ROMÁRIO DUARTE BERNARDES

EFFECT OF THE COMBINATION OF PHYTASE AND PROTEASE ON THE PERFORMANCE OF BROILERS AND ON THE VALUES OF METABOLIZABLE ENERGY AND AMINO ACID DIGESTIBILITY OF DIETS

Dissertation submitted to the Animal Science Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae.*

Adviser: Luiz Fernando Teixeira Albino
Co-adviser: Arele Arlindo Calderano

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BIOGRAFIA

ROMÁRIO DUARTE BERNARDES, filho de José Martins Duarte Filho e Percília Bernardes Duarte, nasceu em Viçosa MG, em 27 de junho de 1996.

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Em março de 2020 iniciou o curso de Mestrado em Zootecnia na área de Nutrição e Produção de Monogástricos submetendo-se a defesa em fevereiro de 2022.

ABSTRACT

BERNARDES, Romário Duarte, M.Sc. Universidade Federal de Viçosa, February, 2022. **Effect of the combination of phytase and protease on the performance of broilers and on the values of metabolizable energy and amino acid digestibility of diets.** Adviser: Luiz Fernando Teixeira Albino. Co-Adviser: Arele Arlindo Calderano.

Three experiments were carried out to evaluate the effect of dietary combination of different enzymes (phytase + protease) on performance, metabolizable energy and amino acid digestibility of broiler chickens fed diets with nutritional reduction. A total of 1.400, 336 and 384 male chickens were distributed in a completely randomized design, in the experiments of performance, metabolism and digestibility, respectively. The treatments were divided as follows: Positive control (PC), Negative control - NC1 (PC minus 0.16% Ca, 0.15% Pav and 0.5% crude protein (CP)), NC2 (PC minus 0.16% Ca, 0.15% Pav and 1% CP), NC1 added with phytase deriving from citrobacter and protease deriving from Bacillus licheniformis (CBE), NC1 added with phytase deriving from E. coli and protease deriving from microbial fermentation (SE), NC2 added with CBE and NC2 added with SE. A protein-free diet was included in the digestibility experiment. The nutritional restriction did not affect feed intake (FI) of birds in the first experiment; however the restriction inhibited body weight gain (BWG) and feed conversion rate (FCR) in all phases. In experiment two, the nutritional restriction decreased AME and AMEn values, although the addition of phytase and protease in diets had improved both parameters, mainly in NC2. NC2 impaired the standardized digestibility of total essential amino acids of animals subjected to experiment three, although the addition of enzymes helped to recover the digestibility to levels similar to PC. In conclusion, the supplementation of phytase in association with protease is effective to improve the performance, energy metabolism and standardized amino acid digestibility of broilers fed diets with nutrient restriction.

Keywords: Broiler. Digestibility. Enzyme.

RESUMO

BERNARDES, Romário Duarte, M.Sc. Universidade Federal de Viçosa, fevereiro de 2022. **Efeito da combinação de fitase e de protease no desempenho de frangos de corte e nos valores de energia metabolizável e na digestibilidade de aminoácidos das dietas.** Orientador: Luiz Fernando Teixeira Albino. Coorientador: Arele Arlindo Calderano.

Três experimentos foram conduzidos para avaliar o efeito da combinação dietética de diferentes enzimas (fitase + protease) no desempenho, energia metabolizável e digestibilidade de aminoácidos de frangos de corte alimentados com dietas com redução nutricional. Um total de 1.400, 336 e 384 frangos machos foram distribuídos em delineamento inteiramente casualizado, nos experimentos de desempenho, metabolismo e digestibilidade, respectivamente. Os tratamentos foram divididos da seguinte forma: Controle Positivo (CP), Controle Negativo - CN1 (CP menos 0,16% Ca, 0,15% Pav e 0,5% proteína bruta (CP)), CN2 (CP menos 0,16% Ca, 0,15% Pav e 1 % CP), CN1 adicionado de fitase derivada de *citrobacter* e protease derivada de *Bacillus licheniformis* (CBE), CN1 adicionado de fitase derivada de *E. coli* e protease derivada de fermentação microbiana (SE), CN2 adicionado de CBE e CN2 adicionado de SE. Uma dieta isenta de proteína foi incluída no experimento de digestibilidade. A restrição nutricional não afetou o consumo de ração (CR) das aves no primeiro experimento; entretanto a restrição inibiu o ganho de peso corporal (GPC) e a conversão alimentar (TCA) em todas as fases. No experimento dois, a restrição nutricional diminuiu os valores de EMA e EMAn, embora a adição de fitase e protease nas dietas tenha melhorado ambos os parâmetros, principalmente no CN2. CN2 prejudicou a digestibilidade padronizada dos aminoácidos essenciais totais dos animais submetidos ao experimento três, embora a adição de enzimas tenha ajudado a recuperar a digestibilidade para níveis semelhantes ao CP. Em conclusão, a suplementação de fitase em associação com protease é eficaz em melhorar o desempenho, metabolismo energético e digestibilidade padronizada de aminoácidos de frangos de corte alimentados com dietas com restrição de nutrientes.

Palavras-chave: Frango de corte. Digestibilidade. Enzima.

SUMÁRIO

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EFFECT OF THE COMBINATION OF PHYTASE AND PROTEASE ON THE PERFORMANCE OF BROILERS AND ON THE VALUES OF METABOLIZABLE ENERGY AND AMINO ACID DIGESTIBILITY OF DIETS.

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Abbreviations: PC, Positive control; NC1, Negative control 1; NC2, Negative control 2; Ca, Calcium; P, phosphorus; SE, phytase deriving from E. coli and protease deriving from microbial fermentation; CBE, phytase deriving from citrobacter and protease deriving from Bacillus licheniformis; FI, feed intake; BWG, body weight gain; FCR, feed conversion rate; AME, apparent metabolizable energy; AMEn, nitrogen balance-corrected metabolizable energy; CP, crude protein; PFD, protein-free diet; DM, dry matter; EN, Excreted Nitrogen; NCon, nitrogen consumption; RN, Retained nitrogen; NB, Nitrogen balance; GE, Gross energy; HPLC, High-Pressure Liquid Chromatography; AIA, acid-insoluble ash; DMI, dry matter intake;

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1. INTRODUCTION

Some antinutritional factors observed in plant-origin ingredients, such as phytate, can limit the use of nutrients in poultry diets and reduce animal performance (Gautier et al., 2018).

Phytate is an indigestible compound capable of forming complexes with minerals and proteins, a fact that impairs their digestion and absorption (Jain et al., 2016). Its incidence in diets limits the use of minerals such as phosphorus, which plays essential role in animal development (Humer et al., 2014). In addition, it limits the use of energy, as well as amino acid digestibility, not only because it bonds to nutrients, or because it hinders the access of digestive enzymes to the bolus, but also due to endogenous losses (Selle et al., 2006) caused by its aggression towards the intestinal mucosa.

Dietary supplementation with exogenous enzymes is often carried out to mitigate these effects, since they act by improving nutrient digestibility and the use of energy deriving from animal feed (Yang et al., 2010; Hahn-Didde, Purdum, 2014). Phytase is an example of exogenous enzyme often used in poultry diets. It derives from genetic changes taking place in microorganisms, mostly in fungi or bacteria belonging to fungal genera Aspergillus niger and Peniophora lycii and to bacterial genus Escherichia coli (Jain et al., 2016).

Phytase acts by hydrolyzing phytic phosphorus; this process results in greater bioavailability of this nutrient, as well as of other minerals such as calcium, magnesium, potassium, and zinc, and it also improves amino acid digestibility due to release of molecules linked to hexaphosphate-inositol (Dersjant-Li, et al., 2014). Protease supplementation in low-protein diets has beneficial influence on broilers' performance (Jiang et al., 2020); however, studies focused on investigating the effect of phytase and protease combination on broiler-associated parameters remains scarce in the literature. Assumingly, phytase acts in protein solubility and digestion processes, whereas protease influences amino acid recovery ratesin the intestine (Cowieson; Roos, 2016).

The hypothesis of the present study is that dietary supplementation with phytase+ protease can help improving the performance of broilers fed on diets with reduced nutritional levels. Thus, the aim was to evaluate the effect of combining different enzymes (phytase + protease) in broiler diets on their performance, metabolizable energy values and amino acid digestibility.

2. MATERIALS AND METHODS

All procedures adopted in the current study were previously evaluated and approved by the ethics committee on the use of farm animals CEUAP/UFV (Registration protocol: 0123/2019); they were in compliance with the ethical principles of animal experimentation established by the National Council for the Control of Animal Experimentation (CONCEA). Experiments were carried out in Viçosa County, Minas Gerais State, Brazil (20°45’57.19’’S, 42°51’35.42’’W; and 682 m altitude).

2.1. ENZYMES

Combinations of enzymes produced by two different companies operating in the Brazilian market were used in the experiments. The first combination comprised phytase-6 deriving from bacterial species Citrobacter braakii- expressed in fungal species Aspergillus oryzae, as well as protease resulting from serine protease preparation (E.C.3.4.21.), which is produced by a genetically modified Bacillus licheniformis strain. The acronym adopted for this combination was (CBE). The second combination encompassed phytase-6 deriving from E. Coli, which was produced based on submerged fermentation and on special granulation technology, as well as thermostable protease deriving from the microbial fermentation of refined advanced strains capable of acting at wide temperature (from 35°C to 42°C) and pH (3.5-7.5) ranges - the acronym set for this combination was (SE).

Phytases are enzymes capable of hydrolyzing the phytic acid molecule to myo-inositol and inorganic phosphates (Pi), which consequently eliminates its anti-nutritional characteristic and makes it available to the animal. Phytase-6 has the ability to completely dephosphorylate phytic acid (Scottá et al., 2014). With regard to microbial proteases, the mechanism of action occurs through competition with trypsin inhibitors for active sites, which consequently reduces the activity of these factors and improves the use of protein and amino acids by the animal. However, these factors can be inactivated as well as eliminated (Aderibigbe et al., 2020).

2.2. EXPERIMENTAL DESIGN, DIETS AND ANIMALS

Experimental treatments applied in the present research comprised positive control (PC; basal diet); negative control 1 (NC1; PC minus 0.16% Calcium, 0.15% available phosphorus and 0.5% crude protein); NC1 added with CBE; NC1 added with SE; negative control 2 (NC2; PC minus 0.16% calcium, 0.15% available phosphorus and 1% crude protein); NC2 added with CBE; NC2 added with SE;

The positive control diet was formulated in compliance with nutritional recommendations by Rostagno et al. (2017); it was divided into initial diet (provided to broilers at the age of1 to 21 days) and growing/finishing diet (provided to them at the age of 22 to 42 days), based on corn and soybean meal (Table 1). The nutritional level of the diet adopted in the positive control was reduced in diets adopted for negative controls. Enzymes were added "*on top*" to the experimental diets by taking into consideration the following values: phytases (50 g/ton) and proteases (200 g/ton).

Table 1. Ingredients and nutrient composition of experimental diets.

Ingredients (%)	Starter (1-21 days)			Growth (22-42 days)		
	PC	NC1	NC2	PC	NC1	NC2
Corn	50.579	54.409	56.313	50.579	62.110	63.758
Soybean meal	41.422	39.183	37.482	32.892	31.271	29.854
Soy oil	3.847	2.960	2.592	4.549	3.791	3.492
Dicalcium phosphate	1.786	0.986	0.997	1.489	0.685	0.694
Limestone	0.924	1.038	1.045	0.715	0.826	0.832
Salt	0.515	0.515	0.516	0.472	0.472	0.472
DL-Methionine, 99%	0.318	0.296	0.310	0.252	0.224	0.236
BioLis. 54.5%	0.136	0.176	0.226	0.253	0.256	0.317
L-Threonine, 98%	0.048	0.012	0.022	0.030	0.012	0.022
Vitamin supplement[1]	0.130	0.130	0.130	0.130	0.130	0.130
Mineral supplement[2]	0.130	0.130	0.130	0.130	0.130	0.130
Choline Chloride, 60%	0.100	0.100	0.100	0.100	0.100	0.100
Salinomycin[3] (12%)	0.055	0.055	0.055	0.055	0.055	0.055
Antioxidant (BHT)	0.001	0.001	0.001	0.001	0.001	0.001
Total	100	100	100	100	100	100
Calculated nutritional composition						
EM. Kcal/kg	3000	3000	3000	3150	3150	3150
Crude protein, %	23.23	22.52	22.06	20.00	19.50	19.00
Calcium, %	0.937	0.777	0.777	0.758	0.598	0.598
Available phosphorus, %	0.440	0.290	0.290	0.374	0.224	0.224
Sodium, %	0.218	0.218	0.218	0.200	0.200	0.200
Digestible Arginine, %	1.460	1.404	1.404	1.224	1.184	1.145

Digestible Gly+Ser., %	1.871	1.808	1.808	1.595	1.551	1.507
Digestible Lysine, %	1.256	1.238	1.238	1.070	1.052	1.052
Digestible Met.+Cys., %	0.929	0.893	0.893	0.792	0.756	0.756
Digestible threonine, %	0.829	0.761	0.761	0.706	0.662	0.645
Digestible tryptophan, %	0.267	0.257	0.248	0.224	0.216	0.209
Digestible Valine, %	0.967	0.936	0.926	0.827	0.805	0.783

[1]Vitamin Supplement - Guarantee levels per kg of feed: Vit. A- 9,375 IU; Vit. D3- 2,375 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- 37.5 mg; B.C. Pantothenic acid- 12.5mg; Vit. K3- 1.88mg; B.C. Folic acid- 0.875mg; Biotin- 0.088 mg. [2]Mineral Supplement - Guarantee levels per kg of feed: Selenium- 0.375 mg; Manganese- 88mg; Iron- 62.5 mg; Zinc- 81.3 mg; Copper-12.5 mg; Iodine- 1.25 mg. [3]Anticoccidia.

In total, 1,400 male Cobb 500 chicks in the age group 01 to 42 days were used to assess animals' performance. They were weighed and distributed into seven treatments by following a completely randomized design, with 10 repetitions and 20 birds per experimental unit. Chicks were housed in masonry shed divided into 1.0 x 2.0 m boxes lined with wood shavings. Animals were subjected to 24-hour light program at 32ºC in their first week of life; this time was gradually shortened based on recommendations in the Cobb® lineage manual. Birds had access to feed and water *ad libitum* throughout the experimental period; the maximum and minimum temperatures inside the facilities were recorded on a daily basis by three thermometers positioned at strategic points, at birds' height.

In total, 720 male Coob 500 chicks were used in experiments conducted to determine apparent metabolizable energy value (AME), nitrogen balance-corrected apparent metabolizable energy value (AMEn) and ileal amino acid digestibility coefficient. Protective circles were lined with wood shavings and equipped with tubular feeders and pendular drinkers to provide birds with free access to water and food. Animals fed on the initial basal diet based on corn and soybean meal, according to recommendations by Rostagno et al. (2017). A 24-hour light program was carried out at ambient temperature of 32°C in the first experimental week; this time was gradually shortened based on recommendations in the Cobb® strain manual.

In total, 336 chicks (14 days old) were weighed and transferred to metallic cages with two-floor compartments, which were arranged in a 68-m2 room with ceiling height of approximately 2.8 m; it was done to determine apparent metabolizable energy value (AME) and nitrogen balance-corrected apparent metabolizable energy value (AMEn). Cages were equipped with nipple drinkers and chute feeders. The adopted treatments were the same as in experiment one, but this one comprised 8 repetitions and 6 birds per experimental unit. The diet provided to the animals was the same diet initially described in Table 1. The experimental period took place from chicks' 14th to 23rd day of life; it adopted 5 days for animals' adaptation and 5 days for total excreta collection in each experimental unit - and 12-hour interval between collections. Plastic-coated aluminum trays were placed under the cages for excreta collection purposes. Collected excreta were placed in plastic bags, identified based on experimental unit, and kept in freezer until the end of the collection period. Feed consumption was measured during the excreta collection period.

In total, 384 chicks (in the age group 18 to 23 days of life) were distributed in a completely randomized design, with 8 treatments and 8 repetitions- with 6 birds per experimental unit- to determine the apparent and standardized digestibility coefficients of amino acids in the diets. Treatments comprised all 7 experimental feeds used in the performance experiment, as well as a protein-free diet (PFD), to determine endogenous losses.

2.3. PERFORMANCE, METABOLIZABLE ENERGY AND AMINO ACID DIGESTIBILITY.

Body weight, feed intake and left over feed rates were recorded for animals (at the ages of 1, 21 and 42 days) in each experimental unit to determine body weight gain (BWG), mean feed intake (FI) and feed conversion (FCR) during experimental periods 1-21 and 1-42 days.

Excrements collected in each cage were weighed at the end of the experimental period and homogenized for the energy test. In order to do so, 200-g samples were pre-dried at 55°C for 72 hours and ground in ball mill (Tecnal Equipamentos para Laboratório, TE-350, São Paulo, Brazil) for 5 minutes, until it turned into a fine mix.

Animals subjected to the digestibility trial were slaughtered to enable collecting the ileal digesta at their 23rd day of life. Their abdominal cavity was opened and all intestinal contents found 40 cm away from the terminal ileum, anterior to the ileocecal junction, were removed. The ileal digesta of animals used in each repetition was combined to form a composite sample for each treatment. Ileal digesta samples were lyophilized at -40°C for 72 hours.

2.4. CHEMICAL ANALYSIS

Diets and excreta were analyzed to determine dry matter (DM) and crude protein (CP) rates (AOAC, 1990). The Kjeldahl method was used to determine nitrogen levels, both in the diets and the excreta, based on official analysis methods (AOAC, 1990). Excreted Nitrogen (EN) was calculated by multiplying the total excretion amount (in DM) by the nitrogen rate found in the excretion (also in DM). The same method was applied to calculate nitrogen consumption (NCon). Retained nitrogen (RN) was calculated by subtracting EN from NCon. Retained nitrogen rate (%RN) was calculated by taking into consideration the amount of nitrogen that was consumed. Nitrogen balance (NB) was obtained based on the amount of consumed nitrogen, minus the excreted nitrogen. Gross energy (GE) values were determined by using a C5001 adiabatic calorimetric pump (IKA-Werke GmbH & Co. KG, Staufen, Germany). AME and AMEn values were calculated based on GE values recorded for food and excreta by using the equation described by Sakomura and Rostagno (2016).

The equations are:

AME= (GE ing – GE exc)/DM ing and

AMEn= (GE ing – GE exc – (8.22*NB))/DM ing

Where:

GE ing = Gross energy ingested;

GE exc = Gross energy excreted;

DM ing = Dry matter ingested;

DM, CP, fecal indicator and indigestibility factor (AOAC, 1990) of ileal digesta collected from broiler chickens were analyzed for digestibility calculation purposes. The laboratory analyses based on HPLC (High-Pressure Liquid Chromatography) were performed to determine amino acid contents in animals' diets and excreta. Apparent and standardized amino acid digestibility rates were calculated by using the acid-insoluble ash (AIA) indigestibility factor, based on equations presented in the book titled “Métodos de Pesquisa em Nutrição de Monogástricos” [Research Methods on Monogastric Nutrition] (Sakomura & Rostagno, 2016). AME, AMEn, and amino acid digestibility coefficients were calculated based on the equations described by Sakomura and Rostagno, (2016).

The equations are:

CDAAapa= ((AA ing – (AA dig * FI1))/AA ing)*100

CDAAest= (AA ing – ((AA dig * FI1) – (AA end* FI2))/ AA ing)* 100

Where:

CDAAapa= apparent amino acid digestibility coefficient;

AA ing= ingested amino acid;

AA dig= digesta amino acid;

FI1= indigestibility factor 1; FI1= AIA diet/ AIA digesta;

FI2= indigestibility factor 2; FI2= AIA protein-free diet/ AIA digesta;

CDAAest= standardized amino acid digestibility coefficient;

AA end= endogenous amino acid;

2.5. STATISTICAL ANALYSIS

The here in adopted statistical model was:

$$Yik = \mu + \tau i + \varepsilon ik$$

Where in:

Yik= value recorded for the response variable observed in the k^{th} repetition of the i^{th} level of the tested factor;

μ = mean value recorded for treatments;

τi = effect of the i^{th} level of the tested factor;

εik = experimental error associated with the observed yik value.

All collected data were subjected to analysis of variance at 5% significance level; it was done by using the ExpDes.pt package of the R statistical software (R Software v. 4.0.4). Data were subjected to Shapiro-Wilk test to determine residuals’ normality; subsequently, they were subjected to analysis of variance (ANOVA).

Dunnett's test at 5% significance level was used to compare means recorded for the control treatment to those of other treatments.

3. RESULTS

3.1. PERFORMANCE

There were no differences amoung the treatments on FI of chickens in starter phase (from 01 to 21 days old). Negative controls have shown lower BWG results, where as enzymes' addition to the diets did not lead to BWG results different from those of PC. All treatments, except for NC1 + SE, recorded worse FCR results than PC.

There were no difference between the treatments and the PC on FI for the whole experimental period (01 to 42 days). NC2 was the only treatment showing difference in BWG during this period; it recorded the worst BWG results. NC1 and NC2 recorded worse FCR results than PC, whereas the other treatments did not show difference in this parameter (Table 2).

Table 2. Results observed for parameters such as feed intake (FI), body weight gain (BWG) and feed conversion rate (FCR) at all three performed experimental stages.

Treatments	Variables from 1 to 21 days			Variables from 1 to 42 days		
	FI (kg)	BWG (kg)	FCR	FI (kg)	BWG (kg)	FCR
PC	1.227	1.049	1.170	4.920	3.245	1.516
NC1	1.243	0.990*	1.256*	4.941	3.146	1.571*
NC1+ CBE	1.250	1.033	1.209*	4.900	3.167	1.547
NC1+ SE	1.238	1.035	1.196	4.942	3.267	1.513
NC2	1.197	0.974*	1.230*	4.769	3.044*	1.567*
NC2 + CBE	1.256	1.035	1.214*	4.933	3.206	1.540
NC2 + SE	1.283	1.057	1.214*	4.954	3.241	1.528
SEM	0.0068	0.0059	0.0046	0.0184	0.0141	0.0044
P-Value	0.3434	0.0464	<0.001	0.7923	0.0489	0.0112

Mean followed by * in the same column differ from CP, based on Dunnett's test, at 5% significance level ($P<0.05$); FI= feed intake; BWG= body weight gain; FCR= feed conversion rate; PC= positive control; NC1= negative control 1; NC2= negative control 2; CBE= phytase deriving from citrobacter added with protease deriving from Bacillus licheniformis; SE = E. coli-derived phytase added with microbial fermentation protease; SEM= standard error mean.

3.2. METABOLIZABLE ENERGY

Treatments NC1 and NC2 recorded AME and AMEn values lower than those of PC. The addition of both enzymes to the diet provided in NC1 helped improving these parameters, which achieved values similar to those of PC. Enzymes' addition to the diet in NC2 led to results higher than those recorded for PC.

Treatments NC1 and NC1 + CBE recorded nitrogen consumption (NCon) similar to that of PC, whereas the other treatments recorded lower NCon values than it. NC1 was the only treatment recording EN similar to that of PC, all other treatments recorded lower EN values than it. Retained nitrogen (RN) in NC1 + CBE recorded higher values than that of PC, whereas the other treatments did not show difference in this parameter (Table 3).

Table 3. Results observed for parameters such as apparent metabolizable energy value (AME), metabolizable energy value corrected for nitrogen balance (AMEn), nitrogen consumption (NCon), excreted nitrogen (EN) and retained nitrogen (RN).

Treatments	Variables					
	DMI (kg)	AME (kcal/kg)	AMEn (kcal/kg)	NCon (g/bird)	EN (g/bird)	RN (%)
PC	0.398	3498.70	3276.80	16.38	5.65	65.52
NC1	0.403	3243.40*	3026.00*	16.05	5.38	66.45
NC1+ CBE	0.397	3549.60	3323.00	15.82	4.65*	69.27*
NC1+ SE	0.383	3548.20	3331.50	15.22*	4.87*	66.25
NC2	0.399	3391.00*	3198.50*	14.29*	4.94*	65.42
NC2+ CBE	0.403	3625.30*	3431.60*	14.11*	4.61*	67.33
NC2+ SE	0.384	3631.90*	3441.20*	13.81*	4.91*	64.44
SEM	0.0022	18.9639	19.2461	0.1474	0.0654	0.3248
P-Value	0.6139	0.0385	0.0394	<0.001	<0.001	<0.001

Mean followed by * in the same column differ from CP, based on Dunnett's test, at 5% significance level (P<0.05); AME= apparent metabolizable energy; AMEn= nitrogen balance-corrected apparent metabolizable energy; NCon= nitrogen consumption; EN= Excreted Nitrogen; RN= Retained nitrogen; PC= positive control; NC1= negative control 1; NC2= negative control 2; CBE= phytase deriving from citrobacter added with protease deriving from Bacillus licheniformis; SE = E. coli-derived phytase added with microbial fermentation protease; SEM= standard error mean.

3.3. AMINO ACID ILEAL DIGESTIBILITY COEFFICIENT

Both negative control treatments impaired the digestibility of amino acids such as arginine, histidine, leucine, lysine and threonine; these two treatments recorded amino acid digestibility results lower than that of PC; however, the addition of both enzymes to animals' diets was capable of improving the digestibility coefficient of all the afore mentioned amino acids. Nutritional reduction did not affect the isoleucine amino acid digestibility coefficient, where as the addition of both enzymes to the investigated diets helped improving these coefficients, which reached values higher than those of PC.

The methionine amino acid digestibility was only impaired in NC2; however, enzyme addition to the diet was capable of improving this parameter. NC2 was the only group recording total essential amino acid digestibility lower than that of PC (Table 4).

Table 4. Results observed for standardized digestibility coefficient of essential amino acids.

Variables	PC	NC1	NC1 +CBE	NC1 + SE	NC2	NC2 + CBE	NC2 + SE	SEM	P-Value
Arginine	94.75	93.27*	94.31	95.21	92.83*	94.99	93.76	0.1508	0.0292
Histidine	92.13	90.32*	92.42	94.01*	89.49*	92.89	92.69	0.2291	0.0421
Isoleucine	93.69	94.05	95.30*	96.76*	93.17	95.76*	95.25*	0.1946	<0.001
Leucine	92.40	90.79*	93.52	94.98*	89.93*	94.04*	93.86*	0.2597	0.0227
Lysine	93.90	92.61*	94.65	95.46*	92.50*	95.59*	94.02	0.1838	<0.001
Methionine	97.89	97.64	98.07	98.71	96.13*	98.89*	98.22	0.1392	0.0376
Phenylalanine	93.17	92.07	94.01	95.46*	91.52*	94.60*	94.22	0.2045	0.0108
Threonine	91.10	88.82*	90.61	93.25	86.83*	91.51	91.03	0.3229	0.0123
Valine	91.98	91.39	92.68	94.39*	90.01*	93.23	92.24	0.2185	0.0405
Total Essential aa	92.75	92.19	93.91	95.27*	91.33*	94.60*	93.88	0.2029	0.0443

Mean followed by * on the same line differ from PC, based on Dunnett's test, at 5% significance level (P<0.05); NC1= negative control 1; NC2= negative control 2; CBE= phytase deriving from citrobacter and protease deriving from Bacillus licheniformis; SE = E. coli-derived phytase and microbial fermentation protease; SEM= standard error mean.

4. DISCUSSION

Significant nutritional reductions without enzyme supplementation can have negative impact on broiler performance, mainly by reducing BWG and FI, and by hindering FCR. The present study recorded decreased PB, P and Ca levels in the analyzed animals; these nutrients play essential role in several metabolic functions in their body. Phosphorus and calcium are essential minerals involved in several metabolic processes such as skeletal development, energy transfer, enzyme activation and basic acid balance, among others (Jlali et al., 2020; Bavaresco et al., 2020). Protein reduction can lead to higher body fat deposition (Chrystal et al. 2020) and result in worsened weight gain and poor feed conversion.

Results observed for BWG and FCR in the stage from 1 to 21 days have explained the enzymes' effect on the assessed parameters, since the reason for using them is to make dietary nutrients available, and to improve their use, to enable better weight gain and feed conversion (Babatunde et al., 2022). The combined use of enzymes may have improved the availability and absorption of nutrients that were not available in traditional feed types, since it promoted the use of amino acids that play important role in animal growth. Among them, one finds lysine, which plays essential role in the synthesis of muscle proteins and acts in the formation of structures such as collagen and digestive enzymes. Results recorded in the stage from 1 to 42 days were similar to each other, for the same reason.

Previous studies about the use of phytase + protease have shown controversial results. According to Cowieson et al. (2019), supplementation with phytase + protease can help improving BWG and FCR, where as Walk and Poernama (2019) have shown that protease supplemented with phytase did not change animals' performance results. Kamel et al. (2015) used protease in diets presenting reduced nutritional levels and concluded that such a supplementation improved parameters such as animals' performance, villus height and villus/crypt ratio. Proteases have proteolysis functions which are characterized by the breakdown of peptides bond of proteins chain. Proteases are classified as endopeptidases or exopeptidases, in other words, they will act hydrolyzing the peptides chains in smaller molecules or hydrolyzing the carboxyl-terminal domain releasing free amino acids, thus, improving the nutrient utilization and animal development (Sakomura et al., 2014).

Results recorded for energy metabolism can be explained by the presence of phytate in the diet, since it can limit the use of phosphorus (Dersjant-Li et al., 2014), which plays key role in energy metabolism processes. Using phytase enables higher P availability to act in energy metabolism, as seen in AME and AMEn values recorded in the current study. Using phytase can also lead to increased use of dietary energy; it mainly happens because phytase favors mucin availability due to higher cell turnover, which is triggered by its presence in the diet (Bao et al., 2013; Cowieson et al., 2017). Furthermore, phytase can act by preventing the formation of binary protein-phytate complexes through hydrolysis, by reducing endogenous protein flow and nitrogen losses, as well as by increasing nitrogen retention (Cowieson et al., 2017; Gallardo et al., 2020).

The incidence of phytate in birds' diet leads to amino acids' complexation, endogenous enzymes' inhibition and to increased endogenous amino acid secretions, a fact that affects their digestibility (Bao et al., 2013). Based on results in the current study, treatments without enzyme addition have shown amino acid digestibility deterioration, where as phytase + protease addition to treatments presenting reduced nutritional levels has improved the digestibility of all essential amino acids.

The importance of essential amino acids lies on the fact that they are not synthesized by the body fast enough to meet maximum performance requirements; thus, they mustbe supplied via diet, which consequently promotes their good digestibility and enables them to perform their functions in the body (Bertechini, 2012). Results observed in the current study based on phytase + protease combination can be explained by the fact that phytase has increased pepsin and trypsin activity (Murugesan et al., 2014), and the microbial protease competed with trypsin inhibitors for the binding sites, which consequently decreased the activity of these antinutritional factors and increased the protein and amino acid utilization by the animal (Aderibigbe et al., 2020). These two enzymes, which play essential role in protein digestion, are inhibited by the presence of phytate in the diet. Protease acts by increasing amino acid digestibility, as well as by improving intestinal parameters, and it can lead to better absorption of nutrients such as amino acids (Kamel et al., 2015).

Another factor linked to phytase addition to birds' diet lies on the fact that this enzyme is associated with lower loss of endogenous amino acids (Yueming et et al., 2014; Gallardo et al., 2020). It happens because phytic acid associates with basic amino acid residues, such as lysine, arginine and histidine, to form large insoluble aggregates when birds eat the prepared diet and it reaches the gastric digestion stage. This effect encourages birds to secrete hydrochloric acid and pepsin in order to restore food solubility and digestibility; this process increases the need of gastric and intestinal mucin, as well as of sodium bicarbonate, to ensure intestinal integrity. Thus, the negative effect of phytic acid on amino acid digestibility is significantly associated with higher loss of endogenous amino acids in the intestine, rather than with direct impacton dietary protein digestibility. This very same factor is also linked to the use of protease in animals' diet since, according to Cowieson and Roos (2016), protease helps reducing endogenous amino acid losses.

5. CONCLUSION

The dietary combination of phytase and protease is effective to improve performance, metabolizable energy and amino acid digestibility of broiler chickens fed nutrients-deficient diets. Although all assessed enzymatic combinations shows good results, the SE combination shows better results in majority of assessed parameters. Therefore, the SE enzymatic combination is recommended for broiler chickens fed diets with nutrient deficiencies.

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