RAULLY LUCAS SILVA

PHOSPHORUS EQUIVALENCY OF THREE PHYTASES OF FUNGAL AND BACTERIAL ORIGIN UNDER DIFFERENT MANUFACTURING PROCESSES FOR BROILERS

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Zootecnia, para obtenção do título de *Magister Scientiae*.

Orientador: Luiz Fernando Teixeira Albino

Coorientadores: Horacio Santiago Rostagno

Arele Arlindo Calderano

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APROVADA: 19 de fevereiro de 2020.

Assentimento:

Raully Lucas Silva
Autor

Luiz Fernando Teixeira Albino
Orientador

Aos meus pais, João Batista e Mairelane por todo amor, compreensão, dedicação e sacrifício em prol da minha educação.

Dedico.

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Biografia

Raully Lucas Silva, filho de João Batista Pereira da Silva e Mairelane Isabel de Souza, nasceu em 13 de maio de 1991, em Manhumirim, Minas Gerais. Ingressou no curso de Zootecnia na Universidade Federal de Viçosa em março de 2011. De agosto de 2013 a dezembro de 2014 foi bolsista pelo programa Ciências sem Fronteiras, cursando parte da graduação na Montana State University – Bozeman – Montana – EUA. Graduou-se em Zootecnia pela Universidade Federal de Viçosa em 26 de Janeiro de 2018. Em março do mesmo ano iniciou o Mestrado em Zootecnia na mesma instituição, realizando suas pesquisas na área de Nutrição e Produção de Monogástricos e submetendo-se à defesa da dissertação em 19 de fevereiro de 2019.

Abstract

SILVA, Raully Lucas, M.Sc., Universidade Federal de Viçosa, February, 2020. **Phosphorus equivalency of three phytases of fungal and bacterial origin under different manufacturing processes for broilers.** Adviser: Luiz Fernando Teixeira Albino. Co-advisers: Horacio Santiago Rostagno and Arele Arlindo Calderano.

The aim of this study was to determine the efficacy and the phosphorus equivalency of three commercial phytases of fungal (Aspergillus niger) and bacterial (*Hafnia* sp., *Yersinia mollaretii* and *Buttiauxella gaviniae*) origin under different manufacturing processes in the diets of broiler chickens from 1 to 21 days of age. A total of 1750 male broiler chickens were randomly distributed in 7 treatments with 10 replicates of 25 birds each. A basal diet was formulated containing 1.8 g/kg of nPP and 4.05 g/kg of total calcium (T1). For treatments 2-4, the basal diet was supplemented with 0.7, 1.4, or 2.1 g per kg of inorganic phosphorus from dicalcium phosphate to create diets with 2.5, 3.2 or 3.9 g/kg of nPP, respectively. The treatments from 5 to 7 received 500 phytase units per kg of 3-fungal phytase powder (phytase A), 6-bacterial phytase powder (phytase B) and 6-bacterial phytase granular (phytase C) respectively. Increasing levels of inorganic phosphorus from dicalcium phosphate at 0.7, 1.4, or 2.1 g per kg improved ($P<0.05$) final body weight by 17, 21.7 and 22.4%; feed intake by 14, 18 and 16.4%; weight gain by 18, 23 and 24%; tibia ash by 27, 43 and 37.3% and tibia P content by 25.4, 36 and 32%. Supplementation with phytase A, phytase B and phytase C contributed to an improvement ($P< 0.05$) of 15.4, 20.8 and 21% in final body weight, 11, 16 and 15.5% in feed intake, 16.3, 22 and 22.2% in weight gain, 10.4, 24 and 41.7% in tibia ash weight and 7, 21.4 and 36% in tibia P, respectively in relation to the basal diet. Linear and quadratic regression equations ($P<0.05$) were used to estimate phosphorus equivalency values of the three phytases. Phytase A, phytase B and phytase C can be used in the diets of broiler chickens from 1 to 21 days of age to partially reduce the addition of dicalcium phosphate source. The supplementation of 500 FTU/kg with phytase A, B and C were determined to be equivalent on average of 0.097, 0.139 and 0.145% of inorganic phosphorus from dicalcium phosphate, respectively.
Keywords: Animal nutrition. Feed additive. Phytase efficacy. Phosphorus equivalency.

Resumo

SILVA, Raully Lucas, M.Sc., Universidade Federal de Viçosa, fevereiro de 2020. **Equivalência de fósforo de três fitases de origem fúngica e bacteriana com diferentes processos de fabricação para frangos de corte**. Orientador: Luiz Fernando Teixeira Albino. Coorientadores: Horacio Santiago Rostagno e Arele Arlindo Calderano.

Objetivou-se com esse estudo determinar a eficácia e a equivalência de fósforo de três fitases comerciais de origem fúngica (*Aspergillus niger*) e bacteriana (*Hafnia sp., Yersinia mollaretii e Buttiauxella gaviniae*) com diferentes processos de fabricação, em dietas para frangos de corte de 1 até 21 dias de idade. Um total de 1750 frangos machos foram distribuídos aleatoriamente em 7 tratamentos com 10 repetições de 25 aves por unidade experimental. A dieta basal foi formulada contendo 1,8 g por kg de fósforo não fítico e 4,05 g por kg de cálcio total (T1). Para os tratamentos de 2 a 4, a dieta basal foi suplementada com 0,7; 1,4 e 2,1 g/kg de fosforo inorgânico de fosfato bicálcico para formar dietas com 2,5; 3,2 e 3,9 g/kg de P, respectivamente. Os tratamentos de 5 a 7 receberam 500 unidades de fitase por kg de uma 3-fitase fúngica em pó (Fitase A), 6-fitase bacteriana em pó (Fitase B) e uma 6-fitase bacteriana granular (Fitase C), respectivamente. O aumento dos níveis de fósforo inorgânico do fosfato bicálcico em 0,7, 1,4 ou 2,1 g por kg melhorou ($P<0,05$) o peso final em 17, 21,7 e 22,4%; consumo de ração em 14, 18 e 16,4%; ganho de peso em 18, 23 e 24%; cinzas da tíbia em 27, 43 e 37,3% e teor de P na tíbia em 25,4, 36 e 32%. A suplementação com as fitases A, B e C, contribuiu para uma melhora significativa ($P<0,05$) de 15,4, 20,8 e 21% no peso final, 11, 16 e 15,5% no consumo de ração, 16,3, 22 e 22,2% no ganho de peso, 10,4, 24 e 41,7% no peso das cinzas da tíbia e 7, 21,4 e 36% na conteúdo de fosforo na tíbia, respectivamente, em relação à dieta basal. Equações de regressão linear e quadrática ($P<0,05$) foram usadas para estimar os valores de equivalência de fósforo das três fitases. As fitases A, B e C podem ser utilizadas nas dietas de frangos de corte de 1 a 21 dias de idade para reduzir parcialmente a adição de fonte de fosfato bicálcico. A suplementação de 500 FTU / kg com a fitase A, B e C foi determinada como equivalente a média de 0,097, 0,139 e 0,145% de fósforo inorgânico a partir de fosfato bicálcico, respectivamente.
Palavras-chave: Nutrição animal. Aditivo alimentar. Eficiência de fitase. Equivalência de fósforo.

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CHAPTER 1

This chapter was written in the form of international scientific paper according to the standards of the Animal Feed Science and Technology Journal.

METABOLISM AND NUTRITION

PHYTASE FOR BROILERS

Phosphorus equivalency of three phytases of fungal and bacterial origin under different manufacturing processes for broilers.

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Abstract

The aim of this study was to determine the efficacy and the phosphorus equivalency of three commercial phytases of fungal (Aspergillus niger) and bacterial (*Hafnia* sp., *Yersinia mollaretii* and *Buttiauxella gaviniae*) origin under different manufacturing processes in the diets of broiler chickens from 1 to 21 days of age. A total of 1750 male broiler chickens were randomly distributed in 7 treatments with 10 replicates of 25 birds each. A basal diet was formulated containing 1.8 g per kg of non-phytate phosphorus and 4.05 g per kg of total calcium (T1). For treatments 2-4, the basal diet was supplemented with 0.7, 1.4, or 2.1 g per kg of inorganic phosphorus from dicalcium phosphate to create diets with 2.5, 3.2 or 3.9 g per kg of P, respectively. The treatments from 5 to 7 received 500 phytase units per kg of 3-fungal phytase powder (phytase A), 6-bacterial phytase powder (phytase B) and 6-bacterial phytase granular (phytase C) respectively. Increasing levels of inorganic phosphorus from dicalcium phosphate at 0.7, 1.4, or 2.1 g per kg improved ($P<0.05$) final body weight by 17, 21.7 and 22.4%; feed intake by 14, 18 and 16.4%; weight gain by 18, 23 and 24%; tibia ash by 27, 43 and 37.3% and tibia P content by 25.4, 36 and 32%. Supplementation with phytase A, phytase B and phytase C contributed to an improvement ($P< 0.05$) of 15.4, 20.8 and 21% in final body weight, 11, 16 and 15.5% in feed intake, 16.3, 22 and 22.2% in weight gain, 10.4, 24 and 41.7% in tibia ash weight and 7, 21.4 and 36% in tibia P, respectively in relation to the basal diet. Linear and quadratic regression equations ($P<0.05$) were used to estimate phosphorus equivalency values of the three phytases. Phytase A, phytase B and phytase C can be used in the diets of broiler chickens from 1 to 21 days of age to partially reduce the addition of dicalcium phosphate source. The supplementation of 500 FTU/kg with phytase A, B and C were determined to be equivalent on average of 0.097, 0.139, 0.145% of inorganic phosphorus from dicalcium phosphate respectively.

Keywords: Animal nutrition. Feed additive. Phytase efficacy. Phosphorus equivalency.

Abbreviations: BW, Body weight; Ca, Calcium; DCP, Dicalcium phosphate; FCR, Feed conversion ratio; FI, Feed intake; FTU, Phytase units; nPP, Non-phytate phosphorus; P, Phosphorus; WG, Weight gain.

1. Introduction

The adequate absorption of phosphorus (P) is important for growing chicks, due to its high demand for bone development and health. The absorption of phosphorus in birds is directly influenced by its type in the feed ingredient. It is known that phosphorus bound on phytate molecule is not an adequate source when we consider the absorption and further utilization by poultry than an inorganic source such dicalcium phosphate (Singh, 2008). The use of inorganic sources of phosphorus to meet the nutritional requirements for non-ruminants animals is very common in poultry production. Dicalcium phosphate is frequently added to diets, however, this practice increases the cost of feed as well as contributes to the excretion of phosphorus in the environment (Smith et al., 1999; Waldroup, 1999; Yan et al., 2003).

The phytase enzyme has been widely used as an important tool to reduce the inclusion of inorganic phosphorus in the diet (Smith et al., 1999; Waldroup, 1999; Yan et al., 2003). Also, the usage of phytase represents one of the best and most economical ways to achieve phosphorus requirements in diets for broiler chickens (Selle and Ravindran, 2007). Due to phytase enzymatic characteristics, phosphorus that was originally attached to the phytate molecule may be released and absorbed by birds, reducing significantly calcium and phosphorus supplementation from inorganic sources (Mullaney et al., 2000, Menezes-Blackburn et al., 2014).

Nowadays, commercial phytases are mainly derived from fungi or bacterial sources and have varying degrees of efficacy in terms of P availability (Camden et al., 2001). Some of those fungal derived phytase are from *Aspergillus niger* which are a 3-

phytase-type, once the initial release of phosphate radicals occur in the C3 position of phytate molecule (Selle and Ravindran, 2007). Others types of phytases are from bacteria source, such as *Hafnia* sp., *Yersinia mollaretii* and *Buttiauxella gaviniae,* which begin the phytate degradation at position C6 of the myo-inositol hexaphosphate (EFSA, 2017). The effect of phytase supplementation on P availability has been well documented (Denbow et al., 1998, Camden et al., 2001, Rutherfurd et al., 2004, Santos et al., 2008). However, phytases from different sources may have different values for P release due to their distinct characteristics, such as resistance to degradation in the gut tract and thermal stability (Onyango et al., 2004).

Several studies have been published in the last few years showing that phytases improve utilization of P phytate by poultry. However, the P release by phytase is relative to the nature of the displaced inorganic P source and also by the type of phytase (Angel et al., 2001, Augspurger et al., 2004, Shaw et al., 2011). The decision on which phytase should be used to formulate poultry diets is difficult due to a lack of clarity of how these characteristics can influence the main phytase site of activity as well as the efficacy of phytate P release (Maenz et al., 1998).

Thus, the main objective with the current study was to evaluate the effects of three commercial phytases and their equivalency relative to P from dicalcium phosphate in birds from 1 to 21 days of age.

2. Material and Methods

The experimental protocol was reviewed and approved by the Federal University of Viçosa Institutional Animal Care and Use Committee (protocol n^{o}. 039/2019) and followed principles established by the Canadian Council on Animal Care Guidelines and Policies (CCAC, 1993).

2.1. Birds and experimental design

The study was a completely randomized design with seven dietary treatments and ten experimental units per treatments. A total of 1750 1-day-old birds male Cobb-500 broiler chicks were obtained from a commercial hatchery (Ribelli Alimentos SA, Matheus Leme, Brazil) to evaluate the effects of 3-phytase derived for *Aspergillus niger* and a hybrid bacterial 6-phytase expressed in *Aspergillus niger* under different manufacturing processes (as detailed in Section 2.3).

The chicks were vaccinated against Bursal Disease and Marek's Disease (Serotype 3, Live Marek's Disease Vector, Material Inc. Athens, GA). Birds were weighed and randomly allocated to dietary treatments and placed into pens equipped with two nipple drinkers and feed dispenser. There were 70 pens with 25 birds/pen and 10 pens, totalizing 10 experimental units per treatment. Experimental diets were fed to the birds ad libitum, from 1 to 21 days of age. Water was freely available and temperature was kept at 32 ºC in the first week and gradually reduced to 22 ºC at 21 days of age, according to Cobb® manual recommendations. During the experimental period an intermittent lighting schedule of 23L:1D was applied.

2.2 Diets and treatments

A P-low corn and soybean based basal diet (Treatment 1) with 1.8 g/kg of non-phytate phosphorus (nPP) and 4.05 g/kg of total calcium (Ca) was formulated, while containing all of the other necessary nutrients to meet the birds' requirements according to the Brazilian Table for Poultry and Swine (Rostagno et al., 2017) as listed in Table 1. For treatments 2–4, the basal diet was supplemented with 0.7, 1.4, or 2.1 g/kg of inorganic phosphorus from dicalcium phosphate (DCP) to create diets with 2.5, 3.2 or 3.9 g/kg of P, respectively. For treatments 5–7 (Table 2) the basal diet was supplemented with 500 unit of phytase (FTU) per kg of a 3-fungal phytase powder (phytase A), 6-bacterial phytase powder (phytase B) and 6-bacterial phytase granular (phytase C) respectively.

2.3. Enzymes

Three different commercial phytases were used to investigate its effectiveness in phosphorus release. The first experimental phytase is encoded by the *Aspergillus ficuum* gene and expressed in a genetically modified strain of an *Aspergillus niger* which is available as a commercial product under the name of Nathupos SD 55000®. This product is in a powder form and it is a 3-phytase, which initiates phosphate hydrolysis at 3-position of the phytate molecule, and had an analyzed enzyme activity of 5000 phytase units/g. The second and third phytases studied were a hybrid 6-phytase preparation, produced by genes of enterobacteria *Hafnia* sp., *Yersinia mollaretii* and *Buttiauxella gaviniae* expressed in *Aspergillius niger* under two different forms (powder or granular) and different concentrations (50000 FTU/g or 10000 FTU/g) which is available as a commercial product under the name Nathupos E 50000 and Nathupos E 10000 G respectively.

Enzymes were included in 9.09, 10 and 50 g/ton for phytases A, B and C, respectively, to achieve one target dose level of 500 FTU/kg feed, where FTU referred to the amount of enzyme required to hydrolyze 1 μmol inorganic P per minute from 0.0051 mol/L sodium phytate at pH 5.5 and a temperature of 37 °C (AOAC, 2000).

The phytases are obtained by submerged aerobic fermentation of the production strain followed by a recovery and downstream processing. The resulting product (liquid concentrate) is mixed with magnesium sulfate and then spray-dried to prepare the powder formulation. The spray-dried product is referred as enzyme SD powder (solid concentrate) utilized in treatment 5.

The phytase B used in treatment 6 contains the liquid concentrate of phytase and magnesium sulfate (2%), wheat bran (97%), and vegetable oil (1%, soya bean). The phytase C used in treatment 7 has a granular formulation. It contains the phytase concentrate (from 1.5% to 2.7%), starch (82%), polyvinylalcohol (1.4%), arabic gum (3%), wax-based coating agent (5.0%) and water (up to 100%).

2.4. Measurements and sampling

Feed intake (FI) was measured from 1 to 21 days of age. Bird weight was recorded on day 14 and day 21 per pen and weight gain (WG) was calculated from 1 to 21 days of age. Mortality was recorded daily and used to calculate mortality corrected feed conversion ratio (FCR). FCR was calculated as total feed consumed per pen and total body weight (BW) including the BW of dead birds. At 21 days of age, 2 birds per experimental unit, which body weight was close to the average weight of each pen, were selected to be slaughtered. Each bird was euthanized and the right and left tibia was removed to evaluate ash (g/kg) and P (g/kg) contents. Tibias were dried at 55 ◦C in a forced-draft oven for 3 days and its contents were determined in a private Lab using the method 942.05 (AOAC, 2000).

2.5. Statistical analysis

Data were based on pens as experimental unit. Data were analyzed as a completely randomized design by one-way analysis of variance (ANOVA) using SAS 9.2 (2010). The statistical model is described as follows:

$$Y_{ij} = \mu + \tau_i + \varepsilon_{ij}$$

where: Y_{ij} is the observation j in group or treatment i, μ is the overall mean, τ_i is the fixed effect of group or treatment i (denotes an unknown parameter) and ε_{ij} is random error associated with the observed value of Y_{ij}.

Differences were considered statistically significant at $P \leq 0.05$. When the analysis of variance was significant ($P \leq 0.05$) a dose-response relationship was determined using a linear and polynomial quadratic model.

Based on the methodology described by Sakomura and Rostagno (2016), the performance and bone characteristics were used to design the standard curve, based on the percentage of available phosphorus in the control diets added to supplemental phosphorus. The results of treatments that were supplemented with phytases were compared with the standard curve to calculate the values of phosphorus release. In the case of the quadratic equation: $Y=a+bX+cX^2$ the equivalence of available phosphorus of phytases was cleared with the following equation.

$$X = \frac{-b \pm \sqrt{b^2 - 4c\,(a - Y)}}{2c}$$

Where Y is the response criterion (performance or bone traits), a, b and c are parameters of the model and X corresponds to the equivalency of phosphorus byphytases

3. Results

In the current study, the phytase supplemented diets were formulated to contain 500 FTU/kg of a 3-fungal phytase powder, 6-bacterial phytase powder and 6-bacterial phytase granular. The supplemental P levels from dicalcium phosphate resulted in linear and quadratic effects on broilers final body weight, body weight gain, feed intake and feed conversion rate. Also, tibia ash and tibia P content had a significantly ($P<0.05$) effect explained for linear and quadratic equations (Table 3).

The growth parameters increased ($P<0.05$) at the levels of 0.7, 1.4 and 2.1 g/kg of phosphorus from dicalcium phosphate. Final body weight improved by 17, 21.7 and 22.4%; feed intake by 14, 18 and 16.4%; weight gain by 18, 23 and 24% respectively. Feed conversion ratio had a decrease by 0.04, 0.06 and 0.08 when P was supplemented in increasing levels.

Increasing the percentage of non-phytate phosphorus in the feeds results in higher contents of bone ash as well P in tibia. Supplementation with dicalcium phosphate improved significantly ($P<0.05$) tibia ash weight by 27, 43 and 37.3% and tibia P by 25.4, 36 and 32% when compared with the basal diet, respectively (Table 3).

The supplementation with 500FTU per kg of the three phytases used in this study improved performance parameters. The inclusion of phytase A, phytase B and phytase C on the broiler diets promoted an increase of 15.4, 20.8 and 21% in body weight, 11, 16 and 15.5% in feed intake, 16.3, 22 and 22.2% in weight gain and a reduction of 0.063, 0.069 and 0.076 points in feed conversation ration, respectively, in relation to the basal diet (Table 3).

Furthermore, for bone characteristics, the supplementation with phytases also showed an improvement. The addition of 500 FTU/kg contributes to increase tibia ash weight by 10.4, 24 and 41.7% and tibia P by 7, 21.4 and 36% respectively in relation to the basal diet. The percentage of tibia ash was improved by 4.03, 9,10 and 8.14% and the percentage of tibia P was improved by 1.3, 6.4 and 4.3% respectively, in relation to the basal diet.

Mortality during the trial was minimal and not associated with dietary treatments.

Regression equations for growth performance and bone mineral contents with increasing levels of P and the calculated equivalence of the studied phytases are presented in Table 4 and Table 5. Linear and quadratic polynomial regression equations were used to determine P equivalency values of phytases when dicalcium phosphate was used as an inorganic P source in broiler diets based on the methodology described by Sakomura and Rostagno (2016). Estimated phosphorus equivalence was conducted with both equations (linear and quadratic). Most measurements had a best fit (highest R^2) with quadratic adjustments.

The inclusion of 500 FTU/kg of phytase A, B and C were determined to be equivalent to the average addition of 0.113, 0.160 and 0.164% of nPP from DCP per kg in broiler diets respectively, when linear equations are used. Meanwhile, the average determined for phytase A, B and C was of 0.080, 0.117 and 0,125% of nPP equivalence from DCP per kg in the diet respectively, when the quadratic polynomial regression was used (Table 4).

4. Discussion

In this study three commercial phytases of different microbial origins and different manufacturing process were studied in order to predict their potential as feed additive in broiler nutrition and to determine the phosphorus release equivalence in diets with low levels of nPP.

According to our results the supplementation of increasing levels of nPP from DCP resulted in linear and quadratic effects on performance and bone parameters of broiler chickens. When dietary levels of phosphorus were increased the performance parameters and bone characteristics improved. As expected, the best responses for the analyzed parameters were with the highest inclusion of inorganic phosphorus. Weight gain showed the greatest difference between the highest phosphorus level and the basal diet. These results are in accordance with Adeola, (2010), Hamdi et al. (2015), and Vieira et al., (2015) which reported linear or quadratic effects on growth performance and bone mineralization in broiler chicks fed with increasing levels of nPP on diets.

Based on broilers growth performance and bone mineralization, the supplementation of phytase from the three sources, phytase A, phytase B and phytase C, were efficient on phosphorus release. Over the total 21-day trial, compared with the basal diet (0.18% nPP) supplemental phytase A, B and C averaged across sources, increased weight gain on 16.3, 22 and 22% and FI on 11, 16 and 15.5%. These results are higher than several studies that have reported improvements on performance when phytase was supplemented at 500 FTU/kg on diets. Onyango et al. (2005) reported an increase of 17% on weight gain and 8.9% on FI of broiler chickens at 8 to 22 days old, when they were fed a P-low diet supplemented with 500 FTU/kg. Also, Zhang et al. (2000) showed an improvement on weight gain of 8.1% and 3.9% for FI over a 28-day trial with chicks fed with P-low diets supplemented with 500FTU/g of an *Aspergillus niger* phytase. Jendza et al. (2006) reported an increase of 87% in the weight gain of birds at 42 days when they were supplemented with 500 FTU/kg of phytase. This result is higher than those found on this trial. However, the difference between our results and those of Jendza et al. (2006) could be explained by the trial's time where the effects of phytase could be more pronounced. Moreover, in our study FCR was improved in treatments supplemented with phytases compared to treatments with low levels of nPP. This result is in accordance with Beiki et al. (2013) and Akter et al. (2016) who reported a better FCR in birds supplemented with 500 FTU/kg of phytase in low-P diets.

Due the fact that bones act as the reservoir of minerals, tibia ash weight and percentage are usually used as criteria to evaluate P bioavailabity of feedstuffs and the efficacy of phytase to release P (Zhang et al. 2000). According to Sakomura and Rostagno (2016) if tibia is mineralized, this indicates that the mineral was available and used by the animal. In this study, reducing the percentage of nPP in the feeds resulted in lower contents of tibia ash as well as P. As expected, tibia ash and P content in bone was highest when birds were fed the diets supplemented with 0,32 and 0.39% of DCP.

Bone characteristics were improved when phytase was supplemented to the low-P diets. Addition of 500 FTU/kg of phytase A, phytase B and phytase C increased tibia ash weight by 10.4, 24 and 41.7% and tibia P content by 7, 21.4 and 36% respectively, in relation to the basal diet. Several studies reported a bone mineral improvement with phytase supplementation (Perney et al. 1993; Sebastian et al. 1996; Denbow et al., 1998 Ahmad et al. 2000 ; Singh et al.,2013)

The growth performance improvement and bone mineralization when phytase is supplemented indicate an increase of inorganic phosphorus liberation from phytate molecule. The efficacy of phytase to improve P availability by hydrolyzing phytate in poultry diets is described by past studies (Simons et al., 1990, Broz et al., 1994, Kornegay et al., 1996). According to Ravidran et al. (2001) the improvement of growth performance parameters is also possible due to an indirect effect of the supplementation with phytase on the energy available for birds. This effect occurs by the increase of nutrient digestibility, hindering the reaction of saponification between the lipids and the minerals of the phytate-mineral complex. Moreover, Sebastian et al. (1996) suggest that a better development of birds fed diets supplemented with phytase can be explained by the increase of digestibility of the starch and protein availability.

In the current study, the phytase added to treatments increase the availability of phosphorus in P-low diets. Similar results were reported by studies where the higher utilization of P from phytate reduced the supplementation of nPP sources in diets for broiler chickens while normal growth is maintained (Broz et al., 1994; Sebastian et al., 1996; Cabahug et al., 1999). Denbow et al. (1995) reported a P release of 47% by 500 FTU of phytase per kg of feed. By using fungal phytase, Simons et al. (1990) showed that the availability of P in low P diet for broilers increased to over 60% and decreased the amount of phosphorus in the excreta by 50%. Likewise, Waldroup et al. (1999) indicated that approximately 50% of the phytate-bound P in a corn-soybean diet was released by phytase.

The linear and quadratic regression equations were used on this trial to determine P equivalence values of phytase A, phytase B and phytase C, when DCP was used as an inorganic source of P in broiler's diets according to the methodology described by past studies (Denbow et al. 1995, Adedokun et al., 2004, Jendza et al. 2006, Sakomura and Rostagno 2016). The P equivalency was calculated with the response criterion that provides best R^2 values considering regression equations. Higher values of R^2 as a reference for choosing performance or bone mineralization to estimate P equivalence values from phytase has been the main criterion for some authors (Ravindran et al. 1995, Denbow et al. 1995, Harper et al. 1997, Adeola, 2010) However, the response criterion that are chosen in data analyses can influence the results obtained (Kornegay and Qian, 1996). Several authors have developed regressions by using treatment means rather than individual observations, which can result in elevated R^2 values (Jendza et al., 2006). In this study, the regression equations were developed by using individual observations as recommended by Jendza et al,. (2006). Thus, our values of R^2 for some variables, especially for bone characteristics, are lower than some results found in the literature (Yi et al., 1996, Yi et al. 1996a, Harper et al. 1997, Adedokun et al., 2004) where the authors utilized the average of responses to calculate R^2. Nevertheless, our results are in accordance with Denbow et al. (1995) and Onyango et al., (2005), who used the same methodology as us to obtain the R^2 values.

Based on the high R^2 values for linear or quadratic equations, WG was the most sensitive indicator to assess P availability. About 0.068, 0.111 and 0.112% of iP would be release in a diet contained 500 FTU units of phytase A, phytase B and phytase C respectively. The P equivalency values obtained in the present study are comparable to those reported by Onyango et al., (2005) and Dessimoni et al., (2019). However, our results of estimated P equivalency values are higher than those reported by past studies (Denbow et al., 1995, Pereira et al., 2012, Vieira et al., 2015). Yi et al., (1996) supplementing 500 FTU/kg of an *Aspergillus niger* phytase to a low-nPP diet for broiler chicks, reported estimated P equivalency values of 0.042%. The authors used a basal diet containing 0.27% of non-phytate phosphorus and 0.9% of total calcium, while we used a basal diet with 0.18% of non-phytate phosphorus and 0.405% of total calcium. These studies strongly suggest that the P equivalency values of phytase are influenced by the nPP and Ca levels. Probably, the high Ca level used in the study of Yi et al., (1996) contributed to the divergent estimates. Possibly, the different responses in studies evaluating phytase efficacy is the high calcium level (Adedokun et al., 2004), which can decrease the efficacy of phytase in birds due to phytate precipitation and the formation of the phytate-Ca-complex in the small intestine (Selle et al., 2009). Also, Tamim et al., (2004) reported that adding Ca at 0.9% into broilers diets resulted in a reduction of 85% in phytate phosphorus hydrolysis.

The higher values for growth performance, bone mineralization and equivalence of P for phytase B and C in comparison to phytase A noted in the present study may be an indication of the superior efficacy of these phytases. Higher P release values of phytase B and C are related with the biological characteristics and manufacturing technology of these enzymes. Some enzymatic properties of the three commercially available phytase products can influence broiler response, such as the fact that all phytases tested have been produced by genetically modified microorganisms. Phytases from five different donor organisms have been used in this trial. The first phytase tested (phytase A) is from fungal origin (*Aspergillus niger* + *Aspergillus ficuum*) and the last two phytase (B and C) are hybrid of fungal and bacterial origin (*Aspergillus niger* + *Hafnia sp., Yersinia mollaretii and Buttiauxella gaviniae*). On the basis of the carbon in the myo-inositol ring of phytate at which hydrolysis is initiated, the commercial phytase products consist either of a 3-phytase (phytase A) or a 6-phytase (B and C). These characteristics are important to explain the higher efficiency of phytase B and phytase C when compared to phytase A.

Differences between a 3-phytase and 6-phytase has been studied by Wodzinski et al. (1996) and Augspurger et al. (2003) who reported that broilers fed diets with 6-phytase had improved weight gain and had higher P digestibility. It has been hypothesized that 6-phytases always completely dephosphorylate phytate whereas 3-

phytases do not. However, Juin et al. (2001) and Sands et al. (2003) reported no difference in phytase source when comparing 3-phytase with a 6-phytase. This indicates that other factors than the site of phytate molecule dephosphorylation may be related to explain the higher efficiency of bacterial source phytases.

Augspurger et al. (2003), showed differences in phosphorus release when they studied the supplementation of fungal and bacterial phytase to a low P diet for broiler chicks. According to Augspurger et al. (2003), the addition of 500 FTU of a bacterial phytase to basal diet released 0.125% bioavailable P compared with 0.026% for the same units of *Aspergillus niger* phytase source. In addition, Augspurger et al. (2004) reported that 500 FTU/kg of a bacterial phytase produced bone ash values that were markedly superior to those resulting from 1000 FTU/kg of a fungal phytase. Likewise, Leeson et al., (2000) comparing a fungal phytase from *Aspergillus niger* with a bacterial phytase from *E. coli* in a P-low diet for broiler chicks, reported that both phytases improved growth parameters, bone ash and bone phosphorus content, however, birds supplemented with *E. coli* phytase had better results for growth parameters and bone phosphorus ($P < 0.01$) than birds fed with *Aspergillus niger*.

Moreover, Elkhalil et al., (2007) reported that phytases from bacterial sources are more stable to protease inactivation than Aspergillus phytase, therefore, the resistance to proteolytic degradation of bacterial phytase is higher than Aspergillus. This suggests that bacterial phytase might act within the gizzard for a longer time period than Aspergillus phytase. Igbasan et al., (2000) in an *in* vitro comparative study of phytase from different microbial origins reported that bacterial phytase when exposed to endogenously secreted protease activities in the host animals had a greater resistant to pepsin and pancreatin than a fungal source of phytase. Thus, the higher activity of phytase B and C is due to their ability to maintain activity in the presence of proteolytic activities.

Overall, the result of this study demonstrated that the supplementation of the three phytases analyzed is capable of partially alleviating the deleterious effect of feeding a P-low diet on growth performance in broiler chicks, as well as influencing the mineralization of bones. Also, it is possible to reduce the amount of DCP added to the feed when phytase are supplemented to the broiler diets. The mean P equivalency values of phytase A, phytase B and phytase C ranged between 0.097, 0.139 and 0.145% of nPP respectively, when 500 FTU/kg is supplemented. However, this study used just one level of FTU for treatments supplement with phytase, which could affect the release of phosphorus. Maybe, a higher supplementation of FTU/kg in diets can reduce even more the addition of DCP and consequently reduce phosphorus excretion by birds.

5. Conclusion

The 3-fungal phytase powder (phytase A), 6-bacterial phytase powder (phytase B) and 6-bacterial phytase granular (phytase C) can be used in the diets of broiler chicks from 1 to 21 days of age to reduce the addition of dicalcium phosphate as a phosphorus source. The supplementation of 500 FTU/kg of phytase A, phytase B and phytase C has the mean equivalency of 0.097, 0.139 and 0.145% of nPP from DCP respectively, without losing efficiency on growth performance and bone mineralization.

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Tables

Table 1. Ingredients and nutrient composition of the diets supplemented with DCP for broilers of 1 to 21 days of age.

Ingredients	Basal Diet	Dicalcium phosphate added diets		
	0.18% nPP	0.25% nPP	0.32% nPP	0.39% nPP
Ground corn	51.705	51.705	51.705	51.705
Soybean meal, 45%	40.720	40.720	40.720	40.720
Soybean oil	3.600	3.600	3.600	3.600
Common salt	0.500	0.500	0.500	0.500
L-Lisyne HCl, 79%	0.158	0.158	0.158	0.158
DL-Methionine, 99%	0.325	0.325	0.325	0.325
L-Threonine, 98%	0.057	0.057	0.057	0.057
L-Valine, 98%	0.013	0.013	0.013	0.013
Choline choride, 60%	0.100	0.100	0.100	0.100
Mineral supplement[1]	0.140	0.140	0.140	0.140
Vitamin supplement[2]	0.140	0.140	0.140	0.140
Salinomycin 12%	0.055	0.055	0.055	0.055
Avilamycin, 20%	0.010	0.010	0.010	0.010
Antioxidant (BHT)[3]	0.010	0.010	0.010	0.010
Limestone (Ca: 37,7%)	0.428	0.600	0.771	0.942
DCP[4]	0.388	0.766	1.146	1.524
Sand[5]	1.651	1.095	0.550	0.001
Total	100.000	100.000	100.000	100.000
Calculated nutrient composition				
Crude protein, %	22.931	22.931	22.931	22.931
AME[6], kcal/kg.	3000.0	3000.0	3000.0	3000.0
Calcium, %	0.405	0.560	0.720	0.877
Non-phytate phosphorus, %	0.180	0.250	0.320	0.390
Total phosphorus, %	0.420	0.490	0.560	0.630
Phytic phosphorus, %	0.240	0.240	0.240	0.240
Sodium %	0.212	0.212	0.212	0.212
Lysine, %	1.256	1.256	1.256	1.256
Threonine,%	0.829	0.829	0.829	0.829
Methionine + Cysteine , %	0.929	0.929	0.929	0.929
Isoleucine, %	0.894	0.894	0.894	0.894
Tryptophan, %	0.263	0.263	0.263	0.263
Arginine, %	1.442	1.442	1.442	1.442
Valine, %	0.967	0.967	0.967	0.967
Glycine + Serine, %	1.846	1.846	1.846	1.846

[1] - Mineral Supplement (Amount per kg of Feed): Manganese 88 mg; Iron - 62.5 mg; Zinc - 81.3 mg; Copper - 12.5 mg; Iodine - 1.25 mg.
[2] - Vitamin Supplement (Amount per kg of Feed): vitamin A - 9375 IU; vitamin D3 - 2375 IU; Vitamin E - 35 IU; Vitamin B1 - 2.5 mg; vitamin B6 - 3.5 mg; Pantothenic Ac - 12.5 mg; Biotin - 0.088 mg; Vitamin K3 - 1.88 mg; Folic acid - 0.875 mg; Nicotinic Acid 37.5 mg; Vitamin B12 - 0.015 mg; Selenium - 0.375 mg.
[3] - Hydroxy butyl toluene
[4] - Dicalcium Phosphate (Ca: 24,5% ; P: 18,5%)
[5] - Enzymes were added in place of washed sand. Considering the contribution of nPP and Ca from phytases: NSD treatment: nPP: 0.115%, Ca: 0.100%; NE treatment: nPP: 0.1495%, Ca: 0.167%; NEG treatment: nPP: 0.1495%, Ca: 0.167%
[6] – Apparent Metabolizable Energy.

Table 2. Ingredients and nutrient composition of the diets supplemented with phytase for broilers for 1 to 21 days of age.

Ingredients	Phytases added diets		
	Phytase A*	Phytase B*	Phytase C*
Ground corn	51.705	51.705	51.705
Soybean meal, 45%	40.720	40.720	40.720
Soybean oil	3.600	3.600	3.600
Common salt	0.500	0.500	0.500
L-Lisyne HCl, 79%	0.158	0.158	0.158
DL-Methionine, 99%	0.325	0.325	0.325
L-Threonine, 98%	0.057	0.057	0.057
L-Valine, 98%	0.013	0.013	0.013
Choline choride, 60%	0.100	0.100	0.100
Mineral supplement[1]	0.140	0.140	0.140
Vitamin supplement[2]	0.140	0.140	0.140
Salinomycin 12%	0.055	0.055	0.055
Avilamycin, 20%	0.010	0.010	0.010
Antioxidant (BHT)[3]	0.010	0.010	0.010
Limestone (Ca: 37,7%)	0.850	0.876	0.876
DCP[4] (Ca: 24,5% ; P: 18,5%)	0.388	1.203	1.203
Sand[5]	1.229	1.203	1.203
Total	100.000	100.000	100.000
Calculated nutrient composition			
Crude protein, %	22.931	22.931	22.931
AME[6], kcal/kg.	3000.0	3000.0	3000.0
Calcium, %	0.564	0.574	0.574
Non-phytate phosphorus, %	0.180	0.180	0.180
Total phosphorus, %	0.420	0.420	0.420
Phytic phosphorus, %	0.240	0.240	0.240
Sodium %	0.212	0.212	0.212
Lysine, %	1.256	1.256	1.256
Threonine,%	0.829	0.829	0.829
Methionine + Cysteine , %	0.929	0.929	0.929
Isoleucine, %	0.894	0.894	0.894
Tryptophan, %	0.263	0.263	0.263
Arginine, %	1.442	1.442	1.442
Valine, %	0.967	0.967	0.967
Glycine + Serine, %	1.846	1.846	1.846

* Phytase A: 3-fungal phytase powder, phytase B: 6-bacterial phytase powder, phytase C:6-bacterial phytase granular

1 - Mineral Supplement (Amount per kg of Feed): Manganese 88 mg; Iron - 62.5 mg; Zinc - 81.3 mg; Copper - 12.5 mg; Iodine - 1.25 mg.

2 - Vitamin Supplement (Amount per kg of Feed): vitamin A - 9375 IU; vitamin D3 - 2375 IU; Vitamin E - 35 IU; Vitamin B1 - 2.5 mg; vitamin B6 - 3.5 mg; Pantothenic Ac - 12.5 mg; Biotin - 0.088 mg; Vitamin K3 - 1.88 mg; Folic acid - 0.875 mg; Nicotinic Acid 37.5 mg; Vitamin B12 - 0.015 mg; Selenium - 0.375 mg.

3 - Hydroxy butyl toluene

4 - Dicalcium Phosphate

5 - Enzymes were added in place of washed sand. Considering the contribution of nPP and Ca from phytases: NSD treatment: nPP: 0.115%, Ca: 0.100%; NE treatment: nPP: 0.1495%, Ca: 0.167%; NEG treatment: nPP: 0.1495%, Ca: 0.167%

6 – Apparent Metabolizable Energy.

Table 3. Performance and tibia characteristics of broilers fed diets with inorganic phosphorus levels (g/kg) from dicalcium phosphate or supplemental with 500 FTU/kg of different commercial phytases from 1 to 21 days of age[a].

Item	Basal diet	Supplemental nPP from DCP (g/kg)				Supplemental Phytase (500 FTU/kg)		
	0.0	0.7	1.4	2.1	SEM	Phytase A*	Phytase B*	Phytase C*
BW g/bird[b,c]	791.0	925.0	963.0	968.0	4.9524	913.0	956.0	958.0
FI g/bird[b,c]	1019.0	1161.0	1199.0	1186.0	6.1674	1131.0	1182.0	1177.0
WG g/bird[b,c]	749.0	882.0	921.0	926.0	4.9344	871.0	914.0	915.0
FCR[b,c]	1.362	1.316	1.302	1.281	0.0029	1.299	1.293	1.286
Tibia ash (%)[b,c]	44.06	46.99	49.47	50.50	0.1391	45.84	48.07	47.65
Tibia ash (mg)[b,c]	3190.0	4050.0	4550.0	4380.0	98,790	3523.0	3960.0	4520.0
Tibia P (%)[b,c]	8.10	8.58	8.67	9.02	0.0720	8.21	8.62	8.45
Tibia P (mg) [b,c]	590.0	740.0	800.0	780.0	16.112	632.0	716.0	800.0
Mortality (%)	1.6	1.6	1.2	0.8		1.2	1.2	1.4

[a] - P = Phosphorus; nPP= non-phytate phosphorusç DCP = Dicalcium phosphate; FTU = Phytase units; BW = Body Weight; FI = Feed Intake; WG = Weight Gain; FCR = Feed Conversion Rate.

* Phytase A: 3-fungal phytase powder, phytase B: 6-bacterial phytase powder, phytase C:6-bacterial phytase granular

[b]Linear effect of DCP (P<0.0001)

[c] Quadratic effect of DCP (P<0.0001)

Table 4. Linear and quadratic regression equations estimated to each variable on response of inorganic phosphorus levels (g/kg) from dicalcium phosphate for broilers from 1 to 21 days of age.

Iten	Equation	Effect	SEM[a]	P-value	R^2
Body weight (kg/bird)	Y=0.8260+0.8141X	Linear	0.0131	<0.0001	71.35
	$Y=0.7937+2.1974X-6.5867X^2$	Quadratic	0.0079	<0.0001	89.66
Weight gain (kg/bird)	Y=0.784+0.8154X	Linear	0.0131	<0.0001	71.55
	$Y=0.7517+2.1933X-6.5612X^2$	Quadratic	0.0079	<0.0001	89.72
Feed intake (kg/bird)	Y=1.0604+0.7713X	Linear	0.0163	<0.0001	59.02
	$Y=1.0220+2.4181X-7.8418X^2$	Quadratic	0.0107	<0.0001	82.93
Feed conversion rate	Y=1.3542-0.3686X	Linear	0.0079	<0.0001	58.65
	$Y=1.3608-0.6493X+1.3367X^2$	Quadratic	0.0077	<0.0001	61.68
Tibia ash (%)	Y=44.4863+31.1171X	Linear	0.3599	<0.0001	82.82
	$Y=44.013+51.4014X-96.592X^2$	Quadratic	0.3298	<0.0001	85.95
Tibia ash (mg)	Y=3.435+5.807X	Linear	1.211.103	<0.0001	59.71
	$Y=3.179+16.783X-52.265X^2$	Quadratic	892.959	<0.0001	78.68
Tibia P (%)	Y=8.164+4.0729X	Linear	0.1030	<0.0001	50.20
	$Y=8.1299+5.5193X-6.8878X^2$	Quadratic	0.1038	<0.0001	50.76
Tibia P (mg)	Y=0.630+0.921X	Linear	227.772	<0.0001	51.32
	$Y=0.588+2.7159X-8.547X^2$	Quadratic	185.253	<0.0001	68.64

[a] SEM = Standard pooled error of the means; (sample size = 10)

Table 5. Phosphorus equivalency values (%) of three commercial phytases[a].

Item	Phytase A		Phytase B		Phytase C	
	Linear	Quadratic	Linear	Quadratic	Linear	Quadratic
BW	0.107	0.068	0.159	0.110	0.162	0.113
WG	0.107	0.068	0.160	0.111	0.161	0.112
FI	0.092	0.055	0.158	0.096	0.151	0.091
FCR	0.149	0.130	0.166	0.152	0.185	0.187
Tibia ash (%)	0.107	0.068	0.159	0.110	0.162	0.113
Tibia ash (mg)	0.107	0.068	0.160	0.111	0.161	0.112
Tibia P (%)	0.092	0.055	0.158	0.096	0.151	0.091
Tibia P (mg)	0.149	0.130	0.166	0.152	0.185	0.187
Mean	0.113	0.080	0.160	0.117	0.164	0.125

[a] - Phytase A: 3-fungal phytase powder, phytase B: 6-bacterial phytase powder, phytase C:6-bacterial phytase granular.

BW: Body weight, WG: Weight gain, FI: Feed intake, FCR: Feed conversion rate.

Attachment

UNIVERSIDADE FEDERAL DE VIÇOSA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS DE PRODUÇÃO
CEUAP/UFV

Campus Universitário – Viçosa, MG – 36570-900 – Telefone:(31) 3899.3275 – e-mail: ceuap@ufv.br – site: www.ceuap.ufv.br

Viçosa, 11 de Junho de 2019

CERTIFICADO

Certificamos que o projeto intitulado **"Equivalência em Fósforo Disponível de Fitases para Dietas de Frangos de Corte"**, protocolo **nº 039/2019**, sob a responsabilidade de **Luiz Fernando Teixeira Albino** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo chordata, subfilo vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 8 de outubro de 2008, do decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo conselho nacional de controle da experimentação animal (concea), e foi apreciado pela comissão de ética no uso de animais de produção da universidade federal de viçosa (ceuap-ufv) em reunião de **17 de Mai. de 2019.**
Finalidade: **(x)Pesquisa** **( )Ensino**
Vigência do Projeto: **de 11 de Junho de 2019 a 03 de Set. de 2019**
Espécie/linhagem: **Frango de corte (*Gallus domesticus*)** Nº de animais: **1750**
Peso: **0,04 Kg** Idade: **1 dia** Sexo: **Macho** Origem: **Setor de Avicultura - DZO/UFV - CNPJ/CPF: 25.944.455/0001-96**

CERTIFICATE

We certify that the project entitled **"Equivalence in Available Phosphorus of Phytases for Broiler Diets"**, protocol **nº 039/2019**, under the responsibility of **Luiz Fernando Teixeira Albino** - which involves the production, maintenance and/or use of animals belonging to the phylum chordata, subphylum vertebrata (except man), for scientific research purposes (or education) - is in accordance with the law nº. 11.794, of October 8, 2008, Decree nº. 6899 of July 15, 2009, and the rules issued by the Brazilian National Council for Animal Experimentation Control (CONCEA), and was approved by the Ethics Commission on the use of farm animals of Universidade Federal de Viçosa (CEUAP-UFV) in its meeting on **May, 17th, 2019.**
Finality: **(x)Research** **( )Education**
Duration of the Project: from **Jun. 11th, 2019** to **Sep, 03th, 2019.**
Species / strain: **Broiler (*Gallus domesticus*)** Nº of animals: **1750**
Weight: **0,04 Kg** Age: **1 day** Sex: **Male** Source: **Setor de Avicultura - DZO/UFV - CNPJ/CPF: 25.944.455/0001-96**

Luciana Navajas Rennó
Coordenadora da CEUAP/UFV