Evaluation of Extra-Phosphoric Effects of Phytase on Growth Performance, Carcass Characteristics, Phytate Degradation, Plasma Inositol Concentrations, Gene Expression of Hypothalamic Appetite Hormones, and Catecholamine Concentrations in Broiler Production

by

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ABSTRACT

Phytase is an enzyme responsible for the hydrolysis of phytate at the carbon-phosphate linkage. Commonly, phytase is added in poultry diets at 500 to 1,000 phytase units (**FTU**)/kg to liberate phosphorus from phytate. However, when adding phytase for phosphorus liberation, previous research viewed the degradation of IP6 as the end goal of phytase supplementation. Unfortunately, lower phytate esters may also have a similar capacity to chelate nutrients, which causes these nutrients to be unavailable to broilers. Increasing phytase dose may be an alternative to rapidly hydrolyze phytate with lower number of phosphate groups. The benefits of adding phytase in a high dose (beyond 1,500 FTU/kg) to broiler diets have been realized through the improvement in growth performance beyond phosphorus liberation. While these benefits have mainly been attributed to the enhancement of nutrient utilization, other contributors such as liberation of inositol, degradation of lower phytate esters, and feed intake stimulation may also warrant investigation as participants of extra-phosphoric effects of phytase. Five experiments were conducted to evaluate the effects of phytase beyond phosphorus liberation in broilers. Experiment 1 was conducted to determine the influence of phytase supplementation on plasma inositol concentrations for its use as a biomarker for phytase efficacy. Experiments 2 and 3 were designed to evaluate the extent of phytate degradation and inositol liberation through high phytase doses (0, 500, 1,500, 4,500, 13,500, and 40,500 FTU/kg). Experiments 4 and 5 aimed to determine if the benefits of extra-phosphoric effects of phytase specifically originate from the increased nutrient intake. In Experiment 1, despite observing phytate degradation and inositol liberation in the gastrointestinal tract of broilers, plasma inositol concentration of broilers was not altered

($P > 0.05$) by phytase supplementation. Phytase inclusion up to 40,500 FTU/kg in Experiments 2 and 3 removed 98% of all phosphate groups of phytate and increased inositol liberation, which resulted in enhanced ($P < 0.05$) growth performance and carcass characteristics of broilers. In Experiment 4, the resulting higher amino acid digestibility and AME_n due to phytase addition was used to calculate nutrient uplifts in Experiment 5. In Experiment 5, additive effects of phytase and nutrient uplifts were observed ($P < 0.05$) on BW gain, FCR, and carcass characteristics. The effects of phytase supplementation on increasing feed intake of broilers approached significance ($P = 0.06$) compared with broilers fed diets without phytase inclusion. Interestingly, hypothalamic dopamine concentration was greater ($P < 0.05$) in broilers provided diets with phytase addition compared with those receiving diets without phytase. Nevertheless, hypothalamic gene expression of appetite hormones was not influenced by phytase and/or nutrient uplifts. Results of this research provide evidence on the extra-phosphoric effects of phytase on phytate degradation, inositol liberation, and feed intake. However, the mechanism of phytase supplementation on feed intake stimulation remains inconclusive, which warrants future research.

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I. INTRODUCTION

Phosphorus is an essential nutrient for poultry due to its role in many biological functions, such as energy storage, nucleic acid formation, protein synthesis, skeletal tissue development, and eggshell formation (Summers, 1997). In broiler diets, most of the phosphorus is supplied using inorganic phosphorus, while the remaining dietary phosphorus can be supplied by plant or animal sources. However, supply of dietary phosphorus from inorganic sources may be limited as phosphate rock reserves are depleting. Moreover, a rising demand to feed chickens without animal sources, such as meat and bone meal and poultry by-product meal, also prompts researchers to further evaluate plant origin phosphorus as an alternative ingredient. Feed ingredients from vegetable origins commonly store phosphorus in the form of phytate (Bohn et al., 2008). Nonetheless, 60 to 70% of this phytate phosphorus is unavailable to birds due to poor phytase secretion and substrate solubility in the small intestine (Cowieson et al., 2011). This limitation can cause a large amount of phosphorus to be excreted to the environment leading to pollution that causes eutrophication and loss of aquatic animals (Correll, 1999). In addition, phytate also possesses anti-nutritive properties. Its phosphate groups are surrounded by 12 highly reactive protons that can bind to cations, such as minerals, amino acids, and starch (Angel et al., 2002; Dersjant-Li et al., 2015). Consequently, phytate can alter enzyme activity and reduce the availability of nutrients leading to poor performance of poultry (Yu et al., 2012).

The advancement of technology has allowed nutritionists to supplement exogenous phytase to broiler diets. Phytase is an enzyme that catalyzes the breakdown of phytate through stepwise hydrolysis of carbon-phosphate ester bonds of phytate resulting in phosphorus liberation (Selle and Ravindran, 2007). The inclusion of phytase in broiler diets allows nutritionists to lower the inclusion of inorganic phosphorus, dietary cost, and phosphorus excretion in the litter. Research has indicated that adding phytase beyond 1,000 phytase units (**FTU**)/kg may provide benefits beyond phosphorus liberation that results in the improvement of growth performance of broilers (Shirley and Edwards, Jr., 2003; Gehring et al., 2013; Beeson et al., 2017; Walk et al., 2018). However, the mechanism of extra-phosphoric effects of phytase on improving growth performance of broilers is not well understood and warrants further investigation. Several possibilities may explain the mode of action of high phytase supplementation in improving growth performance of broilers. The degradation of lower inositol phosphate (**IP**) esters with 3 or 4 phosphate groups may likely be the key in improving broiler performance and meat yield considering the similar capacity of lower IP esters to bind to nutrients (Persson et al., 1998). Further degradation of phytate may also lead to an increase in feed intake translating to increases nutrient intake of broilers (Walk and Olukosi, 2019). In addition, inositol may be involved in cell signaling and membrane functions to increase protein synthesis of broilers (Lee and Bedford, 2016).

The current research hypothesized that feeding broilers with elevated phytase supplementation will provide benefits beyond phosphorus liberation on phytate degradation, inositol liberation, growth performance, and carcass characteristics of broilers. This research was accomplished through 5 experiments by evaluating the

mechanism of extra-phosphoric effects of phytase in 3 areas: plasma inositol concentration, degradation of lower phytate esters, and feed intake stimulation. Experiment 1 was conducted to evaluate plasma inositol concentration as a biomarker for phytase efficacy on phytate degradation in broilers. Inositol is the final product of phytate degradation, which can be effectively absorbed (99.8%) in the small intestine (Croze and Soulage, 2013). This experiment was accomplished by measuring plasma inositol concentrations of broilers fed varying concentrations of phytase. In Experiments 2 and 3, extra-phosphoric effects of phytase were evaluated to determine the extent of phytate degradation by adding elevated phytase doses in broilers diets. This was accomplished by measuring growth performance, meat yield, phytate degradation, and inositol concentrations of broilers. In addition, the effects of dietary inositol were evaluated to determine if the extra-phosphoric effects of phytase originate from inositol liberation. Furthermore, Experiments 4 and 5 were conducted to investigate the influences of high phytase supplementation and nutrient uplifts on growth performance, carcass characteristics, gene expression of appetite hormones, and catecholamine concentrations of broilers during a 6-week production period. Experiment 4 was designed to obtain differences of digestible amino acid concentrations and AME_n between broilers fed phytase-added diets and those that were fed control diets. Difference in digestible amino acid concentrations and AME_n were used as nutrient uplifts in Experiment 5. Nutrient uplifts were used to mimic effects of phytase on increased nutrient intake. Experiment 5 was conducted by comparing the effects of nutrient uplifts and/or phytase supplementation on growth performance, meat yield, and hypothalamic appetite hormones of broilers. Data from these experiments generated new knowledge in the extra

phosphoric effects of phytase and provided nutritionists with better understanding on phytase application in broiler production.

II. LITERATURE REVIEW

PHYTATE

Phytate is a salt form of phytic acid, which has a 6-carbon inositol ring bound to a phosphate group on each of its carbons (Maga, 1982). In plants, phytate serves as principle storage of phosphorus and energy. In corn, phytate is stored in the germ, whereas in wheat and rice, phytate is located in the aleurone layers of the kernel and the outer bran. Conversely, phytate is usually bound to protein bodies in the endosperm of oilseeds (Bohn et al., 2008). The presence of phosphate groups provides the possibility of phytate as an alternative source of phosphorus. Rostagno et al. (2011) reported that phytate phosphorus concentration in various cereal grains and oilseeds ranges from 0.25 to 1.03%. Unfortunately, 60 to 70% of phytate phosphorus is unavailable to birds due to inefficient endogenous phytase secretion and low phytate solubility in the lower gastrointestinal tract of poultry (Cowieson et al., 2011). Consequently, phytate phosphorus may account for 49 to 80% of total phosphorus in the litter when broilers did not receive phytase addition (Lytem and Maguire, 2007). The accumulation of phosphorus in the litter may contribute to the eutrophication of surface waters. Eutrophication may accelerate the growth of algae and aquatic plants and decrease the concentration of dissolved oxygen in the water resulting in the loss of aquatic animals (Correll, 1999).

Phytate can also bind to any available cations such as minerals, amino acids, and starches due to the presence of strong negative charges surrounding its phosphate groups. As a result, phytate may reduce nutrient utilization in broiler diets leading to poor performance and meat yield (Woyengo and Nyachoti, 2013). Previous research noted a 6% reduction in feed intake of broilers when phytate phosphorus concentration was increased from 1.04 to 1.57% resulting in an 11% reduction in body weight and a 9-point increase in feed conversion ratio of broilers from 7 to 25 D of age (Cabahug et al., 1999).

Phytate Interaction with Minerals

Due to the presence of negative charges on the phosphate groups, phytate has the capacity to chelate di- and tri-valent minerals. This chelation may prevent phytate hydrolysis and reduce mineral availability to poultry. The chelation of minerals by phytate is strongly affected by pH, where it occurs at pH less than 3.5. As pH increases, the solubility of phytate-mineral complex decreases, with maximum insolubility at pH 7 (Champagne, 1988). In the gastrointestinal tract of broilers, the gizzard approximately has pH of 3.5, while distal duodenum and jejunum pH is above 6. Hence, phytate-mineral complex usually forms in the gizzard of broilers. As this complex moves to the small intestine, minerals are no longer available for absorption due to low solubility of this complex. Phytate interaction with minerals may also be influenced by binding strength to various minerals. The strength of mineral affinity to phytate at pH 7 was reported as follow $Zn^{2+} > Fe^{2+} > Mn^{2+} > Fe^{3+} > Ca^{2+} > Mg^{2+}$ (Maenz et al., 1999). Despite having a much lower affinity than zinc, dietary calcium in broiler diets may have a greater impact on the extent of phytate hydrolysis because dietary calcium concentration can range from

8 to 40 fold higher than zinc (Angel et al., 2002). Thus, phytate is more likely to bind to calcium than zinc as the ratio of phytate to calcium is higher than phytate to zinc.

Phytate Interaction with Protein

Phytate and protein may interact to form a binary complex at pH below protein isoelectric point (Selle et al., 2012). This interaction occurs through a formation of salt-like linkages between negatively charged phytate and basic amino acid residues carrying a net positive charge, such as arginine, histidine, and lysine (Cosgrove, 1966). As a result, phytate is surrounded by protein residues that is resistant to hydrolysis. Conversely, at pH above isoelectric point of protein, phytate and protein interaction is mediated by divalent cations to form ternary protein-phytate complexes (Cowieson and Cowieson, 2011). In poultry diets, this interaction is usually facilitated by calcium as it is the most abundant mineral in the poultry diet. In addition, phytate can act as a Hofmeister anion when interacting with protein by altering hydrogen bonds surrounding protein (Baldwin, 1996). Phosphate groups of phytate have a strong capacity to form hydrogen bond with water that stabilize protein and reduce its solubility.

The consequence of these direct and indirect interactions of phytate and protein is the reduction in protein utilization (Selle et al., 2012). Specifically, binary phytate-protein complexes reduce protein utilization by resisting to pepsin digestion. As a result, the gastrointestinal tract may compensate this impaired protein digestion by increasing HCl secretion. The hypersecretion of HCl may be countered by increasing mucin secretion to protect the gastrointestinal tract and bicarbonate secretion to buffer HCl. These compensatory mechanisms concomitantly will increase endogenous amino acid losses through mucin secretion and decrease amino acid digestibility as more sodium is utilized

for bicarbonate secretion instead for the sodium-dependent transport system (Cowieson et al., 2004). Onyango et al. (2009) reported the impact of phytate-protein interaction on mucin and endogenous amino acid losses of broilers fed diets either with free phytic acid, magnesium-potassium phytate, or without phytic acid. Broilers fed diets with either form of phytic acid had higher mucin secretion and excretion of threonine, proline, and serine compared with broilers fed diets without phytic acid addition indicating the negative effects of phytate on endogenous amino acid losses. In addition, this study indicated that the magnitude of amino acid excretion was greater when broilers fed diets with magnesium-potassium phytate than with free phytic acid as magnesium-potassium phytate is the predominant phytate found in feed ingredients. A previous study indicated that calcium-magnesium salt of phytic acid had a more pronounced effect on reducing body weight of broilers compared with feeding diets with sodium phytate supplementation (Waldroup et al., 1964).

Phytate Interaction with Starch

Rickard and Thompson (1997) indicated several pathways for phytate to interact with starch. Phytate can directly bind to starch by hydrogen bonds between the oxygen of phosphate group of phytate and hydrogen ion of glucose in starch. In addition, phytate interaction with starch may be facilitated by its interaction with proteins that are closely associated with starch granules creating a phytate-protein-starch complex. Furthermore, phytate can also alter starch digestibility by reduction of amylase activity by binding with calcium, which is required for the activation of amylase (Humer et al., 2014). Negative effects of phytate on starch utilization was reported in humans. Thompson et al. (1987) observed that adding 1.0% phytic acid to dephytinized navy bean decreased sugar release

by 44% compared with dephytinized navy bean that did not receive phytic acid. However, the implication of this report should be taken carefully as anti-nutritive effects of supplemental phytic acid is lower than phytic acid found in feed ingredients (Onyango et al., 2009).

PHYTASE

Phytase is an enzyme that catalyzes the breakdown of phytate by hydrolyzing the carbon-phosphate bonds of phytate (Lei et al., 2013). In poultry, phytase can be secreted in the small intestine with the greatest amount secreted in the duodenum and decreased progressively along the distal portion of the small intestine (Maenz and Classen, 1998). This intestinal brush border phytase was reported to have an optimal pH between 5.0 to 6.5 with the highest activity at pH 6.0. Although intestinal brush border phytase has the potential to hydrolyze phytate, it may be ineffective as phytate is insoluble at high pH. In addition, the secretion of endogenous phytase in the small intestine of poultry is low (Dersjant-Li et al., 2015). Therefore, poultry may need to rely on dietary phytase to assist with phytate degradation. In plants, phytases are commonly used to release phosphorus and inositol in seeds during germination. Plant phytases can also be an alternative to degrade phytate in feed ingredients of chickens; however, corn and soybean meal contain less than 100 FTU/kg phytase (Eeckhout and Paepe, 1994). Furthermore, feed processing and manufacturing may impair plant phytase activity since phytase is susceptible to degradation at high temperatures.

In the early 1960s, an effort was initiated to screen over 2,000 organisms for phytase activity at the International Minerals and Chemicals research laboratory (Kidd et al., 2016). Nelson et al. (1968) were the first to use phytase to treat soybean meal and

found that phytase produced by *Aspergillus ficuum* was able to hydrolyze phytate phosphorus. However, the hydrolysis of phytate phosphorus in soybean meal requires the addition of moisture for enzyme distribution. Hence, the energy required to dry the meal cost more than the revenue generated from phosphorus release. Thus, the approach was not commercially applicable. In 1971, a new technique to add a mold phytase produced by *Aspergillus ficuum* to a complete diet yielded a greater percentage of bone ash of chicks through the hydrolysis of phytate by phytase (Nelson et al., 1971). Simons et al. (1990) noted an increase in phosphorus availability and a reduction in phosphorus excretion by 20 and 22%, respectively, when broilers were fed diets supplemented with 500 FTU/kg phytase. As a result, commercial phytase was produced primarily to mitigate phosphorus pollution, especially in the Netherlands and Maryland, USA (Kidd et al., 2016). Currently, phytase is used in a higher dose for its benefits beyond phosphorus liberation, such as further phytate degradation (Zeller et al., 2015), increased amino acid digestibility (Cowieson et al., 2006) and apparent metabolizable energy (Ravindran et al., 2006), and inositol liberation (Walk et al., 2014).

Factors affecting phytase efficacy

Phytase-related factors

Phytase activity is commonly measured as the quantity of enzyme required to release 1 µmol of monocalcium phosphate from 0.0051 mol/L sodium phytate in one minute at pH 5.5 and 37ºC. This measurement indicates that phytase activity may be influenced by optimum pH and temperature. Igbasan et al. (2000) utilized various phytases derived from *Peniophora lycii*, *Escherichia coli*, *Bacillus subtilis,* and 3 phytases from *Aspergillus* to evaluate factors affecting phytase efficacy. The optimum pH

of each phytase was as follows 5.5, 4.5, 4.5, and 7.0, respectively for *Aspergillus, Peniophora, E. coli, and Bacillus* phytases. Evaluating the optimum pH of phytases is crucial as phytase addition in broiler diets is targeted toward phytate breakdown in the upper gastrointestinal tract of broilers (proventriculus and gizzard), where pH is below 3.5 (Angel and Sorbara, 2014). Therefore, selecting phytase that has optimum pH closer to the pH of the upper gastrointestinal tract of broilers may be beneficial to provide optimum phytate degradation.

The practicality of phytase in broiler diets is also influenced by its thermostability as diets are pelleted at high temperatures (up to 90ºC). Previous research demonstrated that increasing conditioning temperature during pelleting from 60 to 90ºC increased pellet durability index from 39.5 to 84.5% (Abdollahi et al., 2010) indicating that a higher conditioning temperature is more desirable due to higher pellet quality. However, Igbasan et al. (2000) noted that phytase derived from *Aspergillus ficuum* had a 76% reduction in phytase activity from 84 to 19.8%, while *E. coli* phytase activity decreased from 98.8 to 24.4% when conditioning temperature increased from 60 to 80ºC, respectively.

In addition to optimum pH and thermostability, phytase must be able to withstand protease activity in the gastrointestinal tract of broilers to produce optimum growth performance. Because commercial phytases are protein and their activity is targeted in the upper gastrointestinal tract of broilers, these enzymes are susceptible to hydrolysis by digestive proteases. Igbasan et al. (2000) evaluated the effects of phytase incubation in porcine pepsin. After incubation for 60 minutes, *E. coli* phytase had 94.6% of residual phytase activity, while *Aspergillus, Peniophora, and Bacillus* phytases provided 32.2, 1.8, and 19.3% residual phytase activity, respectively. The activity of these phytases was

also evaluated in digesta supernatants from various segments of chicken digestive tract for 60 min. The results indicated that in the gizzard digesta supernatants, only *E. coli* phytase had a residual activity of above 90%, while the activity of other phytases was below 70% indicating a greater resistance of *E. coli* phytase to pepsin digestion in the gizzard of chickens.

One aspect that may also influence the performance of phytase is the Michaelis-Menten constant (**K_m**), which is defined as the concentration of the substrate to reach half of the maximum enzyme reaction velocity. A previous study evaluated enzymatic properties of 7 commercial phytases derived from *Buttiauxella* sp., *Citrobacter braakii*, *Peniophora lycii*, *Aspergillus niger*, and 3 phytases from *E. coli* on the catalytic performance in an in vitro simulation of the digestive tract of poultry (Menezes-Blackburn et al., 2015). This study indicated that commercial phytases may have different K_m ranging from 98 to 427 µM when measured at pH 3.0 and 37ºC. These data are important as the K_m of phytase may influence the degree of phytate degradation. Phytases having a lower K_m can act more rapidly as phytate concentration decreases during phytate hydrolysis compared with higher K_m phytases. The rapid reaction of low K_m phytases is important to prevent the accumulation of phytate. On the contrary, higher K_m phytases require a higher concentration of phytate. Consequently, phytate may accumulate and alter nutrient availability before it can be hydrolyzed by phytase.

Dietary-related factors

The efficacy of phytase is commonly evaluated through its response to phosphorus digestibility, which is directly related to the degradation of phytate represented by percent hydrolysis of IP6. A previous study demonstrated that IP6

hydrolysis in the ileum of broilers increased with the increased addition of phytase. However, the extent of hydrolysis was greater in broilers provided diets containing 0.23% phytate phosphorus compared with those receiving 0.34% phytate phosphorus diet (Li et al., 2017). In addition, the efficacy of phytase may be influenced by various types of feed ingredients. Leske and Coon (1999) evaluated hydrolysis of IP6 by phytase (600 FTU/kg) with 7 dietary treatments formulated primarily with, corn, wheat, and barley at 60% and soybean meal, defatted rice bran, canola meal, and wheat middling at 30%. These researchers indicated that IP6 hydrolysis was the lowest in diets formulated primarily with wheat and the highest when formulated with soybean meal. These differences may be associated with the accessibility of phytate by phytase. Phytate phosphorus in soybeans has been reported to be more accessible for phytase as it is located in the protein bodies (Prattley and Stanley, 1982). In contrast, phytate phosphorus in wheat may not be easily accessible by phytase due to encapsulation within the cell wall of the aleurone layer (Tanaka et al., 1974).

The ability of phytase to liberate phosphorus through phytate degradation is also influenced by the solubility of phytate. Due to the presence of negative charges surrounding its phosphate groups, phytate can form complexes with minerals that are insoluble at pH > 7. In poultry diets, calcium is added at the highest concentration compared with other minerals. Although calcium affinity to phytate is less than zinc, copper, iron, and manganese, the relatively high concentration of calcium can increase the probability of phytate-calcium complexes formation (Angel et al., 2002). Therefore, the concentration of dietary calcium can affect the efficacy of phytase to hydrolyze phytate. Previous research investigating the effects of dietary calcium on phytase efficacy

in broilers indicated that increasing supplemental calcium at 0.5% decreased phytate phosphorus disappearance from 69.2 to 25.4% in diets without phytase addition (Tamim et al., 2004). However, the addition of phytase at 500 FTU/kg to the 0.5% added calcium diet increased phytate phosphorus disappearance of broilers from 25.4% to 58.9% compared with birds fed diets without phytase addition.

Not only the concentration of dietary calcium, but the solubility of calcium source may also influence phytase efficacy in liberating phosphorus from phytate. Kim et al. (2018) investigated the effects of particulate (d_{gw} = 402 µm) and pulverized (d_{gw} < 75 µm) limestone, dietary calcium concentrations, and phytase supplementation on phosphorus digestibility in broilers. These results indicated that phosphorus digestibility was decreased by 21% when using pulverized vs. particulate limestone. Additionally, when phytase was supplemented at 1,000 FTU/kg, phosphorus digestibility of broilers fed particulate limestone did not decrease regardless of calcium concentrations. However, the increase of dietary calcium concentration from 0.6 to 1.0% in broilers receiving pulverized limestone decreased phosphorus digestibility from 66.9 to 50.9%. These data demonstrated that the rate of solubility and concentration of calcium will alter the efficacy of phytase in degrading phytate (Kim et al., 2018). As solubility and concentration of calcium increase, more calcium is accessible for chelation by phytate leading to decreased solubility of phytate. Therefore, increasing phytase doses may be crucial to rapidly remove phosphate groups from phytate before chelation with calcium occurs.

HIGH DOSES OF PHYTASE

The benefits of phytase in liberating phosphorus has been documented previously through the degradation of IP6 to lower phytate esters, IP1, 2, 3, 4, 5, and inositol (Selle and Ravindran, 2007). Phytase is commonly added in poultry diets at 500 to 1,500 FTU/kg to provide phosphorus from phytate by breaking down IP6. However, anti-nutritive effects of phytate may still be present even after IP6 is hydrolyzed. Previous research noted that despite the chelating capacity of phytate to cations decreases in lower phytate esters, IP3, 4, and 5 are still capable of binding to minerals (Persson et al., 1998). Yu et al. (2012) demonstrated in an in vitro study that porcine pepsin activity was inhibited even after IP6 has been completely degraded. In contrast, pepsin activity increased following the degradation of IP3 and IP4. Therefore, the presence of IP3 to IP6 may impair protein digestion through the inhibition of pepsin.

These challenges with lower phytate esters may be ameliorated through increasing phytase doses beyond 1,000 FTU/kg. This approach may allow further degradation of lower phytate esters while concomitantly preventing chelation of nutrients in the gizzard of poultry. Previous research demonstrated that increasing phytase dose to 1,500 FTU/kg in broilers fed a reduced calcium (0.16%) and non-phytate phosphorus (0.15%) diet decreased feed conversion ratio of broilers by 3 points compared with those fed the diet without the reduction of calcium and available phosphorus (Walk et al., 2014). Moreover, a previous study showed that broilers provided reduced calcium (0.14%) and non-phytate phosphorus (0.13%) diets containing 1,600 FTU/kg phytase had 49 g heavier breast meat weight than birds consuming diets without phytase supplementation and calcium and non-phytate phosphorus reduction (Campasino et al., 2014). These data indicated that

supplementing phytase in a high dose (beyond 1,000 FTU/kg) may provide improvements in growth performance and carcass characteristics beyond phosphorus liberation.

The mechanisms involved with extra phosphoric effects of phytase may be attributed to the degradation of lower phytate esters, which subsequently eliminate anti-nutritive effects of phytate. Previous studies demonstrated the accumulation of lower IP esters (IP3 and IP4) when supplementing phytase at 500 to 1,500 FTU/kg (Walk et al., 2014; Beeson et al., 2017; Sommerfeld et al., 2018; Walk et al., 2018). However, when phytase dose was increased to either 3,000 or 4,500 FTU/kg, IP4 concentrations decreased in the ileal digesta (Sommerfeld et al., 2018; Walk et al., 2018). Ultimately, increased phytase addition in broiler diets could decrease the total IP3 to IP5 concentrations in the ileal digesta (Zeller et al., 2015). These data indicated that higher phytase doses may reduce anti-nutritive effects of phytate through the degradation of lower phytate esters.

The improvements in growth performance and meat yield of broilers when fed a high dose of phytase diet may also be the result of increased amino acid digestibility as a consequence of phytate degradation. Gehring et al. (2013) indicated an increase of amino acid digestibility by approximately 2 percentage points when broilers were fed phytase at 2,000 FTU/kg compared with birds fed diets without phytase supplementation. According to Cowieson et al. (2006), increased amino acid digestibility when broilers were fed high doses of phytase may be related to 3 mechanisms of phytate-protein interaction. First, phytase may directly liberate amino acids bound to phytate. Second, phytase may prevent the chelation of phytate with proteases cofactors allowing proteases to effectively digest

protein. Third, phytase may inhibit the interaction of phytate with the gastrointestinal tract to reduce endogenous amino acid losses.

Extra-phosphoric effects of phytase can also be revealed through the increased energy utilization of broilers. This was demonstrated with a 67 kcal/kg increase in apparent metabolizable energy (**AME**) of broilers from 3,277 to 3,344 kcal/kg when increasing phytase addition from 0 to 1,000 FTU/kg, respectively (Ravindran et al., 2006). In addition to a direct liberation of starch, a high phytase dose may also alter AME_n in broilers by decreasing the energy required for gastrointestinal tract maintenance (Cowieson et al., 2006). The efficacy of phytase in hydrolyzing phytate may reduce enterocyte turnover and mucin secretion as a result of limiting the interaction of phytate with the gastrointestinal tract of broilers (Cowieson et al., 2004). A previous study measured sialic acid in the excreta of broilers fed either 1 g/kg phytate, 1,000 FTU/kg phytase, or the combination of 1 g/kg phytate and 1,000 FTU/kg phytase (Cowieson et al., 2004). Sialic acid has been reported to be a suitable marker for mucin production (Larsen et al., 1993). The study indicated that the addition of phytase in the presence of 1 g/kg phytate decreased sialic acid from 5.33 to 2.46 mg/bird compared with broilers fed diets containing 1 g/kg phytate without phytase supplementation, which demonstrates the benefits of phytase in reducing mucin production (Pirgozliev et al., 2007).

INOSITOL

Inositol is a simple 6-carbon ring compound with a hydroxyl group bound to each carbon ($C_6H_{12}O_6$). It is widely distributed in animal tissues and cells, plants, fungi, and some bacteria for many physiological functions (Holub, 1986). Inositol has 9 stereoisomers, but the most active form is myo-inositol (Croze and Soulage, 2013).

Dietary inositol can be supplied in a free form or through complete degradation of phytate. Recently, supply of inositol in poultry diets through phytate degradation has received more attention as phytase supplementation becomes more common (Lee and Bedford, 2016).

Inositol absorption and metabolism

In the small intestine, free inositol may be absorbed in the jejunum and ileum (Walk et al., 2018). Additionally, the kidney has the capacity to reabsorb inositol. The absorption of inositol primarily occurs via transport protein. Several inositol transporters have been identified, such as the sodium-dependent myo-inositol co-transport systems (**SMIT1** and **SMIT2**) and proton-dependent myo-inositol co-transport system (**HMIT**) (Aouameur et al., 2007). A study in broilers reported that increasing phytase doses from 0 to 4,500 FTU/kg increased free inositol concentration in the ileal digesta by approximately 2 fold. As a result, increased gene expression of SMIT2 and HMIT in the jejunum and ileum, respectively, was observed (Walk et al., 2018). The increase in the gene expression of inositol transporters may also demonstrate the increase of inositol absorption in the small intestine.

The catabolism of inositol occurs solely in the kidney (Croze and Soulage, 2013). This process occurs through the glucoronate-xylulose pathway, which is catalyzed by myo-inositol oxygenase to convert myo-inositol to glucoronate. Howard and Anderson (1967) conducted a study to determine inositol catabolism in the rat kidney. These authors noted that nephrectomized rats administered with 0.81 mg of radioactive myo-inositol did not produce radioactive respiratory carbon dioxide indicating that the kidney is the major organ for inositol catabolism. However, the concentration of radioactive

carbon dioxide in rat kidney slices increased within 4 hours of incubation with radioactive myo-inositol with the greatest rate of catabolism occurred within the first hour of incubation. Moreover, the presence of labeled glucoronate in the kidney slices incubated with radioactive myo-inositol confirms that inositol catabolism occurs via the glucoronate-xylulose pathway.

In addition to the complete degradation of phytate, inositol can also be synthesized de novo. The synthesis of inositol in various tissues in the body is catalyzed by an inositol-3-phosphate synthase enzyme. This synthesis is initiated by isomerization of glucose-6-phosphate by inositol-3-phosphate synthases to form myo-inositol 1-phosphate. Then, the phosphate group is dephosphorylated resulting in myo-inositol. A previous study conducted with rats showed that inositol can be synthesized from glucose in the testis, brain, kidney, and liver (Holub, 1986). The synthesis of inositol from glucose was confirmed by Daughaday et al. (1955) using radiolabeled glucose that was administered to immature rats and chick embryos. In this study, these authors observed that radioactive inositol was isolated from the liver and carcass of rats after intraperitoneal administration of radiolabeled glucose. In addition, radioactive inositol was also detected in the chiro-allantonic membrane of chick embryos 64 hours after radioactive glucose was administered.

Functions of inositol

Following its absorption, inositol can be utilized for various physiological functions. Inositol is an important component of phosphatidylinositol (Cooper and Hausman, 2013). The synthesis of phosphatidylinositol requires both cytidine diphosphate-diacylglycerol and inositol and is catalyzed by cytidine diphosphate-

diacylglycerol-inositol 3-phosphatidyltransferase (Croze and Soulage, 2013). The 2 major phosphatidylinositols are phosphatidylinositol 4,5-bisphosphate (**PIP_2**) and phosphatidylinositol 3,4,5-triphosphate (**PIP_3**). Phosphatidylinositol 4,5-bisphosphate is involved in cellular signaling pathways by producing 2 secondary messengers: IP3 and diacylglycerol (**DAG**). These products can be formed by cleaving PIP_2 by phospholipase C. Inositol phosphate 3 is released into the cytosol after the cleaving of PIP_2. The function of IP3 is primarily to stimulate the release of calcium from the endoplasmic reticulum for signaling functions. In contrast, DAG remains associated with the plasma membrane to activate protein kinase C. The role of protein kinase C is to control cell differentiation and growth. In addition, PIP_3 is formed when PIP_2 is phosphorylated by phosphatidylinositide 3-kinase. Phosphatidylinositol 3,4,5-triphosphate is an important messenger for cell proliferation and survival through the activation of Akt, a protein-serine/threonine kinase.

Inositol can also function as a precursor for IP5 formation in the blood of avian species. Unlike mammals that use 2,3-diphosphoglycerate, adult birds use IP5 to control oxygen affinity in the red blood cells (Lutz, 1980). Isaacks et al. (1982) conducted a study to determine the incorporation of inositol to IP5 in 5 d old chicks by incubating blood with radioactive [^{14}C]-inositol for 6 hours. In addition, these researchers measured the resulting inositol concentrations after plasma was separated from blood of chicks. The study demonstrated that the increase in incubation time increased the uptake of inositol by the erythrocyte and the incorporation of inositol to IP5. As a result, inositol in the blood decreased from 14.6 to 10.7 µg/mL in 0 and 6 hours of incubation time,

respectively, indicating the importance of inositol as a precursor for the formation of IP5 in avian red blood cells.

Involvement of inositol in poultry production

Inositol has been reported to have growth-promoting properties when added in broiler diets (Cowieson et al., 2013; Zyla et al., 2013). The supplementation of phytase beyond 1,000 FTU/kg has been reported to liberate inositol in the ileal digesta of broilers (Walk et al., 2014; Beeson et al., 2017). The increase in liberation of inositol in the digesta also increased inositol uptake in the small intestine (Lerner and Smagula, 1979; Walk et al., 2018). Concomitantly, phytase addition beyond 1,000 FTU/kg may increase inositol concentration in the plasma of broilers (Cowieson et al., 2015; Sommerfeld et al., 2018). The elevated plasma inositol concentration may allow for the possibility of measuring plasma inositol as a biomarker for phytase efficacy. Cowieson et al. (2017) conducted a time-series study by repeatedly measuring plasma inositol concentrations in pigs fed diets containing 0, 1,000, and 3,000 FTU/kg. The results indicated plasma inositol reached maximum concentration at 360 minutes after pigs were fed diets with 3,000 FTU/kg phytase supplementation. This study showed the practicality of using plasma inositol as an indicator of phytase efficacy. However, a similar study in poultry is warranted as there may be differences in inositol metabolism between mammalian and avian species.

POSSIBILITY OF PHYTASE EFFECTS ON FEED INTAKE STIMULATION

Appetite is controlled by complex mechanisms that involve the nutritional status of the body, neurotransmitters, and a change in feeding behavior (Richards and Proszkowiec-Weglarz, 2007). Within the arcuate nucleus of the hypothalamus, appetite

is controlled by altering orexigenic (neuropeptide Y (**NPY**) and agouti-related peptide (**AGRP**)) and anorexigenic (proopiomelanocortin (**POMC**) and cocaine and amphetamine regulated transcript (**CART**)) peptide hormones (Boswell et al., 1999; Denbow, 1999; Richards, 2003; Dridi, 2017). Appetite suppression is initiated by the release of α-melanocortin-stimulating hormone (α-**MSH**) from POMC neurons. The release of α-MSH activates melanocortin receptor 4 (**MC4-R**) resulting in decreased feed intake (Dridi, 2017). Conversely, NPY and AGRP increase appetite through direct inhibition of POMC and CART neurons or by intercepting α-MSH and inactivating MC4-R (Dridi, 2017). In addition, adenosine monophosphate-activated protein kinase (**AMPK**) is an energy sensor that alters the stimulation or inhibition of the melanocortin system (Hardie et al., 2006). This enzyme is activated when energy reserve is elevated (Dridi, 2017). Once activated, AMPK inhibits the activity of acetyl-CoA carboxylase, the rate-limiting enzyme to produce malonyl-CoA that initiates fatty acid synthesis (Dridi, 2017). Subsequently, this inhibition also reduces NPY and AGRP and increases POMC gene expressions, which ultimately, decreases feed intake (Richards and Proszkowiec-Weglarz, 2007).

Outside the hypothalamus, cholecystokinin (**CCK**) may also interact with the melanocortin system as a satiety signal through slowing down intestinal motility (Denbow, 1999). Previous research reported that the reduction of feed intake in rats is associated with the secretion of hypothalamic CCK that inhibited NPY gene expression (Bi et al., 2004; Chen et al., 2008). Ghrelin is also an appetite hormone secreted in the proventriculus (Kaiya et al., 2007). However, unlike in mammals where ghrelin increases food intake through the melanocortin system, ghrelin in birds induces anorexia through

the release of corticotropin-releasing factor (Kaiya et al., 2007). In addition to peptide hormones, serotonin and dopamine are catecholamines that may influence appetite through direct or indirect interaction with the melanocortin system. Serotonin has been reported to promote satiety by stimulating MC4-R through activation of POMC neurons and inhibition of NPY and AGRP neurons (Heisler et al., 2006; Lam et al., 2010). Furthermore, dopamine appears to inhibit feed intake through the inhibition of NPY in rats (Kuo, 2002).

Evidence of appetite-stimulating effects of phytase in broilers have been observed when phytase was added to a reduced calcium and phosphorus diet (Campasino et al., 2014; Walk et al., 2014). Reductions in calcium and non-phytate phosphorus (0.14 and 0.13%, respectively) concentrations were reported to decreased feed intake of broilers by 12.7% from 1 to 28 D of age compared with broilers fed the positive control diets (Campasino et al., 2014). When phytase was added at 400 FTU/kg, feed intake of broilers fed the reduced calcium and non-phytate phosphorus diets were restored similar to the positive control-fed birds. However, the response of feed intake due to phytase supplementation may be beyond the restoration of calcium and phosphorus concentrations when added at a higher dose. Additions of dietary phytase ranging from 1,500 to 4,000 FTU/kg were reported to enhance feed intake of broilers by 22 to 207 g from 1 to 14 to 1 to 42 D of age (Walk et al., 2012 and 2014; Gehring et al., 2013; Campasino et al., 2014; Beeson et al., 2017; Lee et al., 2019; and Walk and Olukosi, 2019).

The relationship between phytase supplementation and appetite stimulation through regulation of appetite hormone secretion is unclear (Liu et al., 2014). However, it

is likely that these mechanisms may be linked to benefits of phytase in degrading phytate (Watson et al., 2006). Dos Santos et al. (2014) noted a 20.1% reduction in feed intake broilers from 1 to 21 D of age when increasing dietary phytate phosphorus concentration from 0.18 to 0.29%. The reduction of feed intake may be attributed to a compensatory mechanism to impaired digestion by phytate through increased HCl and mucin secretion as well as decreased passage rate (Cowieson et al., 2004). The reduction in passage rate may alter appetite hormone secretion to depress feed intake (Scanes et al., 2014). A previous study utilizing grass carp indicated that supplementing diets with 0.4% phytic acid increased gene expression of CART and CCK in the brain beyond 2-fold resulting in a 44% reduction of feed intake compared with fish fed diets without supplemental phytic acid (Liu et al., 2014).

In contrast, the influence of phytase in hydrolyzing phytate was reported to decrease digesta transit time in broilers (Watson et al., 2006). Presumably, the reduction of digesta transit time may concomitantly decrease CCK (Scanes et al., 2014) and its inhibition to NPY and AGRP (Bi et al., 2004; Chen et al., 2008; Dunn et al., 2013) leading to increased feed intake. In addition, the impact of phytate degradation on amino acid liberation may also influence the production of appetite hormones as amino acids are major components in biosynthesis of many peptide hormones (Dridi, 2017). Neuropeptide Y is composed of 36 amino acids consisting of Ala, Arg, Asp, Glu, Gly, His, Ile, Pro, Leu, Lys, Ser, Thr, and Tyr (Tatemoto, 1982). In addition, Tyr and Trp are the main components in biosynthesis of dopamine and serotonin, respectively (Meiser et al., 2013; Yabut et al., 2019). It is possible that due to the effects of phytase in stimulating appetite, amino acids liberated from phytate degradation may be indicators

for alteration in gene expression of appetite hormones (Richards and Proszkowiec-Weglarz, 2007).

KNOWLEDGE GAPS IN THE LITERATURE

Benefits of extra-phosphoric effects of phytase in enhancing growth performance and meat yield of broilers have been well documented in the literature (Cowieson et al., 2011; Gehring et al., 2013; Campasino et al., 2014; Walk et al., 2014). Many previous studies have attributed these benefits on the increased nutrient availability (Cowieson et al., 2004; Selle et al., 2012; Yu et al., 2012). However, the contribution of inositol on extra-phosphoric effects of phytase has been inconsistent (Cowieson et al., 2013; Zyla et al., 2013; Pirgozliev et al., 2019). Additionally, it is unclear if inositol liberation in the digesta is translated to the plasma (Cowieson et al., 2015; Sommerfeld et al., 2018). Cowieson et al. (2017) indicated that phytase supplementation in pigs resulted in increased plasma inositol concentrations within 3 hours of feeding experimental diets. However, poultry may have different mechanisms of inositol utilization in the plasma (Lutz et al., 1980). Hence, additional data are necessary to evaluate effects of phytase on inositol liberation and plasma inositol concentrations in broilers.

Many previous studies evaluating the efficacy of phytase on phytate degradation have mostly been focusing on IP6 degradation, while lack of emphasis has been placed on the degradation of lower IP esters, such as IP3 and IP4 (Walk and Bedford, 2016). The influence of lower phytate degradation on growth performance and meat yield of broilers is also unclear as most of previous works were conducted using broilers up to 3 weeks of age (Walk et al., 2014; Zeller et al., 2015; Beeson et al., 2017; Sommerfeld et al., 2018). Moreover, the interaction of age and phytase efficacy on phytate degradation is also

sparse (Olukosi et al., 2020). Therefore, additional research studies are warranted to determine the extent of phytate degradation using doses beyond 1,500 FTU/kg in broilers raised to market age.

In addition, published literature indicated a strong correlation between BW gain and digestible amino acid intake of broilers when fed diets with phytase supplementation (Walk and Olukosi, 2019). This indicated the influence of phytase supplementation on feed intake stimulation (Watson et al., 2006). However, it is unsure whether appetite-stimulating effects of phytase may originate from the elimination of phytate anti-nutritive factors, amino acid liberation, or cell signaling properties of inositol. Furthermore, mechanisms to which phytase alters gene expression of appetite hormones and catecholamines are unknown and require further investigation.

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III. INFLUENCE OF EXOGENOUS PHYTASE SUPPLEMENTATION ON PHYTATE DEGRADATION, PLASMA INOSITOL, ALKALINE PHOSPHATASE, AND GLUCOSE CONCENTRATIONS OF BROILERS AT 28 DAYS OF AGE

ABSTRACT

An experiment was conducted to evaluate effects of supplementing broiler diets with phytase on phytate degradation and plasma inositol concentrations at 28 D of age. Twenty-four Ross × Ross 708 male chicks were placed in battery cages (4 birds per cage) from 1 to 21 D of age and individually from 22 to 28 D of age. Broilers received common diets from 1 to 27 D of age. At 27 D of age, a catheter was placed in the brachial vein of broilers to avoid repeated puncture of the vein during blood collection. At 28 D of age, broilers received 1 of 3 experimental diets formulated to contain 0, 400, and 1,200 phytase units (**FTU**)/kg, respectively in Treatments 1, 2 and 3. Treatment 1 was formulated to contain 0.76% calcium and 0.36% non-phytate phosphorus, whereas calcium and non-phytate phosphorus concentrations in Treatments 2 and 3 were 0.165 and 0.150% lower than Diet 1, respectively. Blood was collected 1 hour prior to feeding experimental diets and from 20 to 240 minutes after feeding experimental diets at 20-minutes intervals with a final blood collection at 480 minutes to determine plasma inositol concentrations. Inositol phosphate (**IP**) ester degradation was determined in

gizzard contents and ileal digesta. Broilers provided the 1,200 FTU/kg phytase diet had 60% less ($P < 0.01$) IP6 concentration in gizzard content (1,264 vs. 4,176 nmol/g) and ileal digesta (13,472 vs. 33,244 nmol/g) compared with birds fed the 400 FTU/kg diet. Adding phytase at 1,200 FTU/kg increased ($P < 0.01$) inositol concentrations in gizzard content and ileal digesta of broilers by 2.5 (2,703 vs. 1,071 nmol/g) and 3.5 (16,485 vs. 4,667 nmol/g) fold, respectively, compared with adding 400 FTU/kg. Plasma inositol concentration of broilers was not different ($P = 0.94$) among the dietary treatments at each collection time. Inositol liberation in the digesta of broilers fed diets with 1,200 FTU/kg phytase did not translate to increased plasma inositol concentrations, which warrants further investigation.

INTRODUCTION

Phytase is supplemented in broiler diets to enhance phosphorus utilization, which concomitantly decreases feed cost by reducing the inclusion of inorganic phosphate (Selle and Ravindran, 2007). Recently, increasing phytase supplementation beyond 1,000 phytase units (**FTU**)/kg in broiler diets led to increased amino acid digestibility, apparent metabolizable energy, and growth performance of broilers beyond phosphorus liberation (Gehring et al., 2013). In addition to these extra-phosphoric effects of phytase, the liberation of inositol may play a key role in altering growth rate of broilers (Walk et al., 2014). Inositol is the final product of complete phytate degradation through a stepwise liberation of all its phosphate groups. However, information regarding the rate of phytate degradation and inositol liberation in broilers fed a phytase supplemented diet is sparse (Cowieson et al., 2017). In order to determine the rate of phytate degradation in broilers

fed phytase beyond 1,000 FTU/kg, digesta inositol phosphate ester and plasma inositol concentration may be utilized as an indicator of phytase efficacy.

Following its liberation, inositol has been reported to be highly absorbed (99.8%) in the small intestine (Croze and Soulage, 2013). Considering its rapid liberation by phytase and efficiency of absorption, it is hypothesized that the inclusion of phytase beyond 1,000 FTU/kg in a broiler diet may result in a rapid increase of inositol concentration in the plasma. Plasma inositol concentration may be used as a biomarker for phytase efficacy, which may allow phytase users to identify problems related to phytase destruction during feed manufacturing as well as to unfold alternatives to enhance phytate degradation (Cowieson et al., 2017). Hence, identifying the optimum time to measure plasma inositol concentration is paramount in utilizing inositol as a biomarker for phytase efficacy. An experiment was conducted to determine phytate degradation and an optimum plasma inositol concentration in broilers fed diets supplemented with phytase within 8 hours of feeding experimental diets.

MATERIALS AND METHODS

All procedures involving care and use of live birds in this experiment were approved by Auburn University Institutional Animal Care and Use Committee (PRN 2017-3067).

Bird Husbandry

Twenty four Ross × Ross 708 male chicks were obtained from a commercial hatchery (Aviagen North America, Huntsville, AL) and received vaccinations for Marek's disease, Newcastle disease, and infectious bronchitis at 1 D of age. Broilers were placed into 6 battery cages (4 birds per cage; 0.12 m^2 per bird; Petersime, Gettysburg,

OH) with each cage having dimensions of 68 × 68 × 38 cm from 1 to 21 D of age. From 22 to 28 D of age, broilers were placed individually in battery cages (0.46 m^2/bird) to prevent other birds from pecking at catheters. Each cage contained a linear feeder and a water trough. Cages were placed in a solid-sided room equipped with forced-air heaters and cooling pads to adjust the temperature. Room temperature at bird placement was set at 33°C and was gradually decreased to 24°C until 28 D of age. A 23L:1D photoperiod was provided from 1 to 7 D of age, thereafter a 20L:4D lighting schedule was utilized. Broilers received feed and water ad libitum throughout the 28 D periods. Broilers were provided common starter and grower diets (corn-soybean meal-based) without phytase addition from 1 to 14 and 15 to 27 D of age, respectively. These diets were formulated to contain AME_n, crude protein, digestible Lys, calcium, and non-phytate phosphorus of 3,053 kcal/kg, 23.3%, 1.23%, 1.01%, and 0.48% for the starter diet and 3,086 kcal/kg, 21.7%, 1.10%, 0.90%, and 0.43% for the grower diet, respectively. Starter and grower diets were provided in mash form.

Novel Catheterization

At 27 D of age, a catheter was placed in the brachial vein of each bird to facilitate repeated blood collections on the same bird. The catheter aided in preventing hematoma due to a repeated puncture of the vein. Lidocaine (2%) was administered subcutaneously to anesthetize the area of catheter placement. A 22-gauge × 1-inch intravenous catheter (B. Braun, Melsungen, Germany) was placed in the brachial vein of each bird. Each catheter was sutured using 3-0 silk string and glued with tissue adhesive to secure the attachment. The catheter was flushed with heparinized saline (0.9% sodium chloride and 100 unit/mL heparin) immediately after placement and every 6 hours following

placement to prevent clotting. Birds were allowed a 15-hour recovery period to become acclimated with the catheter. Feed consumption during the recovery period was measured to ensure broiler well-being.

Dietary Treatments

At 28 D of age, the common grower diet was removed from each cage and broilers were immediately provided experimental diets. Each broiler received 1 of 3 dietary treatments in mash form (Table 3.1). Treatment 1 was the positive control (**PC**) diet formulated to contain adequate calcium (0.76%) and non-phytate phosphorus (0.36%) concentrations without phytase addition according to the primary breeder recommendations (Aviagen, 2016). A negative control (**NC**) basal diet was formulated to contain 0.165 and 0.150% lower calcium and non-phytate phosphorus concentrations, respectively, compared with the PC diet. Treatments 2 and 3 were established by supplementing the NC basal diet with phytase at the expense of sand to contain 400 and 1,200 FTU/kg of diet, respectively. These concentrations were selected to obtain a 3-fold increase of phytase activity to ensure rapid phytate degradation and inositol liberation. Phytase used was an *E. coli* phytase expressed in *Trichoderma reesei* (Quantum Blue 5G, AB Vista, Marlborough, UK). One FTU is defined as the quantity of phytase required to release 1 µmol of monocalcium phosphate from 0.0051 mol/L sodium phytate in 1 minute at pH 5.5 and 37°C (Simons et al., 1990). All experimental diets were formulated with corn and soybean meal as primary ingredients to contain AME_n, crude protein, and digestible Lys at 3,185 kcal/kg, 18.1%, and 1.08%, respectively. Approximately 50 g of diet sample was collected from each bag of feed (approximately 18 kg). Diet sample was mixed thoroughly and a representative sample was analyzed for phytase activity by

ELISA specific for Quantum Blue (ESC, Standard Analytical Method, SAM099; AB Vista) similarly to the method by Engelen et al. (2001).

Blood Collection

A baseline blood collection was performed approximately 1 hour prior to feeding experimental diets without fasting. Then, blood samples were collected at 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, 240, and 480 minutes following feeding of experimental diets to birds. To avoid blood sample contamination with saline, 0.3 mL residual blood was collected using 1 mL syringe (25-gauge needle). Then, a 1 mL of blood sample was collected using 1 mL syringe (25-gauge needle), transferred to a 1 mL heparinized tube (BD Microtainer, BD Vacutainer Systems, Franklin Lakes, NJ), and placed on ice until centrifugation. The catheter was flushed with 0.3 mL heparinized saline (0.9% sodium chloride and 100 unit/mL heparin) after each blood collection to prevent blood clotting. Blood samples were centrifuged at 1,643 × g for 10 minutes to separate plasma from the whole blood and were stored at –20°C until further analysis.

Digesta and Tissue Collection

At the conclusion of blood collections, broilers were euthanized using carbon dioxide asphyxiation followed by cervical dislocation. Gizzard contents were collected by carefully scraping feed contents into a Whirl-Pak bag (Nasco, Fort Atkinson, WI) and ileal digesta samples were collected by gently flushing out the content of the terminal ileum using deionized-distilled water into Whirl-Pak bags (Nasco, Fort Atkinson, WI). The terminal ileum section is defined as the last 1/3 of the section between the Meckel's diverticulum and approximately 4 cm anterior from the ileo-cecal junction (Kluth et al., 2005; Rodehutscord et al., 2012). Both gizzard content and ileal digesta samples were

immediately frozen by submersion in liquid nitrogen for approximately 5 minutes to terminate phytase activity. Samples were kept on ice and stored at –20°C until later analysis.

Segments of the small intestine were separated into duodenum (pancreatic loop), jejunum (from the distal duodenal loop to the Meckel's diverticulum), and ileum (from the Meckel's diverticulum to the ileo-cecal junction) for analysis of intestinal alkaline phosphatase (**ALP**) activity. Each segment was longitudinally cut and gently rinsed with a cold phosphate-buffered saline to remove any digesta material. Tissue samples were wrapped in aluminum foil and placed in Whirl-Pak bags (Nasco, Fort Atkinson, WI). Immediately, samples were frozen by submersion in liquid nitrogen for approximately 5 minutes to terminate alkaline phosphatase activity. Tissue samples were stored at –20°C until further analysis.

Chemical Analyses

Plasma samples were separated into 2 aliquots for analyses. One aliquot was sent to the College of Veterinary Medicine at Auburn University for the determination of plasma ALP using colorimetric assay according to the method by Schumann et al. (2011). Each sample was analyzed in duplicate using Hitachi Cobas C331 (Roche Diagnostic, Indianapolis, IN). The other aliquot was mixed with 1 M perchloric acid in a 1:2 ratio (plasma:$HClO_4$) to precipitate all protein. The solution was centrifuged at 14,000 × g for 10 minutes to collect the supernatant. Samples were analyzed in duplicate for inositol and glucose concentrations using HPLC with pulsed amperometric detection at the University of East Anglia School of Biological Sciences in Norwich, England. Samples were diluted 50 fold in 18.2 mohm × cm water. An aliquot (20 µL) was injected into a 4 mm × 250

mm MetroSep Carb 2 (Metrohm, Runcorn, UK) HPLC column. The column was eluted at a flow rate of 0.5 mL/minute with 150 mM NaOH. Another aliquot (5 µL) was injected onto a 2 mm × 100 mm Metrosep Carb 2 (Metrohm, Runcorn, UK) column with guard column eluted at a flow rate of 0.2 mL/minute with the same solvent. Inositol and glucose peaks were integrated with Chromeleon (ThermoFisher Scientific, Waltham, MA) and DataApex Clarity (DataApex, Prague, Czech Republic) software packages. Concentrations of inositol and glucose were determined by comparing results with standards using a linear least-squares regression.

Samples of gizzard content and ileal digesta were lyophilized (VirTis Genesis 25ES, SP Industries Inc., Warminster, PA) and ground with an electric coffee grinder and were sent to the University of East Anglia School of Biological Science in Norwich, England. Samples were analyzed for inositol hexa-phosphate (**IP6**), inositol penta-phosphate (**IP5**), inositol tetra-phosphate (**IP4**), and inositol tri-phosphate (**IP3**) concentrations using high-performance ion chromatography with postcolumn derivatization and UV detection at 290 nm. In addition, inositol was quantified using HPLC with pulsed amperometric detection (Laird et al., 2016).

Frozen segments of small intestine tissue samples were thawed in ice for 4 hours. The mucosa was gently scraped using a glass slide and placed into a 1.5 mL tube. Mucosa samples were homogenized in a PBS solution with a 20:1 ratio (PBS/mucosa, v/w) using a glass homogenizer. The suspension was subjected for ultrasonication for 15 minutes to further lyse the cell membrane. Homogenates were centrifuged for 15 minutes at 1,500 × g and the supernatant was collected for analysis. Intestinal ALP was analyzed using chicken ALP ELISA kit according to the manufacturer's procedure (ABclonal,

Woburn, MA). Absorbance was measured on a spectrophotometer at 450 nm. (SpectraMax Plus 384, Molecular Devices LLC., San Jose, CA).

Statistical Analyses

Dietary treatments were randomly placed into a randomized complete block design with cage location served as the blocking factor. Individual bird served as the experimental unit with 8 replications. Concentrations of IP3, 4, 5, 6, and inositol in gizzard content and ileal digesta were subjected to a 1-way analysis of variance using the MIXED procedure of SAS (2011) by the following mixed-effects model:

$$Y_{ij} = \mu.. + \tau_i + \beta_j + \varepsilon_{ij}$$

where $\mu..$ is the overall mean; the τ_i are fixed factor level effects of ith dietary treatment (Treatments 1, 2, and 3) such that $\sum \tau_i = 0$; the β_j are identically and independently normally distributed random block effects with mean 0 and variance ${\sigma^2}_\beta$ such that $\sum \beta_j = 0$; and the ε_{ij} are identical and independent random errors that follow a normal distribution with mean 0 and variance σ^2. Statistical significance was considered at $P \leq 0.05$.

Plasma inositol, ALP, and glucose concentrations were analyzed using a 2-way repeated measure analysis of variance with dietary treatment and time as factors. The analysis was conducted using the GLM procedure of SAS (2011) by the following model:

$$Y_{ijk} = \mu ... + \tau_i + \beta_i + (\tau\beta)_{ij} + \pi_{k(i)} + (\beta\pi)_{jk(i)} + \varepsilon_{ijk}$$

where $\mu..$ is the overall mean; the τ_i are fixed factor level effects corresponding to ith dietary treatment (Treatments 1, 2, and 3) such that $\sum \tau_i = 0$; the β_j are effects of jth collection time (0 to 480 minutes) such that $\sum \beta_j = 0$; the $(\tau\beta)_{ij}$ are the interaction of the ith dietary treatment and jth collection time with $\sum(\tau\beta)_{ij} = 0$; the $\pi_{k(i)}$ represent the

random effects of bird *k* nested within dietary treatment *i*; the $(\beta\pi)_{jk(i)}$ are the random interaction effects of bird *k* and collection time *j* nested within dietary treatment *i*; and the ε_{ijk} are random errors that follow a normal distribution with mean 0 and variance σ^2. The baseline plasma concentration was used as a covariate because blood collection was performed prior to feeding experimental diets. Furthermore, orthogonal polynomial contrasts were performed to determine the effects of dietary treatments over time from 20 to 240 minutes after feeding experimental diets. A pre-planned orthogonal contrast was used to determine differences in plasma concentrations between 240- and 480-minute collections. Statistical significance was considered at $P \leq 0.05$.

RESULTS AND DISCUSSION

Experimental diet analyses determined that phytase activity in Treatments 2 and 3 were slightly lower than calculated values (Table 3.1). However, a 3-fold increase in phytase activity from Treatments 2 to 3 was maintained. Analyzed concentrations of total IP6 to IP3 and inositol in experimental diets were in agreement among dietary treatments (Table 3.2). Broilers grew optimally with an average body weight of 1.77 kg prior to catheter placement from 1 to 27 D of age. During a 15-hour recovery period after catheter placement, feed intake was 96 g per bird (CV = 20%). Additionally, the consumption of experimental diets was 70 g per bird (CV = 18%) during an 8-hour experimental period. According to the primary breeder recommendations, daily feed intake of broilers at 28 D of age was 150 g per bird (Aviagen, 2016). Therefore, feed intake of broilers during the 15-hour recovery period and 8-hour experimental period were adequate.

In the gizzard contents, concentrations of IP6 to IP3 and inositol of broilers were not different ($P > 0.05$) between broilers fed diets with phytase addition at 400 FTU/kg

compared with feeding the PC diet (Table 3.2). However, increasing phytase supplementation to 1,200 FTU/kg reduced ($P = 0.001$) IP6 concentration by 70% while increasing ($P = 0.001$) inositol concentrations by 2.5 fold compared with the addition of 400 FTU/kg phytase. Phytase supplementation had no effects ($P > 0.05$) on IP4 and IP5 concentrations in the gizzard content. Conversely, the concentration of IP3 in the gizzard contents of broilers provided the PC diet was 50% lower ($P = 0.028$) than the IP3 concentration in broilers consuming diet supplemented with 1,200 FTU/kg phytase. Total IP3 to IP6 concentrations were not different ($P > 0.05$) between broilers fed the PC diet than those fed diet supplemented with 400 FTU/kg phytase. However, increasing supplementation of phytase to 1,200 FTU/kg decreased ($P = 0.007$) total IP3 to IP6 concentrations by 63% compared with the PC diet.

In the ileal digesta of broilers, the addition of 400 FTU/kg and 1,200 FTU/kg phytase decreased ($P < 0.001$) IP6 concentration by 41 and 76%, respectively, compared with the PC diet (Table 3.2). No differences ($P = 0.11$) were observed among dietary treatments on IP5 concentration in the ileal digesta of broilers. Effects of adding 400 FTU/kg phytase on the concentrations of IP3, IP4, and inositol in the ileal digesta of broilers were not different ($P > 0.05$) than feeding the PC diet. However, supplementing phytase at 1,200 FTU/kg increased ($P < 0.05$) IP3, IP4, and inositol concentrations by 2.2, 2.0, and 3.5 fold, respectively, compared with the PC fed broilers. Similarly, concentrations of IP4 and inositol in ileal digesta of broilers fed diets supplemented with 1,200 FTU/kg phytase were 2.0 and 2.9 fold higher ($P < 0.01$), respectively, compared with those in broilers provided 400 FTU/kg supplemented diet. Phytase supplementations

at 400 and 1,200 FTU/kg decreased ($P < 0.001$) total IP3 to IP6 concentrations by 37 and 64%, respectively, compared with the PC diet.

Phytate degradation in the gizzard is critical as the gizzard is the major site of phytase activity and phytate solubility due to its low pH (Selle et al., 2000; Tamim et al., 2004; Selle and Ravindran, 2007). The solubility of phytate at a low pH allows the interaction of phytase and phytate to cleave the carbon-phosphate bond; hence, reducing anti-nutritive effects of phytate (Zeller et al., 2015). In the current study, there were no differences in IP3 to IP6 and inositol concentrations between broilers fed the control diets and the diet with 400 FTU/kg addition. In contrast, the reduction of total IP3 to IP6 and the increase of inositol when broilers were fed diets with 1,200 FTU/kg phytase indicated the importance of supplementing phytase beyond 1,000 FTU/kg to enhance phytate hydrolysis. In the ileal digesta of broilers, both concentrations of phytase effectively increased phytate degradation, which led to increased inositol liberation. These responses have also been reported in several previous publications, which indicated that the addition of *E. coli* phytase at 500, 1,000, and 1,500 FTU/kg from 1 to 21 D of age decreased IP6 and IP5 in the gizzard contents and ileal digesta of broilers (Walk et al., 2014; Beeson et al., 2017).

Despite the reduction of IP6 due to phytase supplementation, there were accumulations of IP3 and IP4 in the gizzard content and the ileal digesta of broilers. Beeson et al. (2017) also noted that feeding broilers with diets increasing phytase concentrations from 0, 500, and 1,500 FTU/kg resulted in quadratic reductions of IP5 and IP6, but not on IP3 and IP4 in the ileal digesta of 21 D old broilers. Similarly, Zeller et al. (2015) measured the proportion of IP3, IP4, and IP5 to the total IP3 to IP5 concentrations

in the ileum of broilers fed diets containing added monocalcium phosphate with 0, 500, and 12,500 FTU/kg of *E. coli* phytase expressed in *Trichoderma reesei*. These authors observed that when broilers were fed diets with 500 FTU/kg phytase, the greatest accumulation of inositol phosphate ester was IP5, which accounted for 62% of the total IP3 to IP5 concentrations. In contrast, the proportion of IP4 in the ileal digesta was the greatest (39% of the total IP3 to IP5 concentrations) among IP3, IP4, and IP5 when broilers were supplemented with 12,500 FTU/kg phytase. In the current study, the supplementation of 400 FTU/kg phytase resulted in IP5 having the greatest proportion (60%) among the total IP3 to IP5 concentrations. Conversely, providing broilers with 1,200 FTU/kg phytase led to the greatest accumulation of IP4 (45%) among the total IP3 to IP5 concentrations. The accumulation of IP4 even with higher phytase doses may indicate that birds were unable to further degrade phytate to lower inositol phosphate esters. The inability to further degrade lower inositol phosphate esters may likely be due to the tendency of phytase to target higher inositol phosphate esters. Wyss et al. (1999) suggested that lower phytate esters are less effective substrates for phytases derived from *A. niger, A. terreus, and E. coli*. It is possible that these phytases may have a higher K_m, which indicates a lower affinity to lower inositol phosphate esters.

The supplementation of 1,200 FTU/kg phytase reduced total IP3 to IP6 concentrations to approximately 1/3 of the concentrations in the gizzard content and the ileal digesta of broilers fed the PC diet. These data indicated that anti-nutritive effects of phytate may still be present and that it may require a higher dose of phytase supplementation to further break down phytate. Persson et al. (1998) reported that lower inositol phosphate esters also have the capacity to bind to metal ions though it may be

reduced compared with those having a higher number of phosphate groups. For example, an individual IP6 molecule could bind 5.8 and 4.9 copper and zinc ions, respectively, while an individual IP3 molecule could bind 3.1 and 3.0 copper and zinc ions, respectively. Regardless of the number of ions, the ability of these phytate molecules to bind mineral ions may decrease their availability to broilers leading to poor growth performance. Therefore, it may be necessary to further increase phytase doses to decrease anti-nutritive effects of phytate. Walk et al. (2018) demonstrated that increasing phytase supplementation from 0, 500, 1,500, and 4,500 FTU/kg linearly decreased total IP2 to IP6 concentrations in the ileal digesta of broilers resulting in a linear increase in body weight gain demonstrating the benefits of higher doses of phytase to further degrade phytate.

The addition of phytase at either 400 or 1,200 FTU/kg in broiler diets did not influence intestinal ALP concentration in the duodenum, jejunum, and ileum (Table 3.3). In the current study, ALP was measured in the small intestine as it may also contribute to phytate degradation. Despite the increase in phytate degradation and inositol liberation due to increasing phytase concentrations, intestinal ALP in broilers was not altered. It appears that intestinal ALP had high variability, which may be associated with its broad role in hydrolyzing phosphate esters. In addition to being present in phytate, phosphate esters can also be found in the lipopolysaccharide of Gram-negative bacteria. Melo et al. (2016) indicated that ALP might also involve in dephosphorylating lipopolysaccharide of bacteria, which suppresses inflammation caused by bacteria. Additionally, ALP may contribute to hydrolyzing ATP along the small intestine (Zhou et al., 2017). These broad functions of intestinal ALP may be a contributing factor causing the high variability with

intestinal ALP concentrations. Hence, intestinal ALP may not be a suitable indicator of phytate degradation in broilers.

In addition, ALP concentration was also evaluated in the plasma of broilers. The baseline plasma ALP concentration of broilers prior to feeding experimental diets was highly variable ($P < 0.001$) (Figure 3.1). Following the introduction of experimental diets, plasma ALP concentration did not differ ($P = 0.45$) among dietary treatments at any of the collection periods. However, effects of time from 20 to 240 minutes displayed a cubic reduction ($P = 0.007$) of plasma ALP concentration of broilers. Additionally, plasma ALP concentration of broilers was not altered ($P = 0.94$) among dietary treatments between 240 and 480 minutes. Alkaline phosphatase is an enzyme primarily responsible for bone mineralization. Due to its function, ALP concentration in blood circulation can be altered by dietary calcium and phosphorus. For example, plasma ALP concentration in broilers decreased by 5% when increasing dietary non-phytate phosphorus from 4.5 to 5.5 g/kg in a 21-D experimental period (Baradaran et al., 2017). Furthermore, the inclusion of 500 FTU/kg phytase in broiler diet decreased plasma ALP concentration by 24% compared with broilers fed the control diet indicating a reduction of ALP as mineral availability increases (Huff et al., 1998). In contrast, the measurement of plasma ALP concentration over time in the current study did not differ among dietary treatments. Cowieson et al. (2017) did not observe effects of feeding diets supplemented with various phytase concentrations on plasma ALP concentration in pigs over 6 hours. These researchers attributed the lack of differences in plasma ALP concentration to the homeostatic mechanism of plasma phosphorus and calcium, which may take longer than 6 hours.

Plasma inositol concentration of broilers did not vary (P = 0.94) at each collection time despite receiving different dietary treatments (Figure 3.2). Similarly, no dietary treatment effects (P = 0.37) were observed on the concentration of plasma inositol in broilers. However, a cubic increase (P = 0.003) of plasma inositol concentration from 20 to 240 minutes was noted regardless of dietary treatments. From 240 to 480 minutes, there was a 12% reduction from 251 to 222 nmol/mL (P = 0.019) of plasma inositol concentration regardless of dietary treatment (P = 0.30). Interestingly, despite the increase of inositol concentration in the gizzard content and the ileal digesta of broilers due to increasing phytase supplementation, no differences of inositol concentrations among dietary treatments were observed in the plasma of broilers over the time course of this study. In contrast, Cowieson et al. (2015) noted an increase in plasma inositol concentration of broilers with increasing doses of bacterial phytase expressed in *Aspergillus oryzae* from 0, 1,000, 2,000, and 3,000 FTU/kg. Additionally, Sommerfeld et al. (2018) observed an increase of inositol concentration in the ileal digesta of broilers with increasing *E. coli* phytase supplementation from 0, 500, 1,500, and 3,000 FTU/kg. However, the only difference noted in the plasma inositol concentration was a 39% increase when adding 500 FTU/kg phytase compared with the control diet, while no further increase of plasma inositol was noted when increasing phytase to 1,500 and 3,000 FTU/kg. In both studies (Cowieson et al., 2015; Sommerfeld et al., 2018), plasma inositol concentration was measured after feeding diets with phytase addition for 22 D. In the current study, plasma inositol was measured within 8 hours after feeding phytase supplemented diets; hence, it is possible that changes in the plasma inositol concentration may require feeding phytase beyond 8 hours. When fed a higher phytase concentration,

birds may respond by increasing inositol transporters in the small intestine. Walk et al. (2018) demonstrated that broilers receiving microbial phytase added diets at 500, 1,500, and 4,500 FTU/kg from 1 to 21 D of age exhibited higher expressions of sodium/glucose cotransporter 11 and H^+/myo-inositol transporter compared with birds consuming diets without phytase supplementation. The increase of gene expression of inositol transporters may be the factor that caused an increase in plasma inositol concentration when birds were fed increasing phytase doses beyond an 8-hour period.

The lack of response in plasma inositol concentrations among dietary treatments is also in contrast with a previous time-series study in pigs. Cowieson et al. (2017) fed pigs (average body weight of 28.7 kg) with diets containing 0, 1,000, and 3,000 FTU/kg of bacterial phytase expressed in *Aspergillus oryzae* as well as 0.2% inositol. In this study, plasma inositol of pigs was measured at 30 minutes before feeding experimental diets, then at 0, 15, 30, 45, 60, 90, 120, 150, 180, 240, 300, and 360 minutes after feeding experimental diets. These researchers indicated that plasma inositol concentration varies over time due to experimental diets with maximum concentration reached at 120 minutes after pigs were fed diets supplemented with inositol. However, at 360 minutes after feeding experimental diets, the greatest plasma inositol concentration was obtained by feeding diets with 3,000 FTU/kg phytase. The differences between the study by Cowieson et al. (2017) and the current study may likely be associated with species differences in the utilization of inositol post-absorption. Avian hemoglobin has been reported to utilize inositol as a precursor of IP5 for modulating the oxygen affinity of hemoglobin in the red blood cells, while in mammals, this process in facilitated by 2,3-diphosphoglycerate (Lutz, 1980). An *in vitro* study using 5 D old chicken plasma

incubated with inositol for 6 hours demonstrated the increase of inositol uptake by the erythrocyte and inositol incorporation to IP5 with increasing incubation time (Isaacks et al., 1982). These mechanisms led to a reduction of plasma free inositol from 14.6 to 10.7 μg/mL within 6 hours incubation period. In the current study, it is possible that inositol may be rapidly re-phosphorylated after absorption to synthesize IP5, which may explain the reason for the lack of differences in plasma inositol concentration of broilers.

Another possible reason for the lack of response to dietary treatments on plasma inositol concentrations in the current study may be related to the rapid metabolism of inositol. Following absorption, inositol may be used for the formation of phosphatidylinositol. The 2 major phosphatidylinositols in the cell membrane are phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 3,4,5-triphosphate, which function as cell growth, differentiation, proliferation, and calcium mobilization (Cooper and Hausman, 2013). In intestinal epithelial cells, inositol may be important to synthesize phosphatidylinositol due to the rapid turnover of intestinal epithelial cells (Moran, Jr., 2016). A previous study indicated that the mucosa of the jejunum is completely replaced within approximately 48 hours in 2 D old chicks (Imondi and Bird, 1966) indicating a rapid turnover of intestinal epithelial cells and the need for phosphatidylinositol formation. However, careful interpretations must be taken when comparing these data and the current study as broilers in the present study were raised up to 28 D. Moreover, inositol may be metabolized in the liver after absorption in the small intestine. An in vitro study using rat liver cells demonstrated that when liver cells were administered with increasing concentrations of inositol, both inositol uptake and phosphatidylinositol synthesis increased (Prpic et al., 1982). Therefore, in the current study, the increase of

inositol liberation due to higher phytase concentrations may lead to an increase of hepatic inositol uptake and phosphatidylinositol formation, which results in the lack of response of dietary treatments on plasma inositol concentrations, especially when inositol was measured following hepatic metabolism in the brachial vein of birds.

No interactive ($P = 0.74$) effects between dietary treatments and collection time were noted on plasma glucose concentration of broilers (Figure 3.3). In contrast, adding 400 FTU/kg (15,045 nmol/mL) resulted in a higher ($P = 0.038$) plasma glucose concentration of broilers compared with those without phytase (14,422 nmol/mL) and with 1,200 FTU/kg (14,337 nmol/mL) addition regardless the collection time. Furthermore, plasma glucose concentration increased ($P = 0.004$) in a cubic manner from 20 to 240 minutes after feeding experimental diets. There was a 6.8% reduction ($P < 0.001$) in plasma glucose from 240 (15,528 nmol/mL) to 480 minutes (14,478 nmol/mL) collection time regardless the dietary treatments. Phytase addition in broiler diets may affect glucose concentration in the plasma through the reduction of endogenous sodium loss. A previous study indicated that an increase in dietary phytate concentration causes an increase in endogenous sodium losses, while the addition of phytase decreases endogenous sodium loss (Cowieson et al., 2004). Because the majority of glucose absorption in the small intestine is facilitated with sodium-dependent active transport system, the increase in the availability of sodium by phytase addition may also increase glucose concentration in the plasma (Chen et al., 2016). Recent research indicated that the supplementation of phytase at 500 FTU/kg in broiler diets increased blood glucose concentration by 6.7% compared with diets without phytase addition (Cowieson et al., 2013). In the current study, the addition of 400 FTU/kg also increased plasma glucose

concentration of broilers by 4.3% regardless of the collection time compared with those fed diets without phytase addition. Interestingly, when phytase supplementation was increased to 1,200 FTU/kg, no difference in plasma glucose concentration was observed compared with the control-fed birds and this response is not readily explained. However, other studies have also noted the lack of response from adding phytase on blood glucose concentration (Cowieson et al., 2015; 2017), which may be related to the competition of glucose and inositol for similar sodium transport systems in the small intestine (Chukwuma et al., 2016). Therefore, the effects of phytase on blood glucose concentration may require further investigation due to the variable results reported in the literature and the current research.

These data demonstrated benefits of adding phytase at 1,200 FTU/kg as it effectively increased phytate degradation and inositol liberation in the gizzard and small intestine of broilers compared with the addition of 400 FTU/kg. Despite the reduction of phytate concentrations, 1/3 of the total IP3 to IP6 concentrations remained in the small intestine even with the addition of 1,200 FTU/kg suggesting the need for supplementing higher doses of phytase to further degrade phytate. Although increasing phytase concentrations increased inositol concentrations in the gizzard and ileum of broilers, phytase doses did not alter plasma inositol concentrations over time. Future research is warranted to investigate plasma inositol concentration of broilers beyond an 8-hour period after feeding diets with phytase addition greater than 1,200 FTU/kg.

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Table 3.1 Ingredient and nutrient compositions of experimental diets formulated to contain 0, 400, and 1,200 phytase unit (FTU)/kg of phytase fed to broilers at 28 D of age[1]

Item	PC[2]	NC + 400 FTU/kg[2]	NC + 1,200 FTU/kg[2]
Ingredient, % as-fed			
Corn	64.12	65.75	65.75
Soybean meal	27.85	27.60	27.60
Vegetable oil	4.51	3.90	3.90
Dicalcium phosphate	1.48	0.66	0.66
Calcium carbonate	0.91	0.94	0.94
Sodium chloride	0.40	0.40	0.40
DL-Methionine	0.24	0.24	0.24
L-Lysine•HCl	0.14	0.14	0.14
L-Threonine	0.07	0.07	0.07
Vitamin premix[3]	0.05	0.05	0.05
Trace mineral premix[4]	0.10	0.10	0.10
Choline chloride	0.07	0.06	0.06
TBCC[5]	0.02	0.02	0.02
Phytase[6]	---	0.01	0.03
Sand	0.04	0.04	0.02
Calculated composition, %			
AME_n, kcal/kg	3,185	3,185	3,185
Crude protein	18.13	18.13	18.13
Digestible lysine	0.98	0.98	0.98
Digestible sulfur amino acids	0.74	0.74	0.74
Digestible threonine	0.65	0.65	0.65
Total phosphorus	0.60	0.45	0.45
Non-phytate phosphorus	0.36	0.36	0.36
Calcium	0.76	0.76	0.76
Sodium	0.18	0.18	0.18
Analyzed composition, %			
Phytase activity, FTU/kg[1]	< 50	328	902

[1]One unit of phytase activity (FTU) is defined as the quantity of enzyme required to release 1 µmol of monocalcium phosphate from 0.0051 mol/L sodium phytate in 1 minute at pH 5.5 and 37ºC.

[2]PC = Positive Control, NC = Negative Control

[3]Vitamin premix includes per kg of diet: Vitamin A (Vitamin A acetate), 18,739 IU; Vitamin D_3 (cholecalciferol), 6,614 IU; Vitamin E (DL-alpha tocopherol acetate), 66 IU; menadione (menadione sodium bisulfate complex), 4 mg; Vitamin B_{12} (cyanocobalamin), 0.03 mg; folacin (folic acid), 2.6 mg: D-pantothenic acid (calcium pantothenate), 31 mg; riboflavin (riboflavin), 22 mg; niacin (niacinamide), 88 mg; thiamin (thiamin mononitrate), 5.5 mg; biotin (biotin), 0.18 mg; and pyridoxine (pyridoxine hydrochloride), 7.7 mg.

[4]Trace mineral premix include per kg of diet: Mn (manganese sulfate), 120 mg; Zn (zinc sulfate), 100 mg; Fe (iron sulfate monohydrate), 30 mg; Cu (tri-basic copper chloride), 8 mg; I (ethylenediamine dihydriodide), 1.4 mg; and Se (sodium selenite), 0.3 mg.

[5]TBCC = Tri-basic copper chloride (Intellibond C, Micronutrients, Indianapolis, IN)
[6]Quantum Blue 5G, AB Vista, Marlborough, UK (Analyzed as 4,000 FTU/g)

Table 3.2 Inositol and inositol phosphate (IP) ester concentrations in experimental diets, gizzard content, and ileal digesta of broilers fed diets formulated to contain 0, 400, and 1,200 phytase unit (FTU)/kg of phytase at 28 D of age[1, 2]

Experimental Diets[3]	IP6	IP5	IP4	IP3	Inositol	∑IP6-IP3[4]
			Total dietary concentrations, nmol/g[5]			
Positive control	15,762	2,895	1,200	829	1,407	20,686
Negative control + 400 FTU/kg phytase	16,769	1,408	432	553	1,261	19,162
Negative control + 1,200 FTU/kg phytase	18,444	1,850	814	697	1,337	21,805
			Gizzard content, nmol/g			
Positive control	6,144[a]	741	327	280[b]	1,071[b]	7,493[a]
Negative control + 400 FTU/kg phytase	4,176[a]	876	762	407[ab]	1,360[b]	6,221[a]
Negative control + 1,200 FTU/kg phytase	1,264[b]	293	700	580[a]	2,703[a]	2,837[b]
Pooled standard error	826	188	164	77	285	1,020
			Ileal digesta, nmol/g			
Positive control	56,456[a]	6,866	1,613[b]	713[b]	4,667[b]	65,698[a]
Negative control + 400 FTU/kg phytase	33,244[b]	4,907	2,257[b]	958[ab]	5,617[b]	41,301[b]
Negative control + 1,200 FTU/kg phytase	13,472[c]	3,864	4,518[a]	1,590[a]	16,485[a]	23,444[b]
Pooled standard error	5,237	1,034	626	206	1,789	6,395
			Probabilities			
Gizzard content	0.001	0.06	0.14	0.028	0.001	0.007
Ileal digesta	< 0.001	0.11	0.005	0.012	< 0.001	< 0.001

[1]Values represent least-square means of 8 replicate cages with 1 bird per replicate at placement.

[2]One unit of phytase activity (FTU) is defined as the quantity of enzyme required to release 1 µmol of monocalcium phosphate from 0.0051 mol/L sodium phytate in 1 minute at pH 5.5 and 37ºC.

[3]The positive control diet was formulated to contain adequate calcium and phosphorus concentrations. The negative control diet was formulated to contain 0.165 and 0.150% lower calcium and phosphorus concentrations, respectively, compared with the positive control diet.

[4]Total amount of IP3 to IP6 concentrations

[5]Analyzed concentrations of inositol and inositol phosphate esters in experimental diets

[a-c] Means not sharing a common superscript within column in each section differ significantly ($P < 0.05$).

Table 3.3 Intestinal alkaline phosphatase concentrations of broilers fed diets formulated to contain 0, 400, and 1,200 phytase unit (FTU)/kg of phytase at 28 D of age (ng/mL)[1, 2]

Experimental Diets[3]	Duodenum	Jejunum	Ileum
Positive Control	8.42	33.15	87.35
Negative Control + 400 FTU/kg	7.53	35.39	107.39
Negative Control + 1,200 FTU/kg	12.74	36.52	102.37
Pooled standard error	2.40	8.13	16.84
P-Value	0.18	0.88	0.41

[1]Values represent least-square means of 8 replicate cages with 1 bird per replicate at placement.
[2]One unit of phytase activity (FTU) is defined as the quantity of enzyme required to release 1 µmol of monocalcium phosphate from 0.0051 mol/L sodium phytate in 1 minute at pH 5.5 and 37ºC.
[3]The positive control diet was formulated to contain adequate calcium and phosphorus concentrations. The negative control diet was formulated to contain 0.165 and 0.150% lower calcium and phosphorus concentrations, respectively, compared with the positive control diet.

Figure 3.1 Effects of dietary treatments on plasma alkaline phosphatase concentration of broilers at 28 D of age (8 birds per treatment). The positive control diet (—◆—) was formulated to contain adequate calcium and non-phytate phosphorus. A negative control diet was formulated to contain 0.165 and 0.150% lower calcium and non-phytate phosphorus concentrations, respectively. Phytase was added to the negative control diet at 400 (···●··) and 1,200 (- -▲- -) FTU/kg to establish 2 other dietary treatments. Baseline concentration of plasma alkaline phosphatase concentration at 0 minute was used as a covariate. Linear, quadratic, and cubic effects over time from 20 to 240 minutes were analyzed using two-way repeated measure analysis of variance with dietary treatment and time as factors.

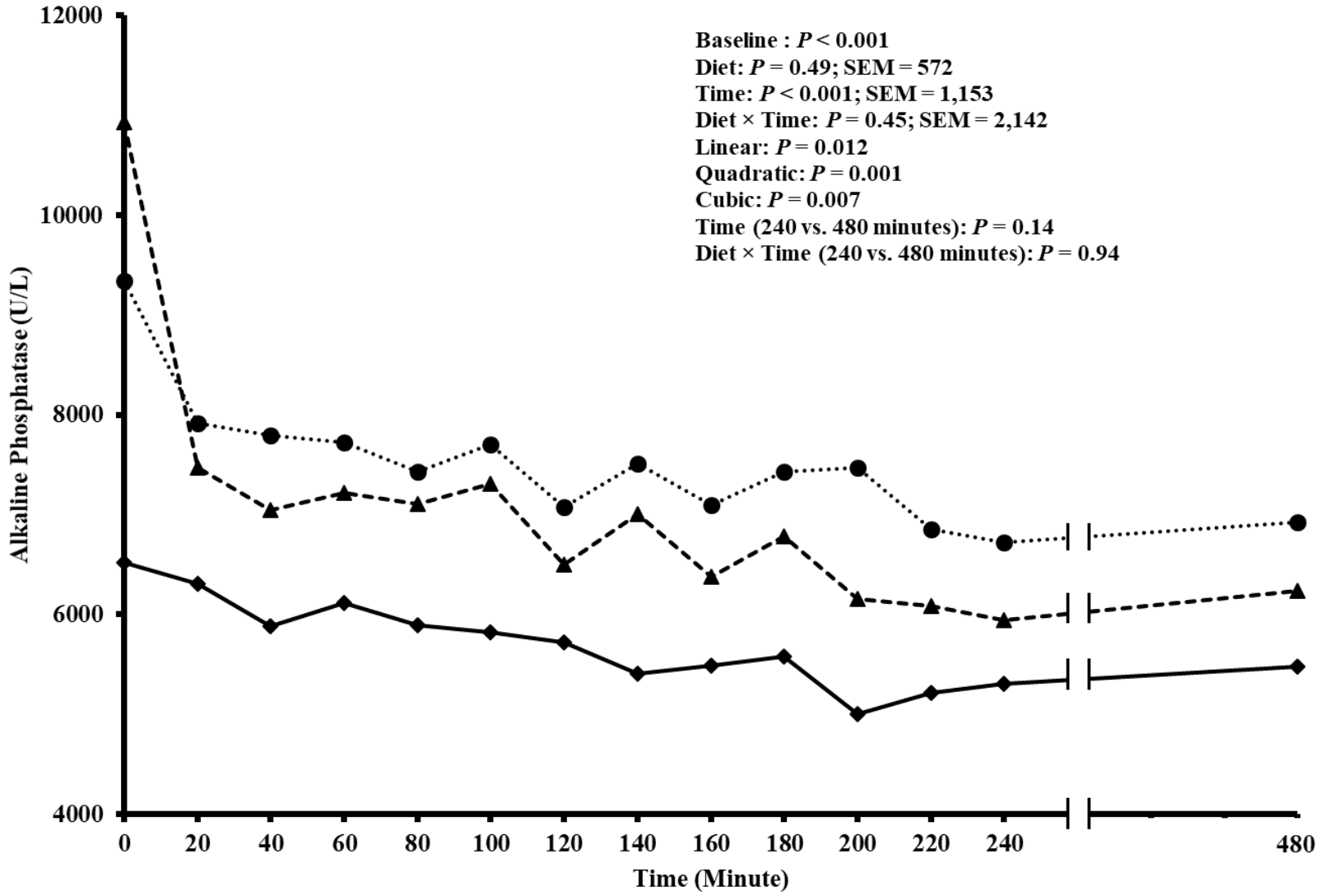

Baseline : P < 0.001
Diet: P = 0.49; SEM = 572
Time: P < 0.001; SEM = 1,153
Diet × Time: P = 0.45; SEM = 2,142
Linear: P = 0.012
Quadratic: P = 0.001
Cubic: P = 0.007
Time (240 vs. 480 minutes): P = 0.14
Diet × Time (240 vs. 480 minutes): P = 0.94
Alkaline Phosphatase (U/L)
12000
10000
8000
6000
4000
0
20
40
60
80
100
120
140
160
180
200
220
240
480
Time (Minute)

Figure 3.2 Effects of dietary treatments on plasma inositol concentration of broilers at 28 D of age (8 birds per treatment). The positive control diet (—◆—) was formulated to contain adequate calcium and non-phytate phosphorus. A negative control diet was formulated to contain 0.165 and 0.150% lower calcium and non-phytate phosphorus concentrations, respectively. Phytase was added to the negative control diet at 400 (···●··) and 1,200 (- -▲- -) FTU/kg to establish 2 other dietary treatments. Baseline concentration of plasma inositol concentration at 0 minute was used as a covariate. Linear, quadratic, and cubic effects over time from 20 to 240 minutes were analyzed using two-way repeated measure analysis of variance with dietary treatment and time as factors.

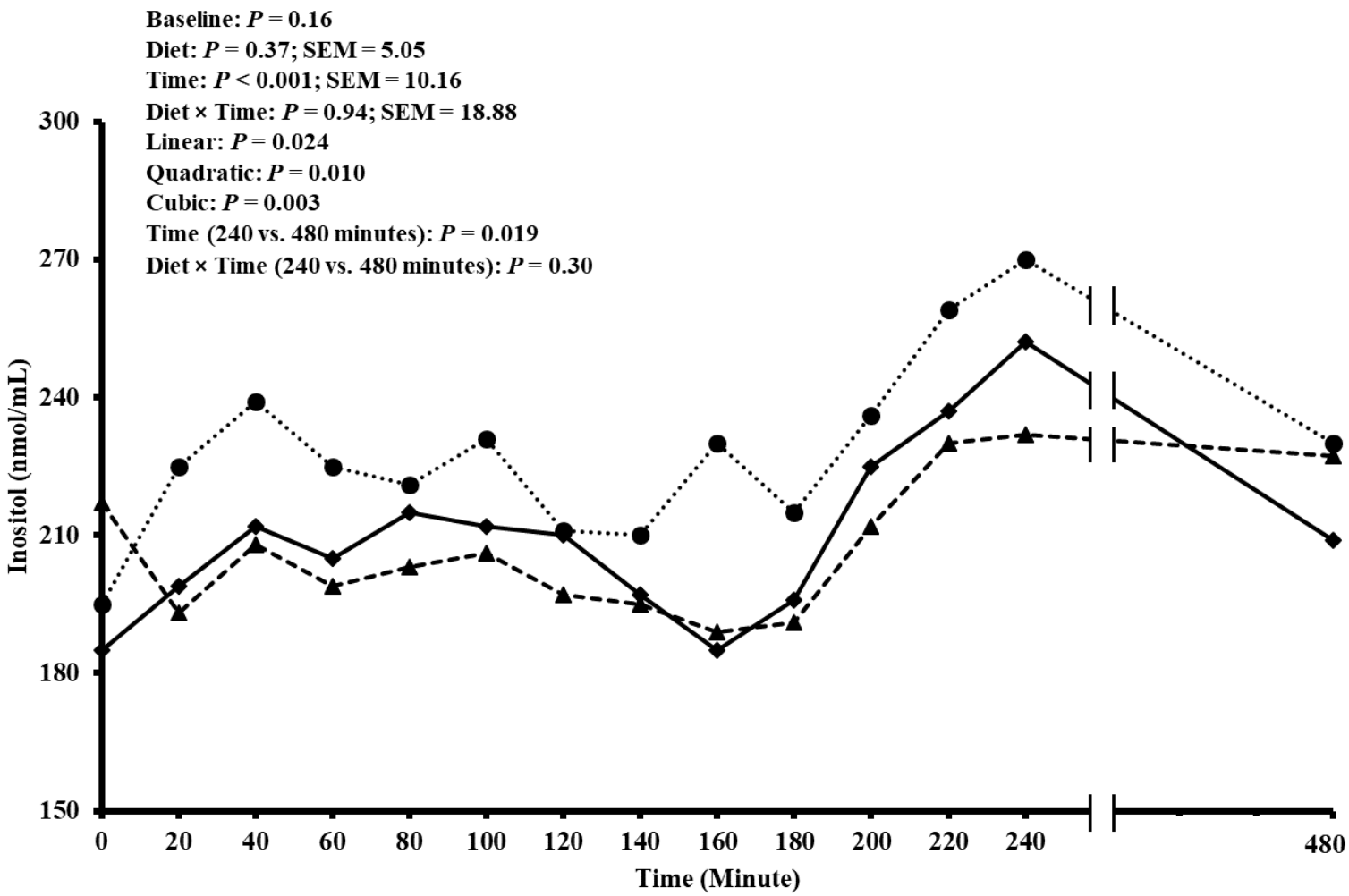
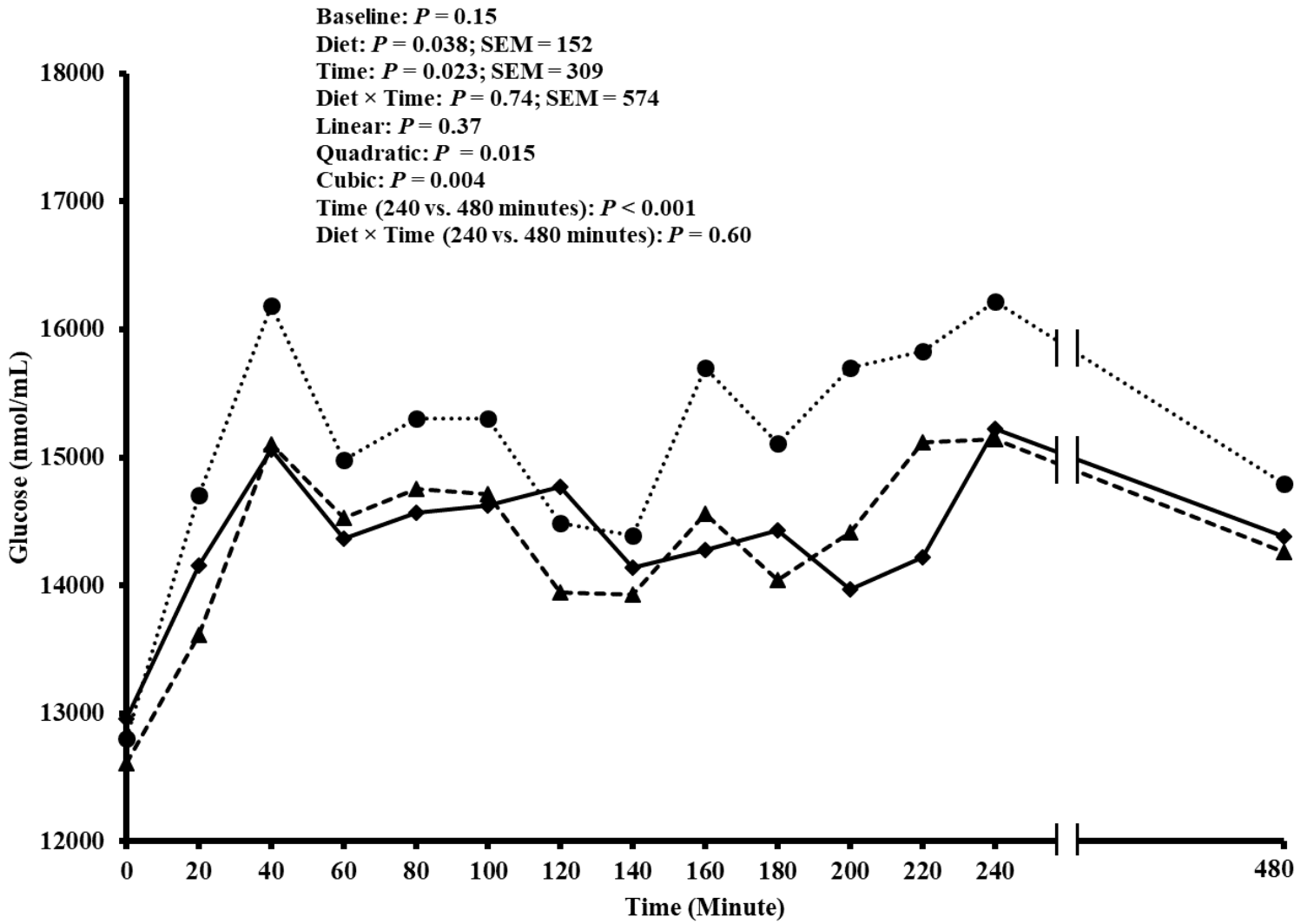

Baseline: P = 0.16
Diet: P = 0.37; SEM = 5.05
Time: P < 0.001; SEM = 10.16
Diet × Time: P = 0.94; SEM = 18.88
Linear: P = 0.024
Quadratic: P = 0.010
Cubic: P = 0.003
Time (240 vs. 480 minutes): P = 0.019
Diet × Time (240 vs. 480 minutes): P = 0.30
Inositol (nmol/mL)
300
270
240
210
180
150
0
20
40
60
80
100
120
140
160
180
200
220
240
480
Time (Minute)

Figure 3.3 Effects of dietary treatments on plasma glucose concentration of broilers at 28 D of age (8 birds per treatment). The positive control diet (—◆—) was formulated to contain adequate calcium and non-phytate phosphorus. A negative control diet was formulated to contain 0.165 and 0.150% lower calcium and non-phytate phosphorus concentrations, respectively. Phytase was added to the negative control diet at 400 (···●··) and 1,200 (- -▲- -) FTU/kg to establish 2 other dietary treatments. Baseline concentration of plasma glucose concentration at 0 minute was used as a covariate. Linear, quadratic, and cubic effects over time from 20 to 240 minutes were analyzed using two-way repeated measure analysis of variance with dietary treatment and time as factors.


Baseline: P = 0.15
Diet: P = 0.038; SEM = 152
Time: P = 0.023; SEM = 309
Diet × Time: P = 0.74; SEM = 574
Linear: P = 0.37
Quadratic: P = 0.015
Cubic: P = 0.004
Time (240 vs. 480 minutes): P < 0.001
Diet × Time (240 vs. 480 minutes): P = 0.60
Glucose (nmol/mL)
18000
17000
16000
15000
14000
13000
12000
0
20
40
60
80
100
120
140
160
180
200
220
240
480
Time (Minute)

IV. INOSITOL AND GRADIENT PHYTASE SUPPLEMENTATION IN BROILER DIETS DURING A 6-WEEK PRODUCTION PERIOD: EFFECTS ON GROWTH PERFORMANCE AND MEAT YIELD

ABSTRACT

An experiment was conducted to evaluate effects of inositol and gradient phytase supplementation on growth performance and meat yield of broilers from 1 to 41 D of age. A total of 1,920 Yield Plus × Ross 708 male chicks were placed in 64 floor pens (30 birds/pen). Each pen received 1 of 8 dietary treatments (8 replicate pens) from 1 to 15, 16 to 29, and 30 to 40 D of age. Treatment 1 was formulated to contain 0.165 and 0.150% lower calcium and phosphorus, respectively, than Treatment 7 (positive control, **PC**). Phytase was added to Treatment 1 at 500, 1,500, 4,500, 13,500, and 40,500 phytase units (**FTU**)/kg to establish Treatments 2 to 6, respectively. Treatment 8 was formulated by adding inositol to Treatment 7 based on the expected inositol liberation in Treatment 6. Feed and birds were weighed at 1, 15, 29, and 40 D of age to determine BW gain, feed intake, and feed conversion. Twelve birds per pen were processed at 41 D of age to determine carcass characteristics. From 1 to 40 D of age, log-quadratic effects of phytase (Treatments 1 to 6) were observed for BW gain ($P = 0.002$) and feed conversion of broilers ($P = 0.018$), whereas feed intake increased log-linearly ($P = 0.045$). The addition of 40,500 FTU/kg phytase increased cumulative BW gain ($P = 0.001$) and decreased

cumulative feed conversion ($P = 0.005$) by 4.7 and 2.6%, respectively, compared with birds fed Treatment 8. Log-quadratic effects of phytase additions were observed for carcass ($P < 0.001$) and breast meat weights ($P = 0.004$). Growth performance and carcass characteristics of broilers fed Treatment 7 were similar ($P > 0.05$) to birds fed Treatment 8. These data demonstrate that the extra-phosphoric effects of phytase may be associated with increased feed intake of broilers. Inositol supplementation did not provide additional benefits to broilers in this study.

INTRODUCTION

Benefits of adding exogenous phytase in broiler diets to liberate phosphorus and other minerals has been previously reviewed in the literature (Selle and Ravindran, 2007; Bedford et al., 2016). However, recent advancement in phytase research has initiated the addition of phytase in greater concentrations to target extra-phosphoric effects of phytase. These effects include increases of amino acid digestibility and apparent metabolizable energy (Ravindran et al., 2001; Gehring et al., 2013), restoration of digestive enzyme activity (Liu et al., 2008), reduction of anti-nutritive effects of phytate (Beeson et al., 2017), and release of inositol (Walk et al., 2014). This practice allows integrators to generate more profit through the reduction of dietary inorganic phosphorus and increased growth and meat yield.

Broiler diets are commonly supplemented with 500 to 1,500 phytase units (**FTU**)/kg to provide phosphorus from the hydrolysis of inositol phosphate (**IP**) 6. As a result, the destruction of IP6 is viewed as the end goal of phytase addition (Bedford and Walk, 2016). However, when focusing on the hydrolysis of IP6 to provide phosphorus, benefits of phytase beyond phosphorus liberation on growth performance and carcass

characteristics may be overlooked. Therefore, optimum growth performance and meat accretion of broilers should also be considered when determining phytase doses to use. Unfortunately, previous research evaluating extra-phosphoric effects of phytase has been mainly conducted up to 3 weeks of age (Walk et al., 2014; Zeller et al., 2015; Beeson et al., 2017). In contrast, the response of extra-phosphoric effects of phytase on growth performance of broilers raised to market age and their carcass characteristics are sparse (Campasino et al., 2014).

Benefits of extra-phosphoric effects of phytase may also arise from the liberation of inositol (Walk et al., 2014; Beeson et al., 2017). Due to its involvement in formation of phosphatidylinositol, inositol may play roles in skeletal muscle development (Lee and Bedford, 2016). However, inconsistency in the response of broilers to inositol supplementation has been observed (Cowieson et al., 2013; Zyla et al., 2013; Farhadi et al., 2017; Sommerfeld et al., 2018; Pirgozliev et al., 2019), which may require further investigation. In this experiement, it was hypothesized that increasing phytase supplementation will enhance growth performance and carcass characteristcis of broilers. Therefore, this experiment was conducted to determine effects supplementing inositol and elevated phytase doses on growth performance and meat yield of broilers during a 41-D production period.

MATERIALS AND METHODS

All experimental procedures regarding live birds were approved by the Institutional Animal Care and Use Committee at Auburn University (PRN 2018-3254).

Husbandry Practices and Measurements

A total of 1,920 Yield Plus × Ross 708 male broilers were obtained from a commercial hatchery (Aviagen North America, Huntsville, AL). All birds were vaccinated for Marek's disease, Newcastle disease, and infectious bronchitis. Birds were randomly distributed into 64 floor-pens (30 birds per pen; 0.07 m^2 per bird) in a solid-sided house equipped with vent boards, exhaust fans, evaporative cooling pads, forced-air heaters, and an electric controller to maintain optimum ventilation and temperature. Each pen was provided with a tube feeder, a nipple drinker line, and used litter. At placement, the house temperature was adjusted to 33°C and gradually decreased to 20°C at 41 D of age. Photoperiod was provided at 23L:1D from 1 to 7 D of age and 20L:4D for the remainder of the experimental period. Additionally, light intensity was set at 30 lux at chick placement and was subsequently dimmed to 10 and 5 lux at 7 and 14 D post-hatch, respectively. The intensity was verified at bird level (30 cm) using a photometric sensor (Extech 407026 Heavy Duty Light Meter, Extech Instruments, Nashua, NH). Three feeding programs were provided from 1 to 15, 16 to 29, and 30 to 41 D of age as starter, grower, and finisher diets, respectively. The starter diet was provided in a crumble form, while the grower and finisher diets were provided in pellet form. Feed and water were provided ad libitum throughout the experimental period.

Broilers and feed were weighed at 1, 15, 29, and 40 D of age to determine BW gain, feed intake, and feed conversion ratio (**FCR**) as a pen basis. The incidence of mortality was recorded daily. At 41 D of age, 12 birds per pen were selected for processing. Feed was withdrawn from each pen 10 hours prior to processing. Selected birds were placed in coops and transported to the Auburn University Pilot Processing

Plant. Broilers were hung on shackles, electrically stunned, exsanguinated, scalded, picked, and manually eviscerated. Following 3 hours of chilling in an ice bath, carcass (without abdominal fat) and abdominal fat weights were recorded. The whole carcass was cut into the front and back half portions and was packed in ice for 18 hours. Pectoralis major (boneless breast) and minor (tenders) muscles were deboned from the front half portion of the carcass and were weighed to determine total breast meat yield. Carcass yield, total breast meat yield, and abdominal fat percentage were calculated relative to the 40-D live BW.

Dietary Treatments

Broilers received 1 of 8 dietary treatments during starter (1 to 15 D), grower (16 to 29 D), and finisher (30 to 41 D, Table 4.1) periods. Corn and soybean meal were used as primary ingredients in each dietary treatment. A negative control (**NC**) diet (Treatment 1) was formulated to contain 0.165 and 0.150% lower calcium and non-phytate phosphorus concentrations, respectively than the positive control (**PC**) diet (Treatment 7). Dietary concentrations of calcium and non-phytate phosphorus in the starter, grower, and finisher periods were formulated based on Aviagen Ross 708 Broiler Nutrition Specification recommendation (Aviagen, 2016). Treatments 2 to 6 were formulated similar to Treatment 1 with additions of *E. coli* phytase from *Trichoderma reesei* at 500, 1,500, 4,500, 13,500, and 40,500 FTU/kg, respectively, at the expense of sand (Quantum Blue 5G, AB Vista, Marlborough, UK). One FTU is defined as the quantity of phytase required to release 1 µmol of inorganic phosphorus from 0.0051 mol/L solution of sodium phytate in 1 minute at pH 5.5 and 37°C (Simons et al., 1990). In Treatment 8, inositol was added to the PC diet (Treatment 7) at 0.26, 0.25, and 0.23% respectively in

the starter, grower, and finisher periods. Inositol was added to evaluate effects of inositol supplementation compared with phytase addition. The amount of inositol addition was calculated based on the expected inositol liberation in Treatment 6 with the assumption that all phytate content in Treatment 6 can be completely degraded, which consequently liberates inositol. Phytate phosphorus concentration was calculated by the amount of feed ingredients multiplied by 0.17 and 0.48% for corn and soybean meal, respectively (AminoDat 5.0, 2016). Then, inositol concentration was calculated by multiplying the concentration of phytate phosphorus in the diet by 0.968, which is the ratio of inositol to phosphorus in phytate. Apparent metabolizable energy in starter, grower and finisher diets was formulated at 3,000, 3,110, and 3,185 kcal/kg, respectively. In addition, amino acid concentrations were formulated at 93% of Aviagen Ross 708 Broiler Nutrition Specifications recommendation (Aviagen, 2016) to mimic commercial practice. Experimental diet samples were collected from each period and were analyzed for phytase activity by ELISA specific for Quantum Blue (ESC, Standard Analytical Method, SAM099; AB Vista) similarly to the method by Engelen et al. (2001).

Statistical Analyses

This study was designed as randomized complete block with pen location as the blocking factor. Individual pen represents the experimental unit with 8 replications. Regression analysis was conducted to determine linear and quadratic effects of phytase supplementations (Treatments 1 to 6) using PROC REG (SAS Institute Inc., 2011). Because phytase doses were not evenly spaced among treatments (0, 500, 1,500, 4,500, 13,500, and 40,500 FTU/kg), these doses were log transformed [$\log_{10}$(FTU+1)] prior to regression analysis to obtain normally distributed data (Shirley and Edwards, 2003;

Gehring et al., 2013). In addition, analysis of variance was performed using PROC MIXED (SAS Institute Inc., 2011) by the following mixed-effects model:

$$Y_{ij} = \mu.. + \rho_i + \tau_i + \varepsilon_{ij}$$

where $\mu..$ is the overall mean; the ρ_i are identically and independently normally distributed random block effects with mean 0 and variance $\sigma^2{}_\rho$; the τ_j are fixed factor level effects corresponding to the j[th] dietary treatment (Treatments 1 to 8) such that $\sum \tau_j$ = 0; and the ε_{ij} are identically and independently normally distributed random errors with mean 0 and a variance σ^2. Pre-planned orthogonal contrasts were used to detect differences between NC vs. PC, Treatment 6 vs. 8, and Treatment 7 vs. 8. Correlation analysis was conducted using PROC CORR by SAS (2011). All statistical significance was considered at $P \leq 0.05$.

RESULTS

Analyzed phytase activity values in experimental diets were in good agreement with the calculated values (Table 4.2). In Treatments 2 to 6, analyzed phytase activity was approximately 93.4% of the calculated values. The activity of phytase in the NC diets and PC diets with inositol addition was less than 65 FTU/kg. However, although the PC diets should contain no phytase inclusion, diet analysis indicated that PC diets in the starter, grower, and finisher contained 695, 238, and 267 FTU/kg phytase. The presence of phytase in the PC diets may be related to diet residue contamination from Treatment 6 as the PC diets were made following the completion of Treatment 6.

Growth Performance

From 1 to 15 D of age, log-quadratic effects of phytase ($P < 0.01$) in Treatments 1 to 6 were observed in all growth performance objectives, where the increase of phytase

doses increased BW gain and feed intake as well as decreased FCR of broilers (Table 4.3). It appears that the optimum growth performance can be obtained by supplementing phytase at 4,500 FTU/kg. The pre-planned orthogonal contrast between NC and PC indicated that broilers fed the PC diet had 5.0% higher ($P < 0.001$) BW gain and 3.3% lower ($P = 0.005$) FCR compared with those provided the NC diets but these responses were not reflected ($P = 0.19$) in the feed intake of broilers. A pre-planned orthogonal contrast between Treatments 6 and 8 was also conducted to evaluate the effects of dietary inositol addition vs. phytase addition at 40,500 FTU/kg, which was expected to liberate a similar amount of inositol. Both BW gain and feed intake of broilers fed diets with inositol supplementation were 5.1 and 3.8% lower ($P < 0.001$), respectively, compared with birds consuming diets with phytase supplementation at 40,500 FTU/kg. Broilers provided diets with inositol addition (Treatment 8) also had 3.6 and 2.5% lower ($P < 0.05$) BW gain and feed intake, respectively, compared with birds fed the PC diets. Mortality was not affected by dietary treatments.

From 1 to 29 D of age, increasing phytase doses from 0 to 40,500 FTU/kg in broiler diets led to log-quadratic increases ($P < 0.01$) of BW gain and feed intake and log quadratic reduction ($P < 0.001$) of FCR (Table 4.4). The pre-planned orthogonal contrast of growth performance responses between broilers provided PC and NC diets did not differ ($P > 0.05$), except the incidence of mortality of broilers fed the NC diets was 2.9% points higher ($P = 0.028$) than birds provided the PC diets. The reason for the higher incidence of mortality of broilers fed the NC diets is unclear; however, it is possible that these mortalities may be related to culls from leg-problems. Inositol addition in broiler diets did not improve ($P > 0.05$) BW gain, feed intake, and FCR compared with those

provided the PC diets. Similarly, broilers consuming PC diets with inositol supplementation had 8.3% lower ($P < 0.001$) BW gain and consumed 3.8% less ($P = 0.005$) feed, while having 6 points higher ($P < 0.001$) FCR compared with broilers fed diets enhanced with phytase at 40,500 FTU/kg.

From 1 to 40 D of age, BW gain and FCR of broilers increased ($P = 0.002$) and decreased ($P = 0.018$), respectively, in log-quadratic manner as phytase doses were increased from 0 to 40,500 FTU/kg in Treatments 1 to 6 (Table 4.5). In contrast, a log-linear effect of phytase ($P = 0.045$) from 0 to 40,500 FTU/kg was observed on the feed intake of broilers, which may indicate that the increase in phytase doses enhanced BW gain of broilers through the stimulation of feed intake. Body weight gain of broilers fed diets supplemented with phytase at 40,500 FTU/kg increased ($P = 0.001$) from 2.784 to 2.914 g, while FCR decreased ($P = 0.005$) by 4 points compared with those fed the PC diets. The NC-fed broilers had a higher ($P = 0.011$) incidence of mortality than birds fed the PC diets. No differences ($P > 0.005$) were observed on all growth performance objectives when comparing birds provided the PC diets and PC diets with inositol addition.

Processing Characteristics

Both carcass and breast meat weights displayed increasing log-quadratic responses ($P < 0.01$) as phytase doses were increased from 0 to 40,500 FTU/kg in Treatments 1 to 6 (Table 4.6). In addition, the increase of phytase doses from 0 to 40,500 FTU/kg increased ($P = 0.042$) abdominal fat weight of broilers, which may be related to the increased BW of broilers as phytase supplementation was increased. The increased carcass yield due to log-quadratic increase of phytase was near significant ($P = 0.053$).

However, no responses ($P > 0.05$) on breast meat yield and abdominal fat percentage were observed when varying dietary treatments. Similarly, pre-planned orthogonal contrast between broilers fed the PC and NC diets did not differ ($P > 0.05$) among carcass characteristic responses. Carcass and breast meat weights of broilers fed diets supplemented with 40,500 FTU/kg phytase were 104 and 26 g heavier ($P < 0.05$), respectively, compared with birds consuming the PC diets with inositol addition. Similarly, the inositol addition to the PC diets did not provide further benefits ($P > 0.05$) on carcass and breast meat weights when compared with birds provided the PC diets.

DISCUSSION

The benefits of supplementing phytase beyond 1,500 FTU/kg have been attributed to the effects beyond phosphorus liberation, such as further phytate degradation (Zeller et al., 2015), increased amino acid digestibility (Gehring et al., 2013) and AME_n (Ravindran et al., 2001), restoration of enzyme functions (Liu et al., 2008), and inositol liberation (Walk et al., 2014). These effects produced improvements in growth performance and meat yield as demonstrated in the current study where phytase doses were increased from 0 to 40,500 FTU/kg. However, it appears that optimum growth performances and carcass characteristics were obtained by supplementing phytase at 4,500 FTU/kg, while no further benefits were observed when supplementing phytase beyond 4,500 FTU/kg. Shirley and Edwards (2003) also observed a quadratic increase in BW gain of broilers from 1 to 16 D of age when increasing phytase doses from 93.75 to 12,000 FTU/kg. Similarly, Walk et al. (2018) noted a linear increase in weight gain of broilers when feeding 0, 500, 1,500, and 4,500 FTU/kg of bacterial phytase during a 21-D production period. These studies were also in agreement with Augspurger and Baker (2004), who

suggested that broilers had an increase in BW gain of 39 g from 302 to 341 g when receiving supplementation of either bacterial or fungal phytase from 500 to 5,000 FTU/kg from 8 to 21 D of age. However, when phytase was increased to 10,000 FTU/kg, BW gain of broiler did not differ with those fed the 5,000 FTU/kg diets, indicating no further benefits of increasing phytase dose.

The current study also demonstrated the increased response of feed intake due to elevated phytase doses in Treatments 1 to 6 in addition to the responses of BW gain and FCR. These data indicated that the extra-phosphoric effects of phytase may not only improve nutrient utilization (Ravindran et al., 2001; Shirley and Edwards, 2003; Gehring et al., 2013), but also stimulate feed intake response. In the current study, correlation coefficients between feed intake and BW gain were 0.73 ($P < 0.001$), 0.80 ($P < 0.001$), and 0.73 ($P < 0.001$), respectively from 1 to 15, 1 to 29 and 1 to 40 D of age. As feed intake increases, digestible nutrient intake also increases leading to the improvement of BW gain. Previously, Walk and Olukosi (2019) conducted a study evaluating the efficacy of *E. coli* phytase from *Trichoderma reesei* in broilers using 0, 2,000 and 4,000 FTU/kg. These authors noted that both apparent ileal amino acid digestibility and digestible amino acid intake increased linearly as phytase doses increased. However, BW gain of broilers had a non-significant correlation ($P > 0.10$, r = –0.14 to –0.29) with apparent ileal amino acid digestibility. In contrast, the increase in digestible amino acid intake was highly correlated ($P < 0.10$, r = 0.33 to 0.72) with BW gain of broilers, which indicated that digestible nutrient intake may be a more suitable indicator of phytase efficacy than nutrient digestibility. Therefore, these data indicated that the benefits of extra-phosphoric effects of phytase may be due to the digestible nutrient intake.

The effects of phytase in stimulating digestible nutrient intake may be associated with its impact on phytate degradation. Phytate has been reported to reduce feed intake in broilers. Dos Santos et al. (2014) reported a 20.1% reduction in feed intake broilers from 1 to 21 D of age when increasing dietary phytate phosphorus concentration from 0.18 to 0.29%. However, when 500 FTU/kg of *E. coli* phytase from *Trichoderma reesei* was added to the high phytate phosphorus diet, there was no difference in feed intake between the high and low phytate phosphorus diets. Additionally, a study by Zeller et al. (2015) provided evidence of phytate degradation by *E. coli* phytase expressed in *Trichoderma reesei*. The supplementation of phytase at 500 and 12,500 FTU/kg in broiler diets led to 78 and 92% inositol phosphate 6 hydrolysis compared with 67% hydrolysis in diets without phytase addition. As a result, broilers receiving diets with 500 and 12,500 FTU/kg phytase consumed 9 and 11 g more feed daily than those without phytase addition from 1 to 21 D of age. These studies and the current study demonstrated that the extra-phosphoric effects of phytase may stimulate nutrient intake in addition to improving nutrient digestibility, which resulted in growth performance enhancement.

The positive influence of phytase on carcass characteristics in the present research has also been noted in a previous study (Schmeisser et al., 2017), which indicated that bacterial phytase supplementation from *Citrobacter braakii* at 1,000 FTU/kg increased breast meat weight of broilers from 90 to 98 g and 220 to 272 g at 21 and 36 D of age, respectively, compared with birds fed reduced calcium and non-phytate phosphorus diets. These researchers indicated that the benefits of phytase on muscle growth may be attributed to several interconnected factors, such as inositol release and upregulation of myogenesis gene expression (Schmeisser et al., 2017). This study indicated that phytase

may increase phospholipase C beta gene expression, which stimulates the release of inositol (1,4,5)-phosphate 3 from phosphatidylinositol 4,5-bisphosphate. Subsequently, calcium can be released from the endoplasmic reticulum activating calmodulin and calcineurin A pathways, which promotes muscle growth (Tokomitsu et al., 1999).

Furthermore, Schmeisser et al. (2017) reported the upregulation of genes involved with insulin growth factor/phosphoinositide 3 kinase pathway, which may be initiated by the release of inositol that mimics the action of glucose (Cowieson et al., 2015). This pathway can activate the mTOR pathway promoting skeletal myogenesis (Cooper and Hausman, 2013). In addition, measurements of gene expression in the breast muscle of broilers fed phytase-added diets displayed an increased myocyte enhancer factor 2 gene expression compared with broilers receiving diets without phytase addition (Schmeisser et al., 2017). Previous research indicated that the myocyte enhancer factor 2 is responsible for the regulation of myoblast determination protein, which regulates muscle differentiation (McKinsey et al., 2002).

It is interesting that the reduction of calcium and non-phytate phosphorus in the current research suppressed BW gain and FCR from 1 to 15 D of age, but not from 1 to 29, 1 to 43 D of age, and carcass characteristics of broilers. These responses are similar to studies by Walk et al. (2013) and dos Santos et al. (2013). Presumably, the younger birds may be more susceptible to a marginal reduction of calcium and non-phytate phosphorus concentrations than the older birds (Walk et al., 2013). A previous study reported that the fastest rate of bone formation and mineralization in broilers occurred from 4 to 18 and 4 to 11 D of age, respectively (Williams et al., 2000). Thus, the reduction in calcium and non-phytate phosphorus in the current study may have a more pronounced effect during

the starter period than the later period.

The lack of differences in growth performance from 1 to 29 and 1 to 40 D of age between broilers provided the NC and PC diets in the present study may also be associated with the concentrations of dietary non-phytate phosphorus. Previous research recommended non-phytate phosphorus concentration of 0.45, 0.29, and 0.23% from 1 to 18, 19 to 32, and 33 to 42 D of age, respectively, for optimum growth performance of broilers (Angel et al., 2005). In the present research, the PC diets were formulated to contain 0.48, 0.44, and 0.39% non-phytate phosphorus from 1 to 14 (starter), 15 to 29 (grower), and 30 to 40 D of age (finisher), respectively. When non-phytate phosphorus was decreased by 0.15% to establish the NC diets, the only concentration lower than that recommended by Angel et al. (2005) was the non-phytate phosphorus in the NC starter diet. Hence, the lack of differences in growth performance from 1 to 29 and 1 to 40 D of age between broilers fed the PC and NC diets may be associated with the high concentration of non-phytate phosphorus even after 0.15% reduction. This indicates that non-phytate phosphorus recommendation by the primary breeder guidelines may be in excess of the requirements (Aviagen, 2016). This concept is supported by Pieniazek et al. (2017), who reported a reduction in BW and an increase in FCR of broilers from 1 to 14, 1 to 28, 1 to 42 D of age when fed NC vs. PC diets. These researchers formulated NC diets to contain 0.28, 0.24, and 0.20% and PC diet to contain 0.45, 0.41, and 0.38% non-phytate phosphorus, respectively from 1 to 14, 15 to 28, and 29 to 42 D of age.

Inositol has been reported to play roles in various physiological mechanisms, such as signaling mechanisms (Cooper and Hausman, 2013), nutrient transport (Jiang et al., 2013), and hepatic lipid metabolism (Pirgozliev et al., 2019). However, the effects of

dietary inositol supplementation on growth performance of broilers have been inconsistent. Cowieson et al. (2013), Zyla et al. (2013), and Sommerfeld et al. (2018) reported improvements in growth performance of broilers when supplementing inositol in the diets. Conversely, Farhadi et al. (2017) and Pirgozliev et al. (2019) did not observe benefits on growth performance of broilers when provided diets with inositol inclusion, which are in agreement with the present research. The reason for this discrepancy of inositol benefits on growth performance of broilers is unclear. Further work should include inositol measurements in the digesta and blood to ascertain whether differences among studies are simply due to differences in basal phytate digestibility, which has been shown to range from 23 to 74% (Rodehutscord et al., 2017), depending upon dietary ingredients and specifications. Clearly, if birds fed diets without phytase supplementation are able to hydrolyze 70% of the IP6 to inositol, then the effects of adding either phytase or inositol to such diet will be limited compared to another diet where IP6 digestibility is only 30%. In addition, inositol concentration for optimizing growth performance of broilers is also not known.

In conclusion, increasing phytase dose up to 4,500 FTU/kg was beneficial for optimizing growth performance and carcass characteristics of broilers. It appears that these improvements may be associated with the extra-phosphoric effects of phytase on stimulating feed intake of broilers, which increased digestible amino acid intake. However, this study indicates that the addition of inositol did not provide further benefits on growth performance and meat yield of broilers.

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Table 4.1 Ingredient and nutrient composition of negative (NC) and positive control (PC) diets fed to broilers during starter (1 to 15 D of age), grower (16 to 29 D of age), and finisher (30 to 41 D of age) periods

	Starter		Grower		Finisher	
Item	NC	PC	NC	PC	NC	PC
Ingredient, %						
Corn	57.47	55.82	61.63	59.98	66.70	65.05
Soybean meal	36.61	36.86	31.69	31.94	26.63	26.88
Vegetable oil	1.18	1.80	2.29	2.91	2.62	3.24
Dicalcium phosphate	1.22	2.04	1.03	1.84	0.84	1.65
Calcium carbonate	1.07	1.05	0.99	0.96	0.90	0.87
Sodium chloride	0.40	0.39	0.40	0.40	0.40	0.40
Builder sand[1]	0.70	0.70	0.70	0.70	0.70	0.70
DL-Methionine	0.30	0.30	0.27	0.27	0.25	0.25
L-Lysine•HCl	0.17	0.17	0.17	0.16	0.17	0.16
L-Threonine	0.09	0.09	0.08	0.08	0.07	0.07
Mineral premix[2]	0.10	0.10	0.10	0.10	0.10	0.10
Vitamin premix[3]	0.10	0.10	0.08	0.08	0.05	0.05
Choline chloride	0.07	0.07	0.07	0.07	0.07	0.07
Titanium dioxide	0.50	0.50	0.50	0.50	0.50	0.50
Xylanase[4]	0.01	0.01	0.01	0.01	0.01	0.01
Calculated nutrient composition, % (unless otherwise noted)						
AME_n, kcal/kg	3,000	3,000	3,110	3,110	3,185	3,185
Crude protein	21.83	21.82	19.80	19.79	17.77	17.77
Digestible lysine	1.19	1.19	1.07	1.07	0.95	0.95
Digestible TSAA[5]	0.88	0.88	0.81	0.81	0.74	0.74
Digestible threonine	0.80	0.80	0.72	0.72	0.63	0.63
Digestible valine	0.89	0.89	0.81	0.81	0.73	0.73
Digestible isoleucine	0.84	0.84	0.75	0.75	0.67	0.67
Calcium	0.80	0.96	0.71	0.87	0.62	0.78
Non-phytate phosphorus	0.33	0.48	0.29	0.44	0.24	0.39
Sodium	0.18	0.18	0.18	0.18	0.18	0.18

[1]The NC basal diet (Treatment 1) was supplemented with phytase at the expense of sand (Quantum Blue 5G, AB Vista Feed Ingredients, Marlborough, UK; analyzed as 7,700 FTU/g) at 500, 1,500, 4,500, 13,500, and 40,500 FTU/kg of diet to create Treatments 2 to 6, respectively. The PC basal diet (Treatment 7) was supplemented with inositol at the expense of sand to generate Treatment 8.[5, 6]

[2]Trace mineral premix include per kg of diet: Mn (manganese sulfate), 120 mg; Zn (zinc sulfate), 100 mg; Fe (iron sulfate monohydrate), 30 mg; Cu (tri-basic copper chloride), 8 mg; I (ethylenediamine dihydriodide), 1.4 mg; and Se (sodium selenite), 0.3 mg.

[3]Vitamin premix includes per kg of diet: Vitamin A (Vitamin A acetate), 18,739 IU; Vitamin D_3 (cholecalciferol), 6,614 IU; Vitamin E (DL-alpha tocopherol acetate), 66 IU; menadione (menadione sodium bisulfate complex), 4 mg; Vitamin B_{12} (cyanocobalamin), 0.03 mg; folacin (folic acid), 2.6 mg: D-pantothenic acid (calcium pantothenate), 31 mg; riboflavin (riboflavin), 22 mg; niacin (niacinamide), 88 mg; thiamin (thiamin mononitrate), 5.5 mg; biotin (biotin),

0.18 mg; and pyridoxine (pyridoxine hydrochloride), 7.7 mg.
[4]Econase XT, AB Vista Feed Ingredients, Marlborough, UK
[5]One unit of phytase activity (FTU) is defined as the quantity of enzyme to liberate 1 μmol of inorganic phosphorus per minute from 0.0051 mol/L sodium phytate at pH 5.5 and 37°C.
[6]Inositol supplementation was calculated based on the maximum inositol liberation from Treatment 6 at 0.26, 0.25, and 0.23%, respectively in the starter, grower, and finisher periods.

Table 4.2 Analyzed activity of phytase (FTU/kg) in the starter (1 to 15 D of age), grower (16 to 29 D of age), and finisher (30 to 41 D of age) diets[1]

Dietary Treatments	Starter	Grower	Finisher
1) Negative control (NC)[2]	65	< 50	< 50
2) NC + 500 FTU/kg	417	519	641
3) NC + 1,500 FTU/kg	1,250	1,250	1,490
4) NC + 4,500 FTU/kg	3,313	3,350	3,960
5) NC + 13,500 FTU/kg	12,480	13,710	12,690
6) NC + 40,500 FTU/kg	36,640	41,290	42,070
7) Positive Control (PC)[2]	695	238	267
8) PC + Inositol[3]	65	< 50	< 50

[1]One unit of phytase activity (FTU) is defined as the quantity of enzyme to liberate 1 µmol of inorganic phosphorus per minute from 0.0051 mol/L sodium phytate at pH 5.5 and 37°C.
[2]The negative control diet was formulated to contain 0.165 and 0.150% lower calcium and phosphorus, respectively, compared with the positive control diet.
[3]Inositol supplementation was calculated based on the maximum inositol liberation from the diet containing the highest phytase concentration.

Table 4.3 Growth performance of broilers fed diets with gradient phytase supplementation from 1 to 15 D of age[1]

Item	BW, kg/bird	BW Gain, kg/bird	Feed Intake, kg/bird	FCR, kg:kg[2]	Mortality, %[3]
Dietary treatment					
1) Negative Control (NC)[4]	0.436	0.397	0.511	1.286	2.9
2) NC + 500 FTU/kg[5]	0.445	0.406	0.511	1.258	2.1
3) NC + 1,500 FTU/kg	0.452	0.413	0.515	1.245	1.6
4) NC + 4,500 FTU/kg	0.470	0.431	0.527	1.223	2.9
5) NC + 13,500 FTU/kg	0.462	0.423	0.525	1.240	2.5
6) NC + 40,500 FTU/kg	0.462	0.424	0.525	1.240	2.5
7) Positive Control (PC)	0.456	0.417	0.518	1.243	1.3
8) PC + Inositol[6]	0.441	0.402	0.505	1.256	1.7
Pooled standard error	0.004	0.004	0.004	0.011	0.9
Source of Variation			*Probabilities*		
Log-linear effect of phytase[7]	< 0.001	< 0.001	0.005	< 0.001	0.55
Log-quadratic effect of phytase[7]	< 0.001	< 0.001	0.006	0.001	0.61
Treatment 1 vs. 7	0.001	< 0.001	0.19	0.005	0.08
Treatment 6 vs. 8	< 0.001	< 0.001	< 0.001	0.28	0.36
Treatment 7 vs. 8	0.012	0.001	0.018	0.39	0.88

[1]Values are least-square means of 8 replicate pens with each pen having 30 birds at placement.
[2]Feed conversion ratio was corrected for mortality.
[3]Mortality was arcsine transformed.
[4]The negative control diet was formulated to contain 0.165 and 0.150% lower calcium and phosphorus, respectively, compared with the positive control diet.
[5]One unit of phytase activity (FTU) is defined as the quantity of enzyme to liberate 1 µmol of inorganic phosphorus per minute from 0.0051 mol/L sodium phytate at pH 5.5 and 37°C.
[6]Inositol supplementation was calculated based on the maximum inositol liberation from the diet containing the highest phytase concentration (Treatment 6).
[7]Phytase levels were $\log_{10}$ transformed before analysis. Log-linear and log-quadratic effects were analyzed for Treatments 1 to 6.

Table 4.4 Growth performance of broilers fed diets with gradient phytase supplementation from 1 to 29 D of age[1]

Item	BW, kg/bird	BW Gain, kg/bird	Feed Intake, kg/bird	FCR, kg:kg[2]	Mortality, %[3]
Dietary treatment					
1) Negative Control (NC)[4]	1.617	1.577	2.165	1.372	5.1
2) NC + 500 FTU/kg[5]	1.645	1.604	2.174	1.356	2.6
3) NC + 1,500 FTU/kg	1.670	1.629	2.210	1.356	1.6
4) NC + 4,500 FTU/kg	1.756	1.715	2.272	1.325	2.9
5) NC + 13,500 FTU/kg	1.737	1.699	2.249	1.325	3.4
6) NC + 40,500 FTU/kg	1.725	1.688	2.234	1.324	2.5
7) Positive Control (PC)	1.621	1.583	2.168	1.370	2.2
8) PC + Inositol[6]	1.587	1.548	2.148	1.388	2.1
Pooled standard error	0.019	0.018	0.024	0.010	1.1
Source of Variation			*Probabilities*		
Log-linear effect of phytase[7]	< 0.001	< 0.001	0.003	< 0.001	0.06
Log-quadratic effect of phytase[7]	< 0.001	< 0.001	0.003	< 0.001	0.08
Treatment 1 vs. 7	0.85	0.83	0.92	0.86	0.028
Treatment 6 vs. 8	< 0.001	< 0.001	0.005	< 0.001	0.65
Treatment 7 vs. 8	0.16	0.15	0.50	0.14	0.74

[1]Values are least-square means of 8 replicate pens with each pen having 30 birds at placement.
[2]Feed conversion ratio was corrected for mortality.
[3]Mortality was arcsine transformed.
[4]The negative control diet was formulated to contain 0.165 and 0.150% lower calcium and phosphorus, respectively, compared with the positive control diet.
[5]One unit of phytase activity (FTU) is defined as the quantity of enzyme to liberate 1 μmol of inorganic phosphorus per minute from 0.0051 mol/L sodium phytate at pH 5.5 and 37°C.
[6]Inositol supplementation was calculated based on the maximum inositol liberation from the diet containing the highest phytase concentration (Treatment 6).
[7]Phytase levels were $\log_{10}$ transformed before analysis. Log-linear and log-quadratic effects were analyzed for Treatments 1 to 6.

Table 4.5 Growth performance of broilers fed diets with gradient phytase supplementation from 1 to 40 D of age[1]

Item	BW, kg/bird	BW Gain, kg/bird	Feed Intake, kg/bird	FCR, kg:kg[2]	Mortality, %[3]
Dietary treatment					
1) Negative Control (NC)[4]	2.848	2.805	4.166	1.486	7.0
2) NC + 500 FTU/kg[5]	2.881	2.837	4.232	1.492	4.2
3) NC + 1,500 FTU/kg	2.868	2.829	4.238	1.498	2.2
4) NC + 4,500 FTU/kg	2.966	2.924	4.318	1.478	4.0
5) NC + 13,500 FTU/kg	2.973	2.927	4.254	1.454	4.7
6) NC + 40,500 FTU/kg	2.956	2.914	4.239	1.455	3.1
7) Positive Control (PC)	2.835	2.790	4.187	1.501	2.2
8) PC + Inositol[6]	2.823	2.784	4.158	1.494	3.2
Pooled standard error	0.034	0.033	0.043	0.011	1.3
Source of Variation			*Probabilities*		
Log-linear effect of phytase[7]	0.003	0.002	0.045	0.050	0.040
Log-quadratic effect of phytase[7]	0.005	0.002	0.14	0.018	0.11
Treatment 1 vs. 7	0.75	0.71	0.69	0.27	0.011
Treatment 6 vs. 8	0.001	0.001	0.11	0.005	0.94
Treatment 7 vs. 8	0.76	0.86	0.56	0.60	0.58

[1]Values are least-square means of 8 replicate pens with each pen having 30 birds at placement.
[2]Feed conversion ratio was corrected for mortality.
[3]Mortality was arcsine transformed.
[4]The negative control diet was formulated to contain 0.165 and 0.150% lower calcium and phosphorus, respectively, compared with the positive control diet.
[5]One unit of phytase activity (FTU) is defined as the quantity of enzyme to liberate 1 μmol of inorganic phosphorus per minute from 0.0051 mol/L sodium phytate at pH 5.5 and 37°C.
[6]Inositol supplementation was calculated based on the maximum inositol liberation from the diet containing the highest phytase concentration (Treatment 6).
[7]Phytase levels were $\log_{10}$ transformed before analysis. Log-linear and log-quadratic effects were analyzed for Treatments 1 to 6.

Table 4.6 Carcass characteristics of broilers fed diets with gradient phytase supplementation from 1 to 41 D of age[1]

Item	Live Weight, kg	Carcass Weight, kg	Carcass Yield, %	Breast Meat Weight, kg	Breast Meat Yield, %	Abdominal Fat Weight, g	Abdominal Fat Percentage, %
Dietary Treatment							
1) Negative Control (NC)[2]	2.917	2.159	74.29	0.780	26.80	26.05	0.89
2) NC + 500 FTU/kg[3]	2.984	2.203	74.17	0.790	26.62	28.17	0.95
3) NC + 1,500 FTU/kg	2.953	2.211	74.91	0.795	26.90	26.51	0.89
4) NC + 4,500 FTU/kg	3.024	2.273	75.11	0.815	26.94	28.17	0.93
5) NC + 13,500 FTU/kg	3.034	2.267	74.68	0.816	26.89	27.97	0.92
6) NC + 40,500 FTU/kg	3.050	2.278	74.73	0.810	26.51	28.48	0.93
7) Positive Control (PC)	2.956	2.200	74.32	0.780	26.34	27.11	0.92
8) PC + Inositol[4]	2.923	2.174	74.32	0.784	26.79	27.57	0.94
Pooled standard error	0.030	0.024	0.33	0.011	0.23	1.02	0.03
Source of Variation				*Probabilities*			
Log-linear effect of phytase[5]	< 0.001	< 0.001	0.054	0.002	0.89	0.042	0.27
Log-quadratic effect of phytase[5]	< 0.001	< 0.001	0.053	0.004	0.83	0.11	0.36
Treatment 1 vs. 7	0.21	0.12	0.94	0.98	0.14	0.39	0.52
Treatment 6 vs. 8	< 0.001	< 0.001	0.23	0.037	0.35	0.44	0.79
Treatment 7 vs. 8	0.26	0.29	0.99	0.74	0.13	0.70	0.49

[1]Values are least-square means of 8 replicate pens with 12 birds selected from each pen for processing.
[2]The negative control diet was formulated to contain 0.165 and 0.150% lower calcium and phosphorus, respectively, compared with the positive control diet.
[3]One unit of phytase activity (FTU) is defined as the quantity of enzyme to liberate 1 µmol of inorganic phosphorus per minute from 0.0051 mol/L sodium phytate at pH 5.5 and 37°C.
[4]Inositol supplementation was calculated based on the maximum inositol liberation from the diet containing the highest phytase concentration (Treatment 6).
[5]Phytase levels were $\log_{10}$ transformed before analysis. Log-linear and log-quadratic effects were analyzed for Treatments 1 to 6.

V. INOSITOL AND GRADIENT PHYTASE SUPPLEMENTATION IN BROILER DIETS DURING A 6-WEEK PRODUCTION PERIOD: 2. EFFECTS ON PHYTATE DEGRADATION AND INOSITOL LIBERATION IN GIZZARD AND ILEAL DIGESTA CONTENTS

ABSTRACT

An experiment was conducted to evaluate effects of dietary phytase and inositol supplementation on phytate degradation in gizzard and ileal digesta contents of broilers from 1 to 43 D of age. One thousand nine hundred and twenty Yield Plus × Ross 708 male chicks were placed in 64 floor pens (30 birds/pen). Each pen received 1 of 8 dietary treatments from 1 to 43 D of age. Treatment 1 was formulated to contain 0.165 and 0.150% lower calcium and phosphorus, respectively, compared with Treatment 7. Treatments 2 to 6 were formulated by adding phytase at 500, 1,500, 4,500, 13,500, and 40,500 phytase units (**FTU**)/kg, respectively to Treatment 1. Treatment 8 was established by adding inositol to Treatment 7 based on the maximum inositol liberation in Treatment 6. At 15, 29, and 43 D of age, gizzard contents, ileal digesta, and blood were collected for analysis of inositol and inositol phosphate (**IP**) 2, 3, 4, 5, and 6 concentrations. Increasing phytase from 0 to 40,500 FTU/kg resulted in log-quadratic reductions ($P < 0.01$) of IP5 and IP6 concentrations in the gizzard and ileal digesta contents of broilers at 15, 29, and 43 D of age. The increase in phytase doses in Treatments 1 to 6 reduced IP3 and IP4

concentrations in a log-quadratic manner ($P < 0.05$) at each collection period in gizzard contents, but only at 43 D of age in the ileal digesta of broilers ($P < 0.01$). Log-quadratic increases ($P < 0.05$) of inositol concentrations were observed in gizzard and ileal digesta contents when increasing phytase activity from 0 to 40,500 FTU/kg at each collection period, which translated to a log-quadratic increase ($P < 0.01$) in plasma inositol concentration of broilers at 15, 29, and 43 D of age. Phytase supplementation up to 40,500 FTU/kg may benefit broilers by reducing phytate concentrations in the gizzard and ileal digesta contents. Moreover, inositol release in the ileal digesta may translate to increased plasma inositol concentration.

INTRODUCTION

Exogenous phytase is supplemented in broiler diets to liberate additional phosphorus (Selle and Ravindran, 2007). Recently, the supplementation of phytase beyond phosphoric effect was reported to also enhance growth performance of broilers (dos Santos et al., 2013; Campasino et al., 2014). These extra-phosphoric effects have been associated with enhancements in nutrient availability through the degradation of phytate to inositol (Beeson et al., 2017). Previous research demonstrated that an *E. coli* phytase addition of up to 3,000 phytase units (**FTU)**/kg increased phytate degradation, inositol liberation, phosphorus, calcium, and amino acid digestibility (Sommerfeld et al., 2018).

The degradation of phytate in broiler diets occurs through a stepwise removal of phosphate groups of phytate from inositol hexakisphosphate (**IP6**) to inositol (Selle and Ravindran, 2007). Many previous studies have demonstrated the efficacy of phytase in degrading IP6 (Tamim et al., 2004). However, it appeared that broilers may have

difficulties in degrading lower phytate esters when provided phytase at 500 FTU/kg, such as inositol triphosphate (**IP3)** and inositol tetraphosphate (**IP4)** (Zeller et al., 2015a,b; Bedford and Walk, 2016). A previous study indicated the accumulation of IP3 and IP4 even when supplementing *E. coli* phytase up to 1,500 FTU/kg (Walk et al., 2014; Beeson et al., 2017). In addition, these lower phytate esters may have similar anti-nutritive effects with IP6 to chelate nutrients and compromise digestive enzyme efficacy leading to poor growth performance of broilers (Persson et al., 1998; Yu et al., 2012). Therefore, it is important to consider phytase efficacy on the extent of phytate degradation not only for IP6 but also for IP3 and IP4 (Bedford and Walk, 2016). Unfortunately, research evaluating the efficacy of phytase to minimize IP3 and IP4 accumulations in the digesta of broilers is sparse (Zeller et al., 2015a,b; Sommerfeld et al., 2018).

The result of further degradation of lower phytate esters is inositol liberation. Following its liberation, inositol can be effectively absorbed in the small intestine (Croze and Soulage, 2013), which has been reported to provide growth-promoting effects (Cowieson et al., 2013; Zyla et al., 2013). Previous research observed an increase in plasma inositol concentrations when feeding broilers with elevated phytase concentrations (Cowieson et al., 2015). These attributes of inositol may allow for its use as an indicator of phytase efficacy to degrade phytate (Cowieson et al., 2017). However, additional data are needed to evaluate the extent of inositol concentration in the plasma of broilers provided phytase supplementation beyond 1,500 FTU/kg. Therefore, this study hypothesized that the increase in dietary phytase concentration will increase phytate degradation and inositol liberation of broilers. The current experiment was designed to

determine effects of elevated phytase supplementation and inositol addition on the extent of phytate degradation and plasma inositol concentrations of broilers.

MATERIALS AND METHODS

All experimental procedures regarding live birds was approved by the Institutional Animal Care and Use Committee at Auburn University (PRN 2018-3254).

Bird Husbandry, Dietary Treatments, and Sample Collections

The current study utilized the same set of birds, husbandry practices, and dietary treatments with the companion manuscript (Tables 5.1 and 5.2). One thousand nine hundred and twenty Yield Plus × Ross 708 male broilers were obtained from a commercial hatchery (Aviagen North America, Huntsville, AL). Birds were randomly distributed into 64 floor-pens with 30 birds per pen (0.07 m^2 per bird). At 14, 28, and 43 D of age, 4, 2, and 2 birds per pen, respectively were sacrificed for necropsy. At least 3 mL of blood was collected from each bird via heart puncture for determination of plasma inositol concentrations. Blood samples were collected into a 4.5 mL heparinized tube (S-Monovette® 4.5 mL LH, Sarstedt, Numbrecht, Germany) and placed on ice until centrifugation. Blood samples were centrifuged at 1,643 × g for 10 minutes to separate plasma from the whole blood and stored at –20°C until further analysis. Gizzard and ileal digesta contents were collected for analyses of phytate breakdown, inositol phosphate ester disappearance, and inositol concentration. Gizzard contents were collected by carefully scraping feed contents into a Whirl-Pak bag (Nasco, Fort Atkinson, WI) and ileal digesta was collected by gently flushing out the content of the terminal ileum using deionized-distilled water into Whirl-Pak bags (Nasco, Fort Atkinson, WI). This section is defined as the terminal 1/3 of the section between the Meckel's diverticulum and

approximately 4 cm anterior from the ileo-cecal junction (Rodehutscord et al., 2012). Both gizzard and ileal digesta samples were immediately frozen by submersion in liquid nitrogen for approximately 5 minutes to terminate any remaining phytase activity. Samples were kept on ice and stored at –20°C until later analysis.

Chemical Analyses

Plasma samples were prepared by mixing with 1 M perchloric acid in a 1:2 ratio (plasma:$HClO_4$) to precipitate all protein. Samples were centrifuged at 14,000 × g for 10 minutes to collect the supernatant and were sent to University of East Anglia School of Biological Sciences in Norwich, England for analysis of inositol concentration using HPLC with pulsed amperometric detection. Samples were diluted 50 fold in 18.2 mohm × cm water. An aliquot (20 µL) was injected into a 4 mm × 250 mm MetroSep Carb 2 (Metrohm, Runcorn, UK) HPLC column. The column was eluted at a flow rate of 0.5 mL/minute with 150 mM NaOH. Another aliquot (5 µL) was injected onto a 2 mm × 100 mm Metrosep Carb 2 (Metrohm, Runcorn, UK) column with guard column eluted at a flow rate of 0.2 mL/minute with the same solvent. Inositol peaks were integrated with Chromeleon (ThermoFisher Scientific, Waltham, MA) and DataApex Clarity (DataApex, Prague, Czech Republic) software packages. Inositol concentration was determined by comparing results with standards using a linear least squares regression.

Samples of gizzard and ileal digesta contents were lyophilized (VirTis Genesis 25ES, SP Industries Inc., Warminster, PA) and ground with an electric coffee grinder. Samples were analyzed at University of East Anglia School of Biological Science in Norwich, England for inositol hexakisphosphate (**IP6**), inositol pentaphosphate (**IP5**), inositol tetraphosphate (**IP4**), inositol triphosphate (**IP3**), and inositol biphosphate (**IP2**)

concentrations using high-performance ion chromatography with postcolumn derivatization and UV detection at 290 nm. Inositol was quantified using HPLC with pulsed amperometric detection (Laird et al., 2016).

Diet and ileal digesta samples were analyzed for titanium dioxide concentration to determine IP6 and total IP2 to IP6 disappearances according to method by Short et al. (1996). Titanium dioxide content in diet samples was analyzed in quadruplicate, while duplicate analyses were conducted for the ileal digesta. Samples of diet (600 mg) and ileal digesta (200 mg) were placed into porcelain crucibles and ashed for 12 hours at 580°C. After ashing, samples were transferred to a 50 mL beaker by rinsing porcelain crucibles using 10 mL sulfuric acid (7.4 M). The solutions were heated (250°C) on a hot plate to dissolve solid particles for approximately 60 minutes. After cooling, the solutions were rinsed using 10 mL distilled water into a glass beaker containing 25 mL distilled water. Twenty mL of hydrogen peroxide (30%) was added to each beaker and the solution was diluted to 100 mL with distilled water. Solutions were kept at room temperature for at least 48 hours prior to absorbance measurement at 410 nm using a spectrophotometer (SpectraMax Plus 384, Molecular Devices LLC., San Jose, CA). Titanium concentration was determined by comparing absorbance results with known standards using a linear least-squares regression.

Calculations

Concentrations of IP6, total IP2 to IP6, and titanium dioxide from feed and ileal digesta analyses were used to calculate percent IP6 and total IP2 to IP6 disappearances using the following equation:

$$IP\ disappearance = \left[1 - \left(\frac{TiO_{2\ Diet}}{TiO_{2\ Digesta}}\right) \times \left(\frac{IP_{Digesta}}{IP_{Diet}}\right)\right] \times 100$$

where $TiO_{2\ Digesta}$ and $TiO_{2\ Diet}$ represent the analyzed concentrations of titanium dioxide in the ileal digesta and diets, respectively, and $IP_{Digesta}$ and IP_{Diet} indicate the analyzed IP6 or total IP2 to IP6 concentrations in ileal digesta and diets, respectively.

Statistical Analyses

This study was designed as randomized complete block with pen location as the blocking factor. Individual pen represents the experimental unit with 8 replications. Regression analysis was conducted to determine linear and quadratic effects of phytase supplementations (Treatments 1 to 6) using PROC REG (SAS Institute Inc., 2011). Because phytase concentrations were not evenly spaced among treatments (0, 500, 1,500, 4,500, 13,500, and 40,500 FTU/kg), these concentrations were log transformed [$\log_{10}$(FTU+1)] prior to regression analysis to obtain a normally distributed data (Shirley and Edwards, 2003; Gehring et al., 2013). In addition, analysis of variance was performed using PROC MIXED (SAS Institute Inc., 2011) by the following mixed-effect model:

$$Y_{ij} = \mu.. + \rho_i + \tau_j + \varepsilon_{ij}$$

where $\mu..$ is the overall mean; the ρ_i are identically and independently normally distributed random block effects with mean 0 and variance $\sigma^2{}_\rho$; the τ_j are fixed factor level effects corresponding to the j^{th} dietary treatment (Treatments 1 to 8) such that $\sum\tau_j = 0$; and the ε_{ij} are identically and independently normally distributed random errors with mean 0 and a variance σ^2. Pre-planned orthogonal contrasts were used to detect differences between NC vs. PC, Treatment 6 (NC with 40,500 FTU/kg addition) vs. 8 (PC with inositol addition), and Treatment 7 (PC) vs. 8 (PC with inositol addition).

Correlation analysis was conducted using PROC CORR (SAS Institute Inc., 2011). All statistical significance was considered at $P \leq 0.05$.

RESULTS

Analyses of phytase activity in Treatments 2 to 6 were approximately 93.4% of the calculated values (Table 5.2). However, analysis of the PC diets in the starter, grower, and finisher periods had phytase activity of 695, 238, and 267 FTU/kg, respectively. Phytase activity in the PC diets may have originated from dietary Treatment 6 phytase residue, which were manufactured prior to the PC diets.

In the gizzard contents of broilers, an increase of phytase concentrations in Treatments 1 to 6 resulted in log-quadratic reductions ($P < 0.05$) of IP3, 4, 5, 6, and total IP3 to IP6 at 15, 29, and 43 D of age (Tables 5.3, 5.4, and 5.5). These responses were accompanied by the log-quadratic increase ($P < 0.05$) of inositol concentration at 15, 29, and 43 D of age. Broilers consuming the PC diets had lower ($P < 0.001$) IP6 and total IP3 to IP6, but higher ($P < 0.001$) IP4 and IP3 concentrations compared with birds provided the NC diets at 15, 29, and 43 D of age. In contrast, inositol concentrations were similar ($P > 0.05$) between broilers fed the PC or NC diets at all collection periods. Phytase supplementation at 40,500 FTU/kg decreased ($P < 0.05$) IP3, 4, 5, 6, and total IP3 to IP6 of broilers compared with birds receiving the PC diets with inositol addition at 15, 29, and 43 D of age. Moreover, higher ($P < 0.05$) IP esters concentrations were also observed in broilers fed the PC diets with inositol compared with birds provided the PC diets at 15, 29, and 43 D of age. Inositol concentration of broilers fed PC diets supplemented with inositol was similar ($P > 0.05$) to birds fed diets with 40,500 FTU/kg phytase at 15, 20, and 43 D of age. However, higher ($P < 0.001$) inositol concentration in broilers fed diets

with inositol supplementation compared with birds fed the positive control diets was only observed at 15 D of age.

In the ileal digesta, increasing supplementation of phytase from 0 to 40,500 FTU/kg in broiler diets decreased ($P < 0.001$) IP5, IP6, and total IP2 to IP6 concentrations in a log-quadratic manner at 15, 29, and 43 D of age (Tables 5.6, 5.7, and 5.8). However, a log-quadratic reduction ($P < 0.001$) of IP4 due to increasing phytase concentrations was obtained only at 29 and 43 D of age, while a log-quadratic IP3 reduction ($P = 0.011$) was observed only at 43 D of age. No log-quadratic reduction ($P > 0.05$) of IP2 was observed at any of the collection period with increasing dietary phytase doses. Increasing phytase doses in Treatments 1 to 6 resulted in log-quadratic increase ($P < 0.001$) of inositol concentrations at each collection period. At 15, 29, and 43 D of age, broilers provided the PC diets had lower ($P < 0.05$) IP6 and total IP2 to IP6 concentrations compared with birds provided the NC diets. Conversely, IP3 and IP4 concentrations were higher ($P < 0.001$) in broilers provided the PC diets compared with those consuming the NC diets. Broilers fed the PC diets had higher ($P = 0.048$) inositol concentration only at 15 D of age than broilers provided the NC diets. Feeding broilers diets formulated with phytase at 40,500 FTU/kg decreased ($P < 0.001$) IP6 and total IP2 to IP6 compared with birds consuming the PC diets with inositol addition at every collection period. However, inositol concentration of broilers fed diets supplemented with phytase (40,500 FTU/kg) was 25, 39, and 41% higher ($P < 0.01$) than in broilers fed the PC diets with inositol addition at 15, 29, and 43 D of age, respectively. Furthermore, broilers receiving PC diets with inositol addition had lower ($P < 0.001$) IP3 and IP4

concentrations but higher ($P < 0.001$) inositol concentrations compared with birds provided the PC diets only at 15, 29 and 43 D of age.

Effects of phytase additions in Treatments 1 to 6 also led to quadratic increase ($P < 0.001$) of apparent ileal disappearance of IP6 and total IP2 to IP6 concentrations at 15, 29, and 43 D of age (Table 5.9). Providing broilers the PC diets resulted in lower ($P < 0.001$) ileal disappearances of IP6 and total IP2 to IP6 concentrations compared with feeding the NC diets or the PC diets with inositol addition. Broilers receiving diets with phytase addition at 40,500 FTU/kg had higher ($P < 0.001$) ileal IP6 and total IP2 to IP6 disappearances than broilers consuming the PC diets with inositol addition.

Moreover, increasing phytase addition from 0 to 40,500 FTU/kg resulted in log-quadratic increase of plasma inositol concentrations of broilers at 15, 29, and 43 D of age (Table 5.10). Broilers receiving the PC diets had higher ($P < 0.01$) plasma inositol concentrations at 15 and 43 D of age than broilers provided the NC diets. The addition of dietary inositol to the PC diets resulted in increased ($P < 0.01$) plasma inositol of broilers compared with those consuming the PC diets only at 15, 29, and 43 D of age. However, plasma inositol concentrations of broilers fed the PC diets with inositol addition were similar ($P > 0.05$) to birds provided diets with 40,500 FTU/kg phytase at all collection periods.

DISCUSSION

The efficacy of phytase in hydrolyzing phytate in the current study was evident as supplementing phytase at 40,500 FTU/kg decreased concentrations of total IP2 to IP6 in both gizzard and ileal digesta contents to less than 10% of the total IP2 to IP6 concentrations in broilers fed diets with 500 FTU/kg phytase addition. Similarly, the

disappearance of total IP esters increased up to 98% when phytase was supplemented at 40,500 FTU/kg indicating the near complete destruction of all phytate esters. The reduction of phytate content in the gastrointestinal tract of broilers may likely be the reason for increased feed intake of broilers, which was presented in the companion paper. Previous research demonstrated that supplementing dietary phytate to grass carps resulted in higher cholecystokinin and cocaine- and amphetamine-regulated transcript, which promote feed intake reduction (Liu et al., 2014). In contrast, the efficacy of phytase in hydrolyzing phytate was observed to increase digesta passage rate and feed intake in broilers (Watson et al., 2006). The increase of feed intake may likely be associated with enhanced BW gain and meat accretion of broilers.

In the current study, increasing IP3 and IP4 concentrations in the gizzard and ileal digesta were observed with the addition of phytase at 1,500 and 4,500 FTU/kg even following reductions of IP5 and IP6 concentrations. As a result, both IP3 and IP4 were the most dominant phytate degradation products among total IP3 to IP6 concentrations. Similarly, Walk and Olukosi (2019) observed that IP4 accounted for the majority (46%) of total IP3 to IP6 in the gizzard digesta of broilers fed diets with 2,000 FTU/kg of *E. coli* phytase from *Trichoderma reesei* at 18 D of age. Interestingly, increasing phytase dose to 4,000 FTU/kg also resulted in the accumulation of IP4 (47% among total IP3 to IP6 concentrations) in the ileal digesta of broilers (Walk and Olukosi, 2019). The accumulation of IP3 and IP4 may occur due to the fact that these lower IP esters are poor substrates for commercial phytases (Wyss et al., 1999; Bedford and Walk, 2016). Previous research indicated that as more phosphate groups are released from a phytate molecule, the binding capacity of phytase to phytate esters decreases, resulting in the

accumulation of lower phytate esters (Menezes-Blackburn et al., 2015). Alternative strategy to reduce the accumulation of IP4 may be implemented by increasing concentration of phytase in broiler diets (Bedford and Walk, 2016). In the present research, the efficacy of phytase in degrading IP3 and IP4 in the gizzard and ileal digesta contents was evident when the concentrations of these phytate esters decreased and inositol concentration increased following the supplementation of 13,500 and 40,500 FTU/kg phytase.

The present research also demonstrated varying magnitudes of phytate degradation between the gizzard and ileal digesta. Log-quadratic reductions of IP3 and IP4 in gizzard digesta of broilers fed Treatments 1 to 6 were observed at all collection periods, whereas log-quadratic reduction of IP4 in the ileal digesta was observed at 29 and 43 D of age and IP3 only at 43 D of age. The inconsistency of phytate degradation in the ileal digesta compared with gizzard contents may be attributed to the change of pH from the gizzard to the small intestine (Schlemmer et al., 2001). The gizzard is the primary site of phytate degradation due to its low pH (Selle and Ravindran, 2007). However, as pH increases in the small intestine, phytate solubility decreases and dietary phytase activity is reduced. In a study using pigs, Schlemmer et al. (2001) indicated that when feeding non-extruded diets, 57% of all phytate hydrolysis products in the gastric chyme are soluble in the liquid phase. Conversely, up to 87% of these IP esters precipitates in the small intestine, presumably with minerals or proteins, resulting in low phytate degradation and unavailability of nutrients. Xu et al. (1992) demonstrated that IP4 reduced the solubility of calcium and zinc by approximately 40 and 90%, respectively, when pH rises from 5 to 6.

The difficulties in degrading lower phytate esters may also be influenced by an interactive effect of phytase dose and age of broilers (Olukosi et al., 2020). In research presented herein, concentrations of IP3 and IP4 in the ileal digesta increased as phytase dose is increased from 0 to 1,500 FTU/kg but decreased when phytase is further increased to 40,500 FTU/kg. However, as the age of broilers increased, the accumulation of IP3 and IP4 concentrations also increased in broilers supplemented with phytase at 1,500 FTU/kg. Data evaluating the efficacy of phytase in degrading lower IP esters in broilers up to 6 weeks of age are lacking. However, Olukosi et al. (2020) demonstrated that broilers at 28 D of age had higher concentrations of IP3 and IP4 than birds at 7 D of age when fed diets with phytase supplementation at either 1,500 or 3,000 FTU/kg.

The higher accumulation of IP3 and IP4 concentrations with the increase of age may be attributed to older birds having greater feed intake and digestive capacity than younger birds. This was evident as the proportion of IP4 to total IP esters in the present study increased from 14, 26, to 34% at 15, 29 and 43 D of age, respectively, in the ileal digesta of broilers fed diets with 1,500 FTU/kg. In addition, the influence of age on phytase efficacy may vary due to young birds having less developed gastrointestinal tract compared with older birds. From 6 to 10 D of age, the small intestine of broilers undergoes a rapid increase in size relative to body weight (Sklan, 2001). Presumably, this may have led to changes in endogenous phytase activity in the small intestine. Morgan et al. (2015) reported that ileal phytase activity and phytate hydrolysis of broilers fed diets without phytase addition increased from 4 to 14 D of age. As dietary phytase may be inactive in the small intestine of broilers, phytate degradation may occur due to the activity of intestinal brush border phytase (Maenz and Classen, 1998).

In the present study, broilers fed the PC diets appeared to have greater phytate degradation and ileal IP disappearance compared with those provided the NC diets and the PC diets with inositol addition. The unexpected presence of phytase in the PC diets may explain the greater occurrence of phytate degradation in broilers provided the PC diets. However, despite unexpectedly having phytase activity, there seems to be accumulation of IP3 and IP4 in the gizzard and ileal digesta contents of broilers fed the PC diets indicating the inability of degrading lower phytate esters with small amount of dietary phytase (695, 238, and 267 FTU/kg in the starter, grower, and finisher diets, respectively). This condition was evident as ileal inositol concentration was similar between broilers fed the NC and PC diets except in the ileal digesta at 15 D of age. Previous research has also reported that adding dietary *E. coli* phytase at 500 FTU/kg resulted in the accumulation of IP4 in the gizzard digesta of broilers (Zeller et al., 2015a).

In addition, negative IP disappearance values were observed in broilers fed the NC diets and PC diets supplemented with inositol at 29 and 43 D of age, but not in broilers at 15 D of age. Negative values of IP disappearance have been observed in previous studies when broilers were fed diets with low or no phytase addition. Perryman et al. (2017) noted a –9.3% ileal IP6 disappearance in broilers while Zeller et al. (2016) reported a –7.0% IP6 disappearance in the crop of broilers fed diets without phytase supplementation. In addition, Olukosi et al. (2020) observed a –19.1% IP6 disappearance in turkeys receiving diets with 500 FTU/kg phytase addition at 28 D of age, but not at 7 D of age.

Negative IP6 disappearance values may occur when there is an elevated ratio of marker in the diet to digesta and/or a decreased ratio of IP6 in the diet to digesta. The

ratio of marker in the diet to digesta may increase due to a slower passage rate, which slows down marker flow to the ileal digesta. A previous study indicated that broilers receiving diets without phytase addition had 20% slower rate of passage than broilers fed phytase-supplemented diets (Watson et al., 2006). In addition, the reduction of IP6 ratio in the diet to digesta may also lead to a negative IP6 disappearance value. This condition may occur either due to reverse peristalsis of the liquid fraction containing IP6 (Sacranie et al., 2007) or more complete digestion of starch and protein with increasing age (Batal and Parsons, 2002) resulting in higher concentration of IP6 in the digesta. In the research reported herein, the increase ileal IP6 concentration in birds fed the NC diets from 42,960 to 56,615 and 60,746 nmol/g at 15, 29, and 43 D of age decreased the ratio of IP6 in the diet to digesta from 0.43 to 0.32 and 0.26, respectively. As the ratio of IP6 in the diet to digesta decreases, the ratio of marker to IP6 may rise to above 1, which produced negative IP disappearance values.

The current research also added free inositol in Treatment 8 to further evaluate its role when phytase is supplemented in broiler diets. The amount of dietary inositol was calculated based on the assumption that all phytate in Treatment 6 can be degraded to inositol. Hence, the amount of free inositol in Treatment 8 should be similar to complete liberation of inositol in Treatment 6 (40,500 FTU/kg). However, inositol concentration in the ileal digesta of broilers fed diets supplemented with phytase (40,500 FTU/kg) was higher than in birds fed the PC diets with inositol addition at all phases. Analysis of dietary treatments in the starter, grower, and finisher phases indicated that phytate phosphorus concentrations in Treatment 6 were 0.34, 0.33, and 0.30%, which translate to 0.33, 0.32, and 0.29% inositol (0.968 ratio of inositol to phytate phosphorus),

respectively. However, the addition of inositol in Treatment 8 was 0.26, 0.25, and 0.23% in the starter, grower, and finisher diets, respectively, which were lower than the expected inositol liberation in Treatment 6. This underestimation may likely be attributed to the variation in phytate phosphorus concentration in feed ingredients. Previous research reported phosphorus concentration in corn and soybean meal, which ranges from 0.16 to 0.26% and 0.28 to 0.45%, respectively (Eeckhout and De Paepe, 1994; Leske and Coon, 1994; Ravindran et al., 1994; Selle and Ravindran, 2007; Tahir et al., 2012). In contrast, phytate phosphorus concentrations used in the present study were 0.17 and 0.48% for corn and soybean meal, respectively (AminoDat 5.0, 2016).

Effects of increasing phytase concentrations on the liberation of inositol in both gizzard and ileal digesta contents resulted in increased plasma inositol concentrations of broilers. Following the liberation, inositol can be absorbed in the jejunum and upper ileum using sodium-dependent and proton-dependent myo-inositol co-transporters (Walk et al., 2018). Previously, the supplementation of phytase has also been reported to increase plasma inositol concentrations of broilers (Cowieson et al., 2015; Sommerfeld et al., 2018). In pigs, plasma inositol concentration was observed to peak 360 minutes after the introduction of feed containing 3,000 FTU/kg of bacterial phytase from *Aspergillus oryzae* (Cowieson et al., 2017) indicating the rapid degradation of phytate by a high phyatse dose.

The response of plasma inositol due to phytase supplementation enabled the use of plasma inositol as a biomarker for phytase efficacy of phytate degradation. In the current research, at 15, 29, and 43 D of age, correlations between plasma inositol and total IP esters in the gizzard contents of broilers provided Treatments 1 to 6 were r = –

0.70, –0.73, and –0.64 ($P < 0.001$) and correlations with total IP esters in the ileal digesta were r = –0.63, –0.77, and –0.68 ($P < 0.001$), respectively. These data demonstrated that the increase of plasma inositol may be attributed to the reduction of total IP esters in the gizzard and ileal digesta contents; thus, allowing plasma inositol to be used as a biomarker for phytase efficacy.

In conclusion, supplementation of phytase up to 40,500 FTU/kg promoted phytate degradation, especially IP3 and IP4. The degradation of IP3 and IP4 is critical, as anti-nutritive effects of these lower IP esters may still be present to chelate nutrients. Moreover, further degradation of IP3 and IP4 was shown to liberate inositol. The reduction of anti-nutritive effects of phytate along with inositol liberation may contribute to the enhancement of growth performance and meat accretion of broilers. Additionally, elevated plasma inositol concentration following its liberation indicates the possibility of its use as a biomarker for phytase efficacy on phytate degradation. This attribute may allow phytase users to adopt plasma inositol as a biomarker for phytase efficacy.

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Table 5.1 Ingredient and nutrient composition of negative (NC) and positive control (PC) diets fed to broilers during starter (1 to 15 D of age), grower (16 to 29 D of age), and finisher (30 to 41 D of age) periods

	Starter		Grower		Finisher	
Item	NC	PC	NC	PC	NC	PC
Ingredient, %						
Corn	57.47	55.82	61.63	59.98	66.70	65.05
Soybean meal	36.61	36.86	31.69	31.94	26.63	26.88
Vegetable oil	1.18	1.80	2.29	2.91	2.62	3.24
Dicalcium phosphate	1.22	2.04	1.03	1.84	0.84	1.65
Calcium carbonate	1.07	1.05	0.99	0.96	0.90	0.87
Sodium chloride	0.40	0.39	0.40	0.40	0.40	0.40
Builder sand[1]	0.70	0.70	0.70	0.70	0.70	0.70
DL-Methionine	0.30	0.30	0.27	0.27	0.25	0.25
L-Lysine•HCl	0.17	0.17	0.17	0.16	0.17	0.16
L-Threonine	0.09	0.09	0.08	0.08	0.07	0.07
Mineral premix[2]	0.10	0.10	0.10	0.10	0.10	0.10
Vitamin premix[3]	0.10	0.10	0.08	0.08	0.05	0.05
Choline chloride	0.07	0.07	0.07	0.07	0.07	0.07
Titanium dioxide	0.50	0.50	0.50	0.50	0.50	0.50
Xylanase[4]	0.01	0.01	0.01	0.01	0.01	0.01
Calculated nutrient composition, % (unless otherwise noted)						
AME_n, kcal/kg	3,000	3,000	3,110	3,110	3,185	3,185
Crude protein	21.83	21.82	19.80	19.79	17.77	17.77
Digestible lysine	1.19	1.19	1.07	1.07	0.95	0.95
Digestible TSAA[5]	0.88	0.88	0.81	0.81	0.74	0.74
Digestible threonine	0.80	0.80	0.72	0.72	0.63	0.63
Digestible valine	0.89	0.89	0.81	0.81	0.73	0.73
Digestible isoleucine	0.84	0.84	0.75	0.75	0.67	0.67
Calcium	0.80	0.96	0.71	0.87	0.62	0.78
Non-phytate phosphorus	0.33	0.48	0.29	0.44	0.24	0.39
Sodium	0.18	0.18	0.18	0.18	0.18	0.18

[1]The NC basal diet (Treatment 1) was supplemented with phytase at the expense of sand (Quantum Blue 5G, AB Vista Feed Ingredients, Marlborough, UK; analyzed as 7,700 FTU/g) at 500, 1,500, 4,500, 13,500, and 40,500 FTU/kg of diet to create Treatments 2 to 6, respectively. The PC basal diet (Treatment 7) was supplemented with inositol at the expense of sand to generate Treatment 8.[5, 6]

[2]Trace mineral premix include per kg of diet: Mn (manganese sulfate), 120 mg; Zn (zinc sulfate), 100 mg; Fe (iron sulfate monohydrate), 30 mg; Cu (tri-basic copper chloride), 8 mg; I (ethylenediamine dihydriodide), 1.4 mg; and Se (sodium selenite), 0.3 mg.

[3]Vitamin premix includes per kg of diet: Vitamin A (Vitamin A acetate), 18,739 IU; Vitamin D_3 (cholecalciferol), 6,614 IU; Vitamin E (DL-alpha tocopherol acetate), 66 IU; menadione (menadione sodium bisulfate complex), 4 mg; Vitamin B_{12} (cyanocobalamin), 0.03 mg; folacin (folic acid), 2.6 mg: D-pantothenic acid (calcium pantothenate), 31 mg; riboflavin (riboflavin),

22 mg; niacin (niacinamide), 88 mg; thiamin (thiamin mononitrate), 5.5 mg; biotin (biotin), 0.18 mg; and pyridoxine (pyridoxine hydrochloride), 7.7 mg.
[4]Econase XT, AB Vista Feed Ingredients, Marlborough, UK
[5]One unit of phytase activity (FTU) is defined as the quantity of enzyme to liberate 1 µmol of inorganic phosphorus per minute from 0.0051 mol/L sodium phytate at pH 5.5 and 37°C.
[6]Inositol supplementation was calculated based on the maximum inositol liberation from Treatment 6 at 0.26, 0.25, and 0.23%, respectively in the starter, grower, and finisher periods.

Table 5.2 Analyzed activity of phytase (FTU/kg) in the starter (1 to 15 D of age), grower (16 to 29 D of age), and finisher (30 to 41 D of age) diets[1]

Dietary Treatments	Starter	Grower	Finisher
1) Negative control (NC)[2]	65	< 50	< 50
2) NC + 500 FTU/kg	417	519	641
3) NC + 1,500 FTU/kg	1,250	1,250	1,490
4) NC + 4,500 FTU/kg	3,313	3,350	3,960
5) NC + 13,500 FTU/kg	12,480	13,710	12,690
6) NC + 40,500 FTU/kg	36,640	41,290	42,070
7) Positive Control (PC)[2]	695	238	267
8) PC + Inositol[3]	65	< 50	< 50

[1]One unit of phytase activity (FTU) is defined as the quantity of enzyme to liberate 1 µmol of inorganic phosphorus per minute from 0.0051 mol/L sodium phytate at pH 5.5 and 37°C.
[2]The negative control diet was formulated to contain 0.165 and 0.150% lower calcium and phosphorus, respectively, compared with the positive control diet.
[3]Inositol supplementation was calculated based on the maximum inositol liberation from the diet containing the highest phytase concentration.

Table 5.3 Concentrations of inositol phosphate (IP) esters and inositol (nmol/g) in the gizzard digesta of broilers at 15 D of age fed diets with gradient phytase supplementation[1]

Item	IP6[2]	IP5[2]	IP4[2]	IP3[2]	∑IP[2]	Inositol
Dietary Treatments						
1) Negative Control (NC)[3]	4,953	520	264	102	5,839	457
2) NC + 500 FTU/kg[4]	3,197	606	625	209	4,636	604
3) NC + 1,500 FTU/kg	639	238	1,042	333	2,252	794
4) NC + 4,500 FTU/kg	116	39	291	153	599	1,486
5) NC + 13,500 FTU/kg	23	9	46	93	171	1,831
6) NC + 40,500 FTU/kg	11	2	12	79	104	2,536
7) Positive Control (PC)	569	193	984	346	2,092	802
8) PC + Inositol[5]	3,429	532	643	180	4,784	2,434
Pooled standard error	232	33	104	30	247	147
Source of Variation			*Probabilities*			
Log-linear effect of phytase[6]	< 0.001	< 0.001	0.32	0.99	< 0.001	< 0.001
Log-quadratic effect of phytase[6]	< 0.001	< 0.001	0.002	0.019	< 0.001	< 0.001
Treatment 1 vs. 7	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.07
Treatment 6 vs. 8	< 0.001	< 0.001	< 0.001	0.017	< 0.001	0.64
Treatment 7 vs. 8	< 0.001	< 0.001	0.016	< 0.001	< 0.001	< 0.001

[1]Values are least-square means of 8 replicate pens, with 4 birds selected per pen for necropsy at 15 D of age. Concentrations of inositol biphosphate (IP2) were below limit of detection.

[2]IP6 = inositol hexakisphosphate, IP5 = inositol pentaphosphate, IP4 = inositol tetraphosphate, IP3 = inositol triphosphate, and ∑IP = total IP6 to IP3

[3]The negative control diet was formulated to contain 0.165 and 0.150% lower calcium and phosphorus concentrations, respectively, compared with the positive control diet.

[4]One unit of phytase activity (FTU) is defined as the quantity of enzyme to liberate 1 µmol of inorganic phosphorus from 0.0051 mol/L sodium phytate per minute from sodium phytate at pH 5.5 and 37°C.

[5]Inositol supplementation was calculated based on the maximum inositol liberation from the treatment containing the highest phytase concentration (Treatment 6).

[6]Phytase concentrations were $\log_{10}$ transformed before analysis. Log-linear and log-quadratic effects were analyzed for Treatments 1 to 6.

Table 5.4 Concentrations of inositol phosphate (IP) esters and inositol (nmol/g) in the gizzard digesta of broilers at 29 D of age fed diets with gradient phytase supplementation[1]

Item	IP6[2]	IP5[2]	IP4[2]	IP3[2]	∑IP[2]	Inositol
Dietary Treatments						
1) Negative Control (NC)[3]	5,516	620	352	163	6,651	322
2) NC + 500 FTU/kg[4]	2,273	520	1,439	451	4,682	297
3) NC + 1,500 FTU/kg	873	287	1,729	608	3,498	286
4) NC + 4,500 FTU/kg	27	16	126	146	316	673
5) NC + 13,500 FTU/kg	20	22	57	121	220	805
6) NC + 40,500 FTU/kg	0	7	11	87	105	841
7) Positive Control (PC)	1,897	484	1,772	468	4,620	803
8) PC + Inositol[5]	4,526	627	455	161	5,768	1,098
Pooled standard error	276	63	163	48	318	297
Source of Variation			*Probabilities*			
Log-linear effect of phytase[6]	< 0.001	< 0.001	0.24	0.45	< 0.001	0.014
Log-quadratic effect of phytase[6]	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.011
Treatment 1 vs. 7	< 0.001	0.11	< 0.001	< 0.001	< 0.001	0.37
Treatment 6 vs. 8	< 0.001	< 0.001	0.045	0.25	< 0.001	0.29
Treatment 7 vs. 8	< 0.001	0.10	< 0.001	< 0.001	0.007	0.31

[1]Values are least-square means of 8 replicate pens, with 2 birds selected per pen for necropsy at 29 D of age. Concentrations of inositol biphosphate (IP2) were below limit of detection.

[2]IP6 = inositol hexakisphosphate, IP5 = inositol pentaphosphate, IP4 = inositol tetraphosphate, IP3 = inositol triphosphate, and ∑IP = total IP6 to IP3

[3]The negative control diet was formulated to contain 0.165 and 0.150% lower calcium and phosphorus concentrations, respectively, compared with the positive control diet.

[4]One unit of phytase activity (FTU) is defined as the quantity of enzyme to liberate 1 μmol of inorganic phosphorus from 0.0051 mol/L sodium phytate per minute from sodium phytate at pH 5.5 and 37°C.

[5]Inositol supplementation was calculated based on the maximum inositol liberation from the treatment containing the highest phytase concentration (Treatment 6).

[6]Phytase concentrations were $\log_{10}$ transformed before analysis. Log-linear and log-quadratic effects were analyzed for Treatments 1 to 6.

Table 5.5 Concentrations of inositol phosphate (IP) esters and inositol (nmol/g) in the gizzard digesta of broilers at 43 D of age fed diets with gradient phytase supplementation[1]

Item	IP6[2]	IP5[2]	IP4[2]	IP3[2]	∑IP[2]	Inositol
Dietary Treatments						
1) Negative Control (NC)[3]	5,255	513	291	178	6,236	352
2) NC + 500 FTU/kg[4]	1,480	323	1,530	536	3,869	443
3) NC + 1,500 FTU/kg	341	50	767	551	1,708	576
4) NC + 4,500 FTU/kg	286	53	362	254	954	827
5) NC + 13,500 FTU/kg	135	18	58	117	327	1,044
6) NC + 40,500 FTU/kg	145	6	25	114	290	1,414
7) Positive Control (PC)	1,837	411	1,549	593	4,390	302
8) PC + Inositol[5]	4,911	727	891	371	6,901	1,026
Pooled standard error	380	70	185	56	504	326
Source of Variation			*Probabilities*			
Log-linear effect of phytase[6]	< 0.001	< 0.001	0.25	0.52	< 0.001	0.025
Log-quadratic effect of phytase[6]	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.043
Treatment 1 vs. 7	< 0.001	0.28	< 0.001	< 0.001	0.010	0.90
Treatment 6 vs. 8	< 0.001	< 0.001	0.002	0.002	< 0.001	0.27
Treatment 7 vs. 8	< 0.001	0.002	0.015	0.007	0.001	0.07

[1]Values are least-square means of 8 replicate pens, with 2 birds selected per pen for necropsy at 43 D of age. Concentrations of inositol biphosphate (IP2) were below limit of detection.

[2]IP6 = inositol hexakisphosphate, IP5 = inositol pentaphosphate, IP4 = inositol tetraphosphate, IP3 = inositol triphosphate, and ∑IP = total IP6 to IP3

[3]The negative control diet was formulated to contain 0.165 and 0.150% lower calcium and phosphorus concentrations, respectively, compared with the positive control diet.

[4]One unit of phytase activity (FTU) is defined as the quantity of enzyme to liberate 1 µmol of inorganic phosphorus from 0.0051 mol/L sodium phytate per minute from sodium phytate at pH 5.5 and 37°C.

[5]Inositol supplementation was calculated based on the maximum inositol liberation from the treatment containing the highest phytase concentration (Treatment 6).

[6]Phytase concentrations were $\log_{10}$ transformed before analysis. Log-linear and log-quadratic effects were analyzed for Treatments 1 to 6.

Table 5.6 Concentrations of inositol phosphate (IP) esters and inositol (nmol/g) in the ileal digesta of broilers at 15 D of age fed diets with gradient phytase supplementation[1]

Item	IP6[2]	IP5[2]	IP4[2]	IP3[2]	IP2[2]	∑IP[2]	Inositol
Dietary Treatments							
1) Negative Control (NC)[3]	42,960	3,779	1,536	440	234	48,949	7,485
2) NC + 500 FTU/kg[4]	39,832	5,482	3,092	697	364	49,467	8,350
3) NC + 1,500 FTU/kg	23,084	5,408	4,631	1,207	326	34,656	12,490
4) NC + 4,500 FTU/kg	3,884	1,318	4,036	1,720	145	11,103	21,143
5) NC + 13,500 FTU/kg	405	122	1,517	951	271	3,266	21,070
6) NC + 40,500 FTU/kg	190	49	202	96	212	749	26,314
7) Positive Control (PC)	17,082	5,634	8,812	2,973	336	34,837	10,642
8) PC + Inositol[5]	47,661	5,637	2,256	612	458	56,623	21,014
Pooled standard error	2,867	690	757	273	104	3,485	1,278
Source of Variation				*Probabilities*			
Log-linear effect of phytase[6]	< 0.001	0.002	0.96	0.34	0.89	< 0.001	< 0.001
Log-quadratic effect of phytase[6]	< 0.001	< 0.001	0.18	0.63	0.27	< 0.001	< 0.001
Treatment 1 vs. 7	< 0.001	0.032	< 0.001	< 0.001	0.46	0.001	0.048
Treatment 6 vs. 8	< 0.001	< 0.001	0.039	0.16	0.10	< 0.001	0.002
Treatment 7 vs. 8	< 0.001	0.91	< 0.001	< 0.001	0.40	< 0.001	< 0.001

[1]Values are least-square means of 8 replicate pens, with 4 birds selected per pen for necropsy at 15 D of age.

[2]IP6 = inositol hexakisphosphate, IP5 = inositol pentaphosphate, IP4 = inositol tetraphosphate, IP3 = inositol triphosphate, IP2 = inositol bisphosphate, and ∑IP = total IP6 to IP2

[3]The negative control diet was formulated to contain 0.165 and 0.150% lower calcium and phosphorus concentrations, respectively, compared with the positive control diet.

[4]One unit of phytase activity (FTU) is defined as the quantity of enzyme to liberate 1 μmol of inorganic phosphorus from 0.0051 mol/L sodium phytate per minute from sodium phytate at pH 5.5 and 37°C.

[5]Inositol supplementation was calculated based on the maximum inositol liberation from the treatment containing the highest phytase concentration (Treatment 6).

[6]Phytase concentrations were $\log_{10}$ transformed before analysis. Log-linear and log-quadratic effects were analyzed for Treatments 1 to 6.

Table 5.7 Concentrations of inositol phosphate (IP) esters and inositol (nmol/g) in the ileal digesta of broilers at 29 D of age fed diets with gradient phytase supplementation[1]

Item	IP6[2]	IP5[2]	IP4[2]	IP3[2]	IP2[2]	∑IP[2]	Inositol
Dietary Treatments							
1) Negative Control (NC)[3]	56,615	5,821	1,393	246	613	64,688	3,189
2) NC + 500 FTU/kg[4]	33,354	7,817	5,758	1,279	536	48,744	4,708
3) NC + 1,500 FTU/kg	13,689	4,451	6,631	1,523	764	27,059	10,294
4) NC + 4,500 FTU/kg	2,145	1,085	5,117	2,827	1,034	11,171	17,243
5) NC + 13,500 FTU/kg	391	109	1,024	546	784	2,854	20,716
6) NC + 40,500 FTU/kg	314	52	283	105	594	1,348	22,879
7) Positive Control (PC)	31,783	9,332	9,468	2,537	1,290	54,409	4,449
8) PC + Inositol[5]	47,115	6,243	2,100	440	773	56,673	16,469
Pooled standard error	3,000	559	803	255	137	3,434	1,180
Source of Variation				*Probabilities*			
Log-linear effect of phytase[6]	< 0.001	< 0.001	0.79	0.34	0.37	< 0.001	< 0.001
Log-quadratic effect of phytase[6]	< 0.001	< 0.001	< 0.001	0.16	0.37	< 0.001	< 0.001
Treatment 1 vs. 7	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.023	0.42
Treatment 6 vs. 8	< 0.001	< 0.001	0.07	0.37	0.31	< 0.001	< 0.001
Treatment 7 vs. 8	< 0.001	< 0.001	< 0.001	< 0.001	0.005	0.61	< 0.001

[1]Values are least-square means of 8 replicate pens, with 2 birds selected per pen for necropsy at 29 D of age.

[2]IP6 = inositol hexakisphosphate, IP5 = inositol pentaphosphate, IP4 = inositol tetraphosphate, IP3 = inositol triphosphate, IP2 = inositol bisphosphate, and ∑IP = total IP6 to IP2

[3]The negative control diet was formulated to contain 0.165 and 0.150% lower calcium and phosphorus concentrations, respectively, compared with the positive control diet.

[4]One unit of phytase activity (FTU) is defined as the quantity of enzyme to liberate 1 µmol of inorganic phosphorus from 0.0051 mol/L sodium phytate per minute from sodium phytate at pH 5.5 and 37°C.

[5]Inositol supplementation was calculated based on the maximum inositol liberation from the treatment containing the highest phytase concentration (Treatment 6).

[6]Phytase concentrations were $\log_{10}$ transformed before analysis. Log-linear and log-quadratic effects were analyzed for Treatments 1 to 6.

Table 5.8 Concentrations of inositol phosphate (IP) esters and inositol (nmol/g) in the ileal digesta of broilers at 43 D of age fed diets with gradient phytase supplementation[1]

Item	IP6[2]	IP5[2]	IP4[2]	IP3[2]	IP2[2]	∑IP[2]	Inositol
Dietary Treatments							
1) Negative Control (NC)[3]	60,746	6,204	1,902	175	609	69,637	1,728
2) NC + 500 FTU/kg[4]	27,367	7,101	7,648	1,733	783	44,632	4,120
3) NC + 1,500 FTU/kg	9,185	2,901	8,357	2,761	795	24,000	8,894
4) NC + 4,500 FTU/kg	2,868	604	3,229	1,425	510	8,637	13,339
5) NC + 13,500 FTU/kg	1,885	296	1,387	311	596	4,475	14,893
6) NC + 40,500 FTU/kg	419	32	126	41	474	1,092	18,548
7) Positive Control (PC)	27,515	6,487	7,324	1,884	1,301	44,512	3,512
8) PC + Inositol[5]	47,628	6,360	2,793	476	977	58,234	13,118
Pooled standard error	3,078	590	1,016	398	178	3,966	1,225
Source of Variation	*Probabilities*						
Log-linear effect of phytase[6]	< 0.001	< 0.001	0.58	0.71	0.51	< 0.001	< 0.001
Log-quadratic effect of phytase[6]	< 0.001	< 0.001	< 0.001	0.011	0.14	< 0.001	< 0.001
Treatment 1 vs. 7	< 0.001	0.68	< 0.001	0.001	0.004	< 0.001	0.26
Treatment 6 vs. 8	< 0.001	< 0.001	0.043	0.39	0.031	< 0.001	0.001
Treatment 7 vs. 8	< 0.001	0.84	< 0.001	0.007	0.16	0.008	< 0.001

[1]Values are least-square means of 8 replicate pens, with 2 birds selected per pen for necropsy at 43 D of age.

[2]IP6 = inositol hexakisphosphate, IP5 = inositol pentaphosphate, IP4 = inositol tetraphosphate, IP3 = inositol triphosphate, IP2 = inositol bisphosphate, and ∑IP = total IP6 to IP2

[3]The negative control diet was formulated to contain 0.165 and 0.150% lower calcium and phosphorus concentrations, respectively, compared with the positive control diet.

[4]One unit of phytase activity (FTU) is defined as the quantity of enzyme to liberate 1 μmol of inorganic phosphorus from 0.0051 mol/L sodium phytate per minute from sodium phytate at pH 5.5 and 37°C.

[5]Inositol supplementation was calculated based on the maximum inositol liberation from the treatment containing the highest phytase concentration (Treatment 6).

[6]Phytase concentrations were $\log_{10}$ transformed before analysis. Log-linear and log-quadratic effects were analyzed for Treatments 1 to 6.

Table 5.9 Ileal disappearance (%) of inositol phosphate 6 (IP6) and total inositol phosphate 6 to 2 (∑IP) of broilers at 15, 29, and 43 D of age fed diets with gradient phytase supplementation[1]

	15 D		29 D		43 D	
Item	IP6	∑IP	IP6	∑IP	IP6	∑IP
Dietary Treatments						
1) Negative Control (NC)[2]	32.97	30.49	–6.34	–9.84	–9.29	–10.22
2) NC + 500 FTU/kg[3]	35.72	26.28	49.90	33.00	41.77	16.46
3) NC + 1,500 FTU/kg	60.24	46.04	76.15	56.74	80.52	56.13
4) NC + 4,500 FTU/kg	92.36	81.43	96.15	82.49	92.46	83.35
5) NC + 13,500 FTU/kg	99.38	95.60	99.12	94.95	96.82	93.48
6) NC + 40,500 FTU/kg	99.69	98.49	99.40	98.05	98.31	97.49
7) Positive Control (PC)	73.75	52.11	48.04	20.53	50.52	29.49
8) PC + Inositol[4]	29.31	24.64	0.61	–2.17	–8.58	–15.22
Pooled standard error	4.54	5.40	3.78	4.93	7.17	6.87
Source of Variation	*Probabilities*					
Log-linear effect of phytase[5]	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Log-quadratic effect of phytase[5]	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Treatment 1 vs. 7	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Treatment 6 vs. 8	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Treatment 7 vs. 8	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

[1]Values are least-square means of 8 replicate pens, with 4, 2, and 2 birds selected per pen for necropsy at 15, 29, and 43 D of age, respectively.

[2]The negative control diet was formulated to contain 0.165 and 0.150% lower calcium and phosphorus concentrations, respectively, compared with the positive control diet.

[3]One unit of phytase activity (FTU) is defined as the quantity of enzyme to liberate 1 μmol of inorganic phosphorus from 0.0051 mol/L sodium phytate per minute from sodium phytate at pH 5.5 and 37°C.

[4]Inositol supplementation was calculated based on the maximum inositol liberation from the treatment containing the highest phytase concentration (Treatment 6).

[5]Phytase concentrations were $\log_{10}$ transformed before analysis. Log-linear and log-quadratic effects were analyzed for Treatments 1 to 6.

Table 5.10 Plasma inositol concentration (μM) of broilers fed diets with gradient phytase supplementation from 1 to 43 D of age[1]

Item	15 D	29 D	43 D
Dietary Treatment			
1) Negative Control (NC)[2]	247	234	234
2) NC + 500 FTU/kg[3]	305	299	260
3) NC + 1,500 FTU/kg	392	396	374
4) NC + 4,500 FTU/kg	517	472	456
5) NC + 13,500 FTU/kg	544	480	450
6) NC + 40,500 FTU/kg	473	458	469
7) Positive Control (PC)	332	276	354
8) PC + Inositol[4]	474	402	526
Pooled standard error	34	29	30
Source of Variation		*Probabilities*	
Log-linear effect of phytase[5]	< 0.001	< 0.001	< 0.001
Log-quadratic effect of phytase[5]	< 0.001	< 0.001	< 0.001
Treatment 1 vs. 7	0.022	0.29	0.005
Treatment 6 vs. 8	0.98	0.15	0.16
Treatment 7 vs. 8	< 0.001	0.002	< 0.001

[1]Values are least-square means of 8 replicate pens with 4, 2, and 2 birds selected per pen for blood collections at 15, 29, and 43 D of age, respectively.
[2]The negative control diet was formulated to contain 0.165 and 0.150% lower calcium and phosphorus concentrations, respectively, compared with the positive control diet.
[3]One unit of phytase activity (FTU) is defined as the quantity of enzyme to liberate 1 μmol of inorganic phosphorus from 0.0051 mol/L sodium phytate per minute from sodium phytate at pH 5.5 and 37°C.
[4]Inositol supplementation was calculated based on the maximum inositol liberation from the diet containing the highest phytase concentration (Treatment 6).
[5]Phytase concentrations were $\log_{10}$ transformed before analysis. Log-linear and log-quadratic effects were analyzed for Treatments 1 to 6.

VI. EFFECTS OF PHYTASE SUPPLEMENTATION AND NUTRIENT UPLIFTS ON GROWTH PERFORMANCE, CARCASS CHARACTERISTICS, HYPOTHALAMIC GENE EXPRESSION OF APPETITE HORMONES, AND CATECHOLAMINE CONCENTRATIONS IN BROILERS FROM 1 TO 43 DAYS OF AGE

ABSTRACT

Experiments were conducted to evaluate extra-phosphoric effects of phytase and nutrient uplifts on growth performance, meat yield, hypothalamic gene expression of appetite hormones, and catecholamine concentrations of broilers. Experiment 4 determined differences of digestible amino acid concentrations and AME_n using 256 Yield Plus × Ross 708 broilers (32 cages, 8 birds/cage) fed diets without or with 4,500 phytase units (**FTU**)/kg inclusion (16 reps/treatment). In Experiment 5, 832 Yield Plus × Ross 708 broilers (32 pens; 26 birds/pen) were provided diets in a 2 × 2 factorial arrangement consisting of 2 nutrient contents (control or nutrient uplifts) and 2 phytase inclusions (0 or 4,500 FTU/kg). Nutrient uplifts were formulated to contain 0.007, 0.015, 0.013, 0.021, 0.024%, and 61 kcal/kg higher digestible SAA, Lys, Thr, Val, Ile, and AME_n (from Experiment 4) respectively, compared with the control diet. Nutrient uplifts were used to mimic extra-phosphoric effects of phytase on nutrient intake. Growth performance was determined at 14, 28, and 40 D of age and carcass characteristics at 41

D of age. At 43 D of age, plasma inositol, hypothalamic gene expression of appetite hormones, and catecholamine concentrations were determined from 4 birds/pen. Additive effects of phytase inclusion and nutrient uplifts resulted in the lowest ($P < 0.05$) feed conversion from 1 to 40 D of age and the heaviest ($P < 0.01$) breast meat weights among dietary treatments. Phytase addition numerically increased feed intake ($P = 0.06$) and BW gain ($P = 0.051$) compared with birds fed diets without phytase from 1 to 40 D of age. Plasma inositol and dopamine concentrations were 2.3 and 1.2 fold higher ($P < 0.01$) in broilers fed phytase-added diets than birds fed diets without phytase inclusion. However, gene expression of neuropeptide Y, agouti-related peptide, proopiomelanocortin, cholecystokinin A receptor, ghrelin, and serotonin concentration were not different ($P > 0.05$) among treatments. These data indicated extra-phosphoric effects of phytase on nutrient intake. However, the mechanism of appetite stimulation by phytase warrants future research.

INTRODUCTION

Phytase supplementation targeting effects beyond phosphorus liberation was reported to enhance growth performance and carcass characteristics of broilers (Gehring et al., 2013; Campasino et al., 2014; Walk et al., 2014; Beeson et al., 2017). Although these effects have been attributed to increased nutrient utilization (Selle and Ravindran, 2007; Selle et al., 2012; Dersjant-Li et al., 2013), benefits of extra-phosphoric effects of phytase may also originate from the stimulation of feed intake (Walk and Olukosi, 2019). A previous study in our laboratory demonstrated that increasing *E. coli* phytase concentration from 0 to 40,500 FTU/kg led to a positive correlation ($r = 0.73$, $P < 0.001$) between feed intake and BW gain of broilers with maximum feed intake of broilers

obtained with 4,500 FTU/kg phytase inclusion (Kriseldi et al., 2019). Similarly, Walk and Olukosi (2019) reported that the increased BW gain of broilers due to *E. coli* phytase addition of 0, 2,000, and 4,000 FTU/kg was associated with increased digestible amino acid intake. Therefore, extra-phosphoric effects of phytase addition may increase feed intake, which translates to increased nutrient intake (Walk and Olukosi, 2019).

Despite the potential benefits of phytase in increasing feed intake, research evaluating mechanisms of extra-phosphoric effects of phytase on nutrient intake is lacking; thus, warrants further investigation. Presumably, changes in feeding behavior may be influenced by the effect of phytase on phytate degradation (Watson et al., 2006; Liu et al., 2014). Nutrients liberated from phytate hydrolysis may serve as indicators or precursors to alter gene expression of appetite hormones or catecholamines leading to changes in appetite (Richards and Proszkowiec-Weglarz, 2007; Dridi, 2017). Therefore, it was hypothesized that broilers provided diets with phytase addition will have increased feed intake through changes in gene expression of appetite hormones and catecholamine concentrations. Two experiments were conducted to determine extra-phosphoric effects of phytase and nutrient uplifts on growth performance, meat yield, hypothalamic gene expression of appetite hormones, and catecholamine concentrations of broilers throughout a 6-week production period.

MATERIALS AND METHODS

All procedures involving live birds were approved by Auburn University Institutional Animal Care and Use Committee (PRN 2018–3394).

Broiler Husbandry and Processing

Experiments were conducted utilizing Yield Plus × Ross 708 male chicks (Aviagen North America, Huntsville, AL). Chicks were obtained from a commercial hatchery post-hatch and were vaccinated for Marek's disease, Newcastle disease, and infectious bronchitis. In Experiment 4, 256 broiler chicks were placed into 32 battery cages (8 birds/cage; 0.06 m^2/bird; Petersime, Gettysburg, OH). Cages were placed in a solid-sided room equipped with exhaust fans, forced-air heaters, and evaporative coolers to adjust room temperature. Each cage was provided with 1 trough feeder and 1 trough waterer. The temperature of the room was set at 33^{o}C at placement and was gradually decreased to 25^{o}C at 23 D of age. Photoperiod was provided at 23L:1D from 1 to 7 D of age and 20L:4D from 8 to 23 D of age, while light intensity was maintained at 30 lux throughout the experimental period. Feed and water were provided ad libitum. Birds and feed were weighed at 1 and 19 D of age to determine BW, BW gain, feed intake, and feed conversion ratio (**FCR**). The incidence of mortality was recorded daily.

In Experiment 5, 832 broiler chicks were placed into 32 floor pens (26 birds/pen; 0.08 m^2/bird) equipped with a tube feeder, a nipple drinker line, and litter (from 2 previous flocks). Birds were housed in a solid-sided house with a negative-pressure ventilation system equipped with exhaust fans, cooling pads, and forced-air heaters to adjust house temperature. At placement, ambient temperature was set at 33^{o}C and was gradually decreased to 20^{o}C at 41 D of age. Light intensity was maintained at 30, 10, and 5 lux from 1 to 7, 8 to 14, and 15 to 41 D of age, respectively. Photoperiod was provided at 23L:1D from 1 to 7 D of age and 20L:4D for the remainder of the experiment. Feed and water were provided ad libitum. Feed and birds were weighed at 1, 14, 28, and 40 D

of age to determine BW, BW gain, feed intake, and FCR. The incidence of mortality was recorded daily.

At 41 D of age, 14 birds per pen were selected for processing. Feed was removed from each pen 10 hours prior to processing. Selected birds were placed in coops and transported to the Auburn University Pilot Processing Plant. Broilers were hung on shackles, electrically stunned, slaughtered, scalded, picked, and manually eviscerated. Following 3 hours of chilling in an ice bath, carcass (without abdominal fat) and abdominal fat weights were recorded. Whole carcasses were then placed in ice for 18 hours. The whole carcass was deboned to determine weights of *pectoralis major* muscle (boneless breast) and *minor* muscle (tenders), wing, drum, and thigh. Meat yields were calculated relative to the 40-D live BW.

Dietary Treatments

In Experiment 4, 2 dietary treatments were fed from 1 to 23 D of age consisting of a control diet and a diet supplemented with 4,500 phytase units (**FTU**)/kg (Table 6.1). Treatment 1 was formulated with calcium and non-phytate phosphorus at 0.96 and 0.48%, respectively. Treatment 2 was formulated similar to Treatment 1 but with 0.165 and 0.150% lower calcium and non-phytate phosphorus concentrations, respectively. *Escherichia coli* phytase expressed in *Trichoderma ressei* was supplemented in Treatment 2 at 4,500 FTU/kg (Quantum Blue 5G, AB Vista, Marlborough, UK). One FTU is defined as the quantity of phytase required to release 1 µmol of monocalcium phosphate from 0.0051 mol/L sodium phytate in 1 minute at pH 5.5 and 37°C (Simons et al., 1990). Phytase concentration was selected based on a previous study in our laboratory, which indicated that optimum growth performance of broilers may be

obtained by supplementing phytase at 4,500 FTU/kg (Kriseldi et al., 2019). Both treatments were formulated with digestible amino acid concentrations at 93% of Aviagen Ross 708 Broiler Nutrition Specifications (Aviagen, 2016) to mimic commercial practice.

In Experiment 5, 4 dietary treatments were provided for starter (from 1 to 14 D of age), grower (from 15 to 28 D of age), and finisher (from 29 to 43 D of age) periods (Table 6.2). Experimental diets were provided in a 2 × 2 factorial arrangement consisting of 2 nutrient contents (control or nutrient uplifts) and 2 phytase inclusions (0 or 4,500 FTU/kg). Treatment 1 was the control diet. Treatment 2 was formulated with nutrient uplifts. Treatment 3 was formulated to contain phytase addition at 4,500 FTU/kg. Treatment 4 was formulated with both nutrient uplifts and phytase supplementation at 4,500 FTU/kg. Diets with nutrients uplifts were formulated to contain higher digestible SAA, Lys, Thr, Val, Ile concentrations, and AME_n at 0.007, 0.015, 0.013, 0.021, 0.024%, and 61 kcal/kg, respectively. Nutrient uplifts were obtained from differences in digestible amino acid concentrations and AME_n between the control and the diet with phytase addition in the Experiment 4. Nutrient uplifts were utilized to mimic effects of phytase on nutrient intake. Additionally, phytase concentration was selected based on a previous study in our laboratory, which indicated that optimum growth performance may be obtained when broilers were supplemented with 4,500 FTU/kg phytase (Kriseldi et al., 2019). Phytase used was an *E. coli* phytase expressed in *Trichoderma reesei* (Quantum Blue 5G, AB Vista, Marlborough, UK). All treatments were formulated to contain 93% amino acid concentrations of Aviagen Ross 708 Broiler Nutrition Specifications (Aviagen, 2016) to mimic moderate amino acid specifications used in the US broiler industry. Feed samples from each experiment were analyzed for phytase activity by

ELISA specific for Quantum Blue (ESC, Standard Analytical Method, SAM099; AB Vista) similarly to the method by Engelen et al. (2001).

Sample Collections, Chemical Analyses, and Calculations

In Experiment 4, feed consumption and excreta output were measured for determination of AME_n of dietary treatments from 20 to 22 D of age. Excreta subsamples were collected from 10 locations of the pan beneath each cage and homogenized to create a pooled sample (approximately 2 kg) for each pen. Representative samples of excreta and feed were lyophilized in a freeze dryer (VirTis Genesis 25ES, SP Industries Inc., Warminster, PA). Dried samples were ground through a coffee grinder to ensure a homogenous mixture. Duplicate 0.8 g samples of feed and excreta were analyzed for gross energy using an adiabatic oxygen bomb calorimeter (Model no. 6300, Parr Instruments, Moline, IA), while nitrogen content (250 mg samples) was measured using a combustion analyzer (Rapid N cube, Elementar, Hanau, Germany) according to Dumas method (method 968.06; AOAC International, 2006). Nitrogen corrected AME for each dietary treatment was calculated using a nitrogen correction factor of 8.73 kcal/g (Titus, 1956) with the following equation:

$$AME_n = \frac{[GE_{Intake}(kcal) - GE_{Excretion}(kcal)] - \{8.73(kcal/g) \times [N_{Intake}(g) - N_{Excretion}(g)]\}}{Feed\ Intake\ (kg)}$$

where, GE_{Intake} and $GE_{Excretion}$ represent the amount of gross energy consumed and excreted, respectively; and N_{Intake} and $N_{Excretion}$ represent the amount of nitrogen consumed and excreted by broilers.

At 23 D of age, ileal digesta from 6 birds per cage was collected to determine the apparent ileal amino acid digestibility of dietary treatments. Ileal digesta from each bird

was collected by gently flushing the terminal ileum of broilers using deionized-distilled water, which was defined as the terminal 1/3 of the section between the Meckel's diverticulum and approximately 4 cm anterior from the ileo-cecal junction (Rodehutscord et al., 2012). Pooled ileal digesta contents were immediately frozen by submersion in liquid nitrogen for approximately 5 minutes to terminate phytase activity. Samples were kept on ice until further analysis.

Ileal digesta samples were lyophilized (VirTis Genesis 25ES, SP Industries Inc., Warminster, PA) and ground with an electric coffee grinder. Samples were analyzed for total amino acid contents in duplicate at the University of Missouri Agricultural Experiment Station Chemical Laboratories using ion exchange chromatography with postcolumn ninhydrin derivatization. Hydrolysates were obtained using acid hydrolysis for all amino acids except for Met, Cys, and Trp, performic acid oxidation followed by acid hydrolysis for Met and Cys, and alkaline hydrolysis for Trp (method 982.30 E(a,b,c); AOAC International, 2006).

Analysis of titanium dioxide content in feed samples were conducted in quadruplicate (600 mg), while ileal digesta samples were analyzed in duplicate (200 mg) (Short et al., 1996). Samples were ashed for 12 hours at 580°C placed in porcelain crucibles. After ashing, samples were rinsed into a 50 mL beaker using 10 mL sulfuric acid (7.4 M). The solutions were heated at 250°C on a hot plate to dissolve solid particles for approximately 60 minutes. After cooling to room temperature, solutions were rinsed using 10 mL distilled water into a glass beaker containing 25 mL distilled water. Twenty mL of hydrogen peroxide (30%) was added to each beaker and the solution was diluted to 100 mL with distilled water. Solutions were kept at room temperature for at least 48

hours prior to absorbance measurement at 410 nm using a spectrophotometer (SpectraMax Plus 384, Molecular Devices LLC., San Jose, CA). Titanium concentration was determined by comparing absorbance results with known standards using a linear least-squares regression. Concentrations of total amino acids and titanium dioxide from feed and ileal digesta analyses were used to calculate apparent ileal amino acid digestibility using the following equation:

$$Apparent\ ileal\ amino\ acid\ digestibility = \left[1 - \left(\frac{TiO_{2\ Diet}}{TiO_{2\ Digesta}}\right) \times \left(\frac{AA_{Digesta}}{AA_{Diet}}\right)\right] \times 100$$

where $TiO_{2\ Digesta}$ and $TiO_{2\ Diet}$ represent the analyzed concentrations of titanium dioxide in the ileal digesta and diets, respectively, and $AA_{Digesta}$ and AA_{Diet} indicate the analyzed total amino acid concentrations in ileal digesta and diets, respectively.

In Experiment 5, 4 birds per pen were randomly selected for necropsy at 28 and 43 D of age, respectively. Bird were euthanized using CO_2 asphyxiation. Samples of hypothalamus were collected to determine gene expression of orexigenic (neuropeptide Y (**NPY)** and agouti-related peptide (**AGRP**)) and anorexigenic (proopiomelanocortin (**POMC**), cholecystokinin A receptor (**CCKAR**), and ghrelin) peptide hormones. In addition, hypothalamic concentrations of dopamine and serotonin were determined. Hypothalamus samples were collected by decapitating the head. The skull was removed to expose the brain. Then, cuts were made to detach the olfactory nerves. The brain was removed, and the hypothalamus was collected into a 1.5 mL tube by referring to the stereotaxic atlas (plate 4.6 to 5.6) indicated as the nucleus infundibuli hypothalami (Kuenzel and Masson, 1988). Samples were immediately frozen by submersion in liquid

nitrogen for approximately 5 minutes to prevent degradation of hormones and stored at –80^{o}C until further analysis.

Hypothalamus samples from 2 birds per pen were utilized to determine concentrations of dopamine and serotonin. Tissue samples were prepared in a 1.8 mL microfuge tube by adding 300 µL of perchloric acid (0.1 M) and 2,3-dihydroxybenzoic acid to each tube for every 10 mg sample. Samples were sonicated until completely homogenized, kept on ice for 10 minutes, and vortexed to mix the homogenate. Samples were centrifuged at 16,000 × g for 15 minutes at 4°C and the supernatant was transferred to a new microfuge tube. The supernatant was centrifuged again with a similar condition to precipitate remaining homogenate. Dopamine and serotonin concentrations were determined using HPLC with amperometric detection according to the method by Yang and Beal (2011). A 2.1 × 250 mm Dionex Acclaim 120 C18 HPLC column (Thermo Fisher Scientific, Waltham, MA) was used with a 3 × 20 mm Supelco Supelguard LC18 guard column (Sigma-Aldrich, St. Louis, MO). The column was eluted at a flow rate of 0.2 mL/minute with a mixture of citrate buffer, methanol, and water. An aliquot of the sample was injected into the column with a 25 µL loop. Dopamine and serotonin peaks were integrated with Dionex Chromeleon™ Chromatography Data System software version 7 (Thermo Fisher Scientific, Waltham, MA). Dopamine and serotonin concentrations were determined by comparing the results with known standards using a linear least-squares regression.

Gene expressions of hypothalamic NPY, AGRP, POMC, CCKAR, and ghrelin were determined from 1 bird per pen (4 replications per treatment). Samples of hypothalamus were weighed and placed into 5 mL tube containing 20 µL β-

mercaptoethanol and RNA-solv reagent (Omega Bio-tek, Inc., Norcross, GA) to lyse cells and protect RNA from degradation. Samples were homogenized in 2 rounds of homogenization for 30 seconds using Tissuemiser homogenizer (Fisher Scientific, Hampton, NH). Subsequently, RNA was separated by transferring the solution through an RNA Homogenizer column (Omega Bio-tek, Inc., Norcross, GA) into a collection tube. The column and tube were centrifuged at 10,000 × g for 1 minute. The solution in the collection tube was kept at room temperature for 5 minutes prior to RNA extraction. Extraction of RNA was performed using EZNA Total RNA Kit II (Omega Bio-tek, Inc., Norcross, GA) according to the manufacturer's recommendation. Total RNA was quantified using DeNovix DS-II Spectrophotometer (DeNovix Inc., Wilmington, DE) with all samples exhibiting an optical density of 260/280 and 260/320 between 1.9 to 2.1. Spectral scans ranging from 200 to 400 nm further verified sample purity as all RNA samples produced smooth curves exhibiting 1 peak at 260 nm.

Total RNA was reverse transcribed by mixing mRNA solution with Nucleus Free water, 4 µL of Reaction mix (qScript cDNA Synthesis Kit, Quantabio, Beverly, MA), and 1 µL of reverse transcriptase. Reverse transcription of RNA into cDNA was conducted using Bio-Rad T100 Thermal Cycler (Bio-Rad, Hercules, CA) with a reaction volume of 40 µL. The reaction was performed in a single cycle with the following steps: incubation at 25°C for 5 minutes followed by 42°C for 30 minutes and enzyme inactivation at 85°C for 5 minutes. The cDNA was stored at -20°C until gene expression assay. Master mix for each gene was prepared to a reaction volume of 20 µL by mixing the cDNA with SYBR Green (Bio-Rad, Hercules, CA), forward and reverse primers (Eurofins Genomics LLC, Louisville, KY; Table 6.3), and nuclease-free water. Primers

were prepared to 10 ng concentrations. Real-Time quantitative PCR was performed using Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) under the following conditions: 1 preincubation step of 3 minutes at 95°C followed by 40 cycles with each cycle consisting of a melting step of 10 seconds at 95°C, an annealing step of 10 seconds at 56°C, and an elongation step of 5 seconds at 65°C. Each sample was run in 3 separate PCR runs with resulting threshold cycle (**CT**) values averaged across values obtained from 3 separate plates. Data were expressed as fold change of the target sample relative to the control sample, normalized to a reference gene (β-actin) calculated using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

At 43 D of age, blood samples (3 mL) were collected from 4 birds per pen via heart puncture to determine plasma inositol concentration. Blood samples were collected into a 4.5 mL heparinized tube (S-Monovette® 4.5 mL LH, Sarstedt, Numbrecht, Germany) and placed on ice until centrifugation. Blood samples were centrifuged at 1,643 × g for 10 minutes to separate plasma from the whole blood and stored at –20°C until further analysis. Plasma samples were prepared by mixing with 1 M perchloric acid in a 1:2 ratio (plasma:$HClO_4$) to precipitate all protein. Samples were centrifuged at 14,000 × g for 10 minutes to collect the supernatant and were sent to the University of East Anglia School of Biological Sciences in Norwich, England for analysis of inositol concentration using HPLC with pulsed amperometric detection. Samples were diluted 50 fold in 18.2 mohm × cm water. An aliquot (20 µL) was injected into a 4 mm × 250 mm MetroSep Carb 2 (Metrohm, Runcorn, UK) HPLC column. The column was eluted at a flow rate of 0.5 mL/minute with 150 mM NaOH. Another aliquot (5 µL) was injected onto a 2 mm × 100 mm Metrosep Carb 2 (Metrohm, Runcorn, UK) column with a guard

column eluted at a flow rate of 0.2 mL/minute with the same solvent. Inositol peaks were integrated with Chromeleon (ThermoFisher Scientific, Waltham, MA) and DataApex Clarity (DataApex, Prague, Czech Republic) software packages. Inositol concentration was determined by comparing results with known standards using a linear least-squared regression.

Statistical Analyses

These experiments were designed as a randomized complete block with cage or pen locations as the blocking factor. Individual cage or pen represents the experimental unit with 16 and 8 replications in Experiments 4 and 5, respectively. In Experiment 4, data were subjected to 1-way ANOVA, whereas in Experiment 5, data were analyzed as 2 × 2 factorial treatment arrangement with 2 factors of nutrient content (control or uplift) and 2 factors of phytase supplementation (0 or 4,500 FTU/kg). Interactive and main effects were analyzed using 2-way ANOVA by MIXED procedure of SAS (2011). The following mixed-effect model was used in both experiments:

$$Y_{ij} = \mu.. + \tau_i + \beta_j + (\tau\beta)_{ij} + \varepsilon_{ijk}$$

where $\mu..$ is the overall mean; the τ_i are fixed effect of i^{th} level of nutrient content factor (control of uplift); the β_j are fixed effects of the j^{th} level of phytase supplementation factor (0 or 4,500 FTU/kg); the $(\tau\beta)_{ij}$ are interactive effect between the i^{th} level of nutrient content factor and the j^{th} level of phytase supplementation factor; and the ε_{ijk} are identically and independently normally distributed random errors with mean 0 and a variance σ^2. In Experiment 5, means were separated by Tukey's HSD test when a significant interaction effect was observed. Pre-planned orthogonal contrasts were conducted between Treatment 1 (control diet) vs. 3 (control diet with phytase addition),

Treatment 2 (control diet with nutrient uplifts) vs. 4 (control diet with nutrient uplifts and phytase addition), and Treatment 3 (control diet with phytase addition) vs. 4 (control diet with nutrient uplifts and phytase addition). However, fold change calculation on gene expression of peptide hormones using $2^{-\Delta\Delta CT}$ method prevented the use of contrast analysis. Hence, only interactive and main effects on gene expression data were presented. Statistical significance was considered at $P \leq 0.05$.

RESULTS

In Experiment 4, analyzed phytase activity in Treatment 2 was 82.0% of the calculated values based on 4,810 FTU/g analyzed phytase source (Table 6.1). In Experiment 5, analyzed phytase activity in Treatments 3 and 4 of starter diets were 78.9 and 76.9% of the calculated values (Table 6.2). The lower analyzed phytase activity may be associated with unexpected lower phytase activity in phytase source used for Treatments 3 and 4 of starter diets. Phytase source used in Treatments 3 and 4 of Experiment 5 was the same phytase used in Treatment 2 of Experiment 4 (analyzed as 4,810 FTU/g). However, phytase activity in phytase source decreased to 3,810 FTU/kg, which may be the reason for the lower phytase activity in Treatments 3 and 4 of starter diets in Experiment 5. Therefore, the inclusion of phytase for Treatments 3 and 4 of grower and finisher diets in Experiment 5 was increased to compensate for the lower phytase activity in the phytase source. This resulted in similar analyzed phytase activity in Treatments 3 and 4 of grower and finisher diets compared with the calculated values.

Experiment 4

Broilers fed the diet formulated with phytase addition at 4,500 FTU/kg had higher ($P < 0.05$) BW gain (0.782 vs. 0.749 kg/bird) and feed intake (0.936 vs. 0.909 kg/bird)

compared with birds fed the control diet from 1 to 19 D of age. However, FCR of broilers receiving the diet with phytase addition (1.199) was similar ($P = 0.22$) with those fed the control diet (1.215). In addition, broilers fed the control diet (2,809 kcal/kg) had lower ($P < 0.001$) AME_n than birds fed the phytase-added diet (2,872 kcal/kg). Apparent ileal digestibility of Arg, Ile, Leu, Asp, Glu, and Pro in the diet formulated with phytase inclusion were higher ($P < 0.05$) than in the control diet (Table 6.4).

Experiment 5

No interactive effects ($P > 0.05$) between nutrient uplifts and phytase supplementation were observed on feed intake and FCR of broilers from 1 to 14 D of age (Table 6.5). However, pre-planned orthogonal contrasts indicated that broilers consuming diets formulated with the combination of nutrient uplifts and phytase inclusion (Treatment 4) had 5 and 6% higher ($P < 0.05$) BW gain than birds fed diets with either nutrient uplifts (Treatment 2) or phytase addition (Treatment 3), respectively. Phytase addition in Treatment 3 decreased ($P = 0.013$) FCR of broilers by 6 points compared with broilers provided the control diet (Treatment 1). Nutrient uplift and phytase inclusion and main effects indicated higher ($P < 0.05$) BW gain and lower ($P < 0.01$) FCR in broilers provided diets with nutrient uplifts and phytase inclusion, respectively, compared with broilers receiving diets without nutrient uplifts and phytase supplementation. Incidence of mortality was higher ($P = 0.034$) in broilers provided diets with nutrient uplifts than those without nutrient uplifts. Interestingly, 75% of mortality of birds provided diets with nutrient uplifts occurred within 7 D post-hatch. The reason for the higher incidence of mortality is not readily available.

From 1 to 28 D of age, the combination of nutrient uplifts and phytase addition resulted in broilers having 118 and 115 g higher ($P < 0.05$) feed intake than birds fed diets with nutrient uplifts or phytase inclusion only, respectively (Table 6.6). Pre-planned orthogonal contrasts detected that broilers provided diets with the combination of nutrient uplifts and phytase supplementation (Treatment 4) had higher ($P < 0.01$) BW gain than those fed diets with either nutrient uplifts (Treatment 2) or phytase addition (Treatment 3). Feed conversion of broilers supplemented with phytase (Treatment 3) was 3.7 points lower ($P = 0.049$) compared with broilers fed the control diet (Treatment 1). Main effects of nutrient uplifts demonstrated benefits in increasing ($P < 0.001$) BW gain and decreasing ($P = 0.004$) FCR of broilers by 5.3 and 2.9% compared with those receiving the control diet. Similarly, BW gain of broilers was 3.8% higher ($P = 0.010$) in broilers fed diets supplemented with phytase compared with those fed the control diet. The incidence of mortality was not impacted ($P > 0.05$) by dietary treatments.

From 1 to 40 D of age, interactive effects were reflected in the FCR of broilers where broilers consuming diets supplemented with the combination of nutrient uplifts and phytase supplementation had 3.3% lower ($P = 0.025$) FCR compared with birds fed diets with phytase inclusion only (Table 6.7). Furthermore, pre-planned orthogonal contrasts indicated increased ($P < 0.05$) BW gain by 3.6 and 4.4% when broilers were fed diets with both nutrient uplifts and phytase inclusion (Treatment 4) compared with birds fed diets either with nutrient uplift (Treatment 2) or phytase addition (Treatment 3), respectively. Main effects of nutrient uplifts supplementation demonstrated an increase ($P = 0.011$) in BW gain of broilers by 3.3% compared with broilers provided diets without nutrient uplifts. In contrast, main effects of phytase addition on increasing feed

intake ($P = 0.06$) and BW gain ($P = 0.051$) approached significance compared with birds fed diets without phytase inclusion. No influence ($P > 0.05$) of dietary treatments was observed on the incidence of mortality.

Carcass, total breast meat, and thigh weights of broilers provided diets with the combination of nutrient uplifts were heavier ($P < 0.05$) among dietary treatments (Table 6.8). Pre-planned orthogonal contrasts revealed that the combination of nutrient uplifts and phytase addition led to greater ($P < 0.05$) weights of carcass, total breast meat, wings, drums, and thighs of broilers fed compared with birds fed diets with either nutrient uplifts or phytase supplementation. Additionally, carcass and total breast meat yields of broilers provided the combination of nutrient uplifts and phytase inclusion were heavier ($P < 0.05$) than birds consuming diets formulated with nutrient uplifts (Treatment 2) but not phytase supplementation (Treatment 3). Thigh yield of broilers fed the combination of nutrient uplifts and phytase addition (Treatment 4) diets was also higher ($P < 0.05$) than those of broilers fed diets either with nutrient uplifts (Treatment 2) or phytase inclusion (Treatment 3). Main effects of nutrient uplifts were apparent in increasing ($P < 0.05$) carcass, wing, drum, thigh, and abdominal fat weights as of broilers compared with providing diets without nutrient uplifts. Likewise, main effects of phytase supplementation increased ($P < 0.001$) carcass, total breast, wing, drum, and thigh weights of broilers compared with diets without phytase supplementation.

Hypothalamic gene expressions of AGRP, CCKAR, ghrelin, NPY, and POMC at 28 and 43 D of age were not influenced ($P > 0.05$) by dietary treatments (Table 6.9). Additionally, interactive effects of dietary treatments and main effects of nutrient uplifts were not observed ($P > 0.05$) in hypothalamic concentrations of dopamine and serotonin

at 28 and 43 D of age (Table 6.10). However, phytase supplementation increased (P = 0.008) hypothalamic dopamine by 20% at 43 D of age. Phytase inclusion also increased ($P < 0.001$) plasma inositol of broilers by 2.3 fold compared with birds fed diets without phytase supplementation.

DISCUSSION

In Experiment 4, benefits of phytase inclusion in broiler diets were evident in the enhancement of nutrient availability (Selle and Ravindran, 2007). Gehring et al. (2013) reported that phytase supplementation at 2,000 FTU/kg was beneficial in increasing digestibility amino acids of broilers compared with birds fed diets without phytase addition from 27 to 32 D of age. The increase of amino acid digestibility in broilers fed phytase-added diets may be attributed to the influence of phytase in hindering protein-phytate interaction (Selle et al., 2012). This mechanism concomitantly decreases mucin secretion and endogenous amino acid losses (Cowieson et al., 2004). Similarly, the degradation of phytate by phytase may also increase AME_n through direct liberation of starch (Rickard and Thompson, 1997). Phytase supplementation from 0 to 12,000 FTU/kg was reported to quadratically increased AME_n of broilers (Shirley and Edwards, 2003).

Experiment 5 indicated additive effects of phytase and nutrient uplifts on increased BW gain, decreased FCR, and increased carcass characteristics of broilers. In agreement, Selle et al. (2007) observed an increase in BW gain and a reduction in FCR of broilers when fed diets with the combination of phytase supplementation and increasing digestible Lys concentration from 1 to 21 D of age (Selle et al., 2007). These additive effects may occur due to a complementary effect of providing higher nutrient contents

and phytase supplementation. Recent investigation indicated that phytase supplementation of *E. coli* phytase from *Trichoderma reesei* at 4,000 FTU/kg increased gene expression of system L amino acid transporter 4 and sodium-coupled neutral amino acid transporter 1 in the jejunum of broilers at 18 D of age (Walk and Olukosi, 2019). The increased amino acid transporters due to phytase supplementation may allow for an increased nutrient uptake when provided diets with nutrient uplifts.

Furthermore, greater nutrient concentrations in diets with nutrient uplifts may allow the increased supply of amino acids and energy as building blocks for enhanced muscle accretion (Wu, 2014; Barzegar et al., 2020), which was promoted by the effect of phytase supplementation. In the current research, phytase inclusion was observed to increase plasma inositol concentration. Inositol has been reported to increase protein synthesis and muscle accretion through the activation of calmodulin/calcineurin A (Tokomitsu et al., 1999; McKinsey et al., 2002) and Akt/mTOR pathways (Hassan et al., 2013). Schmeisser et al. (2017) indicated that supplementing bacterial phytase from *Citrobacter braakii* at 1,000 FTU/kg upregulated gene expression of phosphatidylinositide-3-phosphate kinase and myocyte enhancer factors 2A and A, which involve with protein synthesis in the *pectoralis major* muscle of broilers. Broilers receiving phytase supplementation at 1,000 FTU/kg had heavier breast meat weight compared with birds fed diets without phytase inclusion. Hence, higher nutrient contents may have complemented the effect of phytase on enhancing muscle accretion of broilers.

In addition to the complementary effects of phytase and nutrient uplifts on increased meat accretion, these additive effects may also be associated with feed intake stimulation. In the present research, additive effects of nutrient uplifts and phytase

supplementation was observed on increased cumulative feed intake of broilers from 1 to 28 D of age. Additionally, phytase supplementation numerically ($P = 0.06$) increased feed intake of broilers compared with broilers fed diets without phytase inclusion. Variations within each dietary treatment may have caused the insignificant effect on feed intake. Despite the insignificance, the numerical increase of feed intake is still worth noting as it may indicate a biological response of phytase on feed intake stimulation. In contrast, broilers fed diets with nutrient uplifts did not demonstrate increased feed intake compared with birds fed diets without nutrient uplifts. Therefore, positive responses on growth performance and carcass characteristics may be influenced by increased feed intake of broilers due to phytase supplementation.

Kriseldi et al. (2019) also reported that increased BW gain of broilers due to *E. coli* phytase supplementation from *Trichoderma reesei* was highly correlated ($r = 0.73$, $P < 0.001$) with feed intake from 1 to 40 D of age. Similarly, Walk and Olukosi (2019) noted that BW gain of broilers was correlated ($r = 0.33$ to 0.72, $P < 0.10$) with digestible amino acid intake but not ($r = –0.29$ to 0.14, $P > 0.10$) with apparent ileal digestibility of amino acids. In addition, previous research reported that additions of dietary phytase ranging from 1,500 to 4,000 FTU/kg enhanced feed intake of broilers by 22 and 207 g from 1 to 14 and 1 to 42 D of age (Walk et al., 2012 and 2014; Gehring et al., 2013; Campasino et al., 2014; Beeson et al., 2017; Lee et al., 2019; and Walk and Olukosi, 2019).

The mechanism of appetite stimulation through phytase supplementation is not well elucidated in the literature. Possibly, stimulation of feed intake is associated with the role of phytase on phytate degradation (Liu et al., 2014). A previous study demonstrated

that grass carp provided diets with phytic acid supplementation at 0.4% had higher gene expression of CCK, cocaine- and amphetamine-regulated transcript (**CART**), and ghrelin in the brain, which led to lower feed intake compared with those fed diets without phytic acid addition (Liu et al., 2014). Cholecystokinin has been reported to promote satiety through CCKAR by inhibiting gene expression of NPY and AGRP (Bi et al., 2004; Chen et al., 2008; Dunn et al., 2013) and stimulating gene expression of POMC (Fan et al., 2004). Similarly, CART inhibits feed intake in poultry by lowering the expression of NPY (Tachibana et al., 2003). In addition, ghrelin in avian species has been reported as a suppressor of appetite mediated by corticotropin-releasing factor (Kaiya et al., 2007).

Conversely, the hydrolysis of phytate by microbial phytase supplementation may increase feed intake of broilers through increasing digesta passage rate (Watson et al., 2006). Presumably, the higher digesta passage rate may reduce CCK secretion (Scanes et al., 2014) and decrease the inhibition of NPY and AGRP in the hypothalamus (Bi et al., 2004; Chen et al., 2008; Dunn et al., 2013). However, hypothalamic gene expression of orexigenic (AGRP and NPY) and anorexigenic (CCKAR, ghrelin, and POMC) appetite hormones in the current research were not influenced by dietary treatments. The reason for the lack of differences in hypothalamic gene expression of appetite hormones may be attributed to variation within each treatment. Hence, additional data are warranted to determine the role of phytase on changes in appetite hormone concentrations.

The present research also observed an increased hypothalamic dopamine concentration due to phytase supplementation. The increased dopamine concentration may be related to the role of phytase in liberating inositol. A recent study reported that broilers fed diets with inositol supplementations at 0.38 and 0.35% from 1 to 11 and 12 to

22 D of age, respectively, had higher plasma dopamine concentration compared with broilers fed diets devoid in supplemental inositol (Gonzalez-Uarquin et al., 2020). In the current study, supplementation of phytase increased plasma inositol concentrations of broilers compared with broilers receiving diets without phytase inclusion. However, the relationship between dopamine and feed intake of broilers is inconsistent. In the current research, dopamine concentration was not correlated (r = 0.25, *P* = 0.17) with feed intake of broilers from 1 to 40 D of age. Similarly, Bungo et al. (2010) observed no response of intra-cerebroventricular injection of dopamine on feed intake of broilers after 60 minutes of injection. In contrast, Zendehdel et al. (2019) reported that intra-cerebroventricular injection of dopamine 1 and 2 receptors antagonists increased feed intake of broilers within 120 minutes after injection.

Despite the inconsistency, the elevated dopamine concentration may play a role in muscle accretion. Previous research indicated that the activation of dopamine 1 and 5 receptors with dopamine receptor agonists increased *tibialis anterior* and *medial gastrocnemius* muscle mass in mice (Reichart et al., 2011). This positive effect may be attributed to the increased activity of cyclic adenosine monophosphate in those muscles (Reichart et al., 2011), which allows for the activation of protein kinase A leading to muscle hypertrophy (Berdeaux and Stewart, 2012). This mechanism may have contributed to the effect of phytase on enhancing carcass characteristics in the present research. However, this mechanism has not been elucidated in birds.

This research demonstrated additive effects of phytase supplementation and nutrient uplifts on enhanced growth performance and carcass characteristics of broilers. This attribute may be due to synergistic effects of nutrient uplifts in providing amino

acids and energy as building blocks of muscle accretion and phytase in increasing protein synthesis via inositol liberation and dopamine release. In addition, positive responses on growth performance and carcass characteristics of broilers may be attributed to extra-phosphoric effects of phytase through numerical increase of feed intake. However, the mechanism of appetite stimulation by phytase on appetite hormone concentrations is unclear and warrants additional investigation.

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Table 6.1 Ingredient and nutrient composition of dietary treatments fed to broilers from 1 to 23 D of age, Experiment 4

	Dietary Treatment	
Ingredient, %	1	2
Corn	57.94	59.60
Soybean meal (46%)	35.93	35.62
Vegetable oil	1.15	0.54
Dicalcium phosphate	2.04	1.23
Limestone	1.05	1.08
Sodium chloride	0.39	0.32
DL-Met	0.30	0.30
L-Lys•HCl	0.19	0.20
L-Thr	0.12	0.12
Titanium dioxide	0.50	0.50
Builder sand	0.10	0.10
Mineral premix[1]	0.10	0.10
Vitamin premix[2]	0.10	0.10
Choline chloride	0.07	0.07
Xylanase[3]	0.01	0.01
Phytase[4]	---	0.11
Calculated nutrient composition, % (unless otherwise noted)		
AME_n, kcal/kg	3,000	3,000
Crude protein	21.54	21.54
Digestible Lys	1.19	1.19
Digestible Met	0.60	0.60
Digestible SAA	0.88	0.88
Digestible Thr	0.80	0.80
Digestible Val	0.89	0.89
Digestible Ile	0.81	0.81
Ca	0.96	0.96
Non-phytate P	0.48	0.48
Na	0.18	0.18
Analyzed phytase activity, FTU/kg[5]	85	3,690

[1]Trace mineral premix include per kg of diet: Mn (manganese sulfate), 120 mg; Zn (zinc sulfate), 100 mg; Fe (iron sulfate monohydrate), 30 mg; Cu (tri-basic copper chloride), 8 mg; I (ethylenediamine dihydriodide), 1.4 mg; and Se (sodium selenite), 0.3 mg.

[2]Vitamin premix includes per kg of diet: Vitamin A (Vitamin A acetate), 18,739 IU; Vitamin D_3 (cholecalciferol), 6,614 IU; Vitamin E (DL-alpha tocopherol acetate), 66 IU; menadione (menadione sodium bisulfate complex), 4 mg; Vitamin B_{12} (cyanocobalamin), 0.03 mg; folacin (folic acid), 2.6 mg: D-pantothenic acid (calcium pantothenate), 31 mg; riboflavin (riboflavin), 22 mg; niacin (niacinamide), 88 mg; thiamin (thiamin mononitrate), 5.5 mg; biotin (biotin), 0.18 mg; and pyridoxine (pyridoxine hydrochloride), 7.7 mg.

[3]Econase XT, AB Vista Feed Ingredients, Marlborough, UK

[4]Quantum Blue 5G, AB Vista Feed Ingredients, Marlborough, UK

[5]One unit of phytase activity (FTU) is the quantity of enzyme to liberate 1 µmol of inorganic phosphorus per minute from 0.0051 mol/L sodium phytate at pH 5.5 and 37°C.

Table 6.2 Ingredient and nutrient composition of dietary treatments fed to broilers, Experiment 5

	Starter, 1 to 14 D				Grower, 15 to 28 D				Finisher, 29 to 43 D			
Ingredient, %	1	2	3	4	1	2	3	4	1	2	3	4
Corn	60.01	56.53	61.58	58.10	64.23	60.75	66.28	62.80	67.84	64.36	69.88	66.41
Soybean meal (46%)	34.75	36.58	34.51	36.35	30.02	31.85	29.71	31.54	25.43	27.26	25.12	26.96
Vegetable oil	0.73	2.43	0.14	1.84	1.64	3.34	0.87	2.57	2.50	4.20	1.73	3.43
Dicalcium phosphate	2.18	2.18	1.37	1.36	1.97	1.96	0.91	0.91	1.76	1.75	0.70	0.69
Limestone	0.98	0.97	1.01	1.00	0.90	0.89	0.94	0.93	0.82	0.81	0.86	0.85
Salt	0.39	0.39	0.32	0.32	0.40	0.40	0.30	0.30	0.40	0.40	0.30	0.30
DL-Met	0.31	0.31	0.31	0.31	0.28	0.27	0.27	0.27	0.25	0.25	0.24	0.24
L-Lys•HCl	0.23	0.20	0.23	0.20	0.21	0.19	0.22	0.19	0.20	0.17	0.20	0.17
L-Thr	0.13	0.12	0.12	0.12	0.10	0.10	0.10	0.09	0.08	0.07	0.08	0.07
Mineral premix[1]	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Vitamin premix[2]	0.10	0.10	0.10	0.10	0.08	0.08	0.08	0.08	0.05	0.05	0.05	0.05
Choline chloride	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07
Xylanase[3]	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
L-Val	0.01	0.01	0.01	0.01	---	---	---	---	---	---	---	---
Titanium dioxide	---	---	---	---	---	---	---	---	0.50	0.50	0.50	0.50
Phytase[4]	---	---	0.11	0.11	---	---	0.14	0.14	---	---	0.14	0.14
Calculated nutrient composition, % (unless otherwise noted)												
AME_n, kcal/kg	3,000	3,061	3,000	3,061	3,100	3,161	3,100	3,161	3,180	3,241	3,180	3,241
Crude protein	21.47	22.01	21.49	22.03	19.57	20.10	19.60	20.13	17.69	18.23	17.72	18.25
Digestible Lys	1.19	1.21	1.19	1.21	1.07	1.09	1.07	1.09	0.95	0.96	0.95	0.96
Digestible Met	0.60	0.61	0.60	0.60	0.55	0.55	0.54	0.55	0.50	0.50	0.50	0.50
Digestible SAA	0.88	0.89	0.88	0.89	0.81	0.82	0.81	0.82	0.74	0.75	0.74	0.75
Digestible Thr	0.80	0.81	0.80	0.81	0.72	0.73	0.72	0.73	0.63	0.65	0.63	0.65
Digestible Val	0.89	0.91	0.89	0.91	0.81	0.83	0.81	0.83	0.74	0.76	0.74	0.76
Digestible Ile	0.80	0.82	0.80	0.82	0.73	0.75	0.73	0.75	0.65	0.68	0.65	0.68
Digestible Arg	1.29	1.34	1.29	1.34	1.16	1.20	1.16	1.20	1.03	1.07	1.03	1.07
Ca	0.96	0.96	0.96	0.96	0.87	0.87	0.87	0.87	0.78	0.78	0.78	0.78
Non-phytate P	0.48	0.48	0.48	0.48	0.44	0.44	0.44	0.44	0.39	0.39	0.39	0.39
Na	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18
Analyzed phytase, FTU/kg[5]	< 50	< 50	3,550	3,460	< 50	< 50	4,570	5,020	< 50	< 50	4,620	4,400

[1]Trace mineral premix include per kg of diet: Mn (manganese sulfate), 120 mg; Zn (zinc sulfate), 100 mg; Fe (iron sulfate monohydrate), 30 mg; Cu (tri-basic copper chloride), 8 mg; I (ethylenediamine dihydriodide), 1.4 mg; and Se (sodium selenite), 0.3 mg.

[2]Vitamin premix includes per kg of diet: Vitamin A (Vitamin A acetate), 18,739 IU; Vitamin D_3 (cholecalciferol), 6,614 IU; Vitamin E (DL-alpha tocopherol acetate), 66 IU; menadione (menadione sodium bisulfate complex), 4 mg; Vitamin B_{12} (cyanocobalamin), 0.03 mg; folacin (folic acid), 2.6 mg: D-pantothenic acid (calcium pantothenate), 31 mg; riboflavin (riboflavin), 22 mg; niacin (niacinamide), 88 mg; thiamin (thiamin mononitrate), 5.5 mg; biotin (biotin), 0.18 mg; and pyridoxine (pyridoxine hydrochloride), 7.7 mg.

[3]Econase XT, AB Vista Feed Ingredients, Marlborough, UK

[4]Quantum Blue 5G, AB Vista Feed Ingredients, Marlborough, UK

[5]One unit of phytase activity (FTU) is the quantity of enzyme to liberate 1 µmol of inorganic phosphorus per minute from 0.0051 mol/L sodium phytate at pH 5.5 and 37°C.

Table 6.3 Forward and reverse primer sequences used for Real-Time PCR, Experiment 4

Gene[1]	Direction	Sequences	Accession No.	Reference
β-actin	Forward	GTCCACCGCAAATGCTTCTAA	NM205518.1	Delp et al., 2017
	Reverse	TGCGCATTTATGGGTTTTGTT		
AGRP	Forward	GGTTCTTCAACGCCTTCTGCTA	AB029443.1	Delp et al., 2017
	Reverse	TTCTTGCCACATGGGAAGGT		
CCKAR	Forward	CATTTGAAAACAGCAGAAGCA	NM001081501.1	El-Kassas et al., 2016
	Reverse	CTGCTGAATGACATCACTTGG		
Ghrelin	Forward	GAAGCACTGCCTAACGAAGACA	NM001001131.1	Yi et al., 2015
	Reverse	GGATGCTGAGAAGGAGAATTCCT		
NPY	Forward	CATGCAGGGCACCATGAG	M87294.1	Delp et al., 2017
	Reverse	CAGCGACAAGGCGAAAGTC		
POMC	Forward	GCCAGACCCCGCTGATG	NM001031098.1	Yi et al., 2015
	Reverse	CTTGTAGGCGCTTTTGACGAT		

[1]AGRP = agouti-related peptide, CCKAR = cholecystokinin A receptor, NPY = neuropeptide Y, POMC = proopiomelanocortin

Table 6.4 Apparent ileal digestibility (%) of indispensable and dispensable amino acids in diets fed to broilers with or without phytase supplementation from 1 to 23 D of age, Experiment 4[1]

	Dietary Treatments			
Amino acids	1	2[2]	SEM[3]	*P*-Value
Indispensable				
Arg	89.69	91.23	0.46	0.033
Cys	73.37	74.40	1.55	0.65
His	86.01	87.14	0.65	0.24
Ile	82.14	84.95	0.88	0.042
Leu	83.11	85.79	0.78	0.029
Lys	88.56	89.77	0.49	0.10
Met	93.73	94.45	0.34	0.16
Phe	84.59	86.72	0.72	0.06
Thr	79.64	81.05	0.82	0.24
Trp	79.65	81.05	0.91	0.29
Val	82.52	84.67	0.82	0.08
Dispensable				
Ala	83.60	85.72	0.75	0.07
Asp	82.23	84.26	0.66	0.047
Glu	87.63	89.79	0.52	0.011
Gly	79.87	81.20	0.83	0.19
Pro	84.05	86.50	0.70	0.027
Ser	82.08	84.11	0.77	0.08
Tyr	82.91	84.55	0.77	0.16

[1]Values are least-square means of 8 replicate cages with 8 birds per cage at placement.
[2] Treatment 2 was formulated with the addition of phytase at 4,500 FTU/kg. One unit of phytase activity (FTU) is the quantity of enzyme to liberate 1 µmol of inorganic phosphorus per minute from 0.0051 mol/L sodium phytate at pH 5.5 and 37°C.
[3]Pooled standard error

Table 6.5 Growth performance of broilers fed diets enhanced with nutrient uplifts and/or phytase supplementation from 1 to 14 D of age, Experiment 5[1]

Nutrient Uplifttt[2]	Phytase, FTU/kg[3]	BW, kg/bird	BW Gain, kg/bird	Feed Intake, kg/bird	FCR, kg:kg[4]	Mortality, %[5]
1) Control	0	0.398	0.354	0.452	1.276	0.0
2) Uplifts	0	0.410	0.367	0.442	1.207	1.9
3) Control	4,500	0.406	0.363	0.440	1.215	0.6
4) Uplifts	4,500	0.428	0.385	0.453	1.178	1.5
SEM[6]		0.006	0.006	0.007	0.017	0.8
Main effects of nutrient uplift						
Control		0.402	0.359	0.446	1.245	0.3
Uplifts		0.419	0.376	0.448	1.193	1.7
SEM[6]		0.005	0.005	0.005	0.012	0.6
Main effects of phytase						
	0	0.404	0.361	0.447	1.241	1.0
	4,500	0.417	0.374	0.446	1.197	1.0
	SEM[6]	0.004	0.004	0.005	0.012	0.6
Source of Variation				*Probabilities*		
Nutrient uplift × phytase		0.35	0.32	0.10	0.30	0.80
Treatment 1 vs. 3		0.24	0.26	0.22	0.013	0.54
Treatment 2 vs. 4		0.012	0.011	0.24	0.16	0.75
Treatment 3 vs. 4		0.006	0.006	0.20	0.10	0.18
Main effects of nutrient uplift		0.002	0.002	0.83	0.002	0.034
Main effects of phytase		0.013	0.013	0.89	0.007	0.51

[1]Values are least-square means of 8 replicate pens with each pen having 26 birds at placement.
[2]Diets with nutrient uplifts were formulated to contain 0.007, 0.015, 0.013, 0.021, 0.024%, and 61 kcal/kg higher digestible SAA, Lys, Thr, Val, Ile concentrations, and AME_n (obtained from Experiment 4), respectively, compared with the control diet.
[3]One unit of phytase activity (FTU) is the quantity of enzyme to liberate 1 µmol of inorganic phosphorus per minute from 0.0051 mol/L sodium phytate at pH 5.5 and 37°C.
[4]Feed conversion ratio was corrected for mortality.
[5]Mortality was arcsine transformed.
[6]Pooled standard error

Table 6.6 Growth performance of broilers fed diets enhanced with nutrient uplifts and/or phytase supplementation from 1 to 28 D of age, Experiment 5[1]

Nutrient Uplift[2]	Phytase, FTU/kg[3]	BW, kg/bird	BW Gain, kg/bird	Feed Intake, kg/bird	FCR, kg:kg[4]	Mortality, %[5]
1) Control	0	1.552	1.509	2.052[ab]	1.360	3.2
2) Uplifts	0	1.604	1.561	2.030[b]	1.301	6.5
3) Control	4,500	1.581	1.538	2.033[ab]	1.323	4.7
4) Uplifts	4,500	1.690	1.647	2.148[a]	1.304	2.6
SEM[6]		0.021	0.021	0.031	0.013	1.4
Main effects of nutrient uplift						
Control		1.567	1.523	2.042	1.341	4.0
Uplifts		1.647	1.604	2.089	1.302	4.6
SEM[6]		0.015	0.015	0.022	0.009	1.1
Main effects of phytase						
	0	1.578	1.535	2.041	1.330	3.7
	4,500	1.636	1.593	2.090	1.314	3.2
	SEM[6]	0.015	0.015	0.022	0.009	1.4
Source of Variation				*Probabilities*		
Nutrient uplift × phytase		0.19	0.19	0.033	0.11	0.10
Treatment 1 vs. 3		0.33	0.33	0.66	0.049	0.49
Treatment 2 vs. 4		0.006	0.006	0.010	0.83	0.09
Treatment 3 vs. 4		0.001	0.001	0.015	0.31	0.40
Main effects of nutrient uplift		< 0.001	< 0.001	0.14	0.004	0.64
Main effects of phytase		0.010	0.010	0.11	0.19	0.48

[1]Values are least-square means of 8 replicate pens with each pen having 26 birds at placement.
[2]Diets with nutrient uplifts were formulated to contain 0.007, 0.015, 0.013, 0.021, 0.024%, and 61 kcal/kg higher digestible SAA, Lys, Thr, Val, Ile concentrations, and AME_n (obtained from Experiment 4), respectively, compared with the control diet.
[3]One unit of phytase activity (FTU) is the quantity of enzyme to liberate 1 μmol of inorganic phosphorus per minute from 0.0051 mol/L sodium phytate at pH 5.5 and 37°C.
[4]Feed conversion ratio was corrected for mortality.
[5]Mortality was arcsine transformed.
[6]Pooled standard error
[a-b]Means not sharing a common superscript within column differ significantly ($P < 0.05$).

Table 6.7 Growth performance of broilers fed diets enhanced with nutrient uplifts and/or phytase supplementation from 1 to 40 D of age, Experiment 5[1]

Nutrient Uplift[2]	Phytase, FTU/kg[3]	BW, kg/bird	BW Gain, kg/bird	Feed Intake, kg/bird	FCR, kg:kg[4]	Mortality, %[5]
1) Control	0	2.930	2.887	4.176	1.447[ab]	5.4
2) Uplifts	0	2.993	2.950	4.294	1.456[ab]	6.5
3) Control	4,500	2.967	2.925	4.310	1.475[a]	4.7
4) Uplifts	4,500	3.104	3.055	4.356	1.426[b]	3.6
SEM[6]		0.035	0.036	0.054	0.014	1.4
Main effects of nutrient uplift						
Control		2.948	2.906	4.243	1.461	5.0
Uplifts		3.049	3.003	4.325	1.441	5.0
SEM[6]		0.025	0.025	0.039	0.010	1.0
Main effects of phytase						
	0	2.962	2.918	4.235	1.451	6.0
	4,500	3.035	2.990	4.333	1.450	4.1
	SEM[6]	0.024	0.025	0.038	0.010	1.0
Source of Variation				*Probabilities*		
Nutrient uplift × phytase		0.28	0.35	0.48	0.025	0.51
Treatment 1 vs. 3		0.46	0.46	0.08	0.12	0.25
Treatment 2 vs. 4		0.024	0.037	0.37	0.08	0.76
Treatment 3 vs. 4		0.008	0.015	0.52	0.009	0.55
Main effects of nutrient uplift		0.006	0.011	0.12	0.11	0.84
Main effects of phytase		0.039	0.051	0.06	0.95	0.29

[1]Values are least-square means of 8 replicate pens with each pen having 26 birds at placement.
[2]Diets with nutrient uplifts were formulated to contain 0.007, 0.015, 0.013, 0.021, 0.024%, and 61 kcal/kg higher digestible SAA, Lys, Thr, Val, Ile concentrations, and AME_n (obtained from Experiment 4), respectively, compared with the control diet.
[3]One unit of phytase activity (FTU) is the quantity of enzyme to liberate 1 μmol of inorganic phosphorus per minute from 0.0051 mol/L sodium phytate at pH 5.5 and 37°C.
[4]Feed conversion ratio was corrected for mortality.
[5]Mortality was arcsine transformed.
[6]Pooled standard error
[a-b]Means not sharing a common superscript within column differ significantly ($P < 0.05$).

Table 6.8 Carcass characteristics of broilers fed diets with nutrient uplifts and/or phytase supplementation from 1 to 41 D of age, Experiment 5[1]

Nutrient Uplift[2]	Phytase, FTU/kg[3]	Carcass Weight, g	Carcass Yield, %[4]	Total Breast Weight, g	Total Breast Yield, %[4]	Wing Weight, g	Wing Yield, %[4]	Drum Weight, g	Drum Yield, %[4]	Thigh Weight, g	Thigh Yield, %[4]	Abdominal Fat Weight, g	Abdominal Fat Percent, %[4]
1) Control	0	2,249[b]	74.60	795[b]	26.38	224	7.43	265	8.79	281[b]	9.33	36	1.21
2) Uplifts	0	2,259[b]	74.23	784[b]	25.75	229	7.51	270	8.89	287[b]	9.42	39	1.27
3) Control	4,500	2,269[b]	74.78	805[b]	26.41	228	7.53	269	8.88	285[b]	9.40	35	1.17
4) Uplifts	4,500	2,376[a]	74.84	838[a]	26.38	239	7.54	282	8.90	305[a]	9.62	38	1.21
SEM[5]		19	0.28	9	0.20	2	0.04	3	0.05	4	0.09	1	0.04
Main effects of nutrient uplift													
Control		2,259	74.69	800	26.39	226	7.48	227	8.84	283	9.36	36	1.19
Uplifts		2,317	74.54	811	26.07	234	7.52	276	8.89	296	9.52	39	1.24
SEM[5]		14	0.24	6	0.14	1	0.03	2	0.04	4	0.07	1	0.03
Main effects of phytase													
	0	2,254	74.42	790	26.06	226	7.47	267	8.84	284	9.38	37	1.24
	4,500	2,322	74.81	821	26.40	234	7.53	276	8.89	295	9.51	37	1.19
	SEM[5]	13	0.24	6	0.14	1	0.03	2	0.04	4	0.07	1	0.03
Source of Variation							*Probabilities*						
Nutrient uplift × phytase		0.005	0.24	0.009	0.12	0.09	0.27	0.09	0.40	0.018	0.36	0.72	0.71
Treatment 1 vs. 3		0.43	0.47	0.47	0.89	0.07	0.057	0.16	0.18	0.38	0.52	0.54	0.38
Treatment 2 vs. 4		< 0.001	0.012	< 0.001	0.014	< 0.001	0.65	< 0.001	0.81	< 0.001	0.041	0.90	0.14
Treatment 3 vs. 4		< 0.001	0.82	0.006	0.92	< 0.001	0.93	< 0.001	0.79	< 0.001	0.028	0.029	0.32
Main effects of nutrient uplift		< 0.001	0.38	0.21	0.09	< 0.001	0.22	< 0.001	0.22	< 0.001	0.028	0.006	0.08
Main effects of phytase		< 0.001	0.026	< 0.001	0.08	< 0.001	0.09	< 0.001	0.45	< 0.001	0.06	0.60	0.10

[1]Values are least-square means of 8 replicate pens with 14 birds selected from each pen for processing.
[2]Diets with nutrient uplifts were formulated to contain 0.007, 0.015, 0.013, 0.021, 0.024%, and 61 kcal/kg higher digestible SAA, Lys, Thr, Val, Ile concentrations, and AME_n (obtained from Experiment 4), respectively, compared with the control diet.
[3]One unit of phytase activity (FTU) is the quantity of enzyme to liberate 1 µmol of inorganic phosphorus per minute from 0.0051 mol/L sodium phytate at pH 5.5 and 37°C.
[4]Percent yield was calculated based on weight proportion to bird live weight at 40 d of age.
[5]Pooled standard error
[a-b]Means not sharing a common superscript within column differ significantly ($P < 0.05$).

Table 6.9 Hypothalamic gene expression of appetite hormones in broilers fed diets enhanced with nutrient uplifts and/or phytase supplementation, Experiment 5[1]

Nutrient Uplift[2]	Phytase, FTU/kg[3]	28 D of age					43 D of age				
		AGRP[4]	CCKAR[4]	Ghrelin	NPY[4]	POMC[4]	AGRP[4]	CCKAR[4]	Ghrelin	NPY[4]	POMC[4]
1) Control	0	1.094	1.154	1.304	1.114	1.097	0.889	1.082	1.118	1.173	1.089
2) Uplifts	0	1.070	1.466	0.902	1.257	0.523	1.160	1.103	2.543	1.082	0.666
3) Control	4,500	1.028	1.261	0.864	0.873	1.208	1.402	1.629	2.194	1.372	1.537
4) Uplifts	4,500	0.977	1.612	0.947	1.106	1.352	1.129	1.365	2.311	1.275	1.242
SEM[5]		0.347	0.470	0.370	0.312	0.277	0.574	0.311	0.673	0.318	0.256
Main effects of nutrient uplift											
Control		1.332	1.079	1.336	0.726	1.091	1.138	1.103	1.271	1.174	1.106
Uplifts		1.285	1.401	1.140	0.855	0.888	1.104	1.004	1.863	1.088	0.803
SEM[5]		0.355	0.312	0.410	0.155	0.222	0.338	0.213	0.357	0.220	0.164
Main effects of phytase											
	0	1.099	1.068	1.335	1.568	1.267	1.137	1.134	1.250	1.118	1.155
	4,500	1.018	1.223	1.096	0.723	2.003	1.400	1.553	1.538	1.313	1.829
	SEM[5]	0.287	0.288	0.402	0.544	0.352	0.379	0.264	0.328	0.235	0.239
Source of Variation		*Probabilities*									
Nutrient uplift × phytase		0.99	0.78	0.53	0.77	0.13	0.92	0.39	0.48	0.90	0.17
Main effects of nutrient uplift		0.88	0.30	0.50	0.47	0.45	0.94	0.64	0.26	0.73	0.21
Main effects of phytase		0.75	0.60	0.39	0.26	0.08	0.63	0.10	0.54	0.47	0.07

[1]Values represent the fold change of appetite hormone gene expressions in a target sample relative to the control sample, normalized to a reference gene (β-actin) from 4 replicate pens with 1 bird per pen. These values were calculated using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

[2]Diets with nutrient uplifts were formulated to contain 0.007, 0.015, 0.013, 0.021, 0.024%, and 61 kcal/kg higher digestible SAA, Lys, Thr, Val, Ile concentrations, and AME_n (obtained from Experiment 4), respectively, compared with the control diet.

[3]One unit of phytase activity (FTU) is the quantity of enzyme to liberate 1 µmol of inorganic phosphorus per minute from 0.0051 mol/L sodium phytate at pH 5.5 and 37°C.

[4]AGRP = agouti-related neuropeptide, CCKAR = cholecystokinin A receptor, NPY = neuropeptide Y, POMC = proopiomelanocortin

[5]Pooled standard error

Table 6.10 Hypothalamic catecholamine (ng/mg) and plasma inositol concentrations (μM) of broilers fed diets with nutrient uplifts and/or phytase supplementation, Experiment 5[1]

Nutrient Uplift[2]	Phytase, FTU/kg[3]	28 D of age		43 D of age		
		Dopamine	Serotonin	Dopamine	Serotonin	Plasma Inositol
1) Control	0	0.779	0.784	0.494	0.340	179
2) Uplifts	0	0.708	0.815	0.549	0.404	169
3) Control	4,500	0.670	0.758	0.608	0.409	403
4) Uplifts	4,500	0.715	0.793	0.645	0.453	407
SEM[4]		0.064	0.097	0.040	0.063	23
Main effects of nutrient uplift						
Control		0.724	0.771	0.551	0.374	291
Uplifts		0.711	0.804	0.597	0.428	288
SEM[4]		0.048	0.069	0.031	0.045	16
Main effects of phytase						
	0	0.743	0.799	0.521	0.372	174
	4,500	0.693	0.775	0.627	0.431	405
	SEM[4]	0.048	0.069	0.031	0.045	16
Source of Variation		*Probabilities*				
Nutrient uplift × phytase		0.33	0.98	0.80	0.88	0.75
Treatment 1 vs. 3		0.21	0.85	0.034	0.45	< 0.001
Treatment 2 vs. 4		0.93	0.87	0.07	0.59	< 0.001
Treatment 3 vs. 4		0.59	0.80	0.48	0.63	0.90
Main effects of nutrient uplift		0.83	0.74	0.22	0.40	0.89
Main effects of phytase		0.40	0.80	0.008	0.36	< 0.001

[1]Values are least-square means of 8 replicate pens with 2 birds per pen for catecholamine analysis and 4 birds per pen for plasma inositol analysis.
[2]Diets with nutrient uplifts were formulated to contain 0.007, 0.015, 0.013, 0.021, 0.024%, and 61 kcal/kg higher digestible SAA, Lys, Thr, Val, Ile concentrations, and AME_n (obtained from Experiment 4), respectively, compared with the control diet.
[3]One unit of phytase activity (FTU) is the quantity of enzyme to liberate 1 μmol of inorganic phosphorus per minute from 0.0051 mol/L sodium phytate at pH 5.5 and 37°C.
[4]Pooled standard error

VII. CONCLUSIONS

Experiment 1 was designed to determine extra-phosphoric effects of phytase on phytate degradation, inositol liberation, and plasma inositol concentration of broilers. Increasing phytase concentrations from 0, 400, and 1,200 phytase units (**FTU**)/kg in broiler diets increased phytate degradation and inositol liberation in the ileal digesta of broilers. However, these responses did not translate to plasma inositol concentrations. The lack of response on plasma inositol concentration may indicate several possible inositol metabolisms following liberation. Inositol may be rapidly metabolized in the liver for phosphatidylinositol synthesis or rephosphorylated to inositol phosphate (**IP**) 5 for modulating oxygen affinity in the red blood cell. Hence, future research should be conducted to measure inositol concentrations in the liver as well as determining inositol and IP5 concentrations in the whole blood of broilers.

The lack of changes in plasma inositol concentrations could also be related to the duration of feeding phytase-supplemented diets (8 hours). This duration may not be sufficient to allow changes in plasma inositol concentration. Based on these considerations, Experiments 2 and 3 were conducted to evaluate extra-phosphoric effects of phytase on the extent of phytate degradation, inositol liberation, growth performance, and carcass characteristics of broilers during a 6-week production period. Furthermore, dietary free inositol was utilized as it may also contribute to extra-phosphoric effects of phytase. In Experiment 2, growth performance and carcass characteristics of broilers

were enhanced by increasing phytase doses. These positive responses may be attributed to the influence of phytase in increasing feed intake of broilers, which translated to increased nutrient intake. This was evident as feed intake was correlated (r = 0.73, $P < 0.001$) with BW gain of broilers from 1 to 40 D of age. Contrary to the hypothesis, inositol supplementation did not provide benefits on growth performance and carcass characteristics of broilers in this experiment.

In Experiment 3, increasing phytase concentrations from 0 to 40,500 FTU/kg resulted in log-quadratic reductions of total IP2 to IP6 concentrations in the gizzard and ileal digesta of broilers. More importantly, phytase supplementation beyond 1,500 FTU/kg decreased the accumulation of IP3 and IP4 in both gizzard and ileal digesta contents. In addition, elevated phytase concentrations led to a log-quadratic increase of plasma inositol concentrations of broilers. These data indicated that the enhancement of growth performance and carcass characteristics in Experiment 2 may be contributed by actions of phytase on phytate degradation, inositol liberation, and feed intake stimulation.

The degradation of IP3 and IP4 by phytase indicated the reduction of anti-nutritive capacity of phytate. This attribute is critical as IP3 and IP4 are not suitable substrates for commercial phytases. Hence, supplementing phytase beyond 1,500 FTU/kg may be an effective strategy to reduce anti-nutritive effects of phytate. In addition, the elevated plasma inositol concentration due to phytase supplementation may indicate the contribution of inositol in improving growth performance and carcass characteristics of broilers. The increase of plasma inositol concentrations was also negatively correlated (r = –0.68, $P < 0.001$) with total IP2 to IP6 concentrations in the ileal digesta at 43 D of age. This negative correlation between total IP esters and plasma inositol concentrations may

allow phytase users to use plasma inositol concentration as a biomarker for phytase efficacy on phytate degradation.

Due to the effect of phytase on the positive correlation between BW gain and feed intake of broilers in Experiment 2, Experiments 4 and 5 were conducted to evaluate extra-phosphoric effects of phytase and nutrient uplifts on growth performance, meat yield, hypothalamic gene expression of appetite hormones, and catecholamine concentrations of broilers. Experiment 4 determined differences of digestible amino acid concentrations and AME_n in broilers fed diets with or without phytase supplementation at 4,500 FTU/kg. These differences were used as nutrient uplifts in Experiment 5 to mimic effects of phytase on increasing nutrient intake.

Experiment 5 demonstrated additive effects of supplementing phytase and nutrient uplifts on improving BW gain, FCR, and carcass characteristics of broilers. Moreover, phytase supplementation led to a numerical increase ($P = 0.06$) in feed intake of broilers from 1 to 40 D of age. However, nutrient uplifts had no effect on feed intake of broilers. Phytase supplementation at 4,500 FTU/kg also increased plasma inositol and hypothalamic dopamine concentrations by 2.3 and 1.2 fold compared with birds fed diets without phytase inclusion. In contrast, hypothalamic gene expressions of neuropeptide Y, agouti-related peptide, cholecystokinin A receptor, proopiomelanocortin, ghrelin, and serotonin concentration were not influenced by dietary treatments.

Additive effects of phytase and nutrient uplifts may be realized through synergistic effects of phytase in enhancing protein synthesis through inositol liberation and nutrient uplifts in providing building blocks for muscle accretion. These additive effects may also be influence by the numerical increase of feed intake due to phytase

supplementation. However, this research could not confirm appetite stimulation mechanisms by phytase as gene expression of appetite hormones were not influenced by dietary treatments. Furthermore, despite the increase of hypothalamic dopamine concentrations due to phytase, feed intake was not correlated ($r = 0.25$, $P = 0.17$) with dopamine concentration. Additional investigation is warranted to determine the influence of phytase on appetite hormone concentrations in broilers.

Overall, this research demonstrated that extra-phosphoric effects of phytase may be contributed through phytate degradation, which increased inositol liberation and feed intake of broilers. Effects of phytase on phytate degradation allow for the reduction of anti-nutritive effects of phytate, while inositol may be beneficial in stimulating mechanisms involving protein synthesis. This study also indicated that enhanced growth performance and carcass characteristics of broilers may be observed by the combination of phytase and nutrient uplifts. This combination allowed for complementary effects through the influence of phytase in enhancing nutrient utilization and the role of amino acids and energy for building blocks of muscles.