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Efficacy and phosphorus equivalency values of two bacterial phytases (*Escherichia coli* and *Citrobacter braakii*) allow the partial reduction of dicalcium phosphate added to the diets of broiler chickens from 1 to 21 days of age

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ABSTRACT

The objective of this study was to determine the efficacy and the phosphorus equivalency of two bacterial phytases (*Citrobacter braakii*-derived phytase or genetically-modified *Escherichia coli* phytase) in the diets of broiler chickens from 1 to 21 days of age. A total of 2100 male broiler chickens were randomly distributed in 10 treatments with 10 replicates of 21 chicks each. A basal diet was formulated containing 1.8 g per kg of non-phytate phosphorus and 9 g per kg of total calcium (T1). For treatments 2–4, dicalcium phosphate was added to give 0.9, 1.8, or 2.7 g per kg of additional inorganic phosphorus. The treatments from 5 to 7 received 500, 1000, or 2000 phytase units of *Citrobacter braakii* (FYT) per kg while the treatments from 8 to 10 received 250, 500, or 1000 phytase units of *Escherichia coli* (FTU) per kg. Increasing levels of inorganic phosphorus from dicalcium phosphate at 0.9, 1.8, and 2.7 g per kg improved ($P < 0.05$) feed intake by 52, 71, and 80%; weight gain by 41, 75, and 32%; tibia ash weight by 64, 156, and 185%; and tibia phosphorus by 110, 323, and 378%, respectively. Increasing levels of *Citrobacter braakii*-derived phytase at 500, 1000, and 2000 FYT per kg contributed to a significant improvement ($P < 0.05$) of 52, 55, and 65% for feed intake; 37, 56, and 65% for weight gain; 52, 87, and 133% for tibia ash weight; and 96, 173, and 273% for tibia phosphorus, respectively. Increasing levels of *Escherichia coli*-derived phytase at 250 to 500, and 1000 FTU per kg increased ($P < 0.05$) feed intake by 50, 61, and 70%; weight gain by 49, 60, and 76%; tibia ash weight by 80, 103, and 164%; and tibia phosphorus by 128, 198, and 330%. Linear regression equations ($P < 0.05$) were used to estimate phosphorus equivalency values of the two phytases. The *Citrobacter braakii*- and *Escherichia coli*-derived phytases can be used in the diets of broiler chickens from 1 to 21 days of age to partially reduce the addition of dicalcium phosphate as a phosphorus source. The supplementation of 500, 1000, and 2000 phytase units of *Citrobacter braakii* per kg were determined to be equivalent to the average addition of 0.625, 1.091, and 2.024 g of inorganic phosphorus from dicalcium phosphate per kg in broiler diets, respectively. The supplementation of 250, 500, and 1000 phytase units of *Escherichia coli* per kg were

Abbreviations: Ca, calcium; CBP, *Citrobacter braakii*-derived phytase; ECP, *Escherichia coli*-derived phytase; FI, feed intake; F:G, feed-to-gain ratio; WG, weight gain; P, phosphorus; iP, inorganic phosphorus; DCP, dicalcium phosphate; nPP, non-phytate phosphorus; FYT, phytase units of *Citrobacter braakii*; FTU, phytase units of *Escherichia coli*.

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determined to be equivalent to the average addition of 0.763, 1.307, and 2.395 g of inorganic phosphorus from dicalcium phosphate per kg in broiler diets, respectively.

1. Introduction

In poultry production, inorganic phosphorus sources as dicalcium phosphate (DCP) are added to the feed to meet the dietary phosphorus (P) requirements, but this practice increases the cost of feeding as well as the P excretion, which contributes to environmental issues such as eutrophication in aquatic ecosystems (Smith et al., 1999; Waldroup, 1999; Yan et al., 2003).

Therefore, the development of exogenous phytase is one of the most important discoveries for poultry nutrition in the past few decades, as they allow for a better use of P in non-ruminant animals that consume diets containing vegetal products (Cromwell, 2009). Also, the use of Phytase in animal diets is currently widespread and represents one of the most economic ways of meeting part of the dietary phosphorus requirements (Selle and Ravindran, 2007).

There are several types of Phytase that are commercially available; some of those are fungal-derived Phytase such as from *Aspergillus ficum* and *Aspergillus niger*, which are generally type 3 Phytase (EC 3.1.3.8) as the initial release of phosphate radicals is preferred in the C3 position of the phytate (Selle and Ravindran, 2007). Other commercial phytase are derived from the fungi *Peniphora lycii* or from the bacteria *Escherichia coli*, which begin the phytate degradation at position 6 (EC 3.1.3.26) of the myo-inositol hexaphosphate (Bedford and Partridge, 2010). Moreover, phytase from different sources may have different characteristics, such as a pH for the optimal enzymatic activity, resistance to degradation in the gut tract, and thermal stability (Onyango et al., 2004). These characteristics can affect the P release, and studies to determine the efficacy of new phytase should be ongoing.

There is an *Escherichia coli* 6-phytase (phytase; EC 3.1.3.26) that is produced by a genetically modified strain of *Trichoderma reseii* whose efficacy and equivalency relative to P from dicalcium phosphate should be evaluated based on the methodology described by preliminary studies (Adedokun et al., 2004; Jendza et al., 2006; Adeola, 2010). Additionally, there is a new 6-phytase that is produced by a strain of *Aspergillus oryzae* and expresses 2 synthetic genes that both mimic a phytase gene from a strain of *Citrobacter braakii*, and previous studies were conducted to evaluate its efficacy (Cowieson et al., 2014) and its equivalency relative to P from dicalcium phosphate (Vieira et al., 2015). However, those past studies just offered experimental diets to birds from 8 to 21 days of age. Additionally, they evaluated two or less levels of phytase units (500 or 1000 FYT/kg). Thus, studies evaluating the efficacy of both enzymes with more and different levels of phytase units as well as their equivalency relative to P from DCP in birds fed the experimental diets from 1 to 21 days of age are still needed.

The objective of this study was to determine the efficacy as well as the equivalency values relative to P from dicalcium phosphate of a new *Citrobacter braakii*-derived phytase (expressed in *A. oryzae*) and a genetically-modified *Escherichia coli* phytase (expressed in *Trichoderma reseii*) to partially reduce the addition of dicalcium phosphate in the diets of broiler chickens from 1 to 21 days of age.

2. Materials and methods

The experiment was conducted at the Experimental Poultry Farm of the Animal Science Department, Viçosa, Brazil. Animal care procedures throughout the study followed protocols approved by the Institutional Animal Care and Use Committee (IACUC) guidelines at the UFV number 52/2013.

2.1. Birds and experimental design

Two thousand and one hundred 1-d-old male Cobb 500 broiler chickens with an initial weight of 40 G were used in a 21-d experiment to investigate the effectiveness of a *Citrobacter braakii*- derived phytase (CBP) expressed in *A. oryzae*, which is available as a commercial product under the name of Ronozyme® HiPhos (Ronozyme HiPhos GT, Novozymes A/S, Bagavaerd, Denmark), and a modified *Escherichia coli*-derived phytase (ECP) expressed in *Trichoderma reseii*, which is available as a commercial product under the name of Quantum® Blue (AB Vista Feed Ingredients, Marlborough, Wilts, UK). Birds were randomly distributed into 10 treatments with 10 pens of 21 chicks each. Each pen containing 21 chicks was considered as an experimental unit and consisted of one box with a concrete floor, with dimensions of 1.25 × 1.80 m and a total of 2.25 m^2. The animals were handled in masonry sheds that were 3 m high with cement asbestos shingles, low walls of 50 cm, and a half inch screen (1"/2) that was adapted for animal experimentation. Poultry litter consisting of new sawdust was utilized. Birds were offered feed and water *ad libitum*.

2.2. Diets and treatments

A P-low corn- and soybean-based basal diet (Treatment 1) with 1.8 g per kg of non-phytate phosphorus (nPP) and 9.0 g per kg of total calcium (Ca) was formulated, while containing all of the other necessary nutrients to meet the birds' requirements according to Rostagno et al. (2011) as listed in Table 1.

Table 1
Ingredients and nutrient composition of the diets (g/kg diet as-fed basis).

Ingredients	Basal diet	Dicalcium phosphate-added diets		
Ground corn	565.6	565.6	565.6	565.6
Soybean meal, 46%	324.1	324.1	324.1	324.1
Corn gluten meal, 60%	40.0	40.0	40.0	40.0
Soybean oil	26.3	26.3	26.3	26.3
Common salt	4.9	4.9	4.9	4.9
DL-Methionine, 99%	2.8	2.8	2.8	2.8
L-Lysine HCl, 79%	3.3	3.3	3.3	3.3
L-Threonine, 98%	0.5	0.5	0.5	0.5
L- Valine, 96.5%	0.3	0.3	0.3	0.3
Mineral supplement[a]	1.1	1.1	1.1	1.1
Vitamin supplement[b]	1.1	1.1	1.1	1.1
Salinomycin (12%–66 ppm)	0.6	0.6	0.6	0.6
Choline choride 60%	1.0	1.0	1.0	1.0
Antioxidant (BHT)[c]	0.1	0.1	0.1	0.1
Dicalcium phosphate	3.9	8.9	13.7	18.5
Limestone	16.9	14.7	13.6	9.3
Sand[d]	7.7	4.9	1.2	0.6
Calculated nutrient composition (g/kg diet as-fed basis)[e]				
Crude protein (g/kg)	219.5	219.5	219.5	219.5
Apparent metabolizable energy (AMEn, MJ/kg)	12.8	12.8	12.8	12.8
Calcium (g/kg)	9.0	9.0	9.0	9.0
Non-phytate phosphorus (g/kg)	1.8	2.7	3.6	4.5
Na (g/kg)	2.09	2.09	2.09	2.09
Digestible lysine (g/kg)	12.19	12.19	12.19	12.19
Digestible methionine (g/kg)	5.78	5.78	5.78	5.78
Digestible methionine + cysteine (g/kg)	8.67	8.67	8.67	8.67
Digestible threonine (g/kg)	7.85	7.85	7.85	7.85
Digestible tryptophan (g/kg)	2.27	2.27	2.27	2.27
Calculated total phosphorus (g/kg)	4.14	5.06	5.95	6.84
Analyzed nutrient composition				
Crude protein (g/kg)	218.8	218.4	218.1	218.6
Calcium (g/kg)	9.21	9.12	8.93	9.32
Total phosphorus (g/kg)	4.07	5.00	5.88	6.77

[a] Mineral supplement (supply per kg diet): broilers in pre-starter phase: manganese ($MnSO_4 \cdot H_2O$), 88 mg; iron ($FeSO_4 \cdot H_2O$), 62.5 mg; zinc (ZnO), 81.3 mg; copper ($CuSO_4 \cdot 5H_2O$), 12.5 mg; iodine (KI), 1.25 mg; selenium (Na_2SeO_3), 0.375 mg; broilers in starter phase: manganese ($MnSO_4 \cdot H_2O$), 77 mg; iron ($FeSO_4 \cdot H_2O$), 55 mg; zinc (ZnO), 71.5 mg; copper ($CuSO_4 \cdot 5H_2O$), 11 mg; iodine (KI), 1.10 mg; selenium (Na_2SeO_3), 0.33 mg.

[b] Vitamin supplement (supply per kg diet): broilers: Pre-starter phase: Vitamin supplement (supply per kg diet): vitamin A (trans—retinyl acetate), 9375 IU; vitamin D_3 (cholecalciferol), 2375 IU; vitamin E (all – rac – tocoferol acetate), 35 IU; vitamin K (bisulfate menadione complex), 1.88 mg; vitamin B_1. 2.50 mg; vitamin B_2, 6.25 mg; vitamin B_6, 3.5 mg; pantothenic acid (D–calcium panthenate), 12.5 mg; biotin, 0.088 mg; nicotinic acid, 37.5 g; folic acid, 0.875 mg; vitamin B_{12} (cyanocobalamin), 0.015 mg; broilers in starter phase: Vitamin supplement (supply per kg diet): vitamin A (trans—retinyl acetate), 8250 IU; vitamin D_3 (cholecalciferol), 2090 IU; vitamin E (all – rac – tocoferol acetate), 31 IU; vitamin K (bisulfate menadione complex), 1.65 mg; vitamin B_1. 2.20 mg; vitamin B_2, 5.5 mg; vitamin B_6, 3.08 mg; pantothenic acid (D–calcuim panthenate), 11 mg; biotin, 0.077 mg; nicotinic acid, 33 mg; folic acid, 0.77 mg; vitamin B_{12} (cyanocobalamin), 0.013 mg.

[c] Butylated hydroxytoluene.

[d] Phytases replaced sand in the diets.

[e] Based on ingredients composition of the Brazilian Tables for Poultry and Swine (2011).

For treatments 2–4, the basal diet was supplemented with 0.9, 1.8, or 2.7 g per kg of inorganic phosphorus (iP) from DCP to create diets with 2.7, 3.6 or 4.5 g per kg of P, respectively. For treatments 5–7, the basal diet was supplemented with 500, 1000, or 2000 phytase units of CBP per kg. The basal diet was supplemented with 250, 500, or 1000 phytase units of ECP per kg for treatments 8–10. For CBP, Vieira et al. (2015) defined a unit of phytase (FYT) as the quantity of enzyme that liberates 1 μmol of iP min^{-1} from 5.0 mM sodium phytate at pH 5.5 and 37 °C. For ECP, Adedokun et al. (2004) reported that the unit of phytase (FTU) is defined as the quantity of enzyme that liberates 1 μmol of iP min^{-1} from 5.1 mM sodium phytate at pH 5.5 and 37 °C.

2.3. Measurements and slaughter

The evaluated performance parameters were feed intake (FI, g/bird), weight gain (WG, g/bird), and feed-to-gain ratio (F:G, g/g). At 21 days of age, 4 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 tibia was removed to evaluate ash (g/kg) and P (g/kg) contents.

2.4. Chemical analysis

Tibia samples were collected to determine ash and P content. Feed samples were collected to perform analyses of Nitrogen (N), Ca, P, and the enzyme activity of phytases. Tibia and feed samples were dried at 55 °C in a forced-draft oven for 3 days. Tibia and feed samples were ground through a 1-mm screen. Samples were then used to determine DM content by oven drying at 105 °C for 24 h. Ash content of the samples was determined according to the method 942.05 (AOAC, 2000). Nitrogen

content of the diets was determined by the combustion method 990.03 (AOAC, 2000; model FP2000, Leco Corp., St. Joseph, MI). Calcium and P were determined after wet-ash digestion with nitric and perchloric acids according to the method 935.13 (AOAC, 2000). Calcium in wet-ashed samples was determined by the atomic absorption spectrophotometric method 968.08 (AOAC, 2000) using an atomic absorption spectrometer (AAnalyst 300, Perkin Elmer, Norwalk, CT). Phosphorus concentration was determined using a colorimetric assay (Fiske and Subbarow, 1925). Acid molybdate and Fiske's SubbaRow reducer solution were added to wet-ash samples to perform a phosphor-molybdenum complex. Color intensity was proportional to P concentration and was determined with a spectrophotometer using absorbance at 620 nm (SpectraCount, Model # AS1000, Packard, Meridian, CT). The analyses were performed at the Animal Nutrition Laboratory of the Department of Animal Science, Federal University of Viçosa.

The phytase activities were determined in a private Lab using the method ISO 30024: 2009 (Gizzi et al., 2008) and the method described by Basu et al. (2007).

The method ISO 30024: 2009 has been published (Gizzi et al., 2008) and recognized as a harmonized phytase method by International Standards Organization (Bedford and Partridge, 2010). This method (ISO 30024: 2009) is considered to be a derived version of the one described by Engelen et al. (2001), as the assay determines phytase activity under very similar *in vitro* conditions: it is at the same pH and temperature, uses essentially the same reagents, buffers, substrate preparation, the same detection mechanism, etc. Although there are other procedural differences (the time of the assay is reduced from 60 to 30 min and the new assay is standardized with a phosphate standard curve instead of an enzyme standard curve, for example), one of the main differences compared with the Engelen method is in the extraction procedure (Sheehan, 2010).

The *E. coli* phytase product, Quantum™ Phytase, has shown problems with detection by these methods (Sheehan, 2010). Generally, phytase is easily recovered from mash diets by the Engelen or ISO methods but after pelleting, problems have arisen. A specialized extraction technique at pH 10.0 is therefore required in order to fully solubilize the enzyme into the extraction buffer (Basu et al., 2007). As new phytases come to market, further extraction and assay modify cations may be necessary, so that a truly universal method may not be possible (Sheehan, 2010).

2.5. Statistical analysis

All data were analyzed with one-way ANOVA using the GLM procedure of SAS statistical package (SAS Institute, Inc., 2010).

The statistical model is described as follows:

$$y_{ij} = \mu + \tau_i + \varepsilon_{ij}; \mathrm{i} = 1, \ldots, \mathrm{a}; \mathrm{j} = 1, \ldots, \mathrm{n};$$

where:

y_{ij} = observation j in group or treatment i.

μ = the overall mean.

τ_i = the fixed effect of group or treatment *i* (denotes an unknown parameter).

ε_{ij} = random error with mean 0 and variance σ^2.

Based on the methodology described by Adedokun et al. (2004), orthogonal polynomial contrasts were used to evaluate the performance and tibia characteristics when supplemental levels of iP from DCP (0.9, 1.8, or 2.7 g per kg), phytase units of CBP (500, 1000, or 2000 FYT per kg), or phytase units of ECP (250, 500, or 1000 FTU per kg) were added to the basal diet, which was used as level 0.0 for running orthogonal polynomial contrasts for DCP or CBP or ECP.

The linear response functions that best fit the data were derived from the DCP, CBP, or ECP levels and are listed as follows:

For linear regression equations:

$$Y_s = a_s + b_s \mathrm{x_s} (\text{Linear regression equation for g per kg of iP from DCP}) (1);$$

$$Y_c = a_c + b_c \mathrm{x_c} (\text{Linear regression equation for FYT of CBP per kg}) (2);$$

$$Y_e = a_e + b_e \mathrm{x_e} (\text{Linear regression equation for FTU of ECP per kg}) (3);$$

$$\text{Eq.}(1) = \text{Eq.}(2) : a_s + b_s \mathrm{x_s} = a_c + b_c \mathrm{x_c};$$

Or

$$\text{Eq.}(1) = \text{Eq.}(3) : a_s + b_s \mathrm{x_s} = a_e + b_e \mathrm{x_e}.$$

Where *Y is* the response criterion (performance or bone traits); x_s is the supplemented iP from DCP (g kg^{-1} diet), x_c is the supplemented FYT per kg from CBP, and x_e is the supplemented FTU per kg from ECP; a_s is the intercept of the linear regression for supplemented iP from DCP (g kg^{-1} diet), a_c is the intercept of the linear regression for supplemented FYT per kg from CBP, and a_e is the intercept of the linear regression for supplemented FTU per kg from ECP; b_s is the slope of the response criterion to the supplemented iP from DCP, b_c is the slope of the response criterion to the supplemented FYT per kg from CBP, and b_e is the slope of the response criterion to the supplemented FTU per kg from ECP. The linear response

Table 2
Performance and tibia characteristics of broilers fed diets with inorganic phosphorus levels (g/kg) from dicalcium phosphate or graded levels of FYT/kg of *Citrobacter braakii* or graded levels of FTU/kg of *Escherichia coli* from 1 to 21 days of age[a].

Item	Basal diet	Supplemental iP from DCP, g/kg			Supplemental phytase units from CBP, FYT/kg			Supplemental phytase units from ECP, FTU/kg			SEM[b]
		0.9	1.8	2.7	500	1000	2000	250	500	1000	
Feed Intake, g/bird[c,e,g,h,i,k,l,m,n]	659	999	1129	1184	1003	1019	1090	989	1060	1120	15.13
Weight gain, g/bird[c,e,g,h,i,k,l,m,n]	431	610	756	786	593	672	714	642	689	758	13.62
F:G, g/g[d,f,j]	1.537	1.647	1.498	1.510	1.691	1.522	1.530	1.546	1.545	1.480	0.028
Tibia ash, g/kg[c,g,h,k,l]	305	500	780	869	464	570	710	548	618	806	26.33
Tibia P, g/kg[c,g,h,k,l]	18.0	37.8	75.9	85.8	35.2	49.1	66.9	40.9	53.6	77.3	3.35
Mortality (%)	7.6	3.3	0.5	0.5	3.8	0.0	0.0	2.4	0.0	0.0	1.17

[a] P = phosphorus; iP = Inorganic P; ECP = *Escherichia coli*-derived phytase; FTU = phytase units from ECP; CBP = *Citrobacter braakii*-derived phytase; FYT = phytase units from CBP; basal diet had no iP or supplemental phytase; the basal diet was used as level 0.0 to run orthogonal polynomial contrasts for DCP or CBP or ECP; F:G = feed-to-gain ratio.
[b] SEM = Standard pooled error of the means; (sample size = 100).
[c] Linear Effect of DCP ($P < 0.01$).
[d] Linear Effect of DCP ($P < 0.05$).
[e] Quadratic Effect of DCP ($P < 0.01$).
[f] Quadratic Effect of DCP ($P < 0.05$).
[g] Linear Effect of CBP ($P < 0.01$).
[h] Linear Effect of CBP ($P < 0.05$).
[i] Quadratic Effect of CBP ($P < 0.01$).
[j] Quadratic Effect of CBP ($P < 0.05$).
[k] Linear Effect of ECP ($P < 0.01$).
[l] Linear Effect of ECP ($P < 0.05$).
[m] Quadratic Effect of ECP ($P < 0.01$).
[n] Quadratic Effect of ECP ($P < 0.05$).

equations for supplemented iP from DCP and those for supplemented FYT per kg from CBP or FTU per kg from ECP were set to be equal, and were solved for P equivalency values for their respective variable.

3. Results

In the current study, the phytase-supplemented diets were formulated to contain 500, 1000, or 2000 FYT/kg of CBP or 250, 500, or 1000 FTU per kg of ECP. The analyses tests were determined to be 545, 1380, and 4210 FYT/kg in CBP-supplemented diets and 377, 559, and 1360 FTU/kg in ECP-supplemented diets. These results were higher than expected, particularly for CBP-supplemented diets, but they did not compromise the results since the increase in phytase units levels were kept.

The supplemental P levels from DCP resulted in linear and quadratic effects on broiler WG, FI, and F:G. Tibia ash and tibia P were only linearly affected by DCP levels ($P < 0.05$, Table 2), but tibia ash showed a tendency for quadratic effect. Increasing P levels from DCP of 0.9, 1.8, and 2.7 g/kg improved ($P < 0.05$) FI by 52, 71, and 80%; WG by 41, 75, and 32%; tibia ash weight by 64, 156, and 185%; and tibia P by 110, 323, and 378%, respectively. The increasing FYT levels of CBP per kg resulted in linear and quadratic effects on WG and FI, but only a quadratic effect on F:G and a linear effect on tibia ash and tibia P ($P < 0.05$). The CBP supplementation of 500, 1000, and 2000 FYT/kg contributed to an increase ($P < 0.05$) of 52, 55, and 65% for FI; 37, 56, and 65% for WG; 52, 87, and 133% for tibia ash; and 96, 173, and 273% for tibia P, respectively in relation to the basal diet. The increasing FTU levels of ECP per kg resulted in linear and quadratic effects on WG and FI, but only a linear effect on tibia ash and tibia P ($P < 0.05$). The ECP addition of 250, 500, and 1000 FTU/kg increased ($P < 0.05$) FI by 50, 61, and 70%; WG by 49, 60, and 76%; tibia ash weight by 80, 103, and 164%; and tibia P by 128, 198, and 330%.

In this study, linear regression equations (Table 3) were used to determine P equivalency values of CBP and ECP, when DCP is used as an inorganic P source in broiler diets based on the methodology described by past studies (Adedokun et al., 2004; Adeola, 2010). Also, equivalency P equations were used to determine P equivalency values of CBP and ECP, when DCP is used as an inorganic P source in broiler diets.

Phosphorus equivalency values for phytase units of *Citrobacter braakii* per kg (Table 4) and for phytase units of *Escherichia coli* per kg were calculated by using equivalency equations for WG, FI, tibia ash, and tibia P results, except for F:G, which showed a low r^2 and regression slopes that were not significant ($P = 0.081$, for regression slope of DCP; $P = 0.236$, for regression slope of CBP; and $P = 0.086$, for regression slope of ECP). The levels of 500, 1000, and 2000 FYT/kg of CBP were determined to be equivalent to the average addition of 0.625, 1.091, and 2.024 g of iP from DCP per kg in broiler diets, respectively. Meanwhile, 250, 500, and 1000 FTU/kg of ECP were determined to be equivalent to the average addition of 0.763, 1.307, and 2.395 g of iP from DCP per kg in broiler diets, respectively.

4. Discussion

In this study, DCP was chosen as an iP source. Past studies have used monosodium phosphate as a P source (Adedokun et al., 2004; Jendza et al., 2006; Adeola, 2010) or DCP (Vieira et al., 2015). Cowieson et al. (2014) reported no difference in broiler performance when evaluating three sources of P (phosphates of monocalcium, dicalcium, and tricalcium with

Table 3
Linear regression equations estimated to each variable on response of inorganic phosphorus levels (g/kg) from dicalcium phosphate or graded levels of FYT per kg from *Citrobacter braakii*-derived phytase or graded levels of FTU per kg from *Escherichia coli*-derived phytase[a].

Item	P source	Regression equation[b]	Standard error of the intercept	Intercept Probability	Standard error of the Slope	Slope Probability	r^2 of equation[c]
Feed	DCP	$\hat{Y} = 737.50 + 189.31X$	21.788	<0.01	12.940	<0.01	0.849
intake,	CBP	$\hat{Y} = 782.48 + 0.183X$	27.808	<0.01	0.024	<0.01	0.599
g/bird	ECP	$\hat{Y} = 779.53 + 0.41X$	26.124	<0.01	0.045	<0.01	0.676
Weight	DCP	$\hat{Y} = 464.20 + 134.36X$	15.201	<0.01	9.028	<0.01	0.854
gain,	CBP	$\hat{Y} = 487.61 + 0.131X$	15.041	<0.01	0.013	<0.01	0.725
g/bird	ECP	$\hat{Y} = 501.66 + 0.29X$	17.323	<0.01	0.030	<0.01	0.712
Tibia	DCP	$\hat{Y} = 317.64 + 219.16X$	24.156	<0.01	14.347	<0.01	0.859
ash,	CBP	$\hat{Y} = 341.0 + 0.196X$	18.544	<0.01	0.016	<0.01	0.794
g/kg	ECP	$\hat{Y} = 364.80 + 0.47X$	22.389	<0.01	0.039	<0.01	0.790
Tibia	DCP	$\hat{Y} = 18.13 + 26.86X$	3.233	<0.01	1.920	<0.01	0.837
P,	CBP	$\hat{Y} = 21.29 + 0.024X$	2.290	<0.01	0.002	<0.01	0.791
g/kg	ECP	$\hat{Y} = 22.42 + 0.06X$	2.360	<0.01	0.004	<0.01	0.836

[a] P = phosphorus; DCP = dicalcium phosphate; iP = inorganic P; CBP = *Citrobacter braakii*-derived phytase; FYT = phytase units from CBP; ECP = *Escherichia coli*-derived phytase; FTU = phytase units from ECP.
[b] Linear regression equations were estimated to each variable using individual observations instead the means.
[c] Coefficient of determination.

Table 4
Equivalency equations and phosphorus (P) equivalency values (g/kg) of *Citrobacter braakii*-derived phytase and *Escherichia coli*-derived phytase[a].

Phytase	Item[c]	Equivalency equations[b]	Equivalency P values[d], g/kg		
			500	1000	2000
CBP	Feed intake, g/bird	737.50 + 189.31(iP, g/kg) = 782.48 + 0.183(FYT/kg)	0.720	1.204	2.171
	Weight gain, g/bird	464.20 + 134.36(iP, g/kg) = 487.61 + 0.131(FYT/kg)	0.662	1.149	2.124
	Tibia ash, g/kg	317.64 + 219.16(iP, g/kg) = 341.0 + 0.196(FYT/kg)	0.554	1.001	1.895
	Tibia P, g/kg	18.13 + 26.86(iP, g/kg) = 21.29 + 0.024(FYT/kg)	0.564	1.011	1.905
	Mean		0.625	1.091	2.024
			250	500	1000
ECP	Feed intake, g/bird	737.50 + 189.31(iP, g/kg) = 779.53 + 0.41(FTU/kg)	0.763	1.305	2.388
	Weight gain, g/bird	464.20 + 134.36(iP, g/kg) = 501.66 + 0.29(FTU/kg)	0.818	1.358	2.437
	Tibia ash, g/kg	317.64 + 219.16(iP, g/kg) = 364.80 + 0.47(FTU/kg)	0.751	1.287	2.359
	Tibia P, g/kg	18.13 + 26.86(iP, g/kg) = 22.42 + 0.06(FTU/kg)	0.718	1.277	2.394
	Mean		0.763	1.307	2.395

[a] P = phosphorus; iP = Inorganic P; DCP = dicalcium phosphate; CBP = *Citrobacter braakii*-derived phytase; FYT = phytase units from CBP; ECP = *Escherichia coli*-derived phytase; FTU = phytase units from ECP.
[b] Equivalency equations were calculated for each variable when linear regression of DCP and linear regression of CBP are equal or linear regression of DCP and linear regression of ECP are equal.
[c] All variables were used to estimate P equivalency, excepting to feed-to-gain ratio that showed low r^2. There was not significance for regression slope of DCP ($P = 0.081$), regression slope for ECP ($P = 0.086$), and regression slope of CBP ($P = 0.236$).
[d] Equivalency P values (g per kg) relative to iP from DCP.

potassium phosphate of inorganic phosphorus) in the diets, but tibia ash (%) was higher in birds that were fed MCP than those that were fed either DCP or TCP. However, DCP was used because it is currently added to broiler diets in Brazil.

The supplemental iP levels from DCP resulted in linear and quadratic effects on performance and bone parameters of broiler chickens. These results are in accordance with those observed in several studies (Adedokun et al., 2004; Jendza et al., 2006; Adeola, 2010; Vieira et al., 2015), which reported linear or quadratic influences on performance and bone mineralization of broiler chickens that were fed diets with supplemental iP from different inorganic sources.

In the current study, the results showed that CBP and ECP increase the availability of phosphorus in P-low corn- and soybean-based basal diets. Similar results were reported by past studies (Adeola, 2010; Shaw et al., 2011; Cowieson et al., 2014; Olukosi and Fru-Nji, 2014). Phytase can partly replace dicalcium phosphate as a phosphorus source, according to the FTU or FYT levels in the diet (Nelson et al., 1968). Simons et al. (1990) reported that more than 65% of the P was released by the addition of phytase in broiler diets. Denbow et al. (1995) showed that P released by Phytase ranged from 31 to 58% for 250 to 1000 U phytase per kg of feed. Yi et al. (1996) calculated that up to 37% of the phytate P in soybean meal was released by the addition of 1000 U of phytase/kg of diet. Waldroup et al. (2000) indicated that approximately 50% of the phytate-bound P in a corn–SBM diet was released by phytase.

The supplementation of 500, 1000, and 2000 FYT/kg of CBP showed average improvements of 60.5, 92.75, and 134% compared to the basal diet. Meanwhile, the supplementation of 250, 500, and 1000 FTU/kg of ECP showed average improvements of 76.75, 105.5, and 160% compared to the basal diet. Several studies have reported improvements on performance associated with the use of Phytase in broiler diets. Jendza et al. (2006) reported an 87% increase in the WG of birds at 42 days old when they were supplemented with 500 FTU/kg phytase from *E. coli*. Onyango et al. (2005a) observed an increase of 17 and 20% in the WG of broiler chickens at 8 and 22 days old, respectively, when they were fed a P-low diet supplemented with 500 and 1000 FTU/kg phytase of a second generation *E. coli* phytase. Dilger et al. (2004) reported an increase of 6 and

19%, respectively, in WG of broiler chickens from 8 to 22 days old when they were supplemented with 500 and 1000 FTU/kg phytase derived from *E. coli*.

The linear regression equations were used to determine P equivalency values of CBP and ECP, when DCP is used as an inorganic P source in broiler diets according to the methodology described by past studies (Adedokun et al., 2004; Adeola, 2010). Those authors verified both linear and quadratic responses to graded levels of iP and units of phytase, but they used linear equations rather than quadratic equations to determine P equivalency values. Additionally, the linear regression equations correctly determine P equivalency values and represent a simple method compared to quadratic regression equations or other polynomial degrees.

Past studies have considered higher r^2 values as a reference for choosing performance or bone mineralization criterion to estimate P equivalency values (Adedokun et al., 2004; Adeola, 2010; Vieira et al., 2015). Kornegay and Qian (1996) relayed that the response criterion that are chosen in data analyses can influence the results that are obtained in the trial. However, several authors have developed regressions by using treatment means rather than individual observations, which may have resulted in elevated r^2 values (Jendza et al., 2006). Therefore, in this study, the regression equations were developed by using individual observations as recommended by Jendza et al. (2006). Aiming to estimate the P equivalency values, we chose the response criterion that provided best r^2 values considering regression equations developed by using individual observations instead the average values. Thus, the r^2 values should be reported as use of averages or individual replicates on ongoing studies.

In the current study, mean P equivalency values of CBP were lower than those observed by Vieira et al. (2015), who reported that 500 and 1000 phytase units provided average estimations of 1.00 and 1.66 nPP per kg, respectively. The authors used a basal diet containing 1.4 g per kg of non-phytate phosphorus (nPP) and 8.0 g per kg of total calcium (Ca), while we used a basal diet with 1.8 g per kg of non-phytate phosphorus (nPP) and 9.0 g per kg of total calcium (Ca). Probably, the high Ca level used in the current study contributed to the divergent estimates. One of the possible explanations for 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). Applegate et al. (2003) verified that a Ca level up to 9.0 g/kg can reduce the intestinal activity of a phytase by 9% and phytate hydrolysis by 12%. Also, studies evaluating phytase efficacy currently vary with regards to the P levels in the broiler diets while keeping Ca levels fixed, which influences the Ca:tP ratio in the experimental diets. Lei and Stahl (2000) reported that a high Ca:tP ratio can reduce phytate solubility, thereby increasing its resistance to hydrolysis.

For supplemental phytase units of ECP per kg, this study estimated P equivalency values higher than those reported by past studies. Adedokun et al. (2004) reported that 500 and 1000 FTU/kg provide average estimations of 0.486 and 1.031 g of iP per kg, respectively. Jendza et al. (2006) observed that 500 FTU/kg of ECP was determined to be equivalent to the addition of 0.49 or 1.00 g of iP per kg when using WG and bone ash, respectively. The past studies used to provide experimental diets at day 8 (Dilger et al., 2004; Adedokun et al., 2004; Onyango et al., 2005a; Jendza et al., 2006). Meanwhile, we provided experimental diets at day 1. This suggests that birds fed a P-low diet from 1-d-old demonstrate higher effectiveness and P equivalency values of phytase units of ECP per kg. Birds fed a diet with adequate P levels before receiving experimental diets can reduce their response to phytase supplementation (Onyango et al., 2005a,b) due to the P stores that are accumulated during the first 7 d, which may decrease variability by modifying the response to lower levels of P in the diet (Jendza et al., 2006).

Overall, the results of this study demonstrated that it is possible to reduce the amount of DCP added to the feed when *Escherichia coli*- and *Citrobacter braakii*-derived Phytase are supplemented to the broiler diets. The mean P equivalency values of CBP ranged between 0.625 and 2.024 g/kg of iP from DCP according to the FYT level that is supplemented. This suggests the possibility of reducing the added DCP between 3.378 and 10.940 kg/ton in corn-soybean meal based diets for broiler chickens from 1 to 21 days of age. Also, the mean P equivalency values of ECP ranged between 0.763 and 2.395 g/kg of iP from DCP according to the FTU level that is supplemented. It also suggests the possibility of reducing the added DCP between 4.124 and 12.946 kg/ton in corn-soybean meal based diets for broiler chickens from 1 to 21 days of age. However, this study used a high Ca concentration in the basal diet (9.0 g/kg), which could affect the phytase activity and underestimating the results. Maybe, the possibility of reducing the addition of DCP could be higher using diets with lower Ca concentration.

Thus, the dietary supplementation of these phytases may reduce the use of inorganic phosphorus sources in broiler diets.

5. Conclusions

The *Citrobacter braakii*- and *Escherichia coli*-derived phytases can be used in the diets of broiler chickens from 1 to 21 days of age to partially reduce the addition of dicalcium phosphate as a phosphorus source.

The supplementation of 500, 1000, and 2000 phytase units of *Citrobacter braakii* per kg were determined to be equivalent to the average addition of 0.625, 1.091, and 2.024 g of inorganic phosphorus from dicalcium phosphate per kg in broiler diets, respectively.

The supplementation of 250, 500, and 1000 phytase units of *Escherichia coli* per kg were determined to be equivalent to the average addition of 0.763, 1.307, and 2.395 g of inorganic phosphorus from dicalcium phosphate per kg in broiler diets, respectively.

Conflict of interest statement

The authors declare that there are no conflic of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.anifeedsci.2016.09.008.

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