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Supplemental phytase derived from *E. coli* in different concentrations on performance, bone mineralization and cost of broilers diets

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Abstract: The trial was conducted to evaluate the supplementation of *E. coli* phytase on performance, weight and ash of bones, as well as to determine the bioavailability of P and cost/benefit of its use in diets. A total 1,890 Cobb male day old chicks were assigned to six treatments and seven replicates with 45 birds each, distributed in a completely randomized design. The treatments were: Positive Control; Negative Control (NC1) - reduction of 0.06% $_{av}$ P; Negative Control 2 (NC2) - reduction of 0.12% $_{av}$ P; NC2 + Phytase (120 OTU); NC2 + Phytase (180 OTU); NC2 + Phytase (240 OTU), being 1 OTU equivalent to approximately 2 FTU. With different phytase inclusions, it was possible to verify a gradual increase on body weight gain, feed intake, feed conversion ratio, viability and even the bone characteristics of broilers fed diets containing reduction of P. The closest levels to the highest studied (240 OTU) showed the best results. The replacement of dicalcium phosphate by phytase supplementation is economically viable when the cost per OTU does not exceed US\$ 1.4×10^{-5} , US\$ 1.2×10^{-5} and US\$ 1.0×10^{-5} for the concentrations of 120, 180 and 240 OTU, respectively.

Key words: additives, enzyme, poultry, phytate, bone, litter.

INTRODUCTION

With the shortage of natural sources of inorganic phosphorus and consequently the increase in the price of dicalcium phosphate, the use of phytase in broiler diets becomes a relevant measure for providing the phosphorus of plants to animals. Phytase acts on the phosphate group links the phytic acid molecule, releasing phosphorus and other minerals such as calcium, copper, iron and zinc, as well as energy and amino acids (Silva et al. 2006, Cowieson et al. 2017).

Diets based on corn and soybean meal present small amount of phytate in comparison to other ingredients of vegetable origin (Li et al. 2017). Thus, it is neither necessary nor economically feasible to include high amounts

of phytase to these diets. In addition to greater economic viability, smaller phytase concentrations have some advantages, such as improved retention of non-mineral nutrients as amino acids and energy, whereas high doses have a greater effect on phosphorus retention, but not on these nutrients (Cowieson et al. 2006, Karadas et al. 2010, Walk et al. 2012, Vieira et al. 2015).

Recent studies have found high inclusions of phytase on broilers diets for maximum response (Cowieson et al. 2006, 2017), however the production of concentrated enzyme is difficult to obtain and the inclusion of large amounts becomes economically unviable. In this sense, it is necessary to evaluate the behaviour of gradual phytase inclusion levels up to 240 OTU

(approximately 500 FTU), with the purpose to achieve greater economic gains in production, especially in diets based on corn and soybean meal.

This study aimed to evaluate gradual inclusion levels of *E. coli* phytase (*Pichia pastoris* genus) on performance, weight and bone ash, as well as to determine the bioavailability of P and cost/benefit of its usage in diets P deficient based on corn and soybean meal for broiler chickens.

MATERIALS AND METHODS

Birds and Housing

All animal procedures were approved and conducted under the guidelines of Universidade Estadual Júlio de Mesquita Filho - UNESP, Jaboticabal, São Paulo, Brazil (013022/14).

The trial was conducted at the Laboratory of Poultry Sciences of Department of Animal Science, Faculty of Agriculture and Veterinary Sciences - UNESP, Jaboticabal, Brazil. The broiler chickens were housed in an experimental facility, with tunnel type system to control the environment. The control of temperature, humidity and air exchange were performed automatically by exhaust fans and climate control system, according to the age of the broilers (Cobb Guidelines 2012). During the initial phase, incandescent lamps were used as heating source according to the needs of poultry. Shaving litter was used in all experimental pens, as well as nipple drinkers and tubular feeders. Infant tubular feeders were used until 14 days of age, then substituted by adult tubular feeders.

Water and feed were offered ad libitum during the whole trial period. The lighting program was set at 24 hours of light. All broilers were vaccinated with the challenge of the region: at the hatchery against Marek and Gumboro diseases, at 12 days of age the broilers were

vaccinated against New Castle, administered via water.

Experimental design

A total of 1,890 day-old male broiler chicks (Cobb 500) were weighed (± 45 g each) and distributed in each treatment with similar body weight means. The broilers were distributed in a completely randomized design with six treatments and seven replicates, allocated to 42 pens (1.0 x 3.0 m) of 45 chicks each.

The experimental treatments were: Positive Control (PC) - diet meeting the nutritional requirements of the birds; Negative Control (NC1) - reduction of 0.06% $_{av}$ P; Negative Control 2 (NC2) - reduction of 0.12% $_{av}$ P; NC2 + Phytase (120 OTU); NC2 + Phytase (180 OTU); NC2 + Phytase (240 OTU).

Experimental diets

Diets were formulated to meet the nutritional requirements of broilers based on the recommendations of the Brazilian Tables for Poultry and Swine (Rostagno et al., 2011), except to $_{av}$ P. The Negative Control diets had reduction of $_{av}$ P in comparison to PC. Table I describes the control diets and their nutritional compositions for initial (1-21 days) and grower (22-35 days) phases.

¹7.000.000.000 U.I. vit A; 2.200.000.000 U.I. vit D3; 11.000.000 mg vit E; 1.600.000 mg vit K3; 2.000.000 mg vit B1; 5.000.000 mg vit B2; 3.000.000 mg vit B6; 12.000.000 mcg vit B12; 35.000.000 mg nicotinic acid; 13.000.000 mg pantothenic acid; 800.000 mg folic acid; 100.000.000 mg Antioxidant; 1.000.000 mg. Vehicle Q.S.P. Addition 1.0 kg/t.²8.000.000 mg Cu; 50.000.000 mg Fe; 70.000.000 mg Mn; 50.000.00 mg Zn; 1.200.000 mg I; 200.000 mg Se; 1.000.000 g Vehicle Q.S.P. Addition 1.0 kg/t.³ Phytase replaced by inert ingrediente on NC2 treatments to obtain the diets with 120, 180 and 240 OTU/Kg, being each

Table I. Positive Control (PC), Negative Control 1 (NC1) and Negative Control 2 (NC2) experimental diets for the phases from 1 to 21 and 22 to 35 days of age of the broilers.

Ingredients (%)	1 - 21 days			22 - 35 days		
	PC	NC1	NC2	PC	NC1	NC2
Corn	55.253	55.505	55.757	61.458	61.710	61.962
Soybean meal (45%)	37.142	37.096	37.051	30.904	30.859	30.813
Soy oil	3.669	3.583	3.498	4.064	3.979	3.893
Limestone	1.379	1.333	1.559	1.132	1.358	1.585
Dicalcium phosphate	1.106	1.030	0.682	1.131	0.783	0.434
Salt	0.508	0.508	0.507	0.475	0.475	0.475
DL - Methionine	0.287	0.287	0.287	0.226	0.226	0.226
L - Lysine HCl	0.243	0.244	0.245	0.215	0.216	0.217
L - Threonine	0.074	0.074	0.074	0.055	0.055	0.055
Vitamin premix ¹	0.100	0.100	0.100	0.090	0.090	0.090
Mineral premix ²	0.100	0.100	0.100	0.100	0.100	0.100
Cocciostat	0.050	0.050	0.050	0.050	0.050	0.050
Choline Chloride 60%	0.070	0.070	0.070	0.070	0.070	0.070
Inert ³	0.020	0.020	0.020	0.030	0.030	0.030
TOTAL	100.00	100.00	100.00	100.00	100.00	100.00
Nutritional levels						
Metabolizable Energy (kcal/kg)	3050	3050	3050	3150	3150	3150
Crude protein (%)	22.5	22.5	22.5	20.00	20.00	20.00
Lysine (%)	1.253	1.253	1.253	1.085	1.085	1.085
Methionine+Cystine (%)	0.879	0.879	0.879	0.770	0.770	0.770
Digestible methionine (%)	0.588	0.588	0.588	0.501	0.501	0.501
Digestible threonine (%)	0.805	0.805	0.805	0.705	0.705	0.705
Crude Fibre (%)	2.965	2.967	2.969	2.735	2.737	2.739
Calcium (%)	0.900	0.900	0.900	0.830	0.830	0.830
Available phosphorus (%)	0.345	0.285	0.225	0.295	0.235	0.175
Total phosphorus (%)	0.601	0.541	0.481	0.534	0.474	0.415
Phytic phosphorus (%)	0.231	0.232	0.232	0.222	0.222	0.222
Potassium (%)	0.834	0.834	0.834	0.737	0.737	0.737
Chlorine (%)	0.349	0.349	0.349	0.329	0.329	0.329
Sodium (%)	0.220	0.220	0.220	0.207	0.207	0.207

¹7.000.000.000 U.I. vit A; 2.200.000.000 U.I. vit D3; 11.000.000 mg vit E; 1.600.000 mg vit K3; 2.000.000 mg vit B1; 5.000.000 mg vit B2; 3.000.000 mg vit B6; 12.000.000 mcg vit B12; 35.000.000 mg nicotinic acid; 13.000.000 mg pantothenic acid; 800.000 mg folic acid; 100.000.000 mg Antioxidant; 1.000.000 mg. Vehicle Q.S.P. Addition 1.0 kg/t.

²8.000.000 mg Cu; 50.000.000 mg Fe; 70.000.000 mg Mn; 50.000.00 mg Zn; 1.200.000 mg I; 200.000 mg Se; 1.000.000 g Vehicle Q.S.P. Addition 1.0 kg/t.

³Phytase replaced by inert ingredients on NC2 treatments to obtain the diets with 120, 180 and 240 OTU/Kg, being each OTU (Phytex method) is equivalent to 2 FTU (AOAC/ISO method).

OTU (Phytex method) is equivalent to 2 FTU (AOAC/ISO method).

The phytase included was produced by *E. coli* (expressed in *Kamatagaella/Pichia pastoris* genus), commercial name OptiPhos PF 4.000 OTU/g, produced by Huvepharma. OptiPhos phytase concentration is calculated by Phytex method (USA), and is expressed in OTU. One unit of phytase activity (OTU) is defined as the amount of enzyme that catalyses the release of 1.0 μM of inorganic phosphate per minute from 5.1 mM sodium phytate in pH 5.5 citrate buffer at 37°C, measured as the blue P-molybdate complex colour at 820 nm. In a simpler way, it is possible to consider that each OTU (Phytex method) is equivalent to 2 FTU (AOAC/ISO method).

Performance

At 21 and 35 days old, broilers and feed leftovers were weighed. Performance parameters evaluated were: body weight (BW), feed intake (FI), body weight gain (BWG), feed conversion ratio (FCR) and viability (VIA). Mortality was recorded daily and was used to adjust the total number of chicks to determine FI and FCR.

Analysis of tibias

At 35 days of age, five broilers of each experimental unit were slaughtered and had their tibiae removed, placed in identified plastic bags according to the treatment and replication and frozen. Once thawed, the tibias were stripped and dried in ventilated oven at 100°C for 24 hours. Fat excess was removed by immersing the tibias in ethyl ether, up to the total disappearance of fat. They were dried again in a ventilated oven at 100°C for 24 hours. After the tibias were individually weighed on precision scale, it was determined the defatted dry weight (DTW). Then, they were milled in a ball mill, a sample of about 2g was weighed and

forwarded to muffle furnace with temperature of 600°C for approximately 4h, until the sample became white, in order to determine ashes (Silva and Queiroz 2009).

Phosphorus Bioavailability

Phosphorus bioavailability was calculated through standard curve methodology. To establish the standard curve, the intake of supplementary phosphorus (ISP) of treatments PC, NC1 and NC2 were calculated, according to formula:

$$\text{ISP} = \frac{(\text{feed intake (g)} \times \text{supplementary dietary P (\%)})}{100}$$

Supplementary dietary P was considered null in the most deficient treatment (NC2); 0.06 for NC1; and 0.12% for PC; being these values the difference of avP between NC2 and the other treatments. The ISP was associated to the responses of performance (BWG) at 21 and 35 and bone characteristics [ash (ASH) and dried tibia weight (DTW)] variables to construct the standard curve.

The results of performance and bone characteristics variables of the treatments with phytase addition were confronted with their respective standard curve, in order to obtain the total phosphorus released (PR) in grams. The formula below demonstrates the calculation to obtain the nutritional matrix (bioavailability - BP) of avP of the phytase enzyme, being considered the feed intake (FI) of the broiler and the phosphorus released.

$$\text{BP (\%)} = \frac{\text{PR (g)}}{\text{FI (g)}} \times 100$$

Cost analysis

The cost analysis of the diets was performed to determine the economy of including phytases at different levels. For these calculations it were used data of phosphorus bioavailability of body weight gain and the dicalcium phosphate cost.

The calculation of the amount of inorganic dicalcium phosphate which could be replaced by phytase inclusions in the diet, based on the bioavailability found for each evaluated phytase inclusion. Thus, it was established the maximum cost (limit) for each phytase FTU of each enzyme that does not exceed the cost of inorganic phosphate defined at the evaluation period.

After determining a threshold cost by OUT for each phytase at different inclusions was generated a figure (graph) containing both phytases, being Y axis correspondent to the found phosphorus bioavailability and the X axis to the concentrations of phytases in the diet. For each inclusion of phytase, there is an associated bioavailability and consequently a limit cost of its utilization, expressed in dollars. The price of dicalcium phosphate was recorded on January 2015, with a value of US\$ 0.50/kg (MFRural 2015).

Statistical analysis

Data were analyzed by general linear models procedures of SAS 2002 (SAS Version 9.00). Regression analyses were applied on performance, bone and bioavailable of phosphorus data to determine the best utilization level of phytases, using NC as the

zero level of phytase inclusion, and as levels 120, 180 and 240 OTU for phytase, respectively. Linear (L), quadratic (Q) and quadratic-plateau (QP) models were used to analyse the different enzymes levels on the variables responses.

The QP model was adjusted according to Robbins et al. (2006), $Y_i = L + U (R - Phy_i)^2$, $i = 1, 2, \dots, n_1, n_1 + 1, \dots, n$; in which $(R - Phy_i)^2 = 0$ for $i \geq n_1 + 1$, n_1 is the number of observations up to the breaking point, n is the number of observation pairs, estimated by the equation. The Y_i is the response of the estimated variable to the phytase level in the diet; Phy_i is the level of phytase in the diet; L is asymptotic response of the function; U is slope at the breaking point; and R is the phytase level estimated by the breaking point.

RESULTS

Performance

Increasing levels of phytase were evaluated on FI, WG, FCR and VIA of broilers (Table II). To obtain the concentration of phytase that provides the best performance, the Linear (L), quadratic (Q) or quadratic with plateau (QP) models were

Table II. Feed intake (FI), body weight gain (BWG), feed conversion ratio (FCR) and viability (VIA) of broilers in the period from 1-21 and 1-35 days fed phosphorus deficient diet with increasing levels of phytase and regression analysis.

1 - 21 days				
Variables	NC2	NC2 + (120 OTU)	NC2 + (180 OTU)	NC2 + (240 OTU)
FI (g)	685 ± 27	786 ± 29	817 ± 40	832 ± 26
BWG (g)	504 ± 21	595 ± 27	611 ± 26	621 ± 20
FCR (g/g)	1.36 ± 0.03	1.32 ± 0.02	1.33 ± 0.02	1.34 ± 0.03
VIA ¹ (%)	96.51 ± 3.11	97.46 ± 2.00	99.37 ± 1.08	99.37 ± 1.08
Regression ²				
FI (g)	$FI = 835.2 (\pm 19.8) - 0.0019 (\pm 0.0009) \times [279.3 (\pm 77.6) - Phy]^2$ $R^2 = 0.80; p(<0.0001)$			

Table II. (continuation)

BWG (g)	$BWG = 619.8 (\pm 8.3) - 0.0022 (\pm 0.0008) \times [231.1 (\pm 44.1) - Phy]^2$ $R^2 = 0.82; p(<0.0001)$			
FCR (g/g)	-			
VIA (%)	$VIA = 96.40 (\pm 0.68) + 0.013 (\pm 0.004) Phy$ $R^2 = 0.27; p=0.0042$			
1 - 35 days				
Variables	NC2	NC2 + (120 OTU)	NC2 + (180 OTU)	NC2 + (240 OTU)
FI (g)	1867 ± 139	2235 ± 77	2378 ± 60	2388 ± 77
BWG (g)	1191 ± 67	1.485 ± 39	1570 ± 44	1594 ± 58
FCR (g/g)	1.57 ± 0.05	1.51 ± 0.03	1.51 ± 0.03	1.50 ± 0.02
VIA¹ (%)	73.65 ± 9.72	81.59 ± 5.25	83.22 ± 3.64	84.44 ± 4.00
Regression²				
FI (g)	$FI = 2423.5 (\pm 50.9) - 0.0076 (\pm 0.0026) \times [271.5 (\pm 53.2) - Phy]^2$ $R^2 = 0.86; p(<0.0001)$			
BWG (g)	$BWG = 1610.8 (\pm 26.5) - 0.0061 (\pm 0.0015) \times [263.3 (\pm 37.2) - Phy]^2$ $R^2 = 0.91; p(<0.0001)$			
FCR (g/g)	$FCR = 1.56 (\pm 0.02) - 0.0006 (\pm 0.0003) Phy + 0.000001 (\pm 0.000001) Phy^2$ $R^2 = 0.28; p=0.0302$			
VIA (%)	$VIA = 83.0 (\pm 1.8) - 0.00025 (\pm 0.0003) \times [194.4 (\pm 129.4) - Phy]^2$ $R^2 = 0.32; p=0.0082$			

¹VIA (viability) = 100 – mortality (%). Adjusted by ASEN (SQUARE ROOT (Viability / 100)).²Regression analysis, considering the phytase inclusion levels (0, 120, 180 and 240 OTU/kg), being each OTU (Phytex method) equivalent to 2 FTU (AOAC/ISO method). ns* no significant (P>0.05).

tested and the one that best fit to the variables was used on the regression analysis.

Considering the initial stage of 1 to 21 days (Table II), none of the models was adjusted to FCR, thus there was no response in function of the studied phytase levels. FI and BWG were adjusted by QP ($P<0.05$) model, with plateau at 279 and 231 OTU, respectively. At these concentrations, there was an increase of 22 and 23% in FI and BWG, respectively, compared to negative control, which has no phytase supplementation. VIA was adjusted by a linear model ($P<0.05$) up to 21 days, as the concentration of phytase increased in the diet, there was reduction in mortality.

The performance up to 35 days of age of the broilers (Table II), BWG and FI have also

been adjusted by a QP model, stabilizing at the levels of 272 and 263 OTU, respectively, being 30 and 35% more efficient for these two evaluated variables in relation to negative control diet, without phytase. FCR and VIA at 35 days were adjusted to quadratic model with maximum values (1.60 and 96%) achieved at 300 and 400 OTU, respectively.

Tibias

The DTW and its ash concentration were evaluated as indicative of minerals release by phytase (Table III).

Quadratic-plateau model were adjusted for both evaluated variables. To DTW, the plateau was stabilized in 266 OTU with response of 4.83g,

and this value was 25% higher than negative control. The maximum percentage of ash was determined at 388 OTU, with a response of 38.5% of tibia ash, 18% higher than the negative control. Control diet showed values of 5.23g and 39.13% for DTW and ash, respectively.

Phosphorus bioavailability

The bioavailability of phosphorus was calculated using the standard curve method, considering body weight gain of broiler chickens up to 21 and 35 days of age, were adjusted by QP model (Table IV and V).

The maximum bioavailability found using DTW as parameter was obtained using 145 OTU of enzyme, with 0.054% P released, and 382 OTU to ash in the tibia, with 0.071% P provided by phytase. The plateau of phytase utilization at 21 days was set at 240 OTU with a bioavailability of 0.079% P. Considering up to 35 days, the plateau was reached at 270 OTU with a bioavailability of 0.085% P.

Regarding the bone variables at 35 days of age of the broilers, the standard curve for DTW showed better R^2 when compared to ash (Table VI).

Economic analysis

Economic viability is an important aspect that should be considered when including phytase in poultry diets. To know the level of phytase added that ensures the greatest economic return to the producer is an important tool for the success of the company. Since body weight gain is the most important performance parameter, it was chosen to calculate the economic viability of the phytase, being calculated its cost limit. The cost limit is the price of each u/g of phytase that would be equivalent to the cost of dicalcium phosphate.

The Figure 1 shows cost limit for each phytase FTU, calculated from the P bioavailability obtained for body weight gain, considering the price of dicalcium phosphate as US\$ 0.50. The phytase had higher cost benefit when used at the level of 120 OTU/kg and each OTU can cost up to

Table III. Ash content and dry defatted tibia weight (DTW) of broilers at 35 days fed phosphorus deficient diets with increasing levels of phytase and regression analysis.

Variables	NC2	NC2 + (120 OTU)	NC2 + (180 OTU)	NC2 + (240 OTU)
DTW (g)	3.858 ± 0.122	4.758 ± 0.177	4.772 ± 0.228	4.895 ± 0.123
ASH (%)	32.67 ± 1.45	35.02 ± 1.47	37.61 ± 1.49	37.30 ± 2.33
Regression ¹				
DTW (g)	$DTW = 4.83 (\pm 0.05) - 0.00004 (\pm 0.00001) \times [166.2 (\pm 32.9) - Phy]^2$ $R^2 = 0.87; p(<0.0001)$			
ASH (%)	$ASH = 38.48 (\pm 3.67) - 0.00004 (\pm 0.00005) \times [388.7 (\pm 374.5) - Phy]^2$ $R^2 = 0.56; p(<0.0001)$			

¹Regression analysis of DTW and ASH, considering the phytase inclusion levels (0, 120, 180 and 240 OTU/kg), being each OTU (Phytex method) equivalent to 2 FTU (AOAC/ISO method).

Table IV. Body weight gain (BWG) of broilers fed diets with decreasing levels of available phosphorus to determine the standard curve and bioavailability of phosphorus at 21 days for the treatments with increasing levels of phytase and regression analysis.

Variable	PC	NC1	NC2
BWG	656.5 ± 32.9	653.4 ± 11.1	504.2 ± 21.3
Standard Curve ¹	$BWG = 527.195 (\pm 14.374) + 143.124 (\pm 21.154) x$ $R^2=0.71; p(<0.0001)$		
Phosphorus Bioavailability			
Variables	NC2 +(120 OTU)	NC2 + (180 OTU)	NC2 + (240 OTU)
BWG (g)	0.477 ± 0.190	0.584 ± 0.180	0.657 ± 0.138
BWG (%)	0.060 ± 0.022	0.071 ± 0.019	0.079 ± 0.015
Regression ¹			
BWG (g)	$Bioav(g) = 0.655 (\pm 0.069) - 0.000009 (\pm 0.000004) \times [258.2 (\pm 63.9) - Phy]^2$ $R^2=0.78; p(<0.0001)$		
BWG (%)	$Bioav(\%) = 0.078 (\pm 0.006) - 0.000001 (\pm 0.0000005) \times [240.4 (\pm 46.3) - Phy]^2$ $R^2=0.80; p(<0.0001)$		

¹Standard curve to determine phosphorus bioavailability calculated from supplemental phosphorus intake of treatments PC, NC1 and NC2, when x is the quantity in grams or percentage of phosphorus, which was released by the enzyme for BWG. ²Regression analysis of bioavailable phosphorus, considering the phytase inclusion levels (0, 120, 180 and 240 OTU/kg), being each OTU (Phytex method) equivalent to 2 FTU (AOAC/ISO method).

Table V. Performance of broilers fed diets with decreasing levels of available phosphorus to determine the standard curve and bioavailability of phosphorus at 35 days for the treatments with increasing levels of phytase and regression analysis.

Variable	PC	NC1	NC2
BWG	1704.9 ± 45.3	1627.1 ± 32.2	1191.1 ± 67.2
Standard Curve ¹	$BWG = 1292.00 (\pm 36.50) + 170.17 (\pm 18.60) x$ $R^2=0.82; p(<0.0001)$		
Phosphorus Bioavailability			
Variables	NC2 +(120 OTU)	NC2 + (180 OTU)	NC2 + (240 OTU)
BWG (g)	1.401 ± 0.230	1.903 ± 0.258	2.039 ± 0.340
BWG (%)	0.062 ± 0.009	0.080 ± 0.010	0.085 ± 0.011
Regression ²			
BWG (g)	$Bioav(g) = 2.10 (\pm 0.24) - 0.00002 (\pm 0.000007) \times [302.6 (\pm 65.7) - Phy]^2$ $R^2=0.90; p(<0.0001)$		
BWG (%)	$Bioav(\%) = 0.085 (\pm 0.003) - 0.000001 (\pm 0.0000003) \times [245.7 (\pm 24.1) - Phy]^2$ $R^2=0.95; p(<0.0001)$		

¹Standard curve to determine phosphorus bioavailability calculated from supplemental phosphorus intake of treatments PC, NC1 and NC2, when x is the quantity in grams or percentage of phosphorus, which was released by the enzyme for BWG. ²Regression analysis of bioavailable phosphorus, considering the phytase inclusion levels (0, 120, 180 and 240 OTU/kg), being each OTU (Phytex method) equivalent to 2 FTU (AOAC/ISO method).

Table VI. Ash content and dry deffated tibia weight (DTW) of broilers at 35 days fed diets with decreasing levels of available phosphorus to determine the standard curve and bioavailability of phosphorus at 35 days for the treatments with increasing levels of phytase and regression analysis.

Variable	PC	NC1	NC2
DTW (g)	5.233 ± 0.125	4.982 ± 0.308	3.858 ± 0.122
Ash (%)	39.13 ± 2.12	36.46 ± 4.05	32.67 ± 1.50
Standard Curve ¹	$DBW = 4.014 (\pm 0.103) + 0.683 (\pm 0.080) x$ $R^2 = 0.79; p(<0.0001)$		
Standard Curve ¹	$Ash = 32.891 (\pm 0.933) + 3.223 (\pm 0.727) x$ $R^2 = 0.51; p(<0.0001)$		
Phosphorus Bioavailability			
Variables	NC2 +(120 OTU)	NC2 + (180 OTU)	NC2 + (240 OTU)
DTW (g)	1.089 ± 0.260	1.109 ± 0.333	1.289 ± 0.180
DTW (%)	0.049 ± 0.011	0.047 ± 0.014	0.054 ± 0.007
Ash (g)	0.661 ± 0.457	1.465 ± 0.462	1.603 ± 0.397
Ash (%)	0.029 ± 0.020	0.061 ± 0.019	0.067 ± 0.016
Regression ²			
DTW (g)	$Bioav(g) = 1.199 (\pm 0.063) - 0.000004 (\pm 0.000002) \times [172.1 (\pm 36.0) - Phy]^2$ $R^2 = 0.84; p(<0.0001)$		
DTW (%)	$Bioav(\%) = 0.0503 (\pm 0.002) - 0.000002 (\pm 0.000001) \times [145.3 (\pm 45.1) - Phy]^2$ $R^2 = 0.84; p(<0.0001)$		
Ash (g)	$Bioav(g) = 1.880 (\pm 1.540) - 0.000009 (\pm 0.000002) \times [445.3 (\pm 513.7) - Phy]^2$ $R^2 = 0.59; p(<0.0001)$		
Ash (%)	$Bioav(\%) = 0.0713 (\pm 0.0404) - 0.000005 (\pm 0.0000006) \times [381.8 (\pm 333.4) - Phy]^2$ $R^2 = 0.59; p(<0.0001)$		

¹Standard curve to determine phosphorus bioavailability calculated from supplemental phosphorus intake of treatments PC, NC1 and NC2, when x is the quantity in grams or percentage of phosphorus, which was released by the enzyme for DTW or ash.

²Regression analysis of bioavailable phosphorus, considering the phytase inclusion levels (0, 120, 180 and 240 OTU/kg), being each OTU (Phytex method) equivalent to 2 FTU (AOAC/ISO method)

US\$ 0.000014. But this scenario changes with the price of dicalcium phosphate. If the phytase has a concentration of 4000 u/g, multiplying 5000 by US\$ 0.000010 (each OTU cost of phytase), it is possible to say this phytase may cost up to US\$ 0.04/g or US\$ 40.00/kg, by using a concentration of 240 OTU/kg in diet.

DISCUSSION

For an enzyme acts efficiently, it is necessary to have the specific substrate in the diet and correct

level, as well as its ability to overcome barriers found in the stomach, such as low pH and proteolytic enzymes, such as pepsin (Ravindran 2013). Thus, there are two different implications for the performance variables that have been adjusted by Q and QP models, as follows, the FI and BWG up to 21 days and FI, BWG, FCR and VIA at 35 days. At first, the concentrations of exogenous enzyme were used in this study, the plateau response, or maximum point, may have been a consequence of the reduction in catalytic activity, influenced by enzyme concentration,

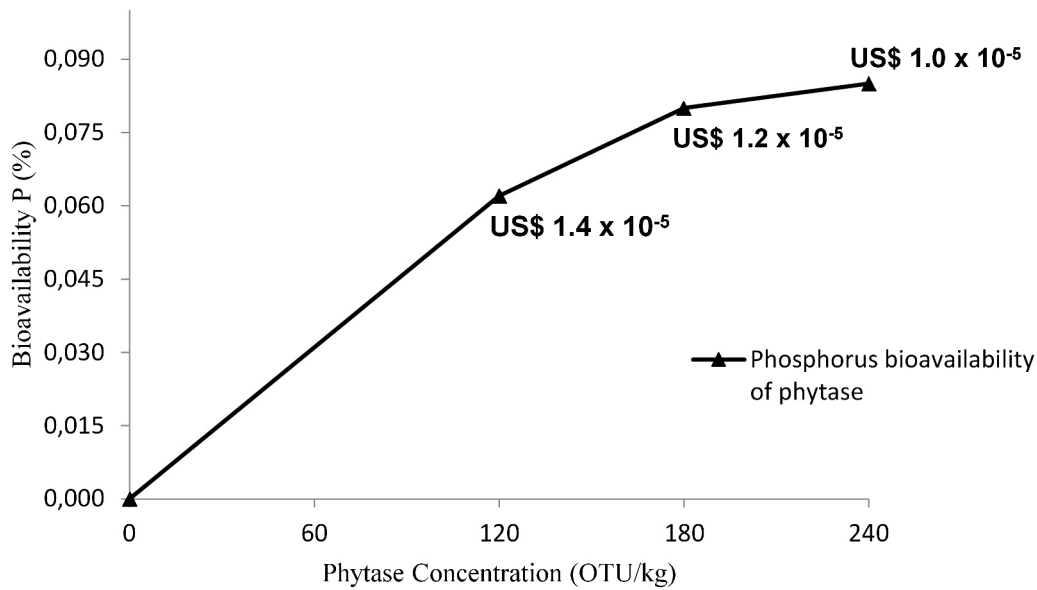


Figure 1. Limit cost per OTU of phytase to replace the inorganic phosphate.

and still having substrate (phytic acid) in the digest, which could be cleaved by phytase.

The phytic acid is soluble at acidic pH, and the beginning of the gastrointestinal tract is the optimal condition for the action of phytase (Li et al. 2015). However, the period that the digest remains under this condition is a short time. Most phytases are not able to degrade the six phosphate groups from the inositol molecule in time and with restrictions in the intestine of poultry (Greiner et al. 2000, Sommerfeld et al. 2018). In this case, it means that the inclusion of higher levels of phytase to the values established by the plateau could provide a higher response than those predicted by the models. Studies with inclusions of up to 24,000 FTU phytase in phosphorus deficient diets have shown performance responses with high phytase concentrations (Shirley & Edwards Jr 2003, Cowieson et al. 2006).

The other implication would be that the maximum set point or plateau predicted by the models would be a result of the reduction or disappearance of phytic acid by the action of phytase, with no further response to higher phytase additions. It is known that as the digesta

moves into the small intestine, there is an increase in pH through the duodenal secretions and phytic acid form crystals by complexing itself with nutrients and precipitates (Selle & Ravindran 2007). This precipitation leads to losses as the reduction in FI and, consequently, decrease in BWG (Santos et al. 2008, Cowieson et al. 2011), as well as the reduction on nutrients availability, resulting in larger environmental excretions (Waldroup et al. 2000). Therefore, the increase in FI, BWG and FCR of the broilers, seen in this study, showed that the phytase was effective to degrade phytic acid, reducing the anti-nutritional effect of phytate.

Some authors attribute the greater BWG to the increased FI with phytase supplementation (Santos et al. 2008, Cowieson et al. 2011, Liu et al. 2014). This study demonstrated increases of up to 30% (272 OTU) and 35% (263 OTU) on FI and BWG at 35 days of age, respectively, in relation to negative control without phytase. Ravindran et al. (2008) evaluating the phytase supplementation in diets P deficient, have also observed a greater response on BWG, FI and FCR. The quadratic model for FCR at 35 days, in this study, generated the optimal level

of phytase usage at 300 OTU, with response of 1.50 versus 1.56 observed with negative control diet. Chung et al. (2013) have also observed a better FCR for broilers fed phytase, however it were not observed improvements in BWG and FI. Several studies corroborate the results of the current study, and indicate that phytase supplementation provides improvements on BWG, FI and FCR (Selle & Ravindran 2007, Lelis et al. 2012, Singh et al. 2013, Vieira et al. 2015, Lee et al. 2017).

Another aspect that is important to highlight is the viability of broilers. As the reduction of P in deficient diets were severe, the mortality of birds reached 26.35% at 32 days for the birds fed diets with P reductions without phytase supplementation. However, as the enzyme was included in the diets, the viability improved. Powell et al. (2011), working with P reductions observed mortality of less than 6% in all treatments with phytase inclusions and 13.89% for the treatment with no enzyme.

Phytase also plays an important role in bone development, through the release of Ca and P, as well as other bivalent minerals that compose this tissue (Augspurger & Baker 2004, Onyango et al. 2005). This greater availability of minerals and effectiveness of phytase activity can be evidenced when the DTW and the ash content of broilers at 35 days are analysed through QP model, with an improvement of 25 and 18%, respectively. Other studies also demonstrate an increase in bone ash content of broilers and hens with phytase in P deficient diets (Selle et al. 2009, Kozłowski & Jeroch 2011, Rutherford et al. 2012).

The minerals deposition in bone is a good parameter for phytase evaluation, since this tissue has the highest concentrations of minerals in the body. Besides the bone integrity is a characteristic which is correlated with the ash content and DTW, this is a problem

faced by the industry in poultry production. The bioavailability of P can be measured by digestibility trials or the standard curve methodology, the latter being easy to perform and the most used (Li et al. 2016). Therefore this methodology was used for the calculation of the P released by the studied levels of phytase in the diets.

The bioavailability of P calculated from the BWG at 21 and 35 days, and from the weight and ashes of defatted tibia, expressed by QP models, explain the higher FI, BWG, weight and ash content as increased the phytase inclusion. Pillai et al. (2006), working with the same enzyme determined the bioavailability of 0.09% P released with 250 FTU/kg (125 OTU), through the ashes. Maybe the methodology used to characterize the enzyme was not considered (Phytex). In this study however, it was observed a lower value of phosphorus release (0.067%) with 240 OTU (500 FTU), considering ash of tibias, and 0.79% considering the BWG. Results presented by Augspurger et al. (2003) agree with our findings, that the phytase supplementation up to 1000 FTU/kg was not able to release quantities exceeding 0.067% P, considering bone ash of broilers. Vieira et al. (2015) observed averaging all values for 500 and 1,000 FTU/kg provided estimations of 0.100 and 0.166%, respectively. Biehl et al. (1995) found 0.116% P bioavailability, using 1200 FTU/kg of phytase (Natuphos). The divergences between the studies are due to variation in the phytic acid content of corn and soybean meal, a combination of foods of the same diet, evolution of the genetic strains of broiler chickens and, mainly, to the progress and quality of the present phytases that have a greater spectrum of action, are resistant to diet processing and act on higher pH and temperature amplitudes. Thus, there is a favorable effect on the metabolic efficiency of broilers in the utilization of nutrients that were

complexed in the form of phytate and phytate-protein interactions.

As demonstrated in Figure 1, the phytase supplementation is economically viable when the cost per OTU/g does not exceed U\$0.000014, U\$0.000012 and U\$0.000010 for the concentrations of 120, 180 and 240 OTU/kg, equivalent to approximately 240, 360 and 500 FTU, respectively. For a phytase at a concentration of 4000 U/g, for example, are needed 60g/ton addition to formulate a diet of 240 OTU/kg. Considering an intermediate price per kilo of phytase (U\$10.00), the cost for each gram of enzyme would be U\$0.01, being the cost for each FTU of U\$0.0000025 below the set limit for the economically feasible use. In this example, would be spent U\$0.60 with phytase compared to U\$2.30 inorganic phosphate, thus a saving of U\$ 1.70 per ton of feed.

CONCLUSIONS

Phytase used in small concentrations demonstrated to be efficient, improving weight gain, feed intake, feed conversion ratio, viability and even the bone characteristics of broilers fed P deficient diets in any tested concentration. However, the ideal inclusion level which presented the best response, is close to the higher level studied, 240 OTU.

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Gabriel Villela Dessimoni conceived the idea presented, carried out the field research, participated in the analytical methods, analyzed and discussed the data observed in the experiment and wrote the article in the initial format as one of the chapters of his Doctoral Thesis. Nilva Kazue Sakomura conceived the idea for carrying out the research, as well as actively coordinating all the processes involved, such as data analysis, methodologies, discussion, writing and final adjustments related to the publication. Daniella Carolina Zanardo Donato participated supporting the realization of all activities relevant to the publication, offering help from conceptualization to the final format of the article. Larissa Vargas, Mirella Melaré and Letícia Pacheco participated in the help in the field experiment, in the laboratory analysis and in all the general support for the successful conduct of the research. Felipe Santos Dalólio participated in the writing and editing process of the article. All authors discussed the results and contributed to the final writing of the manuscript.

