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Broiler feed formulated with phosphorus, calcium, and energy deficiencies supplemented with exogenous enzymes

Rações para frangos formuladas com deficiências de fósforo, cálcio e energia suplementada com enzimas exógenas

Rodrigo de Freitas Jacob[1]*; Luiz Fernando Teixeira Albino[2]; Arele Arlindo Calderano[2]; Romário Duarte Bernardes[3]; Horácio Santiago Rostagno[2]; Maurílio de Lucas Xavier Júnior[1]; Renan Hernandes Lino[4]; Warley Júnior Alves[1]; Samuel Oliveira Borges[4]; Thiago Ferreira Diana[1]

Highlights

Nutrient deficiency impairs broiler performance.
Adding enzymes to reduced-nutrient diets maintains performance and reduces costs.
Enzymes improve energy use, which can reflect on improved performance.
The greater digestibility of amino acids may explain the increase in performance.
Phytases, carboidrases and proteases are able to improve the amino acids values.

Abstract

Three experiments were carried out to verify the effects of the enzyme phytase, alone or combined with an enzyme complex, in diets deficient in available phosphorus (AP), calcium (Ca), and metabolizable energy (ME) on broiler performance, ME, and dietary amino acid digestibility. A total of 1,538 male Cobb 500 broilers were allocated to the three experiments, each of which consisted of five treatments: positive control (PC; basal ration); negative control 1 (NC1; PC minus 0.15% of AP, 0.16% of Ca, and 68 kcal kg-1 ME); negative control 2 (NC2; PC minus 0.15% of AP, 0.16% of Ca, and 101 kcal kg^{-1} of ME); NC1 plus phytase; and NC2 plus phytase plus enzymatic complex. Body weight gain (WG) and feed intake were measured from 1-21

[1] Students of Animal Science Doctoral Course, Department of Animal Science, Unversidade Federal de Viçosa, UFV, Viçosa, MG, Brazil. E-mail: rodrigofj2@gmail.com; maurilio_junior1@hotmail.com; warleyjunioralves@hotmail.com; thiagofnet@hotmail.com
[2] Profs. Drs., Postgraduate Program in Animal Science, Department of Animal Science, UFV, Viçosa, MG, Brazil. E-mail: lalbino@ufv.br; calderano@ufv.br; rostagno@ufv.br
[3] Student of Animal Science Master's Course of the Postgraduate Program in Animal Science, Department of Animal Science, UFV, Viçosa, MG, Brazil. E-mail: romario.bernardes@ufv.br
[4] Students of Animal Science Undergraduate Course, Department of Animal Science, UFV, Viçosa, MG, Brazil. E-mail: renan.lino@ufv.br; samuelborges1001@gmail.com
* Author for correspondence

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days and from 1-42 days, and the corrected feed conversion rate (FCR) for mortality was calculated. In the second and third experiments, the apparent ME corrected for nitrogen balance (AMEn) and standardized digestibility of amino acids, respectively, were determined, for the diets supplemented with phytase and the enzymatic complex. In the first experiment, enzyme supplementation increased ($p < 0.05$) WG at 21 days and 42 days relative to the negative controls. Phytase inclusion improved ($p < 0.05$) FCR at the initial phase compared to the NC1 diet. In the second experiment, enzyme supplementation did not affect ($p > 0.05$) AMEn. In the third experiment, both enzyme treatments improved ($p < 0.05$) the digestibility of amino acids in the supplemented diets compared to the deficient diets. Supplementation with phytase and carbohydrases preserves the performance of broilers fed diets deficient in AP, Ca, and ME and improves amino acid digestibility.

Key words: Broilers. Performance. Phytase. Metabolism. Enzymatic complex.

Resumo

Três experimentos foram conduzidos para determinar o efeito da enzima fitase, sozinha ou associada a um complexo enzimático, em dietas de milho-farelo de soja deficientes em fósforo disponível (Pd), cálcio (Ca) e energia metabolizável (EM) no desempenho de frangos de corte, valores de EM e digestibilidade de aminoácidos da dieta. Para ambos os experimentos, 1.538 frangos Cobb 500 machos foram alocados em 5 tratamentos: controle positivo (CP, ração basal), controle negativo 1 (CN1, CP menos 0,15% de Pd, 0,16% Ca e 68 kcal kg^{-1} EM), negativo controle 2 (CN2, CP menos 0,15% de Pd, 0,16% de Ca e 101 kcal kg^{-1} de ME em relação à dieta CP), CN1 mais fitase (CN1 + F) e CN2 mais fitase mais complexo multienzimático (CN2 + F + E). O ganho de peso corporal e o consumo de ração foram medidos de 1 a 21 dias e de 1 a 42 dias, e a taxa de conversão alimentar (CA) corrigida para mortalidade foi calculada. No segundo e terceiro experimentos, foram analisados os valores de energia metabolizável aparente corrigida para o balanço de nitrogênio (EMAn) e digestibilidade estandardizada de aminoácidos, respectivamente, em adição à fitase e ao complexo enzimático. No primeiro experimento, a suplementação enzimática aumentou ($P < 0,05$) o ganho de peso (GP) aos 21 dias e 42 dias em relação aos controles negativos. A inclusão de fitase melhorou ($P < 0,05$) a taxa de conversão alimentar na fase inicial, em comparação com a dieta CN1. No segundo experimento, a suplementação enzimática não afetou ($P > 0,05$) os valores de EMAn. No terceiro experimento, ambos os tratamentos enzimáticos melhoraram ($P < 0,05$) a digestibilidade dos aminoácidos nas dietas experimentais, em comparação com as dietas deficientes. A suplementação de fitase e carboidrases preserva o desempenho de frangos alimentados com dietas deficientes em Pd, Ca e EM e melhora a digestibilidade dos aminoácidos.

Palavras-chave: Frangos de corte. Desempenho. Fitase. Metabolismo. Complexo enzimático.

Introduction

Corn and soybean meal, the most important ingredients in broiler feed, contain varying concentrations of antinutritional factors that can substantially reduce the digestibility of dietary nutrients, and, therefore, animal performance. Exogenous enzymes are added to feed in an attempt to counter the adverse effects of these factors and improve the nutritional value of the diet.

Phytases are enzymes that catalyze the hydrolysis reaction of the phytate phosphate ester bonds, and thereby not only promote an increase in the bioavailability of phosphorus (P) but also positively affect energy and amino acid values in broiler diets (Dersjant-Li, Awati, Schulze, & Partridge, 2015; Bournazel et al., 2018). Information on the interaction between phytases and carbohydrases is inconclusive. However, it is known that although the target substrates of these enzymes are different in corn and soybean meal, carbohydrases can improve the accessibility of exogenous and endogenous enzymes to the cellular contents of plant tissue. Thus, the more complete breakdown of plant cell walls stimulated by carbohydrases can increase the additive or sub-additive benefits of broiler diets based on corn and soybean meal (Schramm et al., 2017).

Exogenous proteases are enzymes with growing potential for use in diets for non-ruminant animals. They mitigate the negative impacts of factors such as lectins, trypsin inhibitors, and allergenic proteins, and can improve energy use and increase amino acid values in broiler diets (Cowieson & Roos, 2016). The influence of enzyme combinations on performance, amino acid digestibility, and energy of broiler diets has been evaluated by several authors. In most cases, enzymes were added to diets containing deficient levels of calcium (Ca), available phosphorus (AP), and energy, particularly in evaluations of phytase, xylanase, and protease (Walk & Poernama, 2018)

This study tests the hypothesis that supplementation with phytase alone, or in combination with carbohydrases and protease, can improve the use of nutrients and energy in the diet and preserve the performance of broilers fed nutrient- and energy-deficient diets based on corn and soybean meal. Thus, the objective was to evaluate the effects of supplementation with phytase, alone or combined with an enzyme complex, of diets deficient in AP, Ca, and metabolizable energy (ME) on broiler performance, ME, and amino acid digestibility.

Materials and Methods

This study was conducted at the Teaching, Research, and Extension Unit in Production and Nutrition of Poultry of the Animal Science Department, Agricultural Sciences Center, Federal University of Viçosa (UFV), Viçosa, the State of Minas Gerais, Brazil (20°45’57.19’’S, 42°51’35.42’’W and 682 m altitude). All experimental procedures adopted were previously approved by the Ethics Committee on the Use of Farm Animals (process no. 0107/2018) in accordance with the ethical principles of animal experimentation established by the National Council for Animal Experimentation Control (CONCEA) and current legislation.

Two basal diets based on corn and soybean meal were formulated for experiments at the initial and growth/finisher phases, following the nutritional recommendations of Rostagno et al. (2017) (Table 1). The study included three experiments with the same five treatments: positive control (PC; basal ration); negative control 1 (NC1; a reduction by 0.15% of AP, 0.16% of Ca, and 68 kcal kg^{-1} of ME relative to the PC diet); negative control 2 (NC2; a reduction by 0.15% of AP, 0.16% of Ca, and 101 kcal kg^{-1} of ME relative to the PC diet); NC1 with the addition of phytase (NC1 + Phy); and NC2 with the addition of phytase and an enzyme complex (NC2 + Phy + E).

Table 1
Composition and calculated nutritional values of Positive Control (PC) and Negative Controls (NC) diets

Ingredients (%)	1 to 21 days			22 to 42 days		
	PC[1]	NC1[2]	PC[1]	NC1[2]	PC[1]	NC1[2]
Corn	50.531	55.338	57.398	59.240	64.048	66.964
Soybean meal	41.422	39.363	38.307	32.686	30.627	28.823
Soybean oil	3.846	1.867	1.045	4.500	2.526	1.366
Dicalcium phosphate	1.786	0.981	0.985	1.491	0.685	0.694
Limestone	0.924	1.039	0.860	0.715	0.831	0.840
Salt	0.515	0.427	0.427	0.472	0.384	0.384
DL-Methionine (99%)	0.318	0.304	0.304	0.253	0.239	0.245
Biolys HCl (54,6%)	0.135	0.171	0.171	0.160	0.196	0.218
L-Threonine (98%)	0.048	0.035	0.028	0.033	0.019	0.021
Vitamin[4]	0.130	0.130	0.130	0.100	0.100	0.100
Mineral[5]	0.130	0.130	0.130	0.100	0.100	0.100
Choline chloride (60%)	0.100	0.100	0.100	0.100	0.100	0.100
Salinomycin[6] (12%)	0.055	0.055	0.055	0.055	0.055	0.055
BHT[7]	0.010	0.010	0.010	-	-	-
Starch	0.010	0.010	0.010	0.010	0.010	0.010
Total	0.040	0.040	0.040	0.050	0.050	0.050
Corn	100	100	100	100	100	100
Nutritional composition (%)						
Metabolizable energy kcal/kg	3000	2942	2899	3150	3092	3049
Crude protein %	23.228	22.703	22.383	19.934	19.409	18.854
Calcium %	0.930	0.777	0.777	0.758	0.598	0.598
Av. phosphorus %	0.440	0.290	0.290	0.374	0.224	0.224
Sodium %	0.218	0.183	0.183	0.200	0.165	0.165
Dig. lysine %	1.256	1.241	1.218	1.070	1.055	1.032
Dig. methionine + cysteine %	0.929	0.906	0.900	0.792	0.769	0.763
Dig. threonine %	0.829	0.799	0.782	0.706	0.676	0.659

[1]PC = positive control (diet meeting the nutritional requirements of broilers).
[2]NC1 = negative control 1 (PC minus 68 kcal/kg EM; 0,15% AP and 0,16% Ca).

The enzyme complex (SQzyme CEM, Suntaq International Limited, Shenzhen, China), originating from *Trichoderma reesei* (601-17), contained predominantly carbohydrases, and was composed of α-galactosidase, β-mannanase, pectinase, xylanase, acid cellulase, α-amylase, β-glucanase, and protease (Table 2). It was applied at a dose of 100 g/t of diet to provide a guaranteed minimum of 100, 1,500, 1,500, 15,000, 1,200, 150, 2,400, and 2,500 units per kg of diet of α-galactosidase, β-mannanase, pectinase, xylanase, acid cellulase, α-amylase, β-glucanase, and protease, respectively. Phytase was derived from *Escherichia coli* (EC 3.1.3.26), modified by *Pichia pastoris,* and supplied at a rate of 500 FTU kg^{-1} of diet.

Table 2
Definition and enzymatic properties of enzymes added to diets

Enzymes	Definition[1]				
	Molecule released	Substrate	Reaction time	pH	Temperature
Phytase	1 µmol of inorganic P	Sodium Phytate	1 min	5.5	37ºC
α-Galactosidase	1 µmol de p-nitrophenol	p-Nitrophenyl-α-D-Galactopyranoside	1 min	6.0	37ºC
β-Mananase	1 µmol of mannose	Galactomanan	1 min	5.0	70ºC
Pectinase	1 µmol of D-galacturonic acid	Polygalacturonic Acid	1 min	5.0	37ºC
Xilanase	1 µmol de xylose	Xylan	1 min	5.0	50ºC
Celulase	1 µmol de glucose	Filtered paper	1 min	4.8	50ºC
α-Amylase	1 µmol de glicose	Starch	5 min	6.9	40ºC
β-Glucanase	1 µmol de glucose	Carboxymethylcellulose	1 min	4.8	50ºC
Protease	1 µmol of Suc-Ala-Ala-Pro-Phe-N-succinyl Ala-Ala-Pro-Phe-p-nitroanilide	*p*-Nitroaniline	1 min	9.0	37ºC

[1]Quantity of enzyme required to release 1 µmol of a given molecule from the substrate at a given time, pH and temperature.

In the performance experiment, 1,000 one-day-old Cobb 500 male chicks were weighed (43.78 ± 0.025 g each) and distributed in the treatments to ensure that the treatments had similar average body weights. The birds were randomly distributed in the five treatments, each with 10 replicates of 20 chicks each. Each stall containing the 20 chicks was considered an experimental unit and consisted of a box with a concrete floor, with dimensions of 1.25 × 1.80 m and a total area of 2.25 m². The animals were handled in 3 m high masonry sheds with asbestos cement tiles, 50 cm low walls, and a screen with a 0.5-inch mesh adapted for animal experimentation. The performance parameters evaluated were feed intake (FI, g), weight gain (WG, g), and feed conversion rate (FCR, g g^{-1}). Body weight and FI were recorded at 21 days and 42 days. Mortality was recorded daily for subsequent FI correction according to Sakomura and Rostagno (2016). Feed and water were provided *ad libitum* throughout the experimental period. The broiler bed consisted of new sawdust.

In the metabolism experiment, 240 male Cobb 500 broilers were housed in cages containing trays lined with plastic for the collection of total excreta. Each cage was equipped with a bird feeder and waterer for ad libitum access to feed and water. The birds were distributed in a completely randomized design, with five treatments, eight replicates, and six birds per experimental unit. The broilers were initially fed a commercial diet based on corn and soybean meal for 13 days and subsequently introduced to the experimental diets.

The total excreta were collected from day 19 to day 23 to determine the apparent ME corrected for nitrogen balance (AMEn). After an adaptation period of four days (days 14-18), excreta were collected twice a day (at 8 am and 6 pm) for four consecutive days (days 19-23). At the end of the excreta collection period, the samples were thawed at room temperature and homogenized, and the sub-samples were placed in plastic containers and stored in a freezer at −18 ºC until analysis.

Excreta and feed samples were dried at 55 °C in a forced ventilation oven for 72 h. The samples were ground on a 1 mm screen. The dry matter (DM) content was determined by drying the samples overnight in an oven at 105 ºC (Detmann et al., 2012). The Kjeldahl method was used to determine the nitrogen content in diets and excreta according to standard protocols (Detmann et al., 2012). The excreted nitrogen (NE) was calculated by multiplying the total amount excreted in the DM by the percentage of nitrogen in the excreta, also in the DM. The same method was applied to the calculation of the nitrogen consumed (NC). Nitrogen retention (NR) was determined as the amount of NC minus NE. The gross energy content was measured with a C5001 adiabatic calorimetric pump (IKA-Werke GmbH & Co. KG, Staufen, Germany). The AMEn values were calculated based on the analysis of diets and excreta according to Sakomura and Rostagno (2016).

In the digestibility experiment, 288 male Cobb 500 broilers were housed in cages, distributed in a completely randomized design, with five treatments, eight replicates, and six birds per experimental unit. To obtain the coefficients of standardized ileal digestibility (CSIDs) of amino acids, a protein-free diet was used to determine the endogenous excretion of amino acids (Table 3). The birds received the experimental diet from 16 to 20 days of age. Celite® (Celite Corp., Lompoc, CA, USA), a source of acid insoluble ash (AIA), was added to all diets at 10 g kg^{-1} as an indigestible marker, and AIA concentrations were determined by the method of Van Keulen and Young (1977).

After a five-day adaptation period, all birds were killed by cervical dislocation and the abdominal cavity was opened immediately to expose the digestive tract. The 15 cm ileum terminal segment was sectioned 4 cm from the ileocecal junction to access the ileal digesta. The ileal samples were frozen and stored at −20ºC until processing. They were then lyophilized for 72 h at −40ºC in a lyophilizer (LH 0401, Terroni, São Carlos, Brazil).

Dry matter analysis (Detmann et al., 2012) of the diets and ileal digesta was performed for digestibility calculations. Laboratory analysis to determine the amino acid content of diets and excreta was carried out by CBO - Analises Laboratoriais (Campinas, São Paulo, Brazil) using HPLC (high performance liquid chromatography). The digestibility of amino acids was calculated based on the analysis of diets and ileal digesta according to Sakomura and Rostagno (2016).

Table 3
Protein-free diet composition

Ingredients	PFD (%)
Starch	81.240
Sugar	5.000
Soybean oil	5.000
Dicalcium phosphate	2.100
Limestone	0.700
Salt	0.450
Cob[1]	4.000
Vitamin[2]	0.150
Mineral[3]	0.150
Choline cloride (60%)	0.200
BHT[4]	0.010
Acid insoluble ash (Celite™)	1.000
Total	100.000
Crude protein (%)	0.174

[1]Considering cob with 4.4% CP.
[2]Vit. 9375 IU; Vit. D3 2375 IU; Vit E 35 IU; Vit B1 2.50 mg; Vit B2 6.25 mg; Vit B6 3.5 mg; Vit B12 0.015 mg; Nicotinic acid
[3]7.5 mg; Pantothenic Acid 12.5mg; Vit. K3 1.88mg; Folic acid 0.875mg; Biotin 0.088 mg.
[3]Selenium - 0.375 mg; Manganese - 88 mg; Iron 62.5 mg; Zinc 81.3 mg; Copper 12.5 mg; Iodine 1.25 mg.
[4]Antioxidant Butyl Hydroxy Toluene.

Broiler performance, ME, and digestibility of amino acids were analyzed with ANOVA using the software R (R Core Team, 2019). Boxes or cages were considered experimental units. A probability of $p < 0.05$ was considered statistically significant. The Tukey test was used to identify differences between means at the significance level $p < 0.05$.

The statistical model used was

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

where Y_{ij} was the observation of the ith treatment in the jth experimental unit (response variable), μ was the general mean, τ_i was the treatment effect, and ε_{ij} was the experimental error.

Results and Discussion

In the performance experiment (1-21 days), only the birds in the NC1 treatment had a lower FI compared to those in the PC treatment ($p < 0.05$; Table 4). In addition, the FI of birds in the NC2 + Phy + E treatment was higher ($p < 0.05$) than that of the birds in NC2. The decrease in ME was generally offset by the increase in FI. However, the reduction in phosphorus (P) showed the opposite trend by being associated with reduced FI (Walk & Rama Rao, 2020). However, these effects depend on the degree of AP reduction in the diet. For example, Ziarat, Kermanshahi, Mogaddam and Heravi (2020) did not observe a reduction in FI at the initial phase in broilers fed P-deficient diets, and related the lack of response to the P deficiency, which was only 0.11% of AP relative to PC, not being high enough.

Table 4
Effect of exogenous enzymes on broiler performance

Variable	Treatment					SEM	P-value
	PC[1]	NC1[2]	NC2[3]	NC1 + Phy	NC2 + Phy + E		
			1-21 days				
WG (g)	986a	856b	882b	990a	975a	0.00139130	0.001
FI (g)	1263ab	1171c	1191bc	1237abc	1309a	0.00152249	0.001
FCR	1.285ab	1.369c	1.353bc	1.250a	1.343bc	0.001535364	0.001
			1-42 days				
WG (g)	2995a	2916b	2915b	2997a	2998a	0.00446046	0.010
FI (g)	5066a	4812a	4995a	5077a	5061a	0.00160478	0.047
FCR	1.68	1.65	1.71	1.69	1.69	0.00145384	0.437

[a-c]Means in the same row with different letters differ significantly ($P < 0.05$) by Tukey's test.
Abbreviations: E, enzymatic complex; WG, weight gain (g); FI, feed intake (g); FC, feed conversion (g/g).
[1]PC = positive control (diet meeting the nutritional requirements of broilers).
[2]NC1 = negative control 1 (PC minus 68 kcal/kg EM; 0,15% AP and 0,16% Ca).
[3]NC2 = negative control 2 (PC minus 101 kcal/kg EM; 0,15% AP and 0,16% Ca).

The WG of birds in treatments NC1 and NC2 was lower in the period from 1 to 21 days, compared to birds in treatments PC, NC1 + Phy, and NC2 + Phy + E ($p < 0.05$). The negative effect of AP reduction is not only observed in the birds' food intake; AP deficiency can lead to anorexia and, therefore, to less weight gain (Montanhini, Ceccantini, & Fernandes, 2012; Rahimi, Modirsanei, & Mansoori, 2020). In addition to slowing growth, animals become less efficient in using nutrients from the diet. This lower efficiency may be attributed to the negative effect of phytate on the endogenous flow of amino acids (Cowieson & Ravindran, 2007).

However, as hypothesized, when the birds received diets supplemented with phytase or phytase + enzyme complex, their WG did not change relative to that of the birds in the control treatment, and they recovered a part of the lost performance. The results of this study are consistent with those of Dessimoni et al. (2019) who also observed an improvement in performance after adding phytase to diets deficient in P. Likewise, Ribeiro et al. (2016) showed a positive effect on FI and WG of phytase addition to P-deficient diets. Walk, Pirgozliev, Juntunen, Paloheimo and Ledoux (2018) found that the addition of phytase, phytase + xylanase, or the combination of phytase + xylanase + protease to diets deficient in P and Ca restored FI and WG and improved the feeding efficiency of birds. This result can be explained by the positive effect of enzymes on nutrient release from the diet and performance. According to Walk, Santos and Bedford (2014), improvements in FCR can be attributed to the degradation of phytate by phytase and the use of inositol. Other studies have also shown that the inclusion of phytase in broiler diets can improve the ileal digestibility of P, amino acids, and proteins and ME (Rutherfurd, Chung, Thomas, Zou, & Moughan, 2012; Pieniazek et al., 2017).

In addition, the beneficial effects of inclusion of carbohydrases with phytase on bird performance can be justified by the increase in the nutritional value of the diet, which occurs from the moment P deficiency is overcome by phytase and the intake of nutrients is restored, thereby compensating for the reduction in ME (Francesch & Geraert, 2009). Corn and soybean meal are highly digestible ingredients, but they can include significant amounts of non-starch polysaccharides (PNAs; 8-29%). In maize, the main PNAs are arabinoxylans and β-galactomannan, while in soy bran, α-galactosides and β-galactomannan predominate (Choct, 1997; Malathi & Devegowda, 2001; Jamroz, Jakobsen, Knudsen, Wiliczkiewicz, & Orda, 2002; Amerah, 2015; Jaworski, Lærke, Bach Knudsen, & Stein, 2015). In diets based on corn and soybean meal, carbohydrases are associated with the rupture of the cell wall matrix, facilitating the release of encapsulated nutrients and their access by both endogenous and exogenous enzymes, as in the case of phytase access to the molecule phytate (Olukosi, Cowieson, & Adeola, 2007; Diana et al., 2020). This evidence corroborates the hypothesis that the use of a combination of carbohydrases and phytase in broiler diets can potentiate their effects, which can be additive or sub-additive in diets based on corn and soybean meal (Cowieson & Bedford, 2009; Schramm et al., 2017).

The action of exogenous proteases on dietary proteins may improve performance and increase protein digestibility and amino acid absorption in broilers (Fru-Nji, Kluenter, Fischer, & Pontoppidan, 2011; Mahmood et al., 2017). However, performance effects are expected to be evident only in situations where protein digestibility is naturally impaired or when dietary amino acids are reduced (Amerah, Romero, Awati, & Ravindran, 2016). In this study, the activity of protease, supplemented at only 2,500 U/kg, does not seem to have been sufficient to promote additive performance responses in the birds. In addition, the ad libitum supply of feed may have made it difficult to detect the effects of the enzyme. Similarly, Yuan, Wang, Zhang and Wang (2017) did not observe significant improvements in the performance of broilers fed diets based on corn and soybean meal supplemented with carbohydrases (xylanase, beta-glucanase, and cellulase) and protease (at 50,000 U/kg).

Between days 1 and 42, the reduction in the levels of AP, Ca, and ME in the NC diets resulted in significantly worse WG in birds compared to that in birds fed the PC diet ($p < 0.05$). However, FI or FCR responses to the reduction in nutrients were not significant, although they were numerically lower than the responses to the PC diet. Supplementation of the deficient diet with phytase or the enzyme complex caused a body weight gain in birds similar to that with the standard diet. These results agree with the findings of previous studies that phytases or enzyme complexes added to corn and soybean meal-based diets deficient in AP, Ca, and ME improved the growth rate of broilers (Lu et al., 2013; Dessimoni et al., 2019; Rahimi et al., 2020).

There were no treatment effects on AMEn ($p > 0.05$; table 5). The absence of any effect of phytase on AMEn is compatible with the findings of previous studies (Rutherfurd et al., 2012; Akter, Graham, & Iji, 2017). However, this result is at odds with the previous finding that phytase has positive effects on energy retention in birds (Pieniazek et al., 2017; Gallardo, Dadalt, & Trindade, 2018). These conflicting results may be due to several factors, such as the concentration of phytic acid in the diet, types of ingredient used, age of birds, and digestibility coefficients of fat, starch, and amino acids (Cowieson, Aureli, Guggenbuhl, & Fru-Nji, 2015). In addition, it is noteworthy that phytase can improve the net energy of production in broilers without causing significant effects on AMEn (Olukosi & Adeola, 2008). According to Akter et al. (2017), this is because an amount of energy remains locked in protein and fat in the body and this energy is reflected in the value of net energy rather than in the value of AME. This further indicates that the measurement of net energy may be more appropriate for assessing the effect of phytase. The Phy + E combination also did not have an effect on AMEn.

Table 5
Apparent metabolizable energy corrected for nitrogen balance in diets for broilers supplemented with exogenous enzymes

Variable	Treatment					SEM	P-value
	PC[1]	NC1[2]	NC2[3]	NC1 + Phy	NC2 + Phy + E		
EMAn							
Calculated	3000	2942	2899	3000	3000		
Determinated	3062	3023	3012	3049	3061	2.02657	0.665

[a-c]Means in the same row with different letters differ significantly ($P < 0.05$) by Tukey's test.
Abbreviations: E, enzymatic complex; AMEn, apparent metabolizable energy (kcal/kg).
[1]PC = positive control (diet meeting the nutritional requirements of broilers).
[2]NC1 = negative control 1 (PC minus 68 kcal/kg EM; 0,15% AP and 0,16% Ca).
[3]NC2 = negative control 2 (PC minus 101 kcal/kg EM; 0,15% AP and 0,16% Ca).

However, previous studies have shown improvements in nutrient and energy retention with supplementation of poultry diets with phytase + carbohydrase or phytase + carbohydrase + protease (Romero, Parsons, Utterback, Plumstead, & Ravindran, 2013; Murugesan, Romero, & Persla, 2014; Gallardo, Dadalt, Kiarie, & Trindade, 2017; Gallardo, Dadalt, & Trindade, 2019). The main reasons attributed to these observations are better access by digestive enzymes of the plant cell wall matrices, reduction of antinutritional factors, and endogenous losses (Meng & Slominsky, 2005; Olukosi & Adeola, 2008; Gallardo et al., 2017). According to Amerah, Plumstead, Barnard and Kumar (2014), the response of birds to enzymes in terms of energy improvement can vary depending on the energy content of the basal diet, the level of substrate and inherent digestibility, intestinal health, and the effect of enzymes on the microbiota profile. Thus, the high digestibility of the ingredients used in the diets of this study may have contributed to the lack of responses in energy use.

To obtain the CSIDs of amino acids for the different diets, a protein-free diet was used to determine the loss of endogenous amino acids (g/kg DM). The CSIDs of arginine, isoleucine, leucine, lysine, phenylalanine, valine, aspartic acid, glutamic acid, glycine, and serine were similar between treatments PC, NC1, and NC2 (Table 6). However, supplementation of the NC1 treatment with phytase improved the digestibility of arginine, isoleucine, leucine, phenylalanine, aspartic acid, glutamic acid, and serine compared to the NC1 diet without supplementation. The addition of the combined enzymes (Phy + E) improved the digestibility of arginine, isoleucine, lysine, phenylalanine, threonine, aspartic acid, and serine relative to the NC2 diet ($p < 0.05$).

The CSIDs of histidine and alanine in the PC diet were higher than those in the NC1 and NC2 diets. The use of phytase alone or in combination (Phy + E) significantly increased the digestibility coefficient of histidine compared to the respective negative controls; however, only phytase improved the digestibility of alanine relative to the negative control ($p < 0.05$).

The digestibility of the amino acids methionine, threonine, cystine, proline, and tyrosine were significantly different between PC and NC2. However, the digestibility coefficients of these amino acids in PC and NCI were similar. Phytase was able to significantly increase the digestibility coefficient of threonine in diets supplemented with enzymes in contrast to NC1 ($p < 0.05$), while the enzyme combination (Phy + E) significantly improved the digestibility of methionine and threonine in the supplemented diet compared to the NC2 diet ($p <0.05$).

Overall, the enzyme combination (Phy + E) increased the digestibility coefficients of amino acids by 2.62% (from 88.97% to 91.30%) compared to the NC2 diet without supplementation, while the effect solely of phytase was 2.70% greater than that of the NC1 diet without this enzyme (91.68% vs 89.27%).

The positive effects of phytase on protein use observed in this work are consistent with the findings of previous studies that reported improvements in the ileal digestibility of amino acids in broilers fed diets based on corn and soybean meal (Santos, Hruby, Pierson, Remus, & Sakomura, 2008; Rutherfurd et al., 2012; Pieniazek et al., 2017). According to Rutherfurd et al. (2012), the negative effects of phytate on amino acid digestibility are due to the direct association between phytate phosphate groups and the basic amino acid side chains of dietary proteins, which reduces the solubility and digestibility of these nutrients, as well as to the inhibition of digestive enzymes, including pepsin and trypsin, by phytate.

Table 6
Effect of supplementation with exogenous enzymes on the apparent ileal digestibility coefficient of amino acids in broilers

Variable	Treatment					SEM	P-value
	PC[1]	NC1[2]	NC2[3]	NC1 + Phy	NC2 + Phy + E		
Essential amino acids (%)							
Arginine	91.92abc	91.31bc	90.95c	93.22a	92.91ab	0.0356822	0.001
Histidine	90.65a	87.92b	88.13b	90.63a	90.62a	0.0479895	0.001
Isoleucine	91.00abc	89.44bc	89.06c	91.62a	91.19ab	0.0432537	0.004
Leucine	90.37ab	88.83b	88.65b	91.22a	90.47ab	0.0431919	0.005
Lysine	92.21ab	91.59ab	90.77b	93.01a	93.74a	0.0339794	0.003
Methionine	99.55ab	99.06bc	98.47c	99.45ab	100.04a	0.0204844	0.001
Phenilalanine	90.29abc	89.37bc	89.19c	91.69a	91.50ab	0.0440278	0.003
Threonine	89.20ab	86.91bc	85.88c	90.49a	89.24ab	0.0680209	0.001
Valine	90.25a	88.78a	88.41a	90.77a	90.74a	0.0484285	0.022
Total essential amino acids	91.38abc	90.07bc	89.61c	92.19a	91.78ab	0.0417278	0.005
Non-essential amino acids (%)							
Alanine	89.87a	87.32b	87.07b	90.13a	89.55ab	0.0533374	0.001
Aspartic acid	89.26ab	87.64b	87.41b	90.95a	91.41a	0.0576453	0.001
Cysteine	87.51a	85.42ab	83.60b	87.40a	86.84ab	0.0708953	0.019
Glutamic acid	91.63abc	91.13c	91.29bc	93.24a	92.94ab	0.0362221	0.002
Glycine	87.79a	85.26a	85.32a	88.13a	87.43a	0.0584134	0.015
Proline	90.22a	87.83ab	86.99b	90.26a	88.85ab	0.0528881	0.002
Serine	89.21ab	86.84b	87.10b	90.89a	90.29a	0.0618352	0.001
Tyrosine	91.05a	88.73ab	87.78b	90.70a	90.28ab	0.0528718	0.003
Total non-essential amino acids	90.16ab	88.57b	88.41b	91.23a	90.88a	0.0474608	0.002
Total amino acids (%)	90.73abc	89.27bc	88.97c	91.68a	91.30ab	0.0447147	0.002

[a-c]Means in the same row with different letters differ significantly ($P < 0.05$) by Tukey's test.
Abbreviations: E, enzymatic complex.
1PC = positive control (diet meeting the nutritional requirements of broilers).
2NC1 = negative control 1 (PC minus 68 kcal/kg EM; 0,15% AP and 0,16% Ca).
3NC2 = negative control 2 (PC minus 101 kcal/kg EM; 0,15% AP and 0,16% Ca).

However, the adverse effects of phytate on amino acid use are suggested to be largely associated with increased endogenous losses of amino acids in the intestine, rather than with a direct impact on protein retention in the diet (Selle, Creswell, Cadagon, Partridge, & Scott, 2006; Cowieson et al., 2017). Phytate may increase gastric secretions of pepsin and HCl due to the formation of binary protein-phytate complexes in the digestive tract of broilers

(Selle, Cowieson, Cowieson, & Ravindran, 2012). In addition, increased pepsin secretion can trigger higher outflow of protective mucus, resulting in increased mucin secretion in birds (Cowieson, Acamovic, & Bedford, 2004). In this study, phytase supplementation reduced endogenous inputs for protein digestion, which resulted in an improvement in the CSID of the amino acids threonine, leucine, aspartic acid, glutamic acid, glycine, and serine that predominate in endogenous proteins (Truong, Bold, Liu, & Selle, 2015; Siegert et al., 2019; Walk & Poernama, 2018).

Thus, the digestibility of amino acids, such as threonine, serine, aspartic acid, glycine, and leucine, which is substantially improved by phytase, can contribute significantly to the benefit of reduced endogenous flow. However, the improvements in alanine and histidine digestibility, suggested by the data in this study, are not mediated by the reduction in the endogenous flow of amino acids, but must be due to one of the other mechanisms proposed by Rutherfurd et al. (2012).

In addition, some authors consider cysteine, which can be found in the secondary domain of mucin, to be the amino acid most responsive to phytase (Cowieson et al., 2017; Dersjant-Li & Kwakernaak, 2019). However, this amino acid did not respond significantly in this study. The variation in the responses of amino acids may be due to several animal and dietary factors, such as the age of the bird and types of ingredient, level of dietary Ca, source and concentration of phytase, and the presence of antinutritional factors in the diet (Selle & Ravindran, 2007).

In this study, although the phytase, carbohydrase, and protease combination was added to diets with a higher reduction in amino acid specifications, the combined use of these enzymes maintained the standardized ileal digestibility of amino acids at a similar level to that of the phytase-only treatment and the standard diet. Likewise, previous studies have observed the improved “value” of amino acids due to phytase and enzyme complex combinations (Francesch & Geraert, 2009; Gallardo et al., 2019). This improvement was associated with the total or partial breakdown of the cell wall matrix to release starch, fat, protein, minerals, and phytate, allowing access by endogenous enzymes and phytase of their substrates (Slominski, 2011; Karimi et al., 2013; Kiarie, Romero, & Ravindran, 2014).

Conclusion

Supplementation with phytase preserves the performance of broilers fed diets deficient in AP, Ca, and ME and improves the digestibility of amino acids. The addition of phytase in combination with an enzyme complex can increase the growth of birds, although these effects are not always additive.

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