# Limestone particle size, calcium and phosphorus levels, and phytase effects on live performance and nutrients digestibility of broilers

S. Majeed, R. Qudsieh,[2] F. W. Edens and J. Brake[1]

*Prestage Department of Poultry Science, North Carolina State University, Raleigh, NC 27695-7608*

**ABSTRACT** Limestone particle size (**PS**) affects its solubility and thus can influence broiler performance by altering the rate of calcium (**Ca**) release into the gastrointestinal tract. The objective of this research was to determine, using 2 × 2 × 2 factorial arrangement, the influence of PS (fine and coarse) and supplemented phytase (0 and 1,000 FYT/kg) in diets formulated with 2 Ca and $P_i$ levels (positive control [**PC**]; negative control [**NC**]) on live performance, bone ash, and apparent ileal nutrients digestibility (**AID**). Starter PC: 0.9 Ca and 0.45 Pi; NC: 0.72 Ca and 0.03 Pi. Grower PC: 0.76 Ca and 0.38 Pi; NC: 0.58 Ca and 0.23 Pi. The 8 diets were assigned randomly to a total of 1,512 birds, with 21 birds per pen and 9 pens per treatment. The main effects of PS and Ca and $P_i$ levels had no influence on feed intake (**FI**), body weight gain (**BWG**), or feed conversion ratio. Adding phytase improved BWG by 8 g and 50 g and FI by 25 g and 56 g at 0–14 D ($P \leq 0.05$) and 0–35 D ($P \leq 0.05$), respectively. Interaction between Ca and $P_i$ levels and phytase improved BWG and FI for 0–14 D ($P \leq 0.05$) and BWG during 15–28 D ($P \leq 0.05$) for PC without phytase and for PC and NC with phytase when compared with NC without phytase. Birds fed PC without phytase, or either PC or NC with phytase were about 96 g heavier than NC without phytase. Birds fed either PC or NC diet with coarse limestone or PC with fine limestone gained approximately 14 g more ($P \leq 0.05$) than birds fed NC with fine limestone for BWG at 0–14 D ($P \leq 0.05$). Phytase increased tibia bone ash (14 D) by 1% ($P \leq 0.05$). AID of Ca and $P_i$ at 14 D was improved ($P \leq 0.05$) by 66% when phytase was added to coarse limestone. Results indicate that phytase improved broiler performance without being affected by PS. Furthermore, phytase had greater influence on coarse limestone than on fine limestone for bone ash and AID. Ca and $P_i$ levels were the most influential factors in determining bone ash although phytase inclusion could lead to an improvement during early days.

**Key words:** limestone, particle size, broiler, phytase, performance

2020 Poultry Science 99:1502–1514
https://doi.org/10.1016/j.psj.2019.11.009

## INTRODUCTION

Calcium (**Ca**) is the most abundant mineral in the body (Applegate and Angel, 2008) and performs critical functions in 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 oversupplemented because of its being used as a vehicle in various premixes (Kim et al., 2018), which is not taken into account when formulating diets. Limestone is the primary source of Ca in poultry diets; it is readily available and inexpensive (Blount, 2013). One can argue that with accelerated growth of broilers over the years, the Ca requirement must have increased because of a stronger skeleton being required to support greater body mass. Contrary to that supposition, the factual situation is that over the years, Ca requirements have dropped (Driver et al., 2005; Ziaei et al., 2008; Singh et al., 2013) compared with NRC requirements of 1994. The Cobb broiler performance and nutrient supplement

---

© 2019 The Authors. Published by Elsevier Inc. on behalf of Poultry Science Association Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Received July 23, 2019.

Accepted November 4, 2019.

[1] Dr. John Brake passed away before getting the chance to publish this manuscript; his enormous contributions to this manuscript are deeply acknowledged.

[2] Corresponding author: riqudsie@ncsu.edu

guide (Cobb, 2018) and Ross broiler nutrition specification guide (Ross, 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 use Ca than smaller, slower growing broilers of the past.

Phytase initially became commercially available because of legislation designed to limit Pi pollution of the environment in the Netherlands (Selle and Ravindran, 2007) as it had been demonstrated that phytase inclusion reduced Pi excretion (Simons et al., 1990; Paik, 2003). Additional trials have shown phytase to increase amino acid availability (Selle et al., 2000), feed energy profile (Ravindran et al., 1999a, 2000, 2001; Selle et al., 1999, 2001, 2003), and feed intake (**FI**) (Onyango et al., 2005; Bahadoran et al., 2011), which led to its widespread use in broiler feed. It has been well established by now that phytase improves broiler performance, especially in cases of low Pi content in the diets (Nelson et al., 1971; Broz et al., 1994; Cowieson and Adeola, 2005), but more work needs to be done on phytase interaction with various ingredients in the feed. Excess Ca in the diet has been known to bind phytate, resulting in a Ca-phytate complex, which is refractory to hydrolysis by phytase (Wise, 1983), thus negatively affecting phytase activity.

Various studies in layers have attributed Ca retention as a function of limestone particle size (**PS**) solubility (Roland, 1986; Cheng and Coon, 1990; Zhang and Coon, 1997a). Eusebio-Balcazar et al. (2018) demonstrated that coarse PS improved bone integrity of layer hens, which indicated a positive effect of coarse limestone inclusion instead of fine limestone. Compared to layers, fewer studies have been conducted in broilers in terms of PS, although in broilers, PS does affect Ca solubility and availability (Anwar et al., 2016; Kim et al., 2018). It is thought that compared with fine, coarse limestone will be retained longer in the ventriculus (gizzard). Longer retention will increase total dissolution and increase 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, PS has not been given much consideration. The purpose of this experiment was to observe whether PS, 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 North Carolina State University approved Institutional Animal Care and Use Committee protocol (IACUC #16-157). A total of 1,512 male birds of Cobb 500 were placed randomly into 72 pens 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 3 supplemental feed trays available in each pen in addition to 2 tube feeders and one bell drinker. Supplemental drinkers and 2 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 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.

### Experimental Diets

A total of 8 experimental diets were tested in a 2 × 2 × 2 factorial treatment design (Table 1). Factors tested were limestone PS (fine and coarse), phytase (0 and 1,000 FYT/kg), and Ca and $P_i$ levels (positive control [**PC**] and negative control [**NC**]). There was a total of 72 pens with 9 replicate pens and 21 birds per pen for each factorial dietary treatment. Birds were fed crumbled starter (Table 2) from 0–16 D and pelleted grower (Table 3) from 17–35 D *ad libitum*. All diets had titanium dioxide included at the rate of 0.5 g/kg of diet as an inert marker for calculation of apparent ileal digestibility (**AID**). Limestone (Huber engineered material, Quincy, IL) was obtained in 2 PS: fine limestone (Hubercarb Q200) PS was 190 μm, and coarse limestone (Hubercarb 12–40) PS was 900 μm. The PC had Ca of 0.9 and $P_i$ of 0.45 in starter and Ca of 0.76 and $P_i$ of 0.38 in grower. The NC had Ca of 0.72 and $P_i$ of 0.3 in starter and Ca of 0.58 and $P_i$ of 0.23 in grower. The supplemented phytase enzyme was Ronozyme Hiphos 2,500 GT (Koninklijke DSM N.V., Heerlen, Netherland) and was included at the rate of 1,000 FYT/kg. Feed samples were collected during feed manufacturing. Phytase activity measured in the mash feed was on average around 600 FYT/kg and in pelleted feed around 300 FYT/kg. Phytase was added either on top of the PC or with an assigned matrix value of 0.18% for Ca and 0.15% for $P_i$ when added to NC. Pelleting temperature for both starter and grower was 180°C. PS

**Table 1.** Experimental diets design.

| | Calculated Ca:AvP[1] | | |
|---|---|---|---|
| Limestone particle size | Starter | Grower | Phytase[2] |
| Fine | 0.90:0.45 | 0.76:0.38 | 1,000 |
| | 0.90:0.45 | 0.76:0.38 | 0 |
| | 0.72:0.30 | 0.58:0.23 | 1,000 |
| | 0.72:0.30 | 0.58:0.23 | 0 |
| Coarse | 0.90:0.45 | 0.76:0.38 | 1,000 |
| | 0.90:0.45 | 0.76:0.38 | 0 |
| | 0.72:0.30 | 0.58:0.23 | 1,000 |
| | 0.72:0.30 | 0.58:0.23 | 0 |

[1] The Ca and available phosphorus (AvP) levels used in this design are referred to as positive control (PC, 0.9:0.45 and 0.76:0.38) and negative control (NC, 0.72:0.3 and 0.58:0.23).

[2] Phytase (RONOZYME HiPhos) was expected to provide 1,000 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 | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Limestone particle size | Fine | | | | Coarse | | | |
| Ca and AvP level[1] | PC | | NC | | PC | | NC | |
| Phytase | 1,000 | 0 | 1,000 | 0 | 1,000 | 0 | 1,000 | 0 |
| 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 by-product 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®[5] | 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) | 2,958 | 2,958 | 2,958 | 2,958 | 2,958 | 2,958 | 2,958 | 2,958 |
| 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] The Ca and available phosphorus (AvP) levels used in this design are referred to as positive control (PC, 0.9:0.45) and negative control (NC, 0.72:0.3).

[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 (K3), 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 Na2SeO3).

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

[5] Phytase (RONOZYME HiPhos) was expected to provide 1,000 FYT/kg feed and was assigned a matrix value of 0.18% for Ca and 0.15% for AvP.

was measured for limestone and for each treatment at both phases using Ro-TaP sieve shaker following the method ANSI/ASAE S319.3 (ASAE, 2008). All diets had a geometric average PS (Dgw) of 600 μm for both starter and grower. PS range for fine limestone was as follows with percentage in brackets: 297 μm (23%), 212 μm (26%), 150 μm (18%), 103 (15%), 73 μm (9%), and 53 μm (4%). PS range for the coarse limestone was as follows: 1,191 μm (36%), 841 μm (25%), 594 μm (17%), 420 μm (12%), and 297 μm (3%).

The Ca solubility was calculated for both coarse and fine limestone by solubilizing weighed samples of coarse and fine limestone in 0.1 N HCl at 42°C for 10 min with agitation and recovering quantitatively the undissolved residue. The difference between the weighed sample and insoluble residue was calculated to be the solubility and was expressed as a percentage solubility (Zhang and Coon, 1997b). Solubility was 72.8 and 53.0% for fine and coarse limestone, respectively. Calcium concentration in both fine and coarse limestone was also determined; the concentration was 39.5% for fine and 38.9% for coarse.

## Data Collection

Chicks were weighed in groups and individually on 1, 14, 28, and 35 D of age with feed weigh-back on the same days to determine live performance based on body weight (BW), body weight gain (**BWG**), FI, and feed conversion ratio (FCR). FCR and FI were calculated from feed weigh-back and group BW while individual weights were used to calculate coefficients of variation (CV).

**Table 3.** Experimental diet composition and nutrient content (grower).

| | Treatment | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Limestone particle size | Fine | | | | Coarse | | | |
| Ca and AvP level[1] | PC | | NC | | PC | | NC | |
| Phytase | 1,000 | 0 | 1,000 | 0 | 1,000 | 0 | 1,000 | 0 |
| 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 by-product 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®[5] | 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) | 3,025 | 3,025 | 3,025 | 3,025 | 3,025 | 3,025 | 3,025 | 3,025 |
| 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] The Ca and available phosphorus (AvP) levels used in this design are referred to as positive control (PC, 0.76:0.38) and negative control (NC, 0.58:0.23).

[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 (K3), 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 Na2SeO3).

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

[5] Phytase (RONOZYME HiPhos) was expected to provide 1,000 FYT/kg feed and was assigned a matrix value of 0.18% for Ca and 0.15% for AvP.

Ileal content and left tibia were collected from 3 birds/pen at 15 D and 2 birds/pen at 36 D. 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 content was expressed into marked plastic tubes, placed on ice, and frozen to be used for AID. The left tibia was sampled by separating it from the femur at the knee joint and ankle joint removing it from the foot. The tibia was cleaned by removing all the muscles and tendons. Afterward, it was placed in a marked plastic bag and frozen to be used for bone ash analysis.

## *Tibia Bone Ash Analysis*

Defatted tibia bones were dried at 105°C in an oven for 24 h, then were placed in furnace at 600°C for 24 h, and the residues weighed and expressed in % (Hall et al., 2003).

## *Digestibility Analysis*

Ileal digesta and feed samples were freeze-dried at −50°C and ground. Digestibility analysis of both digesta and feed samples for amino acids was performed following the procedure of AOAC Official method 982.30 (AOAC, 2006a), and that for Ca and $P_i$ was conducted using AOAC official method 968.08 (AOAC, 2006b). Titanium content in ileal digesta and feed samples was determined according to Myers et al. (2004).

Ileal digestibility was calculated according to the procedure described by Dilger and Adeola (2006).

**Table 4.** Feed Intake (FI) of male broilers as affected by limestone particle size (PS), phytase enzyme, Ca and $P_i$ levels, and their interactions.

|  |  |  | Age period (D) |  |  |  |  |  |  |  |  |  |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
|  |  |  | FI (g/bird) |  |  |  |  | BWG (g/bird) |  |  |  |  |
| PS[1] | Phytase[2] | Ca and P[3] | 0–14 | 15–28 | 29–35 | 0–28 | 0–35 | 0–14 | 15–28 | 29–35 | 0–28 | 0–35 |
| Main effects |  |  |  |  |  |  |  |  |  |  |  |  |
| Fine |  |  | 544 | 1,962 | 1,424 | 2,506 | 3,930 | 398 | 1,311 | 930 | 1,707 | 2,637 |
| Coarse |  |  | 544 | 1,942 | 1,423 | 2,487 | 3,909 | 398 | 1,305 | 928 | 1,707 | 2,635 |
| *P* value |  |  | 0.90 | 0.30 | 0.86 | 0.38 | 0.42 | 0.99 | 0.58 | 0.89 | 0.10 | 0.91 |
| SE |  |  | 4.2 | 13.3 | 6.9 | 15.2 | 18.0 | 3.0 | 7.7 | 12.0 | 9.9 | 14.6 |
|  | 0 |  | 532$^{B}$ | 1,937 | 1,423 | 2,469$^{b}$ | 3,892$^{b}$ | 392$^{B}$ | 1,297$^{b}$ | 925 | 1,686$^{B}$ | 2,611$^{b}$ |
|  | 1,000 |  | 557$^{A}$ | 1,966 | 1,424 | 2,523$^{a}$ | 3,948$^{a}$ | 404$^{A}$ | 1,319$^{a}$ | 932 | 1,728$^{A}$ | 2,661$^{a}$ |
|  | *P* value |  | 0.00 | 0.13 | 0.86 | 0.01 | 0.03 | 0.01 | 0.05 | 0.67 | 0.00 | 0.02 |
|  | SE |  | 4.2 | 13.3 | 6.9 | 15.2 | 18.0 | 3.0 | 7.7 | 12.0 | 9.9 | 14.6 |
|  |  | PC | 547 | 1,964 | 1,420 | 2,510 | 3,930 | 401 | 1,310 | 938 | 1,715 | 2,652 |
|  |  | NC | 543 | 1,939 | 1,427 | 2,482 | 3,910 | 395 | 1,307 | 920 | 1,700 | 2,619 |
|  |  | *P* value | 0.51 | 0.21 | 0.45 | 0.20 | 0.43 | 0.19 | 0.75 | 0.30 | 0.29 | 0.12 |
|  |  | SE | 4.2 | 13.3 | 6.9 | 15.2 | 18.0 | 3.0 | 7.7 | 12.0 | 9.9 | 14.6 |
| Interactions |  |  |  |  |  |  |  |  |  |  |  |  |
| Fine | 0 |  | 533 | 1,952 | 1,423 | 2,484 | 3,908 | 394 | 1,305 | 928 | 1,694 | 2,622 |
| Coarse | 0 |  | 531 | 1,922 | 1,422 | 2,453 | 3,875 | 390 | 1,290 | 923 | 1,678 | 2,600 |
| Fine | 1,000 |  | 556 | 1,971 | 1,425 | 2,527 | 3,952 | 402 | 1,318 | 932 | 1,720 | 2,652 |
| Coarse | 1,000 |  | 559 | 1,961 | 1,423 | 2,520 | 3,943 | 406 | 1,321 | 933 | 1,736 | 2,669 |
| *P* value |  |  | 0.72 | 0.60 | 0.99 | 0.58 | 0.64 | 0.33 | 0.40 | 0.90 | 0.24 | 0.36 |
| SE |  |  | 6.0 | 18.8 | 9.8 | 21.5 | 25.4 | 4.3 | 10.9 | 17.0 | 14.0 | 20.6 |
|  | 0 | NC | 522$^{C}$ | 1,912 | 1,429 | 2,434 | 3,863 | 382$^{B}$ | 1,281$^{B}$ | 912 | 1,661$^{b}$ | 2,572$^{b}$ |
|  | 0 | PC | 542$^{B}$ | 1,962 | 1,416 | 2,504 | 3,920 | 402$^{A}$ | 1,314$^{A}$ | 939 | 1,771$^{a}$ | 2,650$^{a}$ |
|  | 1,000 | NC | 563$^{A}$ | 1,967 | 1,425 | 2,531 | 3,956 | 408$^{A}$ | 1,333$^{A}$ | 928 | 1,739$^{a}$ | 2,667$^{a}$ |
|  | 1,000 | PC | 551$^{A}$ | 1,967 | 1,423 | 2,516 | 3,940 | 400$^{A}$ | 1,306$^{A,B}$ | 937 | 1,718$^{a}$ | 2,655$^{a}$ |
|  | *P* value |  | 0.01 | 0.18 | 0.56 | 0.06 | 0.16 | 0.00 | 0.01 | 0.59 | 0.01 | 0.03 |
|  | SE |  | 6.0 | 18.8 | 9.8 | 21.5 | 25.4 | 4.3 | 10.9 | 17.0 | 14.0 | 20.6 |
| Fine |  | NC | 539 | 1,931 | 1,428 | 2,470$^{a}$ | 3,898 | 387$^{C}$ | 1,289$^{B}$ | 931 | 1,675$^{C}$ | 2,607 |
| Fine |  | PC | 549 | 1,992 | 1,421 | 2,542$^{b}$ | 3,962 | 409$^{A}$ | 1,334$^{A}$ | 929 | 1,739$^{A}$ | 2,668 |
| Coarse |  | NC | 546 | 1,949 | 1,426 | 2,495$^{a}$ | 3,921 | 403$^{A,B}$ | 1,324$^{A,B}$ | 908 | 1,724$^{A,B}$ | 2,632 |
| Coarse |  | PC | 544 | 1,935 | 1,419 | 2,479$^{a}$ | 3,897 | 393$^{A,B}$ | 1,286$^{B}$ | 947 | 1,690$^{B,C}$ | 2,637 |
| *P* value |  |  | 0.29 | 0.05 | 0.99 | 0.05 | 0.09 | 0.00 | 0.00 | 0.23 | 0.00 | 0.18 |
| SE |  |  | 6.0 | 18.8 | 9.8 | 21.5 | 25.4 | 4.3 | 10.9 | 17.0 | 14.0 | 20.6 |

Means within each column and treatment effect that don't possess any lowercase superscript letter in common differ significantly ($P \leq 0.05$).

Means within each column and treatment effect that don't possess any uppercase superscript letter in common differ significantly ($P \leq 0.01$).

Abbreviations: BWG, body weight gain; SE, standard error of mean.

[1] Limestone particle size: fine (190 µm) and coarse (900 µm).

[2] Phytase: had a dose of 1,000 FYT/kg and a matrix value of 0.18% for Ca and 0.15% for $P_i$.

[3] The Ca and $P_i$ had 2 levels, NC (negative control) had Ca 0.72 and P 0.3 in starter and Ca 0.53 and P 0.23 in grower while PC (positive control) had Ca 0.9 and P 0.45 in starter and Ca 0.76 and P 0.38 in grower.

**Table 5.** Feed conversion ratio (FCR) and mortality of male broilers as affected by limestone particle size (PS), phytase enzyme, Ca and $P_i$ levels, and their interactions.

| | | | Age period (D) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | FCR (g:g) | | | | | Mortality (%) | | | |
| PS[1] | Phytase[2] | Ca and P[3] | 0–14 | 15–28 | 29–35 | 0–28 | 0–35 | 0–14 | 15–28 | 29–35 | 0–35 |
| Main effects | | | | | | | | | | | |
| Fine | | | 1.371 | 1.497 | 1.539 | 1.468 | 1.491 | 0.14 | 0.17$^{b}$ | 0.67 | 0.94 |
| Coarse | | | 1.373 | 1.498 | 1.543 | 1.457 | 1.485 | 0.14 | 1.00$^{a}$ | 0.50 | 1.61 |
| *P* value | | | 0.89 | 0.98 | 0.89 | 0.22 | 0.54 | 1.00 | 0.05 | 0.70 | 0.35 |
| SE | | | 0.01 | 0.01 | 0.02 | 0.01 | 0.01 | 0.14 | 0.29 | 0.31 | 0.51 |
| | 0 | | 1.361 | 1.500 | 1.542 | 1.465 | 1.491 | 0.15 | 0.83 | 0.504 | 1.45 |
| | 1,000 | | 1.383 | 1.495 | 1.539 | 1.461 | 1.485 | 0.13 | 0.34 | 0.662 | 1.10 |
| | *P* value | | 0.17 | 0.69 | 0.91 | 0.67 | 0.56 | 0.94 | 0.24 | 0.72 | 0.63 |
| | SE | | 0.01 | 0.01 | 0.02 | 0.01 | 0.01 | 0.14 | 0.29 | 0.31 | 0.51 |
| | | PC | 1.366 | 1.500 | 1.52 | 1.464 | 1.482 | 0.14 | 0.17$^{B}$ | 0.33 | 0.64$^{B}$ |
| | | NC | 1.378 | 1.494 | 1.56 | 1.461 | 1.494 | 0.14 | 1.00$^{A}$ | 0.83 | 1.91$^{A}$ |
| | | *P* value | 0.45 | 0.64 | 0.15 | 0.73 | 0.27 | 1.00 | 0.01 | 0.26 | 0.01 |
| | | SE | 0.01 | 0.01 | 0.02 | 0.01 | 0.01 | 0.14 | 0.29 | 0.31 | 0.51 |
| Interactions | | | | | | | | | | | |
| Fine | 0 | | 1.355 | 1.498 | 1.536 | 1.466 | 1.491 | 0.02 | 0.32 | 0.68 | 0.96 |
| Coarse | 0 | | 1.367 | 1.501 | 1.549 | 1.463 | 1.491 | 0.23 | 1.33 | 0.33 | 1.94 |
| Fine | 1,000 | | 1.387 | 1.496 | 1.542 | 1.470 | 1.491 | 0.26 | 0.01 | 0.66 | 0.92 |
| Coarse | 1,000 | | 1.380 | 1.493 | 1.537 | 1.452 | 1.478 | 0.00 | 0.67 | 0.67 | 1.28 |
| *P* value | | | 0.58 | 0.15 | 0.78 | 0.41 | 0.51 | 0.18 | 0.67 | 0.69 | 0.67 |
| SE | | | 0.02 | 0.01 | 0.03 | 0.01 | 0.01 | 0.12 | 0.41 | 0.44 | 0.41 |
| | 0 | NC | 1.372 | 1.506 | 1.574 | 1.466 | 1.503 | 0.29 | 1.32 | 0.68 | 2.23 |
| | 0 | PC | 1.350 | 1.494 | 1.511 | 1.464 | 1.479 | 0.00 | 0.33 | 0.33 | 0.66 |
| | 1,000 | NC | 1.385 | 1.483 | 1.549 | 1.457 | 1.485 | 0.02 | 0.66 | 0.99 | 1.60 |
| | 1,000 | PC | 1.382 | 1.507 | 1.529 | 1.465 | 1.485 | 0.28 | 0.00 | 0.33 | 0.61 |
| | *P* value | | 0.58 | 0.15 | 0.46 | 0.53 | 0.25 | 0.13 | 0.70 | 0.72 | 0.70 |
| | SE | | 0.02 | 0.01 | 0.03 | 0.01 | 0.01 | 0.20 | 0.41 | 0.44 | 0.41 |
| Fine | | NC | 1.396$^{a}$ | 1.501 | 1.539 | 1.475 | 1.496 | 0.00 | 0.33 | 0.67 | 0.94 |
| Fine | | PC | 1.346$^{b}$ | 1.493 | 1.538 | 1.462 | 1.486 | 0.28 | 0.00 | 0.67 | 0.94 |
| Coarse | | NC | 1.360$^{a,b}$ | 1.487 | 1.584 | 1.450 | 1.491 | 0.28 | 1.67 | 1.00 | 2.89 |
| Coarse | | PC | 1.386$^{a,b}$ | 1.507 | 1.502 | 1.467 | 1.478 | 0.00 | 0.33 | 0.00 | 0.33 |
| *P* value | | | 0.02 | 0.27 | 0.16 | 0.07 | 0.87 | 0.16 | 0.23 | 0.26 | 0.08 |
| SE | | | 0.02 | 0.01 | 0.03 | 0.01 | 0.01 | 0.20 | 0.41 | 0.44 | 0.71 |

Means within each column and treatment effect that don't possess any lowercase superscript letter in common differ significantly ($P \leq 0.05$).
Means within each column and treatment effect that don't possess any uppercase superscript letter in common differ significantly ($P \leq 0.01$).
Abbreviation: SE, standard error of mean.

[1] Limestone particle size: fine (190 µm) and coarse (900 µm).

[2] Phytase: had a dose of 1,000 FYT/kg and a matrix value of 0.18% for Ca and 0.15% for $P_i$.

[3] The Ca and $P_i$ had 2 levels, NC (negative control) had Ca 0.72 and P 0.3 in starter and Ca 0.53 and P 0.23 in grower while PC (positive control) had Ca 0.9 and P 0.45 in starter and Ca 0.76 and P 0.38 in grower.

## *Data Analysis*

Data were analyzed using the General Linear Model procedure of SAS (Statistical Analysis System, 2017). Means were separated using Tukey's test (Statistical Analysis System, 2017). Significance among main effects, their interactions, and means was based on $P \leq 0.05$ or less. The pen was considered to be the experimental unit.

## RESULTS AND DISCUSSION

The PS of starter and grower feed were similar, being around 600 µm. This is important as different PS of feed can influence the bird's performance as demonstrated by Amerah et al. (2008). PS uniformity was also high among treatments ensuring birds in the trial got similar feed PS. There was no 3-way interaction between the 3 main effects investigated, so only main effects and 2-way interactions between any 2 out of the 3 main effects are discussed. Individual BW data were analyzed for CV calculation at day 0, 14, 28, and 35. All CVs for BW were less than 10%, indicating that birds were uniform.

## *Live Performance*

Live performance results are shown in Table 4 (FI and BWG) and Table 5 (FCR and mortality). The main effects of PS and Ca and $P_i$ levels did not affect FI, BWG, or FCR. Mortality was largely unaffected by the factors with no significant interactions, except for 35 D and 15–28 D in which NC had higher mortality than PC. Overall mortality in the entire trial was less than 2%. In an earlier study (McNaughton et al., 1974), fine PS improved BW of broilers compared with coarse limestone although the coarse PS was

**Table 6.** Tibia bone ash, Ca, and $P_i$ apparent ileal digestibility (AID) of male broilers as affected by limestone particle size (PS), phytase enzyme, Ca and $P_i$ levels, and their interactions.

| | | | Age (D) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Bone ash (%) | | Ca AID (%) | | $P_i$ AID (%) | |
| PS[1] | Phytase[2] | Ca and P[3] | 14 | 35 | 14 | 35 | 14 | 35 |
| Main effects | | | | | | | | |
| Fine | | | 50.0 | 52.5 | $65.1^{A}$ | $54.8^{a}$ | $63.9^{A}$ | $56.5^{A}$ |
| Coarse | | | 50.3 | 52.6 | $56.1^{B}$ | $48.4^{b}$ | $53.4^{B}$ | $52.1^{B}$ |
| *P* value | | | 0.39 | 0.78 | 0.00 | 0.01 | 0.00 | 0.00 |
| SE | | | 0.15 | 0.19 | 1.25 | 1.25 | 1.09 | 0.96 |
| | 0 | | $49.6^{B}$ | 52.3 | $56.7^{B}$ | 52.5 | $55.8^{B}$ | $51.2^{B}$ |
| | 1,000 | | $50.7^{A}$ | 52.7 | $64.4^{A}$ | 50.7 | $61.5^{A}$ | $57.5^{A}$ |
| | *P* value | | 0.00 | 0.11 | 0.00 | 0.28 | 0.00 | 0.00 |
| | SE | | 0.15 | 0.19 | 1.25 | 1.17 | 1.09 | 0.96 |
| | | PC | $50.1^{A}$ | $52.8^{a}$ | $52.4^{B}$ | $49.9^{b}$ | $56.7^{b}$ | $48.4^{B}$ |
| | | NC | $49.4^{B}$ | $52.2^{b}$ | $68.7^{A}$ | $53.3^{a}$ | $60.6^{a}$ | $60.2^{A}$ |
| | | *P* value | 0.00 | 0.02 | 0.00 | 0.05 | 0.01 | 0.00 |
| | | SE | 0.15 | 0.19 | 1.25 | 1.17 | 1.09 | 0.96 |
| Interactions | | | | | | | | |
| Fine | 0 | | 49.7 | 52.4 | $69.7^{A}$ | 55.3 | $63.5^{A}$ | 53.7 |
| Coarse | 0 | | 49.6 | 52.2 | $43.8^{C}$ | 49.6 | $48.1^{B}$ | 48.6 |
| Fine | 1,000 | | 50.5 | 52.5 | $60.5^{B}$ | 54.2 | $64.3^{A}$ | 59.4 |
| Coarse | 1,000 | | 50.9 | 52.9 | $68.3^{A}$ | 47.2 | $58.7^{A}$ | 55.6 |
| *P* value | | | 0.29 | 0.27 | 0.00 | 0.70 | 0.00 | 0.64 |
| SE | | | 0.21 | 0.27 | 1.76 | 1.66 | 1.55 | 1.36 |
| | 0 | NC | $48.4^{C}$ | 52.0 | 66.3 | 54.7 | 57.9 | 57.0 |
| | 0 | PC | $50.9^{A}$ | 52.6 | 47.1 | 50.3 | 53.6 | 45.3 |
| | 1,000 | NC | $50.3^{B}$ | 52.4 | 71.1 | 51.8 | 63.3 | 63.4 |
| | 1,000 | PC | $51.1^{A}$ | 53.1 | 57.7 | 49.6 | 59.7 | 51.5 |
| | *P* value | | 0.00 | 0.78 | 0.10 | 0.50 | 0.82 | 0.93 |
| | SE | | 0.21 | 0.27 | 1.76 | 1.66 | 1.55 | 1.36 |
| Fine | | NC | 49.3 | 51.9 | 71.8 | $54.5^{a}$ | 66.6 | 61.3 |
| Fine | | PC | 50.9 | 53.1 | 58.3 | $55.0^{a}$ | 61.2 | 51.8 |
| Coarse | | NC | 49.4 | 52.5 | 65.6 | $52.0^{a}$ | 54.6 | 59.2 |
| Coarse | | PC | 51.1 | 52.6 | 46.5 | $44.8^{b}$ | 52.1 | 45.0 |
| *P* value | | | 0.66 | 0.05 | 0.11 | 0.02 | 0.36 | 0.09 |
| SE | | | 0.21 | 0.27 | 1.76 | 1.66 | 1.55 | 1.36 |

Means within each column and treatment effect that don't possess any lowercase superscript letter in common differ significantly ($P \leq 0.05$).

Means within each column and treatment effect that don't possess any uppercase superscript letter in common differ significantly ($P \leq 0.01$).

Abbreviation: SE, standard error of mean.

[1] Limestone particle size: fine (190 µm) and coarse (900 µm).

[2] Phytase: had a dose of 1,000 FYT/kg and a matrix value of 0.18% for Ca and 0.15% for $P_i$.

[3] The Ca and $P_i$ had 2 levels, NC (negative control) had Ca 0.72 and P 0.3 in starter and Ca 0.53 and P 0.23 in grower while PC (positive control) had Ca 0.9 and P 0.45 in starter and Ca 0.76 and P 0.38 in grower.

2,360–3,350 µm which is greater than what was used for this study (900 µm). Similarly, Managi and Coon (2007) observed that the best weight gain and FI were with fine PS (137–299 µm) compared with coarse (>500 µm) or very fine (28 µm) PS. Bradbury et al. (2018) came to a conclusion similar to the outcomes of this study, which showed that PS had no significant influence on either BWG or FCR. Findings reported in literature with regard to limestone PS effects are variable depending on the PS investigated.

The phytase main effect was significant for all age periods for BWG except at 29–35 D and for FI at 0–14 D, 0–28 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 $P_i$ (Singh et al., 2003; Shaw et al., 2011; 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 the study by 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 PS 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 than 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. In the present study, improved phytase activity was noted with coarse limestone, but it 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. Supplementing optimum dietary Ca and $P_i$ levels with increasing phytase activity, which releases additional Ca and $P_i$, did not have a significant effect on live performance.

There was an interaction effect between phytase and Ca and $P_i$ levels on live performance. The NC without phytase had lower FI (0–14 D) and BWG (0–14, 15–28, 0–28, and 0–35 D) than PC, NC+phytase, and PC+phytase. Adding phytase to a PC diet did not improve performance compared with 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 (Denbow et al., 1995; Potter et al., 1995). The addition of phytase to NC likely increased phytate 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 $P_i$ levels and PS was significant for BWG (0–14 D, 15–28 D, and 0–28 D). The NC with coarse PS improved live performance compared with the NC with fine PS, which might be associated with the longer ventricular retention of the coarse limestone as well as 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 6) for the PS were not significant for either 14 D or 35 D. This observation was in agreement with results from a recent investigation in which inclusion of dietary limestone of various PS (200 µm, 1,000 µm, 2,000 µm, and 3,000 µm) did not influence tibial bone ash content in 82-week postmolting broiler breeders (Bueno et al., 2016). Neither Joardar (2019) nor Managi and Coon (2007) found a PS effect on tibial ash. Similarly, Bradbury et al. (2018), who studied broiler skeletal integrity, were not able to demonstrate an effect of PS on foot bone ash content.

In this experiment, phytase improved tibial bone ash at 14 D, but the phytase effect was lost at 35 D. Numerous studies have shown phytase improving bone ash content at 14 or 21 D (Qian et al., 1996; Johnston and Southren, 2000; Li et al., 2015). It is generally accepted that dietary phytase results in improved bone ash content 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 wk in chicks, but the phytase effect diminishes as birds age, which would account for lack of phytase influence at 35 D in this study (Olukosi et al., 2007). The high Ca and $P_i$ levels in the PC treatment improved bone ash compared with 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 ash content of tibial bones. Driver et al. (2005) have reported similar results from experiments in which diets with higher Ca and $P_i$ levels resulted in greater tibial bone ash percentage than diets with low Ca and $P_i$ levels in 16-day-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 of 0.9% and $P_i$ of 0.45% compared with starter diets with Ca of 0.8% and $P_i$ of 0.40%. Other studies also have reported greater tibial bone ash content with increasing dietary Ca and $P_i$ levels (Nelson et al., 1990; Onyango et al., 2003).

The interaction between PS and phytase inclusion, as well as the interaction between PS and Ca and $P_i$ levels, did not affect bone ash percentage. However, 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 lowest bone ash percentage compared with the PC treatment, regardless of phytase inclusion. The inclusion of phytase in the NC treatment slightly improved bone ash percentage; however, the improvement did not reach the bone ash percentage of 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 concerning bone mineralization as supplemental phytase in NC diets during the early starter feed phase. Similarly, Gautier et al. (2018) found that broilers fed diets with nonphytate phosphorus (NPP) at 0.53% had a higher bone ash percentage than broilers fed diets with NPP at 0.45%. The addition of phytase to the 0.45% NPP diet improved bone ash percentage but to a lower percentage than the 0.53% NPP diet without phytase. However, the addition of phytase to the PC diet (0.53% NPP) further improved bone ash (Gautier et al., 2018); the same response was not found in the present study.

## Nutrient Digestibility

The AID of Ca, $P_i$, and amino acids was calculated (Tables 6–8). The PS had a significant effect on Ca and $P_i$ digestibility, with fine PS (190 µm) exhibiting better digestibility of both Ca and $P_i$ at 14 and 35 D than coarse PS (900 µm).

Kim et al. (2018) concluded that larger PS had better Ca and $P_i$ digestibility at 28 D, when they defined small PS as 75 µm and large PS as 402 µm. Bradbury et al. (2018) considered fine PS to be <0.5 mm and coarse to be >0.5 mm, and they observed no significant effect of PS on either Ca or $P_i$ digestibility. Anwar et al.

**Table 7.** Amino acid AID of male broilers as affected by limestone particle size (PS), phytase enzyme, Ca and $P_i$ levels, and their interaction at 14 D of age.

| | | | (%) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| PS[1] | Phytase[2] | Ca and P[3] | Lysine | Arginine | Histidine | Leucine | Isoleucine | Methionine | Glycine | Proline | Threonine | Cysteine |
| Main effects | | | | | | | | | | | | |
| Fine | | | $80.1^{a}$ | $84.1^{a}$ | $77.4^{A}$ | $77.9^{a}$ | $77.6^{A}$ | $88.1^{a}$ | $71.2^{A}$ | $75.3^{a}$ | $70.5^{A}$ | $61.0^{A}$ |
| Coarse | | | $75.5^{b}$ | $80.8^{b}$ | $72.8^{B}$ | $73.8^{b}$ | $73.0^{B}$ | $84.6^{b}$ | $67.6^{B}$ | $71.7^{b}$ | $64.3^{B}$ | $52.9^{B}$ |
| *P* value | | | 0.04 | 0.02 | 0.01 | 0.02 | 0.01 | 0.01 | 0.01 | 0.02 | 0.00 | 0.00 |
| SE | | | 1.33 | 1.00 | 1.16 | 1.19 | 1.19 | 0.95 | 1.22 | 1.06 | 1.37 | 1.62 |
| | 0 | | $75.8^{b}$ | $80.8^{b}$ | 73.6 | $74.3^{b}$ | $73.6^{a}$ | 85.5 | $67.5^{b}$ | 72.6 | 66.0 | 55.2 |
| | 1,000 | | $79.8^{a}$ | $84.1^{a}$ | 76.6 | $77.5^{a}$ | $77.1^{b}$ | 87.1 | $71.3^{a}$ | 74.4 | 68.9 | 58.8 |
| | *P* value | | 0.04 | 0.02 | 0.08 | 0.05 | 0.04 | 0.265 | 0.03 | 0.22 | 0.15 | 0.12 |
| | SE | | 1.33 | 1.00 | 1.16 | 1.19 | 1.19 | 0.95 | 1.22 | 1.06 | 1.37 | 1.62 |
| | | PC | 77.0 | 82.2 | 74.9 | 75.2 | 75.0 | 84.9 | 69.4 | 73.4 | 66.6 | 56.1 |
| | | NC | 78.5 | 82.7 | 75.3 | 76.5 | 75.6 | 87.7 | 69.3 | 73.6 | 68.3 | 57.9 |
| | | *P* value | 0.42 | 0.70 | 0.83 | 0.46 | 0.70 | 0.08 | 0.96 | 0.91 | 0.38 | 0.43 |
| | | SE | 1.33 | 1.00 | 1.16 | 1.19 | 1.19 | 0.95 | 1.22 | 1.06 | 1.19 | 1.62 |
| Interactions | | | | | | | | | | | | |
| Fine | 0 | | $80.3^{a}$ | $83.9^{A}$ | $78.0^{A}$ | $78.3^{a}$ | $77.9^{a}$ | 88.6 | $71.8^{A}$ | $76.5^{A}$ | $72.0^{a}$ | $62.4^{a}$ |
| Coarse | 0 | | $71.3^{b}$ | $77.8^{B}$ | $69.3^{B}$ | $70.2^{b}$ | $69.2^{b}$ | 82.5 | $63.2^{B}$ | $68.6^{B}$ | $60.0^{b}$ | $48.0^{b}$ |
| Fine | 1,000 | | $79.9^{a}$ | $84.2^{A}$ | $76.8^{A}$ | $77.5^{a}$ | $77.4^{a}$ | 87.5 | $70.6^{A}$ | $74.1^{A}$ | $69.0^{a}$ | $57.8^{a}$ |
| Coarse | 1,000 | | $79.6^{a}$ | $83.9^{A}$ | $76.4^{A}$ | $77.4^{a}$ | $76.7^{a}$ | 86.7 | $72.0^{A}$ | $74.8^{A}$ | $68.8^{a}$ | $59.7^{a}$ |
| *P* value | | | 0.02 | 0.00 | 0.01 | 0.01 | 0.02 | 0.06 | 0.01 | 0.01 | 0.01 | 0.02 |
| SE | | | 1.88 | 1.42 | 1.64 | 1.68 | 1.69 | 1.35 | 1.73 | 1.50 | 1.68 | 1.69 |
| | 0 | NC | 77.1 | 81.3 | 74.0 | 75.1 | 74.1 | 77.1 | 67.7 | 72.6 | 67.3 | 55.7 |
| | 0 | PC | 74.5 | 80.3 | 73.3 | 73.4 | 73.0 | 74.5 | 67.4 | 72.5 | 64.7 | 54.6 |
| | 1,000 | NC | 79.9 | 84.1 | 76.6 | 77.9 | 77.1 | 79.9 | 71.1 | 74.5 | 69.2 | 60.0 |
| | 1,000 | PC | 79.6 | 84.0 | 76.5 | 77.1 | 77.0 | 79.6 | 71.5 | 74.3 | 68.5 | 57.5 |
| | *P* value | | 0.55 | 0.75 | 0.87 | 0.79 | 0.79 | 0.55 | 0.83 | 0.98 | 0.63 | 0.74 |
| | SE | | 1.88 | 1.42 | 1.64 | 1.68 | 1.69 | 1.88 | 1.73 | 1.50 | 1.94 | 2.30 |
| Fine | | NC | 81.5 | 84.7 | 78.2 | 78.8 | 78.5 | 81.5 | 71.8 | 76.3 | 72.3 | 64.1 |
| Fine | | PC | 78.7 | 83.4 | 76.7 | 77.1 | 76.8 | 78.7 | 70.5 | 74.3 | 68.8 | 58.0 |
| Coarse | | NC | 75.6 | 80.7 | 72.4 | 74.2 | 72.8 | 75.6 | 66.8 | 70.8 | 64.3 | 51.7 |
| Coarse | | PC | 75.3 | 80.9 | 73.2 | 73.4 | 73.2 | 75.3 | 68.4 | 72.5 | 64.4 | 54.1 |
| *P* value | | | 0.51 | 0.59 | 0.48 | 0.78 | 0.79 | 0.51 | 0.41 | 0.23 | 0.37 | 0.07 |
| SE | | | 1.88 | 1.42 | 1.64 | 1.68 | 1.69 | 1.88 | 1.73 | 1.64 | 1.94 | 1.69 |

Means within each column and treatment effect that don't possess any lowercase superscript letter in common differ significantly ($P \leq 0.05$).
Means within each column and treatment effect that don't possess any uppercase superscript letter in common differ significantly ($P \leq 0.01$).
Abbreviations: AID, apparent ileal digestibility; SE, standard error of mean.
[1] Limestone particle size: fine (190 µm) and coarse (900 µm).
[2] Phytase: had a dose of 1,000 FYT/kg and a matrix value of 0.18% for Ca and 0.15% for $P_i$.
[3] The Ca and $P_i$ had 2 levels, NC (negative control) had Ca 0.72 and P 0.3 in starter and Ca 0.53 and P 0.23 in grower while PC (positive control) had Ca 0.9 and P 0.45 in starter and Ca 0.76 and P 0.38 in grower.

(2016), on the other hand, concluded that coarse PS resulted in better true and apparent Ca digestibility with fine PS being <500 µm and coarse PS ranging between 1,000 and 2,000 µm. Guinote and Nys (1991) reported that fine PS (<150 µm) supported better Ca retention than medium PS (600–1,180 µm) and coarse PS (>1,180 µm).

It is accepted that different PSs vary in solubility, and it is well known that in vitro fine PS is more soluble than coarse PS (de Witt et al., 2006; Managi and Coon 2007; Kim et al., 2018). In the ventriculus, it is thought that coarse limestone particles are retained longer as Roland (1986) concluded that PS > 900 µm will be retained longer in the ventriculus of layers, and Zhang and Coon (1997a) also came to the same conclusion. The longer retention time of coarse limestone particles in the ventriculus appears to sustain longer Ca availability to the bird than fine PS (de Witt et al., 2006). The greater and sustained Ca release from coarse limestone particles is at a slower rate than that from fine limestone particles because of differences in solubility. The differences in the solubility of coarse and fine limestone particles will influence $P_i$ and Ca digestibility, with fine PS causing greater variability in Ca and $P_i$ digestibility. One hypothesis to explain the reduction of AID in birds fed coarse PS is that an elevated dietary Ca level will downregulate the Ca transporter in the small intestine, thereby 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 $P_i$ digestibility was affected at 35 D. Phytase degrades phytate in poultry diets, thus improves Ca and $P_i$ availability, which can then result in better live performance (Bougouin et al., 2014; Bradbury et al., 2016; Scholey et al., 2017). Ca and $P_i$ levels also affect Ca and $P_i$ digestibility, where higher inclusion levels in the diet resulted in decreased AID and lower levels improved 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

**Table 8.** Amino acid AID of male broilers as affected by limestone particle size (PS), phytase enzyme, Ca and $P_i$ levels, and their interaction at 35 D of age.

| | | | (%) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| PS[1] | Phytase[2] | Ca and P[3] | Lysine | Arginine | Histidine | Leucine | Isoleucine | Methionine | Glycine | Proline | Threonine | Cysteine |
| Main effects | | | | | | | | | | | | |
| Fine | | | $84.3^{A}$ | $88.7^{A}$ | $83.0^{a}$ | $82.8^{a}$ | $82.5^{A}$ | $83.2^{A}$ | $76.7^{A}$ | $79.8^{A}$ | $75.8^{A}$ | $80.6^{A}$ |
| Coarse | | | $86.2^{B}$ | $87.9^{B}$ | $82.1^{b}$ | $81.9^{b}$ | $81.4^{B}$ | $80.9^{B}$ | $75.4^{B}$ | $77.2^{B}$ | $74.1^{B}$ | $77.5^{B}$ |
| *P* value | | | 0.00 | 0.00 | 0.04 | 0.02 | 0.01 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| SE | | | 0.25 | 0.21 | 0.28 | 0.28 | 0.28 | 0.42 | 0.36 | 0.35 | 0.34 | 0.52 |
| | 0 | | 84.8 | 87.9 | 82.2 | 82.0 | 81.5 | $81.4^{b}$ | $75.3^{b}$ | 78.0 | 74.6 | $78.5^{b}$ |
| | 1,000 | | 85.7 | 88.7 | 82.6 | 82.7 | 82.4 | $82.7^{a}$ | $76.8^{a}$ | 78.9 | 75.3 | $79.8^{a}$ |
| | *P* value | | 0.15 | 0.08 | 0.60 | 0.09 | 0.06 | 0.05 | 0.05 | 0.26 | 0.17 | 0.05 |
| | SE | | 0.25 | 0.21 | 0.28 | 0.28 | 0.28 | 0.42 | 0.36 | 0.35 | 0.34 | 0.52 |
| | | PC | 85.6 | 88.4 | $83.2^{a}$ | 82.6 | 82.5 | $82.5^{A}$ | 76.9 | $79.3^{a}$ | $76.1^{a}$ | $79.6^{A}$ |
| | | NC | 85.0 | 88.0 | $81.6^{b}$ | 81.8 | 81.6 | $81.6^{B}$ | 75.5 | $77.7^{b}$ | $73.8^{b}$ | $78.7^{B}$ |
| | | *P* value | 0.28 | 0.29 | 0.01 | 0.18 | 0.13 | 0.01 | 0.07 | 0.04 | 0.02 | 0.00 |
| | | SE | 0.25 | 0.21 | 0.28 | 0.28 | 0.28 | 0.42 | 0.36 | 0.35 | 0.34 | 0.52 |
| Interactions | | | | | | | | | | | | |
| Fine | 0 | | 85.5 | $88.7^{A}$ | 82.7 | 82.3 | 82.2 | 82.6 | 77.5 | 78.6 | 75.6 | 79.7 |
| Coarse | 0 | | 84.2 | $87.1^{B}$ | 81.7 | 81.7 | 80.8 | 80.3 | 73.4 | 77.5 | 73.6 | 77.3 |
| Fine | 1,000 | | 86.0 | $88.7^{A}$ | 83.2 | 83.2 | 82.8 | 83.8 | 78.0 | 79.4 | 75.9 | 81.5 |
| Coarse | 1,000 | | 85.4 | $88.7^{A}$ | 82.5 | 82.2 | 82.0 | 81.5 | 76.2 | 79.0 | 74.6 | 79.7 |
| *P* value | | | 0.74 | 0.00 | 0.70 | 0.61 | 0.39 | 0.97 | 0.14 | 0.51 | 0.46 | 0.53 |
| SE | | | 0.36 | 0.31 | 0.40 | 0.40 | 0.40 | 0.72 | 0.51 | 0.51 | 0.49 | 0.79 |
| | 0 | NC | 84.2 | 87.3 | $80.9^{b}$ | $80.8^{b}$ | $80.5^{B}$ | 80.3 | $73.5^{B}$ | $76.2^{B}$ | 73.1 | $77.0^{b}$ |
| | 0 | PC | 85.4 | 88.4 | $83.5^{a}$ | $83.2^{a}$ | $83.1^{A}$ | 82.6 | $77.2^{A}$ | $79.9^{A}$ | 76.1 | $80.0^{a}$ |
| | 1,000 | NC | 85.4 | 88.6 | $82.4^{a,b}$ | $82.5^{a}$ | $83.1^{A}$ | 82.9 | $76.5^{A}$ | $78.7^{A}$ | 74.4 | $80.3^{a}$ |
| | 1,000 | PC | 86.0 | 88.7 | $83.4^{a}$ | $82.9^{a}$ | $82.2^{A,B}$ | 82.5 | $77.1^{A}$ | $79.7^{A}$ | 76.1 | $79.3^{a}$ |
| | *P* value | | 0.45 | 0.13 | 0.05 | 0.01 | 0.01 | 0.06 | 0.00 | 0.01 | 0.20 | 0.02 |
| | SE | | 0.36 | 0.31 | 0.40 | 0.40 | 0.40 | 0.72 | 0.51 | 0.50 | 0.49 | 0.79 |
| Fine | | NC | $85.3^{a,b}$ | $87.5^{B}$ | $80.9^{C}$ | $80.9^{B}$ | $80.6^{B}$ | $81.7^{B}$ | $74.0^{B}$ | $76.5^{B}$ | $73.1^{B}$ | $78.9^{B}$ |
| Fine | | PC | $87.2^{a}$ | $89.8^{A}$ | $85.0^{A}$ | $84.7^{A}$ | $84.3^{A}$ | $84.7^{A}$ | $79.4^{A}$ | $81.5^{A}$ | $78.4^{A}$ | $82.3^{A}$ |
| Coarse | | NC | $84.7^{b}$ | $88.4^{B}$ | $82.4^{B}$ | $82.4^{B}$ | $81.9^{B}$ | $80.3^{B}$ | $75.9^{B}$ | $78.3^{B}$ | $74.5^{B}$ | $78.5^{B}$ |
| Coarse | | PC | $83.9^{b}$ | $87.3^{B}$ | $81.8^{B,C}$ | $81.5^{B}$ | $81.0^{B}$ | $80.3^{B}$ | $74.9^{B}$ | $78.1^{B}$ | $73.8^{B}$ | $77.0^{B}$ |
| *P* value | | | 0.02 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| SE | | | 0.36 | 0.31 | 0.40 | 0.40 | 0.40 | 0.72 | 0.51 | 0.50 | 0.49 | 0.79 |

Means within each column and treatment effect that don't possess any lowercase superscript letter in common differ significantly ($P \leq 0.05$).
Means within each column and treatment effect that don't possess any uppercase superscript letter in common differ significantly ($P \leq 0.01$).
Abbreviations: AID, apparent ileal digestibility; SE, standard error of mean.

[1] Limestone particle size: fine (190 µm) and coarse (900 µm).

[2] Phytase: had a dose of 1,000 FYT/kg and a matrix value of 0.18% for Ca and 0.15% for $P_i$.

[3] The Ca and $P_i$ had 2 levels, NC (negative control) had Ca 0.72 and P 0.3 in starter and Ca 0.53 and P 0.23 in grower while PC (positive control) had Ca 0.9 and P 0.45 in starter and Ca 0.76 and P 0.38 in grower.

their intestinal uptake. Phosphate is absorbed in the small intestine by passive diffusion via paracellular phosphate transport and active transport via sodium-dependent phosphate cotransporters (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 (Li et al., 2012; Adedokun and Adeola, 2013). No significant interaction for Ca and $P_i$ AID was observed at 35 D, but there was a significant interaction between PS and phytase enzyme at 14 D for both Ca ($P = 0.0001$) and $P_i$ ($P = 0.0025$).

Along with Ca and $P_i$ digestibility, the AID of 22 amino acids (lysine, methionine, cysteine, threonine, arginine, histidine, taurine, hydroxyproline, aspartic acid, serine, glutamic acid, proline, lanthionine, glycine, alanine, valine, leucine, isoleucine, tyrosine, phenylalanine, hydroxylysine, and ornithine) was determined for 14 D (Table 7) and 35 D (Table 8); results are shown for only 10 out of the 22 amino acids tested (lysine, methionine, cysteine, threonine, arginine, histidine, proline, glycine, leucine, and isoleucine). Similar to Ca and $P_i$, PS had a strong effect on amino acid digestibility, with fine PS 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 PS vs. coarse PS is not yet understood.

Phytase inclusion improved the AID of 10 (arginine, lysine, isoleucine, glycine, leucine, glutamic acid, serine, aspartic acid, hydroxyproline, and phenylalanine) out of 22 amino acids analyzed at 14 D and 5 (hydroxyproline, methionine, cysteine, glycine, and ornithine) of 22 amino acids at 35 D; not all listed amino acids are shown in tables. These observations show that as birds 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., 1999b; Cowieson et al., 2004). Thus, phytase improved AID of certain amino acids by degrading phytate. Furthermore, phytase has an effect on the sodium-dependent transporters, which may also contribute to the observed effects (Truong et al., 2015). The various Ca and $P_i$ ratios did not significantly affect AID of amino acids at 14 D, but Ca and $P_i$ ratios at high levels did positively influence the AID for 6 (hydroxyproline, threonine, cysteine, methionine, ornithine, and histidine) of 21 amino acids at 35 D.

At 14 D, the interaction between PS and phytase was significant; other interactions were not significant. The majority of the amino acids tested showed coarse PS without phytase as the only treatment that was different and had a low AID. Inclusion of phytase in coarse PS treatments resulted in significant improvement in amino acid AID, making it similar to fine PS treatment either with or without phytase. This indicated that phytase enzyme had a stronger influence with coarse limestone PS, which was also observed by Kim et al. (2018) who noted a negative effect of dietary Ca on $P_i$ AID which could be fixed when a larger PS of limestone (400 μm) was used in the presence of phytase, however the effect of fine PS could not be reversed with phytase inclusion. At 35 D, the majority of the amino acids' (hydroxyproline, glutamic acid, proline, glycine, alanine, cysteine, valine, isoleucine, leucine, tyrosine, phenylalanine, hydroxylysine, ornithine, and histidine) AIDs were influenced by the interaction between Ca and $P_i$ levels and phytase. There was also a significant interaction between PS and Ca and $P_i$ levels. Fine PS+PC treatments had greater amino acid AIDs than other treatments.

The results of this experiment demonstrated that phytase inclusion improved live performance (FI and BWG), bone ash (14 D), and nutrient digestibility (except Ca at 35 D), but it is most effective at younger ages; however, adding phytase to diets formulated with sufficient amount of Ca and $P_i$ did not add any benefits. Phytase also improved $P_i$ AID at both 14 D and 35 D, indicating its effectiveness in decreasing $P_i$ content in excreta. On the other hand, PS of 190 μm and 900 μm did not alter tibia bone ash or live performance, although it is possible that NC treatment with coarse PS until 28 D can lead to a live performance similar to the PC treatment with fine limestone PS. The fine PS improved Ca and $P_i$ digestibility, but this did not improve live performance compared with coarse PS. This indicates that transporters in the digestive tract play a huge role in intestinal uptake and that they can be upregulated or downregulated because of FI and nutrient concentration, which can also influence digestibility. Ca and $P_i$ levels were the most influential factors in determining bone ash, although phytase inclusion can lead to an improvement during early days. Results of tibia bone ash and digestibility analyses point to the fact that phytase is more effective when used with NC treatments and with coarse PS.

## REFERENCES

Adedokun, S. A., and O. Adeola. 2013. Calcium and phosphorus digestibility: metabolic limits. J. Appl. Poult. Res. 22:600–608.

Amerah, A. M., V. Ravindran, R. G. Lentle, and D. G. Thomas. 2008. Influence of feed particle size on the performance, energy utilization, digestive tract development, and digesta parameters of broiler starters fed wheat- and corn-based diets. Poult. Sci. 87:2320–2328.

ANSI/ASAE S319. 201 2008. Method of determining and expressing fineness of feed materials by sieving. In: A. S. Engineers (Ed.), American Society of Agricultural and Biological Engineers. 2008 R2012. American Society of Agricultural and Biological Engineers, St. Joseph, MI.

Anwar, N. M., V. Ravindran, P. C. H. Morel, G. Ravindran, and A. J. Cowieson. 2016. Effect of LPS and calcium to non-phytate phosphorus ratio on true ileal calcium digestibility of limestone for broiler chickens. Br. Poult. Sci. 57:707–713.

AOAC 2006a. Official Methods of Analysis Amino Acids Analysis Standard Protein Hydrolysate (SPH) - Item 69, 17th ed. Association of Analytical Communities, Gaithersburg, MD. Reference data: Method 982.30 (45.3.05); NFNAP; NITR; PER.

AOAC 2006b. Official Methods of Analysis Minerals Analysis Flame Atomic Absorption Spectroscopy (FAAS) - Item 58, 17th ed. Association of Analytical Communities, Gaithersburg, MD. Reference data: Method 968.08; NFNAP; MIN.

Applegate, J. T., and R. Angel. 2008. Phosphorus Requirement of Poultry. Purdue University and University of Maryland extension. Accessed Jan. 2019. https://s3.wp.wsu.edu/uploads/sites/346/2014/11/Phosphorus-requirements-for-poultry-final-jhh.pdf.

Bahadoran, R., A. Gheisari, and M. Toghyani. 2011. Effects of supplemental microbial phytase enzyme on performance and phytate phosphorus digestibility of a corn-wheat-soybean meal diet in broiler chicks. Afri. J. Biotech. 10:6655–6662.

Blount, W. 2013. Poultry Nutrition, Food and Feeding. Page 103 in Intensive Livestock Farming. Elsevier, Amsterdam, Netherlands.

Bougouin, A., J. A. D. R. N. Appuhamy, E. Kebreab, J. Dijkstra, R. P. Kwakkel, and J. France. 2014. Effects of phytase supplementation on phosphorus retention in broilers and layers: a meta-analysis. Poult. Sci. 93:1981–1992.

Bradbury, E. J., S. J. Wilkinson, G. M. Cronin, C. L. Walk, and A. J. Cowieson. 2016. Effects of phytase, calcium source, calcium concentration and particle size on broiler performance, nutrient digestibility and skeletal integrity. Anim. Prod. Sci. 58:271–283.

Bradbury, E. J., S. J. Wilkinson, G. M. Cronin, C. L. Walk, and A. J. Cowieson. 2018. Effects of phytase, calcium source, calcium concentration and particle size on broiler performance, nutrient digestibility and skeletal integrity. Anim. Prod. Sci. 58:271–283.

Broz, J., P. Oldale, A.-H. Perrin-Voltz, G. Rychen, J. Schulze, and C. Simoes Nunes. 1994. Effects of supplemental phytase on performance and phosphorus utilisation in broiler chickens fed a low phosphorus diet without addition of inorganic phosphates. Br. Poul. Sci. 35:273–280.

Bueno, I. J. M., D. Surek, C. Rocha, V. G. Schramm, K. Muramatsu, F. Dahlke, and A. Maiorka. 2016. Effects of different limestone particle sizes in the diet of broiler breeders post molting on their performance, egg quality, incubation results, and pre-starter performance of their progeny. Poult. Sci. 95:860–866.

Ceylan, N., S. Cangir, M. Corduk, A. Grigorov, and S. H. Golzar-Adabi. 2012. The effects of phytase supplementation and dietary phosphorus level on performance and on tibia ash and phosphorus contents in broilers fed maize-soya based diets. J. Anim. Feed Sci. 21:696–704.

Cheng, T. K., and C. N. Coon. 1990. Effect of calcium source, particle size, limestone solubility *in vitro*, and calcium intake level on layer bone status and performance. Poult. Sci. 69:2214–2219.

Cobb. 2018. Cobb 500 Broiler performance and nutrition supplement. Accessed Jan. 2019. https://www.cobb-vantress.com/assets/Cobb-Files/product-guides/bdc20a5443/70dec630-0abf-11e9-9c88-c51e407c53ab.pdf.

Cowieson, A. J., and O. Adeola. 2005. Carbohydrates, protease, and phytase have an additive beneficial effect in nutritionally marginal diets for broiler chicks. Poult. Sci. 84:1860–1867.

Cowieson, A. J., T. Acamovic, and M. R. Bedford. 2004. The effects of phytase and phytic acid on the loss of endogenous amino acids and minerals from broiler chickens. Br. Poult. Sci. 45:101–108.

de Witt, F. H., H. J. van der Merwe, J. P. Hayes, and M. D. Fair. 2006. Influence of particle size distribution on in vivo and *in vitro* limestone solubility. S. Afr. J. Anim. Sci. 36:95–98.

Denbow, D. M., V. Ravindran, E. T. Kornegay, Z. Yi, and R. M. Hulet. 1995. Improving phosphorus availability in soybean meal for broilers by supplemental phytase. Poult. Sci. 74:1831–1842.

Dilger, R. N., and O. Adeola. 2006. Estimation of true phosphorus digestibility and endogenous phosphorus loss in growing chicks fed conventional and low-phytate soybean meals. Poult. Sci. 85:661–668.

dos Santos, T., S. Srinongkote, M. Bedford, and C. Walk. 2013. Effect of high phytase inclusion rates on performance of broilers fed diets not severely limited in available phosphorus. Asian-australas. J. Anim. Sci. 26:227–232.

Driver, J. P., G. M. Pesti, R. I. Bakalli, and H. M. Edwards. 2005. Calcium requirements of modern broiler chicken as influenced by dietary protein and age. Poult. Sci. 84:629–1639.

Edwards, Jr, H.M., W. S. Dunahoo, J. L. Carmon, and H. L. Fuller. 1960. Effect of protein, energy and fat content of the ration on calcium utilization. Poult. Sci. 39:1389–1394.

Eusebio-Balcazar, P. E., S. Purdum, K. Hanford, and M. M. Beck. 2018. LPS fed to pullets influences subsequent bone integrity of hens. Poult. Sci. 97:1471–1483.

FASS 2010. Guide for the Care and Use of Agricultural Animals in Research and Teaching, 3rd ed. Federation of Animal Science Society, Champaign, IL.

Gautier, A. E., C. L. Walk, and R. N. Dilger. 2018. Effects of a high level of phytase on broiler performance, bone ash, phosphorus utilization, and phytate dephosphorylation to inositol. Poult. Sci. 97:211–218.

Guinote, F., and Y. Nys. 1991. Effects of particle size and origin of calcium sources on eggshell quality and bone mineralization in egg laying hens. Poult.Sci. 70:583–592.

Hall, L. E., R. B. Shirey, R. I. Bakalli, S. E. Aggrey, G. M. Pesti, and H.M. Edwards, Jr. 2003. Power of two methods for the estimation of bone ash of broilers. Poult. Sci. 82:414–418.

Heaney, R. P., and B. E. C. Nordin. 2002. Calcium effects on phosphorus absorption: implications for the prevention and co-therapy of osteoporosis. J. Am. Coll. Nutr. 21:239–244.

Hurwitz, S., and A. Bar. 1971. Calcium and phosphorus interrelationships in the intestine of the fowl. J. Nutr. 101:677–685.

Joardar, D. 2019. Effect of Dietary Phosphorus and Potassium in Broiler Breeders during Onset of Lay and Effect of Dietary Limestone Particle Size, Potassium and Phytase in Broilers. PhD Diss. NC State, Raleigh.

Johnston, S. L., and L. L. Southern. 2000. The effect of varying mix uniformity (simulated) of phytase on growth performance, mineral retention, and bone mineralization in chicks. Poult. Sci. 79:1485–1490.

Kim, S.-W., W. Li, R. Angel, and M. Proszkowiec-Weglarz. 2018. Effects of LPS and dietary Ca concentration on apparent PI and Ca digestibility in the presence or absence of phytase. Poult. Sci. 97:4306–4314.

Li, J., J. Yuan, Y. Guo, Q. Sun, and X. Hu. 2012. The influence of dietary calcium and phosphorus imbalance on intestinal NaPi-IIb and Calbindin mRNA expression and tibia parameters of broilers. Asian-australasia J. Anim. Sci. 25:552–558.

Li, W., R. Angel, S. W. Kim, E. Jiménez-Moreno, M. Proszkowiec-Weglarz, and P. W. Plumstead. 2015. Impact of response criteria (tibia ash weight vs. percent) on phytase relative non phytate phosphorus equivalence. Poult. Sci. 94:2228–2234.

Managi, M. K., and C. N. Coon. 2007. The effect of calcium carbonate particle size and solubility. Int. J. Poult. Sci. 6:85–90.

McNaughton, J. L., B. C. Dilworth, and E. J. Day. 1974. Effect of particle size on the utilization of calcium supplements by the chick. Poult. Sci. 53:1024–1029.

Morgan, N. K., C. L. Walk, M. R. Bedford, and E. J. Burton. 2015. Contribution of intestinal- and cereal-derived phytase activity on phytate degradation in young broilers. Poult. Sci. 94(7):1577–1583.

Myers, W. D., P. A. Ludden, V. Nayigihugu, and B. W. Hess. 2004. Technical Note: a procedure for the preparation and quantitative analysis of samples for titanium dioxide. J. Anim. Sci. 82:179–183.

National Research Council 1994. Nutrient Requirements of Poultry, 9th rev. Natl. Acad. Press, Washington, DC.

Nelson, T. S., G. C. Harris, L. K. Kirby, and Z. B. Johnson. 1990. Effect of calcium and phosphorus on the incidence of leg abnormalities in growing broilers. Poult. Sci. 69:1496–1502.

Nelson, T. S., T. R. Shieh, R. J. wodzinski, and J. H. Ware. 1971. Effect of supplemental phytase on the utilization of phytate phosphorus by chicks. J. Nutr. 101:1289–1294.

Olukosi, O. A., A. J. Cowieson, and O. Adeola. 2007. Age-related influence of a cocktail of xylanase, amylase and protease or phytase individually or in combination in broilers. Poult. Sci. 86:77–86.

Onyango, E. M., M. R. Bedford, and O. Adeola. 2005. Efficacy of an evolved Escherichia coli phytase in diets for broiler chicks. Poult. Sci. 84:248–255.

Onyango, E. M., P. Y. Hester, R. Stroshine, and O. Adeola. 2003. Bone densitometry as an indicator of percentage tibia ash in broiler chicks fed varying dietary calcium and phosphorus levels. Poult. Sci. 82:1787–1791.

Paik, I. 2003. Application of phytase, microbial or plant origin, to reduce phosphorus excretion in poultry production. Asian-austral. J. Anim. Sci. 16:124–135.

Potter, L. M., M. Potchanakorn, V. Ravindran, and E. T. Kornegay. 1995. Bioavailability of phosphorus in various phosphate sources using body weight and toe ash as response criteria. Poult. Sci. 74:813–820.

Qian, H., H. P. Veit, E. T. Kornegay, V. Ravindran, and D. M. Denbow. 1996. Effects of supplemental phytase and phosphorus on histological and other tibial bone characteristics and performances of broilers fed semi-purified diets. Poult. Sci. 75:618–626.

Ravindran, V., S. Cabahug, G. Ravindran, P. H. Selle, and W. L. Bryden. 2000. Response of broiler chickens to microbial phytase supplementation as influenced by dietary phytic acid and non-phytate phosphorus levels. II. Effects on apparent metabolisable energy, nutrient digestibility and nutrient retention. Br. Poult. Sci. 41:193–200.

Ravindran, V., P. H. Selle, G. Ravindran, P. C. H Morel, A. K. Kies, and W. L. Bryden. 2001. Microbial phytase improves performance, apparent metabolizable energy and ileal amino acid digestibility of broilers fed a lysine-deficient diet. Poult. Sci. 80:338–344.

Ravindran, V., P. H. Selle, and W. L. Bryden. 1999a. Effects of phytase supplementation, individually and in combination, on the nutritive value of wheat and barley. Poult. Sci. 78:1588–1595.

Ravindran, V., S. Cabahug, G. Ravindran, and W. L. Bryden. 1999b. Influence of microbial phytase on apparent ileal amino acid digestibility in feedstuffs for broilers. Poult. Sci. 78:699–706.

Roland, D. A. Sr. 1986. Eggshell quality IV: oystershell versus limestone and the importance of particle size or solubility of calcium source. World's Poult. Sci. J. 42:166–171.

Ross. 2014. Ross 308 broiler nutrient specifications. Accessed Jan. 2019. http://www.garantitavukculuk.com/doc/Ross_nutrition_spec.pdf.

Sabbagh, Y., H. Giral, Y. Caldas, M. Levi, and S. C. Schiavi. 2011. Intestinal phosphate transport. Adv. Chronic Kidney Dis. 18:85–90.

SAS 2017. The SAS System for Windows No. 9.4. SAS Institute, Cary, NC.

Scholey, D., E. Burton, N. Morgan, C. Sanni, C. K. Madsen, G. Dionisio, and H. Brinch-Pedersen. 2017. PI and Ca digestibility is increased in broiler diets supplemented with the high-phytase HIGHPHY wheat. Animal 11:1457–1463.

Selle, P. H., and V. Ravindran. 2007. Microbial phytase in poultry nutrition. A review. Anim. Feed Sci. Technol. 135:1–41.

Selle, P. H., P. H. Pittolo, and W. L. Bryden. 2001. Individual and combined supplementation of wheat-based broiler diets with phytase and xylanase. Proc. Aust. Poult. Sci. Symp. 13:246.

Selle, P. H., V. Ravindran, G. Ravindran, P. H. Pittolo, and W. L. Bryden. 2003. Influence of phytase and xylanase supplementation on growth performance and nutrient utilisation of broilers offered wheat-based diets. Asian–australas. J. Anim. Sci. 16:394–402.

Selle, P. H., V. Ravindran, P. H. Pittolo, and W. L. Bryden. 1999. An evaluation of microbial phytase in sorghum-based broiler diets. Proc. Aust. Poult. Sci. Symp. 11:97–100.

Selle, P. H., V. Ravindran, R. A. Caldwell, and W. L. Bryden. 2000. Phytate and phytase: consequences for protein utilization. Nutr. Res. Rev. 13:255–278.

Shaw, A. L., J. B. Hess, J. P. Blake, and N. E. Ward. 2011. Assessment of an experimental phytase enzyme product on live performance, bone mineralization, and phosphorus excretion in broiler chickens. J. Appl. Poult. Res. 20:561–566.

Simons, P. C. M., H. A. J. Versteegh, A. W. Jongbloed, P. A. Kemme, P. Slump, K. D. Bos, M. G. E. Wolters, R. F. Buedeker, and G. J. Verchoor. 1990. Improvement of phosphorus availability by microbial phytase in broilers and pigs. Br. J. Nutr. 64:525–540.

Singh, A., C. L. Walk, T. K. Ghosh, M. R. Bedford, and S. Haldar. 2013. Effect of a novel microbial phytase on production performance and tibia mineral concentration in broiler chickens given low-calcium diets. Br. Poult. Sci. 54:206–215.

Singh, P. K., V. K. Khatta, R. S. Thakur, S. Dey, and M. L. Sangwan. 2003. Effects of phytase supplementation on the performance of broiler chickens fed maize and wheat based diets with different levels of non-phytate phosphorus. Asian-australasia J. Anim. Sci. 16:1642–1649.

Suttle, F. N. 2010. Calcium. In: In Mineral Nutrition of Livestock, 4th ed. CAB International, Cambridge, MA, USA, pp. 54–56.

Truong, H. H., R. M. Bold, S. Y. Liu, and P. H. Selle. 2015. Standard phytase inclusion in maize-based broiler diets enhances digestibility coefficients of starch, amino acids and sodium in four small intestinal segments and digestive dynamics of starch and protein. Anim. Feed Sci. Tech. 209:240–248.

Venäläinen, E., J. Valaja, and T. Jalava. 2006. Effects of dietary metabolisable energy, calcium and phosphorus on bone mineralisation, leg weakness and performance of broiler chickens. Br. Poult. Sci. 47:301–310.

Wise, A. 1983. Dietary factors determining the biological activities of phytase. Nutr. Abstr. Rev. 53:791–806.

Zhang, B., and C. N. Coon. 1997a. The relationship of calcium intake, source, size, solubility *in vitro* and in vivo, and gizzard limestone retention in laying hens. Poult. Sci. 76:1702–1706.

Zhang, B., and C. N. Coon. 1997b. Improved *in vitro* methods for determining limestone and oyster shell solubility. J. Appl. Poult. Res. 6:94–99.

Ziaei, N., J. H. Guy, S. A. Edwards, P. J. Blanchard, J. Ward, and D. Feuerstein. 2008. Effect of reducing dietary mineral content on growth performance, water intake, excreta dry matter content and blood parameters of broilers. Br. Poult. Sci. 49:195–201.