



# Performance and bone quality of broilers fed diets containing different levels of phosphorus and supplemented with phytase

*Rendimiento y calidad ósea de pollos de engorde alimentados con dietas que contienen diferentes niveles de fósforo y suplementadas con fitasa*

*Desempenho e qualidade óssea de frangos de corte alimentados com dietas com diferentes níveis de fósforo e suplementadas com fitase*

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## To cite this article:

Saldanha MM, Pereira LFP, Abreu ARC, Castro FLS, Clímaco WLS, Araújo ICS, Lara LJC. Performance and bone quality of broilers fed diets containing different levels of phosphorus and supplemented with phytase. *Rev Colomb Cienc Pecu* 2023; 36(2): 66–79. DOI: <https://doi.org/10.17533/udea.rccp.v36n2a2>

## Abstract

**Background:** Although phytase has been widely used in poultry nutrition, the effects of the enzyme on broilers fed low levels of phosphorus are poorly understood. **Objective:** To evaluate the effects of two commercial phytases on live performance and bone quality of broilers fed diets with normal and reduced levels of phosphorus. **Methods:** Two experiments were conducted with four treatments and six repetitions with 30 birds each, for a total of 24 groups. The first experiment (Exp. I) used a reference level of available phosphorus (AP) with four treatments, as follows: Positive control= 0.45% AP starter diet/0.40% AP grower diet without phytase; Phytase X= 0.35% AP starter diet/0.30% AP grower diet + Phytase X; Phytase Y= 0.35% AP starter diet/0.30% AP grower diet + Phytase Y; and Negative control= 0.35% AP starter diet/0.30% AP grower diet, without phytase. In experiment II (Exp. II) the same treatments were used, but AP levels were reduced by 0.10%. The variables analyzed were: performance from one to 35 days, and bone quality at 35 days of age. Both experiments were analyzed using a completely randomized design. **Results:** In Exp. I, the positive control resulted in greater body weight gain (2,558 g;  $p<0,05$ ) compared to Phytase Y (2,470 g) and negative control (2,472 g), and better feed conversion ratio (1.48;  $p<0,05$ ) than the negative control (1.51). However, when phosphorus was reduced in Exp. II, the positive control and treatments with Phytase X showed better results ( $p<0.01$ ) for feed intake (3,608 g and 3,593 g, respectively) and weight gain (2,430 g and 2,400 g, respectively) compared to the negative control (2,889 g of feed intake and 1,915 g of weight gain;  $p<0.01$ ), which also presented low bone ash (36.8%) and phosphorus in the tibia (5.48%;  $p<0.01$ ). **Conclusion:** Reducing AP concentration in diets not added with phytase negatively affects weight gain and feed intake of broilers.

Received: March 1, 2021. Accepted: April 17, 2022

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**Keywords:** *available phosphorus; bone health; broiler; exogenous enzymes; feed conversion; weight gain; growth performance; low phosphorus diet; mineral; nutrition; phytase; poultry.*

## Resumen

**Antecedentes:** Aunque la fitasa ha sido ampliamente utilizada en nutrición aviar, sus efectos en pollos de engorde alimentados con bajos niveles de fósforo son poco comprendidos. **Objetivo:** Evaluar el efecto de dos fitasas comerciales sobre el desempeño y la calidad ósea de pollos de engorde alimentados con dietas con niveles normales y reducidos de fósforo. **Métodos:** Se realizaron dos experimentos con cuatro tratamientos y seis repeticiones de 30 aves cada una, totalizando 24 grupos. En el primero (Exp. I) se utilizó el nivel de referencia de fósforo disponible (Pd) con cuatro tratamientos, así: Control positivo= 0,45% Pd dieta inicial/0,40% Pd dieta de engorde, sin fitasa; Fitasa X= 0,35% Pd dieta inicial/0,30% Pd dieta engorde + Fitasa X; Fitasa Y= 0,35% Pd dieta inicial/0,30% Pd dieta engorde + Fitasa Y; finalmente, Control negativo= 0,35% Pd dieta inicial/0,30% Pd dieta engorde, sin fitasa. En el segundo experimento (Exp. II) se utilizaron los mismos tratamientos, pero reduciendo en 0,10% el nivel de Pd. Las variables analizadas fueron: desempeño de uno a siete días y de uno a 35 días y calidad ósea a los 35 días de edad. Ambos experimentos se analizaron mediante un diseño completamente aleatorizado. **Resultados:** En el Exp. I, control positivo presentó una mayor ganancia de peso corporal (2.558 g;  $p<0,05$ ) en comparación con la Fitasa Y (2.470 g) y el control negativo (2.472 g), y mejor índice de conversión alimenticia (1,48;  $p<0,05$ ) que el control negativo (1,51). Sin embargo, cuando se redujo el nivel de fósforo en el Exp. II, el control positivo y los tratamientos con Fitasa X mostraron mejores resultados ( $p<0,01$ ) para el consumo de alimento (3.608 g y 3.593 g, respectivamente) y la ganancia de peso (2.430 g y 2.400 g, respectivamente) en comparación con el control negativo (2.889 g de consumo de alimento y 1.915 g de ganancia de peso;  $p<0,01$ ), el cual también presentó baja concentración de cenizas óseas (36,8%) y fósforo en tibia (5,48%;  $p<0,01$ ). **Conclusion:** La reducción de la concentración de Pd en dietas no aditivadas con fitasa afecta negativamente la ganancia de peso y el consumo de alimento del pollo de engorde.

**Palabras clave:** *aves; crecimiento; desempeño; dieta baja en fosforo; conversión alimenticia; enzimas exógenas; fitasa; fósforo disponible; ganancia de peso; mineral; nutrición; pollo de engorde; salud ósea.*

## Resumo

**Antecedentes:** A fitase é uma enzima amplamente utilizada na nutrição de frangos de corte. No entanto existem várias opções comerciais e seus efeitos com níveis reduzidos de fósforo, são pouco avaliados. **Objetivo:** Avaliar o efeito da suplementação de fitasas comerciais no desempenho e na qualidade óssea de frangos de corte, alimentados com níveis normais e reduzidos de fósforo. **Métodos:** Dois experimentos foram conduzidos com quatro tratamentos e seis repetições, com 30 aves em cada, totalizando 24 grupos. No primeiro (I) utilizou o nível de referência de fósforo disponível (Pd), totalizando quatro tratamentos: controle positivo= 0,45% Pd dieta inicial/0,40% Pd dieta crescimento, sem fitase; tratamento fitase X= 0,35% Pd dieta inicial/0,30% Pd dieta crescimento + Fitase X; tratamento fitase Y= 0,35% Pd dieta inicial/0,30% Pd dieta de crescimento + Fitase Y; e controle negativo 0,35% Pd dieta inicial/0,30% Pd dieta crescimento, sem fitase. O segundo experimento utilizou os mesmos tratamentos, reduzindo 0,10% o nível de Pd. As variáveis analisadas foram: desempenho de um a sete dias e de sete a 35 dias e qualidade óssea aos 35 dias. Ambos os experimentos foram analisados usando um delineamento inteiramente casualizado. **Resultados:** No Experimento I, o tratamento controle positivo apresentou maior ganho de peso corporal (2.557,86 g;  $p<0,05$ ) em relação a fitase Y (2.470,27 g) e o controle negativo (2.471,73 g) e melhor índice de conversão alimentar (1,48;  $p<0,05$ ) do que o tratamento controle negativo (1,51). Porém, quando o nível de fósforo foi reduzido no Experimento II, o controle positivo e os tratamentos com fitase X apresentaram os melhores resultados ( $p<0,01$ ) para consumo de ração (3.608,0 g e 3.593,1 g, respectivamente) e ganho de peso corporal (2.429,8 g e 2.399,9 g, respectivamente) em comparação ao tratamento controle negativo (2.889,0 g de ingestão de ração e 1.915,3 g de ganho de peso corporal;  $p<0,01$ ) que também apresentou baixa concentração de cinzas ósseas (36,8%) e fósforo na tíbia (5,48%;  $p<0,01$ ). **Conclusões:** A redução da concentração de AP sem o uso de fitase reduz o ganho de peso corporal e o consumo de ração de frangos de corte.

**Palavras-chave:** *conversão alimentar; desempenho; enzimas exógenas; dieta baixa em fósforo; fitase; fósforo disponível; frango de corte; ganho de peso; mineral; nutrição; saúde óssea.*

## Introduction

Phosphorus (P) is an essential mineral for the metabolism, growth and development of broilers, and is especially important for bone structure. According to Scholey *et al.* (2018), broilers currently experience a substantial incidence of bone abnormalities and lameness that hinder performance and cause economic losses to the poultry industry. They attribute these unwanted characteristics to genetic selection for rapid growth. In addition, malabsorption of minerals such as calcium (Ca) and P can also contribute to the incidence of diseases related to bone tissue and performance loss of broilers.

Broiler feeds based on corn and soybean meal have high levels of P (about 0.25% in corn and 0.56% in soybean meal). However, up to 70.0% of this mineral may be present as phytic P, which is poorly absorbed by non-ruminant animals (Karimi *et al.*, 2011). Broiler digestion of phytate is low since they have low endogenous phytase activity for hydrolyzing the phytate complexes present in feed (Woyengo and Nyachoti, 2011). Thus, adding phytase to the diet can improve P digestibility, performance and bone quality of broilers.

Exogenous phytase has become an alternative to feed supplementation with P because it can hydrolyze phytic acid and its salts (phytate), thereby releasing inorganic P and other minerals (Fukayama *et al.*, 2008). Thus, phytase is commonly included in diet formulations to reduce P supplementation, improve diet use and, consequently, reduce excretion of this and other minerals into the environment.

Phytase efficiency can be increased when used in diets with reduced levels of P (Souza *et al.*, 2015). Nonetheless, the use of unconventional doses of phytase to achieve nearly complete phytate hydrolysis resulting in improved performance depends not only on the release of phosphate, but also on the type of phytase and whether or not there is enough P and energy in the diet (Wu *et al.*, 2015).

Thus, this study aimed to evaluate the effects of including two bacterial phytases (*Escherichia coli*)

in diets with normal and reduced levels of P on growth performance and bone quality of broilers.

## Materials and Methods

### *Ethical considerations*

The local Ethics Committee for Animal Research approved the experimental protocol (Protocol 240/2013).

### *Location and experiments*

This study was conducted in the city of Igarapé, Minas Gerais, Brazil (20°4'20" S, 44°18'12" W). Two experiments were conducted each using 720 male broiler chicks (Cobb). Chickens were housed in 24 experimental boxes (12 birds/m<sup>2</sup>) with pendular drinkers and tubular feeders. The birds received 24 h of artificial light (250 W heating lamp) until 14 d of age, after which they received natural light until the end of the experiment. The chicks had *ad libitum* access to feed and water throughout the experiments. Both experiments were analyzed using a completely randomized design, with four treatments and six repetitions with 30 birds. The treatments were defined by the level of available phosphorus (AP) and the inclusion of one of two commercial phytase enzymes (Table 1). The chicks were reared from age one to 35 days.

The commercial enzymes tested were of bacterial origin: phytase X is an *Escherichia coli*-derived 6-phytase developed specifically for use in swine and poultry feeds, while phytase Y is an *E. coli*-derived 6-phytase produced by the yeast *Pichia pastoris* in a submerged fermentation process. 500 units of phytase-FTU- per kg of feed were included, with the nutritional value of the enzymes being 0.10% AP. In addition to the contribution of phytase to AP, the experiments also considered an apparent metabolizable energy of phytase (AME) of 33 kcal/kg, crude protein (CP) of 0.22% and calcium (Ca) of 0.1%.

Diets were formulated using corn, soybean meal and meat and bone meal, and were divided into starter (one to 21 days) and grower diets (22 to 35 days). The nutritional levels of the diets

were calculated using the nutritional values of ingredients according to Rostagno *et al.* (2011). Diet composition, with their respective nutritional values, are provided in tables 2 and 3 for experiments I and II, respectively.

**Table 1.** Treatments used in Experiments I (Normal available P levels) and II (Reduced available P levels).

<b>Experiment I</b>			
<b>Normal available phosphorus levels</b>			
Treatment	Available P (starter feed)	Available P (grower feed)	Enzyme
Positive control	0.45%	0.40%	Without
Phytase X	0.35%	0.30%	With
Phytase Y	0.35%	0.30%	With
Negative control	0.35%	0.30%	Without
<b>Experiment II</b>			
<b>Reduced available phosphorus levels</b>			
Treatment	Available P (starter feed)	Available P (grower feed)	Enzyme
Positive control	0.35%	0.30%	Without
Phytase X	0.25%	0.20%	With
Phytase Y	0.25%	0.20%	With
Negative control	0.25%	0.20%	Without

Enzyme X – *Escherichia coli* derived 6-phytase.

Enzyme Y – *Escherichia coli* derived 6-phytase, produced by the yeast *Pichia pastoris* in a submerged fermentation.

Performance variables (body weight gain (BWG), feed intake (FI), feed conversion ratio (FCR) and productive viability) were evaluated at seven and 35 days of age by recording the weight of the birds, feed supply and surplus on a weekly basis, and the number and weight of birds slaughtered daily.

At 35 days of age, two birds from each replicate -for a total of 12 birds per treatment- were euthanized by cervical dislocation to collect the left and right tibia. Lipid extraction was carried out by immersion in petroleum ether to determine ash percentage in the bones. Ash content was then obtained by calcinating bones in a muffle furnace at 600 °C for six hours and then weighing the sample. Bone ash was used to make a standard solution to determine the percentage of phosphorus according to AOAC, 2005.

The Seedor index was determined by measuring the greatest length of the tibia and dividing the value by its weight, which was obtained using a semi-analytical digital scale (Seedor, 1991). For histopathological analyses, bone tissue samples were previously fixed in 10% buffered formalin for 48 hours and then maintained in 24% formic acid for 15 days for descaling. To prepare slides, the tissues were dehydrated in an increasing series of ethanol, diaphanized in xylol and included in paraffin to obtain 4- $\mu$ m serial sections. The sections were stained using the Hematoxylin-Eosin (HE) technique and analyzed under light microscopy with a Binocular L1000-b-ac optical microscope (Bioval, Curitiba, PR, Brazil).

Bone mineral density ( $\text{g}/\text{cm}^3$ ) was measured in the tibia with a DPX-ALPHA densitometer (Alpha, São Paulo, SP, Brazil). This analysis produced values of bone mineral content (BMC) and bone area, from which bone mineral density (BMD) was obtained by dividing BMC by bone area, since density is the result of the mass/volume ratio. The same bones measured with the densitometer were subjected to a mechanical testing using a universal EMIC test machine (DL 3000 model) (EMIC Equipments and Test Systems Ltda, São José dos Pinhais, PR, Brazil) with load applied at a speed of 5 mm/min and load cell of 2000N in a three-point bending test. The central region of the bone (diaphysis) was selected for application of the load.

The results of the biomechanical analysis were recorded by Instron Series IX software as a graphic of the load vs deformation curve. The analysis of these curves provided bone maximum strength (BMS), bone resilience (BR) and bone rigidity (BRi) for each treatment.

#### *Statistical analysis*

The data from both experiments were analyzed with ANOVA and the means compared using Tukey's test. All analyses were conducted using the SAS software (Statistical Analysis System, Version 9.3, 2003). Statistical significance was  $p < 0.05$ .

**Table 2.** Composition and calculated nutritional values of the diets according to the treatments and phases of Experiment I.

Ingredients (%)	Positive control		Enzyme X		Enzyme Y		Negative control	
	1 to 21	22 to 35	1 to 21	22 to 35	1 to 21	22 to 35	1 to 21	22 to 35
Corn 7.26%	59.50	63.50	60.40	64.50	60.40	64.50	60.40	64.50
Soybean meal 47%	31.20	27.00	32.30	28.00	32.30	28.00	32.30	28.00
MBM 41.86%	5.00	4.30	3.20	2.60	3.20	2.60	3.20	2.60
Soybean oil	2.70	3.50	2.10	2.90	2.10	2.90	2.10	2.00
Limestone 37%	0.33	0.49	0.74	0.80	0.75	0.80	0.75	0.80
Common salt	0.40	0.38	0.42	0.40	0.42	0.40	0.42	0.40
DL-Methionine 99%	0.29	0.25	0.28	0.24	0.28	0.24	0.28	0.24
L-Lysine 98%	0.18	0.19	0.17	0.18	0.17	0.18	0.17	0.18
Mineral vitamin supplement <sup>1,2</sup>	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
L-Threonine	0.09	0.08	0.07	0.06	0.07	0.06	0.07	0.06
Choline chloride 70%	0.05	0.04	0.05	0.04	0.05	0.04	0.05	0.04
Salinomycin*	-	0.05	-	0.05	-	0.05	-	0.05
Nicarbazine+								
Maduracimin*	0.05	-	0.05	-	0.05	-	0.05	-
Halquinol 60%	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005
Enzyme X (5,000 FTU 100 g/T)	-	-	0.1	0.1	-	-	-	-
Enzyme Y (10,000 FTU 50 g/T)	-	-	-	-	0.05	0.05	-	-
Chemical composition (calculated on natural matter basis)								
Met. Energy (kcal/kg)	3,050.0	3,151.0	3,050.0	3,151.0	3,050.0	3,151.0	3,017.0	3,118.0
Crude Protein%	21.5	19.5	21.5	19.5	21.5	19.5	21.3	19.3
Calcium%	0.96	0.89	0.96	0.89	0.96	0.89	0.86	0.79
Available phosphorus%	0.45	0.40	0.45	0.40	0.45	0.40	0.35	0.30
Lysine (digestible)%	1.17	1.06	1.17	1.06	1.17	1.06	1.16	1.05
Methionine (digestible)%	0.59	0.53	0.58	0.53	0.58	0.53	0.58	0.53
Met+Cystine (digestible)%	0.85	0.77	0.85	0.77	0.85	0.77	0.84	0.77

MBM – meat bone and meal; <sup>1</sup>Mineral vitamin supplement (initial phase), each ton contains: 9,000 IU of vit. A; 2,500 IU of vit. D3; 14.0 mg vit. E; 2.0 mg vit. K3; 2.5 mg vit. B1; 6.2 mg vit. B2; 4.0 mg vit. B6; 14.0 mcg vit. B12; 40.0 mg of niacin; 15.0 mg of pantothenic acid; 1.0 mg of folic acid; 10.0 mg of copper; 50.0 mg of iron; 80.0 mg of manganese; 60.0 mg of zinc; 1.2 mg of iodine; 0.2 mg of selenium; <sup>2</sup>Mineral vitamin supplement (grower phase), each ton contains: 7,000 IU of vit. A; 2,000 IU of vit. D3; 11.0 mg vit. E; 1.6 mg vit. K3; 1.6 mg vit. B1; 4.5 mg vit. B2; 2.2 mg vit. B6; 10.0 mcg vit. B12; 32.0 mg of niacin; 12.0 mg of pantothenic acid; 0.8 mg of folic acid; 10.0 mg of copper; 50.0 mg of iron; 80.0 mg of manganese; 60.0 mg of zinc; 1.2 mg of iodine; 0.2 mg of selenium.

FTU- Its activity in FTU is defined as the amount of enzyme that releases 1  $\mu$ mol of inorganic phosphorus/min from 0.0051 mol/L of sodium phytate at pH 5.5 and 37 °C.

\*Coxistac PX 12% e MNGrow.



**Table 3.** Composition and calculated nutritional values of the diets according to the treatments and the growth phases of Experiment II.

Ingredients (%)	Positive control		Enzyme X		Enzyme Y		Negative control	
	1 to 21	22 to 35	1 to 21	22 to 35	1 to 21	22 to 35	1 to 21	22 to 35
Corn 7.26% CP	58.70	62.60	59.70	63.70	597.0	63.70	59.70	63.70
Soybean meal 47% CP	32.80	28.60	33.80	29.60	33.80	29.60	33.80	29.60
MBM 41.86% CP	3.50	2.80	1.70	1.00	1.70	1.00	1.70	1.00
Soybean oil	2.90	3.80	2.30	3.10	2.30	3.10	2.30	3.10
Limestone 37% Ca	0.84	0.10	0.12	0.14	0.12	0.14	0.12	0.14
Common salt	0.42	0.40	0.44	0.42	0.44	0.42	0.44	0.42
DL-Methionine 99%	0.28	0.24	0.27	0.23	0.27	0.23	0.27	0.23
L-Lysine 98%	0.16	0.17	0.15	0.16	0.15	0.16	0.15	0.16
Mineral vitamin supplement <sup>1,2</sup>	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
L-Threonine	0.08	0.07	0.06	0.05	0.06	0.05	0.06	0.05
Choline chloride 70%	0.05	0.04	0.05	0.04	0.05	0.04	0.05	0.04
Salinomycin*	-	0.05	-	0.05	-	0.05	-	0.05
Nicarbazine+								
Maduracimin*	0.05	-	0.05	-	0.05	-	0.05	-
Halquinol 60%	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005
Enzyme X (5,000 FTU 100 g/T)	-	-	0.1	0.1	-	-	-	-
Enzyme Y (10,000 FTU 50 g/T)	-	-	-	-	0.05	0.05	-	-
Chemical composition (calculated on natural matter basis)								
Met. Energy Kcal/kg	3,049.0	3,146.0	3,049.0	3,146.0	3,049.0	3,146.0	3,016.0	3,113.0
Crude Protein%	21.5	19.5	21.5	19.5	21.5	19.5	21.2	19.2
Calcium%	0.96	0.91	0.96	0.91	0.96	0.91	0.86	0.81
Available phosphorus%	0.35	0.30	0.35	0.30	0.35	0.30	0.25	0.20
Lysine (digestible)%	1.17	1.06	1.17	1.06	1.17	1.06	1.16	1.05
Methionine (digestible)%	0.59	0.53	0.58	0.52	0.58	0.52	0.58	0.52
Met + Cystine%	0.85	0.77	0.85	0.77	0.84	0.77	0.85	0.77

MBM – meat bone and meal; CP – Crude Protein; <sup>1</sup>Mineral vitamin supplement (initial phase), each ton contains: 9,000 IU vit. A; 2,500 IU vit. D3; 14.0 mg vit. E; 2.0 mg vit. K3; 2.5 mg vit. B1; 6.2 mg vit. B2; 4.0 mg vit. B6; 14.0 mcg vit. B12; 40.0 mg niacin; 15.0 mg pantothenic acid; 1.0 mg folic acid; 10.0 mg copper; 50.0 mg iron; 80.0 mg manganese; 60.0 mg zinc; 1.2 mg iodine; 0.2 mg selenium; <sup>2</sup>Mineral vitamin supplement (grower phase), each ton contains: 7,000 IU vit. A; 2,000 IU vit. D3; 11.0 mg vit. E; 1.6 mg vit. K3; 1.6 mg vit. B1; 4.5 mg vit. B2; 2.2 mg vit. B6; 10.0 mcg vit. B12; 32.0 mg niacin; 12.0 mg pantothenic acid; 0.8 mg folic acid; 10.0 mg copper; 50.0 mg iron; 80.0 mg manganese; 60.0 mg zinc; 1.2 mg iodine; 0.2 mg selenium.

FTU- Its activity in FTU is defined as the amount of enzyme that releases 1 µmol of inorganic phosphorus/min from 0.0051 mol/L of sodium phytate at pH 5.5 and 37 °C.

\* Coxistac PX 12% e MNGrow.

## Results

In Experiment I, feed intake and feed conversion during day one to seven of age were not influenced by the treatment ( $p>0.05$ ) (Table 4). However, the body weight gain of broilers fed the negative control diet was lower than that of those fed the positive control diet or those supplemented with phytase X ( $p=0.005$ ), and similar to that of birds fed a diet containing phytase Y ( $p>0.05$ ).

In the period from one to 35 days of age, feed intake and viability were similar among all treatments ( $p>0.05$ ) (Table 4). However, lower weight gain was observed in broilers fed the diet supplemented with phytase Y or receiving the negative control diet compared to those receiving the positive control ( $p<0.05$ ). Feed conversion was not influenced by the inclusion of phytases in the diet ( $p>0.05$ ); however, chickens fed the positive control diet had better feed conversion than those fed the negative control diet ( $p<0.05$ ) (Table 4).

Bone quality analysis of broiler chicks at day 35 of Experiment I revealed that the treatments had no effect ( $p>0.05$ ) on bone mineral content, bone area, bone mineral density, Seedor index, maximum strength endured before bone breakage, bone ash and percentage of phosphorus in tibia (Table 5). For bone resilience, however, bones from positive control birds had higher values ( $p<0.05$ ) than bones from negative control birds, the latter not differing from the bones of the other treatments. For bone rigidity, the bones from the phytase X treatment had better rigidity than bones from birds of the other treatments ( $p<0.05$ ).

In Experiment II, during the period from one to seven days of age broilers fed the positive control diet or diet supplemented with phytase X had higher feed intake than broilers fed the diet containing phytase Y or fed the negative control diet ( $p<0.001$ ) (Table 6).

**Table 4.** Effect of phytase supplementation in the diet with normal phosphorus levels on growth performance of broiler chicks.

Treatment	1 to 7 d of age				
	Initial body weight (g)	Feed intake (g)	Body weight gain (g)	Final body weight (g)	Feed conversion ratio
Positive control	42.93	186.40	163.53 <sup>a</sup>	206.46 <sup>a</sup>	1.142
Phytase X	43.56	189.57	167.86 <sup>a</sup>	211.42 <sup>a</sup>	1.131
Phytase Y	42.76	186.11	162.31 <sup>a, b</sup>	205.07 <sup>a, b</sup>	1.153
Negative control	43.25	186.16	156.80 <sup>b</sup>	200.05 <sup>b</sup>	1.191
SEM	0.163	1.31	1.46	1.40	0.005
P-value	0.330	0.453	0.005	0.005	0.128
1 to 35 d of age					
	Feed intake (g)	Body weight gain (g)	Final body weight (g)	Feed conversion ratio	Viability (%)
Positive control	3,782.23	2,557.86 <sup>a</sup>	2,600.79 <sup>a</sup>	1.482 <sup>a</sup>	95.8
Phytase X	3,756.01	2,494.00 <sup>a, b</sup>	2,537.56 <sup>a, b</sup>	1.511 <sup>a, b</sup>	94.2
Phytase Y	3,730.26	2,470.27 <sup>b</sup>	2,513.03 <sup>b</sup>	1.512 <sup>a, b</sup>	94.6
Negative control	3,730.64	2,471.73 <sup>b</sup>	2,514.98 <sup>b</sup>	1.514 <sup>b</sup>	94.0
SEM	15.20	13.20	12.40	0.004	0.52
P-value	0.334	0.005	0.005	0.008	0.683

Values (average  $\pm$  SEM) with no common superscript letters (<sup>a, b</sup>) within columns are different by Tukey's test ( $p<0.05$ ). SEM (Standard Error of the Mean).

**Table 5.** Effect of phytase supplementation in the diet with normal phosphorus levels on bone quality of broilers at 35 days.

	BMC (g)	Area (cm <sup>3</sup> )	BMD (g/cm <sup>3</sup> )	SI	BMS (N)	BR (mJ)	BRi (Mpa)	BA (%)	P (%)
Positive control	2.41	11.56	0.21	2.39	377.68	892.80 <sup>a</sup>	105.03 <sup>b</sup>	43.98	7.12
Phytase X	2.56	11.66	0.22	2.51	380.80	800.60 <sup>a,b</sup>	165.32 <sup>a</sup>	44.97	7.44
Phytase Y	2.67	12.14	0.22	2.17	365.93	868.17 <sup>a,b</sup>	109.31 <sup>b</sup>	45.15	8.01
Negative control	2.28	11.82	0.19	2.42	335.34	701.40 <sup>b</sup>	111.39 <sup>b</sup>	42.87	7.19
SEM	0.08	0.16	0.005	0.09	16.80	40.40	7.16	0.56	0.20
P-value	0.744	0.846	0.066	0.331	0.168	0.029	0.003	0.071	0.234

Values (average  $\pm$  SEM) with no common superscript letters (<sup>a, b</sup>) within columns are different by Tukey's test ( $p < 0.05$ ). Bone mineral content (BMC), bone area (Area), bone mineral density (BMD), Sedor index (SI), maximum strength endured before bone breaking (BMS), bone resilience (BR), bone rigidity (BRi), bone ashes (BA) and percentage of phosphorus in the tibia (P). SEM (Standard Error of the Mean).

**Table 6.** Effect of phytase supplementation in the diet with reduced phosphorus levels on growth performance of broilers chicks.

1 to 7 d of age					
Treatment	Initial body weight (g)	Feed intake (g)	Body weight gain (g)	Final body weight (g)	Feed conversion ratio
Positive control	42.7	177.3 <sup>a</sup>	154.9 <sup>a</sup>	197.6 <sup>a</sup>	1.145
Phytase X	42.1	181.0 <sup>a</sup>	155.1 <sup>a</sup>	197.2 <sup>a</sup>	1.174
Phytase Y	42.9	166.9 <sup>b</sup>	145.7 <sup>b</sup>	188.6 <sup>b</sup>	1.147
Negative control	42.7	158.2 <sup>b</sup>	134.0 <sup>c</sup>	176.7 <sup>c</sup>	1.181
SEM	0.15	2.05	2.22	2.02	0.009
P-value	0.987	<0.001	<0.001	<0.001	0.066
1 to 35 d of age					
Treatment	Feed intake (g)	Body weight gain (g)	Final body weight (g)	Feed conversion ratio	Viability (%)
Positive control	3,608.0 <sup>a</sup>	2,429.8 <sup>a</sup>	2,472.5 <sup>a</sup>	1.486	91.4 <sup>a</sup>
Phytase X	3,593.1 <sup>a</sup>	2,399.9 <sup>a</sup>	2,442.0 <sup>a</sup>	1.498	89.9 <sup>a, b</sup>
Phytase Y	3,347.3 <sup>b</sup>	2,239.1 <sup>b</sup>	2,282.0 <sup>b</sup>	1.494	83.5 <sup>b, c</sup>
Negative control	2,889.0 <sup>c</sup>	1,915.3 <sup>c</sup>	1,958.0 <sup>c</sup>	1.510	76.7 <sup>c</sup>
SEM	62.82	44.05	42.01	0.005	1.53
P-value	<0.001	<0.001	<0.001	0.938	<0.001

Values (average  $\pm$  SEM) with no common superscript letters (<sup>a, b</sup>) within columns are different by Tukey's test ( $p < 0.05$ ). SEM (Standard Error of the Mean).

Weight gain in the first week was influenced by treatment ( $p < 0.001$ ), with the greatest weight gain observed in chickens receiving the positive control feed or feed supplemented with phytase X, followed by chickens that received feed containing phytase Y, and finally those fed the negative control diet. Feed conversion was not influenced by treatment ( $p < 0.05$ ) (Table 6).

In the period from one to 35 days of age, feed intake, weight gain and viability were influenced by treatment ( $p < 0.05$ ) (Table 6). Broilers receiving feed supplemented with phytase X had feed intake similar to chickens fed the positive control diet ( $p > 0.05$ ), and both had higher feed intake compared to the group receiving feed with phytase Y or feed negative control ( $p < 0.05$ ). The



negative control had lower viability than the positive control and phytase X treatment ( $p < 0.05$ ).

Bone quality analysis of broilers at day 35 of Experiment II (Table 7) revealed that the treatments had no effect on bone density, Seedor index, maximum strength endured before bone breakage and BR ( $p > 0.05$ ). However, mineral content and total bone area differed between the phytase X treatment and the negative control ( $p < 0.05$ ), with the former having greater mineral

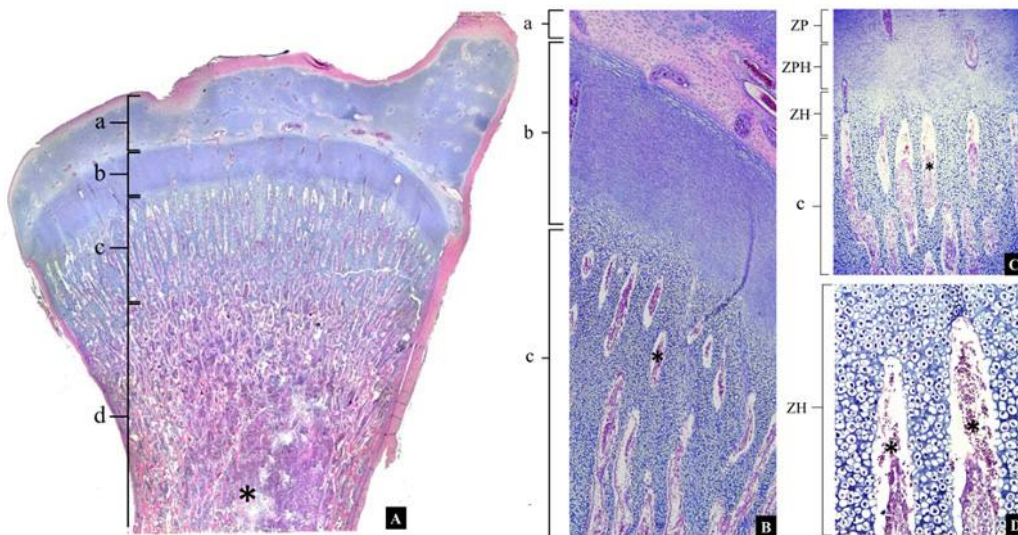
content and total bone area. Percentage of bone ash and phosphorus were lower in the negative control group compared to the other treatments ( $p < 0.05$ ).

Results of the histopathological analysis of the bones in both experiments showed no effect of treatment on bone formation; bones presented regular growth plate and vascularization in the region of the metaphysis (Figure 1).

**Table 7.** Effect of phytase supplementation in the diet with reduced phosphorus levels on bone quality of broilers at 35 days.

Treatment	BMC (g)	Area (cm <sup>3</sup> )	BMD (g/cm <sup>3</sup> )	SI	BMS (N)	BR (mJ)	BRi (Mpa)	BA (%)	P (%)
Positive control	1.5 <sup>a, b</sup>	11.6 <sup>a, b</sup>	0.13	2.13	348.5	894.6	99.5 <sup>b</sup>	42.4 <sup>a</sup>	7.22 <sup>a</sup>
Phytase X	2.2 <sup>a</sup>	12.5 <sup>a</sup>	0.17	2.30	422.3	1018.6	145.8 <sup>a</sup>	45.6 <sup>a</sup>	7.42 <sup>a</sup>
Phytase Y	1.6 <sup>a, b</sup>	12.2 <sup>a, b</sup>	0.13	2.38	344.1	912.6	122.7 <sup>a, b</sup>	43.2 <sup>a</sup>	7.51 <sup>a</sup>
Negative control	1.2 <sup>b</sup>	10.57 <sup>b</sup>	0.11	2.34	292.7	685.6	151.5 <sup>a</sup>	36.8 <sup>b</sup>	5.48 <sup>b</sup>
SEM	0.114	0.255	0.008	0.08	16.90	44.30	6.46	0.872	0.56
P-value	0.038	0.020	0.063	0.089	0.072	0.321	0.016	<0.01	<0.01

Values (average  $\pm$  SEM) with no common superscript letters (<sup>a, b</sup>) within columns are different by Tukey's test ( $p < 0.05$ ). SEM (Standard Error of the Mean).



**Figure 1.** Broiler chicken, normal proximal tibiotarsus. Photos (microscopy with Binocular L1000-b-ac optical microscope -Bioval, Curitiba, PR, Brazil-) A, B and C refer to NCII treatment at the bird's 35<sup>th</sup> day of age. In A, it can be observed epiphysis (a), growth plate or physis (b) and metaphysis (c). In B and C, the zones of the growth plate (b) are observed: proliferation zone (ZP), prehypertrophic zone (ZPH) and hypertrophic zone (ZH). The metaphysis vessels (\*) can be seen in all images, occasionally invading the hypertrophic zone of the growth plate.

## Discussion

In Experiment I, performance results for the two phases (starter and grower) showed that the positive control group was superior to the negative control group for weight gain, even though they had the same feed intake. This finding can be explained by the physiological functions of phosphorus, since it is an essential mineral for growth and structural and metabolic development of broilers. According to Adedokun and Adeola (2013), about 80.0% of ingested P is associated with calcium in bone formation, so it was expected that P would be involved in several stages of broiler life. The negative control group had reduced growth in the starter phase; although P requirement is lower in this period and increases throughout the growth and final stages (Vargas Jr *et al.*, 2003). Demand for P increases with chick growth, while weight gain and feed conversion during days 1-35 were worse for the negative in relation to the positive control. Several studies have shown that phytase addition to broiler diets improves P digestibility and reduces its excretion (Ahmad *et al.*, 2000, Scholey *et al.*, 2018, Leyva-Jimenez *et al.*, 2018) whether or not birds receive reduced dietary levels of P.

In Experiment I, with normal phosphorus, final body weight and feed conversion of phytases X and Y treatments were similar to the negative control group. These can be explained by a variation in the efficiency of phytase activity in the digestive tract of broilers. According to Dersjant-Li *et al.*, (2015), factors such as pH, endogenous protease resistance, dietary phytate levels, feed processing, Ca and Na content, age and intestinal health of birds can affect phytase activity.

In Experiment II, Phytase X results for weight gain in the initial phase (1-7 d) were similar to the positive control and superior to Phytase Y treatment. Thus, although both enzymes can be extracted from the same microorganism, used in the same concentration they can produce different results under reduced levels of dietary P. Leyva-Jimenez *et al.* (2018) also found differences between enzyme results. The different efficiency

between phytases does not necessarily mean one is superior to the other, but may be related to dosage, since both were included at 500 FTU/kg; at a higher dosage it is possible that the enzymes have similar result (Walters *et al.* 2019; Dersjant-Li *et al.* 2021; Sens *et al.* 2021). Accordingly, inclusion levels of these enzymes should be studied, evaluating their efficiency. Still, according to Araújo *et al.* (2008), mineral levels in diets for broilers are often higher than the minimum required, which represents a waste of ingredients and, consequently, elevated cost in broiler production.

According to the feed conversion ratio at 35 days, phytases for broiler chickens can improve the use of phosphorus and guarantee better performance. Scholey *et al.* (2018) reinforced the need for broiler chickens to use P for their bone growth. Regarding bone quality in Experiment I, the positive control performed better than the negative control and was similar to the two enzyme treatments. For example, BR -which measures the resistance of bone to tension and then return to its natural state- was similar between the positive control and the Phytase X treatment, and superior to the negative control.

In Experiment II, broilers revealed sensitivity to reduced phosphorus levels. The negative control and Phytase Y treatment showed worse performance and bone quality (0.25 mg P) compared to the positive control and Phytase X treatment. Feed consumption was lower for the negative control, which is in agreement with Persia and Saylor (2006), who found that increased AP in the diet increases feed consumption. Regarding performance, Leandro *et al.* (2006) stated that chick development in the first week is determinant for weight at 42 days of age. Thus, the low growth of chicks in the initial phase may have been responsible for the low performance observed at 35 days, since the birds did not grow and showed less uniformity. According to Nääs *et al.* (2009), low growth of broiler chickens can reflect behavioral changes, such as not feeding and/or drinking water due to lack of access to feeders and drinkers, which would reduce performance and increase the number of injured

carcasses, thereby reducing viability. Milica *et al.* (2012) observed that reducing dietary AP from 0.43% to as low as 0.10%, both with and without the use of phytase during the final period of growth resulted in increased mortality. They also observed greater efficacy of phytase in diets with reduced phosphorus. It is important to note that when phosphorus levels were reduced in Experiment II from 0.45 to 0.35% AP, a loss in performance was observed, characterized by reduced BWG and FI ( $P < 0.05$ ) regardless of the use of phytase, which shows that the levels of AP used in Experiment I are more adequate for broilers.

Thus, during the initial phase in Experiment II the viability of birds in the negative control was compromised because they did not have good bone and body development due to the low phosphorus levels of the diet and died during the breeding period because they could not reach the drinkers. Studying the inclusion of different phytases in broilers, Wu *et al.* (2015) found no differences for poultry viability during the period of 1-21 days; however, P levels were not reduced. Moreover, our performance results also differed from the findings of Brunelli *et al.* (2012), who did not find differences between groups supplemented or not with phytase; however, they did not include the value of the enzyme in the nutritional matrix and used the enzyme without reducing AP. On the other hand, Laurentiz *et al.* (2009) also observed a decrease in weight gain during the final period of rearing when P levels were reduced without the use of phytases. Thus, it can be concluded that a deficiency of AP for broiler chickens compromises growth performance, and the use of phytases can help increase AP.

These results are in accordance with Cardoso Júnior *et al.* (2010), who found that it was possible to reduce AP by 0.15% and Ca by 0.30% in relation to control feed without impairing performance of broilers during the growth phase when the feed was supplemented with phytase. Santos *et al.* (2011) also found that Ca and AP levels can be reduced to 0.52 and 0.26%, respectively, during the growth phase, and to 0.56

and 0.28%, respectively, during the final phase, if such a reduction is combined with phytase supplementation at the level of 500 FTU/kg of feed. Souza *et al.* (2015) also concluded that the use of phytase improves bird performance for diets with reduced levels of Ca and AP. They observed that Ca reduction in diets supplemented with phytase should be proportional to the reduction of P in order to maintain the proper Ca:P ratio since inadequate levels of Ca may impair the absorption of other minerals, such as P, Zn and Mg.

According to Pereira *et al.* (2012), bone ash content is the best parameter for estimating the amount of AP provided by phytase in diets based on corn and soybean meal. Thus, the amount of AP in the negative control diet was insufficient, with about 2.4% less P retention in tibias in relation to the positive control and the chickens of the phytase treatments. Thus, the use of phytases compensated for the low dietary level of P and increased P retention in tibias, as observed in P content and total ash. According to Scholey *et al.* (2018), the use of low phosphorus during the starter phase causes low mineral deposition, which promotes negative consequences for bone quality and bird performance. In the present study, dietary P reduction was sufficiently compensated for in the later stages of rearing, which can be attributed to the use of phytase, compensating for the AP deficiency and improving bone health and performance. Lelis *et al.* (2010), reported that phytase supplementation (250 and 500 FTU/kg feed) increased P retention in tibia when the nutritional matrix of the enzyme was considered.

Bone health is very important during rearing, capture, transportation to slaughter, and slaughter of broilers (Zhang *et al.*, 2017). The fact that the rate of muscle growth outpaces that of bone growth increases the probability of leg problems for chickens (Almeida Paz *et al.*, 2009). Despite compromising some bone quality variables of broilers, mainly due to low level of AP in the diet, the worst performance is not always associated with poorer bone quality.



Histopathological bone results from both experiments showed that treatment had no effect on bone formation, because they had bones with a regular growth plate and vascularization in the metaphysis region (Figure 1). According to Zhang *et al.* (2017), one of the main bone problems for broiler chickens is the lack of cartilaginous mineralization in the metaphysis of long bones. These problems are due to low vascularization in the metaphysis, which consequently inhibits calcification of the cartilage. Such low mineralization could result in increased frequency of carcass condemnations of broilers.

In view of the results presented herein, broiler chickens, even with normal bone growth, may present apparent problems with bone health. According to Wilson (2010), bruises and fractures among broiler chickens usually occur during capture, transport, discharge and hanging of birds, and can be attributed to bone fragility associated with management errors. Partial condemnations for bruises are the most common condemnations in slaughterhouses, and are due to pre-slaughter management problems (Almeida *et al.*, 2018). Thus, the results of the present study support future studies to evaluate the effect of increased P availability and bone health on traumatic lesions of broiler chickens. Such research is particularly relevant for the final pre-slaughter management phase and the slaughtering process, since bone quality problems may result in increased carcass condemnations.

Therefore, the AP level in broiler feed must be sufficient for optimal performance and bone development. Broiler bones must be strong so that the carcass goes through the slaughter process without bones breaking. The poultry industry is concerned with problems related to bone integrity and resistance, especially because poorly developed and fractured carcasses lead to high discard rates at slaughterhouses.

In conclusion, reduced AP without the use of phytase reduces body weight gain and feed intake of broilers in addition to altering bone parameters, such as phosphorus concentration in the tibia. The efficiency of the commercial phytases studied differed, with Phytase X being more efficient.

## Declarations

### *Acknowledgments*

The authors acknowledge Pró-Reitoria de Pesquisa at Universidade Federal de Minas Gerais (UFMG), Brazil, for providing funds for publication.

### *Funding*

We acknowledge the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) for providing a scholarship.

### *Conflicts of interest*

The authors declare they have no conflicts of interest with regard to the work presented in this report.

### *Author contributions*

Mariana M. Saldanha and Luiz F. P. Pereira were responsible for conducting the experiment. Anna R. C. Abreu, Fernanda L. S. Castro and Winnie L. S. Clímaco assisted in laboratory analysis. Itallo C. S. Araújo assisted in the review and writing of the article. Leonardo J. C. Lara guided the study.

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