ABSTRACT

AYOOLA, AYUUB AYODELE. Impact of Dietary Exogenous Enzyme Supplementation on Endogenous Secretion, Gastrointestinal Health, Nutrients Digestibility and Growth Performance of Poultry. (Under the direction of Dr. Peter Ferket).

Anti-nutritional factors in feed ingredients (ANF) can reduce nutrient utilization and suppresses gut health. Birds typically activate their innate immune system for protection against the adverse effects of ANF, which often involves the secretion of mucin. Although dietary supplementation of exogenous enzymes are commonly used to alleviate the adverse effects of ANF on apparent nutrient digestibility, little is known about how they affect gut health, particularly in relation to enteric mucosa morphological development, and the endogenous nutrient losses due to mucin secretion. The hypothesis of this dissertation centers on the impact of dietary enzyme supplementation on gut mucosa morphology and mucin secretion, in relation to digestibility and nutrient utilization. The primary hypothesis is: dietary enzymes reduce the ANF-inducing effects on enteric mucin secretion, which reduces endogenous nutrient loss and improves apparent nutrient retention and utilization towards growth efficiency. We carried out five studies to examine the hypotheses in turkeys and broilers, using different types of dietary ANF challenges and remedies of exogenous enzymes.

In the first 3 studies, we tested our hypothesis by challenging the birds with dietary non-starch polysaccharides (NSP), and evaluating the response to dietary supplementation of carbohydrases. In the first 2 studies, the NSP challenge was imposed by increasing dietary inclusion levels of DDGS in corn-soybean meal (SBM) diets, supplemented with a blend of

endo-xylanase, alpha-amylase and protease (XAP), and direct fed microbial (DFM). Based on the outcomes of these first 2 experiments, we conducted the third study using the dietary β-mannanase inclusion to resolve the β-mannans ANF effects in high and low energy corn-SBM diets. The fourth and fifth experiment was conducted to further validate that our hypothesis that enteric mucin secretion is also relevant to explain the adverse effect of dietary phytate and the corrective response to phytase supplementation. The objective was to assess the effect of dietary inclusion of phytate, calcium and phytase up to 2000 FTU/kg on intestinal mucin secretion, nutrient digestibility, and growth performance of turkey poults.

The first two experiments revealed that the nutritional value of DDGS in corn-SBM diets for turkeys was not be improved by the XAP and DFM. The growth performance decreased as the DDGS inclusion levels increased. High dietary inclusion of DDGS also impaired jejunum villi development. Although supplementation of XAP and a combination of XAP+DFM had no effect on the BW, XAP improved apparent nutrient digestibility, and reduced intestinal mucin secretion. The results from third experiment showed that increasing dietary energy improved the growth performance, whereas the β-mannanase addition did not have any significant effect of on the growth performance. However, β-mannanase supplementation improved the jejunum villi development, crude protein, fat digestibility, apparent nitrogen retention (ANR), and lowered the ileal mucin secretion, presumably by reducing the endogenous loss of nutrients. In experiment 4 and 5, phytase supplementation significantly reduced the ileal mucin secretion, and improved nutrient digestibility and FCR. Both phytate and calcium enhanced the ileal mucin secretion. Although, the calcium level did

not affect the apparent nitrogen retention (ANR), increasing dietary phytate level reduced the ANR, while the addition of phytase enzyme increased the ANR.

In conclusion ANF, such as NSPs and phytate, impairs the apparent nutrient digestibility and growth performance by suppressing enteric mucosa morphological development, and increasing endogenous loss of nutrients *via* enteric mucin secretion. In contrast, dietary supplementation of exogenous enzymes helps alleviate the adverse effects of ANF on nutrient utilization by directly or indirectly removing the mucosal irritation that stimulates enteric mucin secretion. Excess dietary calcium also enhances enteric mucin secretion as a compensatory response to the formation of insoluble viscous mucoid complexes, which further increases endogenous loss of nutrients and adversely affects apparent nutrient digestibility.

Impact of Dietary Exogenous Enzyme Supplementation on Endogenous Secretion, Gastrointestinal Health, Nutrients Digestibility and Growth Performance of Poultry

by
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DEDICATION

This dissertation is dedicated with deepest sense of gratitude to all my teachers and mentors, past and present, who inspired me throughout these years.

BIOGRAPHY

Ayuub Ayodele Ayoola was born in Lagos, Nigeria. He earned a bachelors degree in Food Science and Technology from the Federal University of Agriculture Abeokuta, Nigeria in 2005. During his undergraduate study, he received the Oyo State of Nigeria Scholar award and graduated among top 5% in his graduating class. After graduation, Ayuub worked briefly in the food manufacturing industry before proceeding to graduate school. He obtained his MSc. in Nutrition and Food Science from NC State University, Raleigh, United States in 2010, and continued immediately with his PhD in Nutrition and Biotechnology (Minor) in spring, 2011. He hopes to utilize the knowledge and skills he acquired to the benefit of humanity.

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Literature Review

1.0 INTRODUCTION.

The US poultry industry is an important component of the economy, and one of the profit-driving forces in the industry is the ability to maximize the utilization of nutrients obtainable from dietary feed ingredients. However despite its contribution, the poultry industry in the USA and the rest of the world continue to strive towards improving their production efficiency and optimize profitability and sustainability. Use of by-product/co-products of human food and biofuels industries as feedstuffs for poultry is becoming a greater necessity as population growth and the need for environmentally sustainable energy increase into the future. However, many of these co-product feedstuffs contain anti-nutritional factors (ANFs) that impair the efficiency of nutrient utilization in poultry and may cause enteric distress (Choct et al., 1996; 1999ab; Cowieson et al., 2004; Selle et al., 2006). Many of adverse effects of these anti-nutritional factors (ANF) can be ameliorated by dietary supplementation of exogenous enzymes. During the recent decade, there has been a dramatic increase in the use of dietary exogenous enzymes as feed supplements, partially driven by the increases in grain and protein meal costs and partially due to advancements in biotechnology and fermentation technology to improve the bio-efficacy of commercial enzymes, and novel developments in enzyme applications. According to a recent global analysis carried out by AB Vista, capturing 500 major end users across 33 countries, the market for carbohydrases and proteases is $500 million USD and $50 million USD, respectfully; and about $450 million USD for phytase. The global market penetration of NSP enzymes has grown especially rapidly during the last 5 years, and now stands at 57% of global monogastric feed supplemented (with poultry penetration much

higher than that for swine). In comparison, more than 80% of all monogastric animal feed issupplemented with a phytase product (Feed Info News Service, March, 3, 2014). Dietary feed components play an important role in the maintenance of a healthy gut (Bedford and Apajalahti, 2001). ANF, such non-starch polysaccharides, serves as potential substrates for the gut microflora, especially those that reside in the hindgut (Apajalahti et al., 1995; Choct et al., 1996). Excessive dietary inclusion levels of ANFs can tilt the microfloral balance away from the symbiotic commensal microflora in the host towards the more pathogenic ones that ultimately adversely affect gut health, animal welfare, and risk the microbial safety of human food products from poultry.

This literature review discusses the available information on the types and chemical composition of ANF. Also examined is the effect of ANF on nutrient digestibility and gastrointestinal health, especially how they interfere with gut morphological development and some enteric secretions. The review discusses the nutritional role and benefits of dietary supplementation of exogenous enzyme for poultry. The last section of this literature review presents some questions and concepts that have not been answered by previous research, and proposes some hypothesis that requires scientific validation.

1.1 ANTI-NUTRITIONAL FACTORS AND NUTRIENTS DIGESTIBILITY

ANFs are frequently referred to as dietary compounds that impede nutrient utilization and metabolism because monogastric animals lacks the physiological capability to degrade or nutritionally overcome these ANFs (Angel et al., 2002; Cowieson et al., 2004). The response of animals that consume ANFs depends on its dietary dosage level, and the age, species and

physiological status of the animal (Odetallah et al., 2002). The anti-nutritional effects may manifest from a slight reduction in growth performance to as extreme as death in some cases. The type and quantity of ANFs in any animal feed depends on the ingredient composition of the feed. Some common dietary ANFs usually found in poultry feed include: non-starch polysaccharides (NSP); phytate; protease inhibitors; and goitrogenic factors. The scope of this literature review is limited to research related to NSP and phytate.

1.1.1 Non-starch polysaccharides

Generally, polysaccharides are macromolecular polymers of monosaccharaides, linked by glycosidic bonds. The monosaccharide unit is typically glucose, which are linked by either α or β (1-4), with (1-6), β (1-3), and (1-4) bonds. The major features that distinguish starch from NSP are the type, number and position of these glucosidic bonds (Hetland et al., 2004). A large percentage of the glycosidic bonds found in NSPs are in the form of β (1-4), and they are mostly found as part of the plants' structural components, and thus are abundant in most grains and plant co-product feed ingredients (Englyst et al., 1992). Most modern poultry feeds formulations are based on grains and co-product meals from grains and oilseed processing, so they contain a significant amount of NSP.

The anti-nutritional effect of NSP stem from the fact that monogastric animals, including poultry, lacks the capability to produce any or enough endogenous enzymes to hydrolyze the glycosidic bonds of NSP (Carré, 1993). Because polysaccharides are a major caloric substrate in poultry nutrition, the metabolizable energy that is available for poultry is directly related to the dietary level of NSP (Santos et al., 2004a,b). Apart from suppressing

digestive capacity, the viscous NSP can encapsulate other nutrients, thus depressing their digestibility in the intestinal lumen and absorption (Choct et al., 1999). The impediment in digestion results in decreased nutrient utilization, along with a simultaneous increase in the feed conversion efficiency. Table 1 shows list of some common NSP that are found in the diet of non-ruminant animals. The most common ones include celluloses, hemicelluloses, pectins, arabinoxylans and β-mannanas (Table 1).

1.1.1.1 Chemical structure and function of arabinoxylans and β-mannans

NSP is a significant component of most feed ingredients, and there is large variation in their structure among different ingredients. NSP are polymers of simple monosaccharides, which are arranged in different orientations and complexities. They can either be branched or linear, with or without charged groups (Smiths and Annison, 1996). The different chemical conformations influence on their physical properties. Among the common NSP that challenge the nutrient utilization and health of poultry are arabinoxylan and β-Mannans.

Arabinoxylan is complex polysaccharide composed of two main sugars: arabinose and xylose, arranged in an extensively branched structure (Martha and Costa, 1995). They are partly soluble in water, and are part of the cell wall polysaccharide components in feed grain ingredients. They consist of a linear β-(1-4) linked xylan backbone, to which α-L-arabinofuranose units are attached as side residues via α-(1 - 3) and/or α- (1-2) linkages (Izydorczyk and Dexter, 2008). According to Fincher and Stone (1986), the thin walls that surround the cells in the starchy endosperm and the aleurone layer in most cereals consist predominantly of arabinoxylans (60-70%), with the exceptions of the endosperm cell walls of

barley (20%), and rice (40%). Furthermore, the non-endospermic tissues of wheat, particularly the pericarp and testa, also have very high arabinoxylan content (64%). Arabinoxylan are found abundantly in grain feed ingredients, thus they may influence the nutritional value of cereal-based diets (Englyst et al., 1992). β-mannans or β-galactomannans are another NSP that are commonly found in the feed ingredients. They are made of polysaccharides with repeating mannose monosaccharide units as a backbone, with galactose and/or glucose attached to the β-mannan backbone (Carpita and McCann, 2000). In some but few cases, the repeating mannose units can be replaced by glucose (Hsiao et al., 2006).

β-mannans are commonly found in different types of plant-based feed ingredients, such as guar gum, guar meal and soybean meal (Rogel and Vohra, 1983). Currently, SBM is the major protein source in most poultry diets (Dierick, 1989). SBM β-mannan has an average galactose: mannose ratio of 1:1.8 (Whistler and Smart, 1953; Whistler and Saarnio, 1957). Apart from being vital structural component of plant cell walls, some pathogenic microflora, such as fungi, bacteria and virus also have β-mannans as part of their cell components (Hsiao et al., 2006). Thus, it could be postulated the presence of β-mannans in the gut may influence the activities of gut microflora.

Table 1. Classification of carbohydrates that are non-digestible by non-ruminant animals[1]

Category	Monomeric residues	Sources
Polysaccharides		
Resistant starch		
Physical inaccessible starch (RS1)	Glucose	Partly milled grains and seeds
Resistant starch granules	Glucose	Raw potato, banana
Retrograded starch	Glucose	Heat-treated starch products
Non-starch polysaccharides (NSP)		
Cell wall NSP		
Cellulose	Glucose	Most cereals and legumes
Mixed linked β-glucans	Glucose	Barley, oats, rye
Arabinoxylans	Xylose, arabinose	Rye, wheat, barley
Arabinogalactans	Galactose, arabinose	Cereal co-products
Xyloglucans	Glucose, xylose	Cereal flours
Rhamnogalacturans	Uronic acids, rhamnose	Hulls of pea
Galactans	Galactose	Soya bean meal, sugar-beet pulp
Non-cell wall NSP		
Fructans	Fructose	Rye
Mannans	Mannose	Coconut cake, palm cake
Pectins	Uronic acids, rhamnose	Apple, sugar-beet pulp
Galactomannans	Galactose, mannose	Guar gum
Oligosaccharides (prebiotics)		
α-Galacto-oligosaccharides	Galactose, glucose, fructose	Soya bean meal, peas, rapeseed meal
Fructo-oligosaccharides	Fructose, glucose	Cereals, Jerusalem artichokes
Transgalacto-oligosaccharides	Galactose, glucose	Feeds additives, milk products

[1]Adapted from review by Montage at al. (2008), with the information extracted from studies by Bach Knudsen (1997) and Englyst et al. (1992).

1.1.1.2 Effects of NSPs on nutrients digestion, endogenous secretion, and gastrointestinal health.

It is due to the unique chemical structure of NSP that they impose their physiological effect on digestion and the enteric ecosystem. There are abundant reports on the anti-nutritive effects of NSP in poultry (Choct and Annison 1992ab; Bedford and Morgan, 1996). Due to their ability to increase viscosity of the intestinal luminal contents in the upper part of the gastrointestinal tract, NSP suppress nutrient utilization, distress gastrointestinal health, and enhance endogenous secretion (Choct et al., 1999; Santos et al., 2004ab). The increased viscosity increases the gastric intestinal transit time of digesta, and it also reduces the access of the digestive enzymes to digesta substrates (starch, protein, and fat) and impedes nutrient absorption (Edwards et al. 1988; Angkanaporn et al., 1994). This is particularly important in monogastric animals like poultry because they lack the ability to secrete NSP-degrading enzymes in sufficient amounts to overcome their anti-nutritional effects (Montagne et al., 2003, 2004). NSP can also bind nutrients, form complexes with digestive enzymes, and influence some regulatory proteins in the gut (Choct et al., 2010). Viscous or soluble NSP especially interfere with the nutritional availability of dietary fat by entrapping fat globules and limiting the access of endogenous lipase to hydrolyze emulsified fat into absorbable fat micelles (Incharoen et al., 2010). Furthermore, NSP can bind bile, and prevent it from emulsifying dietary fat to form fatty acid micelles after lipase hydrolysis. Consequently, bile is not reabsorbed and is subsequently excreted *via* feces. Thus, apart from impeding fat digestion, this situation necessitate the need to synthesis more bile, contributing to endogenous nutrient loss (Vahouny et al., 1981; Ide et al., 1989).

In addition to reducing nutrient utilization, some researchers have reported that NSP can also enhance endogenous secretion and thus promote endogenous nutrient loss. Angkanaporn et al. (1994) reported that the presence of arabinoxylans in the poultry gut leads to an increase in endogenous loss of amino acids. Additionally, Cowieson et al. (2004) reported that NSP may increase endogenous losses by stimulating mucous secretion, with the mucin protein being the major component of mucous. Mucin is rich in some essential amino acids, especially threonine and proline (Lien et al., 1996). Soluble and indigestible NSP has the ability to interact with the intestinal brush border and increase mucin secretion and the thickening of the unstirred water mucous layer (Johnson and Gee 1981). Thus, the secretion of intestinal mucous also has a secondary effect of slowing down the transit of nutrients across the intestinal epithelial layer before absorption by the enterocytes.

The gastrointestinal ecosystem does not escape the adverse effects of NSP. The entrapment of nutrients by the viscous digesta may impact the gastrointestinal ecosystem (Petersen et al., 1999; Santos 2005). This situation provides a favorable environment for the proliferation of competitive gut microflora, and subsequently increases bacterial fermentation of dietary component that should otherwise be digested and absorbed by the host (Bedford and Morgan, 1996). The increased digesta viscosity speeds up the intestinal passage rate of the digesta. Coupled with the lack of endogenous enzyme to degrade NSP, the hindgut is charged with lots of readily fermentable substrate for the hindgut microflora (Choct at al., 1996) and often causes disbacteriosis and pathogenicity (Van Immerseel et al., 2010). The activity of the microflora is enhanced by the anaerobic condition in the distal end of intestinal tract, and it

provides an ideal environment for the proliferation of pathogenic bacteria (Wagner and Thomas, 1987). Choct et al. (1996) observed increased fermentation in the gut of broilers as the dietary inclusion of soluble NSP increased. Diet composition may produce microscopic alterations in the intestinal mucosa, and it is possible that the change in morphology of the gastrointestinal tract may be associated with the irritating effects of dietary NSP (Yamauchi, 2002). Iji et al. (1999) reported NSP can negatively impact gut morphology by increasing crypt depth of both the jejunum and ileum, indicating increased gut epithelial cell turnover rate. The overall effects lead to changes in the gastrointestinal morphology, physiology and the ecosystem of the gut (Angkanaporn et al., 1994).

Although the hindgut microbial fermentation may yield volatile fatty acids (VFAs) that contributes to the net energy availability, there are losses in the efficiency in nutrient utilization for the host animal. The proliferation of the pathogenic anaerobic bacteria, and the drastic changes in the gastrointestinal physiology and morphology may overwhelmed the positive effect of the VFAs. In extreme cases, these changes may lead to the onset of enteric diseases, such as necrotic enteritis (Kaldhusdal and Hofshagen, 1992). The overall effect of this is reduced growth performance of the birds.

1.1.2 Phytate

1.1.3.1 Chemical structure and function

Lowe et al. (1939) discovered the presence of the partially available phytate-phosphorus in animal feed. Ever since, science has discovered the abundance of phytate-

phosphorus in feed ingredients, such as corn and SBM. Phytate or phytate-bound phosphorus is present in most poultry diets. In some literature, phytate is also referred to as phytin and phytic acid; but all are substrates of the microbial enzyme, phytase. Phytate is chemically defined as the mixed salts of phytic acid (*myo*-inositol hexaphosphate; IP_6), while phytic acid is the free form of IP_6 (Selle and Ravindran, 2007). Phytate is found in most feed ingredients because it acts as part of the plant seed's phosphorus reservoir needed for seed germination (Selle and Ravindran, 2007). Approximately 2/3 of the phosphorus present in grains and legumes is in a complex form, which includes phytic acid and phytate-phosphorus (Pirgosliev et al., 2007; Punna and Roland, 1999; Viveros et al., 2000).According to Kasim and Edwards (1998), the major form of phytate in corn, wheat, rice bran and SBM is the IP_6 ester. Table 2 shows the phytate concentration in some common feed ingredients, and it also indicates that the majority of the phosphorus in the plant-based feed ingredients are in form of phytate-phosphorus. The tabulated values indicate that a typical corn-SBM poultry diet contains between 2.5 to 4.0 g kg^{-1}phytate-phoshorous or 8.9 to 14.24 g kg^{-1} phytic acid (Ravindran et al., 1995). In some other ingredients, IP_6 can be bound to some minerals forming chelation complexes known as phytin. The minerals that typically form chelated complexes with phytin include magnesium, calcium, potassium and zinc (Reddy et al., 1982). Based on a 2002 estimate, about 321 million tonnes of feed, and 1 million tonnes of phytate were consumed by broiler and layer chickens (Selle and Ravindiran, 2007). This large quantity of phytate consumed by the poultry industry reveals how much dietary phosphorus is under-utilized and is emitted into the environment with the poultry manure and litter.

Animals, especially monogastric animals, do not possess the capability to secrete sufficient enzyme to degrade the phytate, thus phytate-phosphorus is regarded as an inferior digestible dietary source of phosphorus (Ravindran et al., 1995; Bedford, 2000; Angel et al., 2002; Cowieson et al., 2004). The ANF property of phytate is based on its polyanionic nature (Ravindran et al., 1994; 1995; 1999; Cowieson et al., 2004; Selle et al., 2000). They can dissociate in aqueous solutions (water), making the polymers ionically charged. This chemical configuration enables phytate to chelate cations and other positively charged nutrients (Feil, 2001; Selle and Ravindran, 2007). Although scientific investigations continue about phytate's adverse effects on phosphorus and calcium digestibility, research is beginning to focus on the adverse effects of dietary phytate on other nutrients, such as protein (amino acids), energy, and trace minerals.

Furthermore, because of the significant phosphorus content they contain, poultry manure is normally used to fertilize plants. But excess phosphorus from the manure can easily run off from the farm land into surface water, and it contributes to the eutrophication of aquatic systems by stimulating algae growth, resulting in the mortality of aquatic animals, adverse environmental conditions, increased public health risks, and economic loss (Ryden et al., 1973).

Table 2. Weighted mean (and range) of total P and phytate-P concentrations and proportion of phytate-P of total P in key poultry feed ingredients.

Feed ingredient	Number of data-sets/samples	Total P (g /kg)	Phytate-P (g/kg)	Proportion (%)
Cereals				
Barley	4/41	3.21 (2.73-3.70)[A]	1.96 (1.86-2.20)[A]	61.0 (59-68)[A]
Maize	7/45	2.62 (2.30-2.90)	1.88 (1.70-2.20)	71.6 (66-85)
Sorghum	5/41	3.01 (2.60-3.09)	2.18 (1.70-2.46)	72.6 (65-83)
Wheat	5/97	3.07 (2.90-4.09)	2.19 (1.80-2.89)	71.6 (55-79)
Oilseed meals				
Canola meal	28/5	9.72 (8.79-11.50)	6.45 (4.00-7.78)	66.4 (36-76)
Cottonseed meal	21/3	10.02 (6.40-11.36)	7.72 (4.9-9.11)	77.1 (70-80)
Soyabean meal	6/89	6.49 (5.70-6.94)	3.88 (3.54-4.53)	59.9 (53-68)
By-products				
Rice bran	6/37	17.82 (13.40-27.19)	14.17 (7.90-24.20)	79.5 (42-90)
Wheat bran	25/6	10.96 (8.02-13.71)	8.36 (7.00-9.60)	76.3 (50-87)

Adapted from review by (Selle and Ravindran, 2007). Obtained from studies by Nelson et al. (1968a), Kirby and Nelson (1988), Eeckhout and de Paepe (1994), Ravindran et al. (1994), Viveros et al. (2000), Selle et al. (2003) and Godoy et al. (2005).

[A] Range of values.

1.1.3.2 Effects phytate on nutrient digestion, gastrointestinal health, and endogenous secretion.

There are a lot of reports on the effect of phytate on phosphorus and calcium utilization in poultry (Angel et al., 2002; Cowieson et al., 2004; Selle et al., 2009). Theoretically, the amount of phosphorus included in the diet as phytate-phosphorus from plant-based feed ingredients should be enough to meet the nutritional requirements of animals without the need for supplementation of phosphorus from other sources. However, because of the very limited bioavailability of the phytate-phosphorus, nutritional phosphorus requirements are usually met by the dietary inclusion of more bioavailable sources, such as inorganic sources (i.e. dicalcium phosphate, monocalcium phosphate, or deflourinated rock phosphate) and rendered animal meat and bone meals (Selle et al., 2009).

Phytate is a poly-anionic molecule, and it can chelate cations like calcium to form mineral–phytate complexes (Lonnerdal et al., 1999; Angel et al., 2002; Sandberg, 2002). The polyanionic phytate molecule carries up to twelve negative charges, having a potential to bind six divalent cations such as calcium, zinc and copper atoms, with each varying in chelating affinity towards phytate (Maddaiah et al., 1964; Vohra et al., 1965). Therefore, in addition to reducing phosphorus digestibility, it can also depresses the bioavailability of other minerals. Calcium is a vital and dominant mineral in most poultry diets, thus preventing the *de novo* formation of the insoluble calcium–phytate complexes in the gut at favorable pH levels is important (Wise, 1983 and Angel et al., 2002). Worst still, the insoluble calcium-phytate complexes formed are resistant to enzymatic hydrolysis by dietary exogenous phytase (Taylor,

1965), which further reduces the efficiency of exogenous phytase to degrade phytate (Hill and Taylor, 1954; Shirley and Edwards 2002; Selle et al., 2009). Thus, a balanced dietary calcium level and an appropriate calcium/phosphorus ratio is vital for effective enzymatic degradation of phytate (Lei et al., 1994).

In addition to reducing mineral digestibility, there are postulations that phytate can suppress protein/amino acid utilization in poultry (Cowieson et al., 2004). However, there are limited and variable reports on the exact mechanisms of how phytate impairs dietary amino acid utilization. Selle and Ravindran (2007) listed several factors that could lead to the variation of nutrient digestibility results due to dietary phytate level. These factors include: the dietary levels of calcium and non-phytate-phosphorus; electrolyte balance; feed ingredient; and the choice of inert marker used in the digestibility assays. They also included age of birds, sources and concentrations of phytate in the diet, and the inherent digestibility of dietary amino acids of the test diets.

Phytate can bind protein to form binary protein–phytate complexes, and this process is enhanced by the acidic environment in the stomachs of monogastric animals (Cosgrove, 1966 and Anderson, 1985). Chemically, at pH below the isoelectric point of a protein molecule, polyanionic phytate molecule can bind the protein amino acids components with a strong electrostatic bond. Basic acids residues, such as arginine, histidine and lysine, are usually complexed by phytate, and the initiation process is preceded by a slower and progressive protein–protein aggregation, leading to protein precipitation (Rajendran and Prakash, 1993). The progressive formation of phytate-protein complex stimulates a refractory response to

pepsin activity during digestion of the protein (Selle et al., 2000). This is due to changes in the structural conformation and solubility of the protein (Vaintraub and Bulmaga, 1991). The inhibition of the protein digestion by pepsin is enhanced by the low pH conditions in the proventiculous-gizzard of poultry and the stomach of pigs (Camus and Laporte, 1976; Knuckles et al., 1985; and Knuckles et al., 1989).

There are other reported mechanism by which phytate can affect protein/amino acid utilization in poultry. Ravindran et al. (1999) and Cowieson et al. (2004) observed an increase in endogenous amino acid loss by broilers as dietary phytate concentration increased. They proposed that the observed increase in endogenous amino acid loss was due to the increase in enteric mucin secretion. Furthermore, phytate has been reported to stimulate mucin secretion in rats (Munster et al., 1987). Mucin is a major component of mucous, and it is rich in amino acids such as threonine, proline and serine (Lien at al., 1996). The mechanism behind the increase in enteric mucous was due to an increased HCl secretion, and the refractory effect of phytate-protein complex on pepsin activities in the gastrointestinal tract. The stimulus from the presence of protein keeps stimulating the secretion of pepsin. The animal then responds by activating the innate immune system, secreting mucous to protect the enteric epithelial layer against the activity of the free flowing pepsin.

1.2 SUPPLEMENTAL EXOGENOUS ENZYMES

1.2.1 Use of exogenous enzymes in poultry diet

The use of exogenous enzyme supplementation in poultry diets to improve the nutritional value of feed ingredients has become common practice in the animal production

industry during the last two decades. This is because most monogastric animal diets, including poultry diets, are formulated to contain corn and SBM as the main feed ingredients. These two feed ingredients contain phytate and NSP, which are important substrates for the dietary exogenous enzymes. Thus, suitable dietary supplementation of exogenous enzyme, such as NSPase and phytase, are increasingly employed to improve the nutritional, and the economic value of the feed ingredients by monogastric nutritionists.

1.2.2 NSPase - Nutrients utilization and efficiency.

As aforementioned, NSP are dietary fibers found in plant-based feed ingredients, and can affect nutrient digestibility and growth performance of poultry (Dierick, 1989; Daskiran et al., 2004). Over the last three decades, nutritional biotechnologists have been able to synthesis of some potent dietary exogenous carbohydrases, otherwise known as NSPases (Slominski, 2011). These enzymes have the ability to degrade the non-digestible NSP fed to poultry. Since then, several published research reports have shown that the anti-nutritional effects of NSP in poultry feed can be resolved by supplementing poultry diets with the dietary exogenous NSPases.

Dietary supplementation of NSP hydrolyzing enzymes to diets rich in cereal grains can significantly improve the growth performance of poultry birds (Odetallah et al., 2002). Studies have shown that the metabolizable energy yield for poultry increases when NSP hydrolyzing enzymes are supplemented to the diet (Santos et al., 2004ab). The supplemental enzyme blends enhance the breaking down of the complex NSP into oligosaccharides, which the birds have the metabolic capability to metabolize (Bedford and Classen, 1992; Bedford and Schulze,

1998). In addition to improving NSP digestibility, NSPase, such as xylanase, also has been shown to reduce the relative weight and length of the small intestine of poultry, thereby reducing the gut maintenance requirement for nutrients (Engberg et al., 2004; Wu et al., 2004).

Apart from increasing the amount of carbohydrate substrates that can be metabolized for energy, NSP degrading enzymes have been speculated to have secondary effects to increase the utilization of other nutrients in monogastric animals (Frolich and Asp, 1985). These NSP enzymes ameliorate the nutrient encapsulation effect by reducing digesta viscosity, thereby enhancing nutrient digestion and absorption (Choct and Annison, 1992). However, available published results to support this hypothesis are inconclusive and conflicting. For example, Kim et al. (2005) explained that the improved phosphorus digestibility after addition of exogenous xylanase was presumably associated with the liberation of encapsulated non-phytate phosphorus from fibrous material. In contrast, Leslie et al. (2005) reported that dietary supplementation of xylanase was not effective in improving performance of broiler chickens fed a diet where the first-limiting nutrient is phosphorus, and Cowieson and Adeola (2005) confirmed this observation. The variable results in the literature may depend on the ingredient composition of the test diet, the capability of the enzyme itself, and the microbial source of the enzyme. The microbial source plays an important role in determining the efficiency of enzyme activity. As shown in table 3, there are variable capabilities and activities of enzymes to hydrolyze bonds within the NSP (Bedford and Schulze, 1998). Different enzymes require different optimal temperature and pH among other parameters to achieve optimal enzymatic

efficiency. Nevertheless, the conflicting results reported in the literature clearly demonstrate that effect of the NSPases to increase the nutrient digestibility remains debatable.

Supplementation of poultry feed with dietary exogenous NSPases can also influence the gut ecosystem by stimulating microbial population and diversity. After an enzyme degrades NSP into smaller oligosaccharides, the small oligosaccharides become preferential substrate (i.e. a prebiotic substance) for commensal bacteria fermentation (Choct et al., 1999). As previously described, the end products of the fermentation in the hindgut (ileum, ceca, and colon) includes volatile short chain fatty acids, such as acetic, butyric and propionic acids (Apajalahti et. al., 1995; Apajalahti and Bedford 1999; Bedford and Apajalahti, 2001). These organic acids are effective antimicrobial agents that control the proliferation of hindgut pathogens that promote mucosal inflammation and adversely affect gut health, and favors a more symbiotic microbial population that benefits the host animal.

There are also some reported synergistic effect between NSPases and other non-NSP enzymes, such as phytase and proteases. Although research on the synergy of different enzymes has receive little attention, Cowieson and Adeola (2005) reported that a blend of xylanase, amylase and protease (XAP) improves the growth performance of broiler fed a corn-SBM diet. They observed the digestibility of dietary energy, nitrogen, and dry matter was improved by the XAP supplementation. A blended of XAP enzymes contains xylanase, amylase, and protease, thus the improvements in the nutrient utilization was attributed to better digestibility of starch, the sparing of endogenous amino acids and energy associated with a reduction in endogenous enzyme production (Mahagna et al., 1995; Gracia et al., 2003),

improved access to contents associated with hydrolysis of structural carbohydrates by xylanase (Bedford, 2002), and hydrolysis of proteinaceous anti-nutrients (Huo et al. 1993; Ghazi et al., 2002). Furthermore, NSP enzymes have been hypothesized to increase the efficacy of phytase by eliminating the phytate encapsulation by NSP (Kim et al., 2005). NSP have the capacity to entrap phytate in both feedstuffs and in digesta (Debon and Tester, 2001). However, the information on the effect of NSP degrading carbohydrases on phytase efficacy are inconsistent.

Table 3. Variation in catalytic and physical properties of endoxylanases from 31 species of bacteria and 31 species of fungi

Organisms	Optimal temperature (^oC)	Optimal pH	Molecular Weight	Vmax (U/mg)	Km (mg/ml)
Fungi Min	30	2	13,000	0.37	0.09
Fungi Max	80	7	95,000	$1.4*10^4$	22.3
Bacteria Min	30	4.5	13,000	$1.7*10^{-2}$	0.07
Bacteria Max	105	10	350,000	$1.2*10^4$	17.7

Adapted from a review by Bedford and Schulze, 1998.

1.2.3 Phytase - Nutrient utilization and efficiency.

The importance of phytate in the poultry nutrition has been discussed in detail in the earlier section. In fact, most plant-based animal feeds contain a significant amount of their total phosphorus as phytate-phosphorus (Harland and Morris, 1995). Therefore, dietary exogenous phytase enzyme is added to poultry diets to enable them utilize the phytate-phosphorus more efficiently, and to avoid the associated phosphorus run-off problems (Nelson et al., 1971). Dietary supplementation of exogenous phytase is now seen as a standard practice in the manufacture of most poultry feeds.

Phytases (*myo*-inositol hexaphosphate phosphohydrolases) are enzymes that can hydrolyze the ester bonds between the phosphate groups and the inositol ring in phytate, therefore increasing the dietary available phosphorus (Irving and Cosgrove, 1974; Cowieson et al., 2004ab). Several studies have shown that phytase increases phosphorus digestibility, which ultimately helps reduce the amount of phosphorus run off into surface water bodies (Applegate et al., 2003; Penn et al., 2004; Angel et al., 2006; Leytem et al., 2007). However, there are still opportunities for improving the efficiency of exogenous phytase enzyme currently sold in the market. Collated results (Table 4) from some published data on dietary exogenous phytase shows that only about 20% of the phosphorus bound to phytate in poultry diets (i.e., 0.056% vs. 0.29%) can be released and made nutritionally available by commercial phytase.

Just like NSPases, the microbial source of phytase plays a vital role in determining its activity and efficiency (Igbasan et al., 2000; Simon and Igbasan 2002). Phytase from different

microorganism sources express significant differences in the efficacy of their activity (Segueilha et al. 1992; Shimizu, 1992; Zyla, 1992). They exhibit different properties, depending on the source of the gene and the synthesizing organism, and they behave differently with respect to their in vitro properties (Simon and Igbasan 2002). Currently, the commercial phytase preparations are produced by bacteria and fungi, such as *E.Coli*, and *Aspergillus spp*, respectively (Igbasan et al., 2000). The *Aspergillus spp* phytases express optimal activity at pH 4.5 to 5.5 and temperature of 50 ^{o}C (Wyss et al., 1999; Simon and Igbasan 2002). In contrast, the *E. coli* phytase express optimal activity at pH<4.5 and temperature of 60 ^{o}C (Greiner et al., 1993; Golovan et al., 2000; Simon and Igbasan 2002). The pH optimum for the *Bacillus* phytase was identified at pH 7.0, which makes this the *Bacillus* phytase more active in the small intestine, but the possible formation of phytate-mineral complexes may reduce it efficiency. *E. coli* phytase is most thermal stable, and also insensitive to protein degrading enzymes, such as pepsin and pancreatin (Rodriguez et al. 1999; Igbasan et al., 2000; Simon and Igbasan 2002). These attributes are important in the determination of the application and efficiency of different exogenous phytases as commercial feed additives in manufactured poultry feed.

Aside the microbial source of phytase, there are some dietary components that can influence the efficacy of phytase, such as the mineral composition in the diet formulation, age and species of the bird (Selle and Ravindran, 2007). Phytic acid can bind dietary mineral cations to form insoluble complexes that can resist the hydrolytic activity of phytase (Cowieson et al., 2007; Bryden et al., 2007). This phytate-mineral binding reduces the availability of the

minerals to the animals. Studies have shown that phytase supplementation increases the digestibility and retention of calcium, and improves absorption and retention of other mineral such as magnesium, copper, and iron (Sebastian et al., 1996; Qian et al., 1997). The improved mineral bioavailability is due to the elimination of the formation of insoluble phytate–complexes with the mineral (Tamim et al., 2004), which normally happens in the proximal end of the small intestine. Since most bacterial and fungal phytases are active in the gastric end of the gut (Greiner et al., 1993; Golovan et al., 2000; Simon and Igbasan 2002), phytate hydrolysis by phytase takes place mainly in the fore-stomach (crop, proventriculus, gizzard) where the pH is low enough for optimum phytase activity, and its substrate, phytate, is more water soluble (Simon and Igbasan, 2002; Selle and Ravindran, 2007) and less reactive to dietary cations.

As previously discussed, phytate can also reduce the availability of dietary amino acids in pigs and poultry, either directly through interaction with digestive enzymes or indirect interaction with dietary proteins (Pirgosliev et al., 2007). Therefore, phytase supplementation may improve amino acid utilization by poultry. Some recent studies have shown that in addition to improving mineral digestibility, dietary exogenous phytase supplementation may also improve energy and amino acid digestibility (Ravindran et al., 1999; Selle et al., 2000; Newkirk and Classen, 2001; Ravindran et al., 2001; Rutherfurd et al., 2002; Pirgosliev et al., 2005; Cowieson et al., 2006ab). Another secondary influence of phytase on improving the digestibility of protein is linked to the reduction in endogenous secretion and loss of amino acids. Some recent studies revealed that the inclusion of supplemental phytase in poultry diets significantly reduces endogenous secretion and nutrients loss by reducing the enteric mucin

secretion (Montagne et al., 2000; Cowieson et al., 2004b). Increased enteric mucin secretion is often associated with gut health problems and enteric inflammation (Pirgozliev et al., 2005). Dietary supplementation of phytase may improve gut health, and thus enhance dietary nutrient utilization. The possible association with other nutrients enables nutritionists to assign some extra-phosphoric effects to phytase supplementation in poultry diets, as this approach enables their integration into least-cost formulations (Shelton et al., 2004).

Table 4. Ileal phytate digestibility in chickens fed corn and soybean meal-based diets with and without dietary exogenous phytase supplementation.

Study no.	Trial length (d)	Phytate P (% of diet)	Phytase (U/kg)	Phytate P released[1] (%)			Reference
				Control	Phytase	Difference	
1	21	0.31	250	0.068	0.139	0.071	Camden et al. (2001)
			500		0.149	0.081	
			1,000		0.166	0.098	
2	21	0.28	500	0.070	0.126	0.056	Tamim et al. (2004)
3	21–28	0.30	500	0.030	0.062	0.032	Rutherfurd et al. (2004)
			750		0.062	0.032	
4	21	0.26	1,000	0.076	0.130	0.054	Olukosi et al. (2007)
5	21	0.26	1,000	0.008	0.057	0.049	Leytem et al. (2008)
6	21	0.31	600	0.077	0.109	0.032	Woyengo et al. (2010)
		0.29		0.055	0.111	0.056	

Adapted from a review by Slominski (2011).[1]Calculated based on phytate P digestibility data provided and expressed in actual amounts of phytate P present in the diet**.**

1.3 GASTROINTESTINAL TRACT

1.3.1 Structures and Functions

The GI tract plays an important role in the digestion and absorption of nutrients (Starck, 1993). The distinctive GI tract architecture enhances its ability to perform its functions effectively. It has numerous levels of in-folding, which results in a vast surface area for maximum nutrient absorption (Moffett et al., 1993; Snipes, 1997; DeSesso and Jacobson, 2001). There are also different compartments and layers in the GI tract, and their different structural and physiological attributes define their respective roles in nutrient digestion, absorption and other function (Snyder et al., 1975).

Generally, the GI tract is made up of four different structural and functional layers, including the mucosa, submucosa, muscularis propria, and serosa (Snyder et al., 1975). The mucosa is the innermost layer of the gut wall, and it is made of three sub layers, with the first being the epithelial layer, which faces the intestinal lumen. The epithelial layer is made up of epithelial cells. Villi are fingerlike projections from the epithelial lining of the intestinal mucosa, and it increases the GI tract absorptive surface area. Brush border microvilli projections on the villi further increase the intestinal epithelial surface area to maximize nutrient absorption (Moffett et al., 1993; Snipes, 1997; DeSesso and Jacobson, 2001).

The villi also house the highly differentiating absorptive cells, otherwise known as the enterocytes. Enterocyte cells are the major absorptive cells in the intestine, and the extent of the villi crypt depth is an important indicator of the mitotic activity and turn-over rate of enterocyte cells. Jejunum has the highest number of these cells, which signified their

importance in nutrient digestion and absorption (Figure 1; Noy and Sklan, 1997). Aside the enterocytes, the GI tract villus is also the site of some vital endogenous secretions, and immunoresponses. They house other secretory cells, such as the enteroendocrine and goblet cells. The goblet cells secret mucous, which lubricate and protect the intestinal lining from chemical and physical irritation (Antonioli et al., 1998; Montgomery et al., 1999; Zabielski et al., 2008). The different functions of the cells found in the GI tract emphasize its importance in nutrition absorption and protection of the body.

1.3.2 Gastrointestinal compartments and microbial Diversity

The GI tract microflora population are ubiquitous and heterogeneous, and they are involved in the complex process of nutrients metabolism and animal growth (Choct et al., 1992). They depend on the host for nutrients, and the highly variable pH and oxygen concentration in the gut influences the activities and type of microflora found in different sections of the poultry gut (Smith, 1965; Salanitro et al., 1978). The level of oxygen decreases from the proximal towards the distal part of the gut; thus aerobic microflora are usually present at the proximal end of the gut, while the anaerobic bacteria mostly reside at the distal end. The digesta pH increases progressively from the crop to the ceca and colon end of the poultry gut, with the exception of the proventiculous and gizzard where the pH is relatively lower than in the crop (Fuller, 1977). These changes in the gut pH also influence the type and population of microflora that reside in the specific parts of the gut.

The crop, which serves mainly as a temporary feed storage organ in poultry, has abundant oxygen concentration and a low pH condition. This area of the GI tract is colonized mostly by *Lactobacillus*, *coliform*, and *Streptococcus/Enterococcus* bacteria (Fuller, 1977; Sarra et al., 1985). The *lactobacillus spp,* which are lactic acids producing bacteria, further reduces the crop pH to about 5, and also express some bactericidal effects. The bactericidal effect help to keep the population of pathogenic bacteria, such as *clostridia spp,* at bearable minimum level (Fuller, 1977). Other microflora, including *micrococci*, *staphylococci*, and yeasts, and sometimes aero-tolerant *Clostridium perfringens*, can also be found in the crop, but at a relatively lower populations.

Although some microbial starch degradation can take place in the crop, NSP fermentation is limited by the low pH environment (Santos, 2005). Distal to the crop is the proventiculous, but unlike in the crop, there is little microfloral colonization in this section. This is probably due to the functional property of the proventiculous, which is primarily for peptic degradation of feed materials (Duke, 1986) that requires the secretion of HCl to keep the pH within the range of 1-4. The acidic condition inhibits most microbial activities in this area of the gut. Microflora found in the compartments of the GI tract are mainly acidophilic lactic acid bacteria (Smith, 1965).

The duodenum, jejunum, and ileum form the proximal part of the intestine of the poultry gut. There are a lot of secretory and absorptive cells in the area. Most of the nutrient absorption takes place in this part of the GI tract. Because of the relatively higher pH, the intestine accommodates diverse, and vast bacterial populations that increase towards the

proximal end. These include *E. coli*, *Streptococcus/Enterococcus*, *Staphylococcus* and *Lactobacillus*, anaerobic cocci and species of *Eubacterium*, *Propionibacterium, Clostridium, Gemmiger and Fusobacterium* (Salanitro et al., 1978; Gong et al., 2002). Although, they are found more in the cecum and colon, there are also some butyric acid-producing microflora in the ileal area. Butyric acid is one of the short chain fatty acid (SCFA) that are found in relatively higher concentration in the hind gut of poultry. In addition to contributing to the gut energy demand of epithelial cells lining the epithelial layer of the distal part of the intestine, SCFA also reduces the extent of cell atrophy and enhance immune functions (Duncan et al., 2003; Scheppach and Weiler, 2004). SCFA also enhance the colonization of gut with beneficial commensal microflora, and competitively exclude pathogenic bacteria (Brouns et al., 2002). Thus, they are beneficial in enhancing the GI tract health.

Apart from the physiological condition of each intestinal segment, nutrient availability also plays a vital role in determining the microflora profile in each intestinal segment (Van Dijk et al., 1999). The microflora obtain their nutrients from the substrates available within their intestinal ecosystem, and the nutrients include undigested feed material flowing around in the lumen such as NSP (Montagne et al., 2003). Consequently, gut health can be enhanced by manipulating the nutritional substrates available for microflora fermentation and proliferation (Savory, 1992; Wagner and Thomas, 1987; Van Dijk et al., 1999). The larger population of VFA-producing bacteria reside in the ceca and colon because a lot of undigested nutrients end up in there, and the hindgut have fewer absorptive cells than in the more proximal end of the intestine (Montagne et al., 2003). These undigested nutrients are fermented by the

microflora to produce by-product VFAs and microbial proteins that can be used by the host or other microflora (Choct et al., 1992b; Montagne et al., 2003). However, the low oxygen concentration and the relatively higher pH in the hindgut makes it a more favorable ecosystem for proliferation of pathogenic microflora, which could have a significant on the overall health status of the GI tract (Barnes et al. 1970 and 1979).

1.3.3 The effect of Anti-nutritional factors on gastrointestinal morphology and development.

The gastrointestinal tract is an important organ system, as it facilitates nutrient digestion and absorption. In addition to impacting the diversity of the gut micro floral configuration, dietary compositions can also influence morphological changes, including alteration of villi conformations (Incharoen et al., 2010). Yamauchi et al. (2002) reported that major morphological changes of the GI tract villi are dependent on the presence of nutrients in the intestinal lumen. Thus, a good knowledge of how luminal nutrient contents impact enteric mucosa morphological development and function is vital for effective and efficient nutrients absorption.

The influence of dietary ANF on gut mucosa morphology is variable, depending on the type, physiochemical property, and dietary inclusion level of the ANF, and the age, intestinal site and type of the animal involved (Santos 2005). Phytate, for example, enhances microflora growth which irritate the enteric mucosa and causes inflammations that provokes gut morphological distress (McKay and Baird, 1999). Some studies have demonstrated dietary NSP to affect the development of gut morphology (Bedford and Classen, 1992; Bedford and

Schulze, 1998; Malkki and Virtanen, 2001; Montagne et al., 2003). High intake of NSP promotes an increase in size and length of the digestive organs, including the small intestine, caecum and colon in poultry (Van der Klis and Van Voorst, 1993) and in swine (Jin et al., 1994, Jorgensen et al., 1996; McDonald, 2001 and Iji et al., 2001). In monogastric animals, the indigestible and highly viscous NSP increases the viscosity of intestinal luminal digesta (Bedford and Classen, 1992; Bedford and Schulze, 1998). This viscous NSP entraps nutrients, decreases digesta residence time in the small intestine, and makes those nutrients more available to the hindgut microflora, including pathogens (Gohl and Gohl, 1977; Choct and Annison, 1992).

The proliferation of these pathogenic microorganism in the hindgut adversely effects of the mucosal morphology of the small intestine in poultry (Van der Klis and Van Voorst, 1993; Smits et al., 1997) and swine (McDonald et al., 2001). The activities of pathogenic bacteria enhances the rate of enterocyte losses at the villus apex, leading to villus atrophy. As a compensatory response to replenish the increased enterocyte turn-over rate, there is hypertrophy of the villus crypt depth as enterocyte mitosis increases (Montagne et al., 2003). Southon et al. (1985) reported an increase in jejunum and ileal crypt-cell production rate and mucosal protein synthesis rate when rats were fed non-cellulose NSP. Jin et al. (1994) observed that at high inclusion of fiber in growing pig diet, caused an enlargement of villi, and increased villi crypt depth in the ileal, jejunum, and large intestine. They also reported an increased rate of enterocyte proliferation, and a decrease in villi height/crypt depth ratio, especially in the jejunum and ileum. The increased cell proliferation rate indicates increased enterocyte turnover

rate, migration along the crypt–villus axis, and cell extrusion from the villous apex *via* apoptosis and cell sloughing. These observations were substantiated by McDonald et al. (2001), and similar results were also observed in poultry fed diets with a high dietary inclusion level of NSP (Langhout, 1998; Iji et al., 2001). Increased cell proliferation rate and decreased villus height/crypt depth ratio increases villi maintenance nutrient requirements, and vice versa (Montagne et al., 2003).

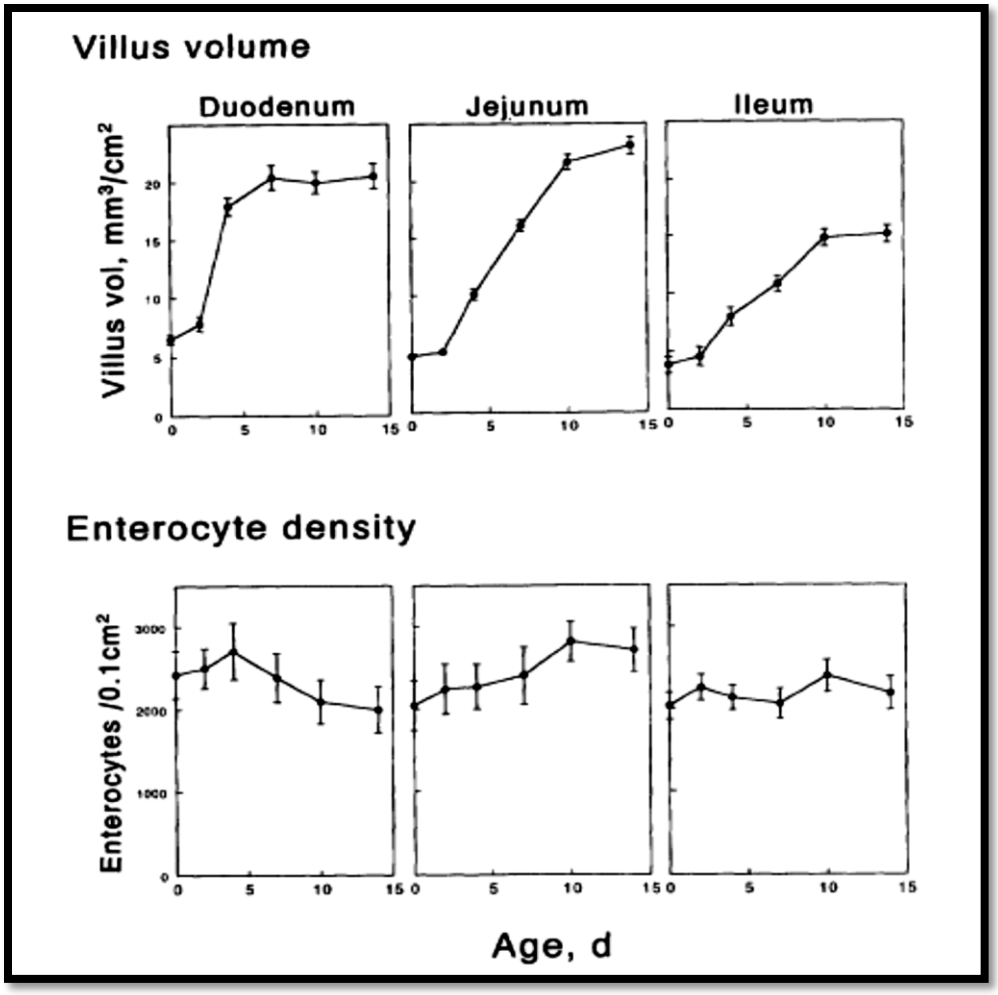

Figure 1. Villus volume (top) and eneterocyte density (bottom) in different intestinal segments at different age. Adapted from review by Noy and Sklan (1997).

1.4 INTESTINAL ENDOGENOUS SECRETIONS

1.4.1 Gastrointestinal immune function and endogenous secretions

Apart from the usual function of the gastrointestinal tract in the digestion and absorption of nutrients, the intestinal mucosal also has a vital defense function (Nagler-Anderson, 2001). Similar to the mucosa lining of the nasal airway, the gut is the first point of contact with undesirous external compounds that are ingested with food particles. It serves as a barrier that protects the host bird against the enteric microflora ecosystem, feed toxins and potent digestive juices secretions. Therefore, the intestinal mucosa plays a vital protection role in upholding the animal health.

This important enteric defense function of the mucosa is accomplished by several types of immunological responses. The first role has to do with assessing risks associated with the undesirous luminal compounds, achieved through the gut-associated lymphoid tissue (GALT) (Nagler-Anderson, 2001). Basically, there are specialized cells in the gut known as M-Cells. These cells are found within organized lymphoid follicles and they examine luminal molecules approaching the epithelial mucosal surface by reverse pinocytosis (Nicoletti, 2000). The potential threats are then removed from the system by subsequent processes (Niedergang and Kraehenbuhl, 2000). The second way by which the gut contributes to the immune system, is through secretion of a protective chemical substance called mucous. During, the normal and everyday processes in the intestinal lumen, the epithelial mucosal layer may be subjected to some luminal irritations from damaging agents and shear forces (Allen and Flemstrom, 2005 and Allen and Pearson, 1993). Mucous covers the entire lining of the mucosa epithelial

layer of the gastrointestinal tract. Mucin is the major glycoprotein component of mucous, and it is secreted from goblet cells (*Iiboshi et al., 1994*). The enteric mucous barrier is made up of two layers (Strugala et al., 2003; Taylor et al., 2004). The luminal (loosely adherent) layer that acts as to lubricate the passage of contents. It entraps damaging agents and sequesters them away from the underlying mucosa (Brownlee, Knight, Dettmar, and Pearson, 2007). The underlying (adherent) layer acts as a selective barrier to luminal factors reaching the mucosa, while still allowing the vital absorptive and secretive roles of the underlying tissues to occur (Pearson, Brownlee, and Taylor, 2004).

As pointed out earlier, there are some postulated theories on how ANF, such as NSP and phytate, can influence the secretion of mucous or mucin in poultry. NSP can influence enteric immunity by stimulating the GALT system and enteric mucous secretion (Barcelo et al. 2000; Sharma and Schumacher, 2001; Shimotoyodome et al., 2001; Brownlee et al., 2003; Strugala et al., 2003; and Brownlee et al., 2005). Phytate has been reported to stimulate mucin secretion through its physical abrasiveness, and the formation of protein-phytate insoluble complex (Cowieson et al., 2004).

1.4.2 Mucin composition and gastrointestinal health

The enteric mucous has a viscoelastic polymer-like characteristic. This physiochemical property is due to its major composition, the gel-forming glycoprotein components known as mucins (*Iiboshi et al., 1994*). Mucins consist of a peptide backbone containing alternating glycosylated and nonglycosylated domains, with *O*-linked glycosylated regions comprising 70–80% of the polymer. *N*-Acetylglucosamine, *N*-acetylgalactosamine, fucose, and galactose

are the 4 primary mucin oligosaccharides (*Iiboshi et al., 1994*). The immune property of enteric mucin rests on its carbohydrate chains. These polysaccharide chains act as specific receptors for the adhesion of gut microflora (Forstner and Forstner, 1994), and they are the basis of classifying enteric mucin. Based on the sugar component, mucin is divided into two major classes: neutral and acidic mucin. The acidic mucin is further divided into sulfated (sulfomucin) or non-sulfated (sialomucin) sub-class. Obviously, the chemical properties of acidic mucin varies from the neutral mucin. The presence of both sulphate and sialic acid conferred acidic mucin with higher acidity and viscosity than neutral mucin (Allen et al., 1982; Van Leeuwen and Versantvoort, 1999).

It has been reported that both commensal and pathogen bacteria adhere to different types of mucin (Montagne et al., 2003). Different parts of the gut secrete different types of mucin (Sheahan and Jervis, 1976; Roberton and Wright, 1997*;* Nieuw Amerongen, 1998*;* Deplancke et al., 2000*).* For example, the neutral mucins seems to be the major subtype expressed in gastric mucosa, while acidic mucins are synthesized throughout the intestinal epithelium and dominates the large intestine (Sheahan and Jervis, 1976). Some reports suggested that acidic mucins protect against bacterial translocation because the sulfated mucins are less degradable by bacterial glycosidases and host proteases (Fontaine et al., 1996; Roberton and Wright, 1997). Acidic mucin enhances the ability of mucous to resist attack by bacterial enzymes, and enhances the elimination of pathogens (Rhodes, 1989). This idea is also consistent with the observation that, goblet cells in intestinal regions densely populated by

microbes express acidic mucins predominantly (Roberton and Wright, 1997*;* Nieuw Amerongen, 1998*;* Deplancke et al., 2000).

The gut microflora influences dynamics of mucin secretion. Several authors have shown that under germfree conditions, the ratio of acidic to neutral mucins in the colon is higher, and sulfomucins appear to increase at the expense of sialylated mucins (Szentkuti et al., 1990; Enss et al., 1992; Sharma and Schumacher, 1995; Meslin et al., 1999). Sharma and Schumacher (1995) also reported fewer sialylated mucins in the small intestine of germfree rats than in conventionally raised rats fed a commercial diet. As different types and populations of microbial organisms colonize different parts of the gut, acidic mucins are most likely secreted in response to the colonization of pathogenic microorganisms, while neutral mucins are most likely secreted in response to the colonization of commensal microorganisms.

In a review by Corfield et al. (2001), it was reported that diseases and enteric inflammations by pathogens can alter the quantitative and/or qualitative changes in mucin production in humans. The interactions between enteric mucin and gut microflora can be either beneficial or detrimental to gut health, depending on the type of bacteria involved (Forstner and Forstner, 1994). Fixation of bacteria by mucin prevents their translocation into the underlying epithelial mucosa, especially the pathogenic bacteria where they can cause inflammation (Mantle and Husar, 1993). However, the strong bond between mucin and bacteria can serves as a way of retaining and increasing their colonization of the gut (Figure 2). Logically, the strong bond will enable the bacteria to resist the clearance by motile and

abrasive waves of intestinal luminal content during nutrients digestion (Mantle and Husar, 1993). The colonization of the gut by the commensal bacteria may be beneficial to gut health, whereas the colonization of pathogenic bacteria may not be in favor of the gut health. Furthermore, bacterial growth rate and penetration of mucus can exceed the rate at which this layer naturally turns over and is eliminated from the gut, thus enhancing bacteria residence time and colonization (Forstner and Forstner, 1994).

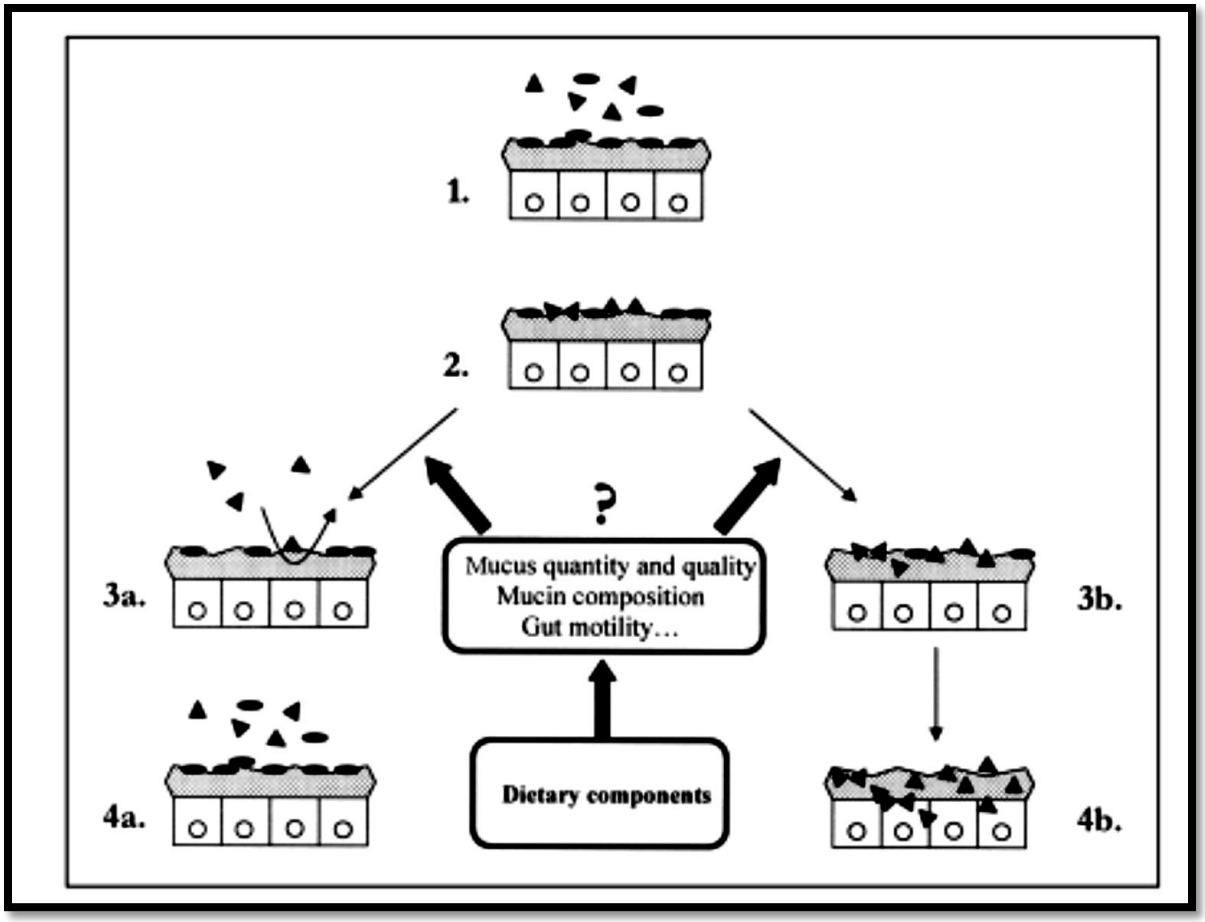

Figure 2. Fate of pathogenic bacteria in the gastrointestinal tract. (1) Commensal bacteria (●) are fixed on the luminal face of the mucus layer preventing fixation by pathogenic bacteria (▲), a phenomenon called the "barrier effect" or "colonization resistance." (2) If the colonization resistance is not complete, pathogenic bacteria can adhere to the mucus layer. This fixation can be beneficial (3a) or detrimental (3b) for the animal. (3a) Fixation of pathogenic bacteria on the mucus restricts their access to the underlying epithelium. Pathogenic bacteria are removed with mucus erosion and infection does not occur (4a). (3b). The rate of bacterial growth exceeds the natural turnover rate of the mucus. Bacterial colonization and proliferation occur leading to intestinal infection (4b). Factors relevant to mucin composition, quality and quantity of mucus, and gut motility influence the beneficial or deleterious outcome. Adapted from Montagne et al., 2004.

1.4.3. Dietary factors that impact enteric mucin secretion and endogenous nutrient loss

NSP and phytate can stimulate enteric mucin synthesis and secretion, but the removal and degradation of the enteric mucous and its constituent mucin in the intestinal lumen leads to endogenous nutrients loss at the terminal ileum of monogastric animals (Nyachoti et al., 1997; Montagne et al., 2003; Cowieson et al., 2004; Montagne et al., 2004). Erosion and enzymatic proteolysis of the mucous layer releases mucin in the GIT lumen (Allen 1981).

The effect of NSP on the loss of mucin in monogastric animals may depend on the solubility of the NSP. Non-soluble NSP increases mucin loss through their abrasive interactions with the intestinal mucous layer, scraping off mucin into the lumen from the intestinal mucosa as it passes down the gut (Leterme et al., 1998). In addition to this physical and direct effect of dietary NSP on enteric mucin dynamics, indirect effects such as enhancement of the proliferation of enteric microflora may also play a role in mucus secretion, and subsequently increase in enteric mucin synthesis. In contrast, phytate increases the intestinal mucin production through mucosa physical irritation (Cowieson et al., 2004). NSP and phytate have been reported to have some secondary effect on the activities and distribution of proteolytic enzymes in the intestinal lumen (Figure 3; Schneeman et al., 1982). This may also contribute to an increase in mucin secretion, and the consequential increase in endogenous nutrient loss.

The increased secretion and loss of mucin forces the animal to preferentially partition more nutrients to meet the gut health maintenance requirements than to meet the productive requirements of growth and reproductive performance. Since mucin is primarily rich in

oligosaccharides and amino acids such as threonine, proline, lysine and serine (Lien et al., 1996), the increasing production of intestinal mucin by ANFs decreases the availability of these amino acids and energy for growth (Cowieson et al., 2004; Leterme et al., 1998). Montagne et al. (2008) suggested that the influence of dietary ingredients on enteric mucin secretion should be taken into consideration when formulating diets for animals. This is because of laid out evidences of the quantitative and qualitative changes in mucin induced by dietary ingredients might have nutritional consequences.

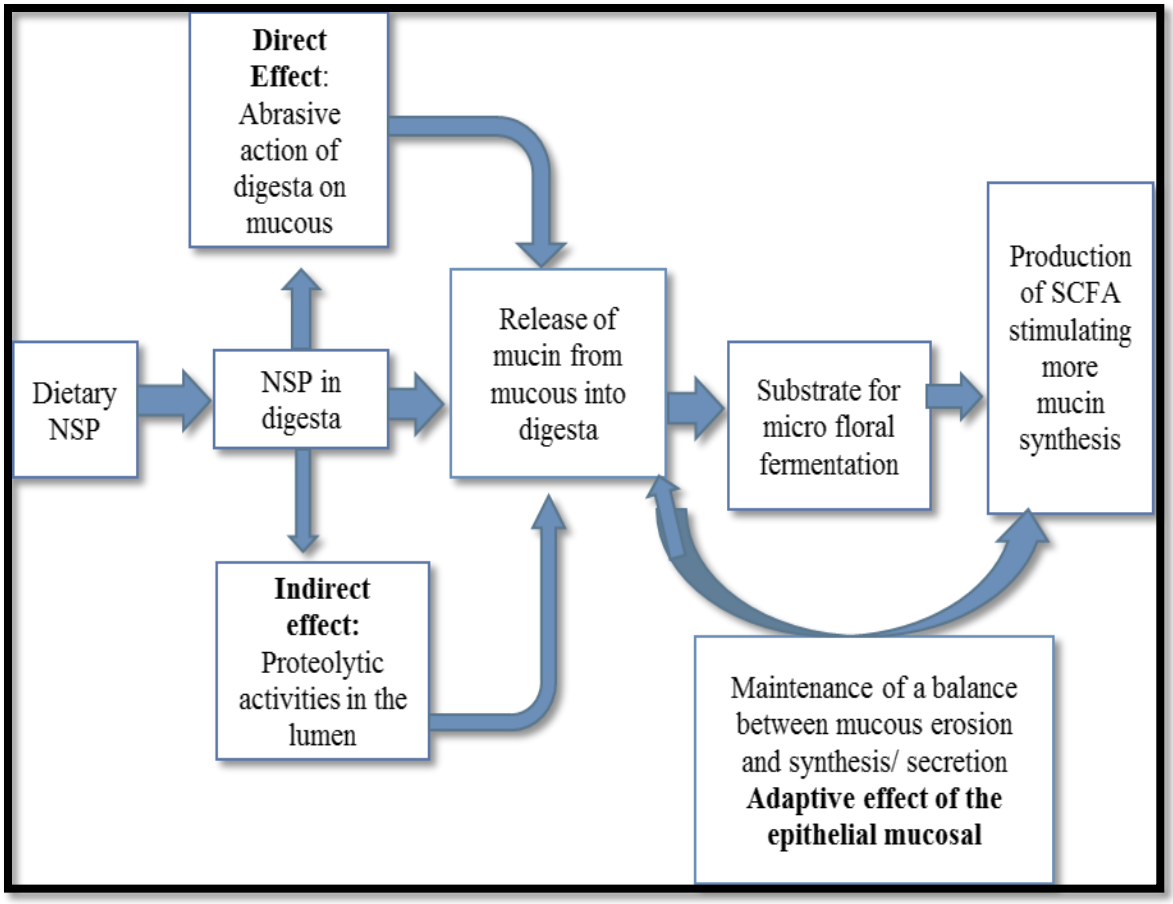

Figure 3. Hypothetical effects of NSPs on the balance between mucus erosion releasing mucin into the gut lumen and synthesis and secretion of mucin from the goblet cells. SCFA= short-chain fatty acids. Adapted and modified after Montagne et al., 2004.

1.5 HYPOTHESIS AND CURRENT STUDIES

U.S. poultry industry is an important component of the United States economy. According to information made available by US Poultry organization, the poultry production was valued at about $38 billion in 2012 (US Poultry, 2014). In fact, the production grew by 8% between 2011 and 2012. Despite the growth, the industry is unrelenting in its efforts to improve production efficiency and human food safety. One of the reasons credited for this impressive market growth is the ability of the industry to maximize the utilization of nutrients obtainable from the feed ingredients through the use of dietary supplementation of exogenous enzymes. Dietary enzyme supplementation help alleviate the adverse effects of ANF, such as NSP and phytate, contained in most poultry feed ingredients (Ravindran et al., 1999; Cowieson et al., 2004; Santos et al., 2004a; 2004 b).

Anti-nutritional factors in feed ingredients not only impair nutrient utilization, and they may also adversely affect gut health. As dietary NSP and phytate challenges gut health, the bird adaptively responds by increasing the secretion of mucins as an immunological defense effort (Choct et al., 1992, 1996; Cowieson et al., 2004). Birds activate their innate immune system by secreting mucous for protection against the adverse physicochemical effect of ANFs and microfloral proliferation, which is encouraged by the presence of the ANF (Choct et al., 1996). The activities of the ANF is further challenged by the bird's inability to produce enough endogenous enzymes to overcome them.

As aforementioned, supplementation of poultry diets with dietary exogenous enzymes has proven effectiveness in countering the adverse effects of ANF. In addition to improving the efficiency of nutrient utilization, there are limited studies that support the postulation that dietary exogenous enzymes can also improve gut health, as defined by proper gut morphological development and reduced enteric mucin secretion that exacerbates endogenous nutrient loss. Therefore, this dissertation addresses the impact of dietary exogenous enzyme supplementation on gut health, nutrient utilization, and growth performance in poultry. Our working hypothesis is that, the supplementation of dietary exogenous enzyme will improve nutrients digestibility and gut health, through proper gut morphology development and reduction of enteric mucin secretion.

The first chapter of this dissertation is a literature review. The first part of the review focused on what is currently known about ANF and how they can interfere with nutrient digestion in poultry. The following section provided information on the usage of some selected dietary exogenous enzyme in poultry industry. The review also discussed poultry GIT morphology and health and how ANF and exogenous enzyme can influence the gut development. The last section focused on endogenous secretion, with an emphasis on enteric mucin secretion and the underlining factors that influence mucin dynamics in the gut.

In chapter 2, we discussed two studies carried out to evaluated how the nutritional value of DDGS in corn-SBM diet can be improved with enzyme blend of endo-xylanase from *T. reesei*, alpha-amylase from *B. licheniformis*, and serine protease from *B. subtilis*, (XAP) and a combination of spores from three defined *B. subtilis* strains (DFM). The objective of these two

trials was to study effect of the dietary factors on gut health, nutrients digestibility, and growth performance in turkey poults. The effect on gut health was assessed by examining changes on gut morphology and intestinal mucin secretion. Based on the research trial results and observations reported in chapter 2, we further tested our hypothesis by using a different type of carbohydrase enzyme in chapter 3. The objective of chapter 3 was to determine the effect of dietary β-mannanase supplementation of turkeys fed high and low energy diets on gut health, nutrients utilization, and growth performance. To further validate the hypothesis and confirm results from previous studies, we evaluated a non-carbohydrases enzyme in chapter 4. The objective of the chapter 4 study was to assess the efficacy of two different phytase sources, included in the diet at levels at up to 2000 FTU/kg, on intestinal mucin secretion, nutrient digestibility, bone mineralization and growth performance. The results of the experiment demonstrated that dietary calcium inclusion level may influence enteric mucin secretion, thereby affecting endogenous nutrients loss. We examined this hypothesis in chapter 5 with the objective to evaluate the effect of different levels of calcium, phytate, and phytase supplementation on intestinal mucin secretion, nitrogen utilization and growth performance in broiler chicks. The results of all experiments are discussed and summarized in the concluding chapter of this dissertation (Chapter 6).

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CHAPTER TWO

The Effect of Supplementing DDGS Diet with Dietary Exogenous Enzymes and Direct-Fed Microbial on Nutrient Digestibility, Gut Health and Growth Performance of Turkey Poults.

2.1 ABSTRACT.

Although dietary inclusion of corn distillers dried grains with solubles (DDGS) in turkey diets may be cost effective, it increases the amount of non-starch polysaccharides (NSP), such as water-insoluble arabinoxylans. NSP not only compromises nutrient digestibility, it may also serve as fermentation substrate for microflora. Two studies were conducted to evaluate the effect of DDGS inclusion level in corn-soybean meal (SBM) diets supplemented with endo-xylanase from *T. reesei*, alpha-amylase from *B. licheniformis*, and serine protease from *B. subtilis*, (XAP), and direct fed microbial (DFM) inform of a combination of spores from three defined *B. subtilis* strains, on nutrient digestibility, gut health and growth performance in turkey poults. The first experiment was a 28 d cage trial, and we evaluated 0 %, 6 %, and 12 %, while the second trial was a 12 wk trial, and we tested 6 % and 12 % inclusion level of DDGS. DDGS inclusion level had transient effect of the growth performance. In the first experiment, compared to the 0 % inclusion level, DDGS inclusion was associated with reduced body weight (BW) of turkey poults on 28 d, and inclusion at 6 % and 12 % had similar BW. In the first experiment, the dietary XAP+DFM supplementation had reduced BW, increased feed intake and FCR. There were no significant interaction effects between the dietary DDGS inclusion level and the feed supplement on growth performances or nutrient digestibility. As dietary DDGS level increased, phosphorus retention, AMEn, ileal crude fat (CF), and ileal crude protein (CP) declined ($P<0.05$). The supplementation of XAP+DFM was associated with the reduced fecal moisture (4.2 %, $P<0.05$), increased ileal digestibility of CF and CP ($P<0.05$), but had no effect on AMEn or ANR. Dietary inclusion

of 18% DDGS impacted the mucosa villi morphological characteristics in comparison to the lower inclusion levels. There was significant increase ileal mucin secretion as the DDGS inclusion level increased, but the dietary supplement of XAP reduced the intestinal secretion. The experiments confirms that growth performance of poults will decrease as dietary inclusion levels of DDGS increases if the diet is not compensated for its inferior protein, fat and P digestibility. Although, dietary supplementation of XAP and a combination of XAP+DFM had no effect on the BW, it improved nutrient digestibility. The supplementation of XAP associated with the reduced intestinal mucin secretion, which may reduce endogenous nutrients loss.

Keywords DDGS, XAP, direct-fed-microbial, nutrient digestibility, turkey poults

2.2 INTRODUCTION.

The production of corn DDGS is increasing in the United States to satisfy governmental biofuels initiative. According to a report released by the Food and Agricultural Policy Research Institute at Iowa State University in 2006/2007, about 15 million tons of DDGS was produced in the United States, and with the passage of Renewable Fuel Standard, it has been estimated that the production of corn DDGS will increase by more than 150% to about 45 million tons by the end of 2014. DDGS is a good source of some vital nutrients that are required in animal production. It has a high concentration of protein, vitamins, and minerals, it contains about 31% crude protein and 10% crude fiber (Spiehs et al., 2002; Belyea et al., 2004). DDGS also has relatively high phosphorus content. According to a NRC report, about 54% of the total phosphorus in corn DDGS is non-phytate phosphorus (NRC, 1994). Shurson (2002) reported that the total phosphorus in some DDGS produced by most modern ethanol plants might even be higher than earlier estimated. The bioavailability of phosphorus in DDGS is higher than in typical plant-sourced feed ingredients because of the microbial phytase associated with the fermentation process involved in ethanol production liberates phytate-bound phosphorus (Singsen et al., 1972; Mahgoub and El Hag, 1997; El Hag et al., 2002). The specie of the animal influences DDGS phosphorus utilization. For example, Singsen et al. (1972) reported that the bioavailability of the phosphorus in DDGS for chickens was about 100%, while the availability of phosphorus in DDGS for swine is within the range of 87% and 92% (Shurson, 2003; Whitney et al. 2001). However, DDGS has a lower metabolizable energy content than corn (2,820 kcal/kg *vs.* 3,420 kcal/kg) (Wagner, 2008). The reduced metabolizable energy in DDGS

is due to its low starch content, but high non-starch polysaccharides (NSP) (soluble and insoluble carbohydrate fiber) relative to the parent corn grain. However, through the use of supplemental NSP enzymes, some of the potential energy contained in DDGS may be made more available to benefit animal nutrition.

Because of the cost benefits and nutritional potential, increasing levels of DDGS are formulated to achieve least-cost formulations in poultry and other animal species. DDGS is more cost effective in ruminant feeds than in monogastric animal feeds because ruminants have a greater digestive capacity through rumen fermentation to utilize the fibrous soluble and insoluble non-starch carbohydrates. For instance, DDGS has about 11% arabinoxylans NSP *versus* 5% found in corn alone (Ward et al., 2008). Having an inferior microbial fermentation capacity, monogastric animals do not have the digestive enzymes to degrade NSP. Furthermore, the soluble and insoluble fibers content in DDGS hold more water in digesta, creating viscous bulk distension within the gut. This increased digesta viscosity binds water-soluble nutrients, entraps fat-soluble nutrient, and may also restrict the access of endogenous enzymes to their respective substrates for digestion (Choct et al. 2010). The overall effect of excessive dietary NSP is a reduction in growth performance and feed efficiency.

Previous researches have shown that the supplementation of poultry diets with enzyme blends of xylanase, amylase, and protease (XAP) can improve nutrient digestibility in poultry (Hong et al., 2002). Amylase improves starch digestion, xylanase reduces gut viscosity and breaks down cereal cell walls, and protease enhances protein digestibility. Ritz et al. (1995) reported an improved growth performance of broilers when their diet was supplemented with

a blend of amylase and xylanase. Ileal nutrient digestibility in broilers was also improved when a corn-soy diet was supplemented with protease and a composite of carbohydrases (Marsman et al., 1997). Similar results also confirm the positive effects of supplemental enzyme blends for poultry (Gracia et al., 2003). Hong et al. (2002) observed an improvement in ileal amino acid digestibility and apparent amino acid retention in ducks by dietary supplementation of a blend of XAP enzymes. They also observed improved growth performance by dietary XAP enzyme supplementation, but AMEn and AME were not significantly affected.

The animal gut is a very complex and diverse ecosystem, and maintenance of gut health is a high nutrient-consuming task. Croom et al. (2000) reported that energy required for gut maintenance accounts for about 25% of the total basal metabolic needs of an animal. The requirements are even higher during an event of enteric distress or microbial pathogen challenge, and this can have a significant impact on the partitioning of energy and other nutrients away from growth, thus reducing the overall feed conversion efficiency. Ferket (2004) reported that good gut health can be maintained by promoting a protective balance between intestinal mucosa mucin secretion and beneficial intestinal organisms that competitively excludes pathogenic microbes. Beneficial microflora can be introduced into the gut of an animal by feed or water supplementation of direct-fed microbials (DFM). Dietary supplementation of DFM helps sustain a symbiotic gut ecosystem that minimizes intestinal inflammation or enteric distress through competitive exclusion of enteric pathogenic bacteria (Snel et al., 2002). Although antibiotics have been used for the past several decades to control

enteric bacteriosis, the increasing public concern about antibiotic-resistant enteric pathogens in humans motivates the quest for sustainable alternatives such as DFM products.

Dietary supplementation of DFM exerts their positive influence on the enteric ecosystem, gut health and growth performance of animals by several means. DFM enhances intestinal mucin secretion to trap pathogenic bacteria, thereby preventing their translocation across the intestinal epithelial membrane of the gut. DFM also enriches the colonization of the intestinal tract with commensal bacteria, which attaches to the mucosa wall by adhering to specific mucosal receptors or to the mucin blanket (Montagne et al., 2003; Caballero-Franco et al., 2007). By stimulating mucin secretion, dietary DFM supplementation indirectly helps increase the resistance to enteric pathogen colonization. However, intestinal mucin secretion leads to an increase in endogenous losses of nutrients in poultry, especially amino acids, such as Threonine, proline, lysine and serine, hence partitioning more nutrients towards the maintenance of animal gut health and leaving less nutrients for growth and productive processes (lien et al., 1996; Leterme et al., 1998; Cowieson et al., 2004).

There is a possible synergistic interaction between dietary supplementation of DFM and carbohydrases on the microbial ecosystem of gastrointestinal tract, as microflora and exogenous and endogenous enzymes both play an important role during digestion. The interaction between DFM and carbohydrases may be considered from two different perspectives. On the one hand, the byproducts of the carbohydrases activity may provide fermentation substrate for the DFM to thrive and multiply. On the other hand, active DFM fermentation may keep enteric pathogens at bay, which ensures the nutrients liberated by

supplemental carbohydrases and endogenous enzymes are efficiently absorbed and utilized for growth and productive purposes. Dietary supplementation of DFM may also metabolize some of the carbohydrases activity byproducts to produce volatile fatty acids, which suppress hindgut pathogens and contribute as an energy source to benefit the host.

Therefore we hypothesized that the adverse effect of DDGS can be overcome by supplementing the poultry diet with dietary exogenous enzymes and DFM. By combining a blend of XAP supplemental enzymes with DFM, the nutritional value of DDGS may be improved by increasing nutrients digestibility and promoting a symbiotic enteric ecosystem. The positive effect of dietary XAP enzyme and DFM supplementation may be most pronounced at high dietary inclusion level of DDGS.

2.3 MATERIALS AND METHODS.

2.3.1 Experiment 1.

2.3.1.1 Experimental Diets.

The experiment was designed as a 3 X 2 factorial arrangement, consisting of three dietary inclusion levels of DDGS (0%, 6%, and 12%), and two XAP[1]+DFM[2] supplemental additive combinations (with and without) in a corn-Soybean meal based diet. The dietary treatment formulations used in this study are shown in Table 2.6.1.1. Three basal diets containing 0%, 6%, and 12% DDGS were mixed and divided into 2 parts; one part supplement

[1] Axtra XAP is a blend of xylanase, amylase and protease. Danisco Animal Nutrition, Marlborough, UK
[2] Enviva Pro DFM. Danisco Animal Nutrition, Marlborough, UK.

with 0.02% XAP+DFM additive premix, and the other with 0.02% vermiculite as non-nutrient filler to occupy space in the formula. The activity of the components of the combined XAP+DFM premix included 2000 U of xylanase, 200 U of amylase, and 4000 U of protease, the DFM is 75000 cfu of *Bacillus subtilis per* gram of premix. All diets were supplemented with 2.0% Celite[3] to serve as a marker for determination of nutrients digestibility. The enzyme activity was confirmed by laboratory analysis[4]. The ingredient and analyzed nutrients compositions of the experimental diets are reported in Table 2.6.1. All experimental diets wereformulated to meet or exceed NRC (1994) requirements for turkeys, and were manufactured at the North Carolina State University Feed Mill Educational Unit (Raleigh, NC).

2.3.1.2 Bird Husbandry and Data Collection.

Four hundred and thirty two Nicholas female poults were obtained from a commercial hatchery[5] and randomly assigned to one of 48 cages with 9 poults per cage[6]. Each bird was identified with a numbered neck-tag in sequence for each replicate cage group. The poults were fed the same basal diet that contain 6% inclusion level of DDGS for the first 14 d, after which they were switched to their respective treatment diets, this was to condition their digestive system to accommodate the DDGS. After 14 d, experimental diets were randomly assigned among 4 blocks of 12 cages at 8 replicate per treatment, and they were fed their

[3] Celite™ (Celite Corp. Lompoc, CA)
[4] Danisco laboratory, Feed Enzyme Services, Brabrand, Denmark
[5] Prestage Farms Hatchery, Clinton, NC.
[6] Alternative Design cages, Alternative Design Manufacturing and Supply, Inc., Siloam Springs, AR

respective treatment diets until 28 d of age. Feed and water were provided *ad libitum*. From 1 to 7 d, the birds were provided 23 h light and 1 h darkness, and 14 h of light and 10 h of darkness after 7 d. Feed consumption, group weight, and number of birds per cage were recorded on 0, 7, 14, 21, and 28 d. Mortality rate and cause of mortality were recorded daily. Weight of each dead bird was recorded to correct the FCR.

2.3.1.3 Fecal Excreta Collection and Analysis.

Excreta were collected on 22 d, 23 d, and 24 d to evaluate fecal nutrients digestibility. Samples from each day were stored at -20^{o} C until analysis. The frozen excreta were later defrosted for approximately 16 h at room temperature before further processing. For each pen, approximately 200 g of representative clean excreta from the three collection days was blended[7] with 100mL of water to obtain a slurry mixture, and was acidified to pH<5.4 with the addition of HCl (0.1N, Fisher Scientific, Fairlawn, NJ 07410) to prevent volatilization of nitrogen during overnight drying. The slurry was dried overnight at 70° C in a forced air convection oven[8]. Once dried, the excreta were grounded to a fine powder, and placed in storage bags at -20° C until further analysis.

2.3.1.3 Ileal Digesta Collection and Analysis.

On the evening of 27 d, before ileal sample collection, the birds had restricted access to feed for at least 6 h by switching off the lights. At 5 am of the following day, the lights were

[7] Waring® Commercial Blender Model 31BL92, Waring Commercial, New Hartford, CT 06057
[8] Blue-M, Model # DC-326F, Serial # DC-509, Blue M, Atlanta, GA

turned on allow the birds *ad libitum* access to feed. Thus, each bird had at least 4 h of *ad libitum* feed consumption before they were euthanized for ileal digesta sample collection. Ileal sections from Meckel's diverticulum to the cecal junction were dissected and contents collected in 125 ml plastic container. The ileal contents were pooled by cage and stored at -20° C until later analysis. The ileal sample were then processed the same way like the fecal sample, and stored in storage bags at -20° C until further analysis.

2.3.1.4 Chemical Analyses.

Representative samples of each dried and grounded excreta and ileum samples were analyzed for dry matter (AOAC, 1995a), crude protein (AOAC, 2006), crude fat (AOAC, 1995b). The gross energy content was determined with an adiabatic oxygen bomb calorimeter[9]. Recovery of acid insoluble ash from the fecal and ileal samples was determined according to the method described by Vogtmann et al. (1975). Ground excreta samples were ashed for approximately 16 h at 600°C in a muffle furnace[10] (AOAC, 1995c) and analyzed for calcium and phosphorus content[11]. Respective diet samples were sent to the NC Department of Agriculture and Consumer Services Food and Drug Protection Division Forage Laboratory (Raleigh, NC) for nutrients composition analysis. The apparent metabolizable energy nitrogen corrected (AME_n) and apparent nitrogen retention (ANR) were calculated according to Lammer et al., (2008) using the following equations:

[9] IKA Calorimeter System C5000 Control, IKA® Werke Labortechnik, Staufen, Germany.
[10] Thermolyn, Model # F-A1730, Serial # 6483, Sybron Corp., Dubuque, IA.
[11] AES mineral analysis at NC State University, Dept. of Soil Science Analytical Service Laboratory

$$AME_n = [(GE_{Diet} - (GE_{Excreta} \times AIA_{Diet} / AIA_{Excreta}) - (8.22 \times N_{Retained})]$$

$$N_{Retained} = N_{Diet} - (N_{Excreta} \times AIA_{Diet}) / AIA_{Excreta}))$$

Where: AMEn (Kcal/g) is the nitrogen corrected apparent metabolizable energy of the diet; GE_{Diet} and $GE_{Excreta}$ were the gross energy of diet and excreta respectively; AIA_{Diet} and $AIA_{Excreta}$ were the concentration of Celite recovered as acid insoluble ash in diet and excreta respectively; 8.22 (Kcal/g) is the energy value of uric acid; and $N_{Retained}$ (g/kg) is the nitrogen retained by bird per kilogram of diet consumed, and N_{Diet} and $N_{Excreta}$ (%) were the nitrogen content of diet and excreta respectively. All values used in this calculation were expressed as grams per kilogram of DM.

$$ANR\ (\%) = [100*(1-(AIA_{Diet} / AIA_{Excreta} * Nitrogen_{Excreta} / Nitrogen_{Diet}))]$$

Where: AIA_{Diet} and $AIA_{Excreta}$ were the concentration of Celite recovered as acid insoluble ash in diet and excreta respectively; $Nitrogen_{Diet}$ and $Nitrogen_{Excreta}$ were the nitrogen content in the dietary and excreta, respectively.

Ileal nutrients digestibility were calculated according to Dilger and Adeola, (2006), using the following equation:

$$Nutrient\ digestibility\ (\%) = 100-[(AIA_{Diet} / AIA_{Excreta)*(}Nutrient_{Ileal} / Nutrient_{Diet})*100]$$

Where: Nutrient digestibility (%) is ileal nutrient digestibility expressed as a percentage; AIA_{Diet} and $AIA_{Excreta}$ are concentration of Celite recovered as acid insoluble ash in diet and excreta respectively; $Nutrient_{Ileal}$ and $Nutrient_{Diet}$ were the ileal and dietary nutrient content. All values used in this calculation were expressed as grams per kilogram of DM.

2.3.2 Experiment 2.

2.3.2.1 Experimental Diets.

The experiment was designed as 2 x 4 factorial, with two inclusion levels of DDGS and 4 levels of different type of feed supplements (Negative control (NC), 2% supplemental fat, XAP and XAP+DFM). Two experimental corn-soybean meal basal diets, containing 6% or 18% DDGS, were pelleted and crumbled and subsequently divided into 4 lots. The first lot was retained as the NC, the second lot was supplemented with 2% supplemental fat, the third lot was supplemented with the enzymes XAP, and the fourth lot was supplemented with the combination of XAP+DFM. The enzyme activities per gram of the XAP blend was 2,000 U of xylanase, 200 U of amylase, and 4,000 U of protease, and the XAP+DFM also contained 75,000 cfu of *Bacillus subtilis* per gram of premix. This same experimental diet preparation procedure was used for all feed phases. All experimental diets were formulated to meet or exceed the NRC (1994) nutritional recommendations for turkeys (Table 2.6.2.1). All diets were manufactured at the North Carolina State University Feed Mill Educational Unit (Raleigh, NC).

2.3.2.2 Bird Husbandry and Data Collection.

Eight hundred and sixty four one-day old female poults[12] were randomly assigned to 48 litter floor pens, at 18 poults per pen, and 6 replicates per dietary treatment. The turkey hens had access to *ad libitum* feed and water for 12 wk of age. Individual body weight and feed

[12] Hybrid Converter Hens, Cold Springs Farm, Thamesford, Ontario, Canada

consumption data were determined at 2, 4, 6, 8, 10, and 12 wk of age. Mortality was recorded as it occurred, and the weights of mortality were used to adjust the feed conversion ratio.

2.3.2.3 Histological Analyses.

At 3 and 6 wk of age, four birds per treatments were euthanized to collect tissue for histological assessment. A 6 cm section from the mid-portion of the jejunum was taken from each bird, gently washed with saline, and fixed in 10% formalin solution. After being submerged in 10% buffered formalin solution for at least 48 h, the 3 cm ileal section was processed. A total of four sections about 2 to 3 mm in length were taken from the 3 cm fixed ileal section of each bird. These smaller sections were placed in tissue cassettes and submerged in 10% buffered formalin solution until processed at the Histopathology Laboratory[13]. The fixed ileal sections were embedded in paraffin wax, and 5 μm thick transverse sections were cut with a microtome. The cut sections were placed on slides and were stained with Lilee Meyer haematoxylin and counter-stained with eosin yellow. A light-microscope[14] was used to visualize the transverse sections placed on slides. The images were captured using a digital camera[15] and analyzed using Image Tool[16]. Villus height, villus apical width at the tip of the villus, villus basal width at the crypt-villus junction, crypt depth, and muscularis depth were

[13] North Carolina State University, College of Veterinary Medicine, Raleigh, NC.
[14] LEICA-DMR light-microscope, Leica Camera AG, Solms, Germany.
[15] Spot-LTCR digital camera, Diagnostic Instruments, Inc., Sterling Heights, MI.
[16] UTHSCSA Image Tool Software, Version 3.0. The University of Texas, San Antonio, TX.

measured on 15 villi per sampled poult. Apparent villus surface area for each section was calculated using the following equation: [((villus tip+villus base)/2)*villus height].

2.3.2.4 Histochemical Analyses- Measurement of Ileal Mucin Secretion.

The epithelial mucin was assessed histochemically with alcian blue stain, based on the affinity of the basic stain for acidic tissue element like mucin (McManusi and Mowry, 1960; Evansee and Kent, 1962). The thickness of the ileal mucus adherent layer was estimated based on the modification of Corne's method (Corne et al., 1974; Kitagawa et al., 1986; Parman et al., 1993). Prior to sampling each bird for histological analysis on 3 and 6 wk of age, a 1-cm section of ileal tissue from each bird was removed and placed in 10 g/L Alcian blue dye solution in buffer containing 160 mmol/L sucrose and 50 mmol/L sodium acetate, pH 5.8. After 6 h of incubation, excess dye was be extracted with 250 mmol/L sucrose. The absorbed dye was extracted from the tissue by incubation in 10 g/L docusate sodium salt solution for 8 h at room temperature. Samples were centrifuged at 700 G, plated on a 96-well plate, and the optical densities were measured at 620 nm using Alcian Blue solution as a standard. The amount of absorbed dye was reported as µg Alcian blue per gram of ileal tissue.

2.3.3 Statistical Analysis.

This experiment was analyzed as a completely randomized design. Pen and cage means were respectively used as the experimental unit. Data were analyzed using JMP[17] software.

[17] Version 10. SAS Institute Cary, NC

ANOVA was used to examine the main effect of dietary treatment factors, and their interaction on parameters evaluated. Means were separated using the LS Means at $P<0.05$.

2.3.4 Animal Ethics.

This experiment was conducted according to the guidelines of the Institutional Animal Care and Use Committee at North Carolina State University. All husbandry practices and euthanasia were performed with full consideration of animal welfare.

2.4 RESULTS.

2.4.1. Experiment 1.

2.4.1.1 Growth Performance.

The main and interactive effects of DDGS and composite of XAP+DFM on growth performance in experiment 1 were presented in Tables 2.6.1.2 and 2.6.1.3. There were neither main effects nor interactions between the DDGS inclusion level and XAP+DFM supplement effect of the body weight at 21 d ($P>0.05$; Table 2.6.1.2). However, on 28 d the inclusion of DDGS at 6% and 12% tend to lower the BW of the poults by 5% when compared with the 0% inclusion level of DDGS ($P<0.05$; Table 2.6.1.2). The addition of a composite of XAP+DFM to the diet was also associated with a reduced BW ($P<0.05$; Table 2.6.1.2). There were no interaction effects observed between DDGS inclusion level and XAP+DFM on BW at 28 d. There were no main or interaction effects observed between DDGS inclusion levels and XAP+DFM on feed intake between 14-21 d and 22-28 d ($P>0.05$; Table 2.6.1.3), but during 22-28 d, the treatments with the supplementation of the blend of XAP+DFM had

approximately 11% less feed intake when compared with those without the supplement ($P<0.05$; Table 2.6.1.3). Similar to the feed intake, there were no main or interaction effects observed between the level of DDGS and XAP+DFM supplement on adjFCR between 14-21 d, and 22-28 d ($P>0.05$; Table 2.6.1.3). However, the supplementation of the blend of XAP+DFM was associated with the 2% improvement of the adjFCR ($P<0.05$; Table 2.6.1.3).

2.4.1.2 Nutrient Digestibility.

The results of the ileal and fecal nutrients digestibility are presented in Tables 2.6.1.4, 2.6.1.5, and 2.6.1.6. Dietary DDGS inclusion level affected the ileal digesta moisture content. Dietary inclusion of 12% DDGS increased ileal digesta moisture content by 4% relative to the 0% DDGS inclusion treatment ($P<0.05$; Table 2.6.1.4), but neither was different from the 6% DDGS inclusion level. However, there were neither a main effect of XAP+DFM supplementation nor interaction effects with DDGS inclusion level on ileal digesta moisture content ($P>0.05$; Table 2.6.1.4).

There were main and interaction effects between the inclusions of DDGS and XAP+DFM on ileal crude protein digestibility. The inclusion of DDGS at both 6% and 12% reduced the crude protein digestibility by 2% and 3%, respectively, when compared to the 0% inclusion level ($P<0.05$; Table 2.6.1.4), but the treatment with the supplementation of the blend of XAP+DFM had about 1% enhancement in crude protein digestibility ($P<0.05$; Table 2.6.1.4). The addition of XAP+DFM to 0% DDGS diet improved ileal crude protein digestibility, and the supplementation of 12% inclusion level of DDGS with the blend of XAP+DFM had an intermediary response on ileal crude protein digestibility. Similar to

response observed in crude protein digestibility, the usage of DDGS at both 6% and 12% inclusion level was associated with a 2% decline in the ileal crude fat digestibility ($P<0.05$; Table 2.6.1.4), but the treatments supplemented with blend of XAP+DFM had a 1% improvement in the ileal crude fat digestibility ($P<0.05$; Table 2.6.1.4). There was no interaction effect between the DDGS inclusion level and XAP+DFM supplementation. There were no significant treatment effects on starch digestibility.

The results for the nutrient digestibility determined from the fecal samples are presented in tables 2.6.1.5 and 2.6.1.6. The inclusion of DDGS at both 6% and 12% did not have any effect on fecal moisture content, neither were there any DDGS Level X XAP+DFM interaction effects observed on fecal moisture content ($P>0.05$; Table 2.6.1.5). However, the XAP+DFM supplemented treatments had a 4% reduction in the fecal moisture content ($P<0.05$; Table 2.6.1.5). There were no treatment effects on fecal crude protein digestibility and apparent nitrogen retention ($P>0.05$; Table 2.6.1.5). The apparent metabolizeable energy corrected for nitrogen was affected by DDGS inclusion level. Treatments with dietary inclusion of 6% and 12% DDGS had 6% lower AMEn when compared to the 0% inclusion level ($P<0.05$; Table 2.6.1.5). Dietary supplementation of XAP+DFM did not affect AMEn, and neither were there any significant interaction effects observed between DDGS levels and XAP+DFM supplementation ($P>0.05$; Table 2.6.1.5).

The results of the fecal mineral digestibility are summarized in table 2.6.1.6. Fecal ash content decreased with the inclusion of dietary DDGS ($P<0.05$; Table 2.6.1.6). XAP+DFM blend supplementation did not affect fecal ash, and neither were any significant interactions

between the inclusion levels of DDGS and XAP+DFM supplementation (P>0.05; Table 2.6.1.6). There were no significant treatment effects on fecal calcium retention (P>0.05; Table 6), but fecal phosphorus retention was affected with the dietary level of DDGS. As compared to the 0% inclusion level of DDGS, both treatments with 6% and 12% inclusion had lower phosphorus retention (P<0.05; Table 2.6.1.6). There were significant treatment effects on both fecal calcium and phosphorus content (P>0.05; Table 2.6.1.6).

2.4.2. Experiment 2.

2.4.2.1 Growth performance.

The biweekly growth performance results are presented in tables 2.6.2.2 to 2.6.2.7. Dietary inclusion level of DDGS did not have any significant effect on the body weight (BW) and body weight gain (BWG) until 12 wk, when treatments with 18% DDGS inclusion had about 2% and 10% lower BW and BWG respectively, as compared to the 6% inclusion treatment (P>0.05; Table 2.6.2.2 and Table 2.6.2.3). The feed supplements had transient effect on BW. Treatments with the addition of 2% supplemental poultry fat had a lower BW, than the other treatment feed supplements (P<0.05; Table 2.6.2.2), but the observed BW effect disappeared after 6 wk of age (P>0.05; Table 2.6.2.2). The addition of 2% supplemental poultry fat to DDGS at either 6% or 18% inclusion level was observed to lower BW and BWG during wk 2 and 4, respectively (P>0.05; Table 2.6.2.2).

Dietary inclusion level of DDGS did not affect feed intake and cumulative feed intake throughout the experiment (P>0.05; Table 2.6.2.4; Table 2.6.2.5). However, when compared to other feed supplements, the treatments with 2% supplemental fat had lower feed intake and

cumulative feed intake, but observed effect disappeared beyond 6 wk of age (P>0.05; Table 2.6.2.4 and Table 2.6.2.5). There were no DDGS level X feed supplement interaction effects on feed intake during the experiment (P>0.05; Table 2.6.2.4 and Table 2.6.2.5).

During the experiment, the DDGS inclusion level and feed supplement affected the adjusted feed conversion ratio (adjFCR) and cumulative adjFCR (Table 2.6.2.6 and Table 2.6.2.7). Poults fed diets containing 6% DDGS had improved FCR as compared to those fed the 18% DDGS diets (P<0.05; Table 2.6.2.6 and Table 2.6.2.7), but the improvement only lasted until 6 wk, after which no treatment effect was observed. During the first 2 wk, the dietary supplementation of XAP and the blend of XAP+DFM improved adjFCR, as compared to the supplemental fat and the negative control (NC) dietary treatments (P<0.05; Table 2.6.2.6). In contrast to other feed supplements, the supplemental fat treatment had lower adjFCR during 4 wk and 6 wk (P<0.05; Table 2.6.2.6), but all the effect faded away after 6 wk (P>0.05; Table 2.6.2.6).

2.4.2.2. Gut Histology.

The effects of dietary inclusion level of DDGS and feed supplements on gut morphology are summarized in Table 2.6.2.8. Although there were no treatment interaction effects observed mucosa morphology of hens at 6 wk of age, some significant treatment main effects were observed. Dietary inclusion of 18% DDGS was associated with a 15% decrease in villus tip width (P<0.05) and an 11% decrease in villi surface area (P<0.05) relative to the 6% DDGS treatment. However, the 18% dietary inclusion of DDGS treatment had a 13% increase in muscularis thickness (P<0.05) relative to the 6% DDGS inclusion treatment.

Dietary inclusion level of DDGS did not have significant effects on the villi height, crypt depth, villi height/crypt depth and villi base width. Furthermore, the feed supplement did not have any main effect on villi tip width, height, villi height/crypt depth, villi base width, muscularis thickness and villi surface area. However, feed supplement factors affected the crypt depth. Poults fed with a blend of XAP and composite of XAP+DFM had reduced crypt depth by about 12% and 11%, respectively, when compared with the supplemental fat and NC ($P<0.05$).

2.4.2.3. Intestinal Mucin Secretion.

The results of the intestinal mucin secretion are also presented in the table 2.6.2.8. Increasing the DDGS level increased the ileal mucin secretion during the 6 wk of the experiment ($P<0.05$; Table 2.6.2.8). However, in comparison to the supplemental fat and NC, dietary supplementation of XAP reduced the intestinal mucin secretion by 24% and 20%, respectively ($P<0.05$); but neither response was different from XAP+DFM.

2.5 DISCUSSION.

The use of DDGS in the poultry feed production is rising and will continue to rise due to the increasing use of corn for ethanol production in order to satisfy regulator biofuels initiatives. DDGS, a co-product of ethanol production, is cost-competitive feed ingredient as it may supply nutrients otherwise supplied by grain (i.e. corn and wheat), protein meals (i.e. soybean meal, canola meal, and rendered by-product meals), and inorganic phosphorus. However, some anti-nutritional factors (namely soluble and insoluble NSPs) and reduced digestible amino acids in DDGS may limiting the dietary inclusion level of DDGS in poultry feed to below ~6%. The general hypothesis tested in the two studies reported herein was that

the supplementation of DDGS with either XAP or a combination of XAP+DFM will increase the nutritional value of DDGS, by increasing nutrient digestibility, improving gut health, and enhancing growth performance. Dietary inclusion of feed supplements containing XAP enzymes blends may degrade the non-starch polysaccharides and poorly digested proteins in the DDGS, thereby improving nutrient utilization and altering substrate dynamics that increases microbial diversity that favor symbiotic bacteria over pathogenic ones in the enteric ecosystem, consequently improving the gut health.

In general, the dietary inclusion of DDGS reduced the growth performance of turkey poultry in both experiments. However, the adverse effects of dietary inclusion of DDGS reduced over time towards the end of the grow-out experiment, suggesting the ability of turkeys to handle a high dietary inclusion DDGS (>15%) is age dependent. Our observation is corroborated by those reported by Lumpkins et al. (2004). They reported that DDGS from modern ethanol plants was an acceptable feed ingredient for broiler diets and could be safely used at a dietary inclusion level of 6% in the starter period, and 12% to 15% in the grower and finisher periods. Perhaps younger birds have limited ability to handle the high dietary inclusion of DDGS because of its inferior protein composition, and protein is especially important for young poultry. It may also be due to the young bird's fragile and immature digestive system and developing enteric ecosystem. Noll et al. (2006) also reported no negative effect on growth performance, namely body weight and FCR, when market turkey toms were fed up to 20%

DDGS in grower and finisher diets. However, Wang et al. 2007) reported that dietary inclusion of up to 25% DDGS had no significant effects on body weight of broilers at any age but it adversely affected their FCR.

Reasons for the variation in the effects of dietary inclusion of DDGS may be related the feed formulation methods, and whether it is based on accurate nutrient matrix values. Formulating feed based on metabolizeable energy and amino acid or crude protein digestibility will have their respective effect or impact on the growth performance outcome. Waldroup et al. (1981) stated that broilers can handle a dietary inclusion level up to 25% DDGS in isocaloric broiler diets without any effect on the growth performance, specifically body weight and FCR. However, they also emphasized the importance of the level of metabolizable energy in the formulation. When dietary energy content was allowed to decrease as the DDGS inclusion level increased above 15%, they observed a decline in growth performance. Similarly, Wang et al. (2007) concluded that the poor performance of broilers fed different levels of DDGS might be due to inadequate estimation of the metabolizeable energy in DDGS, which invariably affect the level of protein intake.

The supplementation of the exogenous XAP and XAP+DFM had a significant main effect on some of growth performance indicators, especially during the earlier growth phases of the experiments. There were no interaction effects between the dietary inclusion level of DDGS and the feed supplements on either the growth performance or nutrient digestibility. This observation was corroborated by Min et al. (2007), who conducted two separate trials with DDGS inclusion levels up to 30%, using two different non-starch polysaccharide enzyme

products. They found no significant interaction effects between the enzyme supplements and DDGS, and concluded that the addition of the enzymes had no apparent beneficial effect on energy digestibility of diets with a high level of DDGS. One possible explanation of this observed inefficiency of supplemental enzymes to improve the nutritional value of DDGS may be associated with the way DDGS is produced as a by-product of yeast fermentation enzymes and metabolism to produce ethanol. The fermentation and subsequent heat-drying processes may structurally dis-configure the residual starch and non-starch polysaccharides, thus impeding the ability of the xylanase and amylase in the XAP supplement to degrade them. The present array of commercial exogenous enzymes used as feed supplements in the animal feed industry are designed to degrade non-starch polysaccharides that are found in unfermented and unheated parent grains and grain milling co-products.

The ethanol production process may not only affect the structural configuration of the non-starch polysaccharide, it also affects the protein digestibility. Protein digestibility is one of the main concerns of using DDGS as a feed ingredient in monogastric animals. In the first experiment, we determine the effect and interaction between DDGS inclusion level and XAP+DFM on nutrients digestibility. The ileal protein and fat digestibility were reduced as the DDGS inclusion increased. However, the supplementation of the diet with XAP+DFM improved the ileal protein and fat digestibility. The reduction in the ileal crude protein digestibility with the inclusion of DDGS is likely because of the formation of maillard reaction indigestible amino acids-carbohydrate complexes (especially lysine) that occurs during fermentation and heat-drying during ethanol production (Fastinger and Mahan, 2006; Stein et

al., 2006). The Maillard reaction reduces the bioavailability of the protein amino acids when they react with a reducing sugar, such as glucose, complexed with the epsilon of amino acid group when subjected to excessive heat (Cromwell et al. 1993). The improvement of protein digestibility by enzyme supplementation was corroborated by Barekatain et al. (2013), who reported that the supplementation of DDGS inclusion with XAP improved the protein digestibility and FCR.

Measured changes in intestinal morphology, such as shorten villi and deep villi crypts, have been used as an indicator of gut health and enteric distress (Yason et al., 1987). Tall mucosal villi increase the surface area available for nutrients absorption (Awad et al., 2008; Luo et al., 2009). There is correlation between the crypt depth and the rate of proliferation of the epithelial cells (Hampson, 1986; Jin, et al., 1994; Brunsgaard, 1998; Yasar and Forbes, 1999). Epithelial regeneration starts from the villi crypt, so a deep crypt is an indication of rapid enterocyte turnover and increased mucosal tissue maintenance requirements (Yason et al., 1987; Paulus et al., 1992). The rapid enterocyte proliferation and the epithelial cell turnover rate greatly impacts protein and energy requirements of the small intestine mucosa (Simon, 1989). Diet composition may produce microscopic alterations in the intestinal mucosa, and it is possible that the change in morphology of the gastrointestinal tract may be associated with dietary NSP levels (Yamauchi, 2002). In the second experiment, we determined the effect of DDGS and XAP, XAP+DFM and supplemental fat on gut health of turkey hens at 6 weeks of age. Relative to the 6% DDGS treatment, dietary inclusion of 18% DDGS adversely affected jejunum mucosal morphology as indicated by reduced villi surface area, tip width and

increased the thickness of the villi muscularis. The poor jejunum morphological development may because of relatively higher NSP content in the 18% DDGS diet. Iji et al. (1999) reported NSP can negatively impact gut morphology by increasing crypt depth of both the jejunum and ileum, promoting GIT cell turnover.

In contrast, some studies have demonstrated that enzyme treatment can influence the intestinal villi morphological development (Brenes et al., 1993; Amat et al., 1996; Luo et al., 2009). In wheat-corn-SBM diet, xylanase supplementation increased villus height of the duodenum, jejunum and ileum. Villus height/crypt depth of the three segments was increased by the supplementation (Luo et al., 2009). Furthermore DFM has been reported to improve growth performance and feed efficiency in poultry (Mountzouris et al., 2007; Samli et al., 2007). Dietary inclusion of DFM may also impact the gut morphology by increasing the jejuna villus and ileal villus height (Cavazzoni et al., 1998; Jin et al., 1998; Zulkifli et al., 2000; Kabir et al., 2004; Chichowski et al., 2007; Samli et al., 2007). In this present study, dietary supplementation of the XAP and XAP+DFM combination had a beneficial effect on gut morphology. The addition of this enzyme blend with or without the DFM reduced the crypt depth, indicating reduced mucosal distress, in comparison to the NC or fat-supplemented diet. Even though the 2% supplemental fat treatment added an extra 150 kcal of energy per kg of diet, mucosa morphology (i.e. villi height, crypt depth, and surface area) was not affected. Evidently, dietary supplementation of XAP and XAP+DFM was not a consequence of greater dietary energy availability, but more a consequents of how it altered the enteric ecosystem and the dietary substrates that support symbiotic enteric microflora fermentation. Establishment of

a symbiotic enteric ecosystem minimizes the inflammatory symptoms of enteric distress caused by the proliferation of enteric pathogens, which is associated with increased intestinal mucin secretion (Fukata et al., 1999; Grizard and Barthomeuf, 1999).

Several research reports demonstrate that the dietary inclusion of NSP enzymes reduces the mucosal blanket secretions in monogastric animals by degrading the substrate that provide nutrients for the pathogenic bacteria. In this present study, we observed that the addition of XAP reduced intestinal mucin secretion. Mehri et al. (2010) reported a reduction in the number of goblet cells in the intestine mucosa after β-mannanase supplementation. Since mucin is secreted from the epithelial goblet cells, dietary supplementation of β-mannanase likely reduced intestinal mucin secretion as a consequence. Goblet cells are found abundantly in the crypt of the villi. Parsaie et al. (2007) observed that shortening of the villi and deepening crypts may also cause increased mucin secretion into the gut lumen, which ultimately enhances endogenous losses. This reference corroborates the observations of our observations; the XAP and XAP+DFM treatments reduced mucosal crypt depth and intestinal mucin secretion. The reduction in the intestinal mucin secretion subsequently leads to a reduction in the endogenous secretions, which is ultimately expressed as improved apparent nitrogen retention.

In the two experiments we conducted, the higher dietary inclusion levels of DDGS had an adverse effect on growth performance of poults than the lower dietary DDGS levels, although turkeys can tolerate the higher DDGS inclusion levels during the growing and finisher feed phases. Regardless, there were minimal interactive effects of the dietary supplementation of XAP, DFM, or added fat towards improving the nutritional value of DDGS. One possible

explanation of this observation may be related to the structural form of the residual anti-nutritional NSPs after corn ethanol fermentation process. During the ethanol production, a process of which DDGS is co-product, the corn is fermented with yeast and subjected to different stages of heating process before the making of the final product. Thus, there is a high probability that the structural configuration of the residual non-starch polysaccharide may be less labile to degradation by supplemental XAP enzymes or DFM fermentation. The outcome of this studies has shown that the nutritional value of DDGS cannot be improved by the current commercial exogenous enzyme, more work still need to be done to synthesis feed additive that will be capable of degrading the NSP content of DDGS.

2.6 TABLES AND FIGURES.

2.6.1 Experiment 1.

Table 2.6.1.1. Dietary ingredients and nutritional compositions.

Ingredients	Starter [a] Diet	Basal Experimental Diets [b]		
		0% DDGS	6% DDGS	12% DDGS
	----------%----------			
SBM	51.04	54.73	51.65	48.62
Corn	31.17	30.84	28.01	25.11
DDGS	6.00	0.00	6.00	12.00
Poultry fat	5.37	6.46	6.43	6.41
Dicalcium phosphate	3.19	2.73	2.60	2.47
Celite™ (insoluble ash)	0.00	2.00	2.00	2.00
Limestone	1.71	1.70	1.78	1.86
Salt	0.35	0.37	0.35	0.33
D,L-Methionine	0.31	0.32	0.31	0.30
Choline chloride	0.20	0.23	0.20	0.18
Mineral premix [2]	0.20	0.20	0.20	0.20
Vitamin premix [3]	0.15	0.15	0.15	0.15
L-Lysine HCl	0.26	0.19	0.24	0.29
Selenium premix[1]	0.05	0.05	0.05	0.05
Phytase	0.00	0.01	0.01	0.01
(XAP+DFM)/ Filler (Vermiculite)	0.00	0.02	0.02	0.02
Analyzed chemical composition				
Dry Matter, %	92.03	92.6	92.76	92.67
Crude Protein, %	29.84	29.8	27.93	28.59
ME, kcal/kg	3000.00	3000.00	3000.00	3000.00
Crude fat, %	7.11	7.05	8.50	8.72
Ash, %	7.80	9.29	9.93	9.39
Acid detergent fiber, %	4.30	3.89	4.38	4.56
Calcium, %	1.64	1.52	1.69	1.66
Phosphorus, %	1.15	1.02	1.02	0.95

[a] Starter diet was fed from 1 to 14 d. [b] Experimental diets were fed from 14 to 28 ds.[1]Se premix provided 0.3 mg Se/kg. [2]Each kilogram of mineral premix supplied: 60 mg Zn as $ZnSO_4 \cdot H_2O$; 60 mg Mn as $MnSO_4 \cdot H_2O$; 40 mg Fe as $FeSO_4 \cdot H_2O$; 5 mg Cu as $CuSO_4$; 1.25 mg I as $Ca(IO_3)_2$; 1 mg Co as $CoSO_4$.[3]Each kilogram of vitamin premix supplied the following: vit A, 13,200 IU; cholecalciferol, 4,000 IU; alpha-tocopherol, 66 IU; niacin, 110 mg; pantothenic acid, 22 mg; riboflavin, 13.2 mg; pyridoxine, 8 mg; menadione, 4 mg; folic acid, 2.2 mg; thiamin, 4 mg; biotin, 0.25 mg; vitamin B_{12}, 0.04 mg; ethoxyquin, 100 mg.

Table 2.6.1.2. Effect of dietary DDGS level and XAP supplementation on body weight [1].

Main effect of treatment		Age (days)		
		14	21	28
		Body weight/bird (g)		
DDGS level (%)				
0		424	783	1269[a]
6		424	777	1206[b]
12		424	762	1205[b]
XAP+DFM level (%)				
0.02		424	783	1191[b]
0.00		424	765	1262[a]
Interactions				
DDGS (%)	XAP+DFM (%)			
0	0.02	424	779	1251
0	0.00	424	787	1286
6	0.02	424	772	1171
6	0.00	424	782	1241
12	0.02	424	743	1152
12	0.00	424	780	1258
Source of variations		P-values		
DDGS		-	0.4151	0.0119
XAP		-	0.1808	0.0008
DDGS x XAP+DFM		-	0.6573	0.3329
SEM(40)[2]		-	12	16

[a,b]Means with different letter superscripts within a column are significantly different (P<0.05).
[1]Values are means of 6 replicates each treatment.
[2]SEM (40) = Standard error of the mean with 40 degrees of freedom.

Table 2.6.1.3. Effect of dietary DDGS level and XAP supplementation on feed intake and adjFCR[1].

Main effect of treatment		Age (days)			
		14-21	22-28	14-21	22-28
		feed intake/bird (g)		adjFCR	
DDGS level (%)					
0		533.89	752	1.50	1.64
6		522.40	789	1.50	1.66
12		519.81	703	1.51	1.71
XAP+DFM level (%)					
0.02		518.43	705[a]	1.42	1.67[b]
0.00		532.29	791[b]	1.51	1.71[a]
Interactions					
DDGS (%)	XAP+DFM (%)				
0	0.02	528.30	714	1.45	1.63
0	0.00	539.48	789	1.55	1.64
6	0.02	525.12	743	1.54	1.65
6	0.00	519.68	834	1.45	1.66
12	0.02	501.89	657	1.49	1.73
12	0.00	537.73	749	1.53	1.70
Source of variations		P-values			
DDGS		0.7687	0.1693	0.9667	0.4716
XAP		0.4157	0.0200	0.0765	0.0400
DDGS x XAP+DFM		0.6058	0.9732	0.3546	0.9306
SEM (40)[2]		15.7	31.26	0.0500	0.0400

[a,b]Means with different letter superscripts within a column are significantly different (P<0.05).
[1]Values are means of 6 replicates each treatment.
[2]SEM (40) = Standard error of the mean with 40 degrees of freedom.

Table 2.6.1.4. Effect of dietary DDGS level and XAP+DFM supplementation on ileal nutrient digestibility[1].

Main effect of treatment		Moisture content	Crude protein digestibility	Crude fat digestibility	Starch digestibility
		----------%----------			
DDGS level (%)					
0		79[a]	85[a]	95[a]	97
6		78[ab]	82[b]	93[b]	95
12		76[a]	82[b]	93[b]	95
XAP+DFM level (%)					
0.02		78	84[a]	94[a]	94
0.00		78	83[b]	93[b]	96
Interactions					
DDGS (%)	XAP+DFM (%)				
0	0.02	79	86[a]	95	97
0	0.00	79	84[b]	95	96
6	0.02	78	82[b]	94	93
6	0.00	78	83[b]	93	97
12	0.02	77	84[ab]	93	93
12	0.00	78	83[b]	93	96
Source of variations		---------- P-values ----------			
DDGS		0.0184	0.0005	0.0111	0.3287
XAP		0.6593	0.0300	0.2882	0.2211
DDGS x XAP+DFM		0.7695	0.0500	0.8570	0.2971
SEM(40)[2]		0.46	0.44	0.45	1.23

[a,b]Means with different letter superscripts within a column are significantly different (P<0.05).
[1]Values are means of 6 replicates each treatment.
[2]SEM (40) = Standard error of the mean with 40 degrees of freedom.

Table 2.6.1.5. Effect of dietary DDGS level and XAP+DFM supplementation on fecal nutrient digestibility[1].

Main effect of treatment		Moisture content	Crude protein digestibility	ANR	AMEn
		----------%----------			Kcal/kg
DDGS level (%)					
0		75	39	56	3007[a]
6		76	37	56	2841[b]
12		75	37	56	2806[b]
XAP+DFM level (%)					
0.02		73[b]	37	56	2924
0.00		77[a]	38	55	2931
Interactions					
DDGS (%)	XAP+DFM (%)				
0	0.02	73	38	58	3033
0	0.00	77	40	54	2980
6	0.02	73	37	53	2805
6	0.00	79	37	56	2876
12	0.02	75	36	57	2935
12	0.00	76	38	55	2937
Source of variations		---------- P-values ----------			
DDGS		0.8546	0.1661	0.6233	0.0112
XAP		0.0300	0.2863	0.6031	0.8704
DDGS x XAP+DFM		0.3094	0.4173	0.1667	0.4891
SEM(40)[2]		1.37	0.78	1.62	37.99

[a,b]Means with different letter superscripts within a column are significantly different (P<0.05).
[1]Values are means of 6 replicates each treatment.
[2]SEM (40) = Standard error of the mean with 40 degrees of freedom.

Table 2.6.1.6. Effect of dietary DDGS level and XAP+DFM supplementation on fecal mineral nutrient digestibility[1].

Main effect of treatment		Ash	Calcium retention	Phosphorus retention	Calcium content	Phosphorus Content
		---------- (%) ----------				
DDGS level (%)						
0		21.25[a]	50	49[a]	2.08	1.75
6		20.63[b]	47	42[b]	2.25	1.75
12		20.42[b]	52	43[b]	2.08	1.62
XAP+DFM level (%)						
0.02		20.67	49	44	2.17	1.63
0.00		21.17	50	45	2.11	1.78
Interactions						
DDGS (%)	XAP+DFM (%)					
0	0.02	21.17	50	50	2.17	1.67
0	0.00	21.33	50	48	2.00	1.83
6	0.02	20.83	44	40	2.33	1.83
6	0.00	20.83	50	45	2.17	1.67
12	0.02	20.00	53	44	2.00	1.40
12	0.00	20.83	51	42	2.17	1.83
Source of variations		---------- P-values ----------				
DDGS		0.0300	0.0300	0.0300	0.0300	0.0300
XAP		0.1771	0.1771	0.1771	0.1771	0.1771
DDGS x XAP+DFM		0.3516	0.3516	0.3516	0.3516	0.3516
SEM(40)[2]		0.21	0.21	0.21	0.21	0.21

[a,b]Means with different letter superscripts within a column are significantly different (P<0.05).
[1]Values are means of 6 replicates each treatment.
[2]SEM (40) = Standard error of the mean with 40 degrees of freedom.

2.6.2 Experiment 2

Table 2.6.2.1. Dietary composition and nutrient content of starter and developer 1 (1-12weeks) diets fed to turkey hens in Experiment 2.

Ingredients	------Starter------		---Developer 1--		----Developer 2---		----Grower------	
	6% DDGS	18% DDGS	6% DDGS	18% DDGS	6% DDGS	18% DDGS	6% DDGS	18% DDGS
	----------%----------							
Soybean Meal	42.65	44.37	35.59	31.07	31.99	26.65	27.16	21.36
Corn	39.25	28.74	46.43	38.83	50.44	43.74	54.67	48.42
DDGS	6.00	18.00	6.00	18.00	6.00	18.00	6.00	18.00
Poultry meal	5.00	3.00	5.00	5.00	5.00	5.00	5.00	5.00
Poultry fat	1.53	2.85	2.17	2.40	2.32	2.45	3.35	3.42
Dical P	2.39	2.60	1.92	1.64	1.51	1.25	1.28	1.02
Limestone	1.23	1.48	1.28	1.46	1.24	1.42	1.16	1.35
Methionine	0.55	0.53	0.41	0.37	0.38	0.35	0.31	0.28
L-Lysine	0.35	0.39	0.29	0.37	0.22	0.33	0.19	0.32
Salt	0.32	0.33	0.30	0.26	0.27	0.24	0.26	0.24
Trace mineral [2]	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Choline Chloride	0.19	0.16	0.19	0.14	0.18	0.14	0.16	0.12
Vitamin premix[4]	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
L-Threonine	0.11	0.13	0.02	0.02	0.02	0.02	0.02	0.05
Selenite Premix[3]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Feed supplement/filler[1]	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Phytase	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Analysed chemical composition	----------%----------							
Dry Matter, %	88.30	89.97	90.35	90.50	90.30	89.38	90.03	89.62
ME Poultry, Kcal/kg	2900	2900	3000	3000	3050	3050	3150	3150
Crude protein*, %	29.60	29.1	26.01	26.49	24.40	23.50	22.48	22.50
Crude fat*, %	5.91	6.18	6.63	7.25	6.13	7.70	7.21	8.23
Crude fiber, %	2.68	3.33	2.55	3.04	2.49	2.97	2.39	2.86
Calcium* %	1.16	0.8	1.18	1.33	1.13	1.16	1.09	1.08
Total Phosphorus*, %	0.79	10.98	0.74	0.74	0.76	0.72	0.69	0.64
Avail. Phos. Poultry	0.80	0.8	0.7	0.70	0.62	0.62	0.57	0.57

Table 2.6.2.1 continued

Na+K-Cl, Meq/kg	320.3	339.9	281.7	270.2	264.59	246.68	237.63	216.33
Arginine, %	1.95	1.89	1.73	1.67	1.62	1.53	1.47	1.37
Lysine, %	1.89	1.89	1.65	1.67	1.49	1.51	1.33	1.35
Methionine, %	0.96	0.94	0.80	0.78	0.75	0.74	0.66	0.65
Met + Cys, %	1.39	1.41	1.20	1.20	1.14	1.14	1.02	1.02
Threonine, %	1.22	1.22	0.99	1.01	0.93	0.94	0.87	0.87
Tryptophan, %	0.33	0.33	0.29	0.28	0.27	0.25	0.24	0.22
Choline, mg/kg	2720	2720	2600	2600	2500	2500	2300	2300

[1]Feed supplements include XAP, XAP+DFM and Supplemental fat. ~2% Supplemental fat was added to create ME difference of 150kcal/kg. Filler was added in diets that had no feed supplement. [2]Each kilogram of mineral premix (.1% inclusion) supplied the following per kg of complete feed: 60 mg Zn as $ZnSO_4 \cdot H_2O$; 60 mg Mn as $nSO_4 \cdot H_2O$; 40 mg Fe as $FeSO_4 \cdot H_2O$; 5 mg Cu as $CuSO_4$; 1.25 mg I as $Ca(IO_3)_2$; 1 mg Co as $CoSO_4$. [3] $NaSeO_3$ premix provided 0.3 mg Se/kg of complete feed. [4]Each kilogram of vitamin premix (.1% inclusion) supplied the following per kg of complete feed: vitamin A, 13,200 IU; cholecalciferol, 4,000 IU; alpha-tocopherol, 66 IU; niacin, 110 mg; pantothenic acid, 22 mg; riboflavin, 13.2 mg; pyridoxine, 8 mg; menadione, 4 mg; folic acid, 2.2 mg; thiamin, 4 mg; biotin, 0.253 mg; vitamin B_{12}, 0.04 mg; ethoxyquin, 100 mg.

Table 2.6.2.2. The effect of dietary energy, DDGS level, and XAP and DFM supplementation on the body weight of turkey hens from 0 to 12 weeks of age.

Main effect of treatment		Body weight (kg)					
		2	4	6	8	10	12
		---------- Weeks----------					
DDGS level (%)							
6		0.37	1.03	2.35	3.96	5.92	7.53[a]
18		0.38	1.04	2.34	3.89	5.88	7.41[b]
Feed supplement							
Negative control (NC)		0.39[a]	1.06[a]	2.34[a]	3.92	5.87	7.42
~2% Supplemental fat (Suppl. fat)		0.35[b]	0.98[b]	2.27[b]	3.85	5.80	7.40
XAP		0.38[a]	1.05[a]	2.38[a]	3.99	5.97	7.54
XAP+DFM		0.38[a]	1.06[a]	2.39[a]	3.94	5.95	7.51
Interactions							
DDGS level (%)	Feed supplement						
6	NC	0.38	1.04[b]	2.32	3.96	5.84	7.44
6	Suppl. fat	0.35	0.99[c]	2.30	3.93	5.85	7.49
6	XAP	0.36	1.05[ab]	2.39	4.11	6.03	7.60
6	XAP+DFM	0.38	1.06[ab]	2.39	3.86	5.96	7.55
18	NC	0.39	1.08[a]	2.33	3.89	5.89	7.39
18	Suppl. fat	0.35	0.97[c]	2.24	3.77	5.75	7.30
18	XAP	0.38	1.06[ab]	2.36	3.87	5.91	7.47
18	XAP+DFM	0.39	1.06[ab]	2.38	4.03	5.94	7.46
Source of variations		---------- P-values ----------					
DDGS		0.2667	0.173	0.430	0.332	0.373	0.015
Feed supplement		<0.001	<0.001	<0.001	0.593	0.062	0.567
DDGS x Feed supplement		0.945	0.045	0.212	0.231	0.570	0.759
SEM(40)[2]		0.003	0.0066	0.020	0.051	0.034	0.067

[a,b]Means with different letter superscripts within a column are significantly different (P<0.05).
[1]Values are means of 6 replicates pens containing 18 hens per pen.
[2]SEM (40) = Standard error of the mean with 40 degrees of freedom.

Table 2.6.2.3. The effect of dietary energy, DDGS level, and XAP and DFM supplementation on the body weight gain of turkey hens from 0 to 12 weeks of age.

Main effect of treatment		Body weight gain (kg)				
		2-4	4-6	6-8	8-10	10-12
		---------- Weeks----------				
DDGS level (%)						
6		0.66	1.23	1.50	1.78	1.54[a]
18		0.67	1.21	1.43	1.80	1.40[b]
Feed supplement						
Negative control (NC)		0.67[a]	1.20	1.46	1.76	1.40
~2% Supplemental fat (Suppl. fat)		0.64[b]	1.22	1.48	1.79	1.49
XAP		0.68[a]	1.23	1.46	1.78	1.51
XAP+DFM		0.68[a]	1.23	1.45	1.82	1.50
Interactions						
DDGS level (%)	Feed supplement					
6	NC	0.65[bcd]	1.20	1.51	1.69	1.48
6	Suppl. fat	0.61[cd]	1.24	1.55	1.78	1.66
6	XAP	0.67[ab]	1.25	1.55	1.69	1.44
6	XAP+DFM	0.67[ab]	1.24	1.38	1.95	1.58
18	NC	0.69[a]	1.20	1.40	1.83	1.32
18	Suppl. fat	0.62[d]	1.21	1.41	1.80	1.33
18	XAP	0.68[abc]	1.22	1.37	1.87	1.6-
18	XAP+DFM	0.67[ab]	1.23	1.53	1.70	1.39
Source of variations		---------- P-values ----------				
DDGS		0.245	0.218	0.263	0.815	0.023
Feed supplement		<0.001	0.331	0.993	0.974	0.583
DDGS x Feed supplement		0.028	0.797	0.238	0.383	0.089
SEM(40)2		0.0051	0.020	0.087	0.067	0.058

[a,b]Means with different letter superscripts within a column are significantly different (P<0.05).
[1]Values are means of 6 replicates pens containing 18 hens per pen.
[2]SEM (40) = Standard error of the mean with 40 degrees of freedom.

Table 2.6.2.4. The effect of dietary energy, DDGS level, and XAP and DFM supplementation on the feed intake of turkey hens from 0 to 12 weeks of age.

Main effect of treatment		Feed intake (kg)					
		0-2	2-4	4-6	6-8	8-10	10-12
		---------- Weeks----------					
DDGS level (%)							
6		0.41	1.13	2.04	3.10	4.83	4.90
18		0.41	1.10	2.04	3.15	4.85	4.82
Feed supplement							
Negative control (NC)		0.44[a]	1.18[a]	2.06[a]	3.09	4.86	4.97
~2% Supplemental fat (Suppl.fat)		0.39[b]	1.03[b]	1.97[b]	3.07	4.80	4.75
XAP		0.42[a]	1.16[a]	2.07[a]	3.17	4.91	4.90
XAP+DFM		0.43[a]	1.13[a]	2.05[a]	3.16	4.80	4.89
Interactions							
DDGS level (%)	Feed supplement						
6	NC	0.44	1.16	2.04	2.99	4.97	5.14
6	Suppl. fat	0.39	1.05	2.02	3.00	4.73	4.77
6	XAP	0.41	1.19	2.05	3.21	4.85	4.91
6	XAP+DFM	0.42	1.56	2.05	3.20	4.77	4.79
18	NC	0.44	1.15	2.07	3.19	4.74	4.79
18	Suppl. fat	0.38	1.01	1.93	3.14	4.87	4.71
18	XAP	0.41	1.12	2.09	3.12	4.97	4.88
18	XAP+DFM	0.41	1.09	2.05	3.12	4.82	4.88
Source of variations		---------- P-values ----------					
DDGS		0.111	0.060	0.914	0.401	0.771	0.137
Feed supplement		<0.001	<0.001	0.028	0.516	0.560	0.053
DDGS x Feed supplement		0.9137	0.431	0.103	0.139	0.192	0.059
SEM(40)[2]		0.004	0.010	0.0345	0.054	0.065	0.056

[a,b]Means with different letter superscripts within a column are significantly different (P<0.05).
[1]Values are means of 6 replicates pens containing 18 hens per pen.
[2]SEM (40) = Standard error of the mean with 40 degrees of freedom.

Table 2.6.2.5. The effect of dietary energy, DDGS level, and XAP and DFM supplementation on the cumulative feed intake of turkey hens from 0 to 12 weeks of age.

Main effect of treatment		Cumulative feed intake (kg)					
		0-2	0-4	0-6	0-8	0-10	10-12
		---------- Weeks----------					
DDGS level (%)							
6		0.41	1.55[a]	3.58	7.01	11.90	17.01
18		0.41	1.51[b]	3.56	7.02	11.98	17.06
Feed supplement							
Negative control (NC)		0.44[a]	1.59[a]	3.66[a]	7.10[a]	12.00	17.22
~2% Supplemental fat (S.fat)		0.39[b]	1.42[b]	3.39[b]	6.67[b]	11.61	16.61
XAP		0.41[a]	1.56[a]	3.62[a]	7.18[a]	12.20	17.37
XAP+DFM		0.41[a]	1.59[a]	3.60[a]	7.11[a]	11.94	16.95
Interactions							
DDGS level (%)	Feed supplement						
6	NC	0.44	1.60	3.64	6.96	12.01	17.31
6	S.fat	0.39	1.43	3.42	6.65	11.39	16.20
6	XAP	0.41	1.58	3.62	7.26	12.25	17.71
6	XAP+DFM	0.42	1.57	3.63	7.16	11.93	16.84
18	NC	0.43	1.58	3.68	7.24	11.92	17.14
18	S.fat	0.38	1.40	3.37	6.68	11.83	17.01
18	XAP	0.41	1.53	3.62	7.09	12.14	17.02
18	XAP+DFM	0.41	1.51	3.57	7.05	11.95	17.07
Source of variations		---------- P-values ----------					
DDGS		0.111	0.002	0.517	0.909	0.587	0.834
Feed supplement		<0.001	<0.001	<0.001	0.001	0.057	0.131
DDGS x Feed supplement		0.9137	0.658	0.665	0.318	0.549	0.163
SEM(40)[2]		0.004	0.009	0.0326	0.089	0.105	0.2321

[a,b]Means with different letter superscripts within a column are significantly different (P<0.05).
[1]Values are means of 6 replicates pens containing 18 hens per pen.
[2]SEM (40) = Standard error of the mean with 40 degrees of freedom.

Table 2.6.2.6. The effect of dietary energy, DDGS level, and XAP and DFM supplementation on the adjFCR of turkey hens from 0 to 12 weeks of age.

Main effect of treatment		adjFCR (kg:kg)					
		0-2	2-4	4-6	6-8	8-10	10-12
		---------- Weeks----------					
DDGS level (%)							
6		1.29[b]	1.61[a]	1.63	1.97	2.82	3.21
18		1.33[a]	1.64[b]	1.65	2.06	2.75	3.44
Feed supplement							
Negative control (NC)		1.32[a]	1.65[a]	1.67[a]	2.03	2.84	3.59
~2% Supplemental fat (Suppl. fat)		1.32[a]	1.58[b]	1.57[b]	1.93	2.72	3.09
XAP		1.28[b]	1.63[a]	1.66[a]	2.07	2.86	3.35
XAP+DFM		1.28[b]	1.62[a]	1.64[a]	2.03	2.71	3.28
Interactions							
DDGS level (%)	Feed supplement						
6	NC	1.37	1.67	1.65	1.92	3.06	3.44
6	Suppl. fat	1.33	1.57	1.59	1.85	2.68	2.83
6	XAP	1.31	1.65	1.63	1.99	3.02	3.57
6	XAP+DFM	1.32	1.64	1.65	2.11	2.49	3.00
18	NC	1.29	1.62	1.69	2.14	2.61	3.73
18	Suppl. fat	1.33	1.58	1.56	2.01	2.75	3.36
18	XAP	1.26	1.60	1.70	2.15	2.69	3.13
18	XAP+DFM	1.25	1.59	1.63	1.94	2.92	3.55
Source of variations		---------- P-values ----------					
DDGS		0.008	0.001	0.287	0.294	0.646	0.118
Feed supplement		0.047	0.0002	0.004	0.723	0.844	0.148
DDGS x Feed supplement		0.196	0.085	0.177	0.435	0.173	0.081
SEM(40)2		0.015	0.010	0.017	0.0906	0.151	0.1423

[a,b]Means with different letter superscripts within a column are significantly different (P<0.05).
[1]Values are means of 6 replicates pens containing 18 hens per pen.
[2]SEM (40) = Standard error of the mean with 40 degrees of freedom.

Table 2.6.2.7. The effect of dietary energy, DDGS level, and XAP and DFM supplementation on the cumulative adjFCR of turkey hens from 0 to 12 weeks of age.

Main effect of treatment		Cumulative adjFCR (kg:kg)					
		0-2	0-4	0-6	0-8	0-10	0-12
		---------- Weeks----------					
DDGS level (%)							
6		1.29[b]	1.49[a]	1.59	1.74	2.02	2.28
18		1.33[a]	1.53[b]	1.59	1.78	2.04	2.32
Feed supplement							
Negative control (NC)		1.32[a]	1.53[a]	1.62[a]	1.77	2.04	2.34
~2% Supplemental fat (Suppl. fat)		1.32[a]	1.49[b]	1.55[b]	1.72	2.01	2.28
XAP		1.29[b]	1.51[ab]	1.59[ab]	1.77	2.05	2.32
XAP+DFM		1.28[b]	1.50[b]	1.58[ab]	1.77	2.03	2.27
Interactions							
DDGS level (%)	Feed supplement						
6	NC	1.36	1.57[a]	1.63	1.73	2.05	2.34
6	Suppl. fat	1.32	1.49[bc]	1.54	1.66	1.97	2.22
6	XAP	1.30	1.54[ab]	1.59	1.74	2.05	2.35
6	XAP+DFM	1.31	1.52[ab]	1.59	1.82	2.02	2.23
18	NC	1.29	1.50[bc]	1.62	1.81	2.03	2.34
18	Suppl. fat	1.33	1.50[bc]	1.57	1.78	2.05	2.33
18	XAP	1.26	1.49[bc]	1.60	1.80	2.05	2.30
18	XAP+DFM	1.25	1.47[c]	1.57	1.713	2.03	2.31
Source of variations		---------- P-values ----------					
DDGS		0.008	<0.001	0.971	0.235	0.265	0.258
Feed supplement		0.047	0.007	0.001	0.662	0.286	0.245
DDGS x Feed supplement		0.196	0.043	0.437	0.063	0.087	0.179
SEM(40)[2]		0.015	0.010	0.017	0.023	0.011	0.029

[a,b]Means with different letter superscripts within a column are significantly different (P<0.05).
[1]Values are means of 6 replicates pens containing 18 hens per pen.
[2]SEM (40) = Standard error of the mean with 40 degrees of freedom.

Table 2.6.2.8. The effect of dietary energy, DDGS level, and XAP and DFM supplementation on the morphological measurements of jejunum villi and mucosa of turkey hens at 42 days of age.

Main effect of treatment		Mucin	Tip width	Height	Crypt depth	Height : Crypt depth	Base width	Muscularis	Surface area
		µg/g	----------µ----------						µ2
DDGS level (%)									
6		679[b]	275[a]	2115	166	12.43	383	251[b]	684224[a]
18		705[a]	233[b]	2118	169	12.20	352	294[a]	606656[b]
Feed supplement									
Negative control (NC)		740[a]	264	2100	176[a]	11.71	376	285	670514
~2% Supplemental fat (Suppl. fat)		770[a]	267	2200	180[a]	11.89	374	274	639138
XAP		589[b]	240	2083	157[c]	13.44	350	266	618222
XAP+DFM		670[ab]	246	2083	159[c]	12.23	370	264	653887
Interactions									
DDGS level (%)	Feed supplement								
6	NC	737	296	2135	177	11.53	390	268	735289
6	Suppl. fat	726	295	2104	185	11.48	408	255	660544
6	XAP	576	252	2044	145	14.06	361	244	630957
6	XAP+DFM	679	260	2175	157	12.64	372	238	710104
18	NC	744	232	2064	176	11.89	360	303	605737
18	Suppl. fat	814	239	2293	174	12.29	340	293	617733
18	XAP	603	228	2122	167	12.81	340	288	605486
18	XAP+DFM	661	233	1991	161	11.82	368	291	597670
Source of variations		---------- P-values ----------							
DDGS		0.045	0.002	0.967	0.614	0.750	0.115	<0.01	0.037
Feed supplement		0.032	0.373	0.580	0.037	0.308	0.778	0.334	0.740
DDGS x Feed supplement		0.871	0.576	0.263	0.423	0.705	0.693	0.885	0.664
SEM(40)[2]		46	12	68	6.519	0.695	19	13	34110

[a,b]Means with different letter superscripts within a column are significantly different (P<0.05).
[1]Values are means of 6 replicates per treatment. [2]SEM (40) = Standard error of the mean with 40 degrees of freedom.

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CHAPTER THREE

Effect of β-Mannanase Supplementation of High and Low Energy (Fat) Diets on Intestinal Mucin Secretion, Gut Morphology, Nutrient Utilization, and Growth Performance of Poults

3.1 ABSTRACT.

β-mannanase is an enzyme that reduces the anti-nutritional effects of β-mannan in the diet of poultry by hydrolyzing the β -1, 4 glycosidic bonds of mannan into smaller, less viscous mannan oligosaccharides. Soybean meal (SBM) protein and hulls, typically included in poultry feeds, contain a significant amount of β-mannan, thus dietary supplementation of β-mannanase may improve the nutrient utilization and gut health of poultry fed corn-SBM diets, especially those containing high fat-energy. The objective of this experiment was to evaluate the effects of a commercial endo-β-D-mannanase (CTCzyme®, CTCBio, Inc., Soul Korea) on growth performance, gut health and ileal mucin secretion of poults fed a low and high energy diets. An experiment was designed as a 2 x 2 factorial arrangement of dietary levels of β-mannanase (0 and ~500 U β-mannanase/kg) and 2 levels of dietary energy differing by 150 kcal ME/kg by the addition of tallow. Four hundred and thirty two 1 d old poults were randomly assigned to 48 cages with 9 poults per cage, and 12 replicate cage per dietary treatment. Feed intake (FI) and body weight (BW) was recorded by pen on 0 d, 7 d, 14 d and 21 d. Individual BW and cage FI were determined on 28 d. The cumulative FI and FCR were calculated for day 7, 14, 21 and 28 d respectively. Jejunum section of gut was collected on 7 d, 14 d and 21 d for gut morphology characterization. On 28 d, 1 cm section from the meckel's diverticulum into the ileal was dissected for mucin secretion quantification, and the Ileal content was collected on 28 day for nutrients digestibility analysis. Cumulative FI (1 to 21 d) and periodic FI (7 to 14 d) was significantly increased among birds fed the high energy diet, and 14 d and 21 d body weights increased accordingly ($P<0.05$) without significant effect on FCR. Fat digestibility and

AMEn was also enhanced by the high energy diet. Although β-mannanase supplementation did not have a significant effect on the growth performance, it did increase the apparent nitrogen retention and fecal dry matter content. On 21 d, β-mannanase supplementation was observed to have a positive effect on jejunum mucosal morphology; β-mannanase supplementation increased villi surface area, basal, tip width and height/crypt depth ratio. In contrast, increased dietary energy just improved villi surface area ($P<0.05$). β-mannanase supplementation reduced enteric mucin secretion and the supplementation of high energy diet with β-mannanase had the lowest ileal mucin secretion ($P<0.05$). Covariance and correlation analysis revealed a positive correlation between the enteric mucin secretion and the ANR ($r^2 = 0.1$, $P<0.05$). Because mucin contains a significant amount of amino acids, dietary treatments that affect the amount of mucin secretion may affect ANR and AMEn results. The results from the experiment showed that increasing the energy improved the FI and BW, whereas the addition of β-mannanase did not have any significant effect of on the growth performance, but it is apparent that the supplementation of diet with β-mannanase corresponded with the improvement in of the some villi morphological parameters, crude protein and fat digestibility, apparent nitrogen retention, and reduction in ileal mucin. The endogenous secretion reduction inform of the enteric mucin secretion may presumably leads to lower endogenous nutrients loss.

Key words Turkey poults, β-mannanase, Energy, Soybean meal.

3.2 INTRODUCTION.

Soybean meal (SBM) is the most common protein source in poultry diets, but it contains some anti-nutritional factors (ANF) that reduce nutrient digestion and utilization. SBM contains a significant amount of ANF, such as and non-starch polysaccharides (NSP). About 23% of the total carbohydrate in SBM are in form of NSP (Chesson, 2000). NSP are plant structural carbohydrates, and exist in different forms. In SBM, 1.3-1.6% of the NSPs are in form of β-mannans (Dierick, 1989; Jackson et al., 1999), and they occur in the forms of glucomannan, galactomannan, glucogalactomannan and glucuronomannans (Aman and Graham, 1990). β-mannans are linear complex polysaccharides with repeating β-1,4 mannose, with α-1,6 galactose and glucose units attached to the β-mannan backbone. The complex structure of β-mannans, like other NSP, enables them to hold a high amount of water, forming viscous gels that entrap fat, protein and starch; thus reducing digestion and nutrient absorption, which adversely effects on growth performance and feed conversion efficiency (Jackson et al., 1999). Furthermore, Choct et al. (1996) speculated that the anti-nutritional effect of NSP is a multifactorial phenomenon. Apart from the apparent and primary effect of the viscous NSP on the on nutrients utilization, there are some secondary effects on the gut ecosystem and microflora that could also have vital repercussions on the efficiency of nutrients utilization by monogastric animals.

Excessive dietary NSP may lead to the development of undesirable pathogenic gut microflora in poultry. Increased fermentation by pathogenic microfloral can occur in the hind gut when a large amount of viscous NSP is present in the diet (Choct et al., 1996). The

proliferation of these NSP-induced pathogens may initiate a mucosal inflammatory response, leading to enteric distress, disbacteriosis, and suppressed growth performance of poultry. NSP gel entrapment make dietary fat and protein in the foregut less digestible and available to the host animal, but more available as fermentation substrates for the hind gut pathogens that cause an inflammatory effect to the host. Consequently, this could have a profound effect on gut health, by changing the mucosal morphological architecture and diverting more nutrients away from productive purposes towards gut maintenance.

Enteric inflammation, due to the proliferation of enteric pathogens, is associated with the activation of the innate immune system, which increases the intestinal mucous synthesis and secretion. The intestinal mucin serves as a mucosal blanket to impede the translocation of pathogens, and to entrap and expel them from the gut. Mucin, which is secreted by the goblet cells, is the protein-rich component of mucous. Viveros et al. (1994) observed that the atrophic shortening and thickening of jejunum villi, and the increased number of goblet cells per villus in birds were found to be associated with increased dietary β-glucans from a diet containing 60% barley in comparison to a conventional corn-SBM diet. Even β-mannans in corn-SBM diets can have significant adverse effects on nutrient digestibility and enteric health (Odetallah et al., 2002a). To reduce these adverse effects, poultry diets may be supplemented with dietary exogenous β-mannanase enzyme.

β-Mannanase breaks the β-1,4 glycosidic bonds of β-mannans into less viscous mannan-oligosaccharides, which allows for better digestion and nutrient absorption. Furthermore, the fermentation of the smaller NSP oligosaccharides by the symbiotic bacteria

in the hindgut produces volatile fatty acids, such as acetic, butyric and propionic acid, which controls the proliferation of pathogenic bacteria, and helps maintain good gut health. The supplementation of NSP enzymes, such as glycanases in NSP-riched diet, markedly elevate cecal volatile fatty acid concentration (Choct et al., 1996). Furthermore, dietary supplementation of a poultry diet containing β-mannan with β-mannanase has been reported to improve nutrient utilization and growth performance, and reduce fecal moisture (Ward and Fodge, 1996; McNaughton et al., 1998; Daskiran et al., 2004; Jackson et al., 2004). Li et al. (2010) demonstrated that the addition of β-mannanase to corn-SBM diets, improved the feed intake and feed conversion ratio of broilers raised to 6 weeks of age. In addition to the improved nutrient utilization, Jackson et al. (2004) hypothesized that an important mode of β-mannanase's action is reduced stimulation of innate immunity associated with a reduction in the β-mannan content of substrate entering the distal part of the intestinal tract.

To our knowledge, there are limited published data available on the effects of β-mannanase on gut health, particularly as it relates to gut morphology and intestinal mucin secretion in turkey poults. We hypothesize that β-mannanase will reduce enteric mucin secretion, benefit gut morphology, and improve nutrient digestibility and growth performance characteristics of commercial turkey hens. The objective of this study was to determine the effect of dietary β-mannanase supplementation of turkeys fed high and low energy diets modified by dietary fat supplementation on growth performance, nutrients utilization, and gut health assessed by mucosa morphometric analysis and intestinal mucin secretion.

3.3 MATERIALS AND METHODS.

3.3.1 Experimental Diet.

The experiment was designed as a 2 x 2 factorial arrangement of two dietary inclusion levels of β-mannanase at (0% and 0.05% CTCzyme®[18], supplying 0 and ~500 U endo-β-D-mannanase/kg, respectively), and two dietary levels of metabolizeable energy (High and Low) that differed by 150 kcal ME/kg by the addition of supplemental beef tallow fat. Beef tallow was chosen as the means to adjust dietary energy because it is less digestible than more polyunsaturated fats for turkey poults (Sell et al., 1981, 1983, and 1986; Leeson and Atteh, 1995). All the experimental diets were corn-SBM based formulations. The high energy basal diet was made and divided into two lots, and each was mixed with either 0.05% of vermiculite, or 0.05% of β-mannanase. A second low energy basal diet (150 kcal/kg less) was also made and divided into two parts, each was also mixed with either 0.05% of vermiculite or 0.05% of β-mannanase. All experimental diets were formulated to meet or exceed NRC (1994) requirements for turkeys (Table 1).

All diets were supplemented with 2.0% Celite[19] to serve as an acid insoluble ash indigestible reference marker for determination of digestibility coefficients. The calculated and analyzed composition of the experimental diets, including the supplemental enzymes, is

[18] Commercial endo-β-D-mannanase, (CTCzyme®, CTCBIO, Inc.). It provides 496 β-mannanase units/kg of diet.
[19] Celite™ (Celite Corp. Lompoc, CA).

reported in Table 1. All diets were produced at the North Carolina State University Feed Mill Educational Unit (Raleigh, NC).

3.3.2 Bird Husbandry and Data Collection.

Four-hundred-thirty-two Nicholas hen poults were obtained from a commercial hatchery[20] and randomly assigned to one of 48 cages with 9 poults per cage[21]. Each bird was identified with a numbered neck-tag in sequence for each replicate cage group. Four experimental treatment groups were randomly assigned among 48 cages. Feed and water was available *ad libitum*. Birds were kept until 27 d or 28 d of age. From 1 to 7 d, the birds were provided 23 h of light to 1 h darkness, and 14 L:10 D after 7 d. Feed consumption, group weight, and number of birds per cage was recorded on 0 d, 7 d, 14 d, and 21 d. Individual bird weights and cage feed consumption were determined on 28 d. Morality rate and cause of mortality were recorded daily and mortality weights were used to adjust the FCR.

3.3.3 Fecal Excreta Collection and Analysis.

Excreta was collected over three 24 h intervals, and pooled to evaluate nutrient digestibility of the diets. Excreta was collected daily from 22 d to 24 d of age and equal portions were stored at -20^{o}C for further analysis. The frozen excreta were allowed to thaw for approximately 16 h at room temperature before further processing. For each pen, approximately 200 g of representative samples free of feathers and other extraneous material,

[20] Prestage Farms Hatchery, Clinton, NC
[21] Alternative Design cages, Alternative Design Manufacturing and Supply, Inc., Siloam Springs, AR

were collected daily for the 3 consecutive days and blended[22] with 100 mL of water to form a slurry. The slurry was then acidified to pH<5.4 with the addition of HCl[23] to prevent volatilization of nitrogen and then placed in pie-tins and dried overnight at 70°C in a forced air convection oven. Once dried, the excreta was blended into a fine powder and placed in storage bags at -20°C until further analysis.

3.3.4 Ileal Digesta Collection and Analysis.

On the evening before ileal sample collection, the birds had restricted access to feed for at least 6 hours. Then starting at 4 a.m. of 28 d, the birds were returned to *ad libitum* access to feed. Thus, each bird had at least 4 h of *ad libitum* feed consumption before they were euthanized for ileal digesta sample collection. Ileal sections from Meckel's diverticulum to the cecal junction were dissected and contents were collected in 200 mL container. The ileal contents of the birds were pooled by cage and stored at -20°C until further analysis. Ileal samples were thawed for approximately 16 h at room temperature and processed the same way as the excreta samples and stored at -20°C until further analysis.

3.3.5 Chemical, Histological and Histochemical Analysis.

3.3.5.1 Chemical analyses.

Representative samples of each dried and grounded excreta and ileum were submitted for analysis of dry matter (AOAC. 1995a), crude protein (AOAC. 2006), and crude fat (AOAC.

[22] Waring® Commercial Blender Model 31BL92, Waring Commercial, New Hartford, CT 06057
[23] 0.1N, Fisher Scientific, Fairlawn, NJ 07410

1995b). The gross energy content was determined with an adiabatic oxygen bomb calorimeter[24]. Recovery of acid insoluble ash was determined according to the method described by Vogtmann et al. (1975). The apparent metabolizable energy nitrogen corrected (AME_n) and apparent nitrogen retention (ANR) was calculated from the nitrogen content of the diets and of excreta according to Lammer et al. (2008) using the following equations:

$$AME_n = [(GE_{Diet} - (GE_{Excreta} \text{ X } AIA_{Diet} / AIA_{Excreta}) - (8.22 \text{ X } N_{Retained})]$$

$$N_{Retained} = N_{Diet} - (N_{Excreta} \text{ X } AIA_{Diet}) / AIA_{Excreta}))$$

Where: AME_n (Kcal/g) is the nitrogen corrected apparent metabolizable energy of the diet; GE_{Diet} and $GE_{Excreta}$ were the gross energy of diet and excreta respectively; AIA_{Diet} and $AIA_{Excreta}$ were the concentration of Celite recovered as acid insoluble ash in diet and excreta respectively; 8.22 (Kcal/g) is the energy value of uric acid; and $N_{Retained}$ (g/kg) is the nitrogen retained by bird per kilogram of diet consumed, and N_{Diet} and $N_{Excreta}$ (%) were the nitrogen content of diet and excreta respectively. All values used in this calculation were expressed as grams per kilogram of DM.

$$ANR\ (\%) = [100*(1-(AIA_{Diet} / AIA_{Excreta} * Nitrogen_{Excreta} / Nitrogen_{Diet}))]$$

Where: AIA_{Diet} and $AIA_{Excreta}$ were the concentration of Celite recovered as acid insoluble ash in diet and excreta respectively; $Nitrogen_{Diet}$ and $Nitrogen_{Excreta}$ were the nitrogen content in the dietary and excreta, respectively.

[24] IKA Calorimeter System C5000 Control, IKA® Werke Labortechnik, Staufen, Germany

Ileal nutrients digestibility were calculated according to Dilger and Adeola, (2006), using the following equation:

$$\text{Nutrient digestibility (\%)} = 100-[(AIA_{Diet}/AIA_{Excreta})*(Nutrient_{Ileal}/ Nutrient_{Diet})*100]$$

Where: Nutrient digestibility (%) is ileal nutrient digestibility expressed as a percentage; AIA_{Diet} and $AIA_{Excreta}$ are concentration of Celite recovered as acid insoluble ash in diet and excreta respectively; $Nutrient_{Ileal}$ and $Nutrient_{Diet}$ were the ileal and dietary nutrient content. All values used in this calculation were expressed as grams per kilogram of DM.

3.3.5.2 Histological analyses.

After being submerged in 10% buffered formalin solution for at least 48 h, the 3 cm ileal section was processed. A total of four sections about 2 to 3 mm in length were taken from the 3 cm fixed ileal section collected from each sampled bird. These smaller sections were placed in tissue cassettes and submerged in 10% buffered formalin solution until processed at the Histopathology Laboratory[25]. The fixed ileal sections were embedded in paraffin wax, and 5 μm thick transverse sections were cut with a microtome. The cut sections were placed on slides and were stained with Lilee Meyer haematoxylin and counter-stained with eosin yellow. A light-microscope[26] was used to visualize the transverse sections placed on slides. The images were captured using a digital camera[27] and analyzed using Image Tool[28] software. Villus height, villus apical width at the tip of the villus, villus basal width at the crypt-villus junction,

[25] North Carolina State University, College of Veterinary Medicine, Raleigh, NC.
[26] LEICA-DMR light-microscope, Leica Camera AG, Solms, Germany.
[27] Spot-LTCR digital camera, Diagnostic Instruments, Inc., Sterling Heights, MI
[28] UTHSCSA Image Tool Software, Version 3.0, the University of Texas, San Antonio, TX

crypt depth, and muscularis depth were measured on 15 villi per sampled poult. The following mathematical formula was used to determine apparent villus surface area [((villus tip+villus base)/2)*villus height].

3.3.5.3 Histochemical analyses- measurement of ileal mucin secretion.

The epithelial mucin was assessed histochemically with Alcian blue stain, based on the affinity of the basic stain for acidic tissue element like mucin (McManusi and Mowry, 1960; Evansee and Kent, 1962). The thickness of the ileal mucus adherent layer was estimated based on the modification of Corne's method (Corne et al., 1974; Kitagawa et al., 1986; Parman et al., 1993). A 1 cm section of ileal tissue from each sampled bird were removed and placed in 10 g/L Alcian blue dye solution in buffer containing 160 mmol/L sucrose and 50 mmol/L sodium acetate, pH 5.8. After 6 h of incubation, excess dye was be extracted with 250 mmol/L sucrose. The absorbed dye was extracted from the tissue by incubation in 10 g/L docusate sodium salt solution overnight at room temperature. Samples were centrifugation at 700 g, plated on 96 well plate, and the optical densities were measured at 620 nm using Alcian Blue solution as a standard. The amount of absorbed dye was reported as µg Alcian blue/g of ileal tissue

3.3.6 Statistical Analysis.

This experiment was analyzed as a completely randomized design. Cage means were used as the experimental unit. Data were analyzed using JMP[29] software. ANOVA was used

[29] Version 10, SAS Institute Cary, NC

to examine the main effect of dietary treatment factors, and their interaction on parameters evaluated. Means were separated using the LS Means at $P<0.05$.

3.3.7 Animal Ethics.

This experiment was conducted according to the guidelines of the Institutional Animal Care and Use Committee at North Carolina State University. All husbandry practices and euthanasia were performed with full consideration of animal welfare.

3.4 RESULTS.

3.4.1 Growth Performance.

Growth performance is an important indicator of dietary treatment efficacy, as it demonstrates how dietary nutrients are utilized and assimilated into body tissue. The results of the growth performance are presented in tables 2 to 5. Dietary supplementation of β-Mannanase did not affect the growth performance. In contrast, dietary energy level did significantly ($P< 0.05$) increase body weight at 14 d and 21 d (Table 2), cumulative feed intake (1-21 d; Table 3), and periodic feed intake (7-14 d; Table 4). The body weight was increased by more than 10% when the energy content in the diet was increased, and the cumulative feed intake and periodic feed intake were increased by 18%. There was no any main or interaction effects on the adjFCR ($P>0.05$; Table 6).

3.4.2 Nutrients Digestibility.

Tables 6 and 7 summarize the treatment effects on nutrient digestibility determined from fecal and ileal samples, respectively. Increasing the dietary energy level increased AMEn and ANR ($P<0.05$; Table 6), but did not affect fecal dry matter digestibility ($P>0.05$; Table 6). The AMEn determined from poults fed the high energy diet was 165 kcal/kg (6.4%) higher than those fed the low energy diet, which is very close to the expected 150 kcal/kg energy difference between the formulated high and low energy diets. ANR was increased by about 7% when the energy content was increased. Treatments with β-mannanase supplementation had 11% drier fecal matter and 4% greater ANR than treatment without β-mannanase supplementation ($P<0.05$; Table 6), but the enzyme supplementation had no effect on AMEn. Poults fed the high energy diet had significantly improved ileal fat content digestibility ($P<0.05$; Table 7), but did there was no dietary energy effect on ileal protein and dry matter digestibility ($P>0.05$, Table 7). Similarly, β-mannanase supplementation improved ileal fat digestibility ($P<0.05$; Table 7), but did not affect ileal dry matter and crude protein digestibility ($P>0.05$; Table 7).

3.4.3 Gut Morphology.

In this study, there were no significant treatment effects on jejunum mucosa morphology observed at 7 d and 14 d (Table 8 and 9), but there was a significant treatment effect observed at 21 d. Increasing dietary energy level was associated with an 18% decrease in the villous base width and a 21% decrease in villus surface area ($P<0.05$; Table 10). In

contrast, dietary supplementation of β-mannanase increased the villous tip width by 36% and villous height/crypt depth by 32% (P<0.05; Table 10), while the villus base width and surface area was increased by about 22.5% and 34%, respectively (P<0.05; Table 10).

3.4.4 Enteric Mucin Secretion.

There was no significant main effects of dietary energy, but poults fed diet containing β-mannanase supplementation had 4% reduction in ileal mucin secretion. Furthermore, the addition of β-mannanase to the high energy diet reduced the quantity of ileal mucin secretion by about 36% when compared to the high energy diet without any supplementation (P<0.05; Table 11), but neither of the diets were different from low energy diets with or without the enzyme supplementation (P>0.05; Table 11).

3.4.5 Relationship between Enteric Mucin Secretion, Apparent Nitrogen Retention and Apparent Metabolizable Energy.

A correlation between enteric mucin secretion and ANR and AMEn may reflect the extent to which the mucin nutrients are being recycled. Correlations between enteric mucin secretion and apparent nitrogen retention (ANR), crude protein digestibility and apparent metabolizable energy (AMEn) are presented in figure 1. Enteric mucin secretion had a positive and significant correlation with ANR (r^2=0.1*;* P<0.05). There was negative and marginally significant correlation between enteric mucin secretion and crude protein digestibility (r^2=0.03*;* P=0.07). AMEn was negatively correlated with enteric mucin secretion, but the relation was not significant (r^2=0.03*;* P=0.13).

3.5 DISCUSSION.

β-mannans are part of the hemicellulose family that are found abundantly in the leguminous plant seeds like soybeans. SBM is an important protein source in animal nutrition, and it contains about 1.6% β-mannans (Jackson et al., 1999; Hsiao et al, 2006). However, *β*-mannans causes decreased growth performance by increasing digesta viscosity and decreasing nitrogen utilization and metabolizeable energy (Dale 1997; Wu et al., 2005; Kong et al., 2011). In some extreme cases, it can also cause enteric inflammation (Daskiran et al., 2004; Lee et al., 2003). This pro-inflammatory phenomenon may limit the dietary inclusion of the SBM in poultry and many other monogastric animals because they lack the endogenous enzymes that can hydrolyze the non-starch polysaccharides, like β-mannan, into less viscous oligosaccharides that promote a more symbiotic gut ecosystem. β-mannanase is an exogenous enzyme that can be supplemented to the diet to improve the β-mannan digestion in poultry, as it hydrolyzes the β-1,4 glycosidic bonds of the β-mannan into mannan-oligosaccharides. The released mannan-oligosaccharides can be metabolized by poultry to improve the energy value of the diet (Verma and McNab 1982; Patel and McGinnis 1985; Teves et al., 1988). Dietary β-mannanase supplementation improved the body weight and feed efficiency of broilers (Daskiran et al., 2004; Jackson et al. 2004). The reduction of the large complex polysaccharides to smaller oligosaccharides, also increases the substrate available for hindgut microfloral fermentation (Choct et al., 1996). Since the microflora plays an important role in the development and maintenance of gut morphology and intestinal secretions (Choct et al., 1992),

the activity of the NSPase enzymes will indirectly affect the geometrics of gut morphology and secretions.

The availability of dietary carbohydrates for newly hatched turkey poults has been shown to increase the rate at which energy metabolism shifts from gluconeogenic to glycolytic pathways (Donaldson 1991, 1994). In addition, Rosenbrough (1976) had demonstrated previously that a high-fat diet will increase glucose-6-phosphatase enzyme activity to sustain gluconeogenesis without dramatically shifting the metabolic system toward glycolysis. As aforementioned, the turkey poult has a limited capability to metabolize a highly saturated fat like beef tallow because it is not easily emulsified and absorbed as micelles. Fat micelle formation and absorption decreases as β-mannan chyme viscosity increases, thus impairing absorption of many of the fat-soluble compounds, including fat-soluble vitamins, pigments, and lipids (Ferket and Veldkamp, 1999). Hypothetically, the supplementation of β-mannanase will improve fat digestibility, especially in high fat diets because it will facilitate better energy utilization due to reduced β-mannan viscosity. Furthermore, the effect of dietary supplementation of NSPases on poultry growth performance is dependent upon the amount and source of dietary fat (Langhout et al., 1997; Jamroz et al., 1999). The 150 kcal diet difference also serves to test if the enzyme improves dietary energy utilization by up to 150 kcal, regardless of dietary fat.

High and low energy basal diets were formulated to differ by 150 kcal/kg. Therefore, the difference in the AMEn of these diets, which was about 165 kcal/kg, confirms the accuracy of our feed formulation were within experimental error, and that any difference in growth

performance, nutrient digestibility, gut morphology, and mucin secretion is solely due to the treatment effect. Increasing the dietary energy level of the turkey poults improved the body weight, cumulative feed intake, and periodic feed intake during the first 21 d, but this did not translate into improved FCR. Evidently, the birds gained the weight because they were eating more, not because they were able to utilize the nutrient more efficiently. Apparently, increasing dietary energy by the addition of fat encouraged the poults to eat more feed, and thus more nutrients were consumed to meet their requirement for growth. In contrast, the enzyme supplementation did not affect feed intake, body weight, and FCR. Odetallah et al. (2002b) observed similar growth performance results with dietary endo-1,4-β-mannosidase supplementation for turkey poults. They observed no improvement in the body weight, feed consumption and FCR after when the corn-SBM diet was supplemented with the enzyme. In addition, Kong et al. (2011) and Mussini et al. (2011) observed similar growth performance results with dietary β-mannanase supplementation for broilers. Our results also substantiated by Zou et al. (2006) and Mehri et al. (2010), who reported no significant benefit of dietary β-mannanase supplementation on the growth performance of young. However, we may have been able to observe improved growth performance by the enzyme supplementation if our trial was continued beyond the 28 d. Odetallah et al. (2002a) suggested that the effects of enzyme supplementation on growth performance is age dependent, being more significant at later ages. The high viscosity effect is more pronounced in older birds because they have a more developed hindgut microflora and fermentation that responds to enzyme effects on substrates. Furthermore, the reduction in feed intake without changing the body weight and FCR after the

enzyme supplementation, implied that the birds were able to obtained sufficient nutrients from less diet intake than those that did not receive the enzyme supplement. The enzyme enabled the birds to utilize the nutrients more efficiently.

In this study, the supplementation of β-mannanase improved nutrient digestibility, which could be attributed to the loss of β-mannan's viscous property within the digesta. Apart from impeding nutrient digestibility, the viscous β-mannan entrap nutrients within its complex interlocking structure, thereby impairing the mixing of nutrient substrate with digestive enzymes, and reducing the rate of nutrient diffusion to the absorptive epithelium (Read, 1986). Thus, β-mannan reduces the overall rate of nutrient (i.e. fat and crude protein) digestion and absorption, and may accelerate digesta passage rate towards the hindgut to "feed" competitive enteric pathogens. We observed significant improvement in the fecal dry matter content and apparent nitrogen retention, which may indicate reduced nutrient availability for the pathogenic hind gut microflora. Consequently, the potential for the proliferation of these competitive pathogens diminishes, along with their ability to cause hindgut distress and inflammatory mucosal leakage.

The increased fecal dry matter is a result of the reduced fecal moisture. This could contribute to better animal welfare by reducing the incidence of possible footpad dermatitis, which has been associated with wet litter conditions in production facilities for broilers (Greene et al., 1985; Martland, 1985) and turkeys (Martland, 1984; Mayne et al., 2007). This is a condition characterized by inflammation and necrotic lesions on the plantar surface of the

footpads and toes (Shepherd et al., 2010). To understand the economic implication of this litter-induced footpad lesion problem, the USA exported 421,000 tons of chicken paws worth about $280,000,000 in 2009 to China alone (Shepherd et al., 2010; Fairchild, 2010). Apart from the drier litter, dietary β-mannanase supplementation also improved ANR and fat digestibility. The improved ANR could be partly or indirectly linked to the improved fat digestibility. Age-dependent profile of enteric microflora has been demonstrated to be influenced by dietary fat (Knarreborg et al., 2002). As digesta viscosity increases more fat ends up in the hind gut, feeding pathogenic bacteria like *C. perfringens* (Collier et al., 2003) that causes inflammation and more mucin secretion and mucosal leakage, which is interpreted as reduced apparent crude protein digestibility. Improved ANR may indicates reduced nitrogen excretion into the litter, which ultimately may be released into the environment as ammonia, especially when litter moisture and pH conditions are excessively high (Lopez and Leeson 1995; Sloan et al., 1995). Excessive ammonia emissions from poultry operations is a point of criticism by the public concerned with environmental sustainability (EPA 1998; Burns et al., 2007). Dietary supplementation of the β-mannanase was significantly associated with the improved ileal fat digestibility, especially when included in the high-fat diet. The β-mannanase likely degraded the viscous β-mannan that would otherwise impede the emulsification of dietary fat and the formation of fat micelles necessary for fat absorption. Dietary β-mannanase supplementation may have enhanced the access of bile and endogenous lipase enzyme emulsify and to hydrolyze the fat to form fatty acid micelles to facilitate absorption.

The viscous nature of β-mannan also causes physiological and morphological changes to the gastrointestinal tract in poultry (Brown et al., 1979; Cassidy et al., 1981; Jacobs, 1983). The nutrient absorptive capacity of the enterocyte cells in the gut depends on the gut mucosal morphology, and dietary NSP like β-mannans may cause mucosal distress, enhance epithelial cell turnover rate, and thus depresses the villi conformations in both the jejunum and ileum in gut (Iji, 1999). Generally, tall and slim villi indicate a healthy mucosal brush boarder with a greater surface area with more functional enterocytes able to absorb nutrients more efficiently with less maintenance requirements than shorter blunted villi with deep crypts. Deep villi crypts indicate a high turnover rate of enterocytes in order to compensate for their shorter life-span and higher extrusion/sloughing rate at the villus tip; thus mucosal maintenance requirements are increased and FCR is ultimately adversely affected (Parsaie, 2007). The supplementation of diets with enzymes can also indirectly influence gut health by affecting the substrate availability for proliferating gut microbes. The fermentation activities of these microbes can either have beneficial or harmful effects on gut health (Yamauchi, 2002). Furthermore, mucin can sometimes indicate the state of intestinal health. This is because gut inflammation is usually accompanied by an increased enteric mucous secretion. Increased enteric mucin secretion has also been associated with endogenous nutrient loss (Cowieson et al., 2006).

We observed in this study that dietary inclusion of β-mannanase benefited jejunum mucosal morphology among poults sampled at 21 d, especially among those fed the high energy (fat) diet. The villus surface area, and tip and base widths were all increased. Although

there are few publications demonstrating the effect of β-mannanase on gut morphology and intestinal mucin secretion, the work of Mehri et al. (2010) and Luo et al. (2009) corroborated with our findings. There was an improvement in the villi surface area and jejunum villi height/crypt depth ratio with the β-mannanase supplementation, although the effect was more pronounced with the inclusion of the enzyme in the lower dietary energy level.

Mehri et al. (2010) observed an increased in villus height and crypt depth in the duodenum when β-mannanase was supplemented to corn-soy diets at 700 g/ton and 900 g/ton. Luo et al. (2009) also reported that supplementation of dietary xylanase improved the villus height and villus height/crypt depth ratio in the ileum and duodenum. Parsaie et al. (2007) concluded that distressed gut morphology, as indicated by the shortened villi and deepened crypts, may cause increased mucosal secretions, which ultimately enhances endogenous losses. In this present study, we observed a reduction in the ileal mucin secretion with dietary β-mannanase supplementation, especially in the high energy (fat) diets. This could indicate a subsequent reduction in the endogenous secretions, while complementing the improved apparent nitrogen retention. Substantiating our results, Mehri et al. (2010) reported that dietary β-mannanase supplementation resulted in a reduction in the number of mucosal goblet cells in broilers. Since mucin is secreted by the goblet cells, this observation agrees with our observations that dietary β-mannanase supplementation significantly reduces intestinal mucin secretion. This response may indicate a subsequent reduction in the endogenous secretions, while complementing the improved ANR.

The correlation between enteric mucin secretions and nutrient utilization reveals some interesting findings. There is a logical explanation for the negative correlation between ileal crude protein digestion and enteric mucin secretion observed in this present study. Some previous studies with rats explained that before nutrients in the small intestinal lumen can interact with digestive and transport sites, it must pass a diffusion barrier that is considered to be mainly composed of glycocalyx and mucins (Smithson et al., 1981; Gerencser et al., 1984). Consequently, the amount and composition of mucin secreted into the lumen may impede digestion and nutrient absorption (Schwartz et al., 1980). Another supporting explanation is the increased demand for threonine and other amino acids for the synthesis of mucin. Although, the negative correlation between enteric mucin secretion and AME was not significant, Pirgozliev et al. (2007) reported similar finding in chickens and turkeys. Mucin secretion's negative correlation with the AMEn could be due to the inability of the hind gut's microflora to ferment sialic acid as a substrate, which is the major carbohydrate component of mucin. In contrast, the improved ANR and its positive correlation with enteric mucin secretion may be due to fermentation of mucin cecal microflora, which recycles the nitrogen to generate microbial proteins (Montagne et al., 2004). The recycling of some of the mucin nutrients by hindgut microflora may indicate that some of the endogenous losses through the stimulation of mucin secretion was not totally lost. Some of the end products of hindgut fermentation include microbial protein and SCFA (Choct et al., 1996). SCFA, such acetate, butyrate and propionate, have the potential to inhibit the proliferation of pathogenic bacteria in the hindgut, thereby suppressing possible inflammation and maintaining healthy gut system (Montagne et

al., 2004). Enteric mucin can be fermented by the gut microfloral, especially at the hind gut (Montagne et al., 2004). The fermentation allows the microbial to recycle some of the mucin nutrients.

Although, dietary supplementation of β-mannanase from CTCzyme® did not clearly influence the growth performance of cage-reared turkey poults, the results of our experiment clearly demonstrate the enzyme's benefit on reducing enteric villi morphological distress and mucin secretion, thus improving apparent utilization of dietary protein, fat and energy. The outcome of experiment demonstrated the importance of NSPase enzyme in poultry nutrition, and this is especially significant when formulating turkey diets with saturated fats such as tallow. In this case, the supplementation of β-mannanase reduced the enteric mucin secretion, which would have gone up because of the impact of poor fat digestion on increasing the proliferation of hindgut pathogens. These pathogenic bacteria cause inflammation, and the bird responds by stimulating mucin secretion at the expense of using nutrients for more productive purposes. Furthermore, apart from the extra income that can be generate from better quality paws, the reduction in the litter moisture content and nitrogen excretion after the enzyme supplementation means that using CTCzyme® may contribute to greater environmental sustainability.

3.6 TABLES AND FIGURES

Table 1. Dietary ingredient composition and nutrient composition of starter diets fed to turkey hens from 1 to 28 days of age.

Ingredients	High Energy		Low Energy	
	% Dietary β-mannanase Supplementation			
	0	0.5	0	0.5
	----------(% of Diet)----------			
Corn	30.56	30.56	34.98	34.9
Soybean Meal	44.84	44.84	44.24	44.24
Soy Hulls	6.11	6.11	6.03	6.03
Poultry meal	5.00	5.00	5.00	5.00
Fat Beef tallow	5.82	5.82	2.09	2.09
Dical phosphate	3.03	3.04	3.03	3.03
Limestone	0.95	0.95	0.95	0.95
Methionine	0.44	0.44	0.43	0.43
Salt	0.32	0.32	0.32	0.32
Lysine	0.26	0.26	0.27	0.27
Choline Chloride	0.22	0.22	0.22	0.22
Trace mineral[2]	0.20	0.20	0.20	0.20
Vitamin premix[4]	0.15	0.15	0.15	0.15
Selenite Premix[3]	0.05	0.05	0.05	0.05
Celite™	2.00	2.00	2.00	2.00
CTCzyme[1]	0.00	0.05	0.00	0.05
Filler (Sand)	0.05	0.00	0.05	0.00
Chemical Analysis (DM basis)				
Dry matter, %	93.34	93.34	92.72	92.72
Crude protein, %	30.06	30.06	30.19	30.19
Gross energy, kcal/kg	4110	4110	3944	3944
Crude fat, %	8.04	8.04	4.92	4.92
Acid detergent fiber, %	6.09	6.09	8.24	8.24
Neutral detergent fiber, %	10.69	10.69	10.47	10.47
Non fiber carbohydrate, %	41.38	41.38	44.76	44.76

[1] Enzyme provided 0.05% of beta-mannanase in diet. [2]Each kilogram of mineral premix (0.1% inclusion) supplied the following per kg of complete feed: 60 mg Zn as $ZnSO_4 \cdot H_2O$; 60 mg Mn as $MnSO_4 \cdot H_2O$; 40 mg Fe as $FeSO_4 \cdot H_2O$; 5 mg Cu as $CuSO_4$; 1.25 mg I as $Ca(IO_3)_2$; 1 mg Co as $CoSO_4$. [3] $NaSeO_3$ premix provided 0.3 mg Se/kg of complete feed. [4]Each kilogram of vitamin premix (.1% inclusion) supplied the following per kg of complete feed: vitamin A, 13,200 IU; cholecalciferol, 4,000 IU; alpha-tocopherol, 66 IU; niacin, 110 mg; pantothenic acid, 22 mg; riboflavin, 13.2 mg; pyridoxine, 8 mg; menadione, 4 mg; folic acid, 2.2 mg; thiamin, 4 mg; biotin, 0.253 mg; vitamin B_{12}, 0.04 mg; ethoxyquin, 100 mg.

Table 2. Effect of dietary energy level and β-mannanase supplementation on body weight of turkey hens.[1]

Main effect of treatment		Age (days)			
		7	14	21	28
		Body weight (g)			
Energy level					
High energy (H)		148	351[a]	631[a]	987
Low energy (L)		142	314[b]	573[b]	1038
β-Mannanase level (%)					
0.05		144	327	600	1027
0.00		146	334	604	998
Interactions					
Energy level	*β-Mannanase level (%)*				
H	0.05	145	345	630	981
H	0.00	150	357	633	994
L	0.05	142	309	571	1073
L	0.00	141	320	574	1002
Source of variations		*P-values*			
Energy		0.1870	<.0001	<.0001	0.0554
β-Mannanase		0.6466	0.1046	0.7213	0.2539
Energy x β-Mannanase		0.5518	0.9623	0.9948	0.1126
SEM(44)[2]		2.233	3.621	4.658	12.801

[a,b]Means with different letter superscripts within a column are significantly different (P<.05).
[1]Values are means of 12 replicates cages containing 9 hens per cage.
[2]SEM = Standard error of the mean, with 44 degree of freedom

Table 3. Effect of dietary energy level and β-Mannanase supplementation on cumulative feed intake per bird of turkey hens [1].

Main effect of treatment		Age (days)			
		1-7	1-14	1-21	1-28
		---------- Cumulative Feed Intake (g) ----------			
Energy level					
High energy (H)		113	375[a]	812[a]	1367
Low energy (L)		109	332[b]	687[b]	1293
β-Mannanase leve(%)					
0.05		111	346	759	1363
0.00		112	361	740	1298
Interactions					
Energy level	*β-Mannanase level (%)*				
H	0.05	111	371	862	1465
H	0.00	115	379	763	1270
L	0.05	111	321	655	1261
L	0.00	108	342	717	1325
Source of variations		---------- P-values ----------			
Energy		0.4849	0.0528	0.0208	0.4725
β-Mannanase		0.7994	0.5068	0.7277	0.5244
Energy x β-Mannanase		0.4714	0.7558	0.1319	0.2117
SEM (44)[2]		2.452	10.932	26.265	50.952

[a,b]Means with different letter superscripts within a column are significantly different (P<.05).
[1]Values are means of 12 replicates cages containing 9 hens per cage.
[2]SEM = Standard error of the mean, with 44 degree of freedom

Table 4. Effect of dietary energy level and β-Mannanase supplementation on feed intake by period of turkey hens.[1].

Main effect of treatment		Period (days)			
		1-7	7-14	14-21	21-28
		---------- Feed Intake by period (g) ----------			
Energy level					
High energy (H)		113	262[a]	413	576
Low energy (L)		109	222[b]	368	630
β-Mannanase level (%)					
0.05		111	236	416	627
0.00		112	249	365	579
Interactions					
Energy level	*β-Mannanase level (%)*				
H	0.05	111	261	470	602
H	0.00	115	264	356	549
L	0.05	111	210	363	652
L	0.00	108	234	375	609
Source of variations		---------- *P-Value* ----------			
Energy		0.4849	0.0428	0.1938	0.3377
β-Mannanase		0.7994	0.4892	0.1367	0.3909
Energy x β-Mannanase		0.4714	0.5914	0.0666	0.9327
SEM(44)[2]		2.452	9.604	16.781	27.919

[a,b]Means with different letter superscripts within a column are significantly different (P<.05).
[1]Values are means of 12 replicates cages containing 9 hens per cage.
[2]SEM = Standard error of the mean, with 44 degree of freedom

Table 5. Effect of dietary energy level and β-mannanase supplementation on adjusted Feed Conversion Ratio (adjFCR) per cage of turkey hens.[1]

Main effect of treatment		Age (days)			
		1-7	1-14	1-21	1-28
		---------- adjFCR ----------			
Energy level					
High energy (H)		1.25	1.29	1.45	1.51
Low energy (L)		1.28	1.32	1.49	1.54
β-Mannanase level					
0.05 %		1.27	1.32	1.48	1.52
0.00 %		1.26	1.30	1.45	1.53
Interactions					
Energy level	*β-Mannanase level (%)*				
H	0.05	1.26	1.31	1.47	1.49
H	0.00	1.24	1.27	1.43	1.53
L	0.05	1.28	1.32	1.50	1.54
L	0.00	1.27	1.33	1.47	1.54
Source of variations		---------- *P-value* ----------			
Energy		0.1837	0.2768	0.2372	0.4956
β-Mannanase		0.5543	0.5925	0.3763	0.6422
Energy x β-Mannanase		0.7823	0.4311	0.8872	0.5813
SEM (44)[2]		0.010	0.016	0.015	0.018

[a,b]Means with different letter superscripts within a column are significantly different (P<.05).
[1]Values are means of 12 replicates cages containing 9 hens per cage.
[2]SEM = Standard error of the mean, with 44 degree of freedom

Table 6. Effect of dietary energy level and β-mannanase supplementation on Dry matter (DM) of feces, Apparent Metabolizable Energy corrected by nitrogen (AMEn), and Apparent Nitrogen Retention (ANR) from fecal cage pool samples from 22, 23, and 24 days of age of turkey hens.[1]

Main effect of treatment		DM Feces (%)	AMEn (Kcal/kg)	ANR (%)
Energy level				
High energy (H)		22	2721[a]	56[a]
Low energy (L)		23	2556[b]	52[b]
β-Mannanase level				
0.05 %		23[a]	2635	55[a]
0.00 %		21[b]	2642	53[b]
Interactions				
Energy level	*β-Mannanase level (%)*			
H	0.05	23	2718	58
H	0.00	21	2723	54
L	0.05	24	2552	53
L	0.00	22	2561	52
Source of variations		----------P-values ----------		
Energy		0.3541	<.0001	0.0005
β-Mannanase		0.0414	0.7898	0.0221
Energy x β-Mannanase		0.7407	0.9380	0.1347
SEM(44)[2]		0.5330	12.98	0.4815

[a,b]Means with different letter superscripts within a column are significantly different (P<.05).
[1]Values are means of 12 replicates cages containing 9 hens per cage.
[2]SEM = Standard error of the mean, with 44 degree of freedom

Table 7. Effect of dietary energy level and β-mannanase supplementation on Dry matter (DM) of ileum content, Crude Protein Digestibility (CP digest.), and Fat Digestibility (Fat Digest.) from pool cage samples of turkey hens at 28 days of age[1].

Main effect of treatment		DM Ileum	CP Digestibilty	Fat Digestibility
Energy level				
High energy (H)		18	84	94[a]
Low energy (L)		17	85	91[b]
β-Mannanase level (%)				
0.05		17	85	94[a]
0.00		18	84	92[b]
Interactions				
Energy level	*β-Mannanase level (%)*			
H	0.05	18	85	94
H	0.00	17	83	94
L	0.05	17	84	93
L	0.00	16	84	92
Source of variations		---------- *P-values* ----------		
Energy		0.8150	0.2017	0.0012
β-Mannanase		0.8361	0.1987	0.0423
Energy x β-Mannanase		0.0677	0.0429	0.5618
SEM(44)[2]		0.133	0.242	0.310

[a,b]Means with different letter superscripts within a column are significantly different (P<.05).
[1]Values are means of 12 replicates cages containing 9 hens per cage.
[2]SEM = Standard error of the mean, with 44 degree of freedom

Table 8. Effect of dietary energy level and β-mannanase supplementation on Jejunum histologic morphology of turkey hens at 7 days of age[1].

Main effect of treatment		Tip Width	Villi Hight	Base width	Crypt	Muscularis Thickness	Surface Area
		---------- Micron ----------					Micron[2]
Energy level							
High energy (H)		121	915	176	102	152	130,551
Low energy (L)		133	848	184	94	137	134,761
β-Mannanase level (%)							
0.05		134	901	180	102	149	136,227
0.00		120	862	180	93	141	129,085
Interactions							
Energy level	*β-Mannanase level (%)*						
H	0.05	132	923	182	103	151	134,500
H	0.00	110	907	170	101	154	126,603
L	0.05	135	880	177	101	147	137,954
L	0.00	131	817	191	86	129	131,568
Source of variations		---------- *P-values* ----------					
Energy		0.2715	0.3622	0.4919	0.2368	0.1974	0.7307
β-Mannanase		0.2158	0.5895	0.9570	0.2633	0.4850	0.5612
Energy x β-Mannanase		0.4222	0.7456	0.3001	0.3931	0.3598	0.9506
SEM(44)[2]		5.11	35.17	5.84	3.55	5.36	5,960

[a,b]Means with different letter superscripts within a column are significantly different (P<.05).
[1]Values are means of 4 replicates each treatment.
[2]SEM = Standard error of the mean, with 44 degree of freedom

Table 9. Effect of dietary energy level and β-mannanase supplementation on Jejunum histologic morphology of turkey hens at 14 days of age[1].

Main effect of treatment		Tip Width	Villi Hight	Base width	Crypt	Muscularis Thickness	Surface Area
		---------- Micron ----------					Micron[2]
Energy level							
High energy (H)		163	1407	280	138	214	317,521
Low energy (L)		143	1420	238	138	228	273,328
β-Mannanase level (%)							
0.05		167	1323	270	133	216	298,400
0.00		140	1504	248	143	226	292,449
Interactions							
Energy level	*β-Mannanase level (%)*						
H	0.05	182	1334	304	135	211	332,840
H	0.00	145	1481	256	142	217	302,203
L	0.05	152	1312	236	132	223	252,059
L	0.00	135	1527	241	145	235	294,597
Source of variations		---------- *P-values* ----------					
Energy		0.2426	0.9160	0.2041	0.9647	0.2864	0.3251
β-Mannanase		0.1241	0.1425	0.5007	0.2490	0.5128	0.8924
Energy x β-Mannanase		0.5397	0.7718	0.4228	0.7141	0.8365	0.4123
SEM(44)[2]		8.10	57.70	15.53	4.02	6.68	21,536

[a,b]Means with different letter superscripts within a column are significantly different (P<.05).
[1]Values are means of 4 replicates each treatment.
[2]SEM = Standard error of the mean, with 44 degree of freedom

Table 10. Effect of dietary energy level and β-Mannanase supplementation on jejunum histologic morphology of turkey hens at 21 days of age[1].

Main effect of treatment		Tip Width	Villi Height	Base Width	Crypt Depth	Muscularis Thickness	Surface Area	Villi Height/ Crypt depth
		---------- Micron ----------					(Micron[2])	
Energy level								
High energy (H)		173	1661	301[b]	244	256	393,523[b]	6.19
Low energy (L)		207	1768	366[a]	222	266	496,505[a]	6.97
β-Mannanase level (%)								
0.05		219[a]	1714	367[a]	224	251	509,870[a]	7.49[a]
0.00		161[b]	1715	300[b]	242	269	380,157[b]	5.70[b]
Interactions								
Energy level	*β-Mannanase level (%)*							
H	0.05	204	1733[ab]	335	232	266	468,246	6.97[ab]
H	0.00	143	1588[b]	266	255	243	318,800	5.43[b]
L	0.05	235	1694[ab]	399	215	236	551,495	7.94[a]
L	0.00	179	1842[a]	334	228	296	441,514	5.92[ab]
Source of variations		---------- *P-values* ----------						
Energy		0.141	0.097	0.011	0.373	0.586	0.003	0.149
β-Mannanase		0.017	0.983	0.009	0.470	0.383	0.001	0.006
Energy x β-Mannanase		0.909	0.030	0.928	0.831	0.060	0.501	0.025
SEM(44)[2]		10	29	11	11	10	14,242	0.35

[a,b]Means with different letter superscripts within a column are significantly different (P<.05).
[1]Values are means of 4 replicates each treatment.
[2]SEM = Standard error of the mean, with 44 degree of freedom

Table 11. Effect of dietary energy level and β-Mannanase supplementation on ileal mucin secretion of turkey hens at 21 days of age[1].

Main effect of treatment		Ileal mucin secretion (μg/g tissue)
Energy level		
High energy (H)		752
Low energy (L)		804
β-Mannanase level		
0.05 %		804[b]
0.00 %		832[a]
Interaction		
Energy level	*β-Mannanase level (%)*	
H	0.05	631[b]
H	0.00	977[a]
L	0.05	791[ab]
L	0.00	873[ab]
Source of variations		---------- *P-values* ----------
Energy		0.1616
β-Mannanase		0.0466
Energy x β-Mannanase		0.0261
SEM (44)[2]		27.51

[a,b]Means with different letter superscripts within a column are significantly different (P<.05).
[1]Values are means of 4 replicates each treatment.
[2]SEM = Standard error of the mean, with 44 degree of freedom

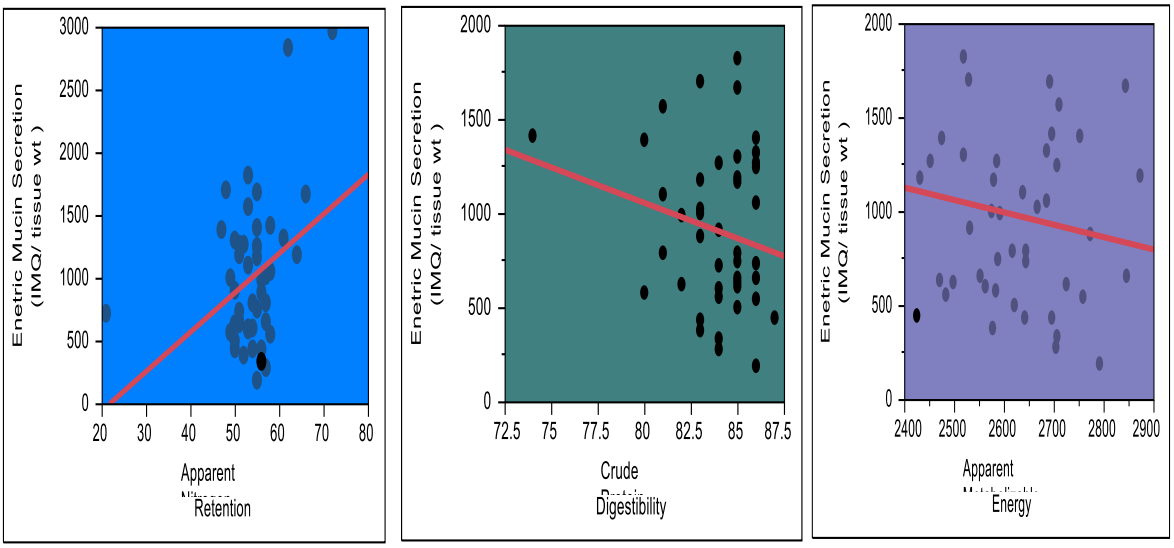

Figure 1. Correlations between enteric mucin secretion and apparent nitrogen retention (ANR), crude protein digestibility and apparent metabolizable energy (AMEn) respectively. Enteric mucin secretion had a positive and significant correlation with ANR (r^2=0.1; P<0.05). There was negative and slightly insignificant correlation between enteric mucin secretion and crude protein digestibility (r^2=0.03; P=0.07). The AMEn was negatively correlated with enteric mucin secretion but the relation was not significant (r^2=0.03; P=0.1284)

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CHAPTER FOUR

Effect of Phytase Source and Supplementation Level on Intestinal Mucin Secretion, Nutrient Utilization, and Growth Performance of Turkey Poults.

4.1 ABSTRACT.

It is well documented in several research reports that dietary supplementation of exogenous phytase enhances phytate-phosphorus availability in poultry, thus reducing the use of inorganic phosphorus supplementation. However, more research is needed to evaluate the efficacy of newer generation phytases, and how they affect nutrient digestibility and gut health at different dosages. This experiment was designed to evaluate effect of two different sources of exogenous phytase on enteric mucin secretion, nutrient digestion and growth performance of turkey poults. The treatments consisted of a positive control (PC), formulated to meet the NRC calcium (Ca) and phosphorus (P) requirements, and a negative control (NC) basal diet formulated with lower Ca and P concentration. The NC basal was divided into 7 equal parts, and each was supplemented with either phytase E or phytase ETB at the following phytase activity levels: 0 FTU/kg, 250 FTU/kg, 500 FTU/kg and 2000 FTU/kg. Phytase ETB was synthesized from a genetically modified version of *E.Coli* that synthesis phytase E. 432 1-day old female poults were randomly assigned to 48 cages at 9 poults per cage, and fed with respective experimental diets and water *ad libitum* for 28 d. Feed intake (FI) and body weight (BW) were recorded on 0 d, 7 d, 14 d, 21 d, and 28 d. Fecal excreta was collected from 22 d to 24 d to evaluate apparent nitrogen and energy utilization. On 28 d, ileal digesta was collected to determine nutrient digestibility, and the right leg for the tibia mineral content. Ileal mucin secretion was measured on 14 d and 28 d. Regardless of the source, dietary phytase inclusion at 2000 FTU resulted in the highest BW ($P<0.05$), and phytase ETB was most effective, especially at 28 d. Dietary treatment effect on FI was inconsistent. Poults fed the diet with 500

FTU Phytase E/kg and 2000 FTU Phytase ETB/kg inclusion had 12% greater 1-14 d FI than those fed the PC ($P<0.001$), with the other treatments having an intermediary effect. But by 28 d, 2000 FTU phytase ETB/kg resulted in the highest FI ($P<0.001$). In comparison to the PC, supplementation of 2000 FTU Phytase E/kg diet yielded 15% and 7% the lower 1-7 d and 1-14 d FCR, respectively ($P<0.05$). But by 28 d, both phytase E and ETB at 2000 FTU/kg diet were equally effective in yielding the lowest FCR, about 5 % lower than poults fed the NC basal diet ($P<0.05$). Similar responses on Ca and P digestibility were observed. Poults fed the NC diet had lower Ca and P digestibility than all other treatments ($P<0.05$). Regardless of the source and level of inclusion, phytase supplementation had similar responses on Ca and P digestibility, such that the enzymes were able to uplift the response of the NC to that of PC ($P<0.05$). PC and NC had the highest and lowest AMEn, respectively, while the phytase supplemented treatments had intermediary effects ($P<0.05$). There were no significant treatment effect on ileal crude protein digestibility, but phytase E at 250 FTU improved the ANR ($P<0.05$). The PC treatment resulted in the highest mucin secretion at 14 d, which was different from NC and the 250 and 500 FTU phytase treatments, irrespective of the source ($P<0.05$). Regardless of the source, the supplementation of the dietary exogenous phytase improved some nutrients digestibility and growth performance, but the lack of additional beneficial response to the higher dosages may be due to limited substrate. Furthermore, probably the higher dietary calcium complexed with phytate in the PC may have caused the anti-nutritional effect of increased mucin secretion

Key words Turkey poults, phytase, nutrient digestibility, mucin

4.2 INTRODUCTION.

The inclusion of feed enzymes in poultry diets to enhance nutrient utilization and improve animal performance has become common practice in the poultry industry within the last two decades. Following the discovery of the presence of phytate-phosphorus in animal feed by Lowe et al. (1939), several studies have reported the abundance of phytate-phosphorus in most poultry feed ingredients, especially corn and soybean meal (SBM), which are the major poultry feed ingredients in the world. Indeed, most plant based animal feed ingredients contain up to 60% of their total phosphorus as phytate-phosphorus (Harland and Morris, 1995). But the utilization of phytate-phosphorus in monogastric animals is limited because they lack the metabolic pathway to synthesize endogenous enzymes to effectively hydrolyze the phosphorus from the phytate (Ravindran et al, 1995; Bedford, 2000).

In order to satisfy the dietary phosphorus requirements of poultry, their diets have long been supplemented with phosphorus from non-plant sources, such as animal by-products and inorganic mineral sources like calcium phosphate. However, with the rising public demand for meat and egg products from poultry fed only vegetable based diets, the restricted use of animal by-products has eliminated this important available phosphorus source in poultry feeds. In addition, rising energy costs has greatly driven up the cost of manufacturing dicalcium phosphate, monocalcium phosphate, and deflourinated phosphate (Hughes et al., 2009), reducing their economic feasibility as alternative. Apart from the cost implication, excessive dietary phosphorus (from both phytate and non-phytate sources) can lead to environmental problems if poultry is not land applied at proper agronomic rates (Keller et al., 2012). Excess

land application of phosphorus from the manure may run off into surface water and contribute to the eutrophication and destabilization of aquatic ecosystems, and associated adverse environmental impacts (Ryden et al., 1973). This situation has promoted the acceptance and sales of dietary exogenous phytase enzymes as feed supplement, which has exceeded that of NSP degrading enzymes (Bedford, 2000).

However, dietary exogenous phytase exhibits different properties, depending on the source of the phytase gene and the synthesizing organism; they behave differently with respect to their in-vitro properties. For example, *Aspergillus spp* fungal phytases have optimal activity between pH 4.5 to 5.5 and temperature of 50 ^{o}C (Wyss et al., 1999; Simon and Igbasan 2002). In contrast, bacterial *E. coli* phytase has an optimum activity at pH <4.5 and temperature of 60 ^{o}C (Greiner et al., 1993; Golovan et al., 2000; Simon and Igbasan 2002), while *Bacillus* phytase has an optimum activity pH 7.0. *E. coli* phytase is the most thermal stable, and also insensitive to protein degrading enzymes, such as pepsin and pancreatin (Rodriguez et al. 1999; Igbasan et al., 2000; Simon and Igbasan 2002). These attributes are important to be put into consideration as newer phytase preparations are developed for future applications as feed additives.

There are some extra-phosphoric effect that has been associated with the dietary supplementation exogenous phytase. Some nutritionists place virtual nutrient matrix values on phytase feed enzymes for protein/amino acids and energy, in addition to calcium and phosphorus (Cowieson et al., 2004). This is because phytate is a polyanionic molecule, which can chelate di- and tri-valent cations (Wise, 1983), and thus can interact with dietary amino

acids/proteins and saccharides (Angel et al., 2002; Cowieson et al., 2004). The bioavailability of these bound nutrients may increase as the dietary supplementation of phytase increases (Cowieson et al., 2006). Furthermore, Cowieson et al. (2004) hypothesized that dietary supplementation of exogenous phytase may reduce the secretion of mucin into the intestinal lumen, thereby reducing endogenous nutrient loss, and improving apparent nutrient digestibility. However, this hypothesis has not been adequately tested and reported in the scientific literature.

Although almost all least-cost formulated commercial poultry feeds are now supplemented with phytase, more research is needed to evaluate the efficacy of newer generation phytases and how they affect nutrient digestibility and gut health at different dosages. Based on the available data, there is considerable opportunity for improvement of phytase activity, either by using higher dietary inclusion levels of phytase, different microbial source, or both (Selle and Ravindran, 2007). We hypothesized that increased dietary inclusion levels will increase the extra-phosphoric effects of dietary phytase supplementation in addition to better phosphorus digestibility. The objectives of this study was to assess the efficacy of two different phytase sources at dietary inclusions from 250 to 2000 FTU phytase/kg on intestinal mucin secretion, nutrient digestibility, bone mineralization, and growth performance.

4.3 MATERIAL AND METHODS.

4.3.1 Experimental Diets.

All experimental diets were formulated to satisfy the Nicholas turkey breed recommendations[30] for all nutrients except for Ca and P as corn-SBM-based diets. The positive control diet was formulated to meet the Ca and P requirements, and the negative control (NC) basal diet was formulated with lower Ca and P concentration (Table 2). The NC basal was divided into 7 equal parts, and each was supplemented with either phytase E or phytase ETB at the following phytase activity levels, 0 FTU[31], 250 FTU, 500 FTU and 2000 FTU/kg diet, respectively (Table 1). Phytase ETB is an improved version of phytase E, it was synthesized from genetically modified *E.Coli* that synthesis phytase E. Phytase ETB is more thermo stable than phytase E, which increases its ability to withstand the high temperature condition of feed production, especially during the pelleting process. The liquid phytase was added post-pelleting with a hand-held sprayer at an application rate of 2-5 micron droplets per second in a double ribbon mixer and mixed for 3 minutes. The enzyme activities in the respective dietary treatment feeds were confirmed by laboratory analysis[32] to be within 10% of target values. All experimental diets included 2.0% Celite[33] to serve as an indigestible marker to determine nutrient digestibility. The ingredients and nutritional composition of the experimental diets is

[30] http://www.aviagenturkeys.com/media/182972/revfeeding_gl_heavylines.pdf
[31] One FTU is defined as the amount of phytase required to hydrolyze one µmol of inorganic phosphorus per minute at pH 5.5, from an excess of 15 µM Na phytate at 37 °C (International Union of Biochemistry, 1979)
[32] Danisco laboratory Feed Enzyme Services, Brabrand, Denmark
[33] Celite™ Corp. Lompoc, CA

presented in Table 2. All diets were manufactured by pellet processing[34] and crumbled at the North Carolina State University Feed Mill Educational Unit (Raleigh, NC).

4.3.2 Bird Husbandry and Data Collection.

Four-hundred-thirty-two day-old Nicholas hen poults were obtained from a commercial hatchery[35], and randomly assigned to 48 cages at 9 poults per cage[36] with 6 replicate cages per treatment. Each bird was identified with a numbered neck-tag according to treatment group. The eight experimental diets were randomly assigned among 6 replicate cages (Table 1). The dietary treatments feed and water were made available *ad libitum* to the poults for 28 d. The birds were provided 23 h of light (L): 1 h of Darkness (D) from 1-7 d, and 14L: 10D from 8-28 d. Feed consumption and group weight per cage were recorded at 0 d, 7 d, 14 d, and 21 d. Individual bird weights and cage group feed consumption were determined on 28 d. Morality rate and cause of mortality were recorded daily, and weight was used to adjust the FCR.

4.3.3 Fecal Excreta Collection and Processing.

Excreta was collected over three 24 h intervals, and pooled to evaluate nutrient digestibility of the diets. Excreta was collected daily from 22 d to 24 d of age and equal portions were stored at -20^{o}C for further analysis. The frozen excreta were allowed to thaw for approximately 16 h at room temperature before further processing. For each pen,

[34] CPM (1 ton/hr) Pellet Mill, PM1112-2. California Pellet Mill, Crawfordsville, IN 47933. Conditioned at 135oF
[35] Prestage Farms Hatchery, Clinton, NC.
[36] Alternative Design cages, Alternative Design Manufacturing and Supply, Inc., Siloam Springs, AR.

approximately 200 g of representative samples free of feathers and other extraneous material, were collected daily for the 3 consecutive days and blended[37] with 100 mL of water to form a slurry. The slurry was then acidified to pH<5.4 with the addition of HCl[38] to prevent volatilization of nitrogen and then placed in pie-tins and dried overnight at 70 °C in a forced air convection oven. Once dried, the excreta was blended into a fine powder and placed in storage bags at -20 °C until further analysis. Ground excreta samples were ashed for approximately 16 h at 600 °C in a muffle furnace[39] (AOAC. 1995a), and analyzed for Ca and P content[40].

4.3.4 Ileal Digesta Collection and Processing.

From the evening of 27 d to about 4 a.m. on 28 d the room lights was turned off, and the birds had restricted access to feed for at least 6 h. After 4 am on 28 d, they were given *ad libitum* access to feed by turning the lights on. Thus, each bird had at least 4 h of *ad libitum* feed consumption before they were euthanized for ileal digesta sample collection. Ileal sections from Meckel's diverticulum to the cecal junction were dissected and contents collected in 200 mL containers. The ileal contents of the birds were pooled by cage, and stored at -20 °C until later analysis. Ileum content samples were defrosted for approximately 16 h at room temperature and processed the same way same as the fecal excreta, and analyzed for calcium and phosphorus content.

[37] Waring® Commercial Blender Model 31BL92, Waring Commercial, New Hartford, CT 06057
[38] 0.1N, Fisher Scientific, Fairlawn, NJ 07410
[39] Thermolyn, Model # F-A1730, Serial # 6483, Sybron Corp., Dubuque, IA.
[40] AES mineral analysis at NC State University, Dept. of Soil Science Analytical Service Laboratory

4.3.5. Tibia Collection for Tibia Ash, Ca, and P Determination.

Immediately after ileal digesta samples were collected from each bird, the entire right leg from 3 euthanized birds per cage was collected and stored in labeled sample bags at -20 °C for subsequent ash content determination of the tibia. The right legs were allowed to thaw for approximately 4 h, and then the tibia was dissected from the muscle, and dried in oven for 24 h. The dried bones were submerged in ether to remove fat for 72 h, and were then removed and dried for 24 h. Each tibia was broken in half, placed in individual crucibles and ashed for approximately 12 h at 600 °C in a muffle furnace (AOAC. 1995a). Ash content of each tibia were analyzed for calcium and phosphorus content.

4.3.6 Chemical Analyses.

Representative samples of each dried and grounded excreta and ileum were submitted for approved analysis of dry matter (AOAC. 1995b), crude protein (AOAC. 2006). The gross energy content was determined with an adiabatic oxygen bomb calorimeter[41]. Recovery of acid insoluble ash was determined according to the method described by Vogtmann et al. (1975). All these were done at the NCSU Nutrition Laboratory. The analysis of the nutritional composition (Table 2) of the treatment were confirmed at the NC Department of Agriculture and Consumer Services Food and Drug Protection Division Forage Laboratory (Raleigh, NC). The apparent metabolizable energy nitrogen corrected (AME_n) and apparent nitrogen retention

[41] IKA Calorimeter System C5000 Control, IKA® Werke Labortechnik, Staufen, Germany

(ANR) of the diets was calculated from the nitrogen content of the diets and of excreta according to Lammer et al. (2008) using the following equations:

$$AME_n = [(GE_{Diet} - (GE_{Excreta} \text{ X } AIA_{Diet} / AIA_{Excreta}) - (8.22 \text{ X } N_{Retained})]$$

$$N_{Retained} = N_{Diet} - (N_{Excreta} \text{ X } AIA_{Diet}) / AIA_{Excreta}))$$

Where: AME_n (Kcal/g) is the nitrogen corrected apparent metabolizable energy of the diet; GE_{Diet} and $GE_{Excreta}$ were the gross energy of diet and excreta respectively; AIA_{Diet} and $AIA_{Excreta}$ were the concentration of Celite recovered as acid insoluble ash in diet and excreta respectively; 8.22 (Kcal/g) is the energy value of uric acid; and $N_{Retained}$ (g/kg) is the nitrogen retained by bird per kilogram of diet consumed, and N_{Diet} and $N_{Excreta}$ (%) were the nitrogen content of diet and excreta respectively. All values used in this calculation were expressed as grams per kilogram of DM.

$$ANR\ (\%) = [100*(1-(AIA_{Diet} / AIA_{Excreta} * Nitrogen_{Excreta} / Nitrogen_{Diet}))]$$

Where: AIA_{Diet} and $AIA_{Excreta}$ were the concentration of Celite recovered as acid insoluble ash in diet and excreta respectively; $Nitrogen_{Diet}$ and $Nitrogen_{Excreta}$ were the nitrogen content in the dietary and excreta, respectively.

Ileal nutrient digestibility were calculated according to Dilger and Adeola, (2006), using the following equation:

$$Nutrient\ digestibility\ (\%) = 100-[(AIA_{Diet} / AIA_{Excreta})*(Nutrient_{Ileal} / Nutrient_{Diet})*100]$$

Where: Nutrient digestibility (%) is ileal nutrient digestibility expressed as a percentage; AIA_{Diet} and $AIA_{Excreta}$ are concentration of Celite recovered as acid insoluble ash in diet and excreta respectively; $Nutrient_{Ileal}$ and $Nutrient_{Diet}$ were the ileal and dietary nutrient content. All values used in this calculation were expressed as grams per kilogram of DM.

4.3.7 Histochemical Analyses: Measurement of Ileal Mucin Secretion.

The epithelial mucin quantity can be an indicator the health status of the gut. It is commonly assessed histochemically with alcian blue stain, based on the affinity of the basic stain for acidic tissue element like mucin (McManusi and Mowry, 1960; Evansee and Kent, 1962). The thickness of the ileal mucus adherent layer was estimated based on the modification of Corne's method (Corne et al., 1974; Kitagawa et al., 1986; Parman et al., 1993). One bird per cage was sampled on 14 d and 3 birds per cage was sampled on 28 d for ileal mucin secretion quantification. A 1-cm section of ileal tissue from each bird was sampled for histo-morphometic analysis. Another 1-cm section placed in 10 g Alcian blue dye /L of pH 5.8 buffered solution containing 160 mmol/L sucrose and 50 mmol/L sodium acetate. After 6 h of incubation, excess dye was be extracted with 250 mmol/L sucrose. The absorbed dye was extracted from the tissue by incubation in 10 g/L docusate sodium salt solution overnight at room temperature. Samples were centrifuged at 700 g, plated on a 96 well plate, and the optical density measured at 620 nm, using a blank Alcian Blue solution as a standard. The amount of absorbed dye is reported as µg Alcian blue/g of ileal tissue.

4.3.8 Statistical Analysis.

This experiment was analyzed as a completely randomized design. Cage means were used as the experimental unit. Data were analyzed using JMP[42] software. ANOVA was used to examine the main effect of dietary treatment factors, and their interaction on parameters evaluated. Means were separated using the LS Means at $P<0.05$. Orthogonal contrast was also used to compare treatment means, and the relationship between different inclusions levels for respective phytase source for each parameter evaluated were analyzed using regression models (JMP software).

4.3.9 Animal Ethics.

This experiment was conducted according to the guidelines of the Institutional Animal Care and Use Committee at North Carolina State University. All husbandry practices and euthanasia were performed with full consideration of animal welfare.

4.4 RESULTS.

4.4.1 Growth Performance.

The results of weekly growth performance are presented in tables 3, 4, 5, and 6. The dietary supplementation of phytase from the two different sources had some significant effects on body weight (BW) of the poults. Poults fed with the dietary supplementation of 2000 FTU phytase/kg diet had the heaviest BW throughout the experimental period, regardless of the

[42] Version 10. SAS Institute Cary, NC

source of phytase. On 21 d and 28 d of the experiment, feeding phytase E at 250 FTU, resulted in lighter BW and BWG, in comparison to the higher inclusion levels at older age ($P<0.05$; Table 5 and 6). Phytase ETB had better effect on BW and BWG, and was most effective at 2000 FTU. Orthogonal contrast analysis revealed that at various levels of dietary inclusion of both enzyme sources, the phytase ETB had the better effect on BW and BWG than their respective inclusion level of phytase E counterpart.

On 7 d, there was no significant treatment effect on feed intake (FI). On 14 d, poults fed the diet supplemented with phytase E at 500 FTU had 12% higher FI than those fed the PC diet, with others having intermediary effect on FI ($P<0.001$; Table 4). Conversely, 2000 FTU phytase ETB/kg diet resulted in the highest FI on 21 d and 28 d ($P<0.001$; Table 5 and 6). Some significant treatment effects were observed on the feed conversion efficiency. During the first two weeks (7 d and 14 d) of the experiment, relative to the PC treatment, poults fed 2000 FTU Phytase E/ kg supplemented diet had 15% and 7% reduction in FCR on 0-7 d and 0-14 d, respectively ($P<0.05$), but the other treatments had intermediary responses on FCR during these periods (Table 3 and Table 4). There was no treatment effect on 0-21 d FCR

($P>0.05$; Table 5), but dietary supplementation of 2000 FTU phytase E or phytase ETB/kg of diet resulted in the lowest 0-28 d FCR. In comparison to the NC treatment, both phytase sources supplemented at 2000 FTU/kg improved 0-28 d FCR by 5% ($P<0.01$; Table 6).

4.4.2 Nutrient Digestibility.

The dietary treatment effects on nutrient digestibility are presented in tables 7 to 10. There were no treatment effects observed on the dry matter and ash content of feces. However, there were significant treatment effects observed on Ca and P digestibility. Poults fed the NC diet had significantly lower Ca digestibility, which was 34.45% lower than the others treatment groups ($P<0.05$; Table 7). In contrast, the dietary inclusion of 250 FTU phytase ETB/kg diet increased Ca digestibility by about 75% in comparison to the NC ($P<0.05$; Table 7). The enzymes did not improved the Ca digestibility when compared with the PC.

Similar to calcium digestibility, the poults fed the NC retained significantly less P than those fed the other dietary treatments ($P<0.01$; Table 7). All dietary phytase inclusion levels resulted in about the same P digestibility, regardless of the source of phytase. Evidently, the all phytase treatments were able to uplift the P digestibility response of the NC to that of PC (Table 7). There was no significant treatment effect on the tibia ash, calcium and phosphorus content ($P>0.05$; Table 8).

The dietary treatment effects on ileal dry matter (DM) and crude protein digestibility (CPd) are presented in Table 9. The dietary inclusion of 250 FTU phytase ETB/kg resulted in highest ileal DM among all treatments. In contrast to the PC and NC treatments, 250 FTU phytase ETB/kg increased ileal DM content by approximately 5 points ($P<0.01$; Table 9). The other phytase treatments had intermediate effects on ileal DM content.

The results of the apparent metabolizable energy (AMEn) and apparent nitrogen retention (ANR) are presented in table 10. The PC and NC treatment groups had the highest and lowest AMEn, respectively ($P<0.01$; Table 10), while all the phytase treatments had intermediary effects. Supplementing the with 500 and 2000 FTU phytase ETB/kg NC basal diet resulted in significantly lower ANR than supplementing phytase E at 250 FTU/kg NC diet ($P<0.01$; Table 10), but neither were significantly different from the other treatments. There were no significant treatment effect on ileal crude protein digestibility.

4.4.2 Ileal Mucin Secretion.

The dietary treatment effects on enteric mucin secretion are presented in table 10. The PC treatment resulted in the highest ileal mucin secretion at 14 d, which was significantly different from NC, supplementing phytase E at 250 and 500 FTU, and supplementing phytase ETB at 250 and 500 FTU ($P<0.01$; Table 11). There was no treatment effect of ileal mucin secretion of 28 d.

4.5 DISSCUSSION.

Our experimental approach was to decrease Ca and P nutrient specifications appropriately in the NC diet, and counter potential reductions in growth performance with phytase supplementation, which has been described to be nutritionally and economically feasible (Selle et al., 2003). The dietary treatments had the same level of phytate-phosphorus of about 0.29%, and the level of Ca and available P was higher in the PC than in the NC and the corresponding phytase-supplemented diets. The objective of this study was to assess the efficacy of two

different phytase sources at different dietary inclusion levels on growth performance, bone mineralization, nutrient digestibility, and intestinal mucin secretion. Logically, the degree of responses to the exogenous phytase supplementation should be more noticeable with increasing levels of dietary phytase inclusion and, presumably, with greater degradation of phytate (Selle et al., 2003; 2007).

Growth performance is one of the indicators used to monitor the efficacy of phytase on P digestibility because of their sensitivity to P availability. Phosphorus is involved in a lot of metabolic activities, and its deficiency will definitely impact the growth performance (Potter, 1988). Dietary phytase supplementation improved the growth performance especially at the 2000 FTU/kg inclusion level, regardless of the phytase source. The positive effect of exogenous phytase enzyme on growth performance has been widely reported. The results from this study was substantiated earlier reports by Onyango et al. (2004) and Denbow et al. (2005). In a 22 d broiler study, Onyango et al. (2004) evaluated three different sources of exogenous phytase source. They observed that the weight gain, feed intake, and FCR were improved by phytase, irrespective of source. The explanation for the improvement in the growth parameter could be related to the different vital metabolic functions of the phosphorus in the body of an animal (Al-Masri, 1995). As aforementioned, phosphorus is an essential mineral for all animals. In the body, about 80% phosphorus is found in the skeletal structures, with the remaining 20% is widely distributed throughout the body in combination with proteins and fats or inform of inorganic salts (Bansal, 1990). Phosphorus is vital for a wide range of metabolic functions, especially being critical in energy and protein metabolism. Phosphorus deficiency

may cause decreased appetite smf increased FCR, leading to a metabolic syndrome known as "pica", in which the animals have a craving for unusual foods, such as wood or other materials. In this present experiment, the addition of the phytase enzyme from either of the two sources improved the feed intake and FCR, especially towards the end of the 28 d experiment, and the response was more pronounced at the higher dosages. Therefore, the improved growth performance observed with the inclusion of the phytase enzymes may likely be associated with to the improved phosphorus bioavailability.

Phytate is a poly-anionic molecule, and it can chelate cations such as calcium (Lonnerdal et al., 1999; Angel et al., 2002; Sandberg, 2002). Thus, in addition to reducing phosphorus digestibility, phytate can also impede the bioavailability of calcium. In this present experiment, the digestibility of calcium and phosphorus were improved by the dietary phytase inclusion, irrespective of the phytase sources. This observation agrees with the results from the broiler experiment reported by Onyango et al. (2004). They reported an increase in the digestibility of calcium and phosphorus after the dietary inclusion of phytase from three different sources. Similarly, Sebastian et al. (1996) and Viveros et al. (2002) also reported an improvement in calcium and phosphorus digestibility with the addition of exogenous phytase to broiler diets. Although, we did not see any significant difference between the two sources of the phytase, Onyango et al. (2004) observed a significant difference among the three sources of phytase evaluated in their broiler study.

In addition to chelating cations, reducing their bioavailability, and suppressing their functionalities as cofactors to enzymatic reactions in the energy and protein metabolism, the

phytic acid structure may also interact with amino acids and carbohydrates, thus reducing their bioavailability in poultry (Lonnerdal et al., 1999; Angel et al., 2002; Sandberg, 2002). As compared to the NC treatment, supplementation from either of the two different phytase sources improved the AMEn. Our results on AMEn validated some previous broiler studies results. After feeding a corn-SBM diet to broiler with phytase, Namkung et al. (1999) observed about 1% improvement in AMEn. Similarly, Ravindran and Bryden (1997) observed a 3% improvement in AMEn after supplementation of broiler corn-SBM diet with phytase. Newkirk and Classen (2001) also reported that AMEn was increased by about 28% in broiler chicks fed a maize-canola meal-SBM based diet supplemented with phytase from 1 to 18 d of age. Furthermore, Farrell et al. (1993) reported a 3% increase in AME after supplementation of broiler diet with phytase. In this current experiment, the AMEn was increased approximately 8% by supplementation of phytase when compared to the NC. The improved AME was also further substantiated by Farrell and Martin (1998), Ravindran et al. (1999), and Camden et al. (2001). The possible explanation for the improved energy metabolism could be because the phytase supplementation had a secondary effect on energy metabolism. The energy-dense inositol ring bounded by the phytate-phosphorus may be released for further metabolism and absorption after the enzyme hydrolysis (Ravindran et al., 2000). Furthermore, Cawley and Mitchell (1968) demonstrated that phytate is a potent inhibitor of α-amylase activity, resulting in reduced starch digestion. Although this impeding effect of phytate on starch digestion may be marginal (Selle and Ravindran, 2007), there are some reported instances of no improvement in AME with phytase supplementation (Onyango et al., 2004; Biehl and Barker, 1997). The

inconsistent response of phytase on AMEn reported in the published literature may be due to differences in diet composition, nutrient formulation constraints, and probably the nature and dosage of the enzyme itself.

In this present study, we also observed an improved apparent nitrogen retention with the phytase supplementation, irrespective of the source. Although Selle and Ravindran (2007) claimed there is no clear underlying mechanisms for improvement in protein digestibility after phytase supplementation, there are a few plausible hypotheses. Selle et al. (2003) reported that the formation of binary protein-phytate complexes in the GI tract, which are refractory to pepsin activity, may be the key mechanism whereby phytate depresses the digestibility of dietary amino acids. Since in the proventriculus and gizzard, the pH is lower than the protein isoelectric point, the negatively-charged phytate reacts with basic amino acids, such as lysine, histidine, and arginine, to form indigestible complexes (Cosgrove, 1966). Therefore, this complex formation most likely takes place in the bird's proventriculus-gizzard because of its highly acidic environment. However exogenous phytase supplementation has the highest enzymatic activity in the crop, which is before the proventriculus in the GI physiology, up to 86% of the enzyme activity is found in the crop, while about 30% is found in the proventiculous (Liebert et al., 1993; Simon and Igbasan, 2002). It follows that phytase partially prevents the formation of protein–phytate complexes by the prior hydrolysis of phytate (Selle and Ravindran, 2007). Another possible mechanism proposed by Ravindran et al. (1999) and later corroborated by Cowieson et al. (2004) is related to phytate's effect to enhance endogenous amino acids loss through the secretion of mucin. Munster et al. (1987) observed a possible

relationship between mucin secretion and pepsin in a rat experiment. They reported that increased pepsin secretion enhances enteric mucin secretion. This may be connected to the refractory action of phytate and protein on pepsin activity, which could lead to an excess of free pepsin in the proventriculus.

In the previous studies reported in this dissertation, we observed that, in addition to increasing nutrient digestibility, dietary supplementation of exogenous enzymes, such as β-mannanase and XAP, reduced endogenous nutrient losses by reducing mucin secretion. The mechanism behind the intestinal mucin reduction is linked to the activities of these enzymes on the dietary anti-nutritional factors, such non-starch polysaccharides, which may irritate the gut and lead to an increase in mucin secretion as a protective measure. The supplementation of turkey poults diets with dietary exogenous phytase reduced the excretion of mucin into the intestinal lumen (Cowieson et al., 2004). In this present experiment, the PC had the highest enteric mucin secretion. But regardless of the source and level, exogenous phytase and NC treatments had lower or similar levels of intestinal mucin. Apparently, the level of phytate-phosphorus was kept constant for all the dietary treatments, while the level of calcium was higher in the positive control (PC) diet. Perhaps it is the higher dietary calcium level complexed with phytate in the PC diet that was responsible for the higher intestinal mucin secretion.

There are limited studies on the role of calcium or the mechanisms by which it can affect the enteric mucin secretion in poultry. Studies with humans suggest that calcium plays an important role in mucin secretion. It was reported that calcium may enhance the formation of mucous "plugs" in glands and ducts by causing polymerization, aggregation, or gel

formation of otherwise normal mucin macromolecules. It has been postulated that the calcium ions bind to anions of goblet cell mucin before uptake by the enterocytes, thereby forming complexes with normal components of secreted mucus. These calcium-mucin complexes become insoluble or highly viscous mucus "plugs", causing ductal obstruction in various glands (Forstner and Forstner, 1975). Furthermore, the excess calcium may also bind to the phytate-protein complex, which may inhibit the action of pepsin, consequently leading to stimulation of mucin secretion by the free flowing pepsin.

In conclusion, we observed that phytase supplementation improved nutrient digestibility and some growth performance parameters, regardless of the source. There was no apparent additional beneficial effects observed by increasing the level of dietary supplementation of the exogenous phytases sources used in this study with turkey poults. This lack of phytase dose response may be due to the limited substrate (phytate-P) in the diet. The result of this present study provide some information on how dietary calcium level affects enteric mucin secretion. Based on our experimental data, dietary calcium may have a critical role on how phytate adversely affects nutrient retention by increasing endogenous losses via increased mucin secretion.

TABLES.

Table 1. Dietary treatment, enzyme identification, and incorporation rate.

Treatment	Description
1	Positive control
2	Negative control
3	Negative control + 250FTU Phytase E
4	Negative control + 500FTU Phytase E
5	Negative control + 2000FTU Phytase E
6	Negative control + 250FTU Phytase ETB
7	Negative control + 500FTU Phytase ETB
8	Negative control + 2000FTU PhytaseETB

Table 2. Dietary ingredient and nutritional compositions of the basal diets.

Ingredients	Positive Control (PC)	Negative Control (NC)
	----------%----------	
Corn	42.19	43.88
Soybean meal	49.14	48.93
Dicalcium phosphate	2.47	1.38
Limestone	1.13	1.31
Soy oil	1.38	0.81
DL-Methionine	0.33	0.32
Salt	0.37	0.38
Trace mineral premix[1]	0.20	0.20
Choline Chloride	0.20	0.20
Lysine	0.28	0.29
Vitamin premix[2]	0.15	0.15
Selenite premix[3]	0.05	0.05
Celite™	2.00	2.00
Phytase	0	0.01
Calculated analysis		
ME, KCAL/KG	2850	2850
Crude protein, %	27.24	27.26
Calcium, %	1.20	1.05
Total phosphorus, %	0.89	0.68
Avail. Phos.	0.60	0.40
Na+K-Cl, MEQ/KG	334.33	332.37
Chemical analysis[4]		
Dry matter, %	89.69	89.64
Crude protein, %	27.99	28.31
Gross energy, KCAL/KG	3784	3708
Ash, %	9.05	9.29
Calcium, %	1.54	1.45
Phosphorus, %	0.94	0.89

[1]Each kilogram of mineral premix (.1% inclusion) supplied the following per kg of complete feed: 60 mg Zn as $ZnSO_4 \cdot H_2O$; 60 mg Mn as $MnSO_4 \cdot H_2O$; 40 mg Fe as $FeSO_4 \cdot H_2O$; 5 mg Cu as $CuSO_4$; 1.25 mg I as $Ca(IO_3)_2$; 1 mg Co as $CoSO_4$.[2]Each kilogram of vitamin premix (.1% inclusion) supplied the following per kg of complete feed: vitamin A, 13,200 IU; cholecalciferol, 4,000 IU; alpha-tocopherol, 66 IU; niacin, 110 mg; pantothenic acid, 22 mg; riboflavin, 13.2 mg; pyridoxine, 8 mg; menadione, 4 mg; folic acid, 2.2 mg; thiamin, 4 mg; biotin, 0.253 mg; vitamin B_{12}, 0.04 mg; ethoxyquin, 100 mg. [3]$NaSeO_3$ premix provided 0.3 mg Se/kg of complete feed.

Table 3. Effect of phytase sources and levels on growth performance of female turkeys at 7 days of age[1].

Treatment		Body Weight (g)	Body Weight gain (g)	Feed Intake (g)	Adj FCR
		---------- 1-7 d ----------			
1	PC	128[b]	71[b]	103	1.46[b]
2	NC	134[ab]	78[ab]	106	1.35[ab]
3	NC+250 FTU (Phytase E)	137[ab]	80[ab]	110	1.40[ab]
4	NC+500 FTU (Phytase E)	139[ab]	81[ab]	115	1.41[ab]
5	NC+2000 FTU (Phytase E)	148[a]	91[a]	113	1.24[a]
6	NC+250 FTU (Phytase ETB)	139[ab]	82[ab]	112	1.36[ab]
7	NC+500 FTU (Phytase ETB)	139[ab]	83[ab]	113	1.37[ab]
8	NC+2000 FTU (Phytase ETB)	147[a]	90[a]	117	1.30[ab]
ANOVA (*P-value*)		0.005	0.010	0.065	0.010
Contrasts	1 vs 2	0.001	0.001	0.001	0.054
	2 vs 3 vs 6	0.167	0.262	0.199	0.635
	2 vs 4 vs 7	0.388	0.411	0.941	0.343
	2 vs 5 vs 8	0.012	0.014	0.642	0.003
	3 vs 6	0.613	0.723	0.966	0.001
	4 vs 7	0.923	0.996	0.135	0.199
	5 vs 8	0.001	0.001	0.063	0.001
Regression Analysis		---------- *P-value* ----------			
Phytase E	Linear	0.159	0.167	0.732	0.044
	Quadratic	0.362	0.393	0.513	0.035
Phytase ETB	Linear	0.024[3]	0.043[4]	0.046[5]	0.209
	Quadratic	0.077	0.125	0.062	0.358
SEM[2] (40)		3	1	1	0.041

[1]Values are means of 6 replicates pens containing 18 hens per pen.
[a-b] Value within different alphabet superscripts are significantly different
[2]SEM (40) = Standard error of the mean with 40 degrees of freedom.
[3]Y=0.135+5.96X
[4]Y=79.76+0.0054X
[5]Y=108.85+0.0004X

Table 4. Effect of phytase sources and levels on growth performance of female turkeys at 14 days of age[1].

Treatment		Body Weight	Body weight gain	Feed Intake	Feed Intake	Adj FCR
		1-14 d	7-14 d	1-14 d	7-14 d	1-14 d
		---------- g ----------				...g:g ...
1	PC	282[b]	158[c]	321[b]	217[b]	1.43[b]
2	NC	293[ab]	154[bc]	330[ab]	224[b]	1.40[ab]
3	NC+250 FTU (Phytase E)	297[ab]	165[abc]	331[ab]	227[ab]	1.36[a]
4	NC+500 FTU (Phytase E)	315[a]	177[a]	361[a]	246[a]	1.40[ab]
5	NC+2000 FTU (Phytase E)	320[a]	178[a]	342[ab]	233[ab]	1.33[a]
6	NC+250 FTU (Phytase ETB)	316[ab]	177[a]	358[a]	246[a]	1.38[ab]
7	NC+500 FTU (Phytase ETB)	314[a]	174[ab]	359[a]	246[a]	1.39[ab]
8	NC+2000 FTU (Phytase ETB)	322[a]	175[a]	365[a]	248[a]	1.37[ab]
ANOVA (*P-value*)		0.001	0.001	0.001	<0.001	0.025
Contrasts	1 vs 2	<0.001	<0.001	0.002	0.012	0.004
	2 vs 3 vs 6	0.168	0.310	0.111	0.117	0.386
	2 vs 4 vs 7	0.676	0.450	0.279	0.221	0.076
	2 vs 5 vs 8	0.473	0.412	0.929	0.731	0.044
	3 vs 6	0.968	0.273	0.818	0.819	0.137
	4 vs 7	0.044	0.003	0.008	0.002	0.147
	5 vs 8	0.020	0.189	0.144	0.110	0.007
Regression Analysis		---------- *P-value* ----------				
Phytase E	Linear	0.184	0.404	0.573	0.493	0.123
	Quadratic	0.102	0.019[1]	0.150	0.058	0.270
Phytase ETB	Linear	0.025	0.130	0.028	0.046	0.454
	Quadratic	0.011[3]	0.011[4]	0.004[5]	0.003[6]	0.755
SEM[2] (40)		3	4	3	5	0.017

[1]Values are means of 6 replicates pens containing 18 hens per pen.
[a-c] Value within different alphabet superscripts are significantly different
[2]SEM (40) = Standard error of the mean with 40 degrees of freedom.
[3]Y=157.31+0.046X-2.02x10-5X2
[4]Y=295.80+0.056X-2.15x10-5X2
[5]Y=161.02+0.043X-1.81x10-5X2
[6]Y=333.43+0.075X-3.006x10-5X2

Table 5. Effect of phytase sources and levels on growth performance of female turkeys at 21 days of age[1].

Treatment		Body Weight	Body weight gain	Feed Intake	Feed Intake	Adj FCR
		1-21 d	14-21 d	1-21 d	14-21 d	1-21 d
		---------- g ----------				....g:g….
1	PC	503[bc]	292[bc]	666[bc]	346[abc]	1.62
2	NC	501[c]	287[bc]	672[bc]	343[bc]	1.63
3	NC+250 FTU (Phytase E)	501[c]	279[c]	665[c]	334[c]	1.61
4	NC+500 FTU (Phytase E)	536[ab]	302[ab]	719[ab]	358[abc]	1.62
5	NC+2000 FTU (Phytase E)	552[a]	326[a]	697[abc]	354[abc]	1.58
6	NC+250 FTU (Phytase ETB)	541[ab]	308[ab]	720[ab]	362[ab]	1.60
7	NC+500 FTU (Phytase ETB)	541[ab]	310[ab]	701[abc]	354[abc]	1.59
8	NC+2000 FTU (Phytase ETB)	563[a]	331[a]	736[a]	371[a]	1.56
ANOVA (*P-value*)		<0.001	<0.001	0.001	0.003	0.158
Contrasts	1 vs 2	0.003	0.014	0.001	0.083	0.103
	2 vs 3 vs 6	0.002	0.001	0.030	0.020	0.148
	2 vs 4 vs 7	0.770	0.395	0.619	0.644	0.262
	2 vs 5 vs 8	0.216	0.038	0.573	0.199	0.235
	3 vs 6	0.230	0.036	0.426	0.187	0.710
	4 vs 7	0.040	0.324	0.028	0.407	1.000
	5 vs 8	0.001	<.001	0.024	0.008	0.012
Regression Analysis		---------- *P-value* ----------				
Phytase E	Linear	0.003	0.017	0.281	0.089	0.053
	Quadratic	0.016[3]	0.044[4]	0.113	0.111	0.160
Phytase ETB	Linear	0.001	0.002	0.010	0.022[8]	0.027
	Quadratic	0.004[5]	0.003[6]	0.007[7]	0.062	0.042[9]
SEM[2] (40)		9	7	13	6	0.018

[1]Values are means of 6 replicates pens containing 18 hens per pen.
[a-c] Value within different alphabet superscripts are significantly different
[2]SEM (40) = Standard error of the mean with 40 degrees of freedom.
[3]$Y=497.41+0.078X-3.014x10-5X^2$
[4]$Y=282.80+0.032X-9.66x10-6X^2$
[5]$Y=506.57+0.10X-3.60x10-5X^2$
[6]$Y=289.16+0.056X-1.79x10-5X^2$
[7]$Y=679.47+0.106X-3.88x10-5X^2$
[8]$Y=349.62+0.0113X$
[9]$Y=1.629+1.07x10-4+3.73x10-8X^2$

Table 6. Effect of phytase sources and levels on growth performance of female turkeys at 28 days of age[1].

Treatment		Body Weight	Body weight gain	Feed Intake	Feed Intake	Adj FCR
		1-28 d	21-28 d	1-28 d	21-28 d	1-28 d
		---------- g ----------				....g:g….
1	PC	866^{bc}	517^{ab}	1,262^{bc}	596^{ab}	1.61^{ab}
2	NC	852^{bc}	509^{b}	1,264^{bc}	592^{ab}	1.65^{b}
3	NC+250 FTU (Phytase E)	851^{c}	515^{ab}	1,234^{c}	569^{b}	1.62^{ab}
4	NC+500 FTU (Phytase E)	914^{ab}	554^{ab}	1,339^{ab}	620^{a}	1.61^{ab}
5	NC+2000 FTU (Phytase E)	938^{a}	554^{a}	1,299^{abc}	602^{ab}	1.57^{a}
6	NC+250 FTU (Phytase ETB)	912^{ab}	547^{ab}	1,332^{ab}	611^{ab}	1.60^{ab}
7	NC+500 FTU (Phytase ETB)	915^{ab}	548^{ab}	1,309^{abc}	596^{ab}	1.62^{ab}
8	NC+2000 FTU (Phytase ETB)	941^{a}	553^{a}	1,358^{a}	621^{a}	1.57a
ANOVA (*P-value*)		0.001	0.001	0.001	0.014	0.019
Contrasts	1 vs 2	0.002	0.001	0.064	0.259	0.019
	2 vs 3 vs 6	0.001	0.005	0.014	0.031	0.012
	2 vs 4 vs 7	0.822	0.794	0.606	0.690	0.011
	2 vs 5 vs 8	0.383	0.897	0.418	0.391	0.137
	3 vs 6	0.122	0.385	0.167	0.086	0.460
	4 vs 7	0.030	0.009	0.048	0.240	0.433
	5 vs 8	0.001	0.026	0.023	0.094	0.001
Regression Analysis		---------- *P-value* ----------				
Phytase E	Linear	0.012	0.026	0.273	0.341	0.017
	Quadratic	0.003[3]	0.002[4]	0.125	0.241	0.041[5]
Phytase ETB	Linear	0.005	0.064	0.010[8]	0.058	0.108
	Quadratic	0.002[6]	0.014[7]	0.019	0.173	0.076
SEM[2] (40)		14	9	2	1	0.016

[1]Values are means of 6 replicates pens containing 18 hens per pen.
[a-c] Value within different alphabet superscripts are significantly different
[2]SEM (40) = Standard error of the mean with 40 degrees of freedom.
[3]$Y=845.27+0.135X-5.19x10^{-5}X^2$
[4]$Y=506.30+0.10X-4.07-5X^2$
[5]$Y=1.64-7.65x10^{-5}X+2.39x10^{-8}X^2$
[6]$Y=859.10+0.160X-5.94x10^{-5}X^2$
[7]$Y=513.77+0.102X-4.13x10^{-5}X^2$
[8]$Y=597.30+0.0112X$

Table 7. Effect of two phytate sources and different inclusion levels on fecal nutrients digestibility of female turkey poults[1].

Treatment		DM Feces	Ash	Ca digestibility	P Digestibility
		----------%----------			
1	PC	23	16	48[b]	50[a]
2	NC	23	16	35[c]	40[b]
3	NC+250 FTU (Phytase E)	23	16	50[ab]	54[a]
4	NC+500 FTU (Phytase E)	22	15	55[ab]	51[a]
5	NC+2000 FTU (Phytase E)	22	16	56[ab]	57[a]
6	NC+250 FTU (Phytase ETB)	22	15	60[a]	52[a]
7	NC+500 FTU (Phytase ETB)	21	15	56[ab]	54[a]
8	NC+2000 FTU (Phytase ETB)	23	16	51[ab]	52[a]
ANOVA (*P-value*)		0.816	0.056	<0.001	<0.001
Contrasts	1 vs 2	0.393	0.985	<0.001	<0.001
	2 vs 3 vs 6	0.717	0.913	<0.001	0.010
	2 vs 4 vs 7	0.467	0.065	<0.001	0.008
	2 vs 5 vs 8	0.274	0.004	<0.001	<0.001
	3 vs 6	0.684	0.600	0.011	0.479
	4 vs 7	0.399	0.016	0.815	0.974
	5 vs 8	0.673	0.004	0.078	0.002
Regression Analysis		---------- *P-value* ----------			
Phytase E	Linear	0.718	0.457	0.004	0.016
	Quadratic	0.920	0.424	0.001[3]	0.053[4]
Phytase ETB	Linear	0.970	0.060	0.400	0.424
	Quadratic	0.774	0.007[5]	0.003[6]	0.062
SEM[2] (40)		1	1	5	2

[1]Values are means of 6 replicates pens containing 18 hens per pen.
[a-c] Value within different alphabet superscripts are significantly different
[2]SEM (40) = Standard error of the mean with 40 degrees of freedom.
[3]$Y=1.81-0.0011X+4.157x10^{-7}X^2$
[4]$Y=1.832-0.00084X+3.58x10^{-7}X^2$
[5]$Y=15.88-0.0019X+1.089x10^{-6}X^2$
[6]$Y=1.65-0.00136X+6.07x10^{-7}X^2$

Table 8. Effect of phytase sources and levels on tibia mineral composition of female turkey poult[1].

Treatment		Ash	Calcium	Phosphorus	Ca/P
		----------%----------			
1	PC	44	42	19	2.21[ab]
2	NC	43	41	18	2.17[c]
3	NC+250 FTU (Phytase E)	43	43	19	2.23[a]
4	NC+500 FTU (Phytase E)	43	41	18	2.20[abc]
5	NC+2000 FTU (Phytase E)	44	41	19	2.17[c]
6	NC+250 FTU (Phytase ETB)	43	41	18	2.18[bc]
7	NC+500 FTU (Phytase ETB)	43	40	18	2.19[abc]
8	NC+2000 FTU (Phytase ETB)	43	43	19	2.18[bc]
ANOVA (*P-value*)		0.251	0.296	0.423	0.040
Contrasts	1 vs 2	0.858	0.836	0.832	0.844
	2 vs 3 vs 6	0.101	0.793	0.693	0.755
	2 vs 4 vs 7	0.375	0.035	0.079	0.293
	2 vs 5 vs 8	0.354	0.416	0.120	0.005
	3 vs 6	0.240	0.535	0.887	0.077
	4 vs 7	0.740	0.042	0.098	0.815
	5 vs 8	0.090	0.217	0.042	0.072
Regression Analysis		---------- *P-value* ----------			
Phytase E	Linear	0.008	0.872	0.591	0.102
	Quadratic	0.024[3]	0.976	0.748	0.175
Phytase ETB	Linear	0.900	0.144	0.084	0.821
	Quadratic	0.776	0.335	0.206	0.886
SEM[2] (136)		1	1	2	0.013

[1]Values are means of 6 replicates pens containing 18 hens per pen.
[a-c] Value within different alphabet superscripts are significantly different
[2]SEM (136) = Standard error of the mean with 136 degrees of freedom.
$3Y=42.86-2.535x10^{-4}X+4.93x10^{-7}X^2$

Table 9. Effect of phytase sources and levels on ileal dry matter (DM) and crude protein digestibility female turkey poult[1].

Treatments		DM Ileum	CP Ileum
		----------%----------	
1	PC	18[b]	87
2	NC	17[b]	86
3	NC+250 FTU (Phytase E)	18[b]	87
4	NC+500 FTU (Phytase E)	19[ab]	87
5	NC+2000 FTU (Phytase E)	18[ab]	87
6	NC+250 FTU (Phytase ETB)	23[a]	86
7	NC+500 FTU (Phytase ETB)	18[ab]	87
8	NC+2000 FTU (Phytase ETB)	17[ab]	87
ANOVA (*P-value*)		0.003	0.873
Contrasts	1 vs 2	0.006	0.306
	2 vs 3 vs 6	0.957	0.279
	2 vs 4 vs 7	0.018	0.630
	2 vs 5 vs 8	0.944	0.377
	3 vs 6	0.055	0.963
	4 vs 7	0.395	0.485
	5 vs 8	0.083	0.800
Regression Analysis		---------- *P-value* ----------	
Phytase E	Linear	<0.001	0.115
	Quadratic	<0.001[3]	0.297
Phytase ETB	Linear	0.215	0.413
	Quadratic	0.260	0.316
SEM[2] (40)		1	5

[1]Values are means of 6 replicates pens containing 18 hens per pen.
[a-b] Value within different alphabet superscripts are significantly different
2SEM (40) = Standard error of the mean with 40 degrees of freedom.
3$Y=16.99+0.0043X-1.29x10^{-6}X^2$

Table 10. Effect of phytase sources and levels on apparent metabolizable energy corrected by nitrogen content (AMEn), and apparent nitrogen retention (ANR) female turkey poults[1].

Treatments		AMEn (Kcal/kg)	ANR (%)
1 PC		2421[a]	45[b]
2 NC		2238[c]	45[b]
3 NC+250 FTU (Phytase E)		2330[ab]	50[a]
4 NC+500 FTU (Phytase E)		2366[ab]	49[ab]
5 NC+2000 FTU (Phytase E)		2420[b]	48[ab]
6 NC+250 FTU (Phytase ETB)		2303[bc]	46[ab]
7 NC+500 FTU (Phytase ETB)		2324[b]	44[b]
8 NC+2000 FTU (Phytase ETB)		2328[b]	44[b]
ANOVA (*P-value*)		<0.001	0.002
Contrasts	1 vs 2	<0.001	0.047
	2 vs 3 vs 6	0.002	0.630
	2 vs 4 vs 7	<0.001	0.251
	2 vs 5 vs 8	<0.001	0.274
	3 vs 6	0.141	0.066
	4 vs 7	0.048	0.963
	5 vs 8	0.020	0.915
Regression Analysis		---------- *P-value* ----------	
Phytase E	Linear	0.8805	0.350
	Quadratic	0.5650	0.098
Phytase ETB	Linear	0.190	0.735
	Quadratic	0.384	0.923
SEM[2] (40)		15	1

[1]Values are means of 6 replicates pens containing 18 hens per pen.
[a-c] Value within different alphabet superscripts are significantly different
[2]SEM (40) = Standard error of the mean with 40 degrees of freedom.

Table 11. Effect of phytase sources and levels on enteric mucin secretion of female turkey poults[1].

Treatments		Ileal Mucin	
		----------µg/g of tissue ----------	
		-------14 d ------	-----28 d-----
1	PC	523[a]	131
2	NC	177[b]	227
3	NC+250 FTU (Phytase E)	206[b]	207
4	NC+500 FTU (Phytase E)	246[b]	183
5	NC+2000 FTU (Phytase E)	288[ab]	263
6	NC+250 FTU (Phytase ETB)	190[b]	240
7	NC+500 FTU (Phytase ETB)	229[b]	163
8	NC+2000 FTU (Phytase ETB)	355[ab]	204
ANOVA (*P-value*)		0.003	0.649
Contrasts	1 vs 2	0.001	0.342
	2 vs 3 vs 6	0.885	0.912
	2 vs 4 vs 7	0.505	0.751
	2 vs 5 vs 8	0.168	0.782
	3 vs 6	0.824	0.477
	4 vs 7	0.803	0.773
	5 vs 8	0.569	0.380
Regression Analysis		---------- *P-value* ----------	
Phytase E	Linear	0.881	0.350
	Quadratic	0.565	0.098
Phytase ETB	Linear	0.190	0.735
	Quadratic	0.384	0.923
SEM[2] (40)		52	51

[1]Values are means of 6 replicates pens containing 18 hens per pen.
[a-b] Value within different alphabet superscripts are significantly different
[2]SEM (40) = Standard error of the mean with 40 degrees of freedom.

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CHAPTER FIVE

Effect of dietary calcium, phytate and exogenous phytase supplementation on enteric mucin secretion, nitrogen utilization and growth performance in male broiler chick

5.1 ABSTRACT

Mucin is an enteric secretion that protects the epithelial mucosa lining against physical, pathogenic, or chemical irritation. Phytate is an antinutritional factor that may irritate the mucosa, and it has been shown to stimulate mucin secretion in poultry; whereas, dietary supplementation of exogenous phytase abates Phytate's antinutritional effect and may reduce enteric mucin secretion. Phytate can also reduce the bioavailability of calcium in the gut. Negatively charged mucin has strong an affinity to complex with cations like calcium, which may further stimulate enteric mucin secretion as a means of compensation. It has been observed that free calcium enhances mucin secretion in human, but there are limited published data on this subject in poultry. It was postulated that increasing the dietary calcium level will enhance enteric mucin secretion. In addition, calcium may enhance the formation of insoluble phytate-protein complexes, thus further increasing enteric mucin secretion. The objective of this trial was to evaluate the effects of calcium, phytate and dietary exogenous phytase on enteric mucin secretion, nitrogen utilization and growth performance. Dietary treatments, consisting of a 2 x 2 x 2 factorial arrangement of two levels of calcium (0.38% and 1.0%), two levels of phytate (0.22% and 0.44%), and two levels of exogenous phytase (0 FTU and 2000 FTU phytase/kg diet) were subjected to broiler chicks until 21 d of age. Four-hundred-eighty day-old male broiler chicks were distributed into 48 Petersime cages containing 10 birds, and six replicates per treatment. Weekly body weight (BW) and feed intake (FI) were measured at 7 d, 14 d, and 21 d. Enteric mucin secretion was determined at 14 and 21 d. Fecal excreta was collected on 18 d, 19 d, and 20 d to measure the apparent nitrogen retention (ANR), and ileal digesta was

collected on 21 d to assess the crude protein digestibility (CPd). BW on 14 d and 21 d increased as dietary calcium was increased. Increasing the phytate-phosphorus increased the FCR, while dietary supplementation of phytase reduced the FCR on 14 d and 21 d ($P<0.05$). Phytate-phosphorus and calcium enhanced the enteric mucin secretion at 14 and 21 d, but the addition of exogenous phytase enzyme reduced the secretion significantly ($P<0.05$). Although, the calcium level did not affect ANR, increasing the phytate-phosphorus reduced the ANR, while the addition of phytase enzyme increased the ANR ($P<0.05$). There was no effects of any of the dietary factors on the CPd ($P>0.05$). In addition to affirming that dietary phytase reduces enteric mucin secretion, this study also confirmed that excess calcium enhances enteric mucin secretion. Increased mucin secretion leads to increased endogenous loss of nutrients, specifically reduced ANR. The outcome of the study demonstrates why it is necessary to balance dietary calcium:phosphorus ratio especially when considering dietary phytate levels and use of supplemental phytase.

Key words Broilers, mucin, calcium, phytate and phytase

5.2 INTRODUCTION

Previous research from our laboratory revealed that the improvement in nutrient digestibility associated with dietary supplementation of exogenous dietary enzymes, such as phytase, β-mannanase and a blend of xylanase, amylase, and protease (XAP), may be due to reduced endogenous losses of nutrients from enteric mucin secretion Chapter 2, 3, and 4). This effect of exogenous enzymes on reducing intestinal mucin secretion is linked to the degrading action of the enzymes on their respective dietary anti-nutritional factors. The anti-nutritional factors, such as non-starch polysaccharides and phytate-phosphorus, may irritate the mucosal lining directly or indirectly by promoting the colonization of irritating microflora. The increase in mucin secretion is a defensive response to this mucosal irritation. In agreement with this hypothesis, Cowieson et al. (2003) reported that dietary supplementation of exogenous phytase reduced the excretion of mucin into the intestinal lumen of poultry in addition to increasing phosphorus digestibility.

Mucin is secreted by the gut goblet cells, and is a highly viscous poly-disperse macromolecule, negatively charged with an asymmetrical shape (Montagne et al., 2000). Enteric mucin forms a protective continuous layer over the epithelial surface, where it separates luminal contents from the underlying mucosa (Forstner and Forstner, 1975). This arrangement protects the epithelial layer against potentially detrimental luminal contents, such as microorganisms, physical and chemical abrasives, and digested feed substances. Intestinal mucin secretion helps to trap or sequester these potentially harmful enteric irritants, thereby preventing their translocation across the intestinal mucosa; but, this comes at a metabolic cost

to the animal (Antonioli et al., 1998; Montgomery et al., 1999; Zabielski et al., 2008). Intestinal mucin secretion leads to an increase in endogenous losses of nutrients (Angkanaporn et al. 1994; Lien et al., 1996; Montagne et al., 2000); therefore, more nutrients are used for gut tissue maintenance, leaving less nutrients available for growth and productive purposes (Cowieson et al., 2003; 2004).

Furthermore, the highly negative ionic charge of mucin enhances its interaction with cations, such as Calcium, Magnesium, Zinc, Copper, and basic amino acids. It has been reported that enteric mucin has a significant ion binding affinity property (Cowieson et al., 2007; Bryden et al., 2007). The ion binding characteristics enhances its ability to regulate the permeability and transport of some nutrients through the intestinal mucosa and into the epithelial cells. Because of mucin's highly negative charge, it may trap specific cations that are destined for absorption. Gibson et al. (1971) observed that mucin has the ability of binding iron and calcium, and this observation was substantiated by Bella and Kim (1973) and Forstner and Forstner (1975). In cystic fibrosis patients for example, there is an abnormally high calcium concentration in mucin secretions (Forstner and Forstner, 1975). Calcium forms complexes with normal components of secreted mucus, which become insoluble or highly viscous mucus (Lev and Spicer 1965; Blomfield et al., 1974).

In addition, calcium may enhance the formation of insoluble phytate-protein complexes (Cheryan, 1980, Reddy et al, 1982). The refractory response of the complex to digestion by pepsin leads to free pepsin in the gastrointestinal tract. The stimulus from the presence protein in the gut lumen keeps stimulating the secretion of pepsin. The animal then responds by

activating the innate immune system, secreting mucous to protect the enteric epithelial layer from the activity of the free flowing pepsin. As a logical consequence, high dietary calcium concentration along with phytate phosphorus in the gut luminal environment ultimately increases the amount of mucin secretion. In one of the previous studies (Chapter 4), we observed that there was an increase in the quantity of enteric mucin secretion as dietary calcium concentration increased. However, there are limited reports on the effect of dietary calcium concentration on enteric mucin secretion in poultry to substantiate our previous observation and proposed hypothesis.

The aim of this current study is to test our hypothesis that is synthesis of enteric mucin secretion is enhanced at high dietary calcium and phytate levels. The objective is to measure the effects of dietary calcium, phytate and exogenous phytase supplementation level on growth performance parameters, apparent nitrogen retention, ileal mucin secretion and crude protein digestibility, apparent nitrogen retention in broiler chicks.

5.3 MATERIALS AND METHODS

5.3.1 Experimental diet.

The experiment was designed as a 2 x 2 x 2 factorial arrangement of treatments, consisting of two dietary inclusion levels of phytate (0.22% and 0.44%), two dietary levels of calcium (0.38% and 1.00%), and two dietary inclusion levels of exogenous phytase[43] (0 and

[43] DSM Phytase, DSM Nutritional Products Ltd, Wurmisweg 576, CH-4303 Kaiseraugst, Switzerland.

2000 FTU[44]/kg). The higher phytate treatments were made by spraying commercial liquid phytate[45] over the respective diets during feed mixing. The dietary treatments were mash corn-SBM based diets. With the exception of the calcium and phytate, all experimental diets were formulated to meet or exceed nutrient requirements for Ross 708 broiler chicks[46]. All diets contained with 1.0 % titanium oxide for estimation of digestibility coefficients. The ingredients and nutritional composition of the experimental diets, including the supplemental enzymes, is reported in Table 2. All diets were produced in the feed mixing room of the Prestage Poultry Department of North Carolina State University (Raleigh, NC).

5.3.2 Bird husbandry and data collection

Ten chicks[47] per replicate group were randomly selected from the hatchery boxes, and weighed. Each bird was identified with a numbered neck-tag in sequence for each replicate cage group. The eight experimental treatments were randomly assigned among 48 Petersime cages (Table 1), and the diets and water were made available for *ad libitum* consumption. Birds were kept until 21 d of age. From 1 d to 7 d, the birds were provided 23 h of light and 1 h darkness per day, and 14L:10D after 7 d. On 7 d, all birds were weighed, and then cage population density was reduced to 4 chicks per cage by removing the outliers to achieve a similarly normal weight distribution among all replicate cages. On day 14 d and 21 d, 3 birds per cage (18 birds per treatment) were weighed and sampled to determine ileal mucin secretion.

[44] One FTU is defined as the amount of phytase required to hydrolyze one μmol of inorganic phosphorus per minute at pH 5.5, from an excess of 15 μM Na phytate at 37 °C (International Union of Biochemistry, 1979)
[45] Phytic acid solution, 50 % (w/w) in H2O. 593648 ALDRIC, Sigma-Aldrich.
[46] http://en.aviagen.com/assets/Tech_Center/Ross_PS/Ross708PS-Nutrition-Specs-2013FINAL.pdf
[47] Hatchery of Department of Poultry Science, North Carolina State University, Raleigh.

All sampled birds were weighed and recorded on pen sheets before culling. The same sampling procedure was repeated for the remaining bird on day 21 d. Weekly cage feed consumption, individual bird weight, and the number of birds per cage was recorded on days 0 d, 7 d, 14 d, and 21 d. Morality weights and the cause of mortality was recorded daily. The weight of each dead bird was used to correct the FCR.

5.3.3 Fecal excreta collection and analysis.

Excreta was collected over 24 h intervals, and pooled at 3 consecutive intervals to evaluate nutrient digestibility of the diets. Collections occurred from 18 d to 20 d of age and were stored at -20^{o} C until later analysis. The frozen excreta were later defrosted for approximately 16 hours at room temperature before further processing. For each pen, approximately 200 g of representative samples free of feathers and other extraneous material, were collected daily for the 3 consecutive days and blended[48] with 100 mL of water to form a slurry. The slurry was then acidified to pH<5.4 with the addition of HCl[49] to prevent volatilization of nitrogen and then placed in pie-tins and dried overnight at 70° C in a forced air convection oven. Once dried, the excreta was blended into a fine powder and placed in storage bags at -20 °C until further analysis.

[48] Waring® Commercial Blender Model 31BL92, Waring Commercial, New Hartford, CT 06057
[49] 0.1N, Fisher Scientific, Fairlawn, NJ 07410

5.3.4 Ileal digesta collection and analysis.

On the evening of day 20, before ileal sample collection, the birds had restricted access to feed for at least 6 hours by turning the room lights off. Then, starting at 4 am of day 21, they were given *ad libitum* access to feed. Thus, each bird had at least 4 hours of *ad libitum* feed consumption before they were euthanized for ileal digesta sample collection. We collected ileal digesta from 3 birds per cage. Briefly, Ileal sections from Meckel's diverticulum to the cecal junction were dissected and digesta contents collected in 200 mL container. The ileal contents of the birds were pooled by cage and stored at -20 °C until further analysis. Ileal samples were defrosted for approximately 16 hours at room temperature. The entire sample were weighed and dried overnight in a forced air oven.

5.3.5 Chemical and Histochemical analysis.

5.3.5.1 Chemical analyses.

Representative samples of each dried excreta and ileum were analysis for crude protein (AOAC, 2006) and the determination of apparent nitrogen retention. The recovery of the indigestible titanium oxide marker was determined according to the method described by Myers et al. (2004). All these were done at the NCSU Nutrition Laboratory. The apparent nitrogen retention (ANR) of the diets was calculated from the nitrogen content of the diets and of excreta according to Lammer et al. (2008), while the Ileal crude protein digestibility was calculated according to Dilger and Adeola, (2006), using the following equation:

$$ANR\ (\%) = (100*(1-(TiO_{2Diet} / TiO_{2Ileal} * Nitrogen_{Excreta} / Nitrogen_{Diet})))$$

Where: TiO_{Diet} and TiO_{Ileal} are concentration of TiO in diet and ileal digesta respectively; $Nitrogen_{Diet}$ and $Nitrogen_{Excreta}$ were the nitrogen content in the dietary and excreta respectively.

$$\text{Crude protein digestibility (\%)} = 100-[(TiO_{2Diet} / TiO_{2Ileal})*(\text{Crude protein}_{Ileal} / \text{Crude protein}_{Diet})*100]$$

Where: Crude protein digestibility (%) is ileal nutrient digestibility expressed as a percentage; TiO_{Diet} and TiO_{Ileal} are concentration of TiO_2 in diet and ileal digesta respectively; $\text{Crude protein}_{ileal}$ and $\text{Crude protein}_{Diet}$ were the ileal and dietary nutrient content. All values used in this calculation were expressed as grams per kilogram of DM.

5.3.5.2 Histochemical analyses- measurement of ileal mucin secretion.

The epithelial mucin is commonly assessed histochemically with alcian blue stain, based on the affinity of the basic stain for an acidic tissue element like mucin (McManusi and Mowry, 1960; Evansee and Kent, 1962). The thickness of the ileal mucus adherent layer was estimated based on the modification of Corne's method (Corne et al., 1974; Kitagawa et al., 1986; Parman et al., 1993). Enteric mucin secretion was estimated from 3 sampled birds per cage. A 1-cm section of ileal tissue from each sampled bird was removed and placed in 10 g/L Alcian blue dye solution in buffer containing 160 mmol/L sucrose and 50 mmol/L sodium acetate, pH 5.8. After 6 h of incubation, excess dye was extracted with 250 mmol/L sucrose. The absorbed dye was extracted from the tissue by incubation in 10 g/L docusate sodium salt

solution overnight at room temperature. Samples were centrifuged at 700 g, plated on 96 well plate and the optical density were measured at 620 nm using Alcian Blue solution as a standard. The amount of absorbed dye was reported as µg Alcian blue/g of ileal tissue.

5.3.6 Statistical analysis

This experiment was analyzed as a completely randomized design. Pen and cage means were respectively used as the experimental unit. Data were analyzed using JMP[50] software. ANOVA was used to examine the main effect of dietary treatment factors, and their interaction on parameters evaluated. Means were separated using the LS Means at $P<0.05$.

5.3.7 Animal ethics

This experiment was conducted according to the guidelines of the Institutional Animal Care and Use Committee at North Carolina State University. All husbandry practices and euthanasia were performed with full consideration of animal welfare.

5.4 RESULTS

5.4.1 Growth performance

The results of the growth performance from this experiment are presented in tables 3 and 4. Although increasing the level of phytate in the diet tended to reduce body weight gain (BWG) of the broiler chicks, there was no statistical significant dietary phytate level effect on BW at 7 d, 14 d and 21 d ($P>0.05$; Table 3). The dietary inclusion of calcium at the higher

[50] Version 10. SAS Institute Cary, NC.

level improved the BW and BWG significantly on 14 d and 21 d ($P<0.05$ Table 3), but dietary calcium inclusion did not affect the BW and BWG on 7 d. BW was increased by approximately 11% and 21% on 14 d and 21 d, respectively, while the BWG was improved by 24% and 25% on 14 d and 21 d, respectively. The supplementation of dietary exogenous phytase did not improved the BW and BWG significantly throughout the experiment ($P>0.05$; Table 3). Furthermore, there were no significant treatment interaction effects on BW and BWG throughout the experiment ($P>0.05$; Table 3).

The treatment effects on feed intake (FI) is presented in tables 4. There was no significant dietary phytate level effect on 1-7 d, 1-14d and 1-21d FI ($P>0.05$; Table 4). Moreover, increasing the dietary inclusion of calcium from 0.38% to 1.00% did not affect 1-7 d FI, but the increased dietary calcium level significantly improved 1-14d and 1-21d FI by 24% and 33%, respectively ($P<0.05$; Table 4). There was no significant main effect of dietary supplementation of exogenous phytase on FI throughout the experiment ($P>0.05$; Table 4). However, supplementing 2000 FTU phytase/kg of the high phytate diet improved the 1-21 FI ($P>0.05$; Table 4), although this treatment combination was not significantly different from the others.

The treatments effects on the FCR are presented in table 4. Dietary calcium inclusion level did not affect the FCR, but both phytate and exogenous dietary phytase supplementation affected the FCR significantly. Increasing the dietary phytate level to 0.44% increased 1-21 d FCR by approximately 7% ($P<0.05$; Table 4). In contrast, the dietary supplementation of

exogenous phytase reduced the 1-21 d FCR by approximately 6%. There were no significant treatment interaction effects observed on FCR (P>0.05; Table 4).

5.4.2 Enteric mucin secretion and nutrients digestibility

The treatment effects on the enteric mucin secretion and nutrient digestibility are presented in table 5. Increasing the level of phytate increased the enteric mucin secretion in the broiler chicks by 7% and 6% at 14 d and 21 d, respectively (P<0.05; Table 5). Increasing the dietary level of calcium resulted in increased enteric mucin secretion by approximately 47% and 34% at 14 d and 21 d, respectively (P<0.05; Table 5). In contrast, dietary exogenous phytase supplementation reduced the enteric mucin secretion by 6 % and 12 % at 14 d and 21 d, respectively, but the reductions were not statistically significant (P>0.05; Table 5). There were no significant treatment interaction effects observed on enteric mucin secretion at 14d and 21 d (P>0.05; Table 5).

The treatment effects on apparent nitrogen retention (ANR) and crude protein digestibility (CPd) are presented in table 5. As dietary phytate level was increased to 0.44%, ANR was reduced by approximately 20% (P>0.05), whereas dietary inclusion of phytase improved ANR by approximately 30% (P>0.05). Increasing the dietary calcium level did not affect the ANR (P>0.05). The addition of enzyme increased the ANR at both phytate levels. There were no significant treatment main or interactions effects observed on CPd (P>0.05; Table 5).

5.5 DISCUSSION

The objective of this study was to evaluate the effect of dietary levels of calcium, phytate, exogenous dietary phytase, and their treatment interactions on enteric mucin secretion in relation to nitrogen utilization and growth performance. The effects of dietary levels of calcium, phytate, and exogenous phytase supplementation on growth performance generally agree with what has been widely reported in the poultry science literature. Increasing the phytate level increased FCR, while the application of dietary exogenous phytase enzyme reduced FCR. Increasing the dietary level of calcium improved the BW. In agreement with our findings, Singh et al. (2003) reported that increasing the dietary phytate levels reduced the body weight gain and increased FCR of broilers. This decrease in body weight gain and increase FCR has been associated with dietary deficiency of non-phytate-phosphorus (Denbow et al., 1995). In contrast, the supplementation of phytase enzyme improves the body weight and reduces the FCR (Singh et al., 2003). Similar observations have been reported by several other researchers (Simons et al., 1990; Cabahug et al., 1999; Rama Rao et al., 1999; Lim et al., 2001; Zyla et al., 2001; Coweison and Adeola, 2005; Ravindran et al., 2006; and Liu et al., 2007).

Calcium plays an essential role in poultry nutrition, which includes bone formation and muscle contractions (Cransberg et al., 2001; Dalmagro, 2012). Calcium turnover determines the optimal calcium level for bone formation, and calcium deficiency will result in skeletal deformations and other mobility problems that affect animal welfare. Our observation of improved body weight as dietary calcium increased was also substantiated by the literature.

After evaluating different dietary calcium concentrations from 0.47 to 1.16%, Leytem et al. (2007) observed a linear relationship between dietary calcium and body weight gain in broilers. Letourneau et al. (2008) also reported better weight gain of broilers by increasing dietary calcium level to 0.9%. Dalmagro (2012) also reported that Heritage broilers showed optimum performance when provided 0.90-0.94% dietary calcium level during the starter growth period. Furthermore, Driver et al. (2006) reported that feeding broilers a diet containing 0.80% calcium improved the weight and quality of dressed carcasses. These recent broiler studies confirm that the NRC (1994) recommended dietary calcium concentration between 0.8 to 0.9% is still relevant for optimal broiler performance. Calcium has the highest concentration among all the mineral components of an adult poultry bone, and represents about of 1.5% of the body weight (Klasing, 1998). Thus, the improved body weight may be partly due to the increased deposition of calcium in the bone. Shivazad et al. (2005) had previously reported that increasing the dietary calcium level increases the bone ash content.

In addition to our observed effects of dietary calcium level on growth performance, we also observed some thought-provoking results on enteric mucin secretion. There is limited study on the effect of dietary calcium level on enteric mucin secretion in poultry. In this study, increasing dietary calcium concentration increased the secretion of enteric mucin secretion, validating the observation from our previous study. The underlying mechanism of how calcium may influence enteric mucin secretion in poultry is not clear. However, a human study report published by Forstner and Forstner (1975) concluded that cystic fibrosis patients, which secret an excessive amount of mucin, also have an abnormally high calcium concentration in the

glycoprotein secretions. This conclusion about calcium's association with the pathology of cyctic fibrosis was validated by Gibson et al. (1971) and Bella and Kim (1973). These researchers explained that calcium forms complexes with the negatively charged glycoprotein components of secreted mucus, which makes it insoluble or highly viscous. This condition is based on preferential ion binding affinity of mucin for free cations (Lev and Spicer, 1965; Blomfield et al., 1974). The level of binding affinity of mucin to free calcium depends on calcium's solubility in the gut. Once complexed with calcium into an insoluble and highly viscous mass, the functional role of mucin as a critical brush boarder barrier is apparently lost, and more mucin must be secreted as a compensatory response. Therefore, we hypothesize that the effect of dietary calcium on enteric mucin secretion depends on calcium's ionic state and solubility, which logically depends on the source of calcium. Further experiments may be required to confirm our hypothesis that mucin secretion responds differently to calcium from different sources.

There are some published reports on effect of phytate and phytase enzyme supplementation on endogenous secretion and loss, especially how they influence enteric mucin secretion dynamics (Cowieson et al., 2004ab, 2006). The underlying mechanism by which phytate influences mucin secretion may be related to the highly reactive nature of phytate, which has been reported to be pH dependent (Liu et al., 2007). In the low pH environment typically experienced at the proximal end (gastric region) of the GI tract of birds, there is strong electrostatic interaction between phytate and protein (Cosgrove, 1980; Anderson, 1985; Thompson, 1986). At low pH, the positively charged proteins, such as the

terminal alpha amino group, epsilon amino group of lysine, or guanidyl group of arginine and histidine residues, can form insoluble complexes with the negatively charged phytate (Barre and Nguyen van Huot, 1965ab; Cheryan, 1980; Reddy et al, 1982). This complex formation changes the protein conformation, thereby reducing its solubility, digestibility, and metabolic function. This phytate-protein complex impedes the solubilization of protein with HCl and its subsequent digestion by pepsin and (Vaintrub and Bulmaga, 1991), leading to an increase in secretion of these digestive components. In order to protect the GI tract from the increased production, there is a feedback mechanism downstream to increase in the protective secretion of mucin by GI epithelium. Just as reported by the researchers above, we observed that mucin secretion increased as dietary phytate level increased, and the dietary supplementation of exogenous phytase, which counters the anti-nutritional effect of phytate, reduced the secretion of enteric mucin.

Intestinal mucin secretion leads to an increase in endogenous losses of nutrients in poultry, hence partitioning more of the ingested nutrients towards maintenance requirements (Cowieson et al., 2004; Leterme et al., 1998). Mucin secretion is a contributor to endogenous loss because once mucin transverses to the distal end of the digestive tract, it becomes substrate for microbial fermentation. Much of its nitrogenous nutrient content is lost and becomes unavailable to the animal. As an obligatory body protein, mucin contains a high proportion of threonine, serine, and proline relative to muscle and other body tissues (Lien et al., 1997). For example, the essential amino acid threonine makes up to 11% of the amino acid backbone of the mucin structure molecule (Lien et al., 1997), so increases in mucin secretion

disproportionally taxes the availability of threonine for growth and maintenance of body tissue proteins. Consequently, excessive dietary phytate, which increases mucin secretion, will indirectly suppress nitrogen utilization efficiency and ANR. Dietary phytate's adverse effect on ANR may have an important economic impact, as it may be reflected in form of reduced meat yield of poultry.

In this study, the ANR was significantly improved by dietary supplementation of phytase, even though we did not observed significant direct improvement in protein digestibility. Apart from reducing endogenous nitrogen loss through mucin secretion, phytase can improve nitrogen utilization efficiency by impeding the formation of insoluble phytate-protein complexes. The dietary supplementation of phytase hydrolyses the phytate before the phytate-protein complex forms. Substantiating our results, Yi et al. (1996) observed the negative effects of phytate on nitrogen utilization was alleviated (ANR improved) by dietary phytase supplementation in turkeys. Similarly, Kemme et al. (1995) reported enhanced nitrogen utilization in swine by dietary phytase supplementation.

The outcome of this study provides another perspective into how enteric mucin secretion can be influenced by feed composition. In addition to affirming that dietary phytase reduces enteric mucin secretion, it also confirmed that excess soluble calcium enhances enteric mucin secretion. Increased enteric secretion of mucin increases endogenous nutrient loss, particularly expressed as reduced nitrogen retention and interpreted as reduced apparent nutrient digestibility. This study demonstrated the importance of balancing calcium and

phosphorus ratio when formulating poultry feed, especially with dietary supplementation of phytase.

5.6 TABLES AND FIGURES

Table 1. Dietary treatments group assignment to Broiler Chicks from 1 to 21 d of age.

Treatment (Diet)	Dietary Phytate Level	Dietary Calcium Level	Dietary Phytase Level
	----------%----------		FTU
A	0.44	1.00	0
B	0.44	1.00	2000
C	0.44	0.38	0
D	0.44	0.38	2000
E	0.22	1.00	0
F	0.22	1.00	2000
G	0.22	0.38	0
H	0.22	0.38	2000

Table 2. Dietary ingredient composition and nutrient content of starter diets fed to broiler chicks from 1 d to 21 days of age.

Ingredients	A	B	C	D	E	F	G	H
	% of Diet							
Corn	64.64	64.64	64.05	64.05	64.64	64.64	64.05	64.05
SBM	20.72	20.72	26.75	26.75	20.72	20.72	26.75	26.75
Poultry meal	8.00	8.00	3.74	3.74	8.00	8.00	3.74	3.74
Poultry fat	2.77	2.77	2.60	2.60	2.77	2.77	2.60	2.60
Limestone	1.39	1.39	0.00	0.00	1.39	1.39	0.00	0.00
Filler	0.27	0.28	0.27	0.28	0.49	0.50	0.49	0.50
Salt	0.38	0.38	0.43	0.43	0.38	0.38	0.43	0.43
Lysine	0.28	0.28	0.22	0.22	0.28	0.28	0.22	0.22
DiCalcium phosphate	0.25	0.25	0.64	0.64	0.25	0.25	0.64	0.64
DL Methionine	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Trace mineral premix[1]	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Choline chloride 60%	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Selenite premix[2]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Vitamin premix[3]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
L-Threonine	0.02	0.02	0.03	0.03	0.02	0.02	0.03	0.03
Phytic acid	0.22	0.22	0.22	0.22	0.00	0.00	0.00	0.00
Phytase	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.00
TiO_2	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Calculated Analysis								
ME Poultry, KCAL/KG	3200	3200	3200	3200	3200	3200	3200	3200
Crude protein, %	20.73	20.73	20.53	20.53	20.73	20.73	20.53	20.53
Crude fat, %	6.05	6.05	5.26	5.26	6.05	6.05	5.26	5.26
Calcium,%	0.95	0.95	0.38	0.38	0.95	0.95	0.38	0.38
Total phosphorus, %	0.74	0.74	0.74	0.74	0.52	0.52	0.54	0.54
Avail. Phos. Poultry	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Sodium, %	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Chloride, %	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35
Na+K-Cl,MEQ/KG	191	191	209	209	191	191	209	209
Arginine, %	1.34	1.34	1.33	1.33	1.34	1.34	1.33	1.33
Lysine, %	1.27	1.27	1.27	1.27	1.27	1.27	1.27	1.27
Methionine, %	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59
MET + CYS, %	0.91	0.91	0.91	0.91	0.91	0.91	0.91	0.91
Threonine, %	0.79	0.79	0.79	0.79	0.79	0.79	0.79	0.79

[1]Each kilogram of mineral premix (.1% inclusion) supplied the following per kg of complete feed: 60 mg Zn as $ZnSO_4 \cdot H_2O$; 60 mg Mn as $MnSO_4 \cdot H_2O$; 40 mg Fe as $FeSO_4 \cdot H_2O$; 5 mg Cu as $CuSO_4$; 1.25 mg I as $Ca(IO_3)_2$; 1 mg Co as $CoSO_4$. [2] $NaSeO_3$ premix provided 0.3 mg Se/kg of complete feed.[3]Each kilogram of vitamin premix (.1% inclusion) supplied the following per kg of complete feed: vitamin A, 13,200 IU; cholecalciferol, 4,000 IU; alpha-tocopherol, 66 IU; niacin, 110 mg; pantothenic acid, 22 mg; riboflavin, 13.2 mg; pyridoxine, 8 mg; menadione, 4 mg; folic acid, 2.2 mg; thiamin, 4 mg; biotin, 0.253 mg; vitamin B_{12}, 0.04 mg; ethoxyquin, 100 mg.

Table 3. Effect of phytate, calcium and phytase supplementation on body weight and body weight gain of broiler chick.[1]

Treatment	Age					
	7 d	14 d	21 d	1-7 d	8-14 d	15-21 d
	Body weight (g)			Body weight gain (g)		
Phytate (%)						
0.22	154	365	758	154	292	344
0.44	155	358	710	154	282	336
Calcium (%)						
0.38	153	344[b]	651[b]	152	256[b]	292[b]
1.00	155	380[a]	785[a]	155	318[a]	388[a]
Phytase (FTU)						
0	156	366	734	155	279	345
2000	152	358	731	152	295	335
[2]SEM(40)	2	6	12	2	6	11
Source of variations	*P-value*					
Phytate	NS[3]	NS	NS	NS	NS	NS
Calcium	NS	NS	NS	NS	<0.001	<0.001
Phytase	NS	NS	NS	NS	NS	NS
Phytate*Calcium	NS	NS	NS	NS	NS	NS
Phytate*Phytase	NS	NS	NS	NS	NS	NS
Calcium*Phytase	NS	NS	NS	NS	NS	NS
Phytate*Calcium*Phytase	NS	NS	NS	NS	NS	NS

[a,b] Means with different letter superscripts within a column are significantly different (P<0.05).
[1]Values are means of 6 replicates cages containing chicks per cage.
[2]SEM = Standard error of the mean with 40 degree of freedom.
[3]NS means no statistical difference

Table 4. Effect of phytate, calcium and phytase supplementation on feed intake and FCR of broiler chick.[1]

Treatment	Age					
	1-7 d	1-14 d	1-21 d	1-7 d	1-14 d	1-21 d
	Feed intake (g)			FCR		
Phytate (%)						
0.22	152	292	525	1.36	1.42	1.52[b]
0.44	153	282	541	1.38	1.41	1.63[a]
Calcium (%)						
0.38	153	256[b]	457[b]	1.40	1.39	1.57
1.00	152	318[a]	609[a]	1.35	1.44	1.57
Phytase (FTU)						
0	152	279	529	1.35	1.39	1.62[a]
2000	153	295	537	1.40	1.44	1.52[b]
SEM	2	8	14	0.02	0.02	0.03
Source of variations	*P-value*					
Phytate	NS[3]	NS	NS	NS	NS	0.02
Calcium	NS	<0.01	<0.01	NS	NS	NS
Phytase	NS	NS	NS	NS	NS	0.04
Phytate*Calcium	NS	NS	NS	NS	NS	NS
Phytate*Phytase	NS	NS	NS	NS	NS	NS
Calcium*Phytase	NS	NS	NS	NS	NS	NS
Phytate*Calcium*Phytase	NS	NS	NS	NS	NS	NS

[a,b]Means with different letter superscripts within a column are significantly different (P<0.05).
[1]Values are means of 6 replicates cages containing chicks per cage.
[2]SEM = Standard error of the mean with 40 degree of freedom.
[3]NS means no statistical difference

Table 5. Effect of phytate, calcium and phytase on ileal mucin secretion, apparent nitrogen retention and ileal protein digestibility of broiler chick[1].

Treatment	Ileal mucin secretion (µg/g tissue)		ANR	Ileal protein digestibility
	------14 d-----	----21 d----	----------%----------	
Phytate (%)				
0.22	504[b]	221[b]	40.67[a]	60.88
0.44	542[a]	236 [a]	32.23[b]	61.56
Calcium (%)				
0.38	423[b]	246[b]	35.43	61.27
1.00	623[a]	328[a]	37.47	61.16
Phytase (FTU)				
0	539[a]	272[a]	31.30[b]	61.79
2000	507[b]	238[b]	41.59[a]	60.64
[3]SEM (40)	41	30	2.15	1.89
Source of variations	---------- *P-value* ----------			
Phytate	0.03	0.04	<0.01	NS
Calcium	<0.01	0.04	NS	NS
Phytase	0.04	0.04	<0.01	NS
Phytate*Calcium	NS[4]	NS	NS	NS
Phytate*Phytase	NS	NS	NS	NS
Calcium*Phytase	NS	NS	NS	NS
Phytate*Calcium*Phytase	NS	NS	NS	NS

[a,b] Means with different letter superscripts within a column are significantly different (P<0.05).
[1]Values are means of 6 replicates cages containing chicks per cage.
[2]ANR= Apparent Nitrogen Retention from fecal sample collected from age 18 to 20 d
[3]SEM = Standard error of the mean with 40 degree of freedom.
[4]NS means no statistical difference

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CHAPTER SIX

Dissertation Summary and Conclusion

6.1 SUMMARY.

The poultry industry makes a great contribution to the economy of the United States and other parts of the world. For example, the US poultry production was valued at about $38 billion in 2012 (US Poultry, 2014), and it has grown by 8% between 2011 and 2012. Despite this aggressive market growth, the industry is unrelenting in its efforts to continually improve production efficiency, sustainability, and the safety and quality of poultry food products. One of the reasons credited for the poultry industry's market growth, was their ability to maximize the utilization of the feed ingredients through the dietary supplementation of exogenous enzymes. Before dietary enzymes supplementation became a common practice, anti-nutritional factors (ANF) in feed ingredients were not only less digestible, but they also reduced nutrient utilization and challenged the health and welfare of poultry.

Exogenous enzymes for two of the most common ANF in poultry feeds, NSP and phytate, was examined in this dissertation. Apart from reducing the metabolizable energy *via* increasing digesta viscosity, NSP entraps nutrients (Petersen et al., 1999; Santos, 2005). The undigested nutrients that escape assimilation in the foregut of poultry provides nutrients for the proliferation and fermentation of microflora in the hindgut (Bedford and Morgan, 1996). Enhanced by the anaerobic condition in the distal end of intestinal tract, excessive dietary NSP impeded foregut digestion of protein, fat, and starch, thus enriching the nutrient load in the hindgut ecosystem that favor the proliferation of pathogenic fermentative bacteria (Wagner and Thomas, 1987). Choct et al. (1996) observed increased fermentation in the hindgut of

broilers as the dietary inclusion of soluble NSP increased. The overall effects leads to changes in the gastrointestinal morphology, physiology and the ecosystem of the gut (Angkanaporn et al., 1994). In extreme cases, these changes may lead to the onset of severe enteric diseases, such as necrotic enteritis in poultry (Kaldhusdal and Hofshagen, 1992). To defend against the invasion of enteric pathogens, the bird responds by activating the innate immune system, stimulating the secretion of mucous to protect its epithelial lining. This involves secretion of mucin from the goblet cells, which functions to trap these pathogens within the web of mucous, preventing their translocation across the epithelial layer (Lien at el., 1996; Montagne et al., 2004). However, this enteric inflammation and mucin secretion comes at a cost of diverting nutrients away from productive purposes, resulting in reduced growth performance.

In addition, there are a lot of well documented reports on the effect of phytate on phosphorus and calcium utilization in poultry (Lonnerdal et al., 1999; Angel et al., 2002; Sandberg, 2002; Cowieson et al., 2004). One polyanionic phytate molecule can bind up six cations, such as calcium, zinc and copper, each with varying chelating affinity towards phytate (Maddaiah et al., 1964; Vohra et al., 1965). Therefore, in addition to reducing phosphorus digestibility, phytate can also depress the bioavailability of other minerals. Because calcium is a vital and the dominant cation in most poultry diets, preventing the *de novo* formation of the insoluble calcium–phytate complexes in the gut is important (Wise, 1983; Angel et al., 2002). Worst still, the formed insoluble calcium-phytate complexes are resistant to enzymatic hydrolysis by dietary exogenous phytase (Taylor, 1965), which further reduces the efficiency of exogenous phytase to degrade phytate (Hill and Taylor, 1954; Shirley and Edwards 2002;

Selle et al., 2009). Thus, appropriately balanced dietary calcium level and calcium:phosphorus ratio is vital for effective enzymatic degradation of phytate (Lei et al., 1994).

Phytate can also reduce apparent protein digestibility by stimulating enteric mucin secretion, and thereby enhancing endogenous nutrient loss (Cowieson et al., 2004). Phytate chelates protein, forming a binary protein–phytate complexes, and the process is enhanced by the acidic environment in the crop and proventriculus of monogastric animals (Cosgrove, 1966 and Anderson, 1985). Basic amino acids residues, such as arginine, histidine and lysine, are usually complexed by phytate (Rajendran and Prakash, 1993). The progressive insoluble complex formed stimulate a refractory response to pepsin activities during digestion of the protein (Selle et al., 2000). This is due to changes in the structural conformation and solubility of the protein (Vaintraub and Bulmaga, 1991). The inhibition of the protein digestion by pepsin is enhanced by the low pH condition of the proventiculous and stomach of poultry and pigs due to the increase in HCl secretion (Camus and Laporte, 1976, Knuckles et al., 1985 and Knuckles et al., 1989). A consequence of this increase in HCl and pepsin secretion is an increase in endogenous nutrients loss by the bird. This is because higher HCl and pepsinogen production has an irritant effect on the gut mucosa, which is compensated by an increased production of mucous as a protective layer. Phytate has been reported to enhance mucin secretion in rats (Munster et al., 1987). The mucus protects the mucosa from auto digestion by pepsin and from erosion by acids. Ravindran et al. (1999) and Cowieson et al. (2004) observed an increase in endogenous amino acid loss with the increase in phytate

concentration. Therefore both NSP and phytate encourages enteric mucin secretion and subsequent endogenous nutrient loss to the bird.

As aforementioned, supplementation of poultry diets with dietary exogenous enzyme has proven effectiveness in countering the adverse effects of ANF. In addition to improving nutrient utilization efficiency, there are some studies that report supplemental enzymes can also improve gut health by facilitating proper morphological development of the enteric mucosa and reduce enteric mucin secretion, thereby reducing endogenous nutrient loss. Therefore, this dissertation addresses the impact of dietary exogenous enzyme supplementation on gut health, nutrient utilization, and growth performance in poultry. Our working hypothesis is that, the supplementation of dietary exogenous enzyme will improve nutrient digestibility and gut health, through proper gut morphology development and reduction of enteric mucin secretion.

This dissertation reports five experiments that tests the hypothesis under different ANF and dietary exogenous enzyme supplementation scenarios. The first 3 of the experiments were designed to test our hypothesis using non-starch polysaccharide ANF and dietary carbohydrases enzymes. The first study was a 28 d battery-cage trial, and we evaluated the effect of DDGS inclusion levels (0, 6 %, and 12 %) in corn-SBM diets supplemented with endo-xylanase from *T. reesei*, alpha-amylase from *B. licheniformis*, and serine protease from *B. subtilis*, (XAP) and a combination of spores from three defined *B. subtilis* strains (DFM) on nutrient digestibility and growth performance in turkey poults. Weekly growth performance

and ileal nutrients digestibility were evaluated on 28 d. The second experiment was an 84 d follow-up growth study in litter floor pens. In this experiment, we evaluated the effect of DDGS inclusion levels of (6% and 18%) in corn-SBM diets supplemented with supplemental fat, XAP, a blend of XAP and DFM on gut health development and growth performance. Weekly growth performance indicators were measured, and the effect on gut morphological development and enteric mucin secretion were evaluated on 21 and 42 d. As dietary DDGS level increased, weekly BW decreased and FCR increased linearly, and digestibility of phosphorus, crude fat, crude protein decreased ($P<0.05$). Dietary supplementation of XAP+DFM had no effect on BW and FCR, but significantly reduced fecal moisture by 4.2% ($P<0.05$). XAP+DFM significantly increased ileal digestibility of CF and CP ($P<0.05$), but had no effect on AMEn or ANR. Higher dietary inclusion of DDGS increased ileal mucin secretion and distressed gut mucosa villi morphology. Dietary supplement of XAP reduced the intestinal mucin secretion.

Based on the outcomes of the first set of experiments, we conducted the third study using another carbohydrases enzyme that degrades β-mannans as the predominant dietary NSP. The experiment was designed as a 2 x 2 factorial arrangement of two dietary inclusion levels of β-mannanase at (0% and 0.05%), and two dietary levels metabolizable energy (High and Low), that differed by 150 kcal ME/kg. The dietary treatments were corn-SBM based diets. We evaluated the effect on weekly growth performance, nutrient digestibility, and ileal mucin secretion at 28 d. The treatment effect on mucosal morphological development was done at 7, 14 and 21 d. Cumulative feed intake (1-21 d) and periodic feed intake (7-14 d) was significantly

increased among birds fed the high energy diet, and 14d and 21 day body weights increased accordingly ($P<0.05$) without significant effect on FCR. Fat digestibility and AMEn was also enhanced by the high energy diet. Although β-mannanase supplementation did not have a significant effect on the growth performance, it did increase the apparent nitrogen retention and fecal dry matter content. On 21 d, β-mannanase supplementation was observed to have a positive effect on jejunum mucosal morphology: β-mannanase supplementation increased villi surface area, basal, tip width and height/crypt depth ratio. In contrast, increasing dietary energy just improved villi surface area ($P<0.05$). Dietary supplementation β-mannanase reduced enteric mucin secretion, and the supplementation of high energy diet with β-mannanase had the lowest ileal mucin secretion ($P<0.05$). Covariance and correlation analysis revealed a positive correlation between the enteric mucin secretion and the ANR ($r^2= 0.1$, $P<0.05$).

To substantiate the hypothesis and to draw clearer conclusions, a non-carbohydrases enzyme and non-NSP ANF were evaluated in the last two experiments. The objectives of the fourth trial was to assess the efficacy of two different phytase sources when supplemented up to 2000 FTU/kg on intestinal mucin secretion, gut morphology, nutrient digestibility, bone mineralization and growth performance. We utilized Phytase E and Phytase ETB. Phytase ETB is an improved version of phytase E, which was synthesized from genetically modified *E.Coli* that synthesized phytase E. Phytase ETB is more thermo stable than phytase E, which increases its ability to withstand the high temperature condition of feed production, especially during the pelleting process. This was a 28 d battery-cage trial, and we evaluated the ileal nutrients digestibility, bone mineralization on 28 d. The effect on enteric mucin secretion was

carried out on 14 and 28 d. Regardless of the source, dietary phytase inclusion at 2000 FTU resulted in the highest BW ($P<0.05$), and phytase ETB was most effective. Dietary treatment effect on FI was inconsistent. Poults fed the diet with 500 FTU Phytase E/kg inclusion had 12% greater 1-14 d FI than those fed the PC (361 g *vs.* 321 g; $p<0.05$), with other treatments having an intermediary effect. But by 28 d, 2000 FTU phytase ETB/kg resulted in the highest FI ($P<0.05$). In comparison to the PC, supplementation of 2000 FTU Phytase E/kg diet yielded 15% and 7% the lower 1-7 d and 1-14 d FCR, respectively ($P<0.05$). But by 28 d, both phytase E and ETB at 2000 FTU/kg diet were equally effective in yielding the lowest FCR, about 5% lower than poults fed the NC basal diet ($P<0.01$). Similar responses on Ca and P digestibility were measured. Poults fed the NC diet had lower Ca and P digestibility than all other treatments ($P<0.05$). Regardless of the source and level of inclusion, the phytase supplementation had similar responses on Ca and P digestibility, such that the enzymes were able to uplift the response of the NC to that of PC ($P<0.05$). PC and NC had the highest and lowest AMEn, respectively, while the phytase supplemented treatments had intermediary effects ($P<0.01$). There was no significant treatment effect on ileal crude protein digestibility, but phytase E at 250 FTU improved the ANR ($P<0.01$). The PC treatment resulted in the highest mucin secretion at 14 d, which was different from NC and the 250 and 500 FTU phytase treatments, irrespective of the source ($P<0.01$).

The interesting result we obtained lead to the fifth experiment. In the 21 d cage study, we examined the effect of dietary calcium, phytate and phytase inclusion level on intestinal mucin secretion, nitrogen utilization efficiency and weekly growth performance in broiler

chicks. The effect on the enteric mucin was determined at 14 and 21 d, while the efficiency of the nitrogen utilization was evaluated at 21 d. Increasing dietary calcium level improved the BW at 14 and 21 d. Increasing the phytate-phosphorus increased the FCR, while supplementation of dietary phytase reduced the 1-14 d and 1-21 d FCR ($P<0.05$). Phytate-phosphorus and calcium enhanced the enteric mucin secretion at 14 and 21 d, but the addition of exogenous phytase enzyme reduced the secretion significantly ($P<0.05$). Although the calcium level did not affect the ANR, increasing the phytate-phosphorus reduced the ANR, while the addition of phytase enzyme increased the ANR ($P<0.05$). There was no effects of any of the dietary factors on the crude protein digestibility ($P>0.05$).

Based on the result from these studies, we concluded that ANF, such as NSPs and phytate, impairs the apparent nutrient digestibility and growth performance by suppressing enteric mucosa morphological development, and increasing endogenous loss of nutrients *via* enteric mucin secretion. In contrast, dietary supplementation of exogenous enzymes helps alleviate the adverse effects of ANF on nutrient utilization by directly or indirectly removing the mucosal irritation that stimulates enteric mucin secretion (Figure 1). Excess dietary calcium also enhances enteric mucin secretion as a compensatory response to the formation of insoluble viscous mucoid complexes, which further increases endogenous loss of nutrients and adversely affects apparent nutrient digestibility. In conclusion, enteric mucin secretion should be considered as a critical component in understanding the mechanisms of action of various feed additives that claim to improve the efficiency of nutrient utilization.

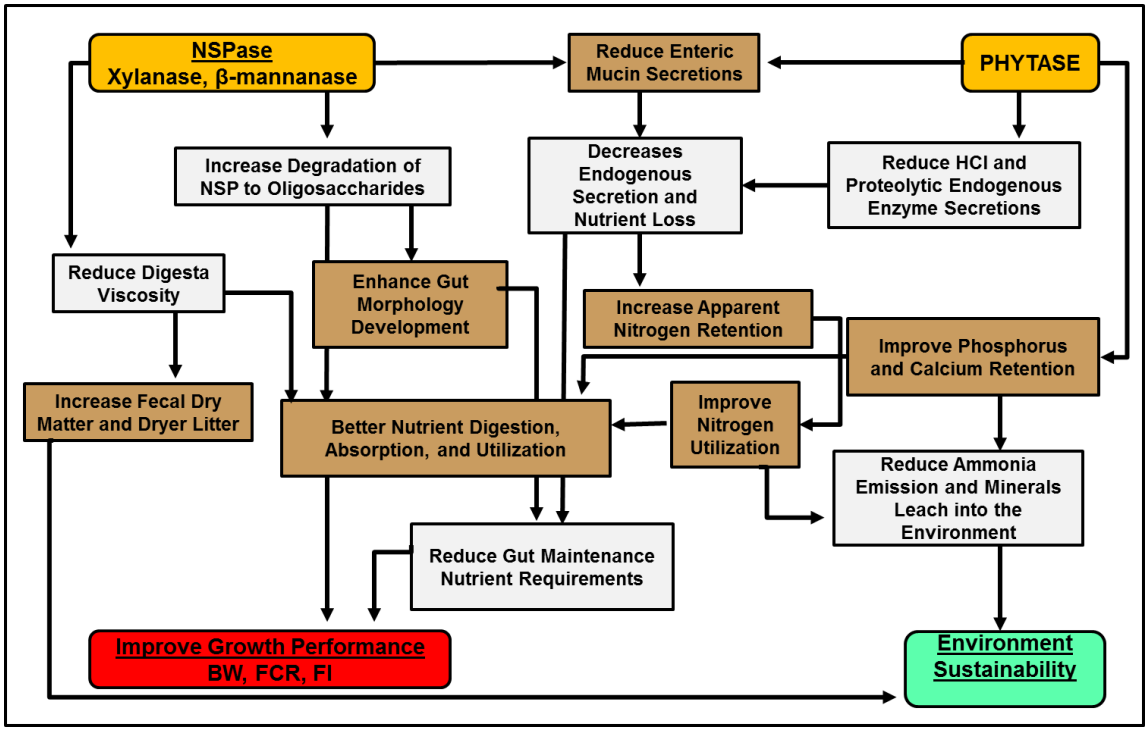

Figure 1. Schematic illustration of experiments results and summary. The yellow boxes depicts the dietary exogenous enzymes tested, the brown boxes shows the effects of the tested enzymes on the evaluated parameters. The gray boxes illustrated the extrapolated relationship between the parameters evaluated in the experiments, based on information available in the literature. The effect on the growth performance is show in the red box, while how exogenous enzyme can influence the sustainability of the environment is depicted in the green box.

6.2. CONCLUSION

Based on the results from the studies from the five experiments carried out to test the validity of our hypothesis, we arrived at the following listed conclusions.

1. Especially at high level, the dietary inclusion of DDGS in turkey diet may leads to reduce growth performance. The nutritional value of DDGS cannot be improved by a blend of XAP or XAP+DFM.

2. Turkey fed high dietary inclusion of DDGS had poorer jejunum villi morphological development, and the high NSP content in DDGS was associated with the increased ileal mucin secretion, and villi epithelial turnover. Although dietary supplementation of XAP and a combination of XAP+DFM had no effect on the BW, it improved nutrient digestibility, gut morphological characteristics, and presumably reduces endogenous nutrient loss by reducing ileal mucin secretion.

3. Increasing the energy level of crumbled diet correlates with an improved the growth performance, whereas the addition of β-mannanase did not have any significant effect of on the growth performance. But it is apparent that the supplementation of diet with β-mannanase was associated with better villi morphological characteristics, crude protein and fat digestibility, ANR, and the

addition of this enzyme presumably diet lowered the endogenous nutrients loss by lowering ileal mucin secretion.

4. Regardless of the source, dietary phytase supplementation was associated with an improved phosphorus and calcium digestibility, and some growth performance parameters. Although, there were no apparent additional beneficial effects at high dosage, which may be due to limited substrate (phytate) in our experimental set up. The positive control diet had highest enteric mucin secretion, which was probably due to the higher calcium concentration compared to the other dietary treatments.

5. Both dietary calcium and phytate enhanced the enteric mucin secretion, but the addition of exogenous phytase reduced the secretion. Although, dietary calcium level did not affect the ANR, increasing the phytate reduced the ANR. However, the addition of phytase enzyme increased the ANR, which was probably due to the reduced endogenous nutrients loss, especially amino acid loss through the ileal mucin secretion.

6. Overall, the outcome of the studies presented in this dissertation substantiate the earlier evidence that NSP and phytate probably increase endogenous nutrient loss by increasing enteric mucin secretion. In contrast, dietary exogenous enzyme counters these adverse ANF by improving nutrients

utilization, and reduced ileal mucin secretion. These results also provided another perspective into how enteric mucin secretion can be influenced by feed composition. It demonstrated that excess dietary calcium may enhances enteric mucin secretion, and perhaps further contributing to increased endogenous nutrient loss, and subsequently reducing dietary nutrient utilization. It underlined another perspective, why there is a need to balance calcium and phosphorus ratio when formulating poultry feed.

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