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Original Article

Effects of Supplementation of Zinc, Manganese, or Copper and Different Phytase Levels in Serum and Bone Acid and Alkaline Phosphatases of Broiler Chicks

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ABSTRACT

The knowledge of biological trace minerals and phytase requirements for modern broiler genotypes is not established and the pressure to reduce their usage in animal feeding due to environmental issues is increasing. Here, the alkaline phosphatase (ALP) and tartarate-resistant acid phosphatase (TRAP) of the tibia and serum of broilers fed with diets containing various levels of phytase and reduced levels of zinc, manganese, and copper was evaluated. The experiment was performed using 1,200 male Cobb broilers raised according to standard commercial husbandry techniques. Data were analyzed as a 4×3 factorial arrangement with four concentrations of zinc (0.34, 0.49, 0.64, and 0.79 ppm), manganese (0.18, 0.43, 0.68, and 0.93 ppm), or copper (0.09, 0.12, 0.15, and 0.18 ppm) and three concentrations of phytase (0, 500, and 1,000 FTU/kg) for age periods of 1-21 and 36-42 days. While the dietary supplementation with copper did not induce a significant effect in bone tissue biochemical markers, serum TRAP activity of 42-day old broilers increased with higher copper levels. Increasing dietary zinc levels linearly increased ALP activity in tibia growth, suggesting that zinc is essential for longitudinal bone growth. Phytase significantly promoted the increase of TRAP and ALP activities, suggesting that manganese increased growth plate activity, accelerated calcification, and remodeled the newly formed tissue into trabecular bone. Although not every enzymatic activity was affected by the treatments, the phytase use, along with trace minerals, improved the animal response to the rapid growth required nowadays and provided the nutrients for adequate bone metabolism.

INTRODUCTION

Genetic selection has increased the production of poultry meat to levels to the highest compared with other sources of animal protein (Dibner *et al.*, 2007; Tickle *et al.*, 2014; Hartcher & Lum, 2020); however, previous studies investigating its nutritional requirements in broilers and turkey poultries are required.

Due to the high demand of nutrients needed to obtain an efficient feed intake, growth, feed conversion, weight gain, and healthy skeletal development of rapidly growing poultry, it is necessary to provide adequate nutrients. Therefore, exogenous enzymes have been used in non-ruminant diets (Bedford, 2000; Ahmed & Tahir, 2015) as an alternative to reduce the effects of anti-nutritional factors, including phytate, which may act as a chelating agent, present in plant seeds and grains, being major constituents of poultry diets (Greiner & Konietzny, 1999; Oh *et al.*, 2004; Santos *et al.*, 2015). Phosphate released from phytate can improve digestion of this compound and reduce or avoid the use of phosphorus in poultry feed (Khalid *et al.*, 2013; Nakagi *et al.*, 2013). In addition, its hydrolysis is dependent on the sources of phytate,

and the impact of phytase on the availability of trace minerals remains to be clarified (Sebastian *et al.*, 1996; Chung *et al.*, 2013; Santos *et al.*, 2015; Manobhavan *et al.*, 2016).

Regarding the trace elements, the positive effect of the addition of metal ions in poultry feed has already been reported by other authors (Holubiev *et. al*, 2020), it has been established that zinc (Zelenka, 2013; Zaghari *et al.*, 2015; Sirri *et al.*, 2016, Cho & Kwun, 2018, Sakara *et al.*, 2021), copper (Leeson, 2009; Liu *et al.*, 2013; Medeiros, 2016) and manganese (Leach & Muenster, 1962; Medeiros, 2016, Sakara *et al.*, 2021) are essential in innumerous metabolic and physiological processes.

Therefore, the use of exogenous enzymes, including phytase, is an alternative strategy to reduce the effects of anti-nutritional factors (Greiner & Konietzny, 1999; Oh *et al.*, 2004; Chung, 2013; Bedford & Apajalahti, 2022). Furthermore, it is an economic and reliable form of improving the digestion of inositol hexaphosphate in the diet of poultry, pigs, and other monogastric animals (Singh *et al.* 2014). It has previously been revealed that exogenous phytase used in diets reduces the need for supplemental zinc (Lei *et al.*, 1993; Khalid *et al.*, 2013; Santos, 2015).

According to Orban *et al.* (1999), the status of bones, particularly leg bones (metatarsus, tibia, and femur), may affect the quality of poultry meat. Nutritionists must consider trace mineral availability, mainly when increased levels of calcium and phosphorus are used, as they are considered antagonists minerals and can induce secondary deficiencies of zinc, copper, and manganese, which are required for normal bone development and remodeling (Orban *et al.*, 1999). In this way, specific markers of bone and blood cells may be used to evaluate metabolic activity of the bone tissue, such as tartrate-resistant acid phosphatase (TRAP) as a specific marker of osteoclastic cells (Pizauro *et al.*, 2002; Pagani *et al.*, 2005; Kirstein *et al.*, 2006) and alkaline phosphatase (ALP) as a specific marker of osteoblasts involved in biological calcification (Cheng *et al.*, 1996; Osyczka & Leboy, 2005; Ansari *et al.* 2021).

In the present study the bone markers, ALP and TRAP, present in the blood serum and tibia of broilers fed diets containing various levels of phytase and reduced levels of zinc, manganese, and copper in two ages were evaluated.

MATERIALS AND METHODS

All procedures were approved by the Ethics Committee on Animal Use of the São Paulo State University (Unesp), School of Agricultural and Veterinarian Sciences, Jaboticabal, and were in accordance with the Ethical Principles in Animal Use of the Brazilian College of Animal Experimentation.

The present study was performed using 1,200 male Cobb broilers, raised according to lineage management manual of Cobb broilers (Nakagi *et al.*, 2013). The diets were available *ad libitum*, containing energy levels of 2,950 and 3,150 kcal ME kg^{-1}. Starter (1-21 days) and finisher (36-42 days) diets were formulated according to the nutritional requirements for chickens (Cruz *et al.*, 2011); each phase and composition of the diets is presented in table 1.

Table 1 – Composition of initial (1-21 d) and final (36 - 42 d) experimental diets.

Ingredients	P total levels	
	Initial	Final
Corn	56.84	59.27
Soybean meal	37.40	32.40
Soybean oil	1.80	4.16
Salt	0.41	0.42
Premix1	0.60	0.60
Dicalcium phosp.	1.76	1.58
Calcitic limestone	1.01	1.03
DL-Methionine	0.16	0.12
Lysine	0.02	0.02
Variable portion	-	0.4
Calculated values		
ME (kcal kg-1)	2.950	3.150
CP (%)	22.00	20.00
Calcium (%)	0.96	0.80
P available (%)	0.45	0.41
P total (%)	0.70	0.63
Lysine (%)	1.24	1.12
Met+cist (%)	0.89	0.81

[1] Mineral and vitamin premix: 10.020 IU vit. A, 2,010 IU vit. D_3, 15 mg vit. E, 2.50 mg vit. K_3, 1.5 mg vit. B_1, 5.01 mg vit. B_2, 1.5 mg vit. B_6, 12 mcg B_{12}, 0.6 mg Folic acid, 0.05 mg Biotin, 35 mg Niacin, Calcium pantothenate 11.22 mg, 6 mg Copper, 45 mg Zinc, Cobalt 0.10 mg, 1.02 mg Iodine, 50 mg Iron, 65 mg Manganese.

The experiments consisted in the effect of the individual variation of each studied trace element: zinc ($ZnSO_4.H_2O$), manganese ($MnSO_4.H_2O$) or copper ($CuSO_4.5H_2O$), as shown in table 2. In each experiment the test of phytase inclusion was also carried out, and the levels used were 0, 100 and 200 $g.ton^{-1}$, ensuring 0, 500 and 1000 FTU kg^{-1} of diet. The enzyme used was Natuphos 5.000® (BASF Animal Nutrition), measured in FTUs, and extracted from *Aspergillus niger*.

At 23 and 42 days of age, four birds from each treatment group were randomly selected for blood collection. The birds were sacrificed by cervical dislocation, and blood samples and the right and left tibias were frozen in liquid nitrogen and stored at

-70°C for determination of ALP and TRAP activities. The epiphyses were homogenized using an OMNI-GLH homogenizer in 5 mM Tris-HCl buffer, pH 7.5, supplemented with 2 mM $MgCl_2$, 1 µM $ZnCl_2$, and 150 mM NaCl (1 g tissue per 3 mL buffer). The homogenate was centrifuged at 10,000 ×*g* for 10 min at 4°C. The obtained supernatant was filtered through glass wool, frozen in liquid nitrogen, and stored at -70°C. The enzyme extract obtained by this procedure was used to determine the alkaline and acid phosphatases of the tibia.

Table 2 – Variations of trace element concentrations in the treatments.

Treatments	Levels of the varied trace element (ppm)		
	Zinc	Manganese	Copper
1	0.34	0.18	0.009
2	0.49	0.43	0.12
3	0.64	0.68	0.15
4	0.79	0.93	0.18

Acid and ALP activities were evaluated at 37°C in 50 mM acetate or 2-amino-2-methyl-1-propanol (AMPOL) buffers at pH 4.5 and 9.4, respectively, and supplemented with 10 mM disodium tartrate through the formation of *p*-nitrophenolate (ε= 17600 mol.L-1 cm-1, pH 13).

The enzymatic reaction was initiated by addition of the enzyme extract to the reaction medium and interrupted by adding 1 mol.L^{-1}NaOH, and the absorbance was determined at 410 nm (Hitachi Spectrophotometer U-2000). The evaluations were performed in triplicate, and the initial velocities remained constant during the incubation time to ensure that substrate hydrolysis was <5%. In each, the determination standards were included to estimate the non-enzymatic hydrolysis of the substrate.

A unit of enzyme activity was defined and expressed as the amount of enzyme that released one nmol of *p*-nitrophenolate per minute and per milligram of protein present in the enzymatic extract, under test conditions. The protein content was determined according to Hartree (1972), using bovine serum albumin (SIGMA®) as the standard.

Data were analyzed statistically as a completely randomized design with a 4×3 factorial (4 elements levels x 3 phytase levels) with four replications of 25 chicks each. Data were submitted to homogeneity and normality analyses according to Everitt (1998) and to analysis of variance with the means compared by the Tukey test. $p<0.05$ was considered to indicate a statistically significant difference. Regression analyses were determined regarding the zinc levels for each phytase level. The statistical tests were performed using the software SAS version 9 (SAS Institute, 1996).

RESULTS AND DISCUSSION

Trace elements, including zinc, copper, and manganese, serve an essential role in growth, development, and maintenance of healthy bones, reproduction, immune system maintenance, and normal metabolism of poultry (Santos *et al.*, 2015; Medeiros, 2016; Sirri *et al.*, 2016). In addition, an inadequate intake of trace elements may lead to abnormal bone development and poor health and chicken growth during the short period required for breeding. This imbalance induces body components to develop without a compensatory increase during this period.

The addition of enzymes in the birds diet may contribute to reduce the discrepancy of tables recommendation, mainly the bivalent micro minerals (Mohanna & Nys, 1999).

In the present study, it was revealed that there was no significant interaction between zinc and phytase levels for the analyzed variables, suggesting that these factors act independently. The activities of serum TRAP and ALP in 21-day old animals differed significantly, with increasing zinc and phytase levels (Table 3). In Table 3, it can be observed that the activities of serum ALP and TRAP in 42-day old animals were not altered by the addition of phytase, although TRAP activity increased with higher levels of zinc.

Regarding the values obtained for the activities of TRAP and ALP observed in the tibia of 21- and 42-day old animals, a significant increase in enzyme activity with higher zinc levels was demonstrated (Table 3). Phytase supplementation significantly increased the ALP activity in 21- and 42-day old animals; conversely, TRAP activity was not affected by this treatment. Similar behavior was observed by Hadley *et al.* (2009), investigating the response of cellular markers of mineralization and bone remodeling in rats fed with diets deficient in zinc. It was observed that acid and ALP activities increased four-fold with zinc concentrations from 2.5 to 30 ppm.

The regression analysis for serum phosphatases regarding the zinc levels for each phytase level revealed R^2 values between 0.35 and 0.59 for acid phosphatase. The highest R^2 value was observed for ALP for the highest phytase level (1,000 FTU) and the values varied from 0.49 to 0.74, which are similar to those observed by Revy *et al.* (2006) in pigs.

Table 3 – F values, means, and coefficient of variation of TRAP and ALP activities from serum and tibia of 21- and 42-day old broilers, fed with different zinc and phytase levels.

Statistics	F-values							
	Serum				Tibia			
	21 days		42 days		21 days		42 days	
	TRAP	ALP	TRAP	ALP	TRAP	ALP	TRAP	ALP
Zinc level	5.7**	26.3**	3.0*	1.4^{ns}	11.0**	10.1**	6.7**	4.7**
Phytase level	4.3*	6.8**	1.9^{ns}	1.6^{ns}	2.2^{ns}	7.2**	0.2^{ns}	13.1**
Zinc x Phytase	1.6^{ns}	2.0^{ns}	2.1^{ns}	4.5^{ns}	9.2^{ns}	0.88^{ns}	2.0^{ns}	9.1^{ns}
Coefficient of variation (%)	20.8	20.7	28.1	34.3	23.7	18.7	27.5	16.9
Zinc level	U mg^{-1}				U mg^{-1}			
0.34	0.43^{c}	0.66^{c}	0.57^{b}	0.74^{ns}	78,36^{b}	634.88^{b}	129.29^{b}	632.07^{b}
0.49	0.61^{b}	1.10^{b}	0.67^{ab}	0.88^{ns}	97.92^{b}	832.08^{a}b	127.35^{b}	751.08^{ab}
0.64	0.74^{ab}	1.17^{b}	0.61^{ab}	0.82^{ns}	103.04^{b}	970.79^{a}	125.60^{b}	778.74^{ab}
0.79	0.77^{a}	1.50^{a}	0.82^{a}	1.02	144.39^{a}	1015.84^{a}	186.88^{a}	856.12^{a}
Phytase level	U mg^{-1}				U mg^{-1}			
0	0.58^{b}	1.00^{b}	0.59	0.74	117.84	734.83^{b}	143.20	612.00^{b}
500	0.61^{ab}	1.04^{b}	0.67	0.96	96.25	870.03^{ab}	136.80	774.80^{a}
1,000	0.72^{a}	1.28^{a}	0.74	0.89	103.90	985.11^{a}	146.70	876.67^{a}

Means in the same column with different case letters differ by Tukey test (p<0.05). *Significant at 5% probability by the F test; ** Significant at 1% probability by the F test. ns, non--significant; TRAP, tartrate-resistant acid phosphatase; ALP, alkaline phosphatase.

Zinc presents ease of complexation with minerals as P, Cr, Na and Fe but mainly with the diet phytate. The soybean and corn are the major components in the diets, around 90%, and these ingredients are rich in fitic acid. The complex of zinc and phytate forms an insoluble and unabsorabable compound and this mechanism reduces zinc availability to animals. To prevent the lack of zinc it is added in high levels in the diets. Phytase reduces the need for supplemental inorganic phosphorus and improves the nutritional value of feedstuffs.

Of note, previous studies have revealed that ALP was expressed mainly in the post-proliferative and terminal stages of osteoblast (Owen *et al.*, 1990; Stein *et al.*, 1990; Weinreb *et al.*, 1990; Mccabe *et al.*, 1995; Kwun *et al.*, 2010), and that this enzyme regulated the mineralization of the extracellular matrix (Ducy *et al.*, 1996; Fedde *et al.*,1999; Anderson *et al.*, 2004) and the expression of genes regulated by Runx2 (Ducy *et al.*, 1999; Ducy, 2000); in addition, it was the main determining factor of differentiation and function of osteoblasts (Komori *et al.*,1997; Otto *et al.*,1997).

The results of the present study revealed that lower levels of zinc induced downregulation of ALP by decreasing the expression of this enzyme (Table 3). The decrease in the activity of TRAP (Table 3) may cause a decrease in the absorption of the site where the trabecular bone should be formed. It was suggested that zinc deficiency might disrupt osteogenesis, decreasing the expression of genes responsible for the biomineralization of the extracellular matrix and the remodeling of the newly formed tissue, which indicated that zinc represents an essential role in bone metabolism (Yamaguchi, 2010; Liu *et al.*, 2013; Santos *et al.*, 2015).

Copper is found in the amorphous mineral formed within the extracellular vesicles in biological calcification, it prevents premature crystallization of the calcium phosphate inside the vesicle, and its collapse (Sauer *et al.*, 1997; Dibner *et al.*, 2007). This trace element, observed in the active site of certain metalloenzymes is crucial for animal growth and for the prevention of many pathological and clinical disorders afflicting various species (Güçlü *et al.*, 2008; Medeiros, 2016).

The analysis of variance of the interaction between copper and phytase levels revealed that there were no significant effects in TRAP activity in the serum of 21-day old and in the tibia of 42-day old broilers (Table 4). In 21-day old animals, a significant interaction was observed between the variables for serum ALP activity (Table 4). The activity of serum TRAP of 42-day old broilers increased significantly with higher copper levels. It was observed that the increase in phytase levels resulted in increased TRAP activity in the tibia of 21-day old broilers, suggesting that the copper complexed with the phytic acid molecule in corn and soybean meals was released.

Regarding the serum and tibia levels of ALP, no significant effects were observed in 42-day old broilers. Similar results were obtained by Baker *et al.* (1999) in 23- and 46-day old animals. It was demonstrated that

Table 4 – F values, means, and coefficient of variation of TRAP and ALP activities from the serum and tibia of 21- and 42-day old broilers fed with different copper and phytase levels.

Statistics	F-values							
	Serum				Tibia			
	21 days		42 days		21 days		42 days	
	TRAP	ALP	TRAP	ALP	TRAP	ALP	TRAP	ALP
Cooper level	0.62^{ns}	26.7**	3.6*	2.22^{ns}	2.22^{ns}	0.62^{ns}	0.18^{ns}	1.15^{ns}
Phytase level	1.4^{ns}	2.59^{ns}	0.18^{ns}	0.78^{ns}	0.78**	0.68^{ns}	1.25^{ns}	1.07^{ns}
Copper x Phytase	1.85^{ns}	5.14**	0.73^{ns}	0.75^{ns}	0.75^{ns}	0.50^{ns}	1.56^{ns}	1.88^{ns}
Coefficient of variation (%)	20.9	22.0	26.3	33.5	33.5	27.6	63.7	19.3
Cooper level	Mean				Mean			
0.09	0,61	1.22	0.69^{b}	0.63	104.38	401.95	104.86	548.98
0.12	0,62	1.63	0.79^{ab}	0.79	111.76	383.65	95.40	537.34
0.15	0,62	1.79	0.89^{ab}	0.87	133.90	367.75	115.15	486.72
0.18	0,67	0.65	0.96^{a}	0.88	125.57	426.77	109.78	548.91
Phytase level	Mean				Mean			
0	0.61	1.60	0.81	0.72	103.61^{b}	370.9	84.96	486.98
500	0.61	1.82	0.83	0.82	109.74^{ab}	397.3	112.73	523.15
1000	0.67	1.99	0.86	0.84	143.40^{a}	416.7	121.20	538.31

Means in the same column with different case letters differ by Tukey test (p<0.05); ns, non-significant; TRAP, tartrate-resistant acid phosphatase; ALP, alkaline phosphatase.

dietary supplementation with copper did not induce a significant effect in these biochemical markers of bone tissue. Pratt *et al.* (1985) also investigated the effect of copper supplementation in the diet of healthy human adults on various biochemical markers of bone tissues and blood samples, including serum ALP levels. These authors also revealed that there was no significant difference in the activity of ALP between adults receiving 10 mg copper/day in the form of copper gluconate compared with those receiving a placebo for 12 weeks. Furthermore, Eaton-Evans *et al.* (1996) investigated the effect of supplementation of 3 mg copper/day in the form of glycine chelate for 2 years in women between 45 and 56-years of age and demonstrated that the levels of total serum ALP were not affected.

In an investigation on the interaction between copper and phytase levels for serum ALP in 21-day old broilers (Table 5), it was observed that the addition of phytase induced changes when the copper level was 0.18 ppm, causing a decrease in activity with increasing phytase levels. When the broiler's diet contained 1,000 U.kg^{-1} of phytase, the addition of copper did not alter the ALP levels; however, when the level of phytase was 500 U/kg or when this enzyme was absent from the diet, the addition of 0.18 ppm of copper induced an increase in ALP activity.

Seymour (1987) demonstrated that excess copper interfered with the metabolism of bone tissue, generally leading to bone loss, rickets, and an abnormal appearance of osteophytes in patients with Wilson's disease. In addition, studies on embryonic chick bone grown in the presence of 2.5 μM copper revealed a significant decrease in collagen content, mainly due to the inhibition of collagen synthesis in the diaphysis and epiphysis (Kaji *et al.*, 1988), and the embryonic bone growing in the presence of concentrations of ≥10 μM copper induced a decrease in ALP activity of the diaphysis. A previous study by Kaji *et al.* (1990) using the same culture system, demonstrated that inhibition induced by copper in the matrix formation and mineral deposition were not affected.

Table 5 – Means of serum alkaline phosphatase activity of 21-day old broilers fed different copper and phytase levels.

Phytaselevel(U/kg)	Copper level (ppm)			
	0.09	0.12	0.15	0.18
0	1.52 ^{Ab}	1.21 ^{Ab}	1.79 ^{Ab}	3.43 ^{Aa}
500	1.87 ^{Ab}	1.22 ^{Ab}	1.49 ^{Ab}	2.69 ^{Ba}
1000	1.99 ^{Aa}	1.23 ^{Aa}	1.61 ^{Aa}	1.82 ^{Ca}

Means in the same column with different case letters differ by Tukey test (p<0.05).

According to Güçlü *et al.* (2008), the increase in serum ALP in animals fed with higher copper levels cannot be attributed to the liberation of bone ALP. This behavior is due to the response of hepatic ALP to the toxicity of copper ion. Almansour (2006) investigated the effect of different doses of copper sulfate bound to casein (10, 100, 250, 500, and 750 ppm) on biochemical markers of antioxidant defense response. They revealed that the excess of copper sulfate bound to casein in quail feeding induced accumulation of this cation in the liver and led to a significant loss in body weight. These authors also revealed that an excess of copper induced alterations in the levels of glutathione peroxidase and superoxide dismutase, enzymes that

act as detoxification agents and are considered markers of oxidative stress. Changes in serum biochemical parameters, lipid profile, and various pathological conditions were also observed; therefore, copper should be used with caution because of its potential role in the pathogenesis of liver disease in humans and poultry (Almansour, 2006; Güçlü *et al.*, 2008). The results obtained in the present study and previous studies do not provide definitive conclusions of whether serum ALP is the best marker to be analyzed in treatments with various levels of copper (Almansour, 2006). This is due to the fact that serum ALP represents numerous isoenzymes activities of the bone, liver, intestine, and kidney, which originated from a single ancestor gene, differing in post-translational glycosylation (Gomez *et al.*, 1995; Pizauro *et al.*, 1995; Goseki-sone *et al.*, 1999). In addition, serum ALP content may vary with the type of feeding and the toxicity of its constituents. Furthermore, authors are not clear whether the values represent total serum ALP activity or if a more sensitive and specific method for the isoform of the bone is required (Delmas, 1992; Karimi *et al.*, 2014). Therefore, it seems reasonable to assume that the behaviors of acid and alkaline phosphatases observed in tibia demonstrate what actually occurs in the biological response of this tissue to the investigated treatments, when the serum ALP activity may be cautiously used as a bone marker.

The results revealed that diet supplementation with copper did not induce a significant effect on the biochemical markers of bone tissues, suggesting that copper deficiency inhibited the biological functions of chondrocytes, whereas the physiological role of osteoclasts was not affected, resulting in the inhibition of the formation and increase in bone resorption (Medeiros, 2016).

Manganese is present in small amounts in most tissues. Levels of 2-4 ppm were identified in bone, liver, pituitary gland, mammary gland, kidney, and pancreas. The physiological and nutritional functions of manganese have been extensively studied in animals (Schroeder *et al.*, 1966; Grieger, 1999; Bao *et al.*, 2009); however, contradictory results have been reported because its deficiency induces specific morphological and physiological alterations depending on the species (Beattie & Avenell, 1992). It has been proposed that birds have a greater requirement for manganese compared with mammals (Settle *et al.*, 1968), and its absorption is influenced by diet components (Halpin *et al.*, 1986).

The activities of TRAP and ALP with increased levels of manganese and phytase are presented in Table 6. Regarding to the serum TRAP levels of 21-day old animals, there was a significant interaction between the parameters (Table 7) and the addition of phytase increased the serum ALP activity in 42-day old broilers. In the tibia of 21-day old animals, a significant interaction was revealed between the levels of phytase and manganese with ALP activity (Table 8). ALP activity of the tibia of 42-day old broilers increased (p<0.05) with a level of 0.93 ppm manganese. In the tibia of 42-day old animals, the addition of phytase significantly promoted the increase of TRAP and ALP activity, suggesting that manganese increased growth

Table 6 – F values, means, and coefficient of variation of TRAP and ALP activities from serum and tibia tissues of 21- and 42-day old broilers fed with different manganese and phytase levels.

Statistics	F-Values							
	Serum				Tibia			
	21 days		42 days		21 days		42 days	
	TRAP	ALP	TRAP	ALP	TRAP	ALP	TRAP	ALP
Manganese level	3.6*	2.51^{ns}	2.05^{ns}	1.40^{ns}	3.6^{ns}	6.19*	1.5^{ns}	6.9**
Phytase level	1.53^{ns}	0.9^{ns}	1.78^{ns}	3.02*	0.18^{ns}	0.21^{ns}	3.9*	6.3**
Manganese x Phytase	3.58**	1.84^{ns}	1.40^{ns}	1.10^{ns}	0.73^{ns}	2.86**	0.36^{ns}	1.7^{ns}
Coefficient of variation (%)	40.0	50.7	24.6	30.0	26.5	21.9	41.8	25.2
Manganese level	Mean				Mean			
0.18	0.46	0.41	0.70	0.77	74.21	641.74	111.45	514.76^{b}
0.43	0.59	0.63	0.71	0.86	77.77	654.51	128.44	529.05^{b}
0.68	0.71	0.70	0.84	0.95	79.18	793.09	136.99	537.73^{b}
0.93	0.79	0.73	0.87	0.98	81.30	892.03	159.14	755.20^{a}
Phytase level	Mean				Mean			
0	0.55	0.59	0.72	0.77^{b}	72.7	728.32	102.91^{b}	485.53^{b}
500	0.66	0.64	0.79	0.89^{ab}	76.3	741.68	142.70^{ab}	592.93^{ab}
1000	0.71	0.65	0.84	1.01^{a}	85.2	766.03	156.28^{a}	674.09^{a}

Means in the same column with different case letters differ by Tukey test (p<0.05); ns, non-significant; TRAP, tartrate-resistant acid phosphatase; ALP, alkaline phosphatase.

plate activity, accelerated the calcification process, and remodeled the newly formed tissue into trabecular bone. Therefore, manganese deficiency negatively affected chondrocyte proliferation, development, and inhibited cell death (Wang *et al.*, 2015).

Regarding the phytase and manganese effects on serum TRAP of 21-day old broilers (Table 7), it was observed that when the manganese levels were 0.93 and 0.43 ppm, the addition of phytase had no significant effect; on the other hand, when the manganese level was 0.18 ppm, the addition of phytase increased TRAP activity, and when the manganese level was 0.68 ppm, the opposite behavior was observed. In the absence of phytase, the addition of manganese above 0.68 ppm increased TRAP activity; however, for broilers fed with a diet of 500 phytase $U.kg^{-1}$, the manganese addition was not sufficient to alter the production of TRAP levels in the blood. The treatments containing 1,000 $U.kg^{-1}$ of phytase and manganese levels of 0.68 and 0.93 ppm demonstrated statistical differences in TRAP activity.

Table 7 – Means of serum tartrate-resistant acid phosphataseactivity of 21-day old broilers fed different manganese and phytase levels.

Phytase level (U/kg)	Manganese level (ppm)			
	0.18	0.43	0.68	0.93
0	0.30 ^{Bb}	0.23 ^{Ab}	1.0 ^{Aa}	0.67 ^{Aab}
500	0.69 ^{Aba}	0.61 ^{Aa}	0.65 ^{Aba}	0.70 ^{Aa}
1000	0.79 ^{Aab}	0.54 ^{Aab}	0.48 ^{Bb}	1.02 ^{Aa}

Means in the same column with different case letters differ by Tukey test ($p<0.05$); ns, non-significant.

The investigation of interactions between the levels of phytase and manganese revealed that for ALP of the tibia of 21-day old broilers (Table 8), the addition of phytase changed the ALP activity when the manganese level was 0.43 ppm, and the use of 1,000 $U.kg^{-1}$of the referred enzyme decreased ALP activity. With the absence of phytase, the treatment with 0.68 ppm manganese induced a decrease in ALP (587.44 $U.mg^{-1}$) level in comparison with 0.43 ppm of manganese (933.48 $U.mg^{-1}$). With a diet including phytase, it was observed that ALP activity increased when the manganese content was 0.18 ppm and the phytase level was 1,000 $U.kg^{-1}$ (Manobhavan *et al.*, 2016)

Bae & Kim (2008) investigated the effect of manganese on bone mineral density and bone metabolism in ovariectomized rats and rats subjected to sham surgery, supplemented with or without 0.001 and 0.01% manganese for twelve weeks. The bone mineral density of the lumbar vertebrae, femur, and tibia, significantly decreased in ovariectomized rats compared with those that underwent sham surgery. The authors also verified that the bone mineral density of the lumbar vertebra significantly increased with increasing concentrations of manganese in rats subjected to sham surgery.

Table 8 – Means of tibia alkaline phosphatase activity of 21-day old broilers fed different manganese and phytase levels.

Phytase level (U/kg)	Manganese level (ppm)			
	0.18	0.43	0.68	0.93
0	859.77 ^{Aab}	933.48 ^{Aa}	587.44 ^{Ab}	683.44 ^{Aab}
500	775.28 ^{Aa}	872.60 ^{Aa}	706.29 ^{Aa}	612.55 ^{Aa}
1000	1041.05 ^{Aa}	573.20 ^{Bb}	669.79 ^{Ab}	629.24 ^{Ab}

Means in the same column with different case letters differ by Tukey test ($p<0.05$); ns, non-significant.

The tibia of poultry have been widely used in experiments to determine the nutritional requirements and bioavailability of minerals from organic and inorganic sources (Mello *et al.*, 2012; An *et al.*, 2016). Bao *et al.* (2009) investigated the effect of supplementation of five different levels of zinc and manganese in the diet of broilers for 35 days, the parameters included were performance, bone size, hardness, and mineral content, and it was demonstrated that lower levels of zinc and manganese significantly inhibited the growth of the tibia and that supplementation with zinc and manganese resulted in a linear increase in growth. On the other hand, Laurentiz (2005) showed that there was no significant difference ($p>0.05$) between the weight gain, feed intake, feed conversion ratio for different trace elements, phytase and doses used in the present study.

The investigation of various levels of phytase and manganese in the diet of broilers revealed that the enzymatic activity of the tibia was more sensitive in detecting changes in acid and ALP levels. The addition of 0.93 ppm manganese in the diet resulted in a significant increase in tibia ALP activity in 42-day old broilers. In the tibia of 42-day old animals, the addition of phytase induced an increase in acid and ALP activities, suggesting that manganese, similar to zinc, increased the growth plate activity and accelerated the calcification and bone remodeling processes. In addition, zinc levels demonstrated a linear increase of tibia growth, suggesting that this trace element may be essential for bone longitudinal growth.

Although not all the enzymatic activities were affected with the different treatments, it was clear that the use of phytase, along with trace minerals, was able to improve the response of the animal to the rapid growth currently required and provided the nutrients necessary for adequate bone metabolism.

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