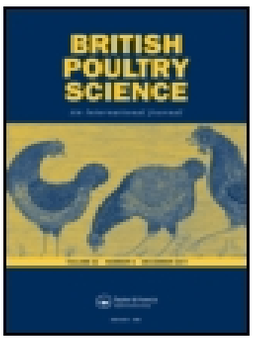

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Influence of bone meal deglatinization and calcium source and particle size on broiler performance, bone characteristics and digestive and plasma alkaline phosphatase activity

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Influence of bone meal degelatinisation and calcium source and particle size on broiler performance, bone characteristics and digestive and plasma alkaline phosphatase activity

Abstract

1. The current experiment was performed to elucidate the effects of degelatinised bone meal (DBM) in combination with different particle sizes of limestone or oyster shell on broiler performance, bone characteristics and digestive and plasma alkaline phosphatase (ALP) activity.

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2. Treatments were applied as a 3 × 3 factorial arrangement with three sources of P (DCP, bone meal and DBM) and three particle sizes (50, 100 and 200 μm) of limestone. Chickens were given either DCP or DBM with oyster shell (523 μm), resulting in a total of 11 treatments with five replicates of eight chicks.

3. Performance criteria were measured weekly. Tibia strength, ash, calcium (Ca) and phosphorus (P) content and plasma P and Ca concentration along with plasma and intestinal alkaline phosphatase (ALP) activity and P digestibility were measured on d 14 and 28.

4. Body weight and FCR were improved in chicks which were fed DBM or oyster shell in comparison to the DCP and limestone respectively ($P \leq 0.05$). Performance was influenced ($P \leq 0.05$) by particle size; with coarser particles BW and feed intake were increased ($P \leq 0.05$). Tibia shear force and P content were reduced ($P \leq 0.001$), whereas tibia shear energy, length, ash and Ca content were increased by substitution of DCP with DBM or bone meal ($P \leq 0.001$; $P \leq 0.05$). A significant difference was observed in the tibia length between the chicks fed oyster shell or limestone with different particles ($P \leq 0.05$). Plasma P concentration was reduced in chicks were fed with DBM, bone meal and lower limestone particle size. Intestinal ALP activity was increased ($P \leq 0.001$) in chicks which were fed DBM, bone meal, oyster shell or coarse particles of limestone. The P digestibility in chicks fed bone meal was lower than that of those fed DBM or DCP ($P \leq 0.01$). Overall, gelatin removal from bone meal improved broiler bone characteristics through the P digestibility and intestinal ALP activity enhancement.

Key words: degelatinisation, P source, Ca source, limestone, bone characteristics, alkaline phosphatase.

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INTRODUCTION

P and Ca are essential minerals and the major mineral components of broiler rations. The former is obtained from the phosphate rock, and the latter from the limestone and oyster shell. The phosphate rock reserves available to supply agricultural demands are diminishing severely in quality and quantity (Von Horn and Sartorius, 2009). Phosphate reserves that were mined were around 12,000 million tons of rock in 2001 according to reports by the FAO (2004). These reserves are much less than the global need for this raw material. Another important issue with phosphate rock is the existence of the heavy metal contamination, which tends to increase with further exploitation of mines (Von Horn and Sartorius, 2009). Furthermore, it has been reported that common processing of phosphate rock for incorporation into livestock diets does not eliminate heavy metals. Cordell and White (2013) mentioned that it is essential to seek possible alternatives to supply agriculture against the long and short-term impacts of global P scarcity in a sustainable manner. Cordell and White (2013) emphasised on the importance not just to improve efficiency of P sources e.g. by phytase supplementation in livestock feed, but to take a systematic approach to move towards sustainable recycling of P from waste materials.

Bone meal is considered a direct recycling ingredient used in the animal feed sector, which contains high P concentrations. Simply, P can be readily recovered from bones *via* low cost and simple technology, by crushing, degreasing and drying under high temperature conditions. However, there is substantial variation both in the P content and digestibility for bone meal, which depends on the chemical and thermo-physical conditions applied during processing (Simons *et al.*,1991; Van der Klis and Versteegh, 1992; Mutucumarana *et al.*, 2014). Van Harn *et al.* (2017) mentioned that digestibility of bone meal P is lower than for P from inorganic phosphate. Nonetheless, they proposed possible increase in bone P digestibility by application of

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a new processing technique (Van Harn *et al.*, 2017). It has been postulated that gelatin, a substance found in bones, prevents P release from the bone matrix. Recently, Van Harn *et al.* (2017) demonstrated that degelatinisation of bone meal increased the pre-caecal P digestibility in broilers, due to increased surface area and crystallinity of bone meal, thus improving P digestion and absorption in the small intestine (Van Harn *et al.*, 2017).

It is well documented that Ca solubility and digestibility in broiler intestines is highly correlated with source and particle size (Shih *et al.*, 2000; Anwar *et al.*, 2017; Kim *et al.*, 2018). Utilisation of P, especially phytate P in broilers, depends mainly on the concentration of Ca in the gastrointestinal tract, principally the amount which is soluble in the upper part of the gastrointestinal tract (Tamim and Angel, 2003; Rutherfurd *et al.*, 2012; Kim *et al.*, 2018). Highly soluble Ca sources produce excessive quantities of Ca in the small intestine lumen, which reduce P utilisation due to less intestinal alkaline phosphatase activity and the formation of Ca-phytate complexes (Tamim and Angel, 2003; Rutherfurd *et al.*, 2012; Manangi *et al.*, 2018). Anwar *et al.* (2017) reported that coarse particles of limestone and oyster shell are less soluble and are more likely to be retained in the gizzard, increasing digestibility of both Ca and P. Conversely, Zhu *et al.* (2018) reported that high Ca level elevated serum alkaline phosphatase activity and reduced bone mineralisation in Pekin ducklings. Anderson *et al.*, (1984) showed that finer particles of limestone caused a greater reduction in weight gain. Likewise, Guinotte and Nys (1991) reported that feed intake and weight gain were increased in chicks fed coarse when compared with fine ground oyster shell.

Limestone and oyster shell are major sources of Ca used in poultry diets (Anwar *et al.*, 2017). Ca is present in the form of carbonate in both sources, but limestone is inorganic Ca of calcitic origin while oyster shell is organic Ca of marine origin (Anwar *et al.*, 2017). Studies have shown

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a huge difference with regards to the availability of Ca in limestone and oyster shell for broiler chickens (Augspurger and Baker, 2004; Anwar *et al.*, 2016a; Anwar *et al.*, 2017). It should be emphasised that earlier studies have mentioned factors which can affect the rate of Ca release from Ca sources, such as solubility and origin, could considerably affect P utilisation or *vice versa* (Tamim and Angel, 2003; Rutherfurd *et al.*, 2012; Manangi *et al.*, 2018). Therefore, the rate of Ca and P release from ingredients plays an important role on their utilisation. Understanding the interaction between Ca and P sources and their particle sizes may open up new opportunities to choose more suitable sources and particle size required for optimum growth and bone mineralisation.

The experiment reported in this paper was conducted to ascertain the utilisation of different P sources with different solubility and digestibility in broiler chicken, and to identify and characterise whether interaction between P and Ca source or particle size in diet results in changes in P utilisation or not. Therefore, the current study was designed for further elucidation of the effects of the Ca particle size and sources in combination with less soluble but highly digestible P from DBM on bone response criteria in broiler. The hypothesis was that using the coarse limestone particles along with steadily but highly digestible P sources (DBM) would eliminate the adverse effects of fine Ca particles and highly soluble Di-Ca phosphate (DCP) on P digestibility and skeletal abnormalities by improving the intestinal phosphatase activity. Moreover, the independent role of limestone particles on P utilisation from different sources and the interaction between common Ca source (oyster shell or limestone) from different P sources (DBM and DCP) was investigated using a 2×2 factorial arrangement by merging two separate experiments into one experimental design.

Materials and methods

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Bone meal, degelatinised bone meal, limestone and oyster shell

A batch of male bull bones (300 kg) was obtained from a local slaughter house and were washed with water and debris was removed. The bone meal was prepared by crushing and drying of fat free bones at 130°C. To producing the DBM, gelatin was removed from bones using the method described by Ulfa *et al.* (2015). Briefly, the cleaned bones were soaked in 1M NaOH and non-collagenous proteins and pigments were removed. The bones were then soaked in hydrochloric acid (HCl) for five days with slow stirring at 50°C. The dissolved gelatin was removed by filtration and the remaining phosphate was precipitated by addition of Ca hydroxide. Thereafter, samples were dried and evaporated at 80°C for 24 hours.

Oyster shell and limestone with different particle sizes (geometric mean diameter 50, 100 and 200 µm), from the same origin and same analysed Ca concentration (391 g/kg) were purchased from a local company. Total P content of the bone meal, DBM and DCP were measured by digestion with hydrochloric and nitric acid and P was determined by the ammonium molybdate method, and colour intensity was determined using a spectrophotometer (UNICO 2150, Germany) measuring absorbance at 340 nm (method 946.06, AOAC International, 2000). The bone meal, DBM, DCP, limestone and oyster shell were digested in perchloric acid and the Ca was quantified by triethanolamine (50%) measuring absorbance at 600 nm. The Ca and P composition of ingredients are shown in Table 1.

Table 1 here

Experimental Design and Diets

In total, 440 one-day-old male Ross-308 broilers with an initial body weight (BW) of 40 ±1.1 g (parent stock aged 39 weeks) were obtained from a local hatchery and were allotted randomly to

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55 experimental pens. The experiment was a completely randomised design in a 3 × 3 factorial arrangement, with three sources of P (DCP, bone meal and DBM) and three particle sizes (50, 100 and 200 μm) of limestone. Furthermore, chickens were given either DCP or DBM with oyster shell (523 μm). so that final dietary arrangement consisted of 11 treatments, of which nine treatments resulted from three sources of P with three particle size of limestone, plus two treatments with oyster shell compared with the limestone (200 μm) using a 2×2 factorial arrangement with five replicates of eight Ross 308 male chicks in each. Control and experimental diets are shown in Table 2.

Table 2 here

For each treatment, a batch of 200 kg of each ration was mixed by using a Twin-Shaft Paddle Mixer. Batches of the resulting 11 experimental diets were then analysed for crude protein, Ca and P content. The birds were reared in pens with wood shavings as litter material over a concrete floor. The room temperature was thermostatically set by automation systems using two heaters and one fogger. Bell drinkers joined the same polyethylene tank were used and water was provided *ad libitum*. Cumulative feed consumption for chicks in each pen was calculated weekly by weighing the total amount of feed placed in pan feeder minus any residual feed. Body weight was measured using a digital weight balance (6.1 kg capacity, model, GF-6100, US). Feed conversion ratio (FCR) for each bird in each pen was calculated by dividing the total weight of consumed feed by the live weight of birds.

Bone Characteristics

Two chicks from each pen were slaughtered by cervical dislocation on 14 and 28 d of the experiment and the left and right tibias of each bird were excised, sealed in plastic bags, and

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stored at -20°C until analysis. Tibia length was measured using a digital caliper (Neiko 01407A, Japan) and its breaking strength was determined with an All-Digital Electronic Universal Testing Machine (Santam Instrument Co. Model-MRT-5, serial no. 628415) fitted with a three point-bend ring with a load cell capacity of 50 kg, a crosshead speed of 5 mm/min, and the span over which the bone was set was 40 mm. The tibia ash and tibia Ca and P content were measured after fat removal by 36-h Soxhlet extraction in ethyl alcohol. Tibia ash content was determined using a muffle furnace for four hours at 600°C. The ash content of tibia was digested with hydrochloric and nitric acids and P was determined by ammonium molybdate method using a spectrophotometer (UNICO 2150, Germany) at an absorbance of 340 nm (method 946.06, AOAC International, 2000). Determination of Ca content in tibia ash was conducted by digestion in perchloric acid, and Ca was quantified by Triethanolamine (50%), and KOH 4N, using a spectrophotometer (UNICO 2150, Germany) with measuring absorbance at 600 nm.

Blood Characteristics

Two chicks from each pen were selected randomly and blood samples were collected into heparinised tubes by puncturing the brachial vein after 4 h starvation on d 14 and 28. Samples were immediately centrifuged for 10 minutes at 3,000 × *g* at 20°C, and frozen at -20°C. Plasma Ca and P concentrations were determined with a Sysmex KX-21N automated haematology analyser using a colorimetric method. Ca was determined with ArsenazoIII method by using a Bionik Diagnostic kit (Lot no. 140335) measuring the colour intensity at wavelength 630 nm at 37°C. For P measurement, plasma samples (free of haemolysis and clots) were rapidly collected into a bottle to avoid elevation of serum P from hydrolysis or leakage of phosphate present in erythrocytes and then transferred into a bottle containing 10 ml of 10% v/v hydrochloric acid to avoid phosphate precipitation, adjusted to pH 2, diluted with distilled water and determined

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directly using an Audit Diagnostic kit (catalogue no. I. 874, Ireland) by reaction with ammonium molybdate as a phosphomolybdate and measuring the colour intensity at wavelength of 340 nm. Alkaline phosphatase activity was measured at 14 and 28 d of age using an automatic biochemical analyser (Hitachi 917, Boehringer Mannheim, Ingelheim am Rhein, Germany) using a Bionik Diagnostic kit (catalog no. A. 110537) measuring the colour intensity at 405 nm.

Intestinal alkaline phosphatase activity measurement

Alkaline phosphatase activity was assayed following the procedure described by Walter and Schutt (1974) at 14 and 28 d of age. Two chicks from each pen were randomly chosen, weighed and slaughtered by cervical dislocation. Before sampling, feed was removed from feeders for 6 h to eliminate digesta from the bird's intestinal lumen. The duodenum and a 10-cm segment of the jejunum adjacent to the distal pancreas, free of residual digesta, were removed and frozen in liquid nitrogen until assay. The intestine samples were thawed at 4°C for 2 h and then homogenised (dilution 1:5, w/v) in cold buffer (50 mM Tris–HCl, pH 8.0 containing 10 mM $CaCl_2$) on ice at 11000 rpm for 2 min. The homogenised samples were centrifuged at 14000 x g for 45 min at 4°C. The resultant supernatants were collected and aliquots were stored at -80°C until analysis. Alkaline phosphatase activity was measured at 37°C by spectrophotometer. Homogenates were incubated with p-nitrophenyl phosphate as the substrate. The increase in absorbance was measured continuously for 30 min at 405 nm.

P digestibility and Gizzard weight

Control and experimental diets were ground before the P analysis. The apparent ileal digestibility of P was calculated based on the formula described by Nyachoti *et al.* (1997) using Cr_2O_3 (2 g/kg in feed) as the inert marker. On d 25 to 26 of the experiment, the digestive tract, along with its contents, was removed aseptically from two chicks and the ileum was separated from Meckel's

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diverticulum up to 1 cm proximal to the ileo-caecal junction, dried with a towel paper; and then the digesta was slowly collected into plastic cups from half of the ileum by flushing with distilled water. Two samples from two chicks in each pen were pooled and frozen until being lyophilised and ground. Digesta and feed samples were ground through a 1-mm screen. Samples were then used to determine dry matter content following drying at 105°C for 24 h. Diets and digesta samples were digested with hydrochloric and nitric acid and P was determined by the ammonium molybdate method (as above). Chromium concentration in the diet and ileal digesta were determined using flame absorption spectrophotometry (Analytikjena, contrAA 700, Germany). On 28 d of age, one chick from each pen with a body weight close to the mean weight of pen was selected and then was slaughtered by cervical dislocation for gizzard weight determination.

Statistical methods

Data were analysed using the MIXED procedure of SAS (SAS Institute, 2003) software. Normal distribution of the residual was tested by the UNIVARIATE procedure of SAS. Orthogonal polynomial contrasts were used to compare the Ca sources (limestone (200 μm) or oyster shell) and effects between birds fed the DCP or DBM as a P source, using the completely randomised design in a factorial arrangement (two sources of Ca × two sources of P). Data from feeding treatments resulting from the factorial arrangement of three sources of P (DCP, bone meal and DBM) and three particle sizes (50, 100 and 200 μm) of limestone were tested as main effects and their interactions using a two-way ANOVA. Where an effect was significant, differences among treatment means were tested using the Tukey's multiple comparison test. The results were reported as means, and differences among treatments were considered significant at a threshold of $P < 0.05$. Plasma alkaline phosphatase activity was subjected to log10 transformation before analysis.

Results

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Performance

Degelatinisation significantly reduced protein content (247 *vs*. 6 g/kg) of bone (Table 1). Significant differences (P≤0.05) were observed between weight gain and FCR of chicks fed DCP, bone meal or DBM at 7 d of age (Table 3 and 4). Both BW and FCR were increased by inclusion of DBM instead of DCP in diets at 7 d of age; however, FCR was improved at 28 d of age in DBM fed chicks. Feed conversion ratio was increased by inclusion of oyster shell in comparison to the limestone on d 7 and 14 (P≤0.05), whereas body weight gain showed no differences among treatments (Table 3; P≥0.05).

Table 3 here

There was no interaction (P>0.05) between P source and Ca source for performance criteria. Feed intake and BW were observed to be higher when chicks were fed coarse particles of limestone on d 14 and 28 (Table 4; P>0.05). There was no significant interaction between the particle size of limestone and P source for performance parameters (Table 4).

Table 4 here

Bone Characteristics

Tibial shear force was reduced by substitution of DCP with DBM at 28 d of age (P≤0.001), whereas shear energy increased in chicks fed with diets contained bone meal and DBM at 14 d of age (P≤0.05; Table 5).

Table 5 here

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Tibia length elongation increased in chicks fed bone meal and DBM instead of DCP only on d 14 ($P \leq 0.001$). Tibia Ca content (on d 14 and 28) was increased and tibia P (on d 14) was decreased by substitution of DCP with DBM (Table 5; $P \leq 0.05$). Likewise, tibia ash (on d 14 and 28) and Ca (on d 28) content were increased and tibia P (on d 14) was decreased by substitution of DCP with DBM or bone meal (Table 6; $P \leq 0.05$). A difference was only observed in tibia length between the chicks fed oyster shell or limestone, but other bone criteria showed no differences in response to the Ca source ($P \leq 0.05$; Table 5). An interaction was observed between P and Ca source for tibia Ca content, which possessed the lowest concentration of Ca with diets contained DCP and oyster shell ($P \leq 0.05$; Table 5). Tibial criteria, except for length, did not change in response to Ca particle size (Table 6). No interaction was found between the P source and limestone particle size for bone measured characteristics.

Table 6 here

Blood Characteristics

Plasma P concentration on d 14 of age and plasma Ca concentration at 28 d of age showed meaningful ($P \leq 0.05$) reduction by substitution of DCP with bone meal or DBM (Table 7 and 8 respectively). Plasma alkaline phosphatase activity did not change either in response to the Ca or P source or limestone particle size (Table 7 and 8 respectively). Oyster shell inclusion decreased plasma P concentration on d 28 (Table 7). An interaction was observed between Ca and P source for plasma P concentration and ALP activity, which was associated with a large reduction of

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plasma P on d 14 and enhancement on d 28 by incorporation of DBM instead of DCP with both the limestone and oyster shell (Table 7).

Table 7 here

A quadratic response was observed for plasma P concentration by increasing limestone particle size at 28 d of age (Table 8). Plasma alkaline phosphatase activity was not affected by changing the limestone particle size. No interaction was found between P source and limestone particle size for plasma Ca and P concentration or alkaline phosphatase activity.

Table 8 here

Intestinal Alkaline Phosphatase Activity

A large difference was seen in alkaline phosphatase activity in response to the P and Ca source (Table 7). Significant increases in ALP activity were seen in broilers fed DBM and bone meal instead of DCP on d 14 and 28 ($P \leq 0.001$; Table 7). ALP activity was influenced by Ca source, with oyster shell resulting in the highest activity (1696 UA/mg of protein) which was observed at 14 d of age ($P \leq 0.05$; Table 7). An interaction was observed between Ca and P source for intestinal ALP activity, which was associated with a reduction seen in chicks fed DCP with limestone ($P \leq 0.05$; Table 7). Similar to plasma P, a significant ($P \leq 0.01$) quadratic response was

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observed for intestinal ALP activity by increasing the limestone particle size on d 14 (Table 8). A significant (P≤0.01) interaction was occurred between P source and limestone particle size for intestinal ALP activity, where no increase in intestinal ALP activity was observed with increasing Ca particle size, when limestone was provided with DCP and DBM. The lowest activity (510 UA/mg of protein) was seen in birds fed DCP plus 50 µm limestone; in contrast the highest activity (2859 UA/mg of protein) was observed in birds fed bone meal with 100 µm limestone at 14 d of age (Table 8). Similar intestinal ALP activity was observed in birds fed bone meal with all limestone particle sizes on d 28 (Table 8).

P digestibility and gizzard weight

The P digestibility in chicks fed bone meal was lesser than that of those fed DBM or DCP (P≤0.01) (Table 8). There was no difference of P digestibility between DCP and DBM fed chicks. Ca source did not alter P digestibility (Table 7). No interaction was observed for P digestibility in regard to Ca and P source (Table 7). Numerically higher P digestibility was obtained with coarse particles of limestone (P≥0.05; Table 8). An interaction was observed between P source and limestone particle size for P digestibility, which was the lowest in chicks fed bone meal containing 50 µm limestone (P≤0.05; Table 8). Gizzard weight did not affect response to the P and Ca sources or limestone particle size (Table 7 and 8).

Discussion

Comparable to the study of Van Harn *et al.* (2017), findings of the current study showed that degelatinisation of bone increases the percentage of P by elimination of gelatin. Likewise, degelatinisation altered P digestibility of bone meal by enhancing the intestinal alkaline phosphatase activity, nonetheless, no remarkable improvement in bone criteria was achieved through a given degelatinised bone meal instead of whole bone meal. In general, greater body weight and lower FCR were obtained when feeding DBM instead of bone meal or DCP.

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Numerous reports have demonstrated that increasing P availability by changing the source or with phytase supplementation is highly correlated with increases in body weight gain and nutrient utilisation in broilers (Dilger *et al.*, 2004; Cowieson *et al.*, 2006; Delezie *et al.*, 2012; Pieniazek *et al.*, 2017). This improvement in growth and FCR by feeding DBM may be partially attributed to the higher P utilisation of this product. Van Harn *et al.* (2017) reported that digestion and absorption of P was increased by the removal of gelatin from bone.

Ca source (oyster shell or limestone) had a significant impact on FCR. When birds were fed diets formulated with limestone, a reduction in FCR was observed. However, no changes in feed intake or weight gain were seen due to Ca source. It is well documented that highly soluble Ca sources increase Ca concentration in the intestinal lumen; consequently, this phenomenon negatively affects mucosal lining and bacterial toxin production (Walk *et al.*, 2012; Paiva *et al.*, 2013). In contrast, in this trial FCR increased when birds were fed oyster shell in comparison to limestone. Considering that oyster shell is less soluble than limestone, the observed effect was not expected in the current trial. The results obtained in this study are in contrast to the previous work, where they used finer particle size of oyster shell (523 μm) (Tamim and Angel, 2003; Rutherfurd *et al.*, 2012; Kim *et al.*, 2018). More recent work by Saunders-Blades *et al.* (2009) showed that large oyster shell particles (>2 mm) provide more surface area for acid reactions and result in higher solubility in oyster shell as compared to similar particle sizes of limestone. Similarly, limestone particle size had a great impact on FCR and body weight in this study. The lowest FCR (on d 7) and highest body weight (on d 14) were obtained in birds fed coarse particles of limestone. As the finer particles of limestone are more soluble, therefore, it is expected to have higher concentrations of Ca in intestinal lumen when fine particles are being

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fed. Paiva *et al.* (2012) demonstrated that highly soluble Ca sources can severely damage intestinal mucosa and impair nutrient digestion and absorption.

In this experiment, both DBM and bone meal significantly increased tibia length elongation, tibia ash and Ca content (on d 28) and reduced P content especially during the early stages (d 14) of life, when the bone growth and remodelling is very notable. Tahir *et al.* (2011) mentioned that the source of P supplement had a significant effect on the P and Ca contents of tibia. Similar results for tibia mineral content were seen in previous trials, where researchers reported a reduction in tibia P and an increase in tibia Ca content in response to P sources (Viveros *et al.*, 2002; Demirel *et al.*, 2007; Tahir *et al.*, 2011). As reported by Van Harn *et al.* (2017), utilisation of P and Ca from degelatinised bone is higher than those of from the DCP and bone meal. Likewise, the higher amount of Ca in DBM (262 g/kg) may cause differences in the P and Ca contents of tibia because of changes in limestone content in diets. Despite the fact that the diets in the current trial have almost the same value of the Ca and P concentration and ratio, the difference in the amount of Ca derived from DBM or limestone could affect digestion and solublisation of Ca in the intestinal lumen. Therefore, it may change the Ca and P absorption by producing the higher amounts of Ca-phosphate complexed with limestone (Harrold *et al.*, 1983).

The results showed that limestone reduced tibia length. McDonald *et al.* (1995) reported that the abnormal Ca to P ratio in the intestinal lumen could increase Ca and reduce P contents of tibia. It is well recognised that an unbalanced Ca to P ratio in the intestinal lumen resulting from the differences in digestibility and solubility of P sources limits the utilisation of P (McDonald *et al.*, 1995; Van der Klis and Versteegh, 1999). It has been demonstrated that factors which increase P digestibility can enhance the availability of P in the gastrointestinal tract for bone growth and development (Qian *et al.*, 1996). Thus, enhanced tibia length elongation by DBM may be offset

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by the occurrence of more nutrient availability, particularly P for bone differentiation and regeneration. However, there were no considerable changes in P digestibility with DBM in comparison to the DCP, although there was an increase in intestinal alkaline phosphatase activity in birds fed DBM, which could enhance the P accessibility for bone growth. However, despite the reduction in P digestibility, tibial length elongation still was noticeable when feeding bone meal. It has been reported that the presence of gelatin in bone meal can prevent bone loss by decreasing bone resorption and can enhance bone growth (Han *et al.*, 2009). Substitution of DCP with DBM increased bone strength by increasing tibia shear force and shear energy. The steady rate of DBM digestion in comparison to the DCP may affect P utilisation and tibia mineralisation and strength.

On d 14, intestinal alkaline phosphatase activity increased considerably in birds fed DBM or bone meal instead of DCP. Feeding oyster shell or coarse particles of limestone enhanced the activity of intestinal alkaline phosphatase. Higher levels of intestinal alkaline phosphatase activity were significantly associated with higher utilisation of P from phosphate sources (McCuaig *et al.*, 1972). It has been shown that excessive Ca can greatly reduce the activity of ALP, and P or vitamin D deficiency can enhance its activity (Motzok, 1963; Holdsworth, 1970). Less soluble highly digestible P from DBM in comparison to the highly soluble DCP may lead to low concentrations of P and Ca in the intestinal lumen and promote intestinal ALP activity. Consequently, digestion of phosphate from DBM may proceed steadily, which supplies a constant flow of P from the intestine to the blood stream, where it provides permanent levels of Ca and P for bone mineralisation and avoid plasma P elevation and excretion of surplus P in urine. The lower plasma P concentration seen in birds fed diet containing DBM or bone meal may be partly due to lower solubilisation of DBM and bone meal as compared to DCP.

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Limestone, especially finer particles, radically reduced intestinal ALP activity. Several studies have shown that fine particles of limestone are more soluble than coarse particles or oyster shell and produce excessive concentrations of Ca in the intestinal lumen, and hence reduce P utilisation through the formation of Ca-phosphate complexes and inhibition of ALP activity (Motzok, 1963; Qian *et al.*, 1996; Rutherfurd *et al.*, 2012; Kim *et al.*, 2018). The interaction between phosphate and Ca source or phosphate source with limestone particle size showed that the highest ALP activity was obtained when DBM or bone meal was fed with oyster shell and coarse limestone particles. Therefore, these results highlighted that solubility of both P and Ca source are important for P and Ca utilisation and intestinal ALP activity. Lower activity of ALP on d 28, may be related to the low requirement of P in older birds. It has been reported that the activity of ALP was much higher in the intestinal mucosa of broiler chicks than in the parent strain (McCuaig *et al.*, 1971).

Plasma ALP activity did not change in this trial. It is well demonstrated that hormones strictly control blood P levels and any increase in plasma P concentration is rapidly brought about by a change in the proportion of high and low-affinity of P binding sites in bone and alleviates plasma concentration of this mineral without any ALP stimulation (Bronner and Stein, 1995).

The P digestibility of DBM was significantly higher than that of bone meal, and was numerically higher than DCP. Van Harn *et al.* (2017) reported a higher P digestibility for degelatinised bone in comparison to bone meal, DCP and MCP. They mentioned that the higher value of P digestibility in degelatinised bone is due to the application of higher heat and pressure used in the processing of this ingredient, which increases surface area, and because of gelatin elimination which increases bone matrix, as P is more available for absorption in the small intestine (Van Harn *et al.* 2017). Increase in bone matrix P availability and surface area could enhance

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accessibility of intestinal ALP to release more of the phosphate moiety from degelatinised bone. Consequently, P digestibility will improve by removal of gelatin from bone.

Neither P and Ca source nor limestone particle size affected gizzard absolute weight, even though it was expected that increasing limestone particle size would increase gizzard weight. In agreement, Anwar *et al.* (2017) reported that the relative weight of gizzard digesta was not affected by Ca source or particle size. It has been reported that gizzard weight was increased with larger corn particle size - from 781 to 2,242 μm (Parsons *et al.*, 2006). The analysis of the mean diameter of particles size of limestone in the current study may indicate that particles were not coarse enough to stimulate gizzard growth.

It can be concluded that removal of gelatin can improve broiler bone quality through P digestibility enhancement and by changing intestinal ALP activity. In addition, digestion of phosphate from DBM occurs at a steady rate, which supplies optimum levels of P in the blood stream and limits P release to the environment *via* urine. Overall, gelatin removal is an effective processing way to enhance the nutritional value of recycled ingredients such as bone meal, which could compensate phosphate resource scarcity challenges in the animal sector.

Conflict of interest statement

The authors declare that there is no conflict of interests.

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Table 1. Dry matter, crude protein, Ca and P (g/kg) of test products

Ingredient	Dry matter	crude protein	Ca	P
Bone meal	962	247	237	116
Degelatinised bone meal (DBM)	914	6	262	178
Di-Ca phosphate	971	-	227	181
limestone	932	-	391	-
Oyster Shell	925	-	361	-

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Table 2. Ingredient and calculated and analysed composition of the diets (g/kg).

Ingredient	DCP[1]		DBM[2]		Bone meal
	Limestone[3]	Oyster Shell	Limestone	Oyster Shell	Limestone
Corn	560.4	560	559.5	560	576
Soybean meal, 42% CP	381	381	381	381	359
Soybean oil	16	16	16	16	16
DBM	-	-	21	21	-
Bone meal	-	-	-	-	30
DCP	20	20	-	-	-
Limestone	9.5	-	9.5	-	6
Oyster Shell	-	10	-	9	-
Sodium chloride	3	3	3	3	3
Vitamin and mineral premix[4]	5	5	5	5	5
DL – methionine, 99%	2.3	2.3	2.3	2.3	2.3
L- lysine HCl, 78%	2	2	2	2	2
L-Threonine	0.7	0.7	0.7	0.7	0.7
Calculated composition (g/kg)					
ME (MJ/kg)	2904	2900	2914	2906	2913
CP	210	216	211	210	209
Lys	13	13.1	12.8	13	12.7
Met	5	4.7	5.1	4.9	5.3
Met + Cys	8.6	8.4	8.7	8.6	8.9
Total P	7.6	7.6	7.5	7.6	7.4
Available P	5	5	5	5	4.9
Ca	10	10	10	10	10
Analysed composition (g/kg)					
CP	203	207	211	208	213
Total P	7.8	7.2	8.3	7.9	8.4
Ca	11.5	10.8	10.4	10	11.3

[1]DCP: Di-Ca phosphate. [2]DBM: Degelatinised bone meal. [3]limestone with different particle size (mean diameter 50, 100 and 200 μm), same origin and same analysed Ca concentration (391) was purchased from a local company. [4]Vitamin and mineral mix supplied the following per kg of diet: transretinol: 13 mg; cholecalciferol: 0.5 mg; a tocopherol acetate: 80 mg; menadione: 3 mg; thiamine: 3 mg, riboflavin: 8 mg; pyridoxine: 5 mg; cyanocobalamin: 0.024 mg; nicotinic acid: 60 mg; folic acid: 2 mg; Ca pantothenate: 15 mg; choline: 1000 mg; Mn: 120 mg; Zn: 1100 mg; Cu: 16 mg; Se: 0.3 mg; I: 1 mg; and Fe: 40 mg.

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Table 3. Influence of Ca and P sources on growth performance[1] of broilers from one to 28 d of age.

Treatments	One to 7 d[2]			One to 14[2]			One to 28 d[3]		
	FCR	FI	BW	FCR	FI	BW	FCR	FI	BW
Phosphate Source									
DCP	0.74[b]	76.12[b]	102.2[b]	1.18	338.44	258.27	1.50[a]	1334	883.91
DBM	0.82[a]	90.15[a]	109.5[a]	1.15	329.16	285.34	1.44[b]	1347	937.22
SEM	0.03	3.36	1.8	0.02	7.34	6.92	0.03	40	30.91
Ca Source									
Oyster Shell	0.84[a]	89.04	105.3	1.20[a]	341.48	283.25	1.49	1376	919.44
Limestone	0.76[b]	81.17	106.0	1.16[b]	331.25	285.99	1.46	1328	907.60
SEM	0.03	3.24	1.7	0.02	7.09	6.68	0.02	38	29.85
Phosphate Source ×Ca Source									
DCP×Oyster Shell	0.80	83.75	102.0	1.20	349.42	289.42	1.52	1331	871.90
DCP×Limestone	0.70	73.58	102.4	1.21	334.79	280.89	1.50	1335	887.91
DBM×Oyster Shell	0.80	88.75	108.6	1.13	327.70	288.10	1.43	1322	927.30
DBM×Limestone	0.80	94.33	109.8	1.20	333.54	277.08	1.47	1421	966.98
SEM	0.04	4.59	2.45	.02	10.02	9.41	0.03	54	42.22
Effect	*P*-value								
Phosphate Source	0.04	0.003	0.003	0.15	0.31	0.99	0.04	0.78	0.17
Ca Source	0.05	0.11	0.77	0.05	0.35	0.78	0.40	0.40	0.78
Phosphate Source× Ca Source (linear)[4]	0.60	0.60	0.85	0.40	0.67	0.40	0.70	0.36	0.53

[a–d]Means within a column without a common superscript significantly differ ($P < 0.05$). [1]Body weight gain (g/period). FI = feed intake (g/period). FCR = feed conversion ratio (g of feed/g of body weigh gain). [2] There were 8 broilers per pen and 5 pens per diet. [3] There were 6 broilers per pen and 5 pens per diet. Pens means were used to calculate each treatment means. [4] Orthogonal polynomial contrasts were used to compare the Ca sources (limestone or oyster shell) effect between birds fed the DCP and DBM as P source.

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Table 4. Influence of P source and Ca particle size on growth performance[1] of broilers from one to 28 d of age.

Treatments	One to 7 d[2]			One to 14[2]			One to 28 d[3]		
	FCR	FI	BW	FCR	FI	BW	FCR	FI	BW
Phosphate Source									
DCP	0.72[b]	73.58[b]	102.36[b]	1.18[b]	334.79	283.88[a]	1.53[ab]	1335	887.91
DBM	0.81[a]	88.75[a]	109.78[a]	1.13[b]	327.70	288.11[a]	1.43[b]	1333	927.30
Bone Meal	0.85[a]	85.81[a]	101.48[b]	1.29[a]	340.84	264.28[b]	1.54[a]	1322	882.43
SEM	0.03	3.01	1.62	0.03	7.57	5.3	0.03	42	31
Ca particle size (μm)									
50	0.84[a]	87.05	103.08	1.20	322.28[b]	268.19[b]	1.51	1266	855.50
100	0.75[b]	77.70	104.16	1.19	335.03[ab]	282.35[ab]	1.47	1343	919.81
200	0.78[ab]	83.38	106.37	1.21	346.02[a]	285.73[a]	1.52	1380	922.33
SEM	0.03	3.01	1.62	0.03	7.57	5.72	0.03	42	31
Phosphate Source ×Ca particle size									
DCP×50	0.70	78.33	104.25	1.17	340.50	288.92	1.52	1333	975.71
DCP×100	0.64	69.33	100.50	1.14	323.29	282.33	1.48	1340	905.63
DCP×200	0.71	73.08	102.32	1.21	340.58	280.42	1.51	1330	882.38
DBM×50	0.90	97.66	107.17	1.16	292.83	256.83	1.45	1222	844.52
DBM×100	0.70	81.16	110.33	1.13	351.85	310.40	1.42	1392	983.32
DBM×200	0.73	87.41	111.83	1.12	333.42	297.08	1.42	1353	954.05
Bone meal×50	0.72	85.16	97.83	1.27	328.50	258.83	1.54	1243	846.27
Bone meal×100	0.83	82.62	101.67	1.29	329.90	254.33	1.49	1298	870.48
Bone meal×200	0.81	89.64	104.95	1.30	364.06	279.33	1.57	1457	930.56
SEM	0.04	5.22	3.81	0.04	13.12	9.25	0.05	73	54
Effect (factorial Analysis)	*P*-value								
Phosphate Source	0.007	0.005	0.003	0.001	0.48	0.01	0.05	0.97	0.50
Ca particle size	0.04	0.11	0.77	0.86	0.11	0.05	0.63	0.18	0.20
Phosphate Source× Ca particle size	0.5	0.62	0.85	0.80	0.08	0.06	0.90	0.46	0.70

[a–d]Means within a column without a common superscript significantly differ ($P < 0.05$). [1]Body weigh gain (g/period). FI = feed intake (g/period). FCR = feed conversion ratio (g of feed/g of body weight gain). [2] There were 8 broilers per pen and 5 pens per diet. [3] There were 6 broilers per pen and 5 pens per diet. Pens means were used to calculate each treatment means.

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Table 5. Influence of Ca and P sources on tibia strength, ash, Ca and P content at 14 and 28 d of age.[2]

Treatments	14 d						28 d					
	Tibia length	Tibia ash	Tibia Ca[1]	Tibia P[1]	Shear force	Shear energy	Tibia length	Tibia ash	Tibia Ca[1]	Tibia P[1]	Shear force	Shear energy
Phosphate Source	(cm)	(g/100 g)	(g/100 g)	(g/100 g)	(N)	(J)	(cm)	(g/100 g)	(g/100 g)	(g/100 g)	(N)	(J)
DCP	37.26[b]	65.79	36.21[b]	17.09[a]	25.51	48.51[b]	60.83	66.41	36.07[b]	16.55	95.94[a]	176.51
DBM	41.97[a]	68.04	36.91[a]	14.81[b]	29.16	65.29[a]	60.91	67.65	37.19[a]	16.25	69.25[b]	124.72
SEM	0.52	1.19	0.21	0.34	3.51	5.93	1.39	0.89	0.33	0.42	9.66	23.38
Ca Source												
Oyster Shell	40.87	67.39	36.59	15.32	28.70	63.35	63.50[a]	67.90	36.64	16.32	86.83	157.26
Limestone	40.53	66.75	36.42	16.16	26.88	54.69	58.91[b]	66.74	36.67	16.43	81.18	148.40
SEM	0.56	1.14	0.27	0.32	3.39	5.73	1.30	0.86	0.32	0.46	11.26	22.58
Phosphate Source ×Ca Source												
DCP×Oyster Shell	39.24	68.48	35.62[b]	16.79	24.80	57.14	63.7	67.63	36.35	16.80	114.53	198.32
DCP×Limestone	39.28	64.89	36.51[a]	17.18	25.74	45.63	59.8	66.01	35.98	16.46	89.74	169.24
DBM×Oyster Shell	42.51	68.62	37.23[a]	13.84	32.58	63.64	63.32	68.17	36.90	15.83	59.93	116.20
DBM×Limestone	41.80	66.29	36.68[a]	15.13	28.02	69.55	60.1	67.48	37.30	16.38	72.62	127.56
SEM	1.6	1.16	0.28	0.46	4.79	8.10	3.9	1.21	0.45	0.57	15.93	25.4
Effect	*P*-value											
Phosphate Source	0.001	0.13	0.05	0.001	0.40	0.03	0.92	0.27	0.01	0.56	0.05	0.08
Ca Source	0.65	0.71	0.57	0.12	0.71	0.31	0.05	0.36	0.98	0.85	0.73	0.79
Phosphate Source× Ca Source (linear)[3]	0.80	0.09	0.03	0.36	0.58	0.73	0.71	0.71	0.43	0.47	0.25	0.54

[a–d]Means within a column without a common superscript significantly differ ($P < 0.05$). [1]Ca: Ca, P: P. [2] There were 2 broilers per pen and 5 pens per diet. Pens means were used to calculate dietary averages. [3] Orthogonal polynomial contrasts were used to compare the Ca sources (limestone or oyster shell) effect between birds fed the DCP and DBM as P source.

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Table 6. Influence of P source and Ca particle size on tibia strength, ash, Ca and P content at 14 and 28 d of age.[2]

Treatments	14 d						28 d					
	Tibia length	Tibia ash	Tibia Ca[1]	Tibia P[1]	Shear force	Shear energy	Tibia length	Tibia ash	Tibia Ca[1]	Tibia P[1]	Shear force	Shear energy
Phosphate Source	(cm)	(g/100 g)	(g/100 g)	(g/100 g)	(N)	(J)	(cm)	(g/100 g)	(g/100 g)	(g/100 g)	(N)	(J)
DCP	39.27[b]	64.89[b]	36.51	17.18[a]	25.75	45.64[b]	59.88	66.01[b]	35.98[b]	16.47	89.74	169.24
DBM	41.79[a]	68.62[a]	36.68	15.13[b]	28.02	63.74[ab]	60.12	67.48[ab]	37.29[a]	16.39	72.62	127.56
Bone Meal	41.32[a]	70.93[a]	35.71	15.93[ab]	32.23	72.76[a]	62.38	69.70[a]	36.35[ab]	16.39	73.13	164.88
SEM	0.42	1.08	0.33	0.52	3.36	6.92	1.23	0.93	0.30	0.28	11.53	23.72
Ca particle size (μm)												
50	41.57[a]	69.04	36.39	16.31	32.55	69.37	59.73	67.57	36.07	16.36	74.54	150.53
100	39.93[b]	68.36	36.30	16.35	28.65	60.93	62.57	67.93	36.71	16.55	77.75	158.94
200	40.89[ab]	67.04	36.20	15.59	24.80	51.83	60.08	67.69	36.85	16.34	83.20	152.20
SEM	0.42	1.08	0.33	0.52	3.30	6.92	1.23	0.98	0.43	0.35	10.67	25.43
Phosphate Source ×Ca particle size												
DCP×50	39.74	64.24	36.45	17.49	25.00	47.61	61.21	67.53	35.52	16.98	101.71	219.58
DCP×100	39.21	64.38	36.18	17.38	26.76	46.38	61.59	65.39	36.12	16.57	76.41	170.04
DCP×200	38.88	66.05	36.88	16.68	25.48	42.92	56.83	65.10	36.28	15.85	91.10	118.09
DBM×50	41.62	70.56	36.02	15.65	30.67	68.67	57.08	65.48	36.52	15.28	69.88	99.02
DBM×100	41.18	68.92	36.88	14.97	27.44	64.08	63.70	69.12	37.25	17.12	74.72	127.41
DBM×200	42.59	66.37	37.12	14.76	25.93	58.47	59.50	67.82	38.10	16.76	93.26	156.24
Bone meal×50	43.36	72.31	36.70	15.78	31.96	91.84	60.89	69.68	36.15	16.82	72.04	133.00
Bone meal×100	39.41	71.79	35.82	16.69	31.74	72.32	62.37	69.28	36.75	15.94	82.11	179.36
Bone meal×200	41.21	68.69	36.60	15.31	22.99	54.10	63.88	70.14	36.15	16.40	65.22	182.27
SEM	0.70	1.78	0.57	0.90	5.80	11.9	2.13	1.62	0.52	0.49	29.98	41.09
Effect (factorial Analysis)						*P*-value						
Phosphate Source	0.001	0.003	0.14	0.05	0.40	0.03	0.30	0.04	0.03	0.97	0.5	0.41
Ca particle size	0.04	0.42	0.92	0.53	0.29	0.22	0.23	0.96	0.20	0.85	0.86	0.96
Phosphate Source× Ca particle size	0.08	0.49	0.13	0.92	0.56	0.70	0.20	0.43	0.61	0.08	0.53	0.33

[a–d]Means within a column without a common superscript significantly differ ($P < 0.05$). [2] There were 2 broilers per pen and 5 pens per diet. Pens means were used to calculate dietary averages.

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Table 7. Influence of Ca and P sources on plasma Ca and P content, plasma and digestive alkaline phosphatase activity and P digestibility at 14 and 28 d of age.[1]

Treatments	14 d					28 d					
Phosphate Source	Plasma Ca (mg/dl)	Plasma P (mg/dl)	Plasma ALP (U/L)	Intestinal ALP[2] (UA/mg of protein)	Gizzard weight (g)	Plasma Ca (mg/dl)	Plasma P (mg/dl)	Plasma ALP (U/L)	Intestinal ALP[2] (UA/mg of protein)	P digestibility (%)	Gizzard weight (g)
DCP	8.35	8.51[a]	2.15	993[b]	10.76	10.99[a]	6.96	2.90	322[a]	78.40	20.20
DBM	8.34	5.30[b]	2.14	1615[a]	9.79	10.31[b]	7.72	2.91	133[b]	81.53	18.22
SEM	0.64	0.68	0.02	163	0.69	0.19	0.52	0.21	49	3.47	1.17
Ca Source											
Oyster Shell	8.06	7.61	2.13	1696[a]	10.06	10.85	5.94[b]	2.71	306	80.77	19.45
Limestone	8.44	6.67	2.15	1173[b]	10.35	10.58	7.81[a]	2.97	202	78.34	19.13
SEM	0.61	0.65	0.02	158	0.67	0.20	0.48	0.21	47	3.78	1.12
Phosphate Source ×Ca Source											
DCP×Oyster Shell	7.79	9.81[a]	2.08[b]	1811[a]	9.83	11.11	4.19[b]	2.67	483[a]	79.17	20.27
DCP×Limestone	8.53	8.08[a]	2.18[a]	721[b]	11.07	10.95	7.89[a]	2.97	269[b]	78.00	20.19
DBM×Oyster Shell	8.35	5.42[b]	2.18[a]	1580[a]	9.62	10.22	7.73[a]	2.96	135[b]	82.76	18.09
DBM×Limestone	8.32	5.26[b]	2.13[a]	1628[a]	10.28	10.60	7.68[a]	2.74	129[b]	80.41	18.63
SEM	0.87	0.85	0.03	223	0.94	0.21	0.7	0.28	65	4.17	1.60
Effect	*P*-value										
Phosphate Source	0.99	0.001	0.61	0.005	0.26	0.01	0.24	0.95	0.005	0.43	0.18
Ca Source	0.67	0.33	0.41	0.03	0.77	0.34	0.01	0.39	0.15	0.87	0.85
Phosphate Source× Ca Source (linear)[3]	0.69	0.02	0.02	0.02	0.94	0.46	0.02	0.88	0.05	0.57	0.80

[a–d]Means within a column without a common superscript significantly differ ($P < 0.05$). [1] There were 2 broilers per pen and 5 pens per diet. Pens means were used to calculate dietary averages. [2]ALP; alkaline phosphatase. [3] Orthogonal polynomial contrasts were used to compare the Ca sources (limestone or oyster shell) effect between birds fed the DCP and DBM as P source.

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Table 8. Influence of P source and Ca particle size on tibia strength, ash, Ca and P content at 14 and 28 d of age.[1]

Treatments	14 d					28 d					
	Plasma Ca	Plasma P	Plasma ALP	Intestinal ALP[2]	Gizzard weight	Plasma Ca	Plasma P	Plasma ALP	Intestinal ALP[2]	P digestibility	Gizzard weight
Phosphate Source	(mg/dl)	(mg/dl)	(U/L)	(UA/mg of protein)	(g)	(mg/dl)	(mg/dl)	(U/L)	(UA/mg of protein)	(%)	(g)
DCP	8.53	8.07^{a}	2.17	720.70^{c}	11.07	10.95	7.89	2.97	269.54^{a}	78.60^{a}	20.19
DBM	8.35	5.26^{b}	2.12	1624.50^{b}	9.62	10.22	7.73	2.96	135.23^{b}	80.14^{a}	18.08
Bone Meal	8.96	4.64^{b}	2.22	2185.70^{a}	9.85	10.68	7.05	2.97	337.89^{a}	57.19^{b}	18.94
SEM	0.59	0.61	0.03	171	0.66	0.24	0.53	0.21	43.63	3.01	1.54
Ca particle size (μm)											
50	8.41	5.74	2.20	1104.50^{b}	10.40	10.62	6.52^{b}	2.94	229.66	76.54	17.41
100	9.20	5.61	2.15	1919.00^{a}	9.86	10.55	8.27^{a}	3.05	222.22	78.12	20.75
200	8.23	6.63	2.17	1509.00^{ab}	10.28	10.68	7.88^{ab}	2.90	290.79	79.88	19.05
SEM	0.59	0.67	0.04	167	0.66	0.24	0.53	0.21	41.21	4.19	1.54
Phosphate Source ×Ca particle size											
DCP×50	7.17	8.34	2.18	510.10^{e}	11.81	11.04	5.68	3.30	373.65^{ab}	77.98^{a}	20.40
DCP×100	10.21	7.61	2.14	971.43^{e}	11.04	10.78	8.56	2.75	134.02^{c}	79.13^{a}	22.56
DCP×200	8.22	8.27	2.21	680.58^{de}	10.38	11.02	9.43	2.86	300.95^{abc}	77.24^{a}	17.60
DBM×50	8.92	4.14	2.10	1244.39^{cde}	8.88	9.78	7.20	2.74	89.45^{c}	80.76^{a}	15.80
DBM×100	8.22	4.98	2.14	1926.52^{abc}	9.25	10.40	8.49	3.28	192.73^{bc}	83.19^{a}	19.30
DBM×200	7.91	6.66	2.13	1708.48^{bcd}	10.74	10.47	7.50	2.86	123.52^{c}	81.27^{a}	19.13
Bone meal×50	9.14	4.73	2.31	1559.04^{cd}	10.52	11.03	6.67	2.88	225.86^{abc}	52.33^{b}	16.03
Bone meal×100	9.18	4.24	2.16	2859.05^{a}	9.30	10.46	7.77	3.06	339.90^{abc}	59.74^{b}	20.36
Bone meal×200	8.56	4.97	2.19	2138.92^{ab}	9.74	10.55	6.71	2.97	447.90^{a}	57.31^{b}	2.60
SEM	1.03	1.06	0.05	296.18	1.14	0.41	0.93	0.37	75.57	5.71	1.67
Effect (factorial Analysis)					*P*-value						
Phosphate Source	0.34	0.002	0.17	0.001	0.23	0.12	0.76	0.99	0.01	0.001	0.63
Ca particle size	0.48	0.45	0.58	0.01	0.83	0.92	0.04	0.90	0.40	0.18	0.33
Phosphate Source× Ca particle size	0.47	0.77	0.48	0.001	0.60	0.59	0.47	0.69	0.03	0.001	0.60

$^{a-d}$Means within a column without a common superscript significantly differ ($P < 0.05$). [1]There were 2 broilers per pen and 5 pens per diet. Pens means were used to calculate dietary averages. [2]ALP; alkaline phosphatase.

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