ABSTRACT

MAJEED, SHUJA. Impact of Ca through Limestone Particle Size and Phytase Matrix Value on Broiler Live Performance, Carcass Quality, Nutrient Digestibility and Bone Ash (Under the direction of Dr. Frank Edens).

Calcium (Ca) is one of the one most abundant minerals in the body and plays crucial roles in body functions. Hence, two experiments were conducted to determine ways to best regulate Ca concentrations and determine management factors that can be adjusted to achieve optimum Ca delivery to broiler chickens, resulting in good performance and health.

The first study was performed to determine the effect of various Calcium: Available Phosphorus (Ca: AvP) ratios and Ca phytase matrix values on broiler live performance and carcass quality. Seven dietary treatments with different Ca: AvP ratio and Ca phytase enzyme matrix value were evalulated. The broilers were reared to 47 d with bodyweight (BW) and feed weigh-back measured at 15, 28, 35 and 47 d. At 47 d, birds were processed to determine carcass weight, white stripping and wooden breast. Diets with phytase inclusion showed greater feed intake (FI) and BW ($P \leq 0.05$), but matrix values did not have any effect on live performance. Decreasing Ca: AvP ratio did not have an effect on live performance parameters and dietary treatments had no significant effect on carcass quality.

In the second study, the effect of limestone particle size and phytase inclusion as phytase units (FTY) on live performance, apparent ileal digestibility (AID) and bone ash content was evaluated in male broilers. The study was designed to be a 2 X 2 X 2 factorial arrangement having two limestone particle sizes (fine [190 μm] and coarse [900

μm]); phytase inclusion (0 FYT/kg and 1000 FYT/kg); and Ca and available phosphorus (P_i) level (positive control [PC] and negative control [NC]). Birds were raised up to 35 d with BW and feed weigh recorded at 14, 28 and 35 d. The ileal content and left tibia of birds were collected at 14 and 35 d to calculate AID and bone ash, respectively. Phytase inclusion improved significantly the body weight gain (BWG) and FI of birds at an early age, and it was most effective when included in the NC diet ($p \leq 0.05$). Limestone particle size did not have any significant effects on live performance although coarse limestone particle size + NC performed better than fine + NC ($P \leq 0.05$). Phytase improved bone ash at 14 d while the PC had the greatest bone ash content at both 14 and 35 d ($p \leq 0.05$). Limestone particle size did not influence bone ash content. AID of Ca, P_i and amino acids was influenced by phytase and limestone particle size with fine limestone and phytase improving AID. Ca and P_i level affected AID of Ca and P_i with the NC die having greater values than the PC diet ($p \leq 0.05$).

In summary it can be discerned that phytase improves live performance, bone ash and AID of birds although it is most effective for young broilers. Ca phytase enzyme matrix values may need a greater difference in order to allow observation of any change in performance and carcass quality. Decreasing Ca: AvP ratios from the standard 2:1 ratio does not have a negative effect on live performance. Fine limestone particle size, although not showing any significant difference in live performance, improved Ca and P_i digestibility. The AID results pointed to greater phytase activity with coarse limestone particle size compared to fine limestone particle size.

Impact of Ca through Limestone Particle Size and Phytase Matrix Value on Broiler Live Performance, Carcass Quality, Nutrient Digestibility and Bone Ash

by
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DEDICATION

I dedicate this to my parents and family for always supporting me.

BIOGRAPHY

Shuja Majeed was born and raised in Pakistan. He graduated with a Doctorate in Veterinary Sciences from the University of Agriculture, Faisalabad in 2014. Afterwards, he joined Pakistan Poultry Farms working as a farm manager at a broiler breeder farm from 2014 - 2016. Feeling the need to learn more about poultry management and nutrition, he decided to pursue the masters in the Poultry Science non-thesis program under the supervision of Dr. John T. Brake. While working in Dr. Brake's lab and through involvement in other graduate students' research, he shifted his interest towards the research aspect of the poultry industry, and that led him to convert his Master of Poultry Science non-thesis degree program to a Master of Science thesis degree program. Unfortunately, Dr. Brake passed away around that time and could not see his degree through. He continued his Master of Science prograam with Dr. Edens. Apart from academics and research, he was a member of Global Pack, played in IMleagues and took part in ISSERV during his master's program.

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Dr. John Brake's contribution to my degree cannot be overstated. His guidance, support and patience was crucial in helping me achieve my target. I believe his lessons will forever stay with me and serve as a great asset in life.

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Introduction

Calcium (Ca) and phosphorus (P_i) are the most essential nutrients involved in many biological processes (Proszkowiec-Weglarz and Angel, 2013). These minerals are the most abundant elements in the body, with 99% of Ca and 80% of P_i stored in the skeleton as hydroxyapatite $Ca_5(PO_4)_3(OH)$ (Applegate and Angel, 2008; Applegate et al., 2008), and both play an important role in bone development and mineralization. Calcium is also involved in blood coagulation, transmission of nerve impulses, the permeability of cell membranes, activation of enzyme systems, glandular secretion, and muscular contraction and calcification of eggshell (Suttle, 2010).

P_i is required for formation of phospholipids, which are essential components of cell membranes. Energy storage and transmission is also dependent on phosphorylated compounds, such as adenosine triphosphate. Nucleic acids contain phosphates in their structure, and a number of hormones, their secondary messengers, and enzymes require phosphorylation for activation. P_i acts as a buffer, which plays an important role in maintaining acid-base balance. Furthermore, 2,3-diphosphoglycerate(2,3-DPG) is a major phosphorus-containing molecule that binds to hemoglobin in red blood cells regulating oxygen delivery to tissues of the body (Knochel et al., 2006).

P_i along with being an essential mineral for animal growth, it is also expensive and has a negative environmental impact (Correll, 1998). Corn and soybean meal (SBM) are major constituents of poultry feed in the USA. Both of these feed ingredients have P_i in phytate form, which poultry can utilize only partially (Dersjant-Li et al., 2014) since there is not sufficient production of endogenous phytase by the birds GI mucosa

(Humer et al., 2014) and the dietary Ca level generally used in broiler diets have negative influence on endogenous phytase as this Ca binds to phytate making it difficult for phytase enzyme to break it down (Tamim and Angel, 2003). This has led to the inclusion of expensive inorganic mineral supplements to fulfill the P_i requirement of poultry. Therefore, P content has to be managed carefully because excess P_i adds cost to feed manufacturing and increases the environmental footprint in a negavitve way, while dietary P_i deficiency results in losses in production, which can be also be viewed as a welfare issue. With the introduction of the dietary enzyme phytase, it has become possible to better optimize P_i availability, which has allowed for improved growth performance and a better understanding of phytase inclusion in the feed matrix has also led to improved P utilization (Broz et al., 1994).

Phosphorus Terms

Total phosphorus (tP or P) includes all forms of phosphorus, and is generally denoted as P (Applegate and Angel, 2008).

Inorganic phosphorus (P_i) is made up of a phosphorus bound to four oxygen molecules. This is the form in which phosphorus is absorbed by animal's gastrointestinal tract (GI) and is present in blood (Schroder et al., 1996).

Phytate phosphorus, is the storage form of the majority of P_i in most plant material. Phytate is a complex of Ca or magnesium (Mg) salts with myoinositol (1, 2, 3, 4, 5, 6-hexakis dihydrogen phosphate) and is regarded as the primary storage form of P_i and inositol in almost all seeds (Cosgrove, 1980).

Non phytate phosphorus (nPP), is a chemically defined substance calculated by subtracting the analyzed phytate P_i content of ingredients from their analyzed tP content

(Angel and Applegate, 2001). The term nPP has been used predominantly in poultry nutrition as an expression of the P_i requirement of birds (NRC, 1994).

Available phosphorus (avP) is a commonly used term to express the available amount of P_i from feed ingredients. avP is also known as relative bioavailable P_i, which is the amount of P_i in feed or feed ingredient that is available at the tissue level for the bird (Plumstead, 2007).

Apparent digestible phosphorus is defined as the difference in amount of P_i consumed from the diet and that remaining in a fecal bolus. If endogenous P_i contribution from intestinal secretion and desquamation of epithelium lining the intestinal tract is taken into account then true digestible P_i value can be obtained (Plumstead, 2007).

Retained phosphorus is the amount of P_i utilized by the bird, and it can be calculated by subtracting the amount of Pi eliminated in the feces and urine from the dietary P_i intake (Plumstead, 2007).

Soluble phosphorus by definition is the P_i fraction that can pass through a 0.45 µm pore filter (NRC, 1993). It has been synonymous with water soluble P_i (WSP) and Water extractable P_i (WEP).

Particulate phosphorus is comprised of particles in soil that do not pass through a 0.45 µm filter and represent the fraction tightly bound to sediment and organic matter by anion adsorption (NRC, 1993; Sturgul and Bundy, 2004).

Sources of Ca and P_i

Calcium is readily found around the world in the form of rocks such as limestone and gypsum (Keshavarz, 1991). Calcium carbonate, available as limestone, is

commonly included in diets for all animals. Limestone is usually the cheapest source of Ca and is available either as a pulverized powder or in a granular form (Blount, 2013).

Oyster shell is another ingredient that can be used to supply Ca to the birds as it is also mainly composed of calcium carbonate though it is more expensive than limestone. This is due to limitations on oyster shell dredging in the Chesapeake region of USA, because of environmental concern and oyster shells being clearly visible in the diet to egg producer which reduces chance of omission during feed manufacture (Leeson and Summers, 2005).

There are no consistent differences in growth rates and percent tibial ash of broilers fed either oyster shell or limestone when particle size of these ingredients is similar (McNaughton et al., 1974).

P_i is a natural element found in rocks, soils and organic matter. It can exist as organic phosphate that is bound to plant or animal tissue, which are primarily formed by biological process or Inorganic phosphate which is not associated with organic material and includes orthophosphate and polyphosphate. Orthophosphate is the more stable and often is used by plants while polyphosphate, known as metaphosphates, are strong complexing agents for some metal ions. In water, polyphosphate is unstable and will convert to orthophosphate. Animal sources include meat, bone and fish meal, which vary in availability from 57-74%, depending on the materials' origin and its production processes that carry contamination risks compared to the plant Phosphorus source that is in phytate form. Fish, meat and bone meal are also considered organic sources for Ca. Most of the calcium-phosphate dietary supplements are considered 100% available

to poultry, but some of the rock phosphate supplements often have much lower availability (Summers, 1997).

The majority of feed grade phosphates used in poultry feeding are chemically processed products (Table 1). One such common product is dicalcium phosphate (DCP), which is produced by reacting phosphoric acid (produced from burning elemental phosphorus or sulfuric acid digestion of phosphate rock) with limestone. Another major group of feed phosphates is defluoriniated phosphate, which is formed by reacting phosphate rocks with phosphoric acid and sodium bicarbonate, then afterwards calcining it to a temperature of 1250°C. This process is more difficult to control compared to the dicalcium phosphate process with its inherent greater variability in biological values (Waldrop, 1996). These mineral supplements are derived from natural rock phosphates, mostly found in Africa, northern Europe, Asia, Middle East and the USA. Natural phosphate is processed because the P_i they contain is not metabolizable by animals and contains impurities such as fluorine, cadmium and arsenic, which can be harmful for animal health. Defluorinated phosphate, dibasic calcium phosphate, and monobasic calcium phosphate are sources for both Ca and P_i.

Table 1. Inorganic Ca and P sources used in animal feed (Neslon et al., 1990)

Source	Total nPP[1] (%)	Ca %
Limestone	-	38.0
Oyster Shell	-	38.0
Monobasic calcium phosphate	20.68	14.56
Dibasic calcium phosphate	18.27	19.30
Defluronated phosphate	18.49	31.80

[1] nPP stands for non-phytate phosphorus

Impact of P_i on the environment

Overtime, ecologists have developed concepts showing that bacterial and plant growth in aquatic systems would be limited by the availability of an essential element and a number of researches have shown that P_i is that element. Bioassays of lake waters from the Great Lakes Region of the USA using the provisional Algal Assay Procedure (USDA, 1969)*, selenastrum capricornutum* cell numbers were found to respond to the addition of P rather than nitrogen (Maloney et al., 1972), showing that most of these lake waters contained limiting concentration of P_i. Another trial, involving addition of P_i to 320 liters of water from Lake Michigan found that P_i was the limiting nutrient (Schelske and Stoermer, 1972). In a more direct approach, in an experimental lake research area in northwestern Ontario, whole lakes were P_i-enriched for a period of years, and these P_i-enriched lakes utilized atmospheric nitrogen and carbon and had significant growth of algae. In the absence of P_i-enrichment and with nitrogen- and carbon-enrichment, there was no significant algal growth (Schindler, 1974, 1975, 1977).

Phosphorous is an essential ingredient for plant growth, and its input has been recognized as necessary for optimal and profitable crop production. Poultry manure, which has an average P_i content of 25 g/kg and litter with average P_i content of 20 g/kg (Barnett, 1994a; Gilbertson et al., 1979), applied to agriculture lands resulted in higher P_i-content than required by the crops. This led to excess P_i accumulation in soil under long-term application. With surface run off, the excess P_i could enrich surface waters (lakes, streams, and estuaries) (Sharpley, 1999), which led to accelerated eutrophication. Eutrophication could result in increased aquatic plant growth with associated oxygen depletion, pH variability, variations in plant species and quality, and food-chain effects. Negative effects of eutrophication include decreased water clarity, clogging of water treatment plant filters by excessive algae growth, and extensive growth of larger plants that interfere with recreation and navigation in water bodies. Additionally, eutrophication have a harmful effect on biodiversity and oxygen availability in the surface waters leading fish kills and loss of other marine life (Sharpley et al., 1993).

Phosphorous is a very biologically active element, and it occurs in a pentavalent form (such as orthophosphate) in aquatic systems. It's delivered to aquatic systems as a mixture of dissolved and particulate form. Only P_i in orthophosphate form can be utilized by autotrophs like algae and plants but this does not mean that other forms pose no threat to these surface waters. Particulate forms of P_i, deposited in the sediments, are acted upon by microbes releasing much of the P_i back into the surface water as orthophosphate (Correll, 1998).

Phytate as storage of P in grains and oil seeds

Phytate, phytin and phytic acid are similar compounds. The most commonly used term, phytate, refers to the mixed salt of the phytic acid. Phytin, refers to the deposited complex of myoinositol hexaphosphosphate or IP6 with potassium, Mg and Ca as it occurs in plants, whereas phytic acid (Figure 1) is the free form of IP6 (Selle and Ravindran, 2007).

Phytic acid is considered as a primary storage form of both phosphate and inositol in almost all seeds (Cosgrove, 1966; 1980), and it is considered a source of P and cations for the germinating seed. Due to phytic acid's strong chelating powers, it has been suggested that it can also act as a carrier or storage for trace mineral during plant growth (Wieldlein, 1951). Gupta and Venkatasubramanian (1975) have proposed that it might also play a role in preventing aflatoxin production in SBM by making zinc (Zn) unavailable to the mold (Reddy et al., 1982). Phytate makes up about 1-2 % by weight of many cereals and oil seeds, in some varieties, its content can reach 3 to 6 %. In soybean, whose meal is used in poultry diets as a rich protein source, 75% of the P_i is in phytate form (Cheryan and Rackis, 1980), and in various cereals phytate P_i content ranges from 50-80% (Hidvegi and Lasztity, 2002). There is no phytate-P_i present in early stages of seed development, but at maturity, it accounts for more than 60% of the total P_i, and the phytate rate of destruction under heat application is low (De Boland et al., 1975). Phytic acid is present in seeds and grains predominantly in bran and outer hull; corn differs from other grains in this aspect as most of the phytic acid in corn is concentrated in the germ (Coulibaly et al., 2011). In seed tissues, phytic acid may bind cations, including K, Mg and Ca but also Fe and Zn to form phytate which is

sequestered in specialized vacuoles termed protein bodies (Lott, 1980; Lott et al., 2002). In cereal grains, phytate is concentrated in globoids within protein bodies, and it is dispersed evenly in the protienacious matrix of protein bodies as protein-phytate complexes or K phytate in species like pea and soybean (Lott, 1980)

However, from the standpoint of poultry production, phytic acid is considered as an anti-nutritional factor because it binds P_i and other important nutrients decreasing their availability. The anti-nutritional effects are not only limited to P_i non-availability (Woyengo and Nyachoti, 2013); phytic acid is a very strong chelating agent with ability to bind important minerals like Zn, copper (Cu), Ca, cobalt (Co), manganese (Mn), iron (Fe), and Mg into insoluble complexes in the digestive tract. Phytate is also able to form enzyme resistant complexes with proteins and interfere with amino acid metabolism (De Rham and Jost, 1979; Ravindran, 1995; Storebakken et al., 1998; Ravindran et al., 2000; Ravindran et al., 2001; Akinmusire and Adeola, 2009; Choct et al., 2010). Phytate is known to inhibit action of proteolytic enzymes, pepsin, and trypsin in the gastrointestinal tract of animals (Knuckles et al., 1989). This resulted in reduced digestion and increased gastrointestinal secretions. It has been shown that the presence of phytic acid in diets for poultry and pigs has generally resulted in reduced growth performance (Ugwuanyi, 2016). Phytic acid reduces apparent mineral digestibility in pigs and broilers, due to reduced dietary availability of those minerals, increased endogenous secretions of these minerals, or both. In poultry, there is an increase in endogenous secretions of Ca, Fe, sodium (Na), and sulphur (S) in broilers that have been precision-fed 50 ml of glucose solution for 48 hours period with addition of 1 g of phytic acid (Cowieson et al., 2004). Reported from the same study, was high

excretion rate of Ca, Mg, Mn and Na over the 48 hour period due to addition of phytic acid. Phytic acid affected enzyme activity as shown by a 6.3% reduction in pepsin activity in the proventriculus (Liu et al., 2009). Phytic acid decreased apparent nitrogen and amino acid digestibility and increased endogenous loss of nitrogen and amino acids in birds. In pig fed corn-soy diet, amino acid digestibility was decreased due to dietary phytic acid (Bohlke et al., 2005, Liao et al., 2005) while diets based on casein and cornstarch showed no phytic acid effect on amino acid digestibility (Woyengoet al., 2009, Knuckles et al., 1989). A 2%-phytic acid-related decrease of 36% in energy digestibility in broilers was associated with reduced digestibility of carbohydrates, lipids and proteins (Ravindran et al., 2006). This indicated that breakdown of phytic acid did not only result in improved performance due to higher availability of P_i and various other minerals but also alleviate anti-nutritional effects of phytate (Woyengo et al., 2012).

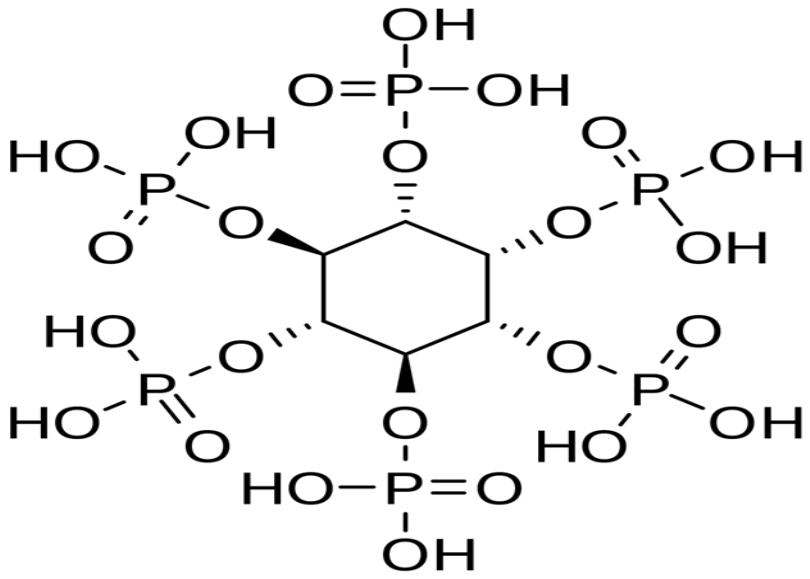

Figure 1. The most accepted phytic acid ($C_6H_{18}O_{24}P_6$) structure as proposed by Anderson (1914).

Requirements of Ca and P_i in broilers

It's uncommon for commercial broilers to be fed the Ca level recommended by the 1994 NRC Nutritional requirement of poultry which are 1% Ca in diet for 0-3 weeks, 0.90% Ca for 3-5 weeks and 0.80% Ca for 6-8 weeks. NRC P requirements are 0.45% P_i for 0-3 weeks, 0.30% P for 3-5 weeks and 0.30% P_i for 6-8 weeks. In a survey of more than 100 feed mills in the USA, it was found that from 1-18 days of age, a 0.09% Ca level was fed, and from 18-33 days, Ca was fed at 0.82% (Driver et al., 2005). One reason for reduction in the Ca level can be due to faster broiler growth and greater feed consumption compared to 1994. Another reason could be that through genetic selection, broilers have become more feed efficient with a reduction in their dietary Ca requirement. Furthermore, Ca is a cheap feed ingredient, and has a tendency to be used as a filler, which, unlike most other feed ingredients, results it being used excessively. Excess Ca levels can interfere with the absorption of other minerals including P_i, Mg, Mn and Zn (NRC, 1994). High levels of Ca are known to chelate lipids and reduce energy levels in diets (Edwards et al., 1960). The Ca requirement might also be a sexually dimorphic trait where it has been reported that males require more Ca than females to maximize tibial ash while females require more Ca for optimum weight gain (Driver et al., 2005). Driver et al. (2005) showed that from 0-16 days of age, the NRC requirement of 1% dietary Ca maximized tibial ash, but 1% dietary Ca for all other parameters is excessive. Rao et al. (2003) predicted Ca requirement for maximized tibia ash was 0.98%, which is similar to NRC and again for other parameters recommended lower levels than NRC (1994). For maximum weight gain, they predicted a dietary Ca level of 0.75%. Cobb-Vantress, Inc. has recommended dietary Ca levels of 0.90% for 0-

10 days, 0.84% for 11-22 days, 0.76% for 23-42 days and 0.76% for 43 days and above for improved growth performance of their chicks. Aviagen has suggested for their Ross broilers a slightly higher dietary Ca recommendation as follows: 0.96% for 0-10 days, 0.87% for 11-24 days, and 0.81% from 25 days and above.

Requirements for P_i in poultry diets are generally expressed as nPP and/or avP, and when calculating a dietary Ca to P_i ratio, excess Ca or P_i in relation to other can reduce absorption due to the formation of an insoluble complex called calcium phosphate, which is either excreted or deposited in soft tissue (Underwood, 1981; Georgievskii et al., 1982). Generally, the ratio of Ca: avP of 2:1 is widely accepted (Rao et al., 2003). Many researchers use maximum bone ash as the criteria to figure out P_i requirement, but it is not confirmed that maximum bone ash would lead to maximum growth or maximum feed efficiency. It might be that maximum bone ash is not required for these parameters but adequate P_i is more than adequate for good performance (Edwards, 1983; Ravindran et al., 1995). While having adequate P_i is essential in poultry diets for good performance, excess P_i can have negative effects such as adding to environmental pollution, and P_i is an expensive ingredient. Thus, it is important to the poultry industry to determine accurate P_i requirement for chickens and their feed accordingly.

Experiments conducted by Yan et al. (2001), supported the NRC requirement for P_i in broiler diets if no phytase enzyme was added, but in other experiments from 3-6 weeks of age, it was determined that 2.6 g/kg (0.26%) nPP (less than NRC requirement), is sufficient to maximize broiler growth (Abudabos, 2012). Rao et al. (2003), for the starter phase, recommended 3 g/kg (0.3%) P_i at Ca: avP ratio of 2:1 for

optimum growth, which again is less than the NRC recommendation. In another experiment conducted with male broilers, it was found that available P_i levels of 4.82 (0.48%) for 1-10 days; 4.10 (0.41%) for 11-21 days; 3.95 (0.39%) for 22-33 days; and 3.19 g/kg (0.31%) for 34-46 days, if the Ca: avP ratio was kept at 2:1, would maximize growth performance (Mello et al., 2012). This showed that avP may be reduced as the bird ages if the Ca: avP ratio was to be kept constant at 2:1. Mello et al. (2012) conducted a similar trial on female broiler and concluded that the optimum level of avP in diet from 1-10 days was 4.59 (0.45%), for 11-21 days was 3.88 (0.39%), for 22 -33 days was 3.07 (0.31%), and for 34-46 days was 2.56 g/kg (0.26%). In another trial, no effect on performance was seen when birds were fed diets with avP at 0.21, 0.26, 0.19 and 0.15%, but tibial ash was low in the 0.15% diet compared to others from 32 to 42 days (Dhandu and Angel, 2003). These results were similar for 42-59 days. Cobb-Vantress, Inc. recommended avP at 0.45% from 0-10 days, 0.42% from 11-22 days, 0.38% from 23-42 days, and 0.38% from 23-42 days and above. Aviagen (Ross broilers) recommended available P_i at 0.48% for 0-10 days, 0.435% for 11-24 days, 0.39% for 25-39 days, 0.390% for 25-39 days and 0.375% for 40 days and above. Various experiments and guides recommend different levels of P_i, especially after 3 weeks of age, which might be due to differences in broiler genetics, experimental design, and/or feed composition, but all these experiments indicated that as the bird ages, its requirement for P_i decreases, especially at finisher and withdrawal phases. Since P_i is required for bone growth and feathering, one possible reason for this could be that as broilers grow and mature, their skeletal system and feathers are developed, which allows for less need for dietary P_i. Furthermore, as the chicken matures from

hatch through about 14 days of age, its inherent ability to potentially interact with phytic acid through its maturation of intestinal alkaline phosphatase activity also increases (Sabatakou et al., 2007).

Dietary Ca: Pi interaction with Ca and P_i regulation in the body

Calcium and phosphorus both perform essential functions in the body. They also interact with each other, and an excess or deficiency of one can influence availability and excretion of the other. Thus, it is important that Ca: P_i ratio in the diet is close to the animal's requirement enabling optimum absorption of both minerals. Historically, the ratio of Ca: Pi was defined for total Ca (tCa) and total P_i (tP) in the diet. With time, the role of phytase in releasing bound P_i found in plant sources became clear and changes were made to Ca:avP, but there has been no movement towards digestible/available Ca, and there are very few data on the actual availability of Ca as opposed to relative availability in feed ingredient sources. Digestibility of Ca is assumed at 100% (Angel, 2013), which is not correct as digestibility of Ca from corn and SBM has been shown to be 20-33% while for Ca from limestone was between 60% and 70% (Tamim and Angel, 2003; Tamim et al., 2004). Calcium digestibility of corn and SBM has been disregarded because it represented only about 20% in a diet containing 1% Ca. In the past, available P was crudely calculated as the sum of tP from inorganic sources, tP from animal sources, and 30% of plant source P (Angel, 2013). As more was understood about phytate this definition was updated but no much effort has been made towards Ca availability and to this day tCa is calculated when formulating diets (Angel, 2013). The latest NRC requirements (1994) termed avP as nPP and recommended Ca:nPP ratio of 2.22-2.67 depending on growth stage. In the poultry industry, the Ca:P_i ratio of 2:1 has

been generally accepted, which Cobb-Vantress, Inc. and Avigen (Ross broilers) also recommended. However, some trials have indicated that a Ca:P_i ratio less than 2:1 is optimum for broiler diet. Driver et al. (2005b) using one concentration of non-phytate P_i of 0.45% and various Ca levels discovered optimum Ca:AvP_i ratio of 1.08, 1.39 and 1.60 for body weight gain, feed to gain ratio and tibia ash, respectively.

Plasma Ca and P_i concentrations are within a narrow physiological range controlled by parathyroid hormone (PTH), active vitamin D_3 (1, 25-dihydroxyvitamin D_3), calcitonin (CT), and their respective receptors localized in small intestine, bone, and kidneys. Calcium deficient diets or an increase in Ca requirement would result in a decreased plasma Ca concentration, which lead to an increased PTH release which in turn activates 1α-hydroxylase in the kidney to produce the active form of vitamin D_3, which results in increased Ca absorption in the small intestine and reabsorption in the kidneys. PTH also causes bone resorption which increases blood Ca level. A low blood P_i level results in increased active D_3 production from the kidney, which increases its absorption from the intestine and its resorption from kidney. However, in contrast to Ca, PTH decreased P_i resorption from kidney, but increased its excretion (Weglarz and Angel, 2013).

Absorption of Ca and P

Calcium absorption is facilitated through both active and passive transport involving three steps: 1. entry across cell well, 2. diffusion through cytoplasm, and 3. exit at the basolateral membrane. Passive transport is characterized by ion movement from the intestinal lumen to the circulation along the chemical gradient through para-cellular means (Bronner, 1998; Bronner and Pansu, 1999; Bronner et al., 1986) and

occurs throughout the length of small intestine (Buckly and Bronner, 1980). When Ca intake is adequate, passive transport is the major way for Ca absorption and is not dependent on Vit D_3 (Rogers et al., 1995). On the other hand, active transport is Vit D_3-dependent and occurs in the upper duodenum. Low dietary Ca or an increase in Ca requirement upregulate active transport. The first step in active intestinal Ca transport or transcelluar entry across the cell wall is facilitated by two epithelial Ca selective anion channel (Hoenderop et al., 2000; Peng et al. 2003). A protein binding agent, Calbindin D28k, is responsible for transcellular diffusion of Ca from brush border membrane to basolateral membrane of duodenal cells (Wasserman and Taylor, 1966). Movement of Ca across the basolateral membrane is mediated through plasma membrane calcium pump (Carafoli, 1991) by either a Na or a Ca exchanger.

Phosphorus is mostly absorbed in bird's intestine as phosphate. Absorption is facilitated through both Na-independent and Na-dependent pathways (Murer and Hildmann, 1981; Hilfiker et al., 1998; Murer et al., 2004). The Na-dependent pathway is not affected by calcium concentration (Murer and Hildmann, 1981, Matsumoto, 1980, Berner et al. 1976), but the Na-independent pathway is unregulated (Danisi et al. 1980, Katai et al. 1999, Katai et al. 1999) and a large phosphorus concentration in a diet could be absorbed through the paracellular pathway, although the intestinal epithelium is not readily permeable to P_i (Cross et al., 1990). Uptake of P_i into the intestinal epithelium requires Na-dependent co-transporters, which is an active process and requires energy (Tenenhouse et al., 2005). In the intestine, a type IIb transporter is present, which regulates P_i uptake, and the expression of this transporter on the brush border

membrane is the rate-limiting step of transcellular P_i uptake (Adeola and Adedokun, 2013).

Phytase Enzyme

Phytase activity was first detected in rice bran nearly a century ago (Suzuki et al., 1907), but attempts of *in vitro* phytase production did not begin in North America until 1962 (Wodzinski and Ullah, 1996). However, it was not until 1991 that the first phytase became commercially available, and this was largely due to a legislation designed to limit P pollution of the environment in the Netherlands (Selle and Ravindran, 2007).

There are various classes of phytase, most extensively studied is histidine acid phosphatases (HAPs); other classes are beta-propeller phytase, known as alkaline phytase, purple acid phytase and protein tyrosine phosphatase. Currently, all of the commercial phytases belong to HAPs. The first generation of commercialized phytase was a fungal phytase launched in 1991, after that launch, new generation of bacterial phytases were developed due to the discovery that *E. coli* phytase were more effective than fungal phytase. It has been observed that new generation phytases possess higher resistance to proteolytic digestion and have a very specific affinity for phytic acid/inositol phosphate 6 (IP6) and inositol phosphate 5 (IP5) (Dersjant et al., 2014).

Phytase has the ability to break down phytate in cereals, legumes and liberate P_i for animal nutrition, it does so by catalyzing the hydrolytic phosphate splitting of phytic acid (IP6) to lower inositol phosphate esters (IP5-IP1) and P_i. In theory, enzymatic hydrolysis of phytate generates series of lower myoinositol phosphate esters through a progression of stepwise dephosphorylating reactions to yield inositol and six P_i moieties. However, axial P_i residue at the C2 position is relatively refractive to hydrolysis

(Wodzinski and Ullah, 1996). Consequently, hydrolysis of phytate by phytase is more likely to yield myoinositol and five P_i moieties.

There are three main types of phytase (Loewus and Murthy, 2000); 3- phytase, which is produced by microbes that hydrolyze the phosphate group at the C3 position, 6- phytase, which is of plant origin and hydrolyze the phosphate group at C6 first and 5-phytase, isolated from pollen and seeds that initiate hydrolysis by removing 5-phosphate of phytic acid (Barietos et al., 1994). An international standard unit does not exist for the measurement of phytase activity; the defined measurement unit of phytase activity depends on assay conditions, which include concentration of substrate (sodium phytate), assay temperature, and pH. In *Aspergillus niger* phytase, which was introduced in 1991, phytase activity was defined as *fytase unit* (FTU), where one FTU is the amount of enzyme that liberates 1 umol of inorganic orthophosphate/min from 0.0051 mol/L of sodium phytate at pH of 5.5 and temperature of 37°C (Engelen et al., 1994). This definition provided a simple benchmark to measure phytase activity. Other units like FYT, U and PU, have been used to represent phytase activity, which is also determined under controlled *in vitro* conditions. An issue associated with these methods of enzyme analysis is still focused on the observation that natural phytate may not be as easily degraded as sodium phytate. As an example, calcium phytate is not as well utilized as sodium phytate *in vivo*, which indicates that calcium phytate is more resistant to degradation (Selle and Ravindran, 2007). Some food animals have the ability to produce limited amounts of intestinal phosphatases that target phytic acid, and phosphatases also occur in grains like wheat, rye and various microorganisms found in the animal's intestine, rumen and soil. Phytase produced from the microbes in the large

intestine might not be of much use to the animal as limited absorption of P takes place in the large intestine. Most cereals and grain produce contain negligible amount of phytase and their optimum pH (4-6) to function is different than the stomach of animals. Additionally, plant enzymes may become inactivated by feed processing as they are more heat labile compared to microbial-origin phytases, and therefore, might not contribute to nutrient release in animal's stomach.

In commercial applications, most of the phytases are of microbial origin (Jackson et al., 1996; Baruah et al., 2004) produced by fungi of the Aspergillus species: *Aspergillus niger* or *Aspergillus oryzae*. Their commercial successes can be attributed to thermal stability and the ability to function at the pH of animal digestive tracts. Other commercially available phytase enzymes are derived from *Peniophora lycii* and *E. coli*, which are 6-phytases compared to the Aspergillus ones, which are 3-phytase. Phytase enzyme may be included in poultry rations as granules or as liquids via post pelleting applications systems to avoid thermos-instability problems at high pelleting temperatures (>80°C). However, there are perceived advantages in inherently heat stable phytase feed enzyme that can withstand steam-pelleting, as indicated by investigations of Wyss et al. (1998) and Garrett et al. (2004).

Little has been done on the poultry gastrointestinal tract site of phytase activity. However, it is likely that phytate hydrolysis mainly occurs in the esophagus (crop) and stomach (proventriculus, and ventriculus) where the pH is more conducive to enzyme activity. The crop is most probably the primary site of phytate degradation by exogenous phytase (Liebert et al., 1993; Takemasa et al., 1996; Kerr et al., 2000). The activity of phytase at various pH and different segments of the intestinal tract is not the only

determinant of phytate hydrolysis. It also depends on where phytate is more soluble, which is at low pH that is relegated to the proventriculus and ventriculus (Campbell and Bedford, 1992). Mucosal phytase activity varies depending on Ca and nPP level (Dersjant-Li et al., 2015). High Ca results in decreased phytase activity believed to be due to formation of insoluble Ca-phytate complex (Wise, 1983), very high dietary Ca (9.0 g/kg) reduces both mucosal phytase activity and ileal phytate degradation (Applegate et al., 2003a). Limestone, which is a major source of Ca in most poultry diets, is known to have high acid binding capacity (Lawlor et al., 2005), and as a result, it binds more acid and increases digesta pH in the proximal gut. The addition of Ca, as limestone, to broiler diets has been shown to increase crop pH (Shafey et al., 1991), which is the main site of phytate degradation by phytase enzyme. This could directly influence phytase efficacy. One possible reason could be that elevation of crop pH resulted in greater amount of mineral-phytate complex including Ca-phytate complexes that reduces susceptibly of hydrolysis of phytate by phytase. Low nPP in the diet causes the bird to increase phosphatase production resulting in more degradation of phytate (McCuaig et al., 1972; McCuaig and Motzok, 1972).

Phytase activity is influenced by various factors such as PH, endogenous protease, and dietary factors (Dersjant-Li et al., 2015) .The optimum pH for plant phytate is about 5.0 (Hill and Tyler, 1954a), it is deactivated below pH 2.5. At the incubation temperature from 15°C to 50°C and at pH 5.1, plant phytase activity has a linear increase. Microbial phytase has 2 pH optima (Heinzl, 1995); at pH 2.5 and at pH 5.5 (Duval, 1996). More details about phytase enzyme characteristic are presented in Table 2.

Table 2. Characteristic of some commercially available phytase supplements (Suttle, 2010)

Origin	Vehicle	Specificity	Optimum PH	Degradability
Aspergillus niger	Fungus	3- phytase	2.5-5.5	High
Peniophora lycii	Fungus	6- phytase	2.5-3.5	High
Escherichia coli	Yeast	6- phytase	2.5-3.5	Low

Transgenic Phytase

Another method of feeding phytase enzyme to animals is through transgenic plants. Genes of microbial origin can be transferred to plants enabling them to produce that specific enzyme. Both prokaryotic and eukaryotic microbes have been used as a source of phytase gene for transformation of plants. Phytase genes from bacteria like *Bacillus subtilis* and *E. coli* have been used to develop transgenic plants, but mostly this involves phytase genes from *Aspergillus niger* and *Aspergillus fumigatus,* which are preferred for developing transgenic plants as their phytases exhibit stability at high temperature and under a wide range of pH. These plant genes have been used to develop plants such as tobacco, corn soybean, rice, wheat, canola and alfalfa. Effect of dietary phytase transgenic corn have been studied in laying hens by Gao et al. (2014). The study concluded that supplementation with transgenic corn had no adverse effect on birds organ weight or serum biochemical parameters and Digestibility of energy,

nitrogen and Ca. The study also concluded that transgenic corn was effective in improving P digestibility in laying hens.

Glycosylation is a process that helps in the folding of proteins to generate active sites, and also maintain thermal stability and activity of enzymes. Glycosylation doesn't have much effect on the substrate specificity but has a significant effect on molecular mass, protein structure, thermal stability and biological synthesis. Phytase genes expressed in transgenic plants exhibit lower phytase activity compared to native enzymes indicating a difference in glycosylation pattern of the gene in plant and fungal systems. Despite the difference is glycosylation patterns, plant-expressed phytase gene enzymes have similar catalytic properties of the native enzyme and are functionally active (Gonita et al., 2012).

Effect of phytase enzyme on broiler performance

As discussed before, P_i can be detrimental to the environment, and inclusion of phytase in diets was initiated to reduce P excretion in an attempt to reduce P_i impact on water quality. Various trials have shown that the feeding phytase reduced P_i excretion. Simons et al. (1990) demonstrated that feeding broilers 1500 FTU/kg phytase activity with less nPP and Ca reduced P excretion by an average of 61% in broilers. Paik (2003) found that inclusion of phytase reduced P_i excretion by 29.6%. Addition of phytase to diets have also been shown to enhance growth performance. In one study of Simons et al. (1990), phytase addition (1500 FTU/kg) to broiler diets containing 4.5 g/kg tP increased weight gain and feed efficiency of broilers from 0-24 days. Cabahug et al. (1999) also reported that inclusion of phytase (400 FTU and 800 FTU/kg) in broiler diets containing 2.3 g/kg (0.23%) nPP improved body weight, feed intake and

conversion ratios of broilers from 7-25 days of age. Generally, responses to phytase in weight gain and feed intake are more consistent than feed efficiency. Feed efficiency effect of phytase has been declining according to Rosen (2003), who attributes it to multiple factors including improvement in broiler strains, feed and management techniques. Selle et al. (1999) ran a trial with broilers from 7 to 25 days in which standard and modified sorghum diets were fed to the birds, with and without addition of phytase at 600 FTU/kg. The modified diets had reduced P_i, Ca, protein and energy density. Addition of phytase did not affect the birds' performance with a standard diet, but it significantly improved the performance in birds that were given a modified diet (Selle and Ravindran, 2007). The extent to which phytase improves protein/amino acid digestibility is variable, and the mechanism of the process is still speculative. It has been suggested that *de novo* formation of binary protein-phytate complexes in the gastrointestinal tract, which are refractory to pepsin activity, may be the reason phytate affects protein availability (Selle et al., 2000), phytate may also increase endogenous amino acid flow, which may again hinder protein availability (Ravindran et al., 1999a; Cowieson et al., 2004). In poultry, the *de novo* formation of binary protein-phytate complex is thought to occur under acidic condition in the proventriculus, exogenous phytase is mostly active in the crop so it's believed that it partially hydrolyses some of phytate before it gets to the proventriculus and binds protein, leading to improved protein/amino acid availability. As discussed above, phytate can bind to starch and lipids along with proteins, and this might reduce energy utilization. Ca-phytate and lipids are believed to form metallic soaps in the gut lumen of poultry, which can be a major constraint in the energy derived from lipids (Leeson, 1993; Atteh and Leeson, 1984).

With phytase supplementation, phytate concentration will be reduced, limiting its ability to bind to these nutrients with promotion of improved energy utilization (Ravindran et al., 1999b, 2000, 2001; Selle et al., 1999, 2001, 2003c, 2005). The dietary level of Ca:P_i are crucial to phytase efficacy as excess Ca can bind to phytate and form insoluble Ca-phytate complex, which is more refractory to hydrolysis by phytase (Wise, 1983; Maenzetal.,1999). Most of the Ca in broiler diets is added as limestone, which is known to have high acid binding capacity (Lawlor et al., 2005), thus increasing digesta pH in the proximal gut. The addition of limestone to poultry diets are known to increase crop pH (Shafey et al., 1991), which is the main site of phytate degradation by exogenous phytase enzyme, this can negatively influence phytase efficacy. Phytase activity also varies among feed ingredients as demonstrated by Leske and Coon (1999) that soy bean and corn had higher phytate hydrolysis by phytase enzyme compared to canola meal and rice bran. Various feed additives can also influence phytase activity in the gastrointestinal tract. Vitamin D_3 (Biehl and Baker, 1997; Mitchell and Edwards, 1996; Driver et al., 2005b) and citric acid (Rafacz-Livingston et al., 2005) have been shown to increase phytate phosphorus utilization in broilers. Chelating agents render phytate more susceptible to phytase (Maenz et al. 1999), while high levels of Zn (Augspurger et al., 2004) and Cu (Banks et al., 2004) in broiler diets have been shown to have negative influence on hydrolyses process of phytate by phytase.

As shown in the early discussion above, phytase is a very useful feed additive. With its addition in the diet, less nPP needs to be supplemented. This not only saves cost as P_i is considered an expensive feed ingredient but also limits P_i excretion in the droppings, hence reducing the environmental impact.

Super Dosing of Phytase

Commercial inclusion rates of phytase range from 300 to 600 FTU/kg, but various studies have been conducted, which have shown benefits of super dosing phytase (Pirgozliev et al., 2007; Cowieson et al., 2011; Broach et al., 2018). The earliest super dosing trial was done by Nelson et al. (1971), where he had treatments ranging from 950 FTU/kg phytase to 7600 FTU/kg. Diets with 950 FTU/kg had weight gain of 156 g, bone ash 33.7% and phytate P_i reduction of 38.9 % while diets with 7600 FTU had 21 d body weight gain of 196 g, bone ash content 40.6% and phytate P_i reduction of 94.4%, indicating an increase in body weight gain and bone ash by 131% and 59%, respectively, compared to the phytase free control. Shirely and Edward (2003) supplemented corn-based diets with up to 12000 FTU/kg phytase and observed a quadratic increase in phytate P_i disappearance with increasing log phytase from around 93 FTU/kg to 12000 FTU/kg. Apparent metabolizable energy and nitrogen retention had similar responses at 12000 FTU/kg although the scale of response to dose increases was less compared to that of phytate destruction. In another trial by Pirgozliev et al. (2007), where the birds were grown until 21 days of age, P_i deficient diets were supplemented with 3 doses of phytase: 250, 500 and 2500 FTU. The birds fed 2500 FTU had greater feed intake and weight gain, 9 and 6 %, respectively, than birds fed 500 FTU. Similar results were shown in a trial conducted by Cowieson et al. (2006), in which supplementation of up to 2000 FTU/kg of phytase improved apparent metabolizable energy and metabolizability of nitrogen, but above that level of supplementation, there was no significant effect. However, super dosing (>1200 FTU/kg) helped with phytate P_i digestibility and tP digestibility.

Matrix Values of phytase

Matrix values indicate additional nutrients that would be released when an exogenous enzyme is added to a diet. In the case of phytase, it would act on phytate in the dietary corn and soybean, hydrolyzing it and releasing Ca, P_i, amino acid and energy, which would have been unavailable previously. The assigned matrix value would be based on knowing how much quantity of each nutrient phytase would release. The phytase matrix values used in practice are for available P_i, Ca, amino acids and energy. In most of the studies, phytate P_i release has been measured by a positive control diet with adequate P_i, a negative control diet which is deficient in P_i, and comparable diets to have which phytase enzyme was supplemented. If the P_i deficient diet with supplemented phytase showed results such as parameters like bone ash, P_i retention, or body weight gain was similar to the positive control, then the enzyme would be releasing phytate P_i comparable to that found in the positive control. Similar methods are employed for other nutrients like Ca and amino acids. However, matrix values for phytase can vary considerably depending on the type of phytase being supplemented, different animal species, dietary composition, age of animal, and other assessment criteria (Dersjant-Li et al., 2014; Almeida et al., 2017). In common practice, phytase is supplemented to most of poultry feeds at a standard inclusion level of 500 FTU/kg for broilers and 300 FTU/kg for layers. The literature states 500 FTU/kg of phytase will release 0.3-1.7 g/kg of P_i while feed enzyme suppliers recommend 500 FTU/kg of enzyme to replace more than 1.0 g of P. Although some studies have shown that 1000-2000 FTU/kg of phytase might be needed to break down more than 60% of phytase in the upper part of the digestive tract to reduce the anti-nutritional effect of phytate and

improve the efficiency of organic P utilization in plant-based ingredients. Almeida et al. (2017) demonstrated that maximum P digestibility was reached at a different level for different feed ingredients. Matrix value determination is an intricate science as there are a lot of variables, different types of phytases will have different activity, and various dietary compositions will affect phytase activity. Thus, it will be hard to have one fixed matrix value for all the formulations, diets, and enzyme types. To be more efficient in using phytase, it would be better to empirically derive the matrix value based on enzyme type, diet composition and animal species.

Most phytase products have a 1:1 relationship between Ca and P matrices regardless of dosage. However, bacterial phytases preferentially target the higher molecular weight esters of inositol phosphate (IP) and so destroy more IP6 and IP5 compared to IP3 and IP4 (Wyss et al., 1999; Greiner and Farouk 2007). Luttrell (1992) and Persson et al., (1998) demonstrated that IP6 and IP5 have greater affinity for chelating Ca compared to lower IPs. IP3 has only 10% chelation capacity of IP1. This might mean that phytase enzyme might release Ca:P in ratio of 2:1 or more rather then 1:1, which is generally assumed when matrices are applied.

Effect of limestone particle size on Broiler performance and tibial ash

In layers, considerable attention has been given to limestone particle size, and generally, it has been shown that coarse particle size have a beneficial effect on eggshell quality (Roland, 1986) and on bone strength (Guinotte and Nys, 1991). Few studies have been done to determine the effect of various limestone particle size on broiler performance, and the results are seldom similar. In a trial done by McNaughton et al. (1974), where 270-pan size limestone particles were included in diets resulted in

improved body weight when compared to 50-40-pan particle size (297-420 µm) and 40-50-pan particle size gave better tibia ash compared to 8-6-pan particle size (2380-3360 µm). In this trial, various sizes of Ca were obtained using United States Bureau of Standards (U.S.B.S) sieve series. Trials by Managi and Coon (2007) demonstrated that fine particle size (137-338 µm) had better body weight gain compared to coarse (1305 µm) and very fine particle size (28 µm). The demonstrated fine particle size performing better than coarse particle size as birds getting fine particle diets had greater feed intake, but Guinote et al. (1991) reported that broilers did not show any benefit in body weight gain, FCR, or tibial bone ash when fed coarse limestone. Anwar et al. (2016) found an *in vitro* solubility coefficient associated with fine (<0.05 mm) limestone to be higher than the coefficient associated with coarse (1-2mm) limestone, which were 0.60, and 0.33, respectively. Anwar et al. (2016) also showed that *in vivo*, the coarse limestone had higher absorption compared to fine, which could be due to a Ca source with less solubility staying in gizzard for a longer time, releasing Ca at slower pace compared to Ca source with high solubility, which passed quickly through the gastrointestinal tract. Zhang and Coon (1997a) concluded that *in vitro* Ca solubility is inversely related to *in vivo* Ca solubility. Managi and Coon (2007) reported similar results in which coarse (1.3 mm) limestone had greater apparent Ca digestibility compared to fine (0.4mm) limestone. This was in contrast to Guinote et al. (1991), who suggested coarse limestone caused low Ca retention because the particles were retained in ventriculus.

Managi and Coon (2007) also showed that phytase enzyme had more activity at pH 2.5 compared to pH 6.5, and diets with increasing Ca particle size had better

phytase activity, which was measured in µg P_i released/unit phytase. They concluded that this observation was the result of larger particles of $CaCO_3$ being less soluble in the crop and anterior gastrointestinal tract. A low soluble $CaCO_3$ was believed to bind in a lesser amount to phytate and form insoluble Ca-phytate complex, which will provide more accessibility to phytate for the phytase enzyme.

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Chapter I. Evaluation of Various Ca: AvP Ratios and Phytase Enzyme Matrix Values Effects on Broiler Live Performance and Carcass Quality

Abstract

This trial was conducted to determine the effect of various calcium (Ca) matrix values of phytase (enzyme) on broiler live performance and carcass quality. Birds were raised to 47 days with body weight (BW) measured at 15, 28, 35 and 47 d. Seven dietary treatments (A – G) were used with A and B being control, C having enzyme supplemented on top. Diets D-G had various matrix values of phytase. The available phosphorus (AvP) matrix value was fixed at 0.15 while the calcium (Ca) value for the enzyme was assigned a different value for each diet. There were a total of 84 pens with each pen having 16 birds and each treatment having 12 replicate pens. Live performance was measured by taking BW, BW gain (BWG), and feed conversion ratio (FCR) adjusted for mortality while carcass quality was determined by scoring for wooden breast and white stripping. Carcass weight and weight of individual carcass parts were recorded. The BWG and feed intake (FI) were higher ($p<0.05$) for diets that had supplemented enzyme, but there were no significant differences among diets with supplemental enzyme. The FCR was improved for diets with enzyme supplemented from 29-35 d ($p\leq0.05$) while for 36-47 d control diets exhibited better FCR ($p\leq0.05$). The trial also showed that adding phytase had no effect on carcass quality. Results of this study showed that giving different matrix values to the enzyme and having decreasing Ca: AvP ratio as birds aged did not have significant effects on live performance or carcass quality.

KeyWords: phytase matrix, Carcass quality, live performance

Introduction

Calcium (Ca) and inorganic phosphorus (P_i) are essential for broilers as these are involved in bone development and various other functions in the body (Wasserman, 1960). When formulating diets, Ca and P_i cannot be considered independently as they play a major role in each other's homeostasis and both are involved in bone formation (Kebreab and Vitti, 2005). Feed ingredients from plant sources are not adequate to meet requirements for these minerals. Therefore, inorganic sources of Ca and P_i are usually supplemented to poultry diets (Adedokun and Adeola, 2013).

Although a significant amount of P_i is present as phytate in plant ingredients, broiler chickens cannot digest readily the phytate (Angel *et al*., 2002). To remedy this problem, phytase enzyme is included in poultry diets. The enzyme digests phytate in corn and soybean resulting in release of P_i (Coelho and Kornegay, 1996), Ca, and other nutrients (Yi *et al*., 1996a; Biehl and Baker, 1997; Farrell *et al*., 1993). The supplementation of phytase in poultry diets ameliorates the need to provide mineral supplements to fulfill the P_i requirement. Phytase supplemented to poultry diets decreases fecal P_i content (Waldroup *et al*., 2000). When the fecal matter is applied to the soil, this helps to prevent eutrophication of surface waters, which is a major environmental concern (Correll, 1998). Mineral supplements, such as dicalcium phosphate (DCP), to provide P_i are expensive, and dietary supplementation of phytase will aid in cost reduction of feed manufacturing. The mechanism and efficacy of an enzyme's activity in making available various nutrients is critical, and many studies have been conducted to reveal the efficacy of nutrient release from phytic acid. Information derived from phytase studies in poultry diets allows nutritionists to better define

compounded diets with greater precision to meet an animal's requirements, reduce compounding costs, and prevent over-formulation of a nutrient. Additionally, Ca can form an insoluble complex with phytate, making it more difficult for the enzyme to degrade it (Taylor, 1965). Thus, if higher matrix values of Ca are attributed to phytase enzyme then less Ca should be supplemented in diets, which might increase the enzyme efficacy and growth performance. Thus the objective of this study was determine wether increasing Ca phytase matrix value and decreasing Ca: AvP ratio would improve broiler live performance and carcass quality.

Materials and Methods

All animal usage conformed to The Guide for Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010) and approved by the N.C.S.U. Instituional Animal Care and Use Committee. Broiler chickens were hatched from eggs obtained from 62 week old Ross YPM x 708 broiler breeders maintained at the site. Chickens were feather sexed at hatching and permanently identified with neck tags. There were 1,344 males placed randomly into 84 pens with 16 birds per pen. Initial litter brooding temperature was set at 35°C and reduced gradually to 27°C by 15 d of age, and held at 27°C until 21 d of age. From 22 d of age until the end of the experiment, temperature was maintained at approximately 24°C.

All diets were formulated to meet or exceed the National Research Council (1994) -suggested requirements. The starter diet was in crumble form while grower and finisher were in pellet form. Phytase enzyme used for this diet was Grainzyme® manufactured by Agrivida. Grainzyme® (GZ) is a transgenic corn that has the phytase enzyme gene inserted into it, which allows the feed compounder to use the special corn

as a source of enzyme for supplementation to poultry diets. The GZ phytase was supplemented at 4lbs/ton, which amounts to 3000 FTU/kg for all the diets with enzyme supplementation. There were 7 treatments (Table 1) with 12 replicates per treatment. Treatments A and B were controls having no GZ phytase. Treatment A had a decreasing Ca:AvPi ratio with the starter diet having the highest and finisher diet having the lowest Ca:AvP_i while treatment B had a fixed Ca:AvP_i ratio in all three feeding phases. Treatment C was similar to B but had GZ phytase supplemented on top during all three feeding phases. Treatments D – G had fixed Ca:AvP_i ratios and GZ phytase (3000 FTU/kg) was supplemented to it during all three feeding phases. In each treatment, a different Ca matrix value for GZ phytase was assigned, and the Ca content in the diet was adjusted accordingly to achieve Ca:AvP_i ratio of 2:1. Starter feed (Table 2) was crumbled, and grower (Table 3) and finisher feeds (Table 4) were pelleted. Calcium particle size analysis was conducted for each treatment and in all three feeding phases (Table 5). One supplemental drinker and two supplemental feed trays were placed in each pen in addition to the normal tube feeder and the single Plasson drinker. Feed trays and the supplemental drinker were removed after the starter feeding phase (16 d). Feed intake (FI) by treatment is shown in Table 6.

Chickens were weighed in groups on d 1, 15, 28, 35, and 47. Pen temperature and mortality were checked twice daily, feeders were shaken once per d to 14 d and twice per d thereafter. Fluorescent light bulbs provided light during the experiment and were lit for 23 h of light during the first week, reduced to 20 hours at 14 d, and to 18 h from 15 -21 d. After 22 d, only natural light was provided.

Sample collection

At 48 d of age, 2 birds per pen were selected on the basis of the average weight of the house. Birds were sacrificed and their carcasses were separated into wholesale components. Body parts were weighed, and whole breasts were chilled and scored two hours later for the presence of wooden breast and white striping. Scoring for wooden breast and white striping was done manually by palpating the breasts; checking subjectively for firmness; and observing for white stripes after carcasses were chilled and rigor had been established. After physical examination of the breasts, overall values from 1-4 were assigned, with 1 representing no wooden breast or white striping development and 4 representing severe wooden breast or white striping. All the scoring was accomplished by one person, who was skilled with the palpation-detection procedure.

Data Analysis

Data analyses were performed using the general linear models procedure of SAS (Statistical Analysis System, 2017). Means were separated using the Least Significant Difference Procedure (Statistical Analysis System, 2017). Data was analyzed as a one way ANOVA and P value ≤ 0.05 were considered significant.

Results and Discussion

Results for FI, BW, BWG, FCR, and mortality are presented in Table 6, 7, 8, 9, and 10. The BW, BWG, and FI were higher ($p<0.05$) for birds with supplemented phytase compared to the controls at 15, 28, and 35 d, but there were no significant

differences among the diets with supplemented GZ phytase. The birds that had GZ phytase supplemented to their diet generally had improved growth performance.

One of the main functions of phytase enzyme is to degrade phytate and make P_i more available. However, as can be seen from the formulation tables (Table 2, 3 and 4), the birds given adequate P_i in diet C while in other diets with GZ phytase supplementation, available P_i was similar to the positive control. This observation shows that the benefit of the enzyme was not due to more available P_i. In previous trials, it has been shown that supplemental enzyme in P_i-deficient diets improved live bird growth performance (Sebastian *et al*., 1996; Simons *et al*., 1990, Broz *et al*., 1994; Nelson *et al*., 1971; Cowieson and Adeola, 2005). Improved growth performance was attributed to greater P_i availability fulfilling the birds' requirement, which facilitated improved live performance. However, little is known about benefits of the enzyme in a P_i adequate diet. Nelson *et al*. (1971) supplemented the enzyme to P_i adequate birds, but those deficient in P_i had a trend toward better BWG compared to the positive control similar to observations by Santos *et al*. (2013) who showed positive control diets with supplemental enzyme facilitated better FCR than positive control without the enzyme. Generally in the broiler industry super dosing of phytase is widely done as it is thought to improve bird's performance and negate the negative effects of pelleting on phytase activity.

In this trial, phytase supplementation resulted in greater body weight and FI compared to the control having only adequate P_i. This can be due to phytase degradation of phytate. Phytate is considered to be an anti-nutritional factor, and it has been shown that its anti-nutritional effects are not only limited to inadequate P_i diets

(Woyengo and Nyachoti, 2013). Phytate binds various minerals and amino acids making them unavailable. Phytate also binds enzymes and increases mucin secretion, thus, resulting in more exogenous losses (Knuckles *et al*., 1989; Choct *et al*., 2010). Increased mucin secretion indicates that phytate may be an irritant in the gut causing reduced appetite. Phytate has also been known to upregulate the expression of somatostatin and down regulate expression of ghrelin (Liu *et al*., 2008). One of the possible reasons for the birds with supplemental phytase having greater FI might be attributed to less phytate in the gut.

The FCRs among treatments were largely non-significant, and this observation was in agreement with various reports (Bahadoran *et al*., 2011; Onyango *et al*., 2005), who strongly suggested a phytase effect on BW and FI but not on FCR. Simons *et al*. (1990) reported improved feed efficiency with 1500 FTU/kg phytase supplementation, but Rosen (2003) derived from multifactorial analyses of phytase feeding trials argued that feed efficiency response to phytase had been declining over recent years due to concurrent improvement in broiler strains, feeds, and management techniques. Although from 0-35 d in this experiment, FCR was improved significantly with enzyme treatments having better FCR that could be explained by less phytate concentration. Yet, from 36-47 days, FCRs were improved for the diets with no enzyme added. It cannot be discounted that as broilers age, their gastrointestinal tracts mature, and one of the maturation effects is the establishment of alkaline phosphatase, which has a potential for degradation of phytate (Sabataku *et al*., 2007).This might also be due to diets with enzyme supplementation having greater BW with poorer FCR compared to

lower BW and better FCR. Larger growing birds require more nutrients for maintenance and growth than smaller birds.

At 47 d, there was a significant difference between BW in control diet A and diet B. Diet A, which had a decreasing Ca:AvP_i ratio had a 15 g greater BW compared to diet B that had a fixed Ca:AvP_i ratio. Broilers given diet A also had greater BWG and FI compared to diet B in the 36-47 d period. This can be explained by the A finisher diet having less metabolizable energy and more crude protein than other diets. Less metabolizable energy means that birds will increase FI to get enough energy as indicated (Lesson *et al.*, 1996). Since the protein level was greater and birds were eating more, they consumed more protein compared to protein intake in other treatments, which resulted in greater BWG from 36-47d.

On the whole, diets with supplemental GZ phytase produced greater BWG compared to the controls (diets A and B). There was no difference in live performance among diets assigned (Diets C-G) different GZ phytase matrix values. The diets had different Ca amounts based on the matrix value of the GZ phytase. It was expected that diets with a higher Ca matrix value due to a GZ phytase low matrix value would have poor performance parameters compared to other diets. Earlier phytase studies have shown phytate precipitation, caused by Ca through formation of insoluble Ca-phytate complexes, are less accessible to phytase (Wise, 1983). There is the possibility of direct depression of phytase enzyme activity in response to excessive Ca competition for the active sites on the enzyme (McCuaig *et al.*, 1972). Additionally, due to a more basic intestinal pH associated with elevated dietary Ca, reduced levels of soluble minerals might interfere with mineral absorption (Shafey and McDonald, 1991).

These observations are in contrast to results reported by Sebastian *et al*. (1996), who suggested that low Ca availability in diet (0.60%) associated with supplemental phytase resulted in better live performance when compared with high Ca availability (1.25%) in phytase-supplemented diets. The P_i was kept constant at 0.3% for both Ca levels. The 1.25% Ca level is greater than the requirement of broilers (NRC, 1994) and excessive Ca intake with a great Ca:AvP_i ratio is known to cause kidney damage and increased incidence of leg abnormalities (McDonald and Shafey, 1987). Kidney damage can lead to calcification of soft tissues where excess Ca can precipitate (Siller, 1981). High Ca:P_i ratio would have also reduced P_i availability (NRC, 1994). These factors might have led to poor performance in the Sebastian *et al*. (1996) trial with high Ca, while low Ca with phytase would have resulted in optimum Ca amount to birds causing better performance. Ca concentration in a diet is crucial because it is highly associated with P_i availability. High matrix values of GZ phytase could cause a lower requirement for supplemental Ca. The Ca requirement according to NRC (1994) should be 0.8-1.0% depending on age and gender, but the 0.76-0.90% Ca level is recommended by the Cobb broiler performance guide (2015). The Ross nutrient specification guide (2014) specifies 0.68-0.96% depending on the age of bird. This indicated that either phytase was releasing Ca as assumed or it was releasing less Ca, which was not supported by the live performance of broilers since calcium requirement is closer to what Driver *et al*. (2005) suggested to be 0.625% Ca to maximize BGW and FCR. Another reason for the difference not being noticed can be that there were only small variations in matrix values.

Results for carcass yield, white striping, and wooden breast score are presented in Table 12. There were no significant differences in percentage carcass yield and white striping and wooden breast scores due to GZ phytase treatment.

The primary goal of this experiment was to compare live performance between decreasing Ca:AvP_i ratio and constant Ca:AvP_i as broilers grew to market age. There were no differences among decreasing or constant Ca:AvP_i ratios, which means that low ratios can be utilized without affecting broiler live performance. This indicates that a Ca:AvP_i ratio, lower than the current standard, can be used for optimized live performance as suggested by Driver *et al.* (2005).

Conclusion

The results of this experiment allowed us to conclude the following:

1. Supplementation of the Grainzyme phytase increased BW and FI without a consistent effect on FCR.
2. Decreasing the Ca:AvP_i ratio produced the same result as a fixed ratio of 2:1, which might indicate that a lower Ca:AvP_i than the standard Ca:AvP_i (2:1) can be used as broilers grow to market age .
3. Assigning a different matrix value to phytase did not affect performance. Greater variation in matrix values might be necessary in order to achieve a significant response.
4. Supplementation of Grainzyme phytase and having various levels of dicalcium phosphate and limestone in the diet did not exert any effects on carcass quality.

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Table 1. Treatment Design.

Trt[1]	Phy	Starter: 0-16 d 2 pounds			Grower: 17-35 d 6 pounds			Finisher: 36-49 d 8 pounds		
		Ca:AvP	Diet Ca and AvP[2]	-Ca/-P matrix	Ca:AvP	Diet Ca and AvP	-Ca/-P matrix	Ca:AvP	Diet Ca and AvP	-Ca/-P matrix
A	-	2:1	1.0/.50	NA	1.85:1	.835/.45	NA	1.7:1	.72/.42	NA
B	-	2:1	1.0/.50	NA	2:1	.9/.45	NA	2:1	.84/.42	NA
C	+	2:1	1.0/.5	On top	2:1	.9/.45	On top	2:1	.84/.42	On top
D	+	2:1	.85/.38	.15/.12	2:1	.75/.33	.15/.12	2:1	.69/.30	.15/.12
E	+	2:1	.89/.38	.11/.12	2:1	.79/.33	.11/.12	2:1	.73/.30	.11/.12
F	+	2:1	.85/.38	.15/.12	2:1	.75/.33	.15/.12	2:1	.73/.30	.11/.12
G	+	2:1	.83/.38	.17/.12	2:1	.73/.33	.17/.12	2:1	.67/.30	.17/.12

[1]: A: descending Ca:AvP (No phytase); B: Fixed 2:1 Ca:AvP (No phytase); C: Fixed 2:1 Ca:AvP with phytase enzyme on top; D – G: Theoretical 2:1 Ca:AvP with phytase enzyme given matrix value. There are two controls (A and B) and a number of comparisons included. P matrix fixed while Ca matrix highest in D and lowest in G.
[2]: The basal numbers shown above are the actual formulation targets without enzyme matrix values added.

Table 2. Experimental diet composition (starter).

	Treatment[1]						
	A	B	C	D	E	F	G
Ingredient (%)							
Corn	55.76	55.76	55.56	57.04	57.00	57.04	57.14
Soybean meal 48%	34.59	34.59	34.59	34.36	34.21	34.36	34.35
Poultry byproduct meal	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Poultry fat	2.45	2.45	2.45	1.90	1.98	1.90	1.86
Dicalcium phosphate	2.23	2.23	2.23	1.48	1.48	1.48	1.48
Limestone	0.64	0.64	0.64	0.68	0.79	0.68	0.63
Sodium chloride	0.50	0.50	0.50	0.50	0.50	0.50	0.50
DL-Methionine	0.26	0.26	0.26	0.26	0.26	0.26	0.26
L-Threonine	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Choline chloride, 60%	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Mineral premix	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Vitamin premix[2]	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Selenium premix[3]	0.05	0.05	0.05	0.05	0.05	0.05	0.05
L-lysine	0.01	0.01	0.01	0.02	0.02	0.02	0.02
Coban[4]	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Grainzyme (GZ203)	0.00	0.00	0.20	0.20	0.20	0.20	0.20

Table 2 (continued). Experimental diet composition (starter).

	Treatment[1]						
	A	B	C	D	E	F	G
Calculated nutrient content (%)							
Metabolizable energy (kcal/kg)	2950	2950	2950	2950	2950	2950	2950
Crude Protien	22.50	22.50	22.50	22.50	22.50	22.50	22.50
Calcium	1.00	1.00	1.00	0.85	0.89	0.85	0.83
Total phosphorus	0.84	0.84	0.84	0.71	0.71	0.71	0.71
Available phosphorus	0.50	0.50	0.50	0.38	0.38	0.38	0.38
Total lysine	1.24	1.24	1.24	1.24	1.24	1.24	1.24
Total methionine	0.59	0.59	0.59	0.59	0.59	0.59	0.59
Analyzed nutrient content (%), as fed							
Crude fat	5.18	5.18	5.22	4.94	4.79	4.94	4.79
Crude protein	23.03	23.03	23.31	23.81	24.55	23.81	22.89
Digestible protein	18.51	18.51	18.82	19.37	20.21	19.37	18.35
Crude fiber	1.40	1.40	1.60	1.50	1.40	1.50	1.60
Total digestible nutrients	67.88	67.88	66.97	69.10	67.44	69.10	67.51
Ash	6.05	6.05	6.27	5.76	5.94	5.76	5.47
Calcium	0.93	0.93	1.08	0.84	1.03	0.84	0.85
Total phosphorus	0.85	0.85	0.93	0.76	0.83	0.76	0.79

[1]Treatments: A: descending Ca:AvP (No phytase); B: Fixed 2:1 Ca:AvP (No phytase); C: Fixed 2:1 Ca:AvP with phytase enzyme on top; D – G: Theoretical 2:1 Ca:AvP with phytase enzyme given matrix value. There are two controls and a number of comparisons included.
[2]Vitamin premix supplied the following per kg of diet: 6,614 IU vitamin A, 1,984 IU vitamin D3, 33 IU vitamin E, 0.02 mg vitamin B12, 0.13 mg biotin, 1.98 mg menadione (K_3), 1.98 mg thiamine, 6.6 mg riboflavin, 11 mg d-pantothenic acid, 3.97 mg vitamin B6, 55 mg niacin, and 1.1 mg folic acid.
[3]Selenium premix provided 0.2 mg Se (as Na_2SeO_3).
[4]Coban supplied monensin sodium at 90 mg/kg of feed.

Table 3. Experimental diet composition (grower).

	Treatment[1]						
	A	B	C	D	E	F	G
Ingredient (%)							
Corn	62.48	62.12	62.00	63.41	63.18	63.41	63.51
Soybean meal 48%	30.02	30.08	30.00	29.84	29.88	29.84	29.83
Poultry byproduct meal	1.50	1.50	1.50	1.50	1.50	1.50	1.50
Poultry fat	2.10	2.23	2.23	1.68	1.77	1.68	1.64
Dicalcium phosphate	2.18	2.18	2.18	1.44	1.44	1.44	1.44
Limestone	0.41	0.58	0.58	0.62	0.72	0.62	0.57
Sodium chloride	0.50	0.50	0.50	0.50	0.50	0.50	0.50
DL-Methionine	0.22	0.22	0.22	0.22	0.22	0.22	0.22
L-Threonine	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Choline chloride, 60%	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Mineral premix	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Vitamin premix[2]	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Selenium premix[3]	0.05	0.05	0.05	0.05	0.05	0.05	0.05
L-lysine	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Coban[4]	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Grainzyme (GZ203)	0.00	0.00	0.20	0.20	0.20	0.20	0.20
Grainzyme (GZ53)	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table 3 (Continued). Experimental diet composition (grower).

	Treatment[1]						
	A	B	C	D	E	F	G
Calculated nutrient content (%)							
Metabolizable energy (kcal/kg)	3000	3000	3000	3000	3000	3000	3000
Crude protein	20.00	20.00	20.00	20.00	20.00	20.00	20.00
Calcium	0.84	0.90	0.90	0.75	0.79	0.75	0.73
Total phosphorus	0.78	0.78	0.78	0.64	0.64	0.64	0.64
Available phosphorus	0.45	0.45	0.45	0.33	0.33	0.33	0.33
Total lysine	1.10	1.10	1.10	1.10	1.10	1.10	1.10
Total methionine	0.52	0.52	0.52	0.52	0.52	0.52	0.52
Analyzed nutrient content (%), as fed							
Crude fat	4.94	5.13	4.88	4.73	4.83	4.73	4.81
Crude protein	20.18	20.02	20.60	19.76	19.67	19.76	20.23
Digestible protein	15.41	15.24	15.86	14.96	14.86	14.96	15.46
Crude fiber	1.60	1.30	1.50	1.80	1.70	1.80	1.80
Total digestible nutrients	67.15	67.51	67.35	67.98	67.92	67.98	68.42
Ash	5.52	5.86	5.99	5.25	5.59	5.25	5.22
Calcium	0.86	1.23	1.00	0.80	0.83	0.80	0.86
Total phosphorus	0.83	1.06	0.92	0.75	0.74	0.75	0.76

[1]Treatments: A: descending Ca:AvP (No phytase); B: Fixed 2:1 Ca:AvP (No phytase); C: Fixed 2:1 Ca:AvP with phytase enzyme on top; D – G: Theoretical 2:1 Ca:AvP with phytase enzyme given matrix value. There are two controls and a number of comparisons included

[2]Vitamin premix supplied the following per kg of diet: 6,614 IU vitamin A, 1,984 IU vitamin D3, 33 IU vitamin E, 0.02 mg vitamin B12, 0.13 mg biotin, 1.98 mg menadione (K_3), 1.98 mg thiamine, 6.6 mg riboflavin, 11 mg d-pantothenic acid, 3.97 mg vitamin B6, 55 mg niacin, and 1.1 mg folic acid.

[3]Selenium premix provided 0.2 mg Se (as Na_2SeO_3).

[4]Coban supplied monensin sodium at 90 mg/kg of feed.

Table 4. Experimental diet composition (finisher).

	Treatment[1]						
	A	B	C	D	E	F	G
Ingredient (%)							
Corn	63.71	67.29	67.09	68.56	68.35	68.35	68.67
Soybean meal 48%	29.83	25.66	25.66	25.43	25.46	25.46	25.41
Poultry byproduct meal	1.50	1.00	1.00	1.00	1.00	1.00	1.00
Poultry fat	1.64	2.10	2.10	1.56	1.64	1.64	1.52
Dicalcium phosphate	1.44	2.10	2.10	1.35	1.35	1.35	1.35
Limestone	0.57	0.55	0.55	0.59	0.69	0.69	0.54
Sodium chloride	0.50	0.50	0.50	0.50	0.50	0.50	0.50
DL-Methionine	0.22	0.19	0.19	0.19	0.19	0.19	0.19
L-Threonine	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Choline chloride, 60%	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Mineral premix	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Vitamin premix[2]	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Selenium premix[3]	0.05	0.05	0.05	0.05	0.05	0.05	0.05
L-lysine	0.03	0.05	0.05	0.06	0.06	0.06	0.06
Coban[4]	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Grainzyme (GZ203)	0.00	0.00	0.20	0.20	0.20	0.20	0.20
Grainzyme (GZ53)	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table 4 (Continued). Experimental diet composition (finisher).

	Treatment[1]						
	A	B	C	D	E	F	G
Calculated nutrient content (%)							
Metabolizable energy (kcal/kg)	3000	3050	3050	3050	3050	3050	3050
Crude protein	20.00	18.00	18.00	18.00	18.00	18.00	18.00
Calcium	0.73	0.84	0.84	0.69	0.73	0.73	0.67
Total phosphorus	0.64	0.74	0.74	0.60	0.60	0.60	0.60
Available phosphorus	0.33	0.42	0.42	0.30	0.30	0.30	0.30
Total lysine	1.10	0.99	0.99	0.99	0.99	0.99	0.99
Total methionine	0.52	0.46	0.46	0.46	0.46	0.46	0.46
Analyzed nutrient content (%), as fed							
Crude fat	5.02	5.13	5.52	4.88	4.85	4.85	4.99
Crude protein	18.48	17.97	18.46	17.59	18.54	18.54	18.02
Digestible protein	13.61	13.09	13.59	12.69	13.68	13.68	13.14
Crude fiber	1.70	1.50	1.60	1.70	1.70	1.70	2.00
Total digestible nutrients	68.71	68.53	69.40	69.66	68.84	68.84	69.10
Ash	5.19	5.72	5.52	4.74	5.08	5.08	4.56
Calcium	0.79	0.98	0.86	0.78	0.78	0.78	0.73
Total phosphorus	0.84	0.86	0.81	0.70	0.67	0.67	0.69

[1]Treatments: A: descending Ca:AvP (No phytase); B: Fixed 2:1 Ca:AvP (No phytase); C: Fixed 2:1 Ca:AvP with phytase enzyme on top; D – G: Theoretical 2:1 Ca:AvP with phytase enzyme given matrix value.
[2]Vitamin premix supplied the following per kg of diet: 6,614 IU vitamin A, 1,984 IU vitamin D3, 33 IU vitamin E, 0.02 mg vitamin B12, 0.13 mg biotin, 1.98 mg menadione (K_3), 1.98 mg thiamine, 6.6 mg riboflavin, 11 mg d-pantothenic acid, 3.97 mg vitamin B6, 55 mg niacin, and 1.1 mg folic acid.
[3]Selenium premix provided 0.2 mg Se (as Na_2SeO_3).
[4]Coban supplied monensin sodium at 90 mg/kg of feed.

Table 5. Geometric mean diameter and standard deviation for mash feed particle size in microns (µm).

	Treatment[1]						
	A	B	C	D	E	F	G
Starter				(µm)			
Dgw[2]	876	876	876	867	807	867	850
Sgw[3]	2.18	2.18	2.18	2.31	2.66	2.31	2.59
Grower							
Dgw	577	546	548	383	460	383	445
Sgw	2.24	2.24	2.17	3.37	3.13	3.37	3.10
Finisher							
Dgw	508	546	569	559	588	588	549
Sgw	2.30	2.18	2.18	2.27	2.29	2.29	2.60

[1]: Treatments: A: descending Ca:AvP (No phytase); B: Fixed 2:1 Ca:AvP (No phytase); C: Fixed 2:1 Ca:AvP with phytase enzyme on top; D – G: Theoretical 2:1 Ca:AvP with phytase enzyme given matrix value. There are two controls and a number of comparisons included.
[2]: Geometric diameter average
[3]: Geometric standard deviation

Table 6. Agrivida-6 live performance (feed intake [FI] in grams).

	Treatment[1]							SE Pooled	P-value
	A	B	C	D	E	F	G		
FI(g)									
Day 0- 15	613	625	663	652	659	653	652	8	0.0001
Day 0 – 28	2268^{B}	2306^{B}	2428^{A}	2372^{A}	2390^{A}	2384^{A}	2399^{A}	23	0.0001
Day 0 – 35	3544^{B}	3587^{B}	3740^{A}	3685^{A}	3705^{A}	3684^{A}	3721^{A}	25	0.0001
Day 0 – 47	6370^{BC}	6365^{C}	6535^{A}	6481^{AB}	6535A	6503^{A}	6538^{A}	41	0.0003
Day 16 – 28	1655^{C}	1680^{BC}	1766^{A}	1720^{AB}	1731^{A}	1731^{A}	1747^{A}	18	0.0001
Day 29 – 35	1276^{B}	1281^{B}	1312^{A}	1313^{A}	1315^{A}	1301^{AB}	1322^{A}	11	0.0182
Day 16 – 35	2930^{B}	2961^{B}	3078^{A}	3033^{A}	3046^{A}	3032^{A}	3068^{A}	20	0.0001
Day 36 – 47	2826	2778	2794	2796	2830	2819	2817	25	0.475

$^{a-c}$Means in a row within each variable that possess different superscripts differ significantly ($P \leq 0.05$).
$^{A-C}$Means in a row within each variable that possess different superscripts differ significantly ($P \leq 0.01$).
[1]Treatments: A: descending Ca:AvP (No phytase); B: Fixed 2:1 Ca:AvP (No phytase); C: Fixed 2:1 Ca:AvP with phytase enzyme on top; D – G: Theoretical 2:1 Ca:AvP with phytase enzyme given matrix value. There are two controls and a number of comparisons included.

Table 7. Agrivida-6 live performance (body weight [BW] in grams).

	Treatment[1]							SE Pooled	P-value
	A	B	C	D	E	F	G		
BW				(g)					
Day 0	47.0	47.1	47.0	46.9	46.8	47.0	47.4	0.3	0.863
Day 15	498.1^{B}	505.3^{B}	542.2^{A}	534.8^{A}	539.4^{A}	532.4^{A}	537.8^{A}	6.1	0.0001
Day 28	1652^{B}	1663^{B}	1755^{A}	1730^{A}	1738^{A}	1736^{A}	1751^{A}	16	0.0001
Day 35	2420^{C}	2445^{C}	2592^{A}	2569^{AB}	2561^{AB}	2555^{AB}	2565^{AB}	16	0.0001
Day 47	3953^{BC}	3938^{C}	4048^{A}	4074^{A}	4009^{AB}	4042^{A}	4026^{A}	24	0.0013

$^{a-c}$Means in a row within each variable that possess different superscripts differ significantly ($P \leq 0.05$).
$^{A-C}$Means in a row within each variable that possess different superscripts differ significantly ($P \leq 0.01$).
[1]Treatments: A: descending Ca:AvP (No phytase); B: Fixed 2:1 Ca:AvP (No phytase); C: Fixed 2:1 Ca:AvP with phytase enzyme on top; D – G: Theoretical 2:1 Ca:AvP with phytase enzyme given matrix value. There are two controls and a number of comparisons included.

Table 8. Agrivida-6 live performance (body weight gain [BWG] in grams).

	Treatment[1]							SE Pooled	P- value
	A	B	C	D	E	F	G		
BWG				(g)					
Day 0 – 15	451.1^{B}	458.3^{B}	495.2^{A}	487.9^{A}	492.6^{A}	485.4^{A}	490.4^{A}	6	0.0001
Day 0 – 28	1605^{B}	1616^{B}	1708^{A}	1683^{A}	1692^{A}	1689^{A}	1703^{A}	16	0.0001
Day 0 – 35	2373^{C}	2398^{C}	2545^{A}	2522^{AB}	2514^{AB}	2508^{AB}	2518^{AB}	16	0.0001
Day 0 – 47	3906^{BC}	3891^{C}	4001^{A}	4027^{A}	3963^{AB}	3995^{A}	3978^{A}	24	0.0012
Day 16 – 28	1154^{C}	1157^{BC}	1213^{A}	1195^{A}	1199^{A}	1203^{A}	1213^{A}	12	0.0001
Day 29 – 35	768^{C}	782^{BC}	837^{A}	839^{A}	822^{A}	819^{A}	815^{AB}	12	0.0005
Day 16 – 35	1922^{B}	1939^{B}	2050^{A}	2034^{A}	2021^{A}	2023^{A}	2027^{A}	14	0.0001
Day 36 – 47	1533^{a}	1493^{ab}	1456^{b}	1505^{ab}	1449^{b}	1487^{ab}	1461^{b}	20	0.04

$^{a-c}$Means in a row within each variable that possess different superscripts differ significantly ($P \leq 0.05$).
$^{A-C}$Means in a row within each variable that possess different superscripts differ significantly ($P \leq 0.01$).
[1]Treatments: A: descending Ca:AvP (No phytase); B: Fixed 2:1 Ca:AvP (No phytase); C: Fixed 2:1 Ca:AvP with phytase PY203 on top; D – G: Theoretical 2:1 Ca:AvP with phytase enzyme given matrix value. There are two controls and a number of comparisons included.

Table 9. Agrivida-6 live performance (feed conversion ratio [FCR]).

	Treatment[1]							SE Pooled	P-value
	A	B	C	D	E	F	G		
FCR[2]				(g:g)					
Day 0 – 15	1.359	1.365	1.339	1.336	1.339	1.345	1.334	0.011	0.399
Day 0 – 28	1.413	1.427	1.421	1.407	1.413	1.411	1.410	0.008	0.688
Day 0 – 35	1.492^{AB}	1.498^{A}	1.477^{BCD}	1.466^{D}	1.475^{BCD}	1.469^{CD}	1.475^{BCD}	0.007	0.006
Day 0 – 47	1.637	1.635	1.635	1.615	1.651	1.630	1.646	0.008	0.001
Day 16 – 28	1.435	1.452	1.455	1.437	1.443	1.438	1.440	0.009	0.801
Day 29 – 35	1.655^{A}	1.641^{AB}	1.594^{C}	1.593^{C}	1.609^{BC}	1.589^{C}	1.619^{BC}	0.013	0.0003
Day 16 – 35	1.525^{A}	1.527^{A}	1.502^{BC}	1.491^{C}	1.506^{ABC}	1.499^{BC}	1.513^{ABC}	0.008	0.03
Day 36 – 47	1.872^{CD}	1.862^{D}	1.920^{ABC}	1.872^{CD}	1.957^{A}	1.913^{ABCD}	1.951^{AB}	0.018	0.0001

[a-c]Means in a row within each variable that possess different superscripts differ significantly ($P \leq 0.05$).
[A-C]Means in a row within each variable that possess different superscripts differ significantly ($P \leq 0.01$).
[1]Treatments: A: descending Ca:AvP (No phytase); B: Fixed 2:1 Ca:AvP (No phytase); C: Fixed 2:1 Ca:AvP with phytase enzyme on top; D – G: Theoretical 2:1 Ca:AvP with phytase enyzme given matrix value. There are two controls and a number of comparisons included.

Table 10. Agrivida-6 live performance (mortality %).

	Treatment[1]							SE Pooled	P-value
	A	B	C	D	E	F	G		
Mortality				%					
Day 0-15	0	0	0.52	0	0	0	1.56	0.4	0.071
Day 0-28	1.04	0	1.04	1.56	0	0	0.52	0.5	0.251
Day 0 – 35	1.56	0.52	2.08	2.60	0.52	1.04	2.60	0.6	0.759
Day 0 – 47	4.17	1.04	4.17	4.69	1.04	3.13	4.69	1.2	0.765
Day 16 – 28	1.0	0.0	1.0	1.6	0.0	0.0	0.5	0.5	0.282
Day 29 – 35	0.5	0.5	0.5	1.0	0.5	1.0	0.5	0.6	0.981
Day 36 – 47	2.60	0.56	2.08	2.08	0.52	2.15	2.12	1.0	0.550

[1]Treatments: A: descending Ca:AvP (No phytase); B: Fixed 2:1 Ca:AvP (No phytase); C: Fixed 2:1 Ca:AvP with phytase enzyme on top; D – G: Theoretical 2:1 Ca:AvP with phytase enzyme given matrix value. There are two controls and a number of comparisons included.

Table 11. Agrivida-6 Carcass and parts weights

	Treatment[1]							SE Pooled	P-value
	A	B	C	D	E	F	G		
Weight (g)									
Fasted live	4198	4219	4223	4202	4194	4180	4219	23	0.05
Hot carcass	3318	3361	3374	3360	3341	3334	3372	19	0.34
Fat pad	42	36	33	33	32	38	36	3	0.14
Legs	1156	1184	1182	1155	1164	1136	1174	14	0.23
Wings	306	303	300	305	308	305	306	4	0.86
Breast skin	128	137	133	132	133	134	134	4	0.28
Pectoralis major	1014	1022	1042	1062	1013	1040	1046	14	0.11
Pectoralis minor	185	190	191	190	194	201	189	6	0.76
Frame and neck	487	491	494	483	498	483	486	9	0.87
Yield (%)									
Carcass	79.1	79.7	79.9	79.9	79.7	79.8	79.9	0.3	0.006
Fat pad	1.3	1.1	1.0	1.0	1.0	1.1	1.1	0.1	0.09
Legs	34.8	35.2	35.0	34.4	34.8	34.1	34.8	0.4	0.41
Wings	9.2	9.0	8.9	9.1	9.2	9.1	9.1	0.1	0.56
Breast skin	3.9	4.1	3.9	3.9	4.0	4.0	4.0	0.1	0.20
Pectoralis major	30.6	30.4	30.9	31.6	30.3	31.2	31.0	0.4	0.24
Pectoralis minor	5.6	5.7	5.7	5.7	5.8	6.0	5.6	0.2	0.70
Frame and neck	14.7	14.6	14.7	14.4	14.9	14.5	14.4	0.3	0.72

[1]Treatments: A: descending Ca:AvP (No phytase); B: Fixed 2:1 Ca:AvP (No phytase); C: Fixed 2:1 Ca:AvP with phytase enzyme on top; D – G: Theoretical 2:1 Ca:AvP with phytase enzyme given matrix value. There are two controls and a number of comparisons included.

Table 12. Agrivida-6 white striping and wooden breast scores

	Treatment[1]							SE Pooled	P-value
	A	B	C	D	E	F	G		
White striping	2.500	2.208	2.500	2.259	2.458	2.292	2.417	0.12	0.24
Wooden breast	2.750	2.708	2.833	2.389	2.750	2.583	2.750	0.18	0.74

[1]Treatments: A: descending Ca:AvP (No phytase); B: Fixed 2:1 Ca:AvP (No phytase); C: Fixed 2:1 Ca:AvP with phytase enzyme on top; D – G: Theoretical 2:1 Ca:AvP with phytase enzyme given matrix value; There are two controls and a number of comparisons included.
[2]Score values from 1-4 illustrate the degree of white striping or wooden breast. A score of 1 represented no wooden breast or white striping while a score of 4 represented severe wooden breast or white striping.

Chapter II. Evaluation of fine and coarse LPS and addition of phytase on live performance, apparent nutrient digestibility and bone ash content of male broilers.

Abstract

Variability in the solubility limestone particle size (LPS) can influence broiler performance by altering the rate of Calcium (Ca) release into the gastrointestinal tract (GIT). The objective of this research was to determinethe influence of LPS (190 µm and 900 µm); supplemented phytase (0 FYT/kg and 1000 FYT/kg); and two Ca and P_i levels (positive control [PC] and negative control [NC]) on broiler live performance, bone ash, and nutrient digestibility. In the starter diet (0-14 d), PC had 0.90 Ca and 0.45 P_i, while NC had 0.72 Ca and 0.30 P_i. In the grower diet (15-35 d), PC had 0.76 and 0.38 while NC had 0.52 and 0.23 for Ca and P_i, respectively. The 8 experimental diets were assigned randomly to a total of 1512 birds, with 21 birds per pen and 9 pens per dietary treatment. Body weight (BW) and feed intake (FI) were determined at 15, 28 and 35 d of age. Body weight gain (BWG) and feed conversion ratio (FCR) were calculated for each period and the overall period (0-35 d). Ileal digesta and left tibia from 3 birds/pen at 14 d and 2 birds/pen at 35 d were sampled. The pen was considered an experimental unit, and data were analyzed using the SAS General Linear Models procedure.

The main effects of LPS and Ca and P_i level had no influence on either BW, BWG, FI, or FCR among all periods of the study. Adding phytase improved BW during all periods ($P \leq 0.05$), BWG and FI at 0-14 d ($P \leq 0.05$) and 0-35 d ($P \leq 0.05$), but the improvement was not significant for FCR ($P \geq 0.05$). The PC and addition of phytase to the NC improved BW, BWG, and FI for 0-14 d ($P \leq 0.05$) and for BWG during the 14-28 d period ($P \leq 0.05$) when compared to the NC without phytase. Birds fed the PC without

phytase, and either the PC or the NC with phytase were about 96 g heavier than the NC without phytase. The NC coarse and PC fine performed better (P≤ 0.05) than PC coarse and NC fine for BW at 14 and 28 d (P≤ 0.05), BWG for 0-14 d and 15-28 d (P≤ 0.05).

Bone Ash results showed LPS having no significant effect while inclusion of phytase at 14 d (P≤0.05) and Ca and Pi level at 14 and 35 d (P≤0.05) improved bone ash. At 14 d the NC had the least bone ash, while PC, irrespective of phytase inclusion, had the greatest bone ash. Addition of phytase to the NC improved bone ash, but it was still less compared to the PC (P≤0.05).

The Apparent Ileal Digestibility (AID) of Ca and P_i were influenced by LPS, Ca and P_i level, and phytase for 14 and 35 d (P≤0.05). Phytase had no effect on Ca AID at 35 d. In a fine particle size diet, inclusion of phytase and NC improved AID compared to coarse particle size, absence of phytase and PC respectively (P≤0.05). AID of amino acids was also significant for LPS and phytase inclusion with fine LPS, phytase having greater value than coarse limestone and no phytase respectively. The results indicate that phytase enzyme improved broiler performance without being affected by LPS, and it also has an influence on bone ash and AID of Ca, P_i and amino acids. Fine also improved AID for Ca and P_i, but it couldn't be confirmed for amino acids as it was thought that the effect might have been due to greater FI. Phytase was observed to have a greater influence on coarse limestone compared to fine for both bone ash and AID. Ca and P_i level was the most influential factor in determining bone ash although phytase inclusion can lead to an improvement during early days.

KeyWords: limestone, particle size, broilers, phytase, performance

Introduction

Calcium (Ca) is the most abundant mineral in the body (Applegate and Angel, 2008) and performs critical functions such as bone mineralization, blood clotting, intracellular signaling and muscle contractions (Suttle, 2010). Being important as it is, excess Ca in the diet can lead to undesirable effects. Excess dietary Ca can interfere with the availability of other minerals such as zinc, magnesium and manganese (NRC, 1994), and Ca can chelate lipids making them unavailable, decreasing the energy profile of feed (Edwards *et al*., 1960). Ca has the capacity to bind with inorganic phosphorus (P_i) and form Ca-phytate complex in the gut, which interferes with P_i availability (Hurwitz and Bar, 1971). In poultry diets, more often than not, Ca is over supplemented due to it being used as a vehicle in various premixes (Kim *et al.,* 2018), which is not taken to account when formulating diets. Also, its primary source in poultry diet is limestone, which is readily available and inexpensive (Blount, 2013). One can also argue, that with accelerated growth of broilers over the years, the Ca requirement must have increased due to a stronger skeleton being required to support greater weight/body mass. Contrary to that supposition, the factual situation is that over the years Ca requirements have dropped (Driver *et al*., 2005a; Ziaei *et al*., 2008: Singh *et al*., 2013) compared to NRC requirements of 1994. The Cobb broiler performance and nutrient supplement guide (2015) and Ross broiler nutrition specification guide (2014) both recommend less Ca than the NRC requirements of 1994. This points to modern fast growing, high-yielding broilers being able to more efficiently utilize nutrients compared to smaller, slower growing broilers of the past.

Limestone is the primary source of Ca in poultry diets and has a major impact on Ca availability. In various studies in layers have attributed Ca retention as a function of LPS solubility (Zhang and Coon, 1997; Roland, 1986: Cheng and Coon, 1990). Eusebio- Balcazar *et al*. (2018) demonstrated that coarse LPS improved bone integrity of layer hens, which indicated a positive effect to coarse limestone inclusion instead of fine limestone inclusion. Compared to layers, fewer studies have been conducted in broilers in terms of LPS although in broilers LPS does affect Ca solubility and availability (Anwar *et al*., 2016; Kim *et al*., 2018). It is thought that compared to fine, coarse limestone will be retained longer in the ventriculus. Longer retention will increase solubility and greater Ca availability associated with slower Ca release, which might be beneficial as there will be less interference with gut pH and P_i digestibility.

In the poultry industry, particularly in broilers, LPS has not been given much consideration. The purpose of this experiment was to observe whether LPS, its interaction with different Ca and P_i levels, and phytase inclusion will have an impact on live performance, digestibility and bone ash of broilers.

Material and Methods

All animal work conformed to The Guide for Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010) and approved by the N.C.S.U. Institutional Animal Care and Use Committee. Broiler chicks were hatched from eggs obtained from 39 week old Cobb 500 broiler breeders maintained at the North Carolina Agricultural Research Service Chicken Educational Unit. Chickens were feather sexed at hatching and permanently identified with neck tags. There were 1,512 males placed randomly into 72 pen with 21 birds per pen. Initial litter brooding temperature was set at 35°C and

was reduced gradually to 27°C by 15 d of age, and was held at 27°C until 21 d of age. From 22 d of age until the end of experiment, temperature was maintained at approximately 24°C. When chicks were placed into the floor pens, there was one supplemental drinker and three supplemental feed trays available in each pen in addition to two tube feeders and one bell drinker. Supplemental drinkers and two supplemental feeders were removed after 7 d while the remaining feeder tray was removed after 14 d. Fluorescent type bulbs provided light during the experiment and were lit for 23 h of light during the first week, reduced to 20 h at 14 d and to 18 h from 15 -21 d. After 22 d, only natural light was provided. Temperature and mortality were checked twice daily. Feeders were shaken once per day up to 14 d and twice per d thereafter.

All diets were formulated to meet or exceed the Cobb Broiler Nutrition Guide requirements, and birds were fed starter from 0-16 days and grower from 17-35 d with 907 g of starter and *ad libitum* grower. All the diets had titanium dioxide inclusion at the rate of 0.5 g/kg of diet for calculation of Apparent Ileal Digestibility (AID). The experiment was arranged as a 2 x 2 x 2 factorial with 8 dietary treatments (Table 1) and had 9 replicates of each dietary treatment. Birds were fed diets having two LPS (fine or coarse), Ca and P_i level positive control [PC] or negative control [NC], and phytase inclusion at either 0 FYT/kg or 1000 FYT/kg. Fine LPS was 190 µm, and coarse LPS had a particle size of 900 µm. The positive control had a Ca- 0.9 to P_i- 0.45 ratio of in starter and Ca- 0.76 to P_i- 0.38 ratio in the grower, while the NC had Ca- 0.72 to P_i- 0.3 in starter and Ca- 0.58 to P_i- 0.23 ratio in grower. The supplemented phytase enzyme was Ronozyme® Hiphos 2500 GT (Koninklijke DSM N.V., Heerlen, the Netherland). The

PC phytase was supplemented without considering matrix values, but in the NC it was assigned a matrix value of 0.18 for Ca and 0.15 for P_i. Starter feed (Table 2) was crumbled, but grower feed (Table 3) was pelleted. Pelleting temperature for both starter and grower was 180°C. Particle size analysis was also conducted on each treatment and in all phases (Table 4) using Ro-TaPi sieve shaker by following the method ANSI/ASAE S319 (ASAE, 2017).

Chicks were weighed in groups and individually on 1, 14, 28, and 35 d of age with feed weigh-back on the same days in order to determine live performance based on body weight (BW), body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR). Feed conversion ratio (FCR) and FI were calculated from feed weigh back and pen means for body weight while Individual bird weights were used to calculate coefficients of variation (CV).

Sample Collection

Ileal content and left tibia of 3 birds/pen at 15 d and 2 birds/pen at 36 d were collected. The ileum, distal to Meckel's diverticulum and about 1 inch proximal to the ileocecal junction, was separated from bird's intestinal tract and its contents were expressed into marked plastic tubes, placed on ice, and frozen the same day. The left tibia was sampled by separating it from the femur at the knee joint and ankle joint removing it from foot. The tibia was cleaned by removing all the muscles and tendons from it. Afterwards, it was placed in marked plastic bags and frozen.

Digestibility analysis

Ileal digesta and feed samples were freeze-dried at -50°C and ground. Digestibility analysis in both digesta and feed samples for amino acid profile was done by AOAC Official method 982.30 (AOAC, 2006) and for Ca and P_i was conducted using AOAC official method 968.08 (AOAC, 1996). Titanium content in ileal digesta and feed samples was determined according to Myers *et al*. (2004).

Ileal Digestibility was calculated according to a procedure described by Dilger and Adeola (2006). The following equation was used to calculate AID (%) of P_i, Ca and amino acids in the diet:

Percentage AID= $\left\{1 - \left[\left(\frac{T_I}{T_O} X \frac{N_O}{N_I}\right)\right]\right\}$ X 100

Wherein the equation each component is as follows:

AID: apparent ileal digestibility (%);

T_I: titanium concentration in the diet;

T_o : titanium concentration in the ileal digesta;

N_o : the concentration of any nutrient that is being calculated in the ileal digesta;

N_I :the concentration of any nutrient that is being calculated in the diet.

Tibia bone ash analysis

Tibia bones were dried at 105°C in an oven for 24 h and weighed. Afterwards, they were wrapped in cheese cloth and soaked in ethyl ether for 48 h to remove fat. The solution was changed once after 24 h. Bones were then dried and the cheese cloth was removed. They were again dried at 105°C for 24 h in an oven and weighed to obtain defatted tibia weight before being placed into tared and labeled crucibles. These

crucibles were then placed for 24 h in furnace at 600°C (Hall *et al*., 2003) and afterwards, cooled to room temperature. The weight of crucible plus ash minus the tared crucible weight was recorded as the tibia ash weight.

The following equation was used to calculate tibia bone ash percentage:

$$\text{Percentage Tibia Bone Ash} = \frac{\text{Tibia Ash weight}}{\text{Defatted tibia weight}} \times 100$$

Limestone Analysis

The Ca solubility was calculated for both coarse and fine limestone (Table 5) by subjecting weighed samples of coarse and fine limestone in 0.1N HCL at 42 °C for 10 min with agitation and recovering quantitively the unsolubilized residue. The difference between the weighed sample and insoluble residue was calculated to be the solubility and was expressed as a percentage solubility (Table 5). Calcium concentration in both fine and coarse limestone (Table 5) was also determined.

Data Analysis

A pen was considered an experimental unit. Using the General Linear Models procedure of SAS (Statistical Analysis System, 2017), the data were analyzed and means were separated. Significance among means was determined using Tukey's Test (Statistical Analysis System, 2017). Significance among main effects, their interactions, and means was based on Pi ≤ 0.05 or less. Data was analyzed as a 2x2x2 factorial with LPS, Ca and P level and phytase being considerd main effects.

Results and Discussion

In the acid solubility medium, fine limestone had greater solubility compared to coarse limestone (72.8% to 53.0%), and Ca concentration in both fine and coarse

limestone was around 39% (Table 5). Particle sizes of starter and grower diets were similar, being around 600µm (Table 4). This is important as different particle sizes of feed can influence the bird's performance as demonstrated by Amerah *et al*. (2008). Particle size uniformity was also high among treatments ensuring birds in the trial got similar feed particle sizes.

Live performance

Live performance results are shown in Tables 6 (BW), 7 (BWG), 8 (FI), 9 (FCR), 10 (Mort) and 11(CV). The main effects of LPS, Ca, and P_i levels did not affect BW, BWG, FI and FCR. However, when the phytase factor was removed and data were analyzed again, Ca and P_i levels did show a significant effect with the PC having greater BW, BWG and FI compared to NC, but LPS did not affect live performance. In an earlier study (McNaughton *et al*., 1974), fine particle size improved BW of broilers compared to coarse limestone although the coarse LPS was 2360- 3350 µm which is significantly greater than what was used for this study (900 µm). Similarly, Managi and Coon (2007) observed the best weight gain and feed intake with fine LPS (137- 299 µm) compared to coarse (>500 µm) or very fine (28 µm) particle size. Bradbury *et al*. (2018) came to a conclusion similar to the outcome of this study, which showed that LPS had no significant influence on either BWG or FCR.

The phytase main effect was significant for all sampling/weighing periods of BW: 14 d (P value= 0.006), 28 d (P value= 0.0036) and 35 d (P value=0.019). The phytase effect was also significant on BWG and FI for 0-15 d and 0-35 d, but it was not significant for FCR. Earlier investigators have shown that phytase improved live performance or was associated with a similar performance in response to adequate Ca

and P_i when it was supplemented in a diet with inadequate Ca and P_i (Shaw *et at*., 2011; Singh *et al*., 2003; Ceylan *et al*., 2012). The BWG and FI show that phytase was most effective for the first 14 d, and as the bird aged, the phytase effect was not significant. This was in agreement with Olukosi *et al.* (2007) who concluded that young chicks have a problem retaining Ca and P_i when given a corn-soy diet. This can be relieved by phytase supplementation. One reason why young birds have a lower Ca and P_i bioavailability might be associated with lower production of endogenous phytase. As birds age, the endogenous phytase activity increases (Morgan *et al*., 2015) leading to a reduced effect of dietary phytase on live performance.

Interaction between LPS and phytase had no significant impact on live performance parameters even though it has been shown, that in the ventriculus, coarse limestone results in more P_i being liberated from phytate (Joardar, 2019). Kim *et al*. (2018) came to the conclusion that phytase when supplemented with coarse limestone could better negate harmful effects of Ca on P_i digestibility, when compared to fine limestone. Managi and Coon (2007) also demonstrated *in vitro* that coarse limestone improved phytase activity, but in a live trial, coarse limestone (> 519 µm) had a negative effect on BWG. Improved phytase activity was noted with coarse limestone, which did not influence live performance. This response could be due to the fact that a higher activity of phytase is required to facilitate an advantage associated with the use of coarse limestone. Birds receiving an optimum dietary Ca and P_i level with increasing phytase activity, which releases additional Ca and P_i, did not have a significant effect on live performance.

Interaction between phytase and Ca and P_i level was significant. The NC without phytase had lower BW (14, 28 and 35 d), BWG (0-14, 15-28 and 0-35 d) and FI (0-14 d) compared to PC, NC +phytase, and PC + phytase. Adding phytase to a PC diet did not improve performance compared to PC without phytase, which showed that the phytase effect is prevalent when broilers are fed a Ca and P_i deficient diet. This was supported by dos Santos *et al*. (2013). Low P_i in diets is associated with decreased BWG and FI (Potter *et al*., 1995; Denbow *et al*., 1995). The addition of phytase to NC likely increased phyate degradation causing greater release of phytate-bound Ca and P_i, which led to mineral levels similar to PC, eliciting a live performance similar to PC. The interaction between Ca and P_i level and LPS was significant for BW (14 d and 28 d) and BWG (0-14 d and 15-28 d). The PC fine LPS produced the better live performance, but the NC fine LPS produced the worst live performance. The NC with coarse LPS produced live performances similar to the live performance of PC with fine LPS. The NC with coarse LPS improved live performance compared to the NC with fine LPS, which might be associated with the longer ventricular retention of the coarse limestone with an associated slower release of Ca (Anwar *et al*., 2016). Longer retention time in the ventriculus would indicate slower Ca release with sustained Ca availability reducing the potential for Ca interference with P_i digestibility, thereby, assuring the likelihood of optimal Ca and P_i for growth and development.

Bone Ash

Bone ash results (Table 12) for the LPS were not significant for neither 14 d nor 35 d. This observation was in agreement with results from a recent investigation in which inclusion of dietary limestone of various particle sizes (200µm, 1000µm,

2000µm and 3000µm) did not influence tibial bone ash content in 82 week post-molting broiler breeders (Bueno *et al*., 2016). Neither Joardar (2019) nor Managi and Coon (2007) found a LPS effect on tibial ash. Similarly, Bradbury *et al*. (2018), studying broiler skeletal integrity, were not able to demonstrate an effect of LPS on percentage foot bone ash.

In this experiment, phytase improved tibial bone ash at 14 d, but at 35 d the phytase effect was lost. Numerous studies have shown phytase improving bone ash percentage at 14 or 21 d (Li *et al*., 2016; Qian *et al*., 1996; Johnston and Southren, 2000). It is accepted generally that dietary phytase results in improved bone ash percentage due to greater availability of Ca and P_i through liberation of these minerals from phytate (Selle and Ravindran, 2007). Dietary phytase is most effective during the first 2-3 weeks in chicks, but the phytase effect diminishes as bird's age, which would account for lack of phytase influence at 35 d in this study (Olukosi *et al*., 2007). The high Ca and P_i level in the PC treatment improved bone ash compared to the low Ca and P_i in the NC treatment at 14 d and 35 d in this study. These observations were expected as Ca and P_i are major constituents of bone, and increasing their levels would result in greater availability leading to a greater percentage tibial bone ash. Driver *et al*. (2005) have reported similar results from experiments in which diets with higher Ca and P_i level resulted in greater tibial bone ash percentage compared to diets with low Ca and P_i levels in 16 d old broilers. Venäläinen *et al*. (2006) observed that Ca and P_i influenced tibial bone ash percentage through 35 d of age when the starter diet had Ca-0.9% and Pi-0.45% levels compared to starter diets with Ca-0.8% and Pi-40% levels.

Other studies also have reported greater tibial bone ash percentage with increasing dietary Ca and P_i levels (Nelson *et al*., 1990; Onyango *et al*., 2003).

The interaction between LPS and phytase inclusion was not significant. Similarly, the interaction between LPS and Ca and P_i levels was not significant, but at 14 d the interaction between phytase and Ca and P_i levels was highly significant ($P = 0.0005$) with the NC + no phytase treatment having the least bone ash percentage compared to the PC treatment, regardless of phytase inclusion, had the greatest bone ash percentage. The inclusion of phytase in the NC treatment improved bone ash percentage, but the bone ash percentage was still less compared to the PC treatment. By 35 d of age, loss of significance of interactions between phytase and Ca and P_i levels was found. These observations suggest that dietary Ca and P_i levels are as important with regard of bone mineralization as supplemental phytase enzyme in NC diets during the early starter feed phase. Similarly, Gautier *et al*. (2017) found that broilers fed diets with non-phytate phosphorus (NPP) at 0.53% had higher bone ash percentage compared broilers fed diets with NPP at 0.45%. The addition of phytase to the 0.45% NPP diet did improve bone ash percentage but to a lower percentage compared to the 0.53% NPP diet. However, the addition of phytase to the PC diet (0.53% NPP) did further improve bone ash (Gautier *et al*., 2017), the same effect was not found in this current study.

Nutrient digestibility

The AID of Ca, P_i, and amino acids was calculated to determine if any correlation could be found between live performance and digestibility (Table II-13). LPS had a significant effect on Ca and P_i digestibility with fine LPS (200µm) exhibiting

better digestibility of both Ca and P_i at 14d and 35d compared to coarse LPS (900µm). Generally, coarse LPS has been associated with higher digestibility. However, the dgw (Geometric diameter average) and sgw (Geometric standard deviation) of coarse and fine LPS has no standard definition.

Kim *et al*. (2018) concluded that larger LPS had better Ca and P_i digestibility, but the LPS used by Kim and colleagues was different than the LPS used in this experiment. They used small LPS at 75 µm and large LPS at 402 µm. Bradbury *et al*. (2018) considered fine LPS to be <0.5 µm and coarse to be >0.5 µm, and they observed no significant effect of LPS on either Ca or P_i digestibility. Anwar *et al*. (2016), on the other hand, concluded that coarse LPS resulted in better true and apparent Ca digestibility with fine LPS being <500 µm and coarse LPS ranging between 1000-2000 µm. Guinote and Nys (1990) reported that fine LPS (<150 µm) supported better calcium retention than medium LPS (600-1180 µm) and coarse LPS (>1180 µm).

It is accepted that different LPSs vary in solubility, and it is well known that *in vitro* fine LPS is more soluble than coarse LPS (Managi and Coon 2007; Witt *et al*., 2005; Kim *et al*., 2018). In the ventriculus, it is thought that coarse limestone particles are retained longer as Roland (1986) concluded that LPS > 900µm will be retained longer in the ventriculus of layers, and Zhang and Coon (1997) also came to the same conclusion that larger limestone particles will be retained longer in the ventriculus of layers. The longer retention time of coarse limestone particles in the ventriculus appears to sustain longer Ca availability to the bird compared to fine LPS as it will pass through the ventriculus relatively quicker (Witt *et al*., 2006). The greater and

sustained Ca release from coarse limestone particles is at a slower rate compared to that from fine limestone particles due to differences in solubility. The physical differences in the solubility of coarse and fine limestone particles, and this chemical difference in solubility will influence phosphorus and Ca digestibility with fine LPS influencing greater variability in Ca and P_i digestibility. An elevated dietary Ca level will down regulate the Ca transporter in the small intestine reducing Ca uptake (Li *et al*., 2012) while additional Ca can bind to P_i precipitating the insoluble calcium phosphate, which passes through the intestinal tract undigested (Heaney and Nordin, 2002).

Inclusion of phytase enzyme improved both Ca and P_i digestibility at 14 d while only phosphorus digestibility was affected at 35 d. Phytase degrades phytate in poultry diets and improves Ca and P_i availability in poultry, which can then result in better live performance (Bougouin *et al*., 2014; Scholey *et al*., 2017; Bradbury *et al*., 2016). Ca and P_i levels also affect Ca and P_i digestibility with higher levels in the diet resulting in decreased AID and lower levels improving AID at 14 d and 35 d of age. These responses were likely due to a stoichiometric mixture of Ca and P_i, which regulates their intestinal uptake. Phosphate is absorbed in the small intestine by passive diffusion via paracellular phosphate transport and active transport via sodium-dependent phosphate co-transporters (Sabbagh *et al*., 2011). Calcium transport is mediated primarily via the calcitriol dependent Calbindin-D28k in the chick intestine. The Ca and P_i transporters are regulated according to chymal Ca and P_i content, with high concentrations causing low transporter activity and low concentrations causing high transporter activity (Adedokun and Adeola, 2013; Li *et al*., 2012). There was a significant interaction between LPS and phytase enzyme at 14 d for AID of P_i. Coarse

LPS had the worst AID compared to other treatments and addition of phytase enzyme made its AID similar to other treatments.

Along with Ca and P_i digestibility, the AID of 21 amino acids was determined for 14 d (Table 14) and 35 d (Table 15). Similar to Ca and P_i, LPS had a strong effect on amino acid digestibility with fine LPS improving digestibility of all the amino acids for both 14 d and 35 d. The reason for this divergence in amino acid digestibility with fine particle size versus coarse particle size is not yet understood. Nevertheless, it was hypotheisezed that calcium interaction with phytate might be responsible for the values as Ca- phytate complex is harder to degrade and phytate had been known to increase endogenous amino acid losses (Cowieson *et al*., 2004) or difference in FI might have also resulted in the difference in AID as observed by Suttle et al. 2012.

Phytase inclusion improved the AID of 10 out of 21 amino acids analyzed at 14 d and 5 of 21 amino acids at 35 d. These observations show that as bird's age, the phytase inclusion effect dissipates significantly. The phytate molecule appears to form a protein-phytate complex, which interferes with protease activity that can degrade the complex proteins (Selle *et al*., 2000). Phytate has been reported to increase endogenous amino acid flow, which can negatively impact amino acid availability (Ravindran *et al*., 1999a; Cowieson *et al*., 2004). Thus, phytase improved AID of certain amino acids by degrading phytate. The various Ca and P_i ratios did not affect significantly AID of amino acids at 14 d, but Ca and P_i ratios at high levels did influence positively at 35 d the AID for 6 of 21 amino acids. This can be due to high variability in these amino acid readings, which were observed during data analysis.

At 14 d only the interaction between LPS and phytase was significant. The majority of the amino acids tested showed coarse particle size without phytase as the only treatment that was different and had a low AID. Inclusion of phytase to coarse particle size treatments resulted in significant improvement in amino acid AID making it similar to fine particle size treatment either with or without phytase. This indicated that phytase enzyme had a stronger influence with coarse limestone particle size, which was also observed by Kim *et al*. (2018), who noted a negative effect of fine particle size Ca (75 µm) on P_i AID which could be reversed when a larger particle size Ca (400 µm) was used. At 35 d the majority of the amino acid AIDs were influenced by an interaction between Ca and P_i levels and phytase. There was also a significant interaction between LPS and Ca and P_i levels. Fine LPS + the PC (diets A and B) treatments had greater amino acid AIDs compared to other treatments, which led to these above-mentioned interactions. Removing PC control as a factor and running the data as 2x2 factoral resulted in LPS having no effect in AID but the other results stayed the same. Thus the effect of LPS on d 35 remained inconclusive.

Conclusion

The results of this experiment led to the following conclusions:

1. Phytase inclusion improved live performance, bone ash and nutrient digestibility, but it is most effective at younger ages.
2. Phytase improved P_i AID on both 14 d and 35 d demonstrating phytase effectiveness in the decreased P_i content in the excreta.
3. In broilers, LPS of 200 µm and 900 µm did not alter tibia bone ash or live performance although it is possible that NC treatment with coarse LPS until 28 d

can lead to a live performance similar to the PC treatment with fine limestone particle size.

4. Tibia bone ash and digestibility analyses point to phytase as being more effective in NC treatment with coarse LPS compared to NC treatment with fine limestone particle size.
5. Supplementation of phytase to PC treatments did not improve live performance.
6. Fine LPS improved Ca and P_i digestibility, but this did not improve live performance compared to coarse LPS indicating there might be other factors influencing digestibility.

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Table 1. Experimental diets design.

Treatment	Limestone blend	Calculated Ca:AvP		Phytase
		Starter	Grower	
A	100% fine	0.9:0.45	0.76:0.38	+
B		0.9:0.45	0.76:0.38	-
C		0.72:0.3	0.58:0.23	+
D		0.72:0.3	0.58:0.23	-
E	100% coarse	0.9:0.45	0.76:0.38	+
F		0.9:0.45	0.76:0.38	-
G		0.72:0.3	0.58:0.23	+
H		0.72:0.3	0.58:0.23	-

Phytase (RONOZYME® HiPhos) was expected to provide 1000 FYT/kg feed and was assigned a matrix value of 0.18% for Ca and 0.15% for AvP.

Table 2. Experimental diet composition and nutrient content (starter)

	Treatment[1]							
	A	B	C	D	E	F	G	H
Ingredient (%)								
Corn	55.66	55.66	55.66	55.66	55.66	55.66	55.66	55.66
Soybean meal 48%	36.59	36.59	36.59	36.59	36.59	36.59	36.59	36.59
Poultry byproduct meal	1.29	1.29	1.29	1.29	1.29	1.29	1.29	1.29
Poultry fat	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50
Deflouridated phosphorus	1.55	1.55	0.68	0.68	1.55	1.55	0.68	0.68
Limestone fine	0.49	0.49	0.70	0.70	0.00	0.00	0.00	0.00
Limestone coarse	0.00	0.00	0.00	0.00	0.49	0.49	0.70	0.70
Sodium chloride	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
DL-Methionine	0.23	0.23	0.23	0.23	0.23	0.23	0.23	0.23
Choline chloride, 60%	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Mineral premix	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Vitamin premix[2]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
L-lysine	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09
Selenium premix[3]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Coban[4]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
L-Threonine	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Titanium	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Ronozyme HiPhos®	0.04	0.00	0.04	0.00	0.04	0.00	0.04	0.00
Sand	0.00	0.04	0.66	0.70	0.00	0.04	0.66	0.70
Calculated nutrient content (%)								
Metabolizable energy (kcal/kg)	2958	2958	2958	2958	2958	2958	2958	2958
Crude protein	22.50	22.50	22.50	22.50	22.50	22.50	22.50	22.50
Calcium	0.90	0.90	0.72	0.72	0.90	0.90	0.72	0.72
Total phosphorus	0.67	0.67	0.52	0.52	0.67	0.67	0.52	0.52
Available phosphorus	0.45	0.45	0.30	0.30	0.45	0.45	0.30	0.30
Total lysine	1.30	1.30	1.30	1.30	1.30	1.30	1.30	1.30
Total methionine	0.58	0.58	0.58	0.58	0.58	0.58	0.58	0.58
Analyzed nutrient content (%), as fed								
Crude fat	4.01	3.99	3.87	3.95	3.91	4.29	4.10	3.86
Crude protein	21.24	21.34	21.68	20.91	22.31	21.13	21.89	21.18
Crude fiber	3.30	3.40	3.60	3.40	3.50	3.30	3.40	3.40
Ash	5.13	4.84	4.62	4.89	5.44	5.59	5.27	5.12
Calcium	0.77	0.80	0.65	0.59	0.86	0.95	0.79	0.67
Total phosphorus	0.63	0.62	0.52	0.49	0.66	0.69	0.58	0.52

[1]Treatments: A-D have fine limestone with A (with phytase) and B (without phytase) being positive control while C (with phytase) and D (without phytase) being negative control. E-H have coarse limestone with E (with phytase) and F (without phytase) being positive control and G (with phytase) and H (without phytase) being negative control.
[2]Vitamin premix supplied the following per kg of diet: 6,614 IU vitamin A, 1,984 IU vitamin D3, 33 IU vitamin E, 0.02 mg vitamin B12, 0.13 mg biotin, 1.98 mg menadione (K_3), 1.98 mg thiamine, 6.6 mg riboflavin, 11 mg d-pantothenic acid, 3.97 mg vitamin B6, 55 mg niacin, and 1.1 mg folic acid. [3]Selenium premix provided 0.2 mg Se (as Na_2SeO_3).
[4]Coban supplied monensin sodium at 90 mg/kg of feed

Table 3.Experimental diet composition and nutrient content (Grower)

	Treatment[1]							
	A	B	C	D	E	F	G	H
Ingredient (%)								
Corn	65.45	65.45	65.45	65.45	65.45	65.45	65.45	65.45
Soybean meal	27.69	27.69	27.69	27.69	27.69	27.69	27.69	27.69
Poultry byproduct meal	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Poultry fat	1.58	1.58	1.58	1.58	1.58	1.58	1.58	1.58
Deflouridated phosphorus	1.13	1.13	0.26	0.26	1.13	1.13	0.26	0.26
Limestone fine	0.41	0.41	0.60	0.60	0.00	0.00	0.00	0.00
Limestone coarse	0.00	0.00	0.00	0.00	0.41	0.41	0.60	0.60
Sodium chloride	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
DL-Methionine	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19
Choline chloride, 60%	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Mineral premix	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Vitamin premix[2]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
L-lysine	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Selenium premix[3]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Coban[4]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
L-Threonine	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Titanium	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Ronozyme HiPhos®	0.04	0.00	0.04	0.00	0.04	0.00	0.04	0.00
Sand	0.00	0.04	0.68	0.72	0.00	0.04	0.68	0.72
Calculated nutrient content (%)								
Metabolizable energy (kcal/kg)	3025	3025	3025	3025	3025	3025	3025	3025
Crude protein	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00
Calcium	0.76	0.76	0.58	0.58	0.76	0.76	0.58	0.58
Total phosphorus	0.58	0.58	0.43	0.43	0.58	0.58	0.43	0.43
Available phosphorus	0.38	0.38	0.23	0.23	0.38	0.38	0.23	0.23
Total lysine	1.15	1.15	1.15	1.15	1.15	1.15	1.15	1.15
Total methionine	0.51	0.51	0.51	0.51	0.51	0.51	0.51	0.51
Analyzed nutrient content (%), as fed								
Crude fat	4.01	4.02	4.22	4.10	4.21	4.21	3.92	3.84
Crude protein	21.04	20.84	21.00	20.39	20.22	19.88	22.25	20.85
Crude fiber	3.10	3.00	3.60	3.90	3.30	3.40	3.80	8.00
Ash	4.62	4.85	5.14	5.01	4.92	4.88	5.15	5.05
Calcium	0.77	0.83	0.59	0.52	0.82	0.83	0.64	0.61
Total phosphorus	0.67	0.68	0.54	0.48	0.65	0.67	0.53	0.51

[1]Treatments: A- D have fine limestone with A (with phytase) and B (without phytase) being positive control while C (with phytase) and D (without phytase) being negative control. E-H have coarse limestone with E (with phytase) and F (without phytase) being positive control and G (with phytase) and H (without phytase) being negative control.
[2]Vitamin premix supplied the following per kg of diet: 6,614 IU vitamin A, 1,984 IU vitamin D3, 33 IU vitamin E, 0.02 mg vitamin B12, 0.13 mg biotin, 1.98 mg menadione (K_3), 1.98 mg thiamine, 6.6 mg riboflavin, 11 mg d-pantothenic acid, 3.97 mg vitamin B6, 55 mg niacin, and 1.1 mg folic acid. [3]Selenium premix provided 0.2 mg Se (as Na_2SeO_3).
[4]Coban supplied monensin sodium at 90 mg/kg of feed

Table 4. Particle size analysis of feed.

	Treatment[1]							
	A	B	C	D	E	F	G	H
Starter				(µm)				
Dgw[2]	615	614	646	598	584	647	685	660
Sgw[3]	2.91	2.75	2.76	2.72	2.92	2.55	2.43	2.40
Grower								
Dgw	600	607	567	611	637	632	601	631
Sgw	2.89	2.73	2.78	2.61	2.67	2.57	2.48	2.53

[1]Treatments: A-D have fine limestone, with A (with phytase) and B (without phytase) being positive control while C (with phytase) and D (without phytase) being negative control. E-H have coarse limestone, with E (with phytase) and F (without phytase) being positive control and G (with phytase) and H (without phytase) being negative control

[2]Dgw: Geometric diameter average. Indicates the particle size of ingredients

[3]Sgw: Geometric standard deviation. Indicates the variation in a sample

Table 5. Limestone solubility and mineral concentration analysis.

Limestone Particle size[1]	Solubility	Ca concentration	PI concentration
		%	
Fine	72.8	39.50	0.03
Coarse	53.0	38.90	0.01

[1]Limestone particle size: Two limestone particle sizes, fine having a size of 190-micrometer and coarse having a size of 900-micrometer

Table 6. Body Weight (BW) of male broilers as affected by limestone particle size (LPS), phytase enzyme, Ca and PI level and their interactions.

LPS[1]	Phytase[2]	Ca and P[3]	BW			
			BW0	BW14	BW28	BW35
Main effects			(g/bird)			
Fine	-	-	43.5	441.2	1750.5	2680.4
Coarse	-	-	43.5	441.2	1750.5	2678.2
P- value			0.645	0.994	0.999	0.913
SE			0.12	3.01	9.9	14.6
-	0	-	43.4	435.1^{B}	1729.3^{B}	2654.4^{b}
-	1000	-	43.6	447.3^{A}	1771.7^{A}	2704.2^{a}
	P- value		0.387	0.006	0.0036	0.019
	SE		0.12	3.01	9.9	14.6
-	-	PC	43.5	444.1	1758.1	2695.8
-	-	NC	43.6	438.3	1743.0	2662.8
		P -value	0.504	0.179	0.284	0.116
		SE	0.12	3.01	9.9	14.6
Interactions						
Fine	0	-	43.5	437.2	1737.6	2665.1
Coarse	0	-	43.5	433.0	1721.0	2643.8
Fine	1000	-	43.5	445.2	1763.4	2695.8
Coarse	1000	-	43.6	449.4	1780.0	2712.5
P- value			0.988	0.328	0.240	0.361
SE			0.18	4.27	14.0	20.7
-	0	NC	43.9	425.2^{B}	1703.9^{b}	2615.5^{b}
-	0	PC	43.5	445.0^{A}	1754.7^{a}	2693.4^{a}
-	1000	NC	43.5	451.4^{A}	1782.0^{a}	2710.2^{a}
-	1000	PC	43.6	443.1^{A}	1761.5^{a}	2698.1^{a}
	P -value		0.893	0.001	0.013	0.033
	SE		0.18	4.27	14.0	20.7
Fine	-	NC	43.4	430.3^{C}	1718.5^{B}	2649.8
Fine	-	PC	43.6	452.1^{A}	1782.6^{A}	2711.1
Coarse	-	NC	43.5	446.3^{AB}	1767.4^{AB}	2675.8
Coarse	-	PC	43.6	436.1^{BC}	1733.6^{B}	2680.5
P -value			0.586	0.0004	0.0001	0.176
SE			0.18	4.27	14.0	20.7

[a, b] means within each column that possess different superscript differ significantly (P≤ 0.05)

[A-C] means in a row within each variable that possess different superscripts differ significantly ($P \leq 0.01$)

[1]Limestone particle size: Two limestone particle sizes, fine having a size of 190-micrometer and coarse having a size of 900-micrometer

[2] Phytase: had a dose of 1000 FYT/kg and a matrix value of 0.18% for Ca and 0.15% for P_i

[3] Ca and P_i had two levels, NC (negative control) had Ca: 0.72 and P:0.3 in starter diets and Ca: 0.53, P_i: 0.23 in grower while PC (positive control) had Ca: 0.9 and P:0.45 in starter and Ca: 0.76, P: 0.38 in grower

Table 7. Body weight gain (BWG) of male broilers as affected by limestone particle size (LPS), phytase enzyme, Ca and PI level and their interactions.

LPS[1]	Phytase[2]	Ca and P[3]	Period (d) 0-14	15-28	29-35	0-28	0-35
Main effects					(g/bird)		
Fine	-	-	397.7	1311.4	929.9	1707.1	2637.0
Coarse	-	-	397.7	1305.3	927.6	1707.0	2634.6
P- value	-	-	0.99	0.577	0.894	0.997	0.909
SE	-	-	3.01	7.7	12.0	9.9	14.6
-	0	-	391.7^{B}	1297.3^{b}	925.12	1685.9^{B}	2611.0^{b}
-	1000	-	403.6^{A}	1319.4^{a}	932.42	1728.1^{A}	2660.6^{a}
	P- value		0.006	0.047	0.670	0.003	0.019
	SE		3.02	7.7	12.0	9.9	14.6
-	-	PC	400.5	1310.1	937.7	1714.5	2652.2
-	-	NC	394.9	1306.6	919.9	1699.5	2619.4
		P -value	0.188	0.7460	0.299	0.287	0.117
		SE	3.02	7.7	12.0	9.9	14.6
Interactions							
Fine	0	-	393.8	1305.0	927.5	1694.2	2621.7
Coarse	0	-	389.6	1289.6	922.8	1677.6	2600.3
Fine	1000	-	401.6	1317.9	932.4	1719.9	2652.2
coarse	1000	-	405.8	1321.0	932.5	1736.4	2668.9
P- value			0.328	0.402	0.889	0.239	0.361
SE			4.27	10.9	17.0	14.0	20.6
-	0	NC	381.9^{B}	1280.5^{B}	911.6	1660.5^{b}	2572.1^{b}
-	0	PC	401.5^{A}	1314.1^{A}	938.7	1771.2^{a}	2649.9^{a}
-	1000	NC	407.9^{A}	1332.7^{A}	928.2	1738.5^{a}	2666.7^{a}
-	1000	PC	399.5^{A}	1306.2^{AB}	936.7	1717.8^{a}	2654.5^{a}
	P -value		0.002	0.008	0.587	0.013	0.033
	SE		4.27	10.9	17.0	14.0	20.6
Fine	-	NC	386.9^{C}	1289.0^{B}	931.4	1675.1^{C}	2606.5
Fine	-	PC	408.5^{A}	1333.9^{A}	928.5	1739.0^{A}	2667.5
Coarse	-	NC	402.8^{AB}	1324.2^{AB}	908.4	1724.1^{AB}	2632.3
Coarse	-	PC	392.6^{AB}	1286.4^{B}	946.9	1690.1^{BC}	2636.9
	P -value		0.0004	0.0004	0.229	0.0009	0.177
	SE		4.27	10.9	17.0	14.0	20.6

$^{a, b}$ means within each column that possess different superscript differ significantly (P≤ 0.05)

$^{A-C}$ means in a row within each variable that possess different superscripts differ significantly (P ≤ 0.01)

[1]Limestone particle size: Two limestone particle sizes, fine having a size of 190-micrometer and coarse having a size of 900-micrometer

[2] Phytase: had a dose of 1000 FYT/kg and a matrix value of 0.18% for Ca and 0.15% for P_i

[3] Ca and P_i had two levels, NC (negative control) had Ca: 0.72 and P: 0.3 in starter diets and Ca: 0.53, P: 0.23 in grower while PC (positive control) had Ca: 0.9 and P:0.45 in starter and Ca: 0.76, P: 0.38 in grower

Table 8. Feed Intake (FI) of male broilers as effected by limestone particle size (LPS), phytase enzyme, Ca and PI level and their interactions.

LPS[1]	Phytase[2]	Ca and P[3]	FI				
			FI0-14	FI 15-28	FI 28-35	FI 0-28	FI 0-35
Main effects			(g/bird)				
Fine	-	-	544.1	1961.6	1424.2	2505.7	3930.0
Coarse	-	-	544.1	1941.8	1422.5	2486.7	3909.2
P- value			0.900	0.296	0.864	0.378	0.418
SE			4.21	13.3	6.93	15.2	18.0
-	0	-	531.9^{B}	1937.1	1422.5	2469.0^{b}	3891.5^{b}
-	1000	-	557.0^{A}	1966.4	1424.2	2523.0^{a}	3947.6^{a}
	P- value		0.0001	0.125	0.863	0.013	0.031
	SE		4.22	13.3	6.9	15.2	18.0
-	-	PC	546.5	1963.5	1419.6	2510.0	3929.6
-	-	NC	542.5	1939.0	1427.1	2482.4	3909.5
		P -value	0.505	0.213	0.447	0.203	0.432
		SE	4.21	13.3	6.9	15.2	18.0
Interactions							
Fine	0	-	532.6	1951.9	1423.4	2484.4	3907.8
Coarse	0	-	531.2	1922.2	1421.6	2453.4	3875.1
Fine	1000	-	555.6	1971.3	1425.0	2526.9	3952.0
Coarse	1000	-	558.5	1961.4	1423.4	2519.9	3943.3
P -value			0.723	0.603	0.992	0.580	0.638
SE			5.97	18.8	9.8	21.5	25.4
-	0	NC	521.9^{C}	1912.4	1429.2	2434.3	3863.4
-	0	PC	541.9^{B}	1961.8	1415.8	2503.7	3919.5
-	1000	NC	563.0^{A}	1967.4	1425.0	2530.5	3955.5
-	1000	PC	551.0^{A}	1967.3	1423.4	2516.3	3939.7
	P -value		0.009	0.176	0.555	0.058	0.163
	SE		5.97	18.8	9.8	21.5	25.4
Fine	-	NC	538.9	1931.1	1427.9	2470.0^{a}	3897.9
Fine	-	PC	549.2	1992.2	1420.5	2541.5^{a}	3961.9
Coarse	-	NC	546.0	1948.8	1426.3	2494.8^{a}	3921.1
Coarse	-	PC	543.6	1934.9	1418.7	2478.8^{a}	3897.3
P -value			0.287	0.051	0.997	0.045	0.089
SE			5.96	18.8	9.8	21.5	25.4

$^{a, b}$ means within each column that possess different superscript differ significantly (P≤ 0.05)

$^{A-C}$ means in a row within each variable that possess different superscripts differ significantly ($P \leq 0.01$)

[1]Limestone particle size: Two limestone particle sizes, fine having a size of 190-micrometer and coarse having a size of 900-micrometer

[2] Phytase: had a dose of 1000 FYT/kg and a matrix value of 0.18% for Ca and 0.15% for P_i

[3] Ca and PI had two levels, NC (negative control) had Ca:0.72 and P:0.3 in starter diets and Ca: 0.53, P: 0.23 in grower while PC (positive control) had Ca: 0.9 and P:0.45 in starter and Ca: 0.76, P: 0.38 in grower

Table 9. Feed Conversion Ratio (FCR[4]) of male broilers as affected by limestone particle size (LPS), phytase enzyme, Ca and PI level and their interaction.

LPS[1]	Phytase[2]	Ca and P[3]	Period (d) 0-14	15-28	29-35	0-28	0-35
Main effects					(g:g)		
Fine	-	-	1.371	1.497	1.539	1.468	1.491
Coarse	-	-	1.373	1.498	1.543	1.457	1.485
P- value			0.889	0.982	0.891	0.222	0.543
SE			0.011	0.009	0.02	0.006	0.007
-	0	-	1.361	1.500	1.542	1.465	1.491
-	1000	-	1.383	1.495	1.539	1.461	1.485
	P- value		0.167	0.685	0.906	0.670	0.560
	SE		0.011	0.009	0.02	0.006	0.007
-	-	PC	1.366	1.500	1.52	1.464	1.482
-	-	NC	1.378	1.494	1.56	1.461	1.494
		P -value	0.445	0.635	0.149	0.726	0.274
		SE	0.011	0.009	0.02	0.006	0.007
Interactions							
Fine	0	-	1.355	1.498	1.536	1.466	1.491
Coarse	0	-	1.367	1.501	1.549	1.463	1.491
Fine	1000	-	1.387	1.496	1.542	1.470	1.491
Coarse	1000	-	1.380	1.493	1.537	1.452	1.478
P -value			0.576	0.152	0.757	0.410	0.513
SE			0.016	0.012	0.030	0.009	0.01
-	0	NC	1.372	1.506	1.574	1.466	1.503
-	0	PC	1.350	1.494	1.511	1.464	1.479
-	1000	NC	1.385	1.483	1.549	1.457	1.485
-	1000	PC	1.382	1.507	1.529	1.465	1.485
	P -value		0.582	0.152	0.460	0.533	0.248
	SE		0.016	0.012	0.03	0.009	0.01
Fine	-	NC	1.396[a]	1.501	1.539	1.475	1.496
Fine	-	PC	1.346[b]	1.493	1.538	1.462	1.486
Coarse	-	NC	1.360[ab]	1.487	1.584	1.450	1.491
Coarse	-	PC	1.386[ab]	1.507	1.502	1.467	1.478
P -value			0.021	0.270	0.161	0.065	0.871
SE			0.016	0.012	0.029	0.009	0.01

[a, b] means within each column that possess different superscript differ significantly (P≤ 0.05)

[A-C] means in a row within each variable that possess different superscripts differ significantly ($P \leq 0.01$)

[1]Limestone particle size: Two limestone particle sizes, fine having a size of 190-micrometer and coarse having a size of 900-micrometer

[2] Phytase: had a dose of 1000 FYT/kg and a matrix value of 0.18% for Ca and 0.15% for P_i

[3] Ca and P_i had two levels, NC (negative control) had Ca:0.72 and P:0.3 in starter diets and Ca: 0.53, P: 0.23 in grower while PC (positive control) had Ca: 0.9 and P:0.45 in starter and Ca: 0.76, P: 0.38 in grower

[4]FCR: adjusted for mortality

Table 10. Mortality (Mort) of male broilers as affected by limestone particle size (LPS), phytase enzyme, Ca and PI level and their interactions.

LPS[1]	Phytase[2]	Ca and P[3]	Period (d) 14	35	15-28	29-35
Main effects				(%)		
Fine	-	-	0.14	0.94	0.17[b]	0.67
Coarse	-	-	0.14	1.61	1.00[a]	0.50
P- value			1.000	0.355	0.05	0.703
SE			0.14	0.51	0.29	0.31
-	0	-	0.15	1.45	0.83	0.504
-	1000	-	0.13	1.10	0.34	0.662
	P- value		0.936	0.628	0.237	0.718
	SE		0.14	0.51	0.29	0.31
-	-	PC	0.14	0.64[B]	0.17[B]	0.33
-	-	NC	0.14	1.91[A]	1.00[A]	0.83
		P -value	1.000	0.008	0.005	0.256
		SE	0.14	0.51	0.29	0.31
Interactions						
Fine	0	-	0.02	0.96	0.32	0.68
Coarse	0	-	0.23	1.94	1.33	0.33
Fine	1000	-	0.26	0.92	0.01	0.66
Coarse	1000	-	0.00	1.28	0.67	0.67
P -value			0.184	0.659	0.672	0.690
SE			0.12	0.71	0.41	0.44
-	0	NC	0.29	2.23	1.32	0.68
-	0	PC	0.00	0.66	0.33	0.33
-	1000	NC	0.02	1.60	0.66	0.99
-	1000	PC	0.28	0.61	0.00	0.33
	P -value		0.134	0.659	0.701	0.718
	SE		0.20	0.71	0.41	0.44
Fine	-	NC	0.00	0.94	0.33	0.67
Fine	-	PC	0.28	0.94	0.00	0.67
Coarse	-	NC	0.28	2.89	1.67	1.00
Coarse	-	PC	0.00	0.33	0.33	0.00
P -value			0.159	0.079	0.228	0.256
SE			0.20	0.71	0.41	0.44

[a, b] means within each column that possess different superscript differ significantly (P≤ 0.05)

[A-C] means in a row within each variable that possess different superscripts differ significantly ($P \leq 0.01$)

[1]Limestone particle size: Two limestone particle sizes, fine having a size of 190-micron meter and coarse having a size of 900-micron meter

[2] Phytase: had a dose of 1000FYT/kg and a matrix value of 0.18% for Ca and 0.15% for P_i

[3] Ca and P_i had two levels, NC (negative control) had Ca: 0.72 and P: 0.3 in starter and Ca: 0.53, P: 0.23 in grower while PC (positive control) had Ca: 0.9 and P: 0.45 in starter and Ca: 0.76, P: 0.38 in grower

Table 11. Coefficient of Variation (CV[4]) of male broilers as affected by limestone particle size (LPS), phytase enzyme, Ca and PI level and their interactions.

			Period (d)			
LPS[1]	Phytase[2]	Ca and P[3]	0	14	28	35
Main effects				(%)		
Fine	-	-	7.43	10.45	8.55	7.27
Coarse	-	-	7.88	9.92	9.19	7.70
P- value			0.130	0.280	0.145	0.338
SE			0.21	0.35	0.31	0.32
-	0	-	7.50	10.29	8.85	7.76
-	1000	-	7.82	10.10	8.89	7.22
	P- value		0.294	0.701	0.929	0.230
	SE		0.21	0.35	0.31	0.32
-	-	PC	7.64	10.17	8.99	7.61
-	-	NC	7.67	10.21	8.74	7.36
		P -value	0.904	0.939	0.571	0.575
		SE	0.21	0.35	0.31	0.32
Interactions						
Fine	0	-	9.97	9.97	8.83	7.61
Coarse	0	-	10.60	10.60	8.67	7.91
Fine	1000	-	9.87	9.87	8.27	6.94
Coarse	1000	-	10.32	10.32	9.51	7.49
P -value			0.743	0.856	0.172	0.779
SE			0.30	0.49	0.43	0.45
-	0	NC	7.62	10.48	8.66	7.81
-	0	PC	7.38	10.09	9.04	7.71
-	1000	NC	7.73	9.94	8.83	6.91
-	1000	PC	7.90	10.26	8.94	7.52
	P -value		0.490	0.471	0.762	0.437
	SE		0.30	0.49	0.43	0.45
Fine	-	NC	7.84	10.07	8.32	7.05
Fine	-	PC	7.94	9.77	8.78	7.50
Coarse	-	NC	7.84	10.35	9.17	7.68
Coarse	-	PC	7.34	10.57	9.20	7.73
P -value			0.648	0.597	0.624	0.658
SE			0.30	0.49	0.43	0.45

[a, b] means within each column that possess different superscript differ significantly (P≤ 0.05)

[A-C] means in a row within each variable that possess different superscripts differ significantly (*P*≤ 0.01)

[1]Limestone particle size: Two limestone particle sizes, fine having a size of 190-micrometer and coarse having a size of 900-micrometer

[2] Phytase: had a dose of 1000FYT/kg and a matrix value of 0.18% for Ca and 0.15% for Pi

[3] Ca and PI had two levels, NC (negative control) had Ca: 0.72 and P: 0.3 in starter diets and Ca: 0.53, P: 0.23 in grower while PC (positive control) had Ca: 0.9 and P: 0.45 in starter and Ca: 0.76, P: 0.38 in grower while PC (positive control) had Ca: 0.9 and P: 0.45 in starter and Ca: 0.76, P: 0.38 in grower

[4] CV: Calculated on body weight and having a range of 10%

Table 12. Tibial Bone Ash of male broilers as affected by limestone particle size (LPS), phytase enzyme, Ca and PI level and their interactions.

LPS[1]	Phytase[2]	Ca and P[3]	Period (d) 14	35
Main effects			(%)	
Fine	-	-	50.01	52.47
Coarse	-	-	50.27	52.55
P- value			0.386	0.777
SE			0.15	0.19
-	0	-	49.64^{B}	52.26
-	1000	-	50.71^{A}	52.73
	P- value		0.0001	0.106
	SE		0.15	0.19
-	-	PC	50.10^{A}	52.83^{a}
-	-	NC	49.35^{B}	52.19^{b}
		PI -value	0.0001	0.022
		SE	0.15	0.19
Interactions				
Fine	0	-	49.66	52.40
Coarse	0	-	49.61	52.17
Fine	1000	-	50.50	52.54
Coarse	1000	-	50.92	52.92
P -value			0.285	0.266
SE			0.21	0.27
-	0	NC	48.41^{C}	52.00
-	0	PC	50.86^{A}	52.56
-	1000	NC	50.29^{B}	52.37
-	1000	PC	51.13^{A}	53.09
	P -value		0.0005	0.776
	SE		0.21	0.27
Fine	-	NC	49.30	51.88
Fine	-	PC	50.85	53.06
Coarse	-	NC	49.39	52.50
Coarse	-	PC	51.14	52.60
P -value			0.657	0.052
SE			0.21	0.27

$^{a, b}$ means within each column that possess different superscript differ significantly (P≤ 0.05)

$^{A-C}$ means in a row within each variable that possess different superscripts differ significantly (*P*≤ 0.01)

[1]Limestone particle size: Two limestone particle sizes, fine having a size of 190-micrometer and coarse having a size of 900-micrometer

[2] Phytase: had a dose of 1000 FYT/kg and a matrix value of 0.18% for Ca and 0.15% for P_i

[3] Ca and P_i had two levels, NC (negative control) had Ca: 0.72 and P: 0.3 in starter diets and Ca: 0.53, P: 0.23 in grower while PC (positive control) had Ca: 0.9 and P: 0.45 in starter and Ca: 0.76, P: 0.38 in grower

Table 13. Ca and Pi Apparent Ileal digestibility (AID) of male broilers as affected by limestone particle size (LPS), phytase enzyme, Ca and P_i level and their interactions.

			14d		35d	
LPS[1]	Phytase[2]	Ca and P[3]	Ca	P	Ca	P
Main effects				(%)		
Fine	-	-	65.05^{A}	63.87^{A}	54.75^{a}	56.52^{A}
Coarse	-	-	56.05^{B}	53.37^{B}	48.41^{b}	52.09^{B}
P- value			0.0001	0.0001	0.011	0.0002
SE			1.25	1.09	1.25	0.96
-	0	-	56.72^{B}	55.79^{B}	52.49	51.16^{B}
-	1000	-	64.38^{A}	61.46^{A}	50.67	57.46^{A}
	P- value		0.0001	0.0005	0.278	0.0001
	SE		1.25	1.09	1.17	0.96
-	-	PC	52.40^{B}	56.65^{b}	49.91^{b}	48.42^{B}
-	-	NC	68.69^{A}	60.60^{a}	53.26^{a}	60.20^{A}
		P -value	0.0001	0.013	0.048	0.0001
		SE	1.25	1.09	1.17	0.96
Interactions						
Fine	0	-	69.65^{A}	63.49^{A}	55.34	53.70
Coarse	0	-	43.79^{C}	48.09^{B}	49.64	48.62
Fine	1000	-	60.45^{B}	64.26^{A}	54.16	59.35
Coarse	1000	-	68.31^{A}	58.65^{A}	47.18	55.57
P -value			0.0001	0.0025	0.702	0.637
SE			1.76	1.55	1.66	1.36
-	0	NC	66.33	57.94	54.73	56.99
-	0	PC	47.1	53.63	50.25	45.33
-	1000	NC	71.06	63.25	51.78	63.41
-	1000	PC	57.71	59.66	49.57	51.51
	P -value		0.100	0.819	0.50	0.93
	SE		1.76	1.55	1.66	1.36
Fine	-	NC	71.79	66.59	54.47^{a}	61.25
Fine	-	PC	58.32	61.17	55.03^{a}	51.80
Coarse	-	NC	65.60	54.61	52.04^{a}	59.15
Coarse	-	PC	46.49	52.13	44.79^{b}	45.03
P -value			0.114	0.355	0.022	0.091
SE			1.76	1.55	1.66	1.36

$^{a, b}$ means within each column that possess different superscript differ significantly (P≤ 0.05)

$^{A-C}$ means in a row within each variable that possess different superscripts differ significantly ($P \leq 0.01$)

[1]Limestone particle size: Two limestone particle sizes, fine having a size of 190-micrometer and coarse having a size of 900-micrometer

[2] Phytase: had a dose of 1000 FYT/kg and a matrix value of 0.18% for Ca and 0.15% for Pi

[3] Ca and P_i had two levels, NC (negative control) had Ca: 0.72 and P: 0.3 in starter and Ca: 0.53, P: 0.23 in grower while PC (positive control) had Ca: 0.9 and P: 0.45 in starter and Ca: 0.76, P: 0.38 in grower

Table 14. Amino Acid AID of male broilers as affected by limestone particle size (LPS), phytase enzyme, Ca and P_i level and their interaction.

LPS[1]	Phytase[2]	Ca and P[3]	14 d				
			Lys	Arg	Hist	Leu	Iso Leu
Main effects			%				
Fine	-	-	80.09[a]	84.07[a]	77.40[A]	77.92[a]	77.62[A]
Coarse	-	-	75.46[b]	80.81[b]	72.81[B]	73.79[b]	72.98[B]
P- value			0.04	0.024	0.007	0.017	0.008
SE			1.33	1.00	1.16	1.19	1.19
-	0	-	75.80[b]	80.84[b]	73.64	74.27[b]	73.55[b]
-	1000	-	79.75[a]	84.05[a]	76.57	77.45[a]	77.05[a]
	P- value		0.04	0.024	0.077	0.05	0.04
	SE		1.33	1.00	1.16	1.19	1.19
-	-	PC	77.02	82.17	74.93	75.24	74.98
-	-	NC	78.53	82.72	75.28	76.49	75.62
		P -value	0.42	0.70	0.83	0.46	0.70
		SE	1.33	1.00	1.16	1.19	1.19
Interactions							
Fine	0	-	80.32[a]	83.92[A]	78.02[A]	78.34[a]	77.88[a]
Coarse	0	-	71.28[b]	77.76[B]	69.25[B]	70.16[b]	69.22[b]
Fine	1000	-	79.86[a]	84.24[A]	76.78[A]	77.47[a]	77.37[a]
Coarse	1000	-	79.64[a]	83.86[A]	76.37[A]	77.43[a]	76.73[a]
P -value			0.02	0.004	0.01	0.012	0.02
SE			1.88	1.42	1.64	1.68	1.69
-	0	NC	77.13	81.33	73.95	75.12	74.10
-	0	PC	74.47	80.33	73.33	73.42	73.01
-	1000	NC	79.93	84.09	76.62	77.85	77.14
-	1000	PC	79.57	84.01	76.53	77.05	76.96
	P -value		0.545	0.75	0.87	0.79	0.789
	SE		1.88	1.42	1.64	1.68	1.69
Fine	-	NC	81.46	84.73	78.15	78.79	78.45
Fine	-	PC	78.72	83.43	76.65	77.06	76.80
Coarse	-	NC	75.60	80.70	72.42	74.18	72.78
Coarse	-	PC	75.32	80.92	73.21	73.41	73.17
	P -value		0.514	0.590	0.480	0.776	0.789
	SE		1.88	1.42	1.64	1.68	1.69

[a, b] means within each column that possess different superscript differ significantly (P≤ 0.05)

[A-C] means in a row within each variable that possess different superscripts differ significantly ($P \leq 0.01$)

[1]Limestone particle size: Two limestone particle sizes, fine having a size of 190-micrometer and coarse having a size of 900-micrometer

[2] Phytase: had a dose of 1000 FYT/kg and a matrix value of 0.18% for Ca and 0.15% for P_i

[3] Ca and P_i had two levels, NC (negative control) had Ca: 0.72 and P: 0.3 in starter diets and Ca: 0.53, P: 0.23 in grower while PC (positive control) had Ca: 0.9 and P: 0.45 in starter and Ca: 0.76, P: 0.38 in grower

Table 14 (Continued). Amino Acid AID of male broilers as affected by limestone particle size (LPS), phytase enzyme, Ca and P_i level and their interaction.

			14 d				
LPS[1]	Phytase[2]	Ca and P[3]	Meth	Gly	Pro	Threo	Cys
	Main effects				%		
Fine	-	-	88.05[a]	71.17[A]	75.33[a]	70.53[A]	61.03[A]
Coarse	-	-	84.58[b]	67.63[B]	71.66[b]	64.33[B]	52.88[B]
P- value			0.013	0.005	0.017	0.002	0.001
SE			0.95	1.22	1.06	1.37	1.62
-	0	-	85.54	67.51[b]	72.56	66.01	55.16
-	1000	-	87.09	71.29[a]	74.43	68.86	58.75
	P- value		0.255	0.033	0.218	0.148	0.124
	SE		0.95	1.22	1.06	1.37	1.62
-	-	PC	84.93	69.44	73.40	66.58	56.05
-	-	NC	87.71	69.34	73.57	68.29	57.87
		P -value	0.08	0.96	0.913	0.382	0.43
		SE	0.95	1.22	1.06	1.19	1.62
	Interactions						
Fine	0	-	88.58	71.78[A]	76.54[A]	72.02[a]	62.37[a]
Coarse	0	-	82.50	63.23[B]	68.55[B]	60.00[b]	47.96[b]
Fine	1000	-	87.53	70.55[A]	74.09[A]	69.04[a]	57.81[a]
Coarse	1000	-	86.65	72.03[A]	74.76[A]	68.67[a]	59.69[a]
P -value			0.058	0.005	0.005	0.012	0.02
SE			1.35	1.73	1.50	1.68	1.69
-	0	NC	77.13	67.65	72.62	67.33	55.69
-	0	PC	74.47	67.37	72.50	64.69	54.63
-	1000	NC	79.93	71.05	74.53	69.24	60.04
-	1000	PC	79.57	71.52	74.32	68.47	57.46
	P -value		0.545	0.830	0.978	0.632	0.741
	SE		1.88	1.73	1.50	1.94	2.30
Fine	-	NC	81.46	71.83	76.33	72.26	64.06
Fine	-	PC	78.72	70.50	74.33	68.80	57.99
Coarse	-	NC	75.60	66.87	70.82	64.31	51.67
Coarse	-	PC	75.32	68.39	72.49	64.36	54.10
P -value			0.514	0.414	0.225	0.368	0.07
SE			1.88	1.73	1.64	1.94	1.69

[a, b] means within each column that possess different superscript differ significantly (P≤ 0.05)

[A-C] means in a row within each variable that possess different superscripts differ significantly ($P \leq 0.01$)

[1]Limestone particle size: Two limestone particle sizes, fine having a size of 190-micrometer and coarse having a size of 900-micrometer

[2] Phytase: had a dose of 1000 FYT/kg and a matrix value of 0.18% for Ca and 0.15% for P_i

[3] Ca and P_i had two levels, NC (negative control) had Ca: 0.72 and P: 0.3 in starter diets and Ca: 0.53, P: 0.23 in grower while PC (positive control) had Ca: 0.9 and P: 0.45 in starter and Ca: 0.76, P: 0.38 in grower

Table 15. Amino Acid AID of male broilers as affected by limestone particle size (LPS), phytase enzyme, Ca and P_i level and their interaction.

			35 d				
LPS[1]	Phytase[2]	Ca and P[3]	Lys	Arg	Hist	Leu	Iso Leu
Main effects					%		
Fine	-	-	84.28^{A}	88.65^{A}	82.96^{a}	82.79^{a}	82.45^{A}
Coarse	-	-	86.23^{B}	87.86^{B}	82.11^{b}	81.93^{b}	81.41^{B}
P- value			0.002	0.001	0.04	0.017	0.008
SE			0.25	0.21	0.28	0.28	0.28
-	0	-	84.84	87.86	82.24	82.02	81.47
-	1000	-	85.67	88.65	82.55	82.70	82.40
	P- value		0.15	0.084	0.604	0.09	0.064
	SE		0.25	0.21	0.28	0.28	0.28
-	-	PC	85.55	88.40	83.16^{a}	82.63	82.51
-	-	NC	84.96	87.96	81.63^{b}	81.79	81.57
		P -value	0.28	0.292	0.013	0.183	0.125
		SE	0.25	0.21	0.28	0.28	0.28
Interactions							
Fine	0	-	85.47	88.66^{A}	82.72	82.34	82.16
Coarse	0	-	84.18	87.06^{B}	81.71	81.69	80.78
Fine	1000	-	86.03	88.65^{A}	83.20	83.23	82.75
Coarse	1000	-	85.39	88.65^{A}	82.50	82.17	892.04
P -value			0.74	0.004	0.704	0.605	0.39
SE			0.36	0.31	0.40	0.40	0.40
-	0	NC	84.22	87.31	80.90^{b}	80.78^{b}	80.47^{B}
-	0	PC	85.37	88.41	83.53^{a}	83.24^{a}	83.08^{A}
-	1000	NC	85.37	88.58	82.36^{ab}	82.49^{a}	83.09^{A}
-	1000	PC	86.04	88.73	83.35^{a}	82.91^{a}	82.17^{AB}
	P -value		0.452	0.13	0.05	0.013	0.009
	SE		0.36	0.31	0.40	0.40	0.40
Fine	-	NC	85.26^{ab}	87.50^{B}	80.88^{C}	80.90^{B}	80.61^{B}
Fine	-	PC	87.21^{a}	89.81^{A}	85.04^{A}	84.67^{A}	84.30^{A}
Coarse	-	NC	84.68^{b}	88.38^{B}	82.38^{B}	82.37^{B}	81.85^{B}
Coarse	-	PC	83.89^{b}	87.33^{B}	81.84^{BC}	81.49^{B}	80.98^{B}
	P -value		0.02	0.001	0.0001	0.0001	0.0001
	SE		0.36	0.31	0.40	0.40	0.40

$^{a, b}$ means within each column that possess different superscript differ significantly (P≤ 0.05)

$^{A-C}$ means in a row within each variable that possess different superscripts differ significantly ($P \leq 0.01$)

[1]Limestone particle size: Two limestone particle sizes, fine having a size of 190-micrometer and coarse having a size of 900-micrometer

[2] Phytase: had a dose of 1000 FYT/kg and a matrix value of 0.18% for Ca and 0.15% for P_i

[3] Ca and P_i had two levels, NC (negative control) had Ca: 0.72 and P: 0.3 in starter diets and Ca: 0.53, P: 0.23 in grower while PC (positive control) had Ca: 0.9 and P: 0.45 in starter and Ca: 0.76, P: 0.38 in grower

Table 15 (continued). Amino Acid AID of male broilers as affected by limestone particle size (LPS), phytase enzyme, Ca and P_I level and their interaction.

			35 d				
LPS[1]	Phytase[2]	Ca and P[3]	Meth	Gly	Pro	Threo	Cys
Main effects					%		
Fine	-	-	83.17^{A}	76.7^{A}	79.79^{A}	75.75^{A}	80.58^{A}
Coarse	-	-	80.89^{B}	75.4^{B}	77.15^{B}	74.12^{B}	77.49^{B}
P- value			0.001	0.0007	0.002	0.001	0.003
SE			0.42	0.36	0.35	0.34	0.52
-	0	-	81.43^{b}	75.33^{b}	78.04	74.59	78.49^{b}
-	1000	-	82.65^{a}	76.80^{a}	78.93	75.27	79.84^{a}
	P- value		0.05	0.048	0.26	0.172	0.05
	SE		0.42	0.36	0.35	0.34	0.52
-	-	PC	82.50^{A}	76.9	79.29^{a}	76.10^{a}	79.64^{A}
-	-	NC	81.57^{B}	75.53	77.65^{b}	73.76^{b}	78.68^{B}
		P -value	0.01	0.071	0.043	0.02	0.001
		SE	0.42	0.36	0.35	0.34	0.52
Interactions							
Fine	0	-	82.58	77.47	78.59	75.59	79.70
Coarse	0	-	80.27	73.35	77.48	73.59	77.28
Fine	1000	-	83.77	77.96	79.40	75.90	81.46
Coarse	1000	-	81.51	76.16	78.96	74.64	79.70
P -value			0.972	0.135	0.509	0.455	0.529
SE			0.72	0.51	0.51	0.49	0.79
-	0	NC	80.26	73.48^{B}	76.15^{B}	73.11	77.03^{b}
-	0	PC	82.59	77.19^{A}	79.92^{A}	76.08	79.95^{a}
-	1000	NC	82.86	76.46^{A}	78.65^{A}	74.42	80.33^{a}
-	1000	PC	82.47	77.13^{A}	79.72^{A}	76.12	79.34^{a}
	P -value		0.063	0.004	0.009	0.199	0.02
	SE		0.72	0.51	0.50	0.49	0.79
Fine	-	NC	81.67^{B}	74.01^{B}	76.50^{B}	73.05^{B}	78.90^{B}
Fine	-	PC	84.69^{A}	79.42^{A}	81.50^{A}	78.44^{A}	82.27^{A}
Coarse	-	NC	80.33^{B}	75.93^{B}	78.30^{B}	74.48^{B}	78.47^{B}
Coarse	-	PC	80.33^{B}	74.91^{B}	78.14^{B}	73.76^{B}	77.03^{B}
P -value			0.002	0.0001	0.0001	0.0001	0.001
SE			0.72	0.51	0.50	0.49	0.79

$^{a, b}$ means within each column that possess different superscript differ significantly (P≤ 0.05)

$^{A-C}$ means in a row within each variable that possess different superscripts differ significantly ($P \leq 0.01$)

[1]Limestone particle size: Two limestone particle sizes, fine having a size of 190-micrometer and coarse having a size of 900-micrometer

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Summary and Conclusion

Based on the two researches performed it can be concluded that phytase improved live performance in birds. Phytase fascilitated improved BW and FI but did not affect FCR. In the first experiment the effect of phytase was significant for a longer period compared to the second experiment, which can be attributed to different type of phytase supplemented in each experiment. The first experiment also had a higher dose of phytase compared to second experiment (3000 FTU/kg vs 1000 FYT/kg). Providing various Ca phytase matrix values and limestone particle sizes did not influence live performance as was expected, but through the first 28 d NC + the coarse limestone diet resulted in similar live performance to PC + the fine limestone diet, which suggested that a lower Ca amount can be supplemented when coarse limestone is utilized. Lower Ca: AvP can be utilized without negative effects on broiler live performance.

Bone ash, which is a determinant of skeletal integrity, was improved with both phytase and PC, although a phytase effect was observed only in day 14, and its improvement was less compared to PC. This indicated that Ca and P_i levels are more critical to bone ash, although it is possible that if a higher dose of phytase were to be supplemented bone ash % might have been similar to PC.

AID of Ca and P_i was improved by fine limestone particle size, phytase inclusion, and NC. Amino acid AID also was improved by phytase enzyme and fine limestone at 14d. Improved digestibility for P_i is beneficial to the environment as less of it will be excreted and greater amino acid digestibility is also beneficial as it'll leads to better growth. Although greater digestibility is better since birds get to utilize more nutrients, it

should also be noted that transporters in the digestive tract play a huge role in digestibility and there is up- and down-regulation based on feed intake and nutrient concentration, which also influences digestibility.