the impact of exogenous amylase on starch and energy utilization in specific intestinal sections. Therefore, the hypothesis of the current study was that starch and energy digestibility would vary between intestinal sections and would be affected by exogenous amylase administration. The objective of the current study was to evaluate the influence of dietary α-amylase supplementation on the digestibility of starch and energy in the different intestinal sites in broiler chickens from d 0 to 21 post hatching.

5.3 Materials and Methods

The protocol of the animal experiment was reviewed and approved by the Purdue University Animal Care and Use Committee (#: 1112000389).

5.3.1 Experimental Birds, Housing, and Diets

A total of 240 male 0-d-old broiler chicks (Cobb 500, Siloam Springs, AR) were obtained from a commercial hatchery. The birds were individually tagged, weighed, and raised in heated battery brooders (model SB 4 T; Alternative Design Manufacturing, Siloam Springs, AR) with temperature and lighting maintained as previously described by Park et al. (2017). Birds were allotted to 3 dietary treatments (Table 5.1) in a randomized complete block design, consisting of 8 replicates and 10 birds per replicate. The diets contained 3 levels of α-amylase supplementation (0, 80 or160 KNU/kg diet of Ronozyme HiStarch, DSM Nutritional Products, Switzerland). All diets were corn-soybean (**SBM**) based and formulated to meet breeder nutrient specifications. Mash diets and water were provided *ad libitum* throughout the experimental period. Titanium dioxide was used as an indigestible marker, and all diets contained phytase (Ronozyme HiPhos, DSM Nutritional Products, Switzerland) at 1,000 FYT/kg.

5.3.2 Sampling Procedures

On d 19 post hatching, trays under the cages were lined with waxed paper for a 3-d excreta collection. On d 21 post hatching, all birds per cage were individually weighed and euthanized by CO_2 asphyxiation and exsanguination. Entire ileal and jejunal segments were excised from each bird. Specifically, each of jejunum and ileum was divided into 2 sections of equal length, namely: anterior jejunum (AJ), posterior jejunum (PJ), anterior ileum (AI) and posterior ileum (PI). The digesta was collected from each section by flushing with distilled water into plastic containers and stored at –20°C prior to analysis. For viscosity measurement, the entire jejunal content from 1 bird per replicate with median BW was gently squeezed into plastic tubes and stored at –20°C prior to analysis.

5.3.3 Viscosity Measurements

The jejunal digesta was thawed on ice and approximately 10 g of sample per replicate was placed in a 50 mL plastic centrifuge tube, vortexed for 10 s and centrifuged at 10,000 × g for 10 min at 4°C. The supernatant was transferred into a 2-mL sample cup and placed in a water bath (Precision, GCA Corp., College Park, MD) that had been preheated to 40°C until the temperature of the sample equilibrated with that of the water in the water bath. The viscosity, in centipoise (cP),

of these samples was determined using a viscometer (Vibro viscometer, model SV-1A, A&D Instruments Ltd, Oxfordshire, United Kingdom).

5.3.4 Intestinal Morphological Analysis

Mid-jejunal segments were collected from 1 bird per replicate with median BW, flushed with ice-cold 10% phosphate-buffered saline (VWR International, Radnor, PA) and fixed in 10% neutral buffered formalin (VWR International, Radnor, PA) for approximately 30 d. Subsequently, the samples were dehydrated with ethanol (VWR International, Radnor, PA), cleared with Sub-X® (Polysciences, Inc., Warrington, PA) and placed in paraffin (Polyfin paraffin, Sigma Polysciences, St. Louis, MO). The segments (5 μm) were stained with hematoxylin and eosin at the Purdue Histology and Phenotyping Laboratory (Purdue University, West Lafayette, IN USA). Villus height and crypt depth were measured from 5 complete, vertically oriented villi per slide and subsequently, the villus height to crypt depth ratio was calculated. All measurements were performed under a binocular light microscope (National Optical and Scientific Instruments, Inc., Schertz, TX).

5.3.5 Chemical Analyses

The intestinal digesta and excreta samples were freeze-dried for 96 h and subsequently ground to pass through a 0.5-mm screen (Retsch ZM 100, GmbH, Haan, Germany). Diets, intestinal digesta and excreta samples were analyzed for DM analysis by drying overnight at 105°C (Precision Scientific Co., Chicago, IL; method 934.01; AOAC, 2006). Nitrogen content of the samples was subsequently determined by combustion (TruMac N; LECO Corp., St. Joseph, MI, USA; method 990.03; AOAC, 2000) with EDTA as a calibration standard. Gross energy (**GE**) concentration in diets, ileal digesta and excreta samples was determined by isoperibol bomb calorimeter (Parr 1261; Parr 105 Instrument Co., Moline, IL). Megazyme total starch determination kit (Method 996.11; AOAC, 2000) was used to analyze samples for starch. Titanium concentration was measured on a UV spectrophotometer following the method of Short et al. (1996).

The apparent digestibility of nutrient in the intestinal digesta and excreta were calculated with the index method, according to the following equation:

$$AD, \% = 100- [(Ti_I/Ti_O) \times (N_O/N_I) \times 100]$$

where AD is the apparent digestibility of nutrient, Ti_I is Titanium concentration in diets; Ti_O is Titanium concentration in output (intestinal digesta or excreta); N_O is the concentration of nutrient in the intestinal digesta or excreta, and N_I is the concentration of a nutrient in the diet.
All digestibility values are expressed as grams per kilogram of DM.

The digestible energy and AME (kcal/kg DM) of the diet was calculated as the product of the coefficient and GE concentrations (kcal/kg) in the diet. The AMEn was calculated by correcting for zero N retention using a factor of 8.22 kcal/g (Hill and Anderson, 1958), as described by Zhang and Adeola (2017).

5.4 Results

The effect of α-amylase supplementation on growth performance of broiler chickens is presented in Table 5.2. Increasing dietary α- amylase supplementation led to linear and quadratic increases ($P < 0.01$) in BW at 21d and linear increases ($P < 0.01$) in BW gain from 0 to 21d. Feed efficiency in the overall period of the study (d 0 – 21) was linearly improved ($P < 0.01$) with increasing levels of α- amylase. In Table 5.3, increasing dietary α- amylase supplementation resulted in linear increases in the digestibility of starch at the PJ ($P < 0.05$), AI **(***P* < 0.01), PI ($P <$ 0.01) and total tract ($P < 0.05$). There was a quadratic response ($P < 0.01$) of increasing α- amylase supplementation on starch digestibility in the PJ and AJ. There were linear and quadratic increases ($P < 0.01$) in the digestibility of energy (DE; %) at the PJ, AI and PI sites associated with increasing α- amylase concentration. There was no effect of α- amylase supplementation on AME (kcal/kg). However, there was a tendency (P = 0.06) for improvement in AMEn (kcal/kg) as a result of α-amylase supplementation. Increasing α- amylase supplementation resulted in linear ($P < 0.01$) and quadratic ($P < 0.05$) response in the villus height and linearly reduced ($P < 0.05$) the viscosity in the jejunum (Table 5.4). Figure 5.1 – 5.4 shows the nutrient disappearance in the gastrointestinal tract of birds fed diets supplemented with α-amylase. As digesta flows from AJ to PJ site, an increasing α- amylase supplementation resulted in linear improvements ($P < 0.01$) in the disappearance of starch and DE. In contrast, increasing α- amylase supplementation resulted in a linear decrease ($P < 0.01$) in starch disappearance with digesta flow from PI to total tract site.

5.5 Discussion

The need to improve and optimize the efficiency of starch digestion is an integral part of animal nutrition. Starch digestion in the digestive tract of livestock is affected by both intrinsic and external factors (Silveira et al., 2007; Witt et al., 2010) and although starch is mainly digested in the small intestine (Wiseman, 2006), variations along discrete intestinal regions may impact the overall starch utilization and animal performance, and also the intestinal microbiota activities (Bolhius et al., 2008; Zijlstra et al., 2012). In the current study, we examined differences in starch and energy digestibility at different intestinal sites and the extent to which exogenous amylase supplementation may influence these digestibility responses.

The results from the current study showed that exogenous α-amylase supplementation improved the overall BW gain and feed efficiency of the birds at 21 d post hatching. This observation is similar to previous reports in which α-amylase was either supplemented separately (Jiang et al., 2008) or included in a cocktail (Olukosi et al., 2008) improved the weight gain and feed efficiency of the 21-d-old birds. Similarly, Stefanello et al. (2019) reported improvements in growth performance when broiler chickens were fed corn-SBM-based diets supplemented with graded concentrations of α-amylase. The improvement in growth performance may be associated with the observed increases in starch and energy digestibility in the gastro-intestinal tract as a result of α-amylase supplementation and corroborates several previous reports for corn-SBM based diets (Gracia et al., 2003; Cowieson et al., 2019; Stefanello et al., 2019; Woyengo et al., 2019). Although broiler chickens have high innate capacity to digest dietary starch, as observed in the current study, it could be limited by several factors such as inadequacies in endogenous amylases, the nature of the starch crystals and issues around extraction of glucose from the lumen via Na-dependent transport systems. Krogdahl and Sell (1989) suggested that poultry develop an increased capacity to digest starch as the intestinal tract matures, by increasing pancreatic amylase production in response to elevated starch intake. However, Noy and Sklan (1995) found that production of amylase in the pancreas is not clearly correlated with the levels of starch digestion. Comparing birds at 14 and 42 days of age, they found that although starch intake increased by over 200%, pancreatic amylase output increased by only 95%. Croom et al (1999) previously noted that intestinal mass and pancreatic tissue become increasingly smaller proportion of the metabolic weight of birds as they grow older. This has led to the assumption that birds may be responsive to augmentation of endogenous amylase systems with exogenous microbial amylase supplementation.

While the cooperativity of exogenous and endogenous amylase is not entirely clear, previous work by Pedersen et al (2015) showed extensive pore formation and collapse of starch granule structure when only pancreatin was used concurrently with exogenous bacterial amylase and vice versa. This could partly explain the improvements in intestinal starch digestibility in the birds as a result of exogenous amylase supplementation in the current study. Given that the jejunum is the site with the largest capacity for nutrient absorption in birds, it is possible that the exogenous amylase action on starch degradation upregulated the extraction of glucose monomers from the lumen via Na-dependent transport systems which resulted in linear increase in starch disappearance from the AJ to PJ sites.

Interestingly, there was no effect of exogenous amylase on starch and energy digestibility in the anterior jejunum compared with other intestinal sites. There was a noteworthy tendency for a decrease in starch and energy digestibility with increasing α- amylase supplementation in the anterior jejunum. This suggests a delayed effect of the exogenous amylase on nutrient digestibility or an ineffective mixing of substrates and digestive enzymes. This asynchrony in response could also be due to variations in the digesta transit and retention times within the intestinal segments. Alternatively, it is possible that the relatively higher concentration of pancreatic amylase, which is secreted into the duodenum, carried over to the anterior jejunum, essentially masking any additional effect of the exogenous amylase. Another possibility is that the exogenous amylase works best in the posterior sections of the gastrointestinal tract but downregulates pancreatic amylase output which thus resulted in lower starch digestibility in the anterior jejunum.

Furthermore, the current study showed a shift in the site of starch digestibility from the distal to the proximal intestinal segments and this corroborates a previous report by Svihus (2014). At the end of the anterior jejunum, approximately 70% of dietary starch have been digested, which further confirms the high innate ability of the chicken to digest starch, as previously described (Moran, 1982). The differences in the digestibility indices, which diminished with digesta flow towards the distal parts suggests high variation in digestion rates within the intestine. Weurding et al (2001) posited that although the amount of starch digested at different intestinal sites differs, variations in the rates of digestion may have metabolic consequences that influence feed efficiency. For example, Weurding (2002) observed that slowly digestible starch in broiler diets benefits feed efficiency and Liu et al. (2014) noted the benefit of starch-protein digestibility dynamics on improvements in feed efficiency. This mechanism is not entirely clear but may be associated with

energy metabolism in intestinal epithelial cells. While glucose is a more effective energy source for the enterocytes in the intestinal tract, amino acids (notably glutamine) are readily catabolized as an alternative energy source, especially in the absence of glucose. Therefore, higher rates of starch digestion in proximal intestinal regions may be deleterious to amino acid digestion and overall feed efficiency of the bird. However, contrary to this, birds fed the control diet had higher starch disappearance towards the more distal intestinal regions but had lower feed efficiency compared to the enzyme supplemented groups. While this observation remains unclear, it could be attributed to the relatively lower starch and energy digestibility of the control birds, further limited by a decrease in the absorptive capacity in the jejunum, compared to the enzyme supplemented groups. Given the role of the jejunum as the site of maximal intestinal absorption, improvements in nutrient digestion and absorptive capacity by supplemental enzymes could favor growth performance of the birds.

Although the current study showed that exogenous amylase substantially shifts the site of starch digestion to the posterior jejunum (about 15% improvement from AJ to PJ), there were also localized improvements in the more distal regions. This suggests a protein sparing effect of the exogenous amylase by generating more sustained circulating levels of glucose to the lower small intestine, which would spare amino acids from catabolism and therefore increase feed efficiency and energy utilization. About 98% of the dietary starch was digested at the end of the ileum, which is consistent with previous reports (Svihus, 2001; Hetland et al., 2003; Svihus et al., 2004; Zalenka and Ceresnakora 2005). Although relatively high, exogenous amylase supplementation led to a linear increase in starch digestibility at the posterior ileum. This high capacity of broiler chickens for starch digestion suggests a balance of the gut absorptive capacity with post-absorptive tissue metabolism. Croom et al (1999) previously suggested that intensive genetic selection for growth in broiler chickens may have uncoupled intestinal nutrient delivery from increased post-absorption nutrient demand, and therefore absorption of nutrients could be a potential rate-limiting factor in survival, growth, and feed conversion in birds. However, the improvement in starch digestion by the exogenous amylase, at the end of the ileum is marginal (about 1.8%) and may not fully explain the increased feed efficiency and body weight responses of the birds. While this remains unclear, it is possible that variations in starch digestion rates along specific intestinal sections (Weurding, 2002), and improvement in the absorptive capacity of the jejunum by the exogenous amylase via increased villi length, could enhance the utilization of other nutrients in the diet (e.g., dietary fat

and protein). For instance, Jiang et al. (2008) noted that supplemental amylase increased the amylase, protease and trypsin activity in the duodenum and jejunum, which marked implications on growth performance. Furthermore, it has been reported that supplemental amylases could improve fat digestibility (Yuan et al., 2017). This suggests that growth performance responses by exogenous enzymes may not always be solely related to greater degradation of the target substrates (Viera et al., 2015), and could partly explain our observation.

The undigested starch fractions, which contains predominantly resistant starch, may also serve as substrate for the exogenous amylase. Schramm et al. (2016) noted a significant increase (75% vs. 81%) in the digestibility of the resistant starch fraction in a corn-SBM based diets not supplemented versus supplemented with an exogenous amylase. This could possibly explain the improvements observed in the total tract starch digestibility with exogenous amylase administration, which corresponds to slight increases in AME (kcal/g) and AMEn (kcal/g). This observation is consistent with previous reports by Svihus (2011) and Stefanello et al. (2019) of a strong correlation between AME and total tract starch digestibility. Although undigested starch may also serve as substrate for bacteria present in the hind gut, starch fermentation is energetically less efficient than enzymatic starch digestion in the small intestine (Dierick et al., 1989). Additionally, Kussaibati et al. (1982) reported a similarity in the undigested starch fraction between conventional and germ-free chicks. Therefore, this undigested portion could provide more substrate for the exogenous enzyme. However, it is possible that even when glucose is successfully produced from starch in the hind gut, it may exceed the absorptive capacity of the bird consequently resulting in no changes in energy utilization.

The current study showed a reduction in the viscosity of the jejunal digesta in response to an increasing α- amylase supplementation. This is contrary to previous reports that show a lack of effect of α- amylase supplementation on intestinal digesta viscosity (Garcia et al., 2003). The reason for this observation is not clear as corn-SBM-based diets are low in non-starch polysaccharides and should not present viscosity issues when compared with barley or wheat. However, starch is an extremely heterogeneous structure (Tester et al., 2004), and the ratio between amylose and amylopectin in starch determines whether starch may be categorized as high amylose or waxy. Waxy starch, which has a high proportion of amylopectin relative to amylose, tend to be more amorphous and soluble. However, the chain length and organization of internal unit chains of amylopectin influence the gelatination and pasting properties of starch (Vamadevan and Bertoft,

2020), which could contribute to viscosity (Klaochanpong et al., 2015). Pirgozliev et al. (2010) previously reported a reduced growth performance and higher viscosity of the jejunal digesta when birds were fed a maize starch mixture with lower amylose content. Although modest, the reduction in viscosity in the current study could be as a result of the disruption in the structure and composition of the native starch granule.

It is well established that viscosity of intestinal content interferes with digestion and absorption (Cowieson, 2010). Therefore, it is not far-fetched to assume that the improvements in starch and energy digestibility by exogenous amylase may have been partially mediated by reducing the digesta viscosity and a greater access to digestive enzymes. Furthermore, exogenous amylase increased the villus height in the jejunal tissue by about 30%. It is safe to assume that this increase in absorptive capacity would have marked implications on nutrient utilization in the chicken intestine, as was observed in the study.

In conclusion, the current study showed significant improvements in the growth performance and nutrient utilization of broiler chickens fed diets supplemented with α-amylase. Although the digestibility of starch and energy varied with intestinal site, the efficacy of the α-amylase supplementation was greater within the jejunum compared with other intestinal regions. Given the potential impact of feed form on bird's responses, the results from this study would require careful interpretation. While pelleted feed, as opposed to the mash, increases feed consumption and efficiency in birds, factors such as variations in pellet quality could affect the digestibility of starch, and other nutrients. Moreover, the pelleting process remains a potentially aggressive process on the stability of exogenous feed enzymes. Therefore, further studies are suggested to evaluate and compare the influence of exogenous amylase on the dynamics of intestinal starch digestion using pelleted feed.

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Table 5.1 Ingredient and calculated nutrient composition of experimental diets, as-fed basis.

	α- amylase, KNU/kg		
Ingredients, g/kg	0	80	160
Corn	555.6	535.6	515.6
Soybean meal	360.0	360.0	360.0
Soybean oil	5.5	5.5	5.5
Monocalcium phosphate[1]	11.0	11.0	11.0
Limestone[2]	13.0	13.0	13.0
Salt	3.0	3.0	3.0
Vitamin-mineral premix[3]	3.0	3.0	3.0
DL-Methionine	2.0	2.0	2.0
L-Lysine HCl	1.9	1.9	1.9
Amylase premix[4]	0.0	20.0	40.0
Titanium dioxide premix[5]	25.0	25.0	25.0
Phytase premix[6]	20.0	20.0	20.0
Total	1000.0	1000.0	1000.0
Calculated Nutrients & Energy			
Crude protein, g/kg	228.2	228.2	228.2
ME, kcal/kg	3005.5	3005.5	3005.5
Ca, g/kg	7.8	7.8	7.8
P, g/kg	6.2	6.2	6.2
Non-phytate P, g/kg	3.6	3.6	3.6
Ca: total P	1.3	1.3	1.3
Ca: non-phytate P	2.2	2.2	2.2
Starch, g/kg	439.6	439.6	439.6
Total amino acids, g/kg			
Arg	14.8	14.8	14.8
His	6.0	6.0	6.0
Ile	9.4	9.4	9.4
Leu	19.4	19.4	19.4
Lys	13.7	13.7	13.7
Met	5.5	5.5	5.5
Cys	3.7	3.7	3.7
Phe	10.7	10.7	10.7
Tyr	8.8	8.8	8.8
Thr	8.5	8.5	8.5
Trp	3.0	3.0	3.0
Val	10.4	10.4	10.4
Met + Cys	9.1	9.1	9.1
Phe + Tyr	19.5	19.5	19.5
Analyzed composition			
Amylase (KNU/kg)[7]	LOQ	61	134

Table 5.1 continued

[1] 16% Ca, 21% P. [2] 38% Ca.
[3] Supplied the following per kg diet: vitamin A, 5,484 IU; vitamin D3, 2,643 ICU; vitamin E, 11 IU; menadione sodium bisulfite,4.38 mg; riboflavin, 5.49 mg; pantothenic acid, 11 mg; niacin, 44.1 mg; choline chloride, 771 mg; vitamin B12, 13.2 ug; biotin, 55.2 ug; thiamine mononitrate, 2.2 mg; folic acid, 990 ug; pyridoxine hydrochloride, 3.3 mg; I, 1.11 mg; Mn, 66.06 mg; Cu, 4.44 mg; Fe, 44.1 mg; Zn, 44.1 mg; Se, 300 ug.
[4] Ronozyme HiStarch contained 600 KNU/g. 1 g of HiStarch added to 149 g of corn supplied 4 KNU/g of premix. 20 g premix delivered 80 KNU/kg of feed and 40 g premix delivered 160 KNU/kg of feed.
[5] Prepared as 1 g titanium dioxide added to 4 g corn.
[6] Ronozyme HiPhos contained 5000 FYT/g. 1 g of HiPhos added to 99 g of ground corn, supplied 50 FYT/g of premix. 20 g delivered 1000 FYT/kg of feed. 1000 FYT/kg supplied 1.5 g P/kg and 1.7 g of Ca/kg.
[7] LOQ = limit of quantification.

Table 5.2 Effect of graded amylase supplementation on growth performance of broiler chickens[1]

	α- amylase, KNU/kg					
Item	0	80	160	SEM	Linear	Quadratic
BW, g						
d 0	36.3	36.3	36.3	0.02	0.416	0.636
d 7	124.0	128.4	126.2	2.27	0.497	0.258
d 14	381.8	395.7	392.0	6.78	0.304	0.306
d 21	806.0	884.0	870.4	11.96	0.002	0.007
d 0 to 7						
BW gain, g/bird	87.7	92.1	89.9	2.27	0.501	0.256
Feed intake, g/bird	128.1	129.4	116.8	11.86	0.508	0.638
Gain: Feed, g/kg	694.5	755.9	826.1	60.73	0.149	0.958
d 0 to 14						
BW gain, g/bird	345.5	359.4	355.7	6.78	0.305	0.316
Feed intake, g/bird	435.8	452.8	426.5	18.56	0.727	0.356
Gain: Feed, g/kg	798.5	808.4	839.2	33.64	0.407	0.803
d 0 to 21						
BW gain, g/bird	769.6	837.1	834.1	14.69	0.007	0.070
Feed intake, g/bird	1073.8	1094.6	1049.6	28.20	0.554	0.357
Gain: Feed, g/kg	718.3	766.7	796.1	13.36	0.001	0.571

[1]Data are least square means of eight replicate cages per diet.

Table 5.3 Efficacy of dietary α- amylase supplementation on nutrient digestibility in different intestinal sites of the broiler chicken[1].

	α- amylase, KNU/kg					
Intestinal site[2]	0	80	160	SEM	Linear	Quadratic
Anterior Jejunum						
DMD, %	42.6	43.3	36.8	2.12	0.077	0.188
DE, %	32.3	30.1	26.2	2.14	0.063	0.769
DE, kcal/g	1.417	1.307	1.175	0.095	0.093	0.923
N, %	49.8	47.4	49.0	1.92	0.781	0.413
Starch, %	71.1	69.6	66.0	1.71	0.053	0.629
Posterior Jejunum						
DMD, %	60.7	60.0	56.6	0.87	0.005	0.227
DE, %	54.8	62.6	61.1	1.06	0.001	0.003
DE, kcal/g	2.403	2.724	2.736	0.047	<0.001	0.017
N, %	68.7	65.3	66.3	1.24	0.189	0.177
Starch, %	79.9	85.0	83.3	0.90	0.018	0.008
Anterior Ileum						
DMD, %	69.8	74.1	74.9	0.49	0.639	0.035
DE, %	69.6	74.1	74.9	0.50	<0.001	0.009
DE, kcal/g	3.048	3.221	3.354	0.022	<0.001	0.477
N, %	76.8	76.9	76.5	0.74	0.757	0.816
Starch, %	92.8	95.8	95.7	0.25	<0.001	<0.001
Posterior Ileum						
DMD, %	74.5	73.5	72.3	0.39	0.001	0.861
DE, %	72.9	76.0	76.3	0.38	<0.001	0.008
DE, kcal/g	3.194	3.306	3.417	0.017	<0.001	0.977
N, %	81.7	79.4	80.3	0.67	0.189	0.077
Starch, %	97.1	97.8	98.9	0.24	<0.001	0.420
Total tract						
DMD, %	74.4	73.8	73.5	1.17	0.586	0.910
AME, %	75.9	76.7	77.0	1.02	0.460	0.877
AME, kcal/g	3.328	3.335	3.452	0.045	0.072	0.335
N, %	72.9	73.1	73.9	0.17	0.568	0.829
AMEn, %	70.6	71.5	71.7	0.94	0.428	0.781
AMEn, kcal/g	3.095	3.109	3.213	0.041	0.063	0.388
Starch, %	98.1	98.7	98.7	0.17	0.038	0.147

[1]Data are least square means of eight replicate cages per diet.

[2]DMD, dry matter digestibility; DE, digestibility energy; AME, apparent metabolizable energy; AMEn, nitrogen-corrected apparent metabolizable energy.

Table 5.4 Villus height, crypt depth, villus height to crypt depth ratio and viscosity of the jejunal digesta of broiler chickens fed diets supplemented with graded levels of α- amylase[1]

	α- amylase, KNU/kg					
Item	0	80	160	SEM	Linear	Quadratic
Villus height, μm	709.6	914.3	937.8	30.82	<0.001	0.031
Crypt depth, μm	99.8	115.9	107.6	7.87	0.495	0.225
VH:CD[2]	7.5	8.1	9.0	0.73	0.179	0.872
Viscosity	3.0	2.8	2.8	0.06	0.023	0.388

[1]Data are least square means of eight replicate cages per diet.
[2]VH, villus height; CD, crypt depth.

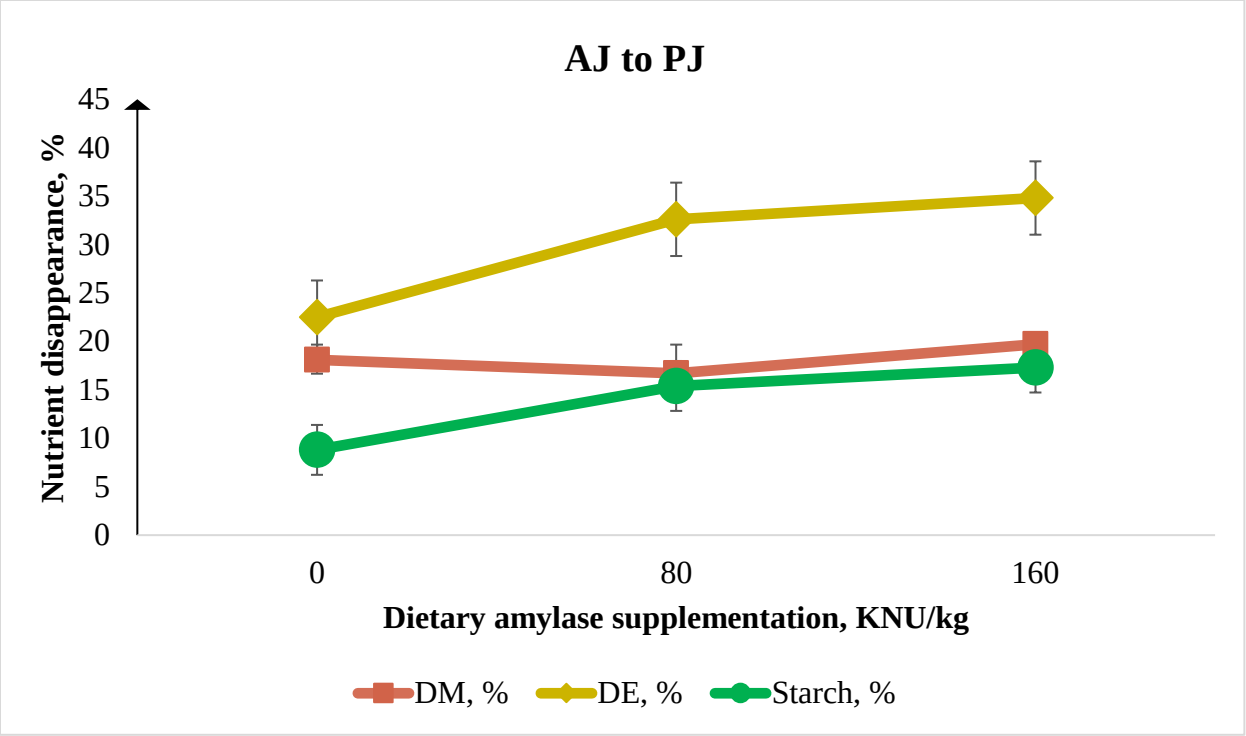

Figure 5.1 Disappearance of dry matter (DM), digestible energy (DE) and digesta starch from anterior jejunum (AJ) to posterior jejunum (PJ) in the broiler chicken intestine. There were linear increases ($P < 0.01$) in starch (%) and DE (%) disappearance with increasing α- amylase supplementation. Error bars are standard error of mean of 8 observations.

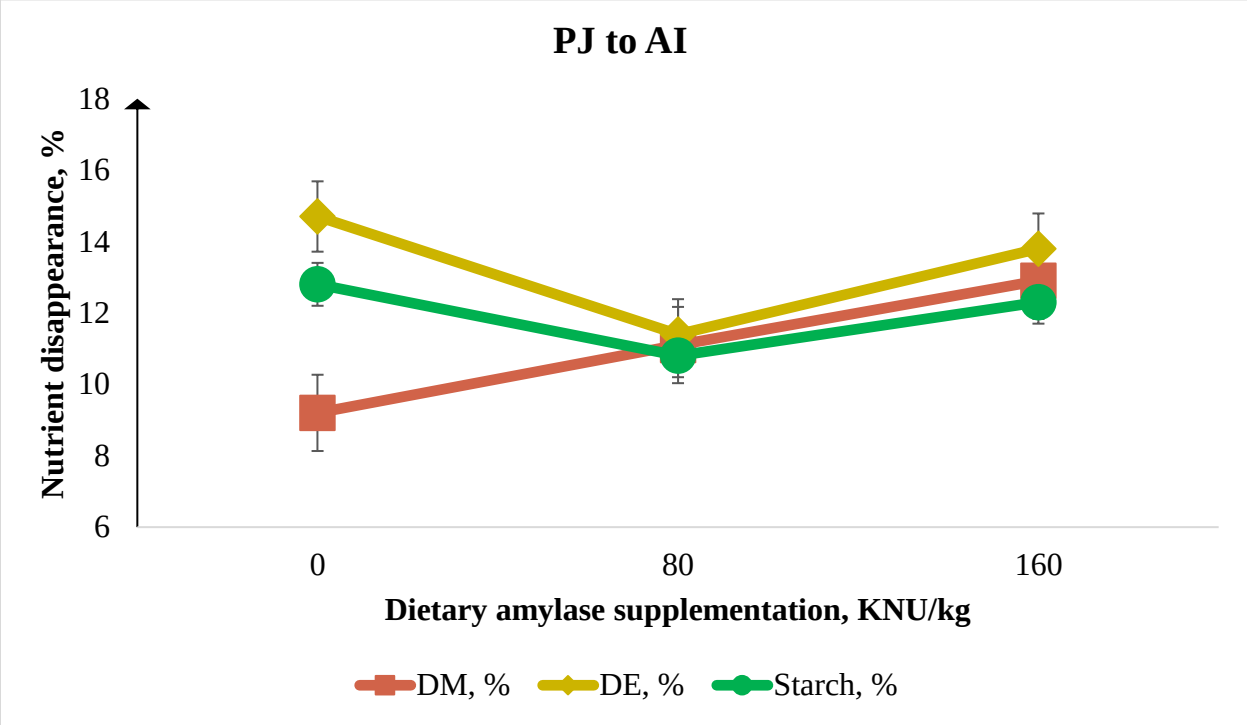

Figure 5.2 Disappearance of dry matter (DM), digestible energy (DE) and digesta starch from posterior jejunum (PJ) to anterior ileum (AI) in the broiler chicken intestine. There was a linear increase ($P < 0.05$) in DM (%) and quadratic response ($P < 0.05$) in DE (%) disappearance with increasing α- amylase supplementation. Error bars are standard error of mean of 8 observations.

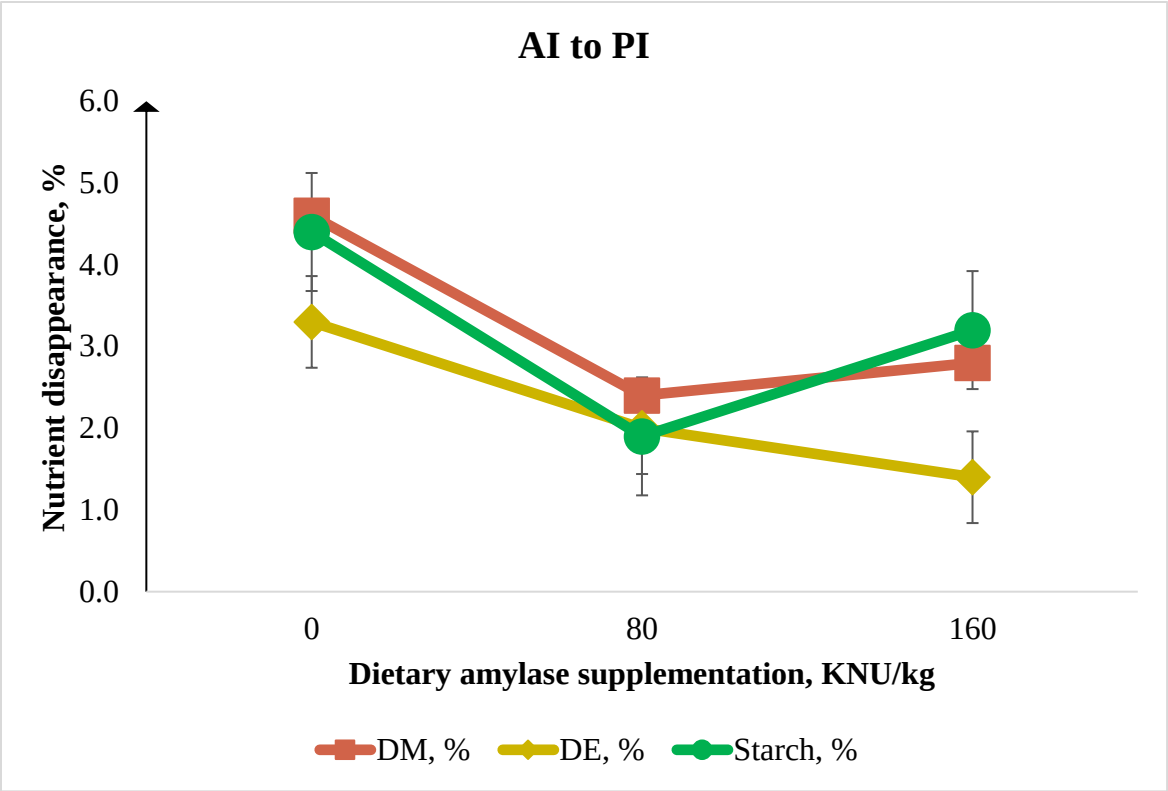

Figure 5.3 Disappearance of dry matter (DM), digestible energy (DE) and digesta starch from anterior ileum (AI) to posterior ileum (PI) in the broiler chicken intestine. Increasing α- amylase supplementation resulted in linear and quadratic responses ($P < 0.05$) in DM (%) disappearance. There was a linear decrease ($P < 0.05$) in DE (%) and a quadratic response ($P < 0.01$) in starch (%) disappearance with increasing α- amylase supplementation. Error bars are standard error of mean of 8 observations.

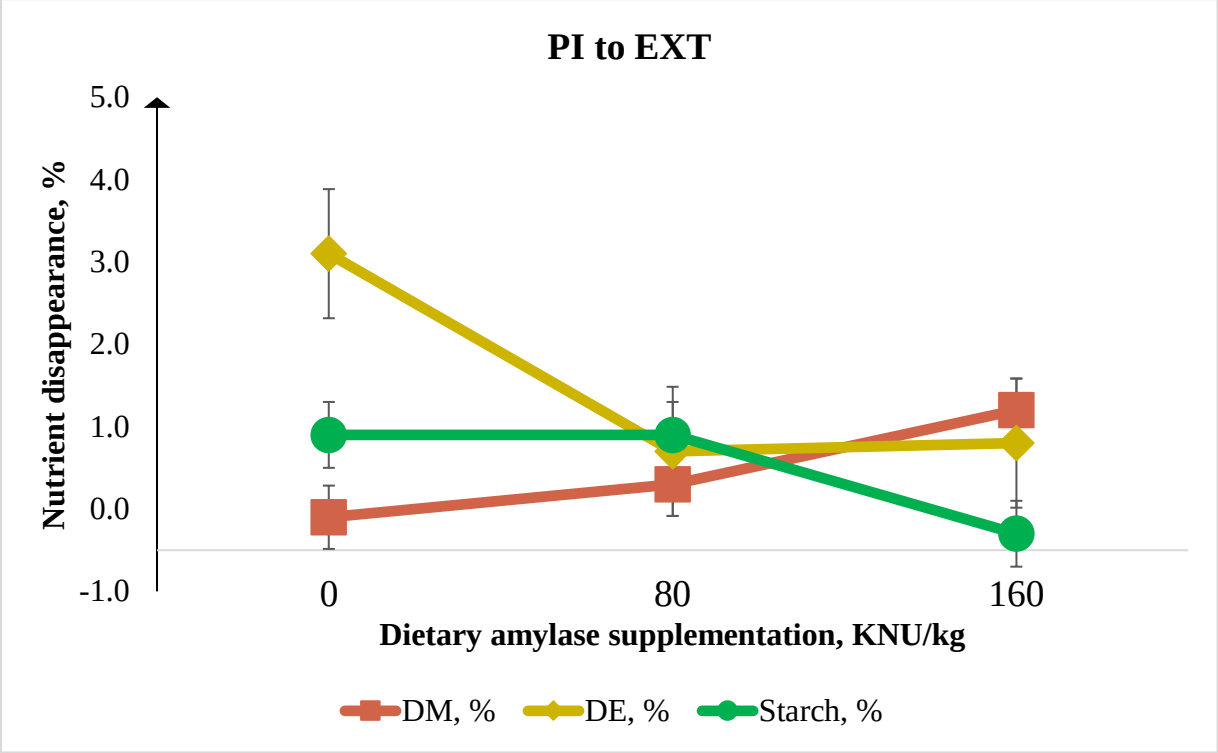

Figure 5.4 Disappearance of dry matter (DM), digestible energy (DE) and digesta starch from posterior jejunum (PI) to excreta (EXT) in the broiler chicken intestine. There was linear decrease ($P < 0.01$) in starch (%) disappearance with increasing α- amylase supplementation. Error bars are standard error of mean of 8 observations.

CHAPTER 6. DIETARY PHOSPHORUS LEVEL REGULATES APPETITE THROUGH MODULATION OF GUT AND HYPOTHALAMIC EXPRESSION OF ANOREXIGENIC GENES IN BROILER CHICKENS

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6.1 Abstract

Two experiments were designed to elucidate gut and hypothalamic molecular regulation of appetite by dietary phosphorus (P) concentration in broiler chickens. Birds (192 Cobb-500 broiler chickens) were randomly assigned to 3 experimental diets in experiment 1 (Exp. 1) and 24 broiler chickens were randomly assigned to 3 treatment groups in Exp. 2. Each diet comprised 8 replicate cages, with either 8 birds (Exp. 1) or 1 bird (Exp. 2) per replicate cage. In Exp. 1, diets contained 1.2 (P-deficient), 2.8 (P-marginal) or 4.4 (P-adequate) g/kg non-phytate P (nPP). In Exp. 2, birds fed the P-adequate diet were pair-fed (PF) to the feed consumption levels of birds fed the P-deficient diet. Feed intake and BW gain ($P < 0.001$) decreased in birds fed the P-deficient diet in Exp. 1. Birds fed the P-deficient diet had similar feed intake and BW gain with PF group fed the P-adequate diet (Exp. 2) but was significantly lower ($P < 0.001$) than birds fed the P-adequate diets. Sodium-phosphate cotransporter (NaPi-IIb) mRNA was upregulated ($P < 0.05$) in both experiments. Conversely, cholecystokinin (CCK) mRNA was downregulated ($P < 0.01$) in birds fed P-deficient diets. Anorexia-related hypothalamic cholecystokinin receptor (CCKAR) and melanocortin receptors (MC3R and MC4R) were upregulated ($P < 0.05$) in birds fed P-deficient diets, in both experiments. The current data show that dietary P deficiency decreases feed intake in broiler chickens by altering the expression of anorexigenic genes in the gut and hypothalamus of broiler chickens.

Key words: appetite, chicken, gut, hypothalamus, phosphorus

6.2 Introduction

Voluntary feed intake in farm animals is very complex and is tightly regulated by the brain–gut axis acting through hypothalamic integration. The intrinsic crosstalk between the

hypothalamus and the gastrointestinal tract ensures that energy balance is maintained. Generally, there are two primary populations of neurons integrating peripheral signals of nutritional status and influencing appetite through the release of signaling molecules, originating in the hypothalamus (Richards, 2003). These signals include the orexigenic neuropeptides; neuropeptide Y (NPY), agouti-related peptide (AgRP), peptide YY (PYY) (Takeuchi et al., 2000; Ando et al., 2001) and anorexigenic neuropeptides; pro-opiomelanocortin (POMC), cocaine- and amphetamine-regulated transcript (CART), corticotropin-releasing hormone (CRH), cholecystokinin (CCK), and ghrelin (Richards, 2003; Furuse et al., 1999). Ghrelin and CCK are highly expressed in the gastrointestinal tract and are essential to feed intake regulation in broilers and layers (Richards, 2003).

Previous reports show that feeding response in animals is affected by dietary phosphorus (P) concentrations. Maynard (1951) stated that a specific deficiency of P has a specific effect in causing loss of appetite. Similarly, Osborne and Mendel (1918) reported that a lack of sufficient dietary P deficiency causes cessation or growth retardation in rats. Recently in broiler chickens, several authors (Dilger et al., 2004; Adeola, 2010; Rousseau et al., 2016; Imari et al., 2020) have reported a drastic decrease in feed intake in birds fed diets deficient in dietary P. However, potential regulation of central and peripheral appetite regulators by dietary P remains unknown and warrants investigation.

Hence, in the current study, our hypothesis was that P deficiency directly affects feed intake response in broiler chickens through alterations in the expression of gut and brain appetite regulators. Data obtained from the current study show that dietary P deficiency decreases feed intake in broiler chickens though alteration in the expression of anorexigenic genes indicating a delicate relationship between dietary P concentration and appetite regulation and opens new insights into other aspects of P metabolism in the chicken.

6.3 Materials and Methods

Protocols of animal experiments were reviewed and approved by the Purdue University Animal Care and Use Committee (#: 1112000389).

6.3.1 Experimental Diets, Birds, Housing, and Design

Three corn-soybean meal-based diets (Table 6.1) were formulated to contain 1.2 (P-deficient), 2.8 (P-marginal) or 4.4 (P-adequate) g/kg non-phytate phosphorus (nPP). Male day-old broiler chicks (Cobb 500, Siloam Springs, AR) were obtained from a commercial hatchery. The birds were individually tagged, weighed, and raised in heated battery brooders (model SB 4 T; Alternative Design Manufacturing, Siloam Springs, AR) with temperature and lighting maintained as previously described (Aderibigbe et al., 2020). The birds were fed a common starter diet, formulated to meet breeder nutrient specifications, for 14 or 18 d. Experiment 1 (Exp. 1) investigated the impact of dietary P concentration on feed intake response and appetite regulatory genes in broiler chickens. The second experiment (Exp. 2), a pair-feeding (PF) trial, was designed to investigate any direct relationship between dietary P-deficiency related feed intake reduction observed in Exp. 1 and appetite regulation in broiler chickens.

6.3.1.1 Experimental 1

One-hundred and ninety-two birds were allotted to the 3 diets (Table 6.1) in a randomized complete block design consisting of 8 replicate cages and 8 birds per cage on d 14 post hatching. Experimental diets, fed as mash, and water were provided *ad libitum* to all birds. Feed intake records were taken every 12 hours and the experiment was terminated on d 18 when there was a significant decrease (approximately 30% reduction) in the average feed intake of birds fed the P-deficient diet (1.2 g/kg nPP) compared to other groups.

6.3.1.2 Experimental 2

A successive PF trial was conducted to delineate any direct impact of dietary P deficiency on the expression of brain-gut appetite regulators in broiler chickens without the confounding effect of differences in amount of feed consumed. On d 18 post hatching, 24 birds previously fed the P-adequate diet (4.4 g/kg nPP) were weighed and individually allotted to three treatment groups in a randomized complete block design consisting of 8 replicate cages and 1 bird per cage. Birds in treatment group 1 were fed *ad libitum* a P-deficient diet (Table 6.1) formulated to contain 1.2 g/kg nPP. Birds in treatment group 2 were fed a P-adequate diet (Table 6.1) formulated to contain 4.4 g/kg nPP but pair-fed to the amount of feed consumed by the birds in treatment group 1 to

equalize the feed intake of the two groups. The average daily feed intake of birds fed the P-deficient diet was offered to the PF group in subsequent days. Birds in the third treatment group were fed *ad libitum* a P-adequate diet formulated to contain 4.4 g/kg nPP.

6.3.2 Sampling Procedures

The studies were terminated on d 18 or 21 post hatching in Exp. 1 or 2, respectively. In both experiments, birds were individually weighed and 1 bird (with median BW in Exp. 1) per replicate was selected for sample collection. First, the birds were anesthetized with sodium pentobarbital via cardiopuncture and then decapitated. The hypothalamus was dissected from the ventral surface of the brain. Two transverse cuts were made at the apex of the optic chiasm and the rostral margin of the mammillary bodies. Next, 2-mm bilateral cuts were made on either side of the midline and the whole hypothalamus removed according to the chicken brain atlas (Yuan et al., 2009). The hypothalamus was immediately snap frozen in liquid nitrogen and stored at –80°C for RNA extraction. For the gut markers of appetite regulation, whole jejunal segments was removed, flushed gently with ice-cold 10% phosphate-buffered saline (VWR International, Radnor, PA). The segments were cut longitudinally in half exposing the lumen, and mucosal contents were scraped with a metal spatula. Mucosal contents were immediately placed in 2 mL of Trizol reagent (Invitrogen, Grand Island, NY) and stored at -80°C prior to RNA isolation.

6.3.3 Total RNA Extraction, Reverse Transcription and Real-Time PCR Analysis

Expression of genes in the hypothalamus and jejunal mucosa was quantified using quantitative real-time polymerase chain reaction (qPCR). Trizol reagent (Invitrogen, San Diego, CA) was used to extract the total RNA following the manufacturer's protocol. RNA concentrations were determined by Nano-Drop 1000 (Thermo Scientific, Wilmington, DE) by taking the optical density at 260 nm and 280 nm. RNA with A260/A280 ratio above 1.8 was retained, and RNA integrity was verified by 1% agarose gel electrophoresis. Afterwards, 2 mg of total RNA from each sample were reverse transcribed into cDNA product with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). The cDNA was then diluted 1:10 with nuclease-free water (Ambion) and stored at -20°C until use. Real-time qPCR was performed with Bio-Rad CFX machine (Biorad, Tamecula, CA) with the SYBR green-based mix (Biotool, Houston, TX)

in a total reaction volume of 20 μL and programmed at the following cycling parameters: 10 min at 95°C; 40 cycles of 95°C for 30 s, primer-specific annealing temperature for 30s, and 72°C for 30s. A melt curve analysis was performed for each gene at the end of the PCR run. Nucleotide sequences for genes of interest used in the current study are listed in Table 6.2. Primers were designed with the Primer Blast software (NCBI-NIH, Bethesda, MD). Samples were analyzed in duplicate, and a difference lesser than or equal to 5% was considered acceptable. Relative gene expression was subsequently calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with normalization against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the housekeeping gene.

6.3.4 Statistical Analyses

Using values from previous publications on feed intake response of broiler chickens to dietary P deficiency, we determined (Dell et al., 2002) that 8 replicates per treatment would be sufficient for the impact of dietary P level on feed intake to achieve 80% power with a significance level of 0.05. Data were analyzed using the Proc GLM procedures of SAS (SAS Inst. Inc., Cary, NC) for randomized complete block design with dietary treatment as the main effect. Initial body weight was used as the blocking factor. Cage was used as the experimental unit for all analyses. For gene expression, fold change of dietary treatments was expressed relative the P-adequate diet, which was set at 1. Statistical significance was declared at $P \leq 0.05$, with $0.05 < P \leq 0.10$ considered as a tendency. When the main effect of the dietary treatment was significant, means were separated using Tukey's HSD. Graphs were generated using GraphPad Prism 5.0 software.

6.4 Results

6.4.1 Growth Performance, Feed, and P Intake

There were significant decreases ($P < 0.001$) in feed intake, final BW and BW gain in birds fed P-deficient diet (1.2 g/kg nPP) compared to other treatments (Exp. 1; Table 6.3). However, there was no difference between birds fed diets containing 2.8 and 4.4 g/kg nPP. Additionally, the total P intake decreased ($P < 0.001$) with decreasing dietary P level. In Exp. 2 (Table 6.4), response of birds fed P-deficient diets (1.2 g/kg nPP) was not different from the PF group, but similar to Exp. 1, had lower final BW ($P < 0.01$), BW gain ($P < 0.001$) and feed intake ($P < 0.001$) compared

to birds fed P-adequate diets (4.4 g/kg nPP). Total P intake was similar between PF group and birds fed P-adequate diets (4.4 g/kg nPP) but were higher ($P < 0.001$) than in birds fed P-deficient diets (1.2 g/kg nPP).

6.4.2 Relative Gene Expression of Appetite Regulators

Figures 6.1 to 6.4 depict the relative mRNA expression of intestinal P transporter and appetite regulatory genes in Exp. 1. The expression of NaPi-IIb mRNA was upregulated ($P < 0.05$) in birds fed P-deficient diets (1.2 g/kg nPP) compared to other treatments. Conversely, expression of intestinal CCK mRNA was downregulated ($P < 0.001$) in birds fed P-deficient diets (1.2 g/kg nPP). However, mRNA expression of intestinal Ghrelin and PYY, or hypothalamic POMC was unaffected by dietary P concentration. There was a tendency for an effect of dietary P concentration on hypothalamic mRNA expressions of NPY ($P = 0.094$) and AGRP ($P = 0.069$). Hypothalamic expressions of CCK receptor (CCKAR), and melanocortin receptors (MC3R and MC4R) were upregulated ($P < 0.05$) in birds fed P-deficient diets but was similar between the birds fed marginal (2.8 g/kg nPP) and P-adequate (4.4 g/kg nPP) diets.

Figures 6.5 to 6.8 show the relative mRNA expression of intestinal P transporter and appetite regulatory genes in Exp. 2. Like Exp. 1, mRNA expression of NaPi-IIb was upregulated ($P < 0.001$) and CCK was downregulated ($P < 0.01$), respectively, in birds fed P-deficient diets (1.2 g/kg nPP) compared to other treatments. The expression of intestinal Ghrelin and PYY, or hypothalamic NPY, AGRP and POMC mRNA were unaffected by dietary P concentration. However, like in Exp. 1, hypothalamic CCKAR, MC3R and MC4R were upregulated ($P < 0.01$) in birds fed P-deficient diets compared to other dietary treatments.

6.5 Discussion

Our current data suggest a strong relationship between appetite regulation in broiler chickens and dietary P deficiency. This was observed as differential expression of anorexigenic genes in the gut and hypothalamus of chickens fed P-deficient diets, compared to other treatment groups, which supports our stated hypothesis. However, that we detected mRNA alterations does not necessarily imply that expression of the protein parallels the transcript. Importantly, there may be other factors

associated with appetite that contribute to differences in feeding behavior that were not evaluated in the current study.

Appetite regulation is essential in animals because of its importance in determining the levels and consumption pattern of nutrients that are important for sustenance of life. Appetite is controlled by complex mechanisms that involve the nutritional status of the body, neurotransmitters, and a change in feeding behavior (Richards and Proszkowiec-Weglarz, 2007). Like mammals (Leibowitz and Wortley, 2004; Bellinger and Langley-Evans, 2005), a variety of orexigenic and anorexigenic neuropeptides have been identified in birds (Kuenzel, 1994; Richards, 2003). However, response to the neuropeptides have been observed to differ between chicken breeds. For instance, although the orexigenic AGRP stimulates feeding behavior in layer chicks, it has no such effect in broiler chicks (Tachibana et al., 2001) whereas the anorexigenic effects of CRH are stronger in layer chicks than in broiler chicks (Tachibana et al., 2007). The mechanisms underlying such differences between species remain unclear. In addition to its major function as a digestive and absorptive organ, the gut also plays an important role in short-term regulation of feed intake via hormones such as CCK, and ghrelin, which transmit meal-related signals to the hypothalamus (Jensen, 2001). Previously, reports have shown that dietary macronutrients such as amino acids, fat, carbohydrates, and calcium can affect appetite in animals (Anderson, 1979; White et al., 2003; McConn et al., 2018). However, few reports exist in literature on P-related appetite in animals, especially in broiler chickens which show a drastic decrease in feed intake when fed a P-deficient diet. To our knowledge, the current study has not been reported before regarding the possible role of gut-brain axis for appetite regulation in response to dietary P deficiency-induced feed intake reduction in broiler chickens.

Consistently, in both experiments, and like previous reports (Dilger et al., 2004; Adeola, 2010; Rousseau et al., 2016; Imari et al., 2020), birds fed P-deficient diets had a drastic decrease in feed intake. Although the reason for this observation have remains unclear, one possible explanation is that as blood phosphate concentration becomes low over time, due to mild chronic hypophosphatemia, bones become weak because of increased resorption, which may eventually lead to loss of appetite in the birds. However, this seems counterintuitive, as birds will typically increase feed intake to satisfy requirement for a nutrient that is deficient in the diet. This is especially true for Ca (Wilkinson et al., 2013), and in fact, birds would more likely adjust their feed intake to satisfy a Ca appetite as dietary P fluctuates and scarcely the other way around

(Wilkinson et al., 2014; Rousseau et al., 2016). For instance, in the study by Wilkinson et al. (2014), birds fed diets with 5.5 g/kg of nPP consumed 37% more of a Ca source than birds fed 2.5 g/kg of nPP, indicating that birds were not only responding to dietary Ca concentration but also to the amount of nPP in the mixed ration. It is likely that birds increased their consumption of the Ca source as dietary nPP concentration increased to avoid narrow Ca:nPP ratios.

Although there were marked reduction in feed and P intake in birds fed P-deficient diets, there was an upregulation of intestinal NaPi-IIb. On a functional level, upregulation of intestinal P absorption due to a deficient-P diet has been described in many species (Caverzasio et al., 1987; Cross et al., 1990; Danisi and Murer, 1991) and are associated with the abundance of NaPi-IIb in the apical membrane. A low P diet would lead to a rapid decrease of the plasma phosphate concentration and activation of the renal 1,25-hydroxylase, resulting in an increased level of vitamin D3 (Matsumoto et al., 1980, Portale et al., 1989). Therefore, adaptation of small intestinal phosphate transport to low P diet has been explained to occur via vitamin D3 (Cross et al., 1990). In the current study, intestinal transcripts of satiety signals such as ghrelin and PYY were unaltered in response to dietary P concentration, which suggests that these hormones may not be directly involved in P appetite. In vertebrates, peripheral PYY is purported to act as a satiety factor released from the gastrointestinal tract after feeding to curb appetite via afferent vagal Y-receptors or directly within the arcuate nucleus of the hypothalamus (Batterham et al., 2002; Batterham and Bloom, 2003; Simpson et al., 2012). However, unlike in mammals where ghrelin increases food intake through the melanocortin system, ghrelin in birds induces anorexia through the release of corticotropin-releasing factor (Kaiya et al., 2002; Saito et al., 2002). Interestingly, intestinal CCK expression was downregulated in birds fed P-deficient diet. This observation is contrary to the known anorexigenic function of CCK (Denbow, 1994; Kuenzel, 1994; Jensen, 2001) and therefore does not explain the decreased feed intake of birds fed the P-deficient diet. This observation warrants further investigation.

Because satiety signals in the gut are relatively short-lived when compared to central appetite regulators, we also investigated appetite regulatory genes in the brain. In the current study, hypothalamic gene expression of orexigenic (AgRP and NPY) and anorexigenic (POMC) appetite hormones were not influenced by dietary treatments. Although, there was a tendency for an alteration in NPY and AGRP expressions, this was not consistently observed in the PF trial and therefore any potential effect of dietary P level was discarded. The reason for the lack of effect in

these genes remains unclear and warrants further investigations. NPY is a very potent orexigenic peptide in mammals in birds (Kuenzel et al., 1987) and AgRP plays a similar role (Tachibana et al., 2001). However, AGRP does not influence feed intake in broiler chicks compared to layer-type chicks (Tachibana et al., 2001; Honda et al., 2007). Although expression of the anorexigenic POMC gene was not influenced by dietary P level, and therefore could not explain the reduction in feed intake response of the birds fed the P-deficient diets, a processed peptide of POMC, the α-melanocyte-stimulating hormone (**α-MSH**) is strongly anorexigenic in broiler chickens (Tachibana et al., 2007; Honda et al., 2007; Honda et al., 2012) and may play a role in the P appetite responses in the birds.

In the current study, there was upregulation of hypothalamic CCKAR, MC3R and MC4R in birds fed P-deficient diets. Upregulation of CCKAR directly contradicts the peripheral CCK expression, which was downregulated in birds fed P-deficient diets. While this observation is unclear, this could be due to negative feedback of the hypothalamic receptor to CCK production in the gut. Alternatively, neuronal synthesis of CCK in the brain have been reported (Innis et al., 1979; Jonson et al., 2000) and may stimulate the CCKAR independently of the CCK originating in the gut. Perhaps, decreased appetite in birds fed P-deficient diet is due more to changes in hypothalamic, and less to peripheral, anorexigenic CCK concentrations. It is also important to state that high-growth haplotypes, like broiler chickens, have been associated with a decreased level of CCK satiety signaling compared with the other genotypes (Dunn et al., 2013). The melanocortin system plays a critical role in whole body energy balance, in addition to appetite-regulatory effects and are mediated via the MCRs (Seeley et al., 2013). In mammals, the anorexigenic effect of α-MSH is mediated by hypothalamic melanocortin receptor; MC4R (Adan et al., 2006; Lee et al., 2006). In addition to MC4R, both MC3R and MC5R are expressed in the chicken brain (Takeuchi and Takahashi, 1998; Ka et al., 2009), although α-MSH has more affinity to MC4R in the chicken brain than MC3R or MC5R (Ling et al., 2004). The melanocortin receptors are strongly anorexigenic in chickens (Tachibana et al., 2001), and could likely explain the decreased feed intake response in birds fed the P-deficient diet. This may suggest a central role of melanocortin receptors in P-induced appetite regulation. However, as previously noted, POMC expression was unaltered by dietary P intake, and therefore presumably the α-MSH concentrations. Because α-MSH concentrations was not measured in the current study, it is not totally clear how the melanocortin receptors are activated to cause the observed changes in feed intake response in the

birds. This potentially suggests possible involvement of other genes in dietary P-induced appetite regulation.

One strong candidate is lipocalin-2 (LNC-2), a bone-specific osteoblast-derived hormone, with strong anorexigenic functions (Yoshikawa et al., 2011; Mosialou et al., 2017). Previously thought to be an adipokine, expression profiling showed that LNC-2 is expressed at least 10-fold higher in bone than in fat or any other tissue (Yan et al., 2007). Given the central role of osteoblasts in plasma P regulation, and our current results, the physiological role of LNC-2 in P-induced appetite regulation cannot be overlooked. Additionally, it has been reported that increased circulating levels of LNC-2 can cross the blood-brain barrier and act directly on anorexigenic melanocortin receptors with similar affinity to α-MSH (Mera et al., 2018). Although this would require a separate investigation, our current results suggest a potential role for LNC-2 in dietary P induced appetite: First, elevated plasma 1,25 $(OH)_2$ D upregulates NaPi-IIb in response to dietary P deficiency and this increases the intestinal utilization of available P. Additionally, 1,25 $(OH)_2$ D directly stimulates osteoblasts proliferation in the bone (Stern, 1990; Van Leeuwen et al., 2001). 1,25 $(OH)_2$ D also promotes bone resorption by increasing the number and activity of osteoclasts (Suda et al., 1992) in the presence of receptor activator of nuclear factor-κB ligand (RANKL) (Kogawa et al., 2010). Osteoclasts perform bone resorption by dissolution and degradation of hydroxyapatite and other organic material releasing Ca and P into the blood to maintain homeostatic balance. Increased osteoblast proliferation increases circulating LNC-2 levels, which crosses the blood-brain barrier. This act on the melanocortin receptors to decrease feed intake in the birds. Taken together, there appears to be strong evidence for a potential role for LNC-2 in the response to P deficiency in this study.

In summary, these results confirm the impact of dietary P deficiency to induce feed intake suppression in broiler chickens. Notably, dietary P deficiency downregulated CCK gene expression in the gut but upregulated the anorexigenic CCKAR, MC3R and MC4R genes in the hypothalamus. The current study provides valuable information towards the understanding of appetite regulation in broiler chickens. However, altered gene expression may not always translate to changes in protein synthesis, therefore these data warrant careful interpretation. Given its direct action on melanocortin anorexigenic pathway, a possible role of osteoblast-derived LNC-2 in phosphorus-induced appetite regulation in broiler chickens requires further investigation.

6.6 References

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Table 6.1 Ingredient and calculated nutrient composition of experimental diets, as-fed basis.

	Non-phytate P, g/kg		
Ingredients	1.2	2.8	4.4
Corn	597.7	574.5	560.7
Soybean meal	340.0	340.0	340.0
Soybean oil	20.0	30.0	30.0
Monocalcium phosphate[1]	0.0	7.5	15.3
Limestone[2]	4.3	10.0	16.0
Salt	4.0	4.0	4.0
Vitamin-mineral premix[3]	3.0	3.0	3.0
DL-Methionine	2.0	2.0	2.0
L-Lysine HCl	2.9	2.9	2.9
L-Threonine	1.1	1.1	1.1
Titanium dioxide premix[4]	25.0	25.0	25.0
Total	1,000.0	1,000.0	1,000.0
Calculated composition[5], g/kg			
Crude protein	222.1	220.1	219.0
ME, kcal/kg	3,162.2	3,165.7	3,117.9
Ca	2.7 (2.8)	6.0 (6.6)	9.6 (9.8)
Total P	3.8 (3.8)	5.3 (5.5)	6.9 (7.0)
Non-phytate P	1.2	2.8	4.4
Ca: total P	0.7	1.1	1.4
Ca: non-phytate P	2.2	2.2	2.2
Total amino acids, g/kg			
Arg	14.2	14.1	14.0
His	5.8	5.7	5.7
Ile	9.0	8.9	8.9
Leu	18.9	18.7	18.5
Lys	13.9	13.9	13.8
Met	5.4	5.3	5.3
Cys	3.6	3.5	3.5
Phe	10.3	10.2	10.2
Tyr	8.5	8.4	8.4
Thr	9.2	9.2	9.1
Trp	2.9	2.9	2.9
Val	10.0	9.9	9.9
Met + Cys	8.9	8.9	8.8
Phe + Tyr	18.8	18.6	18.5

[1] 16% Ca, 21% P. [2] 38% Ca.

[3] Supplied the following per kg diet: vitamin A, 5,484 IU; vitamin D3, 2,643 ICU; vitamin E, 11 IU; menadione sodium bisulfite,4.38 mg; riboflavin, 5.49 mg; pantothenic acid, 11 mg; niacin, 44.1 mg; choline chloride, 771 mg; vitamin B12, 13.2 ug; biotin, 55.2 ug; thiamine mononitrate, 2.2 mg; folic acid, 990 ug; pyridoxine hydrochloride, 3.3 mg; I, 1.11 mg; Mn, 66.06 mg; Cu, 4.44 mg; Fe, 44.1 mg; Zn, 44.1 mg; Se, 300 ug.

[4] 1g of titanium dioxide added to 4 g corn. [5] Analyzed values are in bracket

Table 6.2 Gene-specific primers used in real –time quantitative PCR

Gene	Accession No.	Forward	Reverse	Size (bp)
CCK	NM_001001741.1	GGAAGGAAGGAGGAAGCGAT	GAGGGAGCTCACCGACAG	75
NPY	NM_205473.1	TCAAGCCCAGAGACACTGAT	GGGTCTTCAAACCGGGATCT	80
AGRP	NM_001031457.1	CGGTTGTGCACGTTGCC	CTGAGGTCCGAGGTGAGGAT	96
PYY	NM_001361182.1	GGTATGGGAAGCGCAGCA	CGTCGATGTCGGACCACAG	87
POMC	NM_001031098.1	TCCATGGAGCATTTCCGCTG	GGTAACTCTCAGCCGACTCC	100
Ghrelin	NM_001001131.1	CCTCTCTGCTAACCTGTCTGG	AGGCAGTGCTTCAAATGTGT	72
NaPi-IIb	NM_204474.2	CACCTCTTGTTGGCATTGGTG	GCTGTTGTGGTTGTGCCAAT	85
CCKAR	NM_001081501.1	ACCAGCATCGCCAAATACGA	GTTGCAACGGCACTTTCCTT	79
MC4R	NM_001031514.1	CAAGCGTGTAGGGGTCATCA	CAGATGATGACAACGCTGCTG	99
MC3R	XM_004947236.3	CCGTTCCACCGTTCACCTAA	GGGACCTTGGTGTGGGATTT	75
GAPDH	NM_204305.1	GCTGAATGGGAAGCTTACTG	AAGGTGGAGGAATGGCTG	216

CCK, cholecystokinin; NPY, neuropeptide Y; AGRP, agouti-related peptide; PYY, peptide YY; POMC, proopiomelanocortin; NaPi-IIb, sodium-phosphate cotransporter; CCKAR, cholecystokinin receptor; MC3R, melanocortin-3 receptor; MC4R, melanocortin-4 receptor; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase

Table 6.3 Growth performance of broiler chickens in Exp. 1, from d 14 to 18 post hatching[1]

	Non-phytate P, g/kg				
Item	1.2	2.8	4.4	SEM	P-value
d 14 BW	412.8	412.4	412.3	0.46	0.421
d 18 BW	577.0[b]	651.9[a]	675.9[a]	10.11	< 0.001
BW gain, g/bird	164.2[b]	239.5[a]	262.7[a]	10.21	< 0.001
Feed intake, g/bird	222.7[b]	299.8[a]	321.7[a]	16.12	< 0.001
Gain: Feed, g/kg	772.7	814.9	826.3	50.42	0.735
Total P intake[2], g/bird	0.85[c]	1.59[b]	2.22[a]	0.073	< 0.001

[1] Data represent least square means of 8 replicate cages

[2] Arithmetic product of feed intake (g/bird) and dietary P (g/kg) concentration

[a,b] Means within the same row with different superscripts are different at $P < 0.05$

SEM, standard error of the mean

Table 6.4 Growth performance of broiler chickens in Exp. 2 (Pair-feeding trial), from d 18 to d 21 post hatching[1]

	Non-phytate P, g/kg				
Item	1.2	PF[2]	4.4	SEM	P-value
d 18 BW	707.5	708.4	707.8	5.29	0.993
d 22 BW	884.6[b]	878.8[b]	973.6[a]	19.58	0.006
BW gain, g/bird	177.1[b]	170.4[b]	265.8[a]	18.97	0.005
Feed intake, g/bird	290.3[b]	302.9[b]	404.7[a]	14.1	< 0.001
Gain: Feed, g/kg	615.7	562.8	656.6	48.05	0.535
Total P intake[3], g/bird	1.10[b]	2.09[a]	2.14[a]	0.058	< 0.001

[1] Data represent least square means of 8 replicate cages

[2] PF, pair-fed group in which birds on the 4.4 g non-phytate/kg diet were pair-fed to the feed intake of the birds on the 1.2 g non-phytate/kg diet

[3] Arithmetic product of feed intake (g/bird) and total dietary P (g/kg)

[a,b] Means within the same row with different superscripts are different at $P < 0.05$

SEM, standard error of the mean

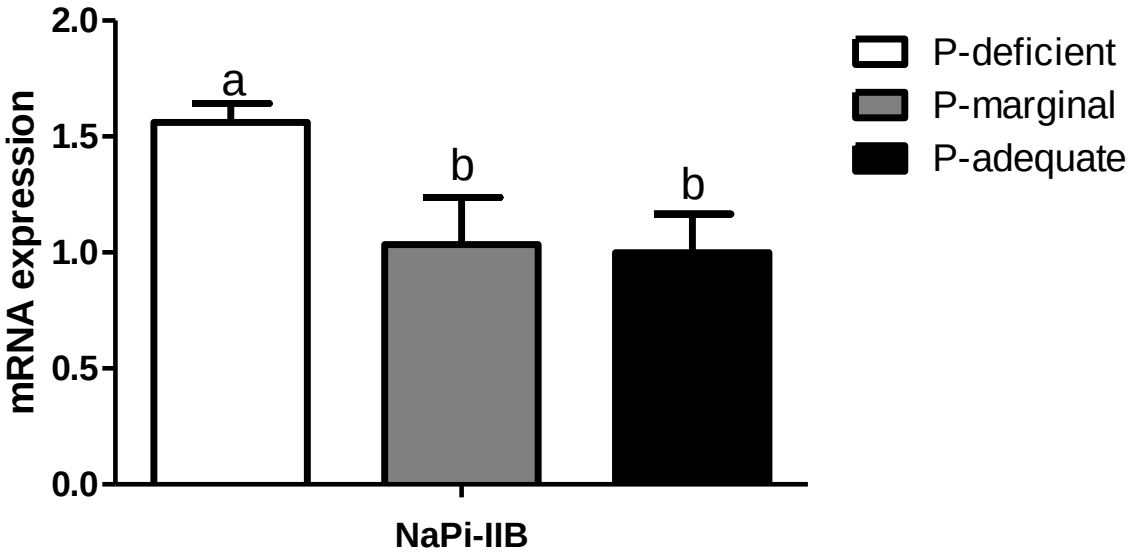

Figure 6.1 Effect of dietary phosphorus (P) level on mRNA expression of sodium-phosphate cotransporter (NaPi-IIb) in jejunal mucosa in experiment 1. Gene expression represents fold change relative to the adequate P treatment. Error bars represents means ± SEMs (n = 8). [a,b] indicates significant difference ($P < 0.05$) between the given pair of treatment.

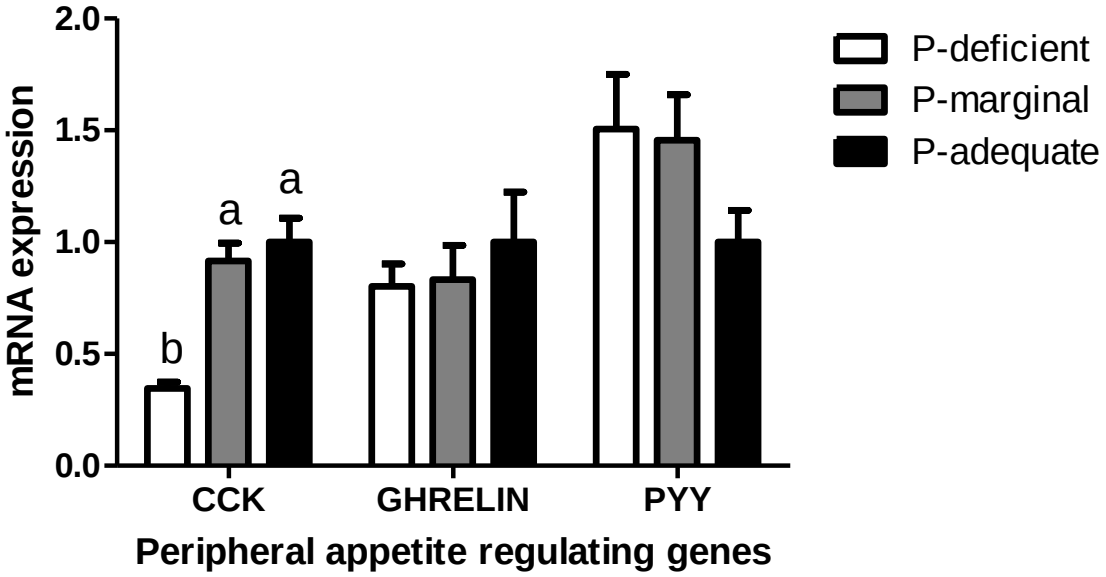

Figure 6.2 Effect of dietary phosphorus (P) level on peripheral appetite regulating genes in jejunal mucosa in experiment 1. Gene expression represents fold change relative to the adequate P treatment. Error bars represents means ± SEMs (n = 8). [a,b] indicates significant difference ($P < 0.05$) between the given pair of treatment. Abbreviations: CCK, Cholecystokinin; PYY, peptide YY.

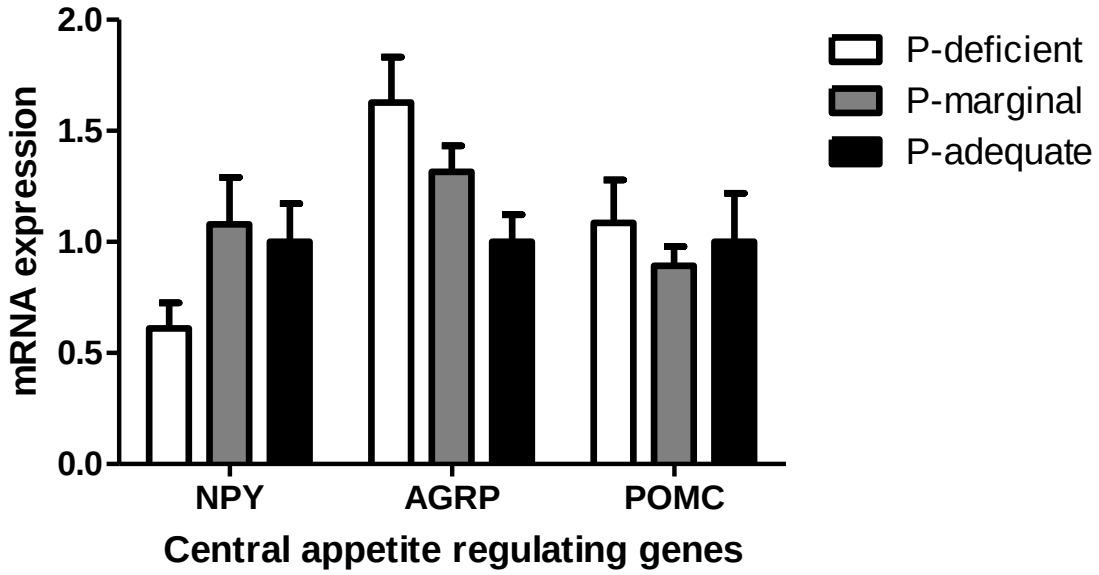

Figure 6.3 Disappearance of dry matter (DM), digestible energy (DE) and digesta starch from anterior ileum (AI) to posterior ileum (PI) in the broiler chicken intestine. Increasing α- amylase supplementation resulted in linear and quadratic responses ($P < 0.05$) in DM (%) disappearance. There was a linear decrease ($P < 0.05$) in DE (%) and a quadratic response ($P < 0.01$) in starch (%) disappearance with increasing α- amylase supplementation. Error bars are standard error of mean of 8 observations.

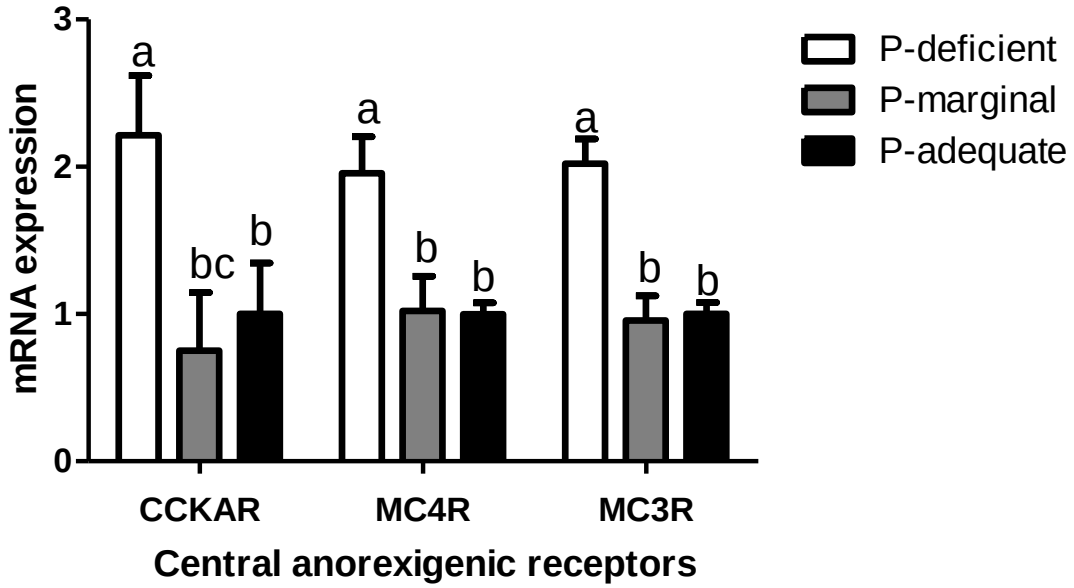

Figure 6.4 Effect of dietary phosphorus (P) level on mRNA expression of receptors of anorexigenic pathway in the hypothalamus in experiment 1. Gene expression represents fold change relative to the adequate P treatment. Error bars represents means ± SEMs (n = 8).

[a,b] indicates significant difference ($P < 0.05$) between the given pair of treatment. Abbreviations: CCKAR, cholecystokinin receptor; MC3R, melanocortin-3 receptor; MC4R, melanocortin-4 receptor

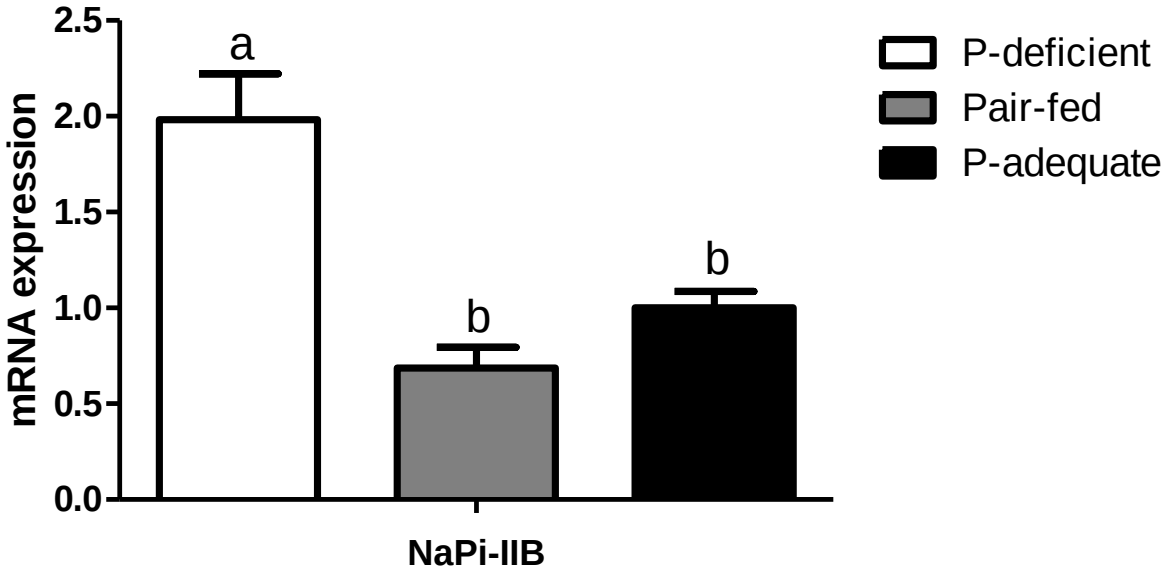

Figure 6.5 Effect of dietary phosphorus (P) level on mRNA expression of sodium-phosphate cotransporter (NaPi-IIb) in jejunal mucosa in experiment 2 (Pair-feeding study). Gene expression represents fold change relative to the adequate P treatment. Error bars represents means ± SEMs (n = 8). ^{a,b} indicates significant difference ($P < 0.05$) between the given pair of treatment.

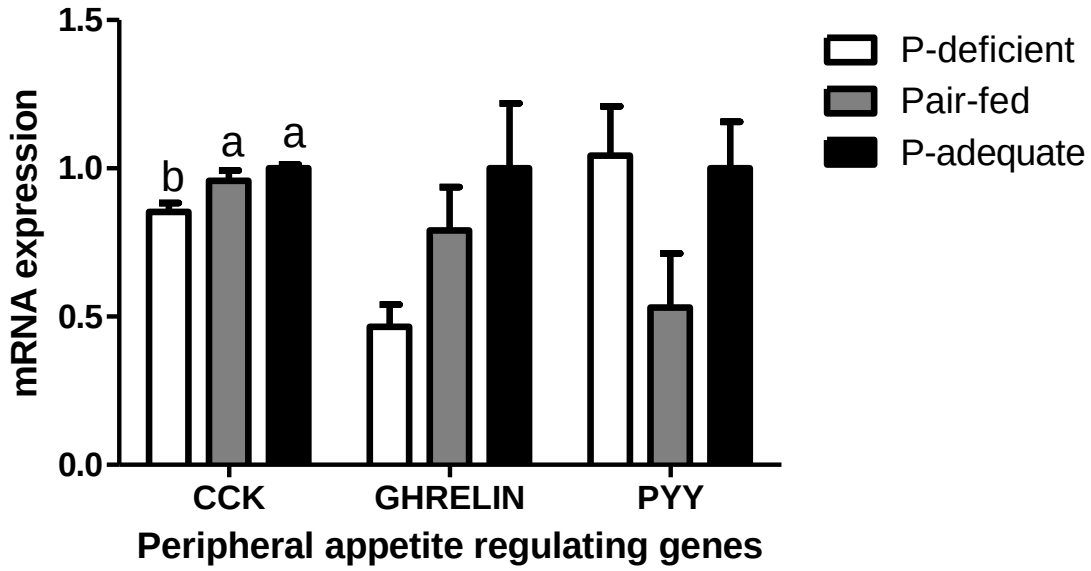

Figure 6.6 Effect of dietary phosphorus (P) level on peripheral appetite regulating genes in jejunal mucosa in experiment 2 (Pair-feeding study). Gene expression represents fold change relative to the adequate P treatment. Error bars represents means ± SEMs (n = 8). [a,b] indicates significant difference ($P < 0.05$) between the given pair of treatment. Abbreviations: CCK, Cholecystokinin; PYY, peptide YY.

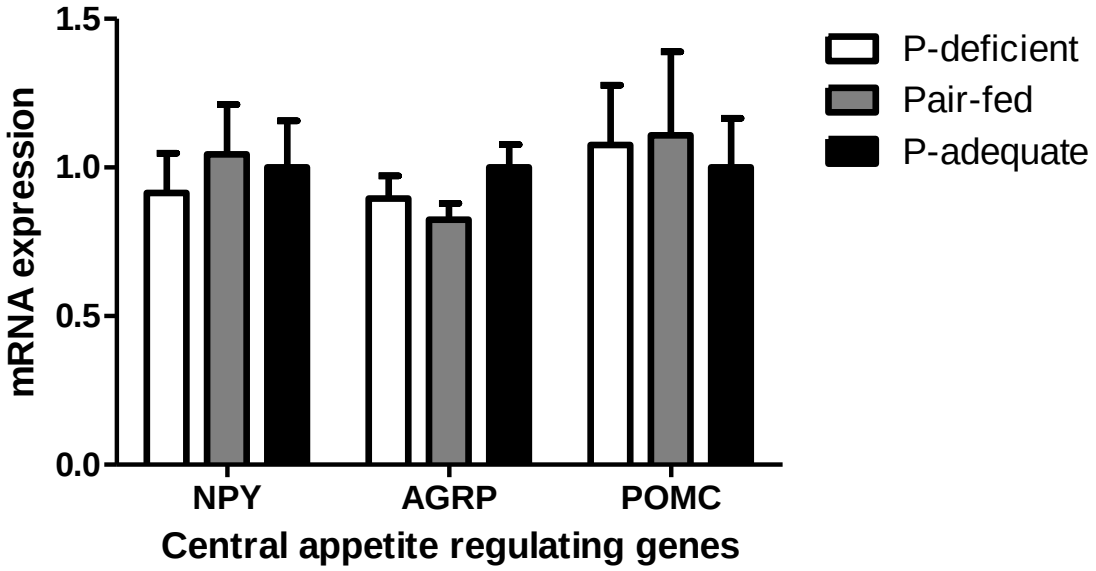

Figure 6.7 Effect of dietary phosphorus (P) level on central appetite regulating genes in the hypothalamus in experiment 2 (Pair-feeding study). Gene expression represents fold change relative to the adequate P treatment. Error bars represents means ± SEMs (n = 8). Abbreviations: NPY, neuro-peptide Y; AGRP, Agouti-related peptide; POMC, proopiomelanocortin

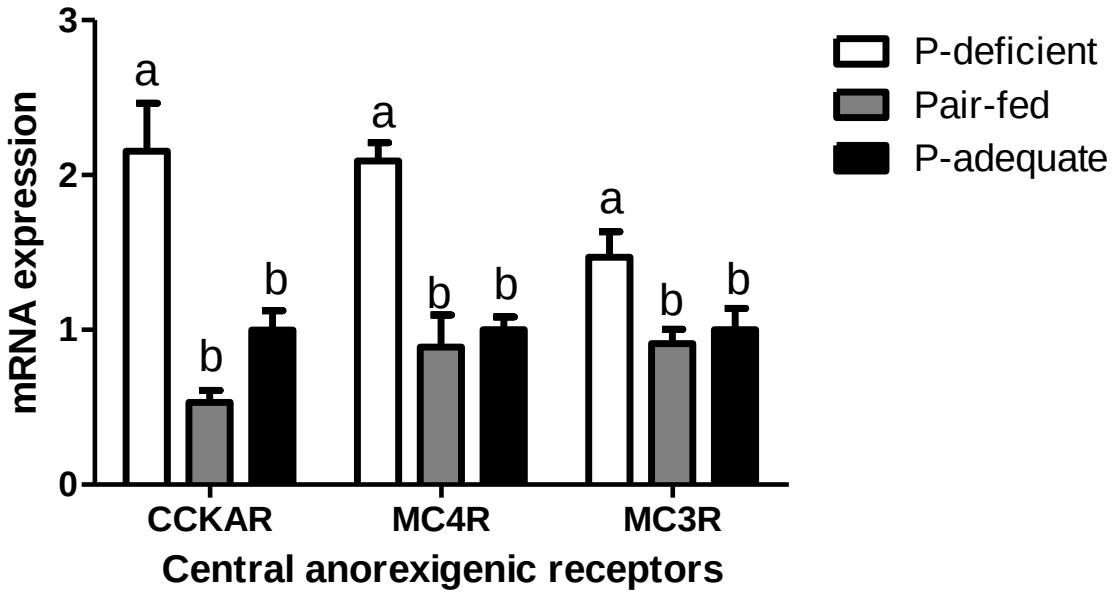

Figure 6.8 Effect of dietary phosphorus (P) level on mRNA expression of receptors of anorexigenic pathway in the hypothalamus in experiment 2 (Pair-feeding study). Gene expression represents fold change relative to the adequate P treatment. Error bars represents means ± SEMs (n = 8). ^{a,b} indicates significant difference ($P < 0.05$) between the given pair of treatment. Abbreviations: CCKAR, cholecystokinin receptor; MC3R, melanocortin-3 receptor; MC4R, melanocortin-4 receptor

CHAPTER 7. SUMMARY

7.1 Summary

As summarized by Bedford (2000), the purposes for enzyme use in poultry diets is manifold. This includes, but no limited to, the need to improve the nutritional value of animal feedstuff, which would also help to reduce nutrient excretion to the environment. Collectively, this improves animal feed efficiency and health, and from a commercial standpoint, would potentially expand the profit margin of the producer. Exogenous enzyme use in poultry production is globally accepted, but as reviewed exhaustively in chapter 1, the effect of the added enzyme is influenced by several factors including diet type, ingredient-specific anti-nutritional factors, breed, and age of the birds. Other limiting factors include the chemical characteristic and product quality of the exogenous enzyme. Although there have been considerable advances made to enzyme development in the past two decades, the efficacy of exogenous enzymes is rather inconsistent and seems riddled with a general lack of data replicability. Bedford (2000) identified some key areas where significant improvements can be made during enzyme development. First is the need for improved optimization of substrate identification by specific enzyme or group of enzymes under development. A more precise identification and association with target substrate will improve the efficacy of the enzyme. Another is the intrinsic characteristics and resilience of the enzyme, which describes the ability of the enzyme to withstand the rigors of aggressive feed processing techniques and the shifting chemical conditions of the animal gut environment.

Identifying the economic impact of TI on nutritional value of SBM (Liener, 1994, Rawlings et al., 2004; Erdaw et al., 2017), and hinging on recorded successes of exogenous protease in broiler nutrition (Costa et al., 2008; Erdaw et al 2017), the experiment in chapter 2 was designed to assess the impact of exogenous protease in ameliorating the effect of soybean TI in broiler chickens. Although, there were no interactions between protease and TI in most of the data, exogenous protease caused reduction in the pancreas weight in the birds at d 21, which had been hypertrophied by TI intake. Except for methionine, exogenous protease increased the AID of N and all AA and suggests that the efficacy of the protease may depend on the digestibility of AA in the original feed material. Secretion of digestive enzyme activity in the pancreas was not affected by protease or dietary TI supplementation and this was contrary to observations in the duodenum.

At the mRNA level, protease supplementation downregulated mucin but upregulated the cationic amino acid transporter (SLC7A-2) in the jejunum. The study showed no direct impact of protease administration on TI amelioration in broiler chickens and that most of the responses observed seem more likely due to a general improvement in dietary protein digestion.

A follow-up study was designed and conducted in chapter 3 to assess the contribution of TI intake to endogenous flow of AA and digestibility of inorganic minerals in broiler chickens fed the NFD. The extent of endogenous secretion and recycling of nutrient in the digestive tract has marked implications on nutrient utilization potential of the animal. More importantly, the level of endogenous AA flow depends on the presence of ANF like TI in the gut (Barth et al., 1993; Schulze, 1994). Although previous *in vitro* trials suggests that an involvement of exogenous protease on the direct degradation of TI, however this has not been substantiated *in vivo.* Results showed no effect of protease on recovery of endogenous AA flow. Contrary to AA results, the ileal digestibility of minerals such as Mn, Ca, Mg, Fe, and Cu were affected by dietary TI. The impact of exogenous protease on improvement in mineral digestibility was not consistent, and this variability in response may be as a result of the propensity for complex mineral interactions in the gut, and also due to the nature of the NFD that was fed to the birds. Dietary TI intake caused increased excretion of crude mucin and suggests a continuous erosion of the mucus layer possibly to get rid of the TI irritant. Surprisingly, TI intake had no effect on pancreas weight or enzyme secretion, and although this remains unclear, it may be attributed to the lack of N in the NFD for sustained TI inhibition of trypsin which is needed to stimulate a pancreatic response. As emphasized in the chapter, the diets were protein-free, and not an ideal representation of a conventional diet system, therefore additional investigation is required.

The objective of the experiment conducted in chapter 4 was to evaluate the impact of dietary amylase supplementation on growth and nutrient utilization in broiler chickens in 4 age groups. The results showed that growth performance of birds improved with dietary amylase addition. Furthermore, the AID and total tract retention of starch and gross energy was improved by amylase supplementation. Importantly, there was an interaction between exogenous amylase and growth phase on AID of starch. Consistently, d 11 to 21 bird group responded differently to amylase supplementation compared to other growth phases. This includes increase in digesta viscosity and pancreatic amylase secretion with amylase supplementation. The reason for these response deviations during d 11 to 21 is unclear and warrants further investigations. In conclusion, the study

indicates that the nutritional benefit obtained from exogenous amylase administration in broiler chickens depends on the growth phase of the birds.

In chapter 5, the contribution of exogenous amylase to starch and gross energy digestibility in specific intestinal sites of broiler chickens was investigated. A previous report (Weurding et al., 2001) showed that rate and magnitude of starch utilization in broiler chickens vary by intestinal site, with metabolic consequences on feed utilization. What's unclear, however, is the contribution of dietary amylase addition to starch utilization at the various intestinal regions. At the posterior jejunum and anterior ileum, there were linear and quadratic responses in starch and gross energy digestibility due to increasing amylase supplementation. Although total tract starch retention was already high (~ 98%), it increased linearly with amylase supplementation. There were linear increases due to exogenous amylase in starch disappearance and digestible energy with digesta flow from anterior to posterior jejunum, suggesting a more localized effect of the enzyme within the jejunum. This result agrees with Svihus (2014) and suggest a tendency for amylase to prioritize starch digestibility within the proximal gut for maximum absorption of the simple sugars. Nevertheless, there were improvements in starch digestibility in distal intestinal regions, suggesting an effect of the amylase to cause sustained circulating glucose levels, which could also have a protein-sparing effect at the hind gut. Due to the impact of the physical form of feed on the rate of feed consumption and nutrient utilization by birds, further research is required using pelleted or crumbled feed.

In chapter 6, two consecutive trials were conducted to evaluate the impact of dietary P deficiency on molecular appetite regulation in broiler chickens. In trial 1, birds were randomly assigned to 3 experimental diets with 3 different concentrations of non-phytate P (g/kg): 1.2 (P-deficient), 2.8 (P-marginal) or 4.4 (P-adequate). In trial 2, the birds offered the P-deficient diet was pair-fed to the feed intake level of the P-adequate group to determine the direct impact of dietary P deficiency on appetite regulation. Result showed feed intake reductions in birds fed P-deficient diet and an upregulation of NaPi-IIb mRNA, which further suggests high sensitivity to available P. The expression of peripheral CCK mRNA was downregulated as a result of P deficiency, which contradicts the known anorexigenic function of CCK and warrants further investigation. However, hypothalamic mRNA expressions of CCK and melanocortin receptors, which are strongly anorexigenic, were upregulated in response to P deficiency. The observed alterations in gene expression, however, requires careful interpretation and may not always translate to protein

synthesis. Because the expression of POMC mRNA remained unaltered in the brain irrespective of dietary P level, the concentration of α-MSH may also have remained unchanged. Therefore, the stimulating ligands or hormones causing the upregulation of melanocortin receptors is unclear and would require further investigation.

The data presented in this dissertation emphasizes the benefit of exogenous enzyme use in poultry nutrition, and its impact on the nutritional value of feed ingredients. Variations in the responses to feed enzymes may be related to factors such as compatibility with intestinal pH, digesta transit time and digestive capacity. Deviation towards cheaper non-conventional alternative feedstuff also results in considerable challenges that limits the efficacy of the exogenous enzymes. The final study of this dissertation showed that the feeding response in broiler chickens depends on the level of dietary phosphorus and suggests a complex interaction of phosphorus with anorexigenic sensors in the gut and brain.

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