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Original Article

High Doses of Phytase Alleviate the Negative Effects of Calcium and Phosphorus Imbalance on Growth Performance and Bone Mineralization in Broiler Chickens

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ABSTRACT

This study investigated the effect of calcium (Ca) and phytase interaction on growth performance and bone quality in 1–42-day-old broiler chickens. A total of 624 female one-day-old Ross 308 broilers were allotted to 13 treatments with four replicates and 12 birds per replicate. A 2 × 6 factorial experiment was designed to test the combinations of 0.50% and 1.00% Ca with 0, 500, 1,000, 2,500, 5,000, and 10,000 FTU/kg phytase in the basal diet (0.25% non-phytate phosphorus, NPP). The control diet contained adequate Ca and phosphorus (P). Dietary Ca, phytase, and their interaction affected growth performance and bone mineralization of broilers at 1–42 days of age (p<0.05). The broilers fed with 1.00% Ca had lower body weight gain (BWG) and feed intake (FI) compared with the birds fed with 0.50% Ca (p<0.05). The BWG, FI, leg bone weight, and ash weight of the broilers fed with 0.25% NPP were lower than those of birds fed with the control diet (p<0.05). The addition of 500–10,000 FTU/kg phytase improved growth rate and leg bone quality, especially at 1.00% Ca (p<0.05). No differences were observed in growth performance and bone quality of 42-day-old broilers fed with 1.00% Ca + 2,500–10,000 FTU/kg phytase and the control diet (p>0.05). These data indicated that high doses of phytase (2,500–10,000 FTU/kg) alleviate the negative effects of Ca and P imbalance (Ca-to-NPP ratio = 4.0) on growth performance and bone mineralization of broiler chickens.

INTRODUCTION

Calcium (Ca) and phosphorus (P) are essential minerals in poultry diets. Dietary Ca or P deficiency results in poor growth performance and bone quality in broiler chickens (Han *et al.*, 2018; Li *et al.*, 2020). Limestone, dicalcium phosphate, and monocalcium phosphate are used as feed additives to meet the Ca and P requirements of poultry in China. In poultry feed, total P includes organic P (i.e., phytate P) and inorganic P (i.e., non-phytate P, NPP). NPP is calculted as: NPP = total P – phytate P. NPP is easily used by poultry, whereas phytate P can't be used effectively.

The optimal dietary Ca-to-NPP ratio is approximately 2.0 in broilers (Rama Rao *et al.*, 2007; Han *et al.*, 2016; Diaz-Alonso *et al.*, 2019), in which dietary Ca and P are considered balanced, and birds obtain greater growth performance. The recommended dietary Ca and NPP levels for 1–21-day-old broilers are 1.00% and 0.45%, respectively (NRC, 1994). In Ca- and P-balanced diet, decreasing the Ca and NPP levels to 0.76% and 0.38% maximizes growth performance of broilers at 1–24 days without negative effects on bone ash and strength (Kiani & Taheri, 2020). These data revealed that Ca and P contents can be appropriately reduced in the balanced diets of broilers.

Dietary Ca and P imbalance has negative effects on growth performance and bone mineralization of broilers (Li *et al.*, 2012). An increase in Ca-to-NPP ratio from 2.1 to 3.8 decreases the BWG and FI of broilers fed with P-deficient diets (Qian *et al.*, 1997). Low P diets are formulated in poultry production to reduce P pollution to the environment. However, the question is how to maintain the growth performance of the broilers fed with P-deficient diets.

Approximately 67% of total P in cereals is presented as phytate P, which can not be effectively utilized by broilers (Steiner *et al.*, 2007). Phytase is used to hydrolyze phytate P in poultry diets. Two kinds of phytase (i. e., endogenous and exogenous phytases) have been reported in poultry. Endogenous phytase is obtained from poultry intestinal mucosa (Maenz & Classen, 1998; Morgan *et al.*, 2015), whereas exogenous phytase is obtained from commercial microbial product, cereal, and its by-products (Xiong *et al.*, 2005; Steiner *et al.*, 2007). Intestinal endogenous phytase can hydrolyze phytate P in broiler diets (Applegate *et al.*, 2003; Tamim *et al.*, 2004). However, only a small amount of phytate P is degraded by endogenous intestinal phytase. Thus, exogenous phytase should be added to poultry diets. In recent years, commercial microbial phytase has been produced and widely used in poultry feed. The addition of phytase increases phytate P hydrolysis and total P retention and improves growth performance and bone quality in broilers fed with P-deficient diets (Shirley & Edwards, 2003; Augspurger & Baker, 2004; Manangi & Coon, 2008). However, the effects of phytase supplementation on growth performance of broilers fed with diets with balanced and imbalanced Ca-to-NPP ratios has not been examined.

Therefore, this study aimed to investigate the effects of dietary phytase levels on growth performance, bone mineralization, and blood mineral concentration in 1- to 42-day-old broilers fed with Ca-inadequate and -adequate diets.

MATERIAL AND METHODS

Animals, diet, and management

All animal experimental procedures used in the present study were approved by the Animal Care and Use Committee of Henan Agricultural University and Shangqiu Normal University.

Phytase was supplied by Guangdong VTR Bio-Tech Co., Ltd. (Zhuhai, China). Microbial phytase was obtained from the *Trichoderma* strain and expressed in yeast *Pichia pastoris*. The product contained 5,000 FTU/g phytase, where 1 FTU is equivalent to 1 phytase unit, which represents the amount of enzyme that liberates 1 µmol P per min from 0.0051 mol/L sodium phytate at 37 °C and pH 5.50. The enzyme was added to the diets in powder form.

On the day of hatch, a total of 624 female Ross 308 broilers were randomly allotted to 13 treatment groups with four stainless-steel replicate cages (190 cm × 50 cm × 35 cm) and 12 birds per replicate. A 2 × 6 factorial experiment was designed to test the combinations of 0.50% and 1.00% Ca with 0, 500, 1,000, 2,500, 5,000, and 10,000 FTU/kg phytase in the basal diet containing 0.25% NPP (Table 1). The control diet contained 1.00% Ca and 0.45% NPP for broilers aged 1–21 days and 0.90% Ca and 0.35% NPP for birds aged 22–42 days. The boilers were provided access to mash feed and water *ad libitum*. The lighting program consisted of 23 h of light and 1 h of darkness on days 1–3, 20 h of light and 4 h of darkness on days 4–21, and 18 h of light and 6 h of darkness on days 22–42. Room temperature was controlled at 33 °C on days 1–3, 30 °C on days 4–7, 27 °C on days 8–21, and 24 °C on days 22–42.

Sample collection

The broilers were weighed on days 1, 21, and 42. All broilers that died spontaneously during the experiment were weighed, and the weight was used to correct the FI. Two chickens per replicate (eight broilers per treatment) were selected randomly for blood and bone collection. Blood samples (5 mL) were collected into tubes with anticoagulant by cardiac puncture on days 21 and 42, and then were centrifuged for 10 min at 3,000 × g at 20 °C to separate plasma. The broilers were euthanized by cervical dislocation after blood sample collection. The femur, tibia, and metatarsus were excised and frozen at –20 °C.

Sample analysis

Blood Ca and P concentrations were determined using a Shimadzu CL-8000 analyzer (Shimadzu Corp., Kyoto, Japan) following the manufacturer's instructions. Leg bones were cleaned, placed in a container with ethanol for 48 h to remove water and polar lipids, and then extracted in anhydrous ether for 48 h to remove non-polar lipids (Hall *et al.*, 2003). The bones were dried at 105 °C for 24 h before weighing. Bone ash weight and percentage content were determined by burning the leg bones in a muffle furnace for 48 h at

Table 1 – Ingredients and nutrient composition of the basal diets.

Item	Days 1–21			Days 22–42		
	Control	0.50% Ca	1.00% Ca	Control	0.50% Ca	1.00% Ca
Ingredient (%)						
Corn	56.97	60.73	57.91	62.31	65.01	62.19
Soybean meal (43% CP)	32.00	32.00	32.00	28.00	28.00	28.00
Soybean oil	1.60	1.60	1.60	2.60	2.60	2.60
Swine lard	1.32	0.00	0.99	0.95	0.00	0.99
Soybean protein concentrate (63% CP)	3.99	3.47	3.86	2.67	2.30	2.69
Limestone	1.36	0.67	2.09	1.46	0.70	2.13
Dicalcium phosphate	1.94	0.71	0.73	1.35	0.73	0.74
L-lysine·HCl (98%)	0.14	0.14	0.14	0.14	0.14	0.14
DL-methionine (98%)	0.14	0.14	0.14	0.08	0.08	0.08
Trace mineral premix[1]	0.01	0.01	0.01	0.01	0.01	0.01
Vitamin premix[2]	0.03	0.03	0.03	0.03	0.03	0.03
Choline chloride (50%)	0.20	0.20	0.20	0.10	0.10	0.10
Sodium chloride	0.30	0.30	0.30	0.30	0.30	0.30
Total	100.00	100.00	100.00	100.00	100.00	100.00
Nutrients composition (%)						
Metabolizable energy (kcal/kg)	2,975	2,975	2,975	3,065	3,065	3,065
Crude protein	21.24	21.24	21.24	19.15	19.15	19.15
Calcium	1.00	0.50	1.00	0.90	0.50	1.00
Analyzed calcium	0.98	0.51	0.95	0.86	0.47	0.94
Total phosphorus	0.69	0.49	0.49	0.57	0.48	0.47
Analyzed total phosphorus	0.64	0.46	0.47	0.55	0.46	0.45
Non-phytate phosphorus	0.45	0.25	0.25	0.35	0.25	0.25

[1]The trace mineral premix provided the following nutrients (per kg of diet): 80 mg iron, 40 mg zinc, 8 mg copper, 60 mg manganese, 0.35 mg iodine, and 0.15 mg selenium.

[2]The vitamin premix provided the following nutrients (per kg of diet): 8,000 IU vitamin A, 1,000 IU vitamin D_3, 20 IU vitamin E, 0.5 mg menadione, 2.0 mg thiamine, 8.0 mg riboflavin, 35 mg niacin, 3.5 mg pyridoxine, 0.01 mg vitamin B_{12}, 10.0 mg pantothenic acid, 0.55 mg folic acid, and 0.18 mg biotin.

600 °C. The Ca and total P contents in the diets and bones were determined through the method of Han *et al.* (2009).

Statistical analysis

Replicate means were used as the experimental units. All data in the 13 treatments was analyzed by using one-way ANOVA procedure of SAS software (SAS Institute, 2002). Two-way ANOVA procedure was used to evaluate the main effect of dietary Ca and phytase interaction. Means were compared using Tukey test for significant probability values ($p<0.05$).

RESULTS AND DISCUSSION

Growth performance

Dietary Ca, phytase, and their interaction affected the BWG and FI of broiler chickens aged 1–21 and aged 1–42 days ($p<0.05$), but did not affect the feed conversion ratio (FCR) ($p>0.05$) (Table 2). The increase in dietary Ca from 0.50% to 1.00% negatively affected growth performance of broilers. The broilers fed with 1.00% Ca (Ca-to-NPP ratio = 4) had lower BWG and FI compared with the birds fed with 0.50% Ca (Ca-to-NPP ratio = 2) ($p<0.05$). These results were in accordance with those reported by previous research (Applegate *et al.*, 2003; Tamim *et al.*, 2004; Rama Rao *et al.*, 2007; Manangi and Coon, 2008; Amerah *et al.*, 2014; Han *et al.*, 2016), in which the highest growth rate of broiler was observed at dietary Ca-to-NPP ratio of 2.0 and the increase of Ca-to-NPP ratio from 2.0 to 7.0 decreased phytate P degradation, BWG, and the FI in the broilers fed with P-deficient diets.

Broilers fed with the negative diet (1.00% Ca, 0.25% NPP) had lower BWG and FI than those fed with the control diet ($p<0.05$). As an essential mineral, P deficiency decreases the growth rate of broilers (Shirley & Edwards, 2003; Manangi & Coon, 2008; Han *et al.*, 2018). Thus, inorganic P or phytase should

Table 2 – Effects of dietary Ca and phytase levels on growth performance of broiler chickens at 1–42 days of age.[1]

Ca (%)	Phytase (FTU/kg)	BWG[2] (g)			FI[2] (g)			FCR[2]			Mortality (%)		
		Days 1–21	Days 22–42	Days 1–42	Days 1–21	Days 22–42	Days 1–42	Days 1–21	Days 22–42	Days 1–42	Days 1–21	Days 22–42	Days 1–42
Control	0	699^{ab}	1649^{a}	2348^{ab}	1103^{a}	3398^{ab}	4501^{ab}	1.58	2.06	1.92	0	0	0
0.50	0	584^{d}	1520^{ab}	2104^{cd}	912^{c}	3056^{bc}	3968^{d}	1.56	2.01	1.89	5.0	2.5	7.5
0.50	500	676^{ab}	1700^{a}	2376^{a}	1059^{a}	3421^{ab}	4480^{ab}	1.57	2.01	1.89	0	0	0
0.50	1,000	667^{ab}	1728^{a}	2395^{a}	1040^{ab}	3386^{ab}	4426^{abc}	1.56	1.96	1.85	0	0	0
0.50	2,500	699^{ab}	1700^{a}	2399^{a}	1075^{a}	3403^{ab}	4478^{ab}	1.54	2.00	1.87	0	0	0
0.50	5,000	643^{bcd}	1699^{a}	2342^{ab}	1063^{a}	3340^{ab}	4403^{abcd}	1.65	1.97	1.88	0	0	0
0.50	10,000	696^{ab}	1717^{a}	2413^{a}	1096^{a}	3372^{ab}	4468^{ab}	1.57	1.96	1.85	0	0	0
1.00	0	486^{e}	1347^{b}	1833^{e}	788^{d}	2665^{c}	3453^{e}	1.62	1.98	1.89	10.0	7.5	17.5
1.00	500	596^{cd}	1485^{ab}	2081^{d}	951^{bc}	3028^{bc}	3979^{cd}	1.60	2.05	1.92	2.5	0	2.5
1.00	1,000	656^{abc}	1469^{ab}	2125^{bcd}	1014^{ab}	3113^{ab}	4127^{bcd}	1.55	2.12	1.94	0	0	0
1.00	2,500	691^{ab}	1629^{a}	2320^{abc}	1058^{a}	3340^{ab}	4398^{abcd}	1.53	2.05	1.90	0	0	0
1.00	5,000	679^{ab}	1631^{a}	2310^{abcd}	1108^{a}	3180^{ab}	4288^{bcde}	1.63	1.95	1.86	0	0	0
1.00	10,000	723^{a}	1724^{a}	2447^{a}	1109^{a}	3504^{a}	4613^{a}	1.53	2.03	1.89	0	0	0
SEM		9	22	27	14	38	48	0.01	0.01	0.01	0.5	0.5	0.8
Main effect													
0.50		661^{a}	1677^{a}	2338^{a}	1041^{a}	3329^{a}	4370^{a}	1.58	1.99	1.87	0.8	0.4	1.3
1.00		639^{b}	1548^{b}	2186^{b}	1005^{b}	3138^{b}	4143^{b}	1.58	2.03	1.90	2.1	1.3	3.3
	0	535^{d}	1434^{b}	1969^{c}	850^{d}	2861^{b}	3710^{c}	1.59^{ab}	2.00	1.89	7.5	5.0	12.5
	500	636^{c}	1593^{ab}	2229^{b}	1005^{c}	3224^{a}	4229^{b}	1.59^{ab}	2.03	1.90	1.3	0	1.3
	1,000	662^{bc}	1599^{ab}	2260^{b}	1027^{bc}	3249^{a}	4276^{ab}	1.55^{b}	2.04	1.90	0	0	0
	2,500	695^{ab}	1665^{a}	2360^{ab}	1066^{abc}	3372^{a}	4438^{ab}	1.53^{b}	2.03	1.88	0	0	0
	5,000	661^{bc}	1665^{a}	2326^{ab}	1086^{ab}	3260^{a}	4346^{ab}	1.64^{a}	1.96	1.87	0	0	0
	10,000	710^{a}	1721^{a}	2430^{a}	1103^{a}	3438^{a}	4540^{a}	1.55^{b}	2.00	1.87	0	0	0
Source of variation													
Ca		0.005	<0.001	<0.001	0.004	<0.001	<0.001	0.914	0.115	0.139	0.057	0.361	0.043
Phytase		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.007	0.527	0.856	<0.001	0.012	<0.001
Ca ×Phytase		0.002	0.169	0.003	0.001	0.041	0.007	0.452	0.370	0.512	0.138	0.519	0.035

[1]The control diet contained 1.00% Ca and 0.45% NPP for broilers aged 1–21 days and 0.90% Ca and 0.35% NPP for birds aged 22–42 days. The negative diet contained 1.00% Ca and 0.25% NPP for birds aged 1–42 days.

[2]BWG = body weight gain, FI = feed intake, and FCR = feed conversion ratio.

[a–e]Means in the same column without a common superscript differ ($p<0.05$).

be added in the diets to meet the P requirement for growth performance of broilers.

The addition of 500 FTU/kg phytase to 0.50% Ca increased the BWG and FI of broilers ($p<0.05$). The increase in phytase levels from 500 to 10,000 FTU/kg did not affect the BWG, FI, and FCR ($p>0.05$). By contrast, the addition of 500–10,000 FTU/kg phytase to 1.00% Ca linearly enhanced the BWG and FI of broilers ($p<0.05$). No differences were observed in the growth rate of 42-day-old broilers fed with 2,500–10,000 FTU/kg phytase ($p>0.05$). The growth performances of the broilers fed with 0.50% Ca + 500–10,000 FTU/kg phytase and 1.00% Ca + 2,500–10,000 FTU/kg phytase were equivalent to those of the birds fed with control diet.

The addition of phytase improves growth performance of broiler chickens (Shirley & Edwards, 2003; Farhadi *et al.*, 2017; Pieniazek *et al.*, 2017; McCormick *et al.*, 2017; Gautier *et al.*, 2018; Babatunde *et al.*, 2019). Ca and P in broiler diets are considered balanced when the Ca-to-NPP ratio is approximately 2.0 (Rama Rao *et al.*, 2007; Han *et al.*, 2016). Low levels of phytase improve growth performance of broilers when dietary Ca-to-NPP ratio is 2.0 (Driver *et al.*, 2005; Walk *et al.*, 2012). By contrast, high doses of phytase (5,000–10,000 FTU/kg) are needed to maintain the growth of broilers when dietary Ca-to-NPP ratio is 4.6–7.5 (Shirley & Edwards, 2003; Augspurger & Baker, 2004). Similar results were noted in the present study. No differences were observed in the BWG, FI, and FCR of the broilers fed with 0.50% Ca + 500 FTU/kg phytase, 1.00% Ca + 10,000 FTU/kg phytase, and control diet. Hence, a small amount of phytase (500 FTU/kg) in Ca- and P-balanced diet (Ca-to-NPP ratio = 2.0) is adequate for broiler growth, but high doses of phytase (2,500–10,000 FTU/kg) are needed to alleviate the negative effects of Ca and P imbalance (Ca-to-NPP ratio = 4.0) on growth performance of broilers.

Bone mineralization

Dietary Ca, phytase, and their interaction affected leg bone mineralization of broiler chickens ($p<0.05$) (Tables 3, 4, and 5). Increasing dietary Ca from 0.50% to 1.00% decreased bone quality in broilers aged 21 days. The percentages of ash and Ca in the femur, tibia, and metetarsus of 21-day-old broilers fed with 1.00% Ca (Ca-to-NPP ratio = 4.0) were lower than those of birds fed with 0.50% Ca (Ca-to-NPP ratio = 2.0) ($p<0.05$). Our results agreed with those reported by Qian *et al.* (1997), in which the increase in Ca-to-NPP ratio from 2.1 to 3.8 decreased the toe ash percentage of broilers. Hence, dietary Ca and P imbalance deteriorates the bone development of poultry.

Dietary P-deficiency influenced bone development. The bone weight, ash weight, and percentage content of ash in the femur and tibia of 42-day-old broilers fed the negative diet (1.00% Ca and 0.25% NPP) were lower than those of birds fed with the control diet ($p<0.05$). These results were in accordance with those reported by Viveros *et al.* (2002), Han *et al.* (2018), and

Table 3 – Effects of dietary Ca and phytase levels on femur mineralization of broiler chickens at 21 and 42 days of age.

Ca (%)	Phytase (FTU/kg)	Weight (g)		Ash (g)		Ash (%)		Ca (%)		*p* (%)	
		Day 21	Day 42	Day 21	Day 42	Day 21	Day 42	Day 21	Day 42	Day 21	Day 42
Control	0	1.36^{a}	4.30^{ab}	0.64^{a}	1.96^{a}	47.53^{a}	45.87^{ab}	17.03^{ab}	17.08^{ab}	8.72^{a}	9.10^{a}
0.50	0	1.08^{bcd}	3.58^{bc}	0.47^{cd}	1.51^{bc}	45.68^{ab}	42.51^{d}	16.95^{ab}	15.61^{c}	8.31^{a}	7.64^{b}
0.50	500	1.23^{abc}	4.31^{ab}	0.58^{abc}	1.86^{ab}	47.42^{a}	43.61^{bcd}	17.48^{a}	16.00^{bc}	8.56^{a}	8.16^{ab}
0.50	1,000	1.15^{abcd}	4.17^{ab}	0.54^{abc}	1.89^{ab}	46.66^{a}	44.79^{abcd}	17.61^{a}	16.83^{abc}	8.34^{a}	8.29^{ab}
0.50	2,500	1.19^{abcd}	3.87^{ab}	0.54^{abc}	1.76^{ab}	45.83^{ab}	44.91^{abcd}	17.69^{a}	16.54^{abc}	8.36^{a}	8.57^{ab}
0.50	5,000	1.07^{cd}	3.99^{ab}	0.50^{bc}	1.77^{ab}	46.76^{a}	43.60^{bcd}	16.84^{ab}	16.84^{abc}	8.32^{a}	8.33^{ab}
0.50	10,000	1.33^{ab}	3.88^{ab}	0.63^{ab}	1.55^{bc}	47.41^{a}	42.47^{d}	17.13^{ab}	16.26^{abc}	8.53^{a}	8.13^{ab}
1.00	0	0.92^{d}	3.04^{c}	0.34^{d}	1.29^{c}	42.28^{b}	42.69^{cd}	14.80^{c}	15.82^{bc}	6.64^{c}	7.52^{b}
1.00	500	1.04^{cd}	3.58^{bc}	0.45^{cd}	1.57^{bc}	42.81^{b}	44.36^{abcd}	15.58^{bc}	15.97^{bc}	7.31^{bc}	8.03^{ab}
1.00	1,000	1.24^{abc}	3.77^{abc}	0.56^{abc}	1.67^{ab}	45.28^{ab}	44.58^{abcd}	16.24^{abc}	16.34^{abc}	8.08^{ab}	8.01^{ab}
1.00	2,500	1.33^{ab}	3.82^{abc}	0.63^{ab}	1.71^{ab}	47.19^{a}	45.38^{abc}	17.22^{a}	16.58^{abc}	8.42^{a}	8.47^{ab}
1.00	5,000	1.21^{abc}	4.22^{ab}	0.58^{abc}	1.97^{a}	47.06^{a}	46.79^{a}	17.14^{ab}	17.39^{a}	8.47^{a}	8.90^{a}
1.00	10,000	1.38^{a}	4.54^{a}	0.66^{a}	2.05^{a}	48.24^{a}	45.40^{abc}	17.56^{a}	17.10^{ab}	8.44^{a}	9.10^{a}
SEM		0.02	0.07	0.01	0.03	0.31	0.23	0.14	0.10	0.09	0.10
Main effect											
0.50		1.17	3.97	0.54	1.73	46.63^{a}	43.65^{b}	17.28^{a}	16.35	8.40^{a}	8.18
1.00		1.19	3.83	0.54	1.71	45.48^{b}	44.87^{a}	16.42^{b}	16.53	7.89^{b}	8.34
	0	1.00^{c}	3.31^{b}	0.40^{c}	1.40^{b}	43.98^{c}	42.60^{b}	15.88^{b}	15.71^{c}	7.48^{b}	7.58^{b}
	500	1.14^{bc}	3.95^{a}	0.51^{b}	1.72^{a}	45.11^{bc}	43.99^{ab}	16.53^{ab}	15.98^{bc}	7.93^{ab}	8.10^{ab}
	1,000	1.19^{ab}	3.97^{a}	0.55^{b}	1.78^{a}	45.97^{abc}	44.68^{a}	16.93^{a}	16.59^{ab}	8.21^{a}	8.15^{ab}
	2,500	1.26^{ab}	3.85^{a}	0.59^{ab}	1.73^{a}	46.51^{ab}	45.14^{a}	17.46^{a}	16.56^{ab}	8.39^{a}	8.52^{a}
	5,000	1.14^{bc}	4.11^{a}	0.54^{b}	1.87^{a}	46.91^{ab}	45.19^{a}	16.99^{a}	17.11^{a}	8.39^{a}	8.61^{a}
	10,000	1.36^{a}	4.21^{a}	0.65^{a}	1.80^{a}	47.82^{a}	43.94^{ab}	17.34^{a}	16.68^{ab}	8.48^{a}	8.61^{a}
Source of variation											
Ca		0.663	0.130	0.716	0.715	0.017	<0.001	<0.001	0.231	<0.001	0.935
Phytase		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.002
Ca ×Phytase		0.006	<0.001	<0.001	<0.001	0.002	0.006	<0.001	0.211	<0.001	0.173

$^{a–d}$Means in the same column without a common superscript differ ($p<0.05$).

Table 4 – Effects of dietary Ca and phytase levels on tibia mineralization of broiler chickens at 21 and 42 days of age.

Ca (%)	Phytase (FTU/kg)	Weight (g)		Ash (g)		Ash (%)		Ca (%)		*p* (%)	
		Day 21	Day 42	Day 21	Day 42	Day 21	Day 42	Day 21	Day 42	Day 21	Day 42
Control	0	1.79^{ab}	5.89^{ab}	0.86^{ab}	2.66^{ab}	48.00^{ab}	47.37^{ab}	17.55^{ab}	17.57^{ab}	8.82^{ab}	8.53^{abcd}
0.50	0	1.32^{ef}	5.07^{bcd}	0.61^{d}	2.29^{bc}	46.26^{b}	45.17^{bc}	16.67^{abc}	16.02^{c}	7.87^{bc}	7.71^{d}
0.50	500	1.54^{bcde}	5.95^{ab}	0.72^{bcd}	2.65^{ab}	46.97^{ab}	45.42^{bc}	16.85^{abc}	16.40^{bc}	8.53^{ab}	7.92^{cd}
0.50	1,000	1.60^{abcd}	5.50^{bc}	0.75^{abcd}	2.59^{ab}	47.24^{ab}	48.50^{a}	17.42^{ab}	17.42^{abc}	8.54^{ab}	8.05^{bcd}
0.50	2,500	1.70^{abc}	5.25^{bc}	0.82^{abc}	2.45^{abc}	48.18^{ab}	46.93^{abc}	17.51^{ab}	17.11^{abc}	8.00^{ab}	8.17^{abcd}
0.50	5,000	1.40^{de}	5.11^{bcd}	0.68^{cd}	2.41^{abc}	47.68^{ab}	48.41^{a}	17.44^{ab}	16.48^{abc}	8.43^{ab}	8.02^{bcd}
0.50	10,000	1.66^{abc}	5.30^{bc}	0.79^{abc}	2.46^{abc}	47.69^{ab}	46.72^{abc}	17.43^{ab}	16.80^{abc}	8.12^{ab}	8.95^{ab}
1.00	0	1.13^{f}	4.16^{d}	0.44^{e}	1.86^{c}	38.65^{d}	44.79^{c}	14.56^{d}	16.55^{abc}	6.68^{c}	8.10^{bcd}
1.00	500	1.47^{cde}	4.75^{cd}	0.63^{d}	2.23^{bc}	43.11^{c}	47.24^{ab}	16.04^{c}	17.59^{ab}	7.63^{bc}	8.62^{abcd}
1.00	1,000	1.62^{abcd}	4.99^{bcd}	0.75^{abcd}	2.31^{bc}	47.22^{ab}	46.27^{abc}	17.94^{a}	17.60^{ab}	8.75^{ab}	8.54^{abcd}
1.00	2,500	1.81^{a}	5.29^{bc}	0.83^{abc}	2.48^{abc}	47.55^{ab}	47.07^{abc}	16.47^{bc}	17.51^{abc}	7.85^{bc}	9.02^{ab}
1.00	5,000	1.60^{abcd}	5.60^{bc}	0.79^{abc}	2.58^{ab}	47.77^{ab}	46.19^{abc}	17.01^{abc}	17.41^{abc}	8.80^{ab}	9.12^{a}
1.00	10,000	1.84^{a}	6.76^{a}	0.87^{a}	3.01^{a}	48.68^{a}	48.15^{a}	17.98^{a}	17.93^{a}	9.11^{a}	8.88^{abc}
SEM	3.4	0.03	0.10	0.02	0.05	0.38	0.20	0.14	0.11	0.11	0.10
Main effect											
0.50		1.53	5.36	0.73	2.48	47.34^{a}	46.86	17.22^{a}	16.70^{b}	8.25	8.14^{b}
1.00		1.58	5.26	0.72	2.41	45.50^{b}	46.62	16.67^{b}	17.43^{a}	8.14	8.72^{a}
	0	1.22^{c}	4.62^{c}	0.53^{d}	2.07^{b}	42.45^{c}	44.98^{b}	15.62^{c}	16.29^{b}	7.27^{b}	7.91^{b}
	500	1.50^{b}	5.35^{b}	0.68^{c}	2.44^{ab}	45.04^{b}	46.33^{ab}	16.44^{b}	16.99^{ab}	8.08^{a}	8.27^{ab}
	1,000	1.61^{ab}	5.25^{b}	0.75^{bc}	2.45^{ab}	47.23^{a}	47.38^{a}	17.68^{a}	17.51^{a}	8.65^{a}	8.30^{ab}
	2,500	1.76^{a}	5.27^{b}	0.82^{ab}	2.46^{a}	47.86^{a}	47.00^{a}	16.99^{ab}	17.31^{a}	7.93^{ab}	8.59^{ab}
	5,000	1.50^{b}	5.35^{b}	0.74^{c}	2.50^{a}	47.72^{a}	47.30^{a}	17.23^{a}	16.94^{ab}	8.62^{a}	8.57^{ab}
	10,000	1.75^{a}	6.03^{a}	0.83^{a}	2.74^{a}	48.19^{a}	47.43^{a}	17.70^{a}	17.36^{a}	8.62^{a}	8.92^{a}
Source of variation											
Ca		0.178	0.389	0.520	0.402	<0.001	0.381	<0.001	<0.001	0.431	0.002
Phytase		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.004	<0.001	0.034
Ca ×Phytase		0.004	<0.001	<0.001	0.003	<0.001	<0.001	<0.001	0.478	0.001	0.466

[a–f]Means in the same column without a common superscript differ ($p<0.05$).

Li *et al.* (2020), in which P-deficient diet caused lower tibia weight, ash weight, and percentage contents of ash, Ca, and P in broilers. Broilers are sensitive to insufficient P in diets. P deficiency damages the bone growth and mineralization of broilers.

The addition of phytase did not enhance bone weight and ash weight in the femur, tibia, and metatarsus of 42-day-old broilers at 0.50% Ca ($p>0.05$). By contrast, adding phytase improved leg bone development at 1.00% Ca ($p<0.05$). The bone weight and ash weight in the three leg bones of broilers at 42 days were linearly increased by dietary phytase levels at 1.00% Ca ($p<0.05$). The interactions between dietary Ca and phytase were observed in the weight, ash weight, and ash percentage content of the leg bones in 42-day-old broilers ($p<0.05$). No differences in bone weight and ash weight were observed among the broilers fed with 0.50% Ca + 500–5,000 FTU/kg phytase, 1.00% Ca + 2,500–10,000 FTU/kg phytase, and the control diet ($p>0.05$).

The addition of phytase improves tibia ash weight and percentage in broilers fed with low-P diets (Shirley & Edwards, 2003; Augspurger & Baker, 2004; Han *et al.*, 2009; Walk *et al.*, 2012; Pieniazek *et al.*, 2017; McCormick *et al.*, 2017; Gautier *et al.*, 2018). In the present study, the addition of phytase did not affect the leg bone mineralization of 42-day-old broilers at 0.50% Ca. By contrast, phytase linearly improved the bone quality of the tibia, femur, and metatarsus of broilers at 1.00% Ca. These data revealed that the efficacy of phytase is affected by the Ca-to-NPP ratio, and high doses of phytase (2,500–10,000 FTU/kg) are required to alleviate the negative effect of dietary Ca and P imbalance on the bone mineralization of broilers.

Table 5 – Effects of dietary Ca and phytase levels on metatarsus mineralization of broiler chickens at 21 and 42 days of age.

Ca (%)	Phytase (FTU/kg)	Weight (g)		Ash (g)		Ash (%)		Ca (%)		*p* (%)	
		Day 21	Day 42	Day 21	Day 42	Day 21	Day 42	Day 21	Day 42	Day 21	Day 42
Control	0	1.02^{a}	3.27^{a}	0.47^{a}	1.36^{a}	46.12^{a}	41.61^{ab}	16.83^{ab}	15.14^{ab}	8.11^{a}	7.40
0.50	0	0.80^{abc}	2.80^{ab}	0.35^{bc}	1.16^{ab}	43.58^{abc}	41.79^{ab}	16.64^{ab}	15.18^{ab}	7.54^{ab}	7.45
0.50	500	0.91^{ab}	3.27^{a}	0.41^{abc}	1.40^{a}	44.96^{ab}	42.79^{ab}	16.88^{ab}	15.57^{ab}	8.02^{a}	7.82
0.50	1,000	0.91^{ab}	2.90^{ab}	0.41^{abc}	1.24^{ab}	45.58^{ab}	42.81^{ab}	16.94^{ab}	16.29^{ab}	8.10^{a}	7.76
0.50	2,500	0.91^{ab}	2.92^{ab}	0.42^{abc}	1.28^{ab}	45.64^{ab}	44.17^{a}	16.80^{ab}	16.39^{a}	8.07^{a}	7.89
0.50	5,000	0.78^{bc}	2.96^{ab}	0.36^{bc}	1.28^{ab}	45.80^{a}	43.42^{ab}	16.66^{ab}	16.28^{ab}	8.16^{a}	8.06
0.50	10,000	0.95^{ab}	2.94^{ab}	0.44^{abc}	1.19^{ab}	46.03^{a}	40.67^{b}	17.04^{ab}	15.38^{ab}	8.15^{a}	7.62
1.00	0	0.66^{c}	2.39^{b}	0.24^{d}	1.00^{b}	36.33^{d}	41.81^{ab}	12.90^{d}	15.23^{ab}	5.85^{c}	7.43
1.00	500	0.84^{abc}	2.78^{ab}	0.34^{c}	1.13^{ab}	40.54^{c}	41.50^{ab}	14.53^{cd}	14.69^{b}	6.77^{b}	7.64
1.00	1,000	0.99^{ab}	3.08^{a}	0.41^{abc}	1.25^{ab}	41.83^{bc}	40.79^{ab}	15.44^{bc}	15.45^{ab}	7.08^{b}	7.75
1.00	2,500	0.97^{ab}	3.03^{ab}	0.43^{abc}	1.24^{ab}	44.50^{ab}	40.99^{ab}	16.57^{ab}	15.46^{ab}	7.56^{ab}	7.60
1.00	5,000	0.94^{ab}	3.22^{a}	0.44^{ab}	1.34^{a}	44.79^{ab}	41.68^{ab}	15.96^{abc}	15.91^{ab}	7.35^{ab}	8.05
1.00	10,000	1.02^{a}	3.34^{a}	0.48^{a}	1.37^{a}	47.38^{a}	41.71^{ab}	17.36^{a}	15.45^{ab}	8.07^{a}	7.52
SEM		0.02	0.05	0.01	0.02	0.44	0.22	0.19	0.11	0.10	0.05
Main effect											
0.50		0.88	2.96	0.40	1.26	45.26^{a}	42.61^{a}	16.83^{a}	15.85^{a}	8.01^{a}	7.77
1.00		0.90	2.97	0.39	1.22	42.56^{b}	41.41^{b}	15.46^{b}	15.37^{b}	7.11^{b}	7.67
	0	0.73^{b}	2.59^{b}	0.29^{c}	1.08^{b}	39.96^{d}	41.80	14.77^{c}	15.20	6.70^{c}	7.44^{b}
	500	0.88^{a}	3.03^{a}	0.37^{b}	1.27^{a}	42.75^{c}	42.14	15.71^{bc}	15.13	7.40^{b}	7.73^{ab}
	1,000	0.95^{a}	2.99^{ab}	0.41^{ab}	1.25^{ab}	43.70^{bc}	41.80	16.19^{ab}	15.87	7.59^{ab}	7.76^{ab}
	2,500	0.94^{a}	2.97^{ab}	0.42^{ab}	1.26^{a}	45.07^{abc}	42.58	16.69^{ab}	15.93	7.82^{ab}	7.75^{ab}
	5,000	0.86^{ab}	3.09^{a}	0.40^{b}	1.31^{a}	45.29^{ab}	42.55	16.31^{ab}	16.09	7.75^{ab}	8.06^{a}
	10,000	0.98^{a}	3.14^{a}	0.46^{a}	1.28^{a}	46.70^{a}	41.19	17.20^{a}	15.42	8.11^{a}	7.57^{ab}
Source of variation											
Ca		0.270	0.899	0.623	0.273	<0.001	0.003	<0.001	0.022	<0.001	0.314
Phytase		<0.001	0.005	<0.001	0.007	<0.001	0.269	<0.001	0.034	<0.001	0.024
Ca ×Phytase		0.016	0.008	<0.001	0.008	<0.001	0.033	<0.001	0.495	0.002	0.950

$^{a–d}$Means in the same column without a common superscript differ (p<0.05).

Blood mineral concentration

Dietary Ca, phytase, and their interaction affected blood P concentration in broilers at 21 days of age (p<0.05) but did not affect blood Ca concentration (p>0.05) (Table 6). The broilers fed with 1.00% Ca had higher plasma Ca concentration but lower plasma P concentration than the birds fed with 0.50% Ca (p<0.05). Dietary Ca level affects the blood mineral concentration of broilers (Han *et al.*, 2016; Li *et al.*, 2020), in which increasing the Ca level enhanced blood Ca but decreased blood P concentration in 21-day-old chickens. These data indicated that high dietary Ca-to-NPP ratio resulted in blood Ca and P imbalance.

The plasma P concentration in 21-day-old broilers fed with the negative diet (1.00% Ca and 0.25% NPP) was lower than that in the birds fed with the control diet (p<0.05). These results were in accordance with those reported by previous research (Han *et al.*, 2009; Li *et al.*, 2020), in which P deficiency decreased the blood P concentration, and supplemental P restored the blood P concentration of broilers.

The addition of phytase increased blood P concentration in 21-day-old broilers at 1.00% Ca (p<0.05). Phytase did not affect plasma Ca and P concentrations in 42-day-old broilers (p>0.05). Our results agreed with those reported by Viveros *et al.* (2002), Shirley & Edwards (2003), and Han *et al.* (2009), in which the addition of phytase increased plasma P concentration of broilers fed with P-deficient diet. Broilers grow fast from hatching to growth phase. A large amount of P is needed to maintain growth rate. Blood mineral concentration is sensitive to phytase addition. By contrast, the mineral metabolism of broilers is relatively stable from grower to finisher phase and blood mineral concentration can be balanced through self-regulation.

Table 6 – Effects of dietary Ca and phytase levels on plasma mineral concentration in broiler chickens.

Ca (%)	Phytase (FTU/kg)	Ca (mg/100mL)		*p* (mg/100mL)	
		Day 21	Day 42	Day 21	Day 42
Control	0	9.05[ab]	10.31	6.72[ab]	7.48
0.50	0	8.00[b]	10.20	6.56[ab]	7.85
0.50	500	9.04[ab]	9.65	6.66[ab]	7.82
0.50	1,000	9.04[ab]	10.33	6.91[a]	8.02
0.50	2,500	9.16[ab]	9.71	6.52[ab]	7.05
0.50	5,000	10.02[ab]	9.20	6.20[ab]	7.15
0.50	10,000	9.58[ab]	10.37	5.91[ab]	7.46
1.00	0	11.38[a]	9.47	3.68[c]	7.73
1.00	500	9.87[ab]	9.31	5.26[b]	7.37
1.00	1,000	8.69[ab]	10.32	6.74[ab]	7.81
1.00	2,500	8.59[ab]	10.43	6.81[a]	7.66
1.00	5,000	8.98[ab]	9.68	6.98[a]	7.27
1.00	10,000	8.93[ab]	9.81	6.59[ab]	6.97
SEM		0.18	0.12	0.14	0.13
Main effect					
0.50		9.14	9.91	6.46[a]	7.56
1.00		9.41	9.84	6.01[b]	7.47
	0	9.69	9.84	5.12[b]	7.79
	500	9.46	9.48	5.96[ab]	7.59
	1,000	8.87	10.33	6.82[a]	7.91
	2,500	8.88	10.07	6.67[a]	7.35
	5,000	9.50	9.44	6.59[a]	7.21
	10,000	9.26	10.09	6.25[a]	7.21
Source of variation					
Ca		0.446	0.784	0.016	0.767
Phytase		0.667	0.339	<0.001	0.623
Ca ×Phytase		0.008	0.570	<0.001	0.889

[a–c]Means in the same column without a common superscript differ (p<0.05).

CONCLUSIONS

In conclusion, low doses of phytase (500–1,000 FTU/kg) are adequate for growth and bone development of the broilers fed with Ca- and P-balanced diets (0.50% Ca, 0.25% NPP, and Ca-to-NPP ratio = 2.0). However, high doses of phytase (2,500–10,000 FTU/kg) are needed to alleviate the negative effects of Ca and P imbalance (1.00% Ca, 0.25% NPP, and Ca-to-NPP ratio = 4.0) on growth performance and bone mineralization of broiler chickens from 1 to 42 days of age.

CONFLICT OF INTERESTS STATEMENT

No potential conflict of interest was reported by the authors.

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