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Addition of Amylase from Aspergillus Awamori to the Diet of Broiler Chickens

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ABSTRACT

Two experiments were performed to evaluate the hematological and blood biochemistry parameters, biometry of digestive organs, enzyme activities, protein content and absolute weight of the pancreas of broilers fed pre-starter and pre-starter diets supplemented or not with amylase from *Aspergillus awamori*. In total, 120 male Cobb chicks were housed in heated cages in each experiment. A completely randomized experimental design, with two treatments (feed with and without amylase) and six replicates per treatment of 10 birds each was applied. The data were subjected to analysis of variance using the F-test at 5% probability level. The dietary amylase addition did not affect hematological and blood biochemistry parameters and the biometry of the gastrointestinal tract of 7- and 21-d-old broilers, nor the absolute weight, enzyme activities or protein concentration of the pancreas of 7-d-old broilers. However, the inclusion of amylase in the diet reduced amylase activity and pancreatic protein concentration in 21-d-old broilers. The application of amylase to broiler chicken pre-starter and starter feeds is not justified given the pancreatic amylase activity and protein concentrations.

INTRODUCTION

Avian hematology is a guiding tool in various physiological situations, but hematology alone does not provide sufficient information to determine the factors that affect the development of animals (Noriega, 2000). Advances in this area still hold opportunities for research, especially studies on the influence of species, breed, age, and other factors on hematological parameters (Borsa *et al.*, 2006). Moreover, blood parameters must be determined under the conditions the animal is reared (Borsa, 2006; Minafra *et al.*, 2010).

The cardiovascular system of birds is closed and represents approximately 7% body weight of an individual (Macari & Luquetti, 2002). The cardiovascular system is particularly sensitive to physiological changes, reflecting the health status of an animal. It is influenced by several factors, such as nutrition, climate and management, and therefore, it is an important indicator of the metabolic response of poultry (Minafra *et al.*, 2010). Possibly due to those factors, the blood parameter values reported by various authors are highly variable (Borsa, 2009). The hemogram is an important tool to diagnose the physiological status of animals. According to Nakage (2007), the hematological profile of broilers has only recently begun to receive greater attention from researchers.

The growth rate exceeds the rate of weight gain during the development and maturation of the digestive system (Jin *et al.*, 1998), influencing digestive processes, including enzyme production, and lipid digestion and absorption (Lima *et al.*, 2007).

The role of the pancreas enzymes for the digestion of proteins, carbohydrates, and lipids is essential. Another important function of the pancreas is the secretion of proteolytic enzymes – primarily trypsin – that break down proteins (Furlan *et al.*, 2002).

According to Moran Jr. (1985) and Sakomura *et al.* (2004), the processes of adaptation and maturation of intestinal absorptive cells, the maturation of the organs that produce and release digestive enzymes, feed types, nutrient chemical composition and complexation are important factors that affecting lipid digestion.

The objective of this study was to evaluate the effects of the inclusion of an amylase produced by *Aspergillus awamori* in broiler starter and pre-starter diets on the hematological and blood biochemical parameters, biometry of the digestive organs, and enzyme activities, protein content, and absolute weight of the pancreas.

MATERIALS AND METHODS

This study was approved by the Research Ethics Committee of the Federal Institute of Education, Science and Technology of Goiás (*Instituto Federal de Educação, Ciência e Tecnologia Goiano*) on 12 May 2011, under number 006/2012.

The amylase was produced in the Enzymology and Digestive Physiology Laboratories of the Institute of Biological Sciences (Instituto de Ciências Biológicas/ ICB II), of the Federal University of Goiás (*Universidade Federal de Goiás*), using an *Aspergillus awamori* strain obtained from the Molecular Biology Laboratory of the University of Brasília (*Universidade de Brasília – UnB*).

Two experiments were carried out. In total, 120 male Cobb broilers were used in each experiment. Birds were distributed in a completely randomized experimental design with two treatments, six replicates and 10 chicks per experimental unit. The average body weights of the birds were 41.83 ± 1.28 g and 153.9 ± 14.6 g on days 1 (phase 1) and 8 (phase 2), respectively. Birds were housed in three four-tiered galvanized steel cages with 0.80 x 0.75 x 0.25 m (l x w x h) divisions each, equipped with trough drinker and feeders. Cages were located in a brick house of Poultry Production Section of the Federal Institute of Goiás - Ceres campus, surrounded by a 0.50 m high concrete wall and a wire mesh, protected from the external environment by a woven plastic curtain. The roof height was 1.80 m.

The poultry house and cages were cleaned and disinfected according to general standards prior to the arrival of the chicks. Each cage level was heated by a 100-W incandescent lamp on days 1-7 (phase 1) and with a 60 W bulb on days 8- 21 (phase 2) of the rearing period. House temperature and humidity were daily monitored using a minimum and maximum thermometer (Incoterm®) and a digital thermometer (Incoterm®). When necessary, curtains were moved to provide adequate temperature to the birds. Feeders were filled three times a day, and water troughs were filled two times a day. Metallic trays were placed under the trays to remove the excreta.

The experimental feeds (Table 1) were based on corn and soybean meal, and formulated according to the nutritional recommendations and feed composition proposed by Rostagno *et al.* (2011). Feeds were manufactured in the experimental feed mill of the School of Veterinary and Animal Science of the Federal University of Goiás.

Table 1 – Composition (%) and calculated nutritional levels of the pre-starter and starter diets.

Ingredient	Pre-starter (1 to 7 days)	Starter (8 to 21 days)
Corn	55.24	59.35
Soybean meal	38.26	34.78
Vegetable oil	2.24	2.21
Dicalcium phosphate	1.91	1.51
Limestone	0.90	0.91
Salt	0.50	0.47
DL-Methionine	0.36	0.29
L-Lysine HCL	0.28	0.22
L-Threonine	0.11	0.06
Vitamin supplement[1]	0.10	0.10
Trace mineral supplement[1]	0.05	0.05
Anticoccidial agent[2]	0.05	0.05
Total	100.00	100.00
Calculated Nutritional Level		
Crude protein (%)	22.2	20.8
Metabolizable energy (Mcal/kg)	2.95	3.00
Dig. Lysine - poultry (%)	1.31	1.17
Dig. Threonine - poultry (%)	0.85	0.76
Methionine + Cystine (%)	0.94	0.84
Dig. Tryptophan - poultry	0.25	0.23
Calcium (%)	0.92	0.81
Available phosphorus (%)	0.47	0.39
Chlorine (%)	0.33	0.31
Sodium (%)	0.22	0.21

[1]Vitamin and trace mineral supplements (guaranteed levels per kg of product): Vitamin A 11,000,000 IU; Vit. D3 2,000,000 IU; Vit. E 16,000 IU; Vit. B1 1,200 mg; Vit. B2 4,500 mg; Vit. B6 2,000 mg; Vit. B12 16,000 mcg; Pantothenic Acid 9,200 mg; Biotin 60 mg; Niacin 35,000 mg; Selenium 250 mg; Copper 18,000 mg; Iron 60,000 mg; Iodine 2,000 mg; Manganese 106,000 mg; and Zinc 120,000 mg.

[2]Monensin (Coban®200, Elanco)

The pre-starter (phase 1) and starter (phase 2) diets were manufactured 24 hours before the beginning of each experiment to prevent fungal contamination. In both experiments, the control diet included all

ingredients (Table 1). The other diet was manufactured without soybean meal, which was daily sprayed with 120 mL amylase/kg diet, and then added to the remaining ingredients and homogenized. Water and feed were offered *ad libitum* in both experiments.

Hematological and blood biochemistry parameters

At 21 days of age, 4 mL of blood was collected from two birds per treatment (representing the average of the group) by venipuncture of the brachial vein using disposable syringes and needles. Two mL of blood per bird were stored in a tube with EDTA (ethylenediaminetetraacetic acid) to determine the counts of total leukocytes, erythrocytes, thrombocytes, hemoglobin concentration, and packed cell volume and 2 mL were stored in a tube with a coagulation activator for serum separation and quantification of serum chemistry profile.

The analyses were performed at the Clinical Pathology Laboratory of the Veterinary Hospital of the School of Veterinary and Animals Sciences of the Federal University of Goiás.

Packed cell volume was obtained by filling capillary tubes up to 2/3 of their volume with blood. The tubes were then flame sealed using a Bunsen burner and centrifuged at 15,000 rpm for five minutes. The capillaries with the blood sediment were then placed on a measurement chart calibrated from 0 to 100 (%) and the packed cell volume was estimated.

Hemoglobin was measured by the cyanomethemoglobin method, using the Hemoglobina Labtest® reagent and the BIO-2000 Bioplus® semiautomatic biochemical analyzer.

Total leukocyte, erythrocyte and thrombocyte counts were determined using 20 µL of the blood sample diluted in 1.98 mL of Natt-Herrick solution and, after five minutes, the suspension was homogenized, and the Neubauer chamber was filled. The cells were counted under a light microscope at 40X magnification.

Based on obtained erythrocyte count, hemoglobin concentration, and packed cell volume, Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), and the Mean Corpuscular Hemoglobin Concentration (MCHC) were calculated. Mean corpuscular volume (MCV) estimates the average size (volume) of the erythrocytes in femtoliters (fL). Mean corpuscular hemoglobin (MCH) reflects the average weight of hemoglobin in the erythrocyte in picograms (pg). The average corpuscular hemoglobin concentration (MCHC) evaluates the percentage (%) of hemoglobin found in the erythrocytes.

Total protein, albumin, cholesterol, triglyceride, uric acid, creatinine, calcium ASX, aspartate aminotransferase (AST), alanine aminotransferase (ALT), α-amylase, phosphorus, magnesium and chloride levels were measured using Biotécnica® reagents and a COBAS MIRA Plus automated biochemical analyzer from Roche®.

Biometry of the digestive organs

At seven and 21 days of age, one bird per replicate, representing the average weight of the experimental unit, was selected, subjected to fasting for six hours, and sacrificed by cervical dislocation. Gastrointestinal tract (GIT) viscera were collected and the following measures were taken:

- liver weight – weight of the liver with the gall bladder;
- pancreas weight – after separation from the duodenal loop;
- small intestine weight – including the beginning of the duodenum until the end of ileum;
- large intestine weight – including the ceca, colon and rectum;
- gizzard (with remaining content) weight – the gizzard was collected after measuring GIT length;
- proventriculus weight – separated after measuring GIT length;
- small intestine length – measured from the end of the gizzard to the beginning of the ceca;
- large intestine length – including the colon and rectum.

The values obtained were used to calculate relative organ weight according to the formula: Relative organ weight = (organ weight/live weight) x 100.

Enzyme activities, protein content, and absolute weight of the pancreas

The pancreases of the birds sacrificed for the biometric studies were used to determine amylase and lipase enzyme activities, and pancreas protein content and absolute weight. Immediately after collection, the pancreases were frozen in liquid nitrogen. Subsequently, the pancreas of each individual was ground, and homogenized with 1 mL of distilled water. The sample was centrifuged at 8000 rpm at 40°C for 10 minutes. The supernatant was collected and protein concentration and enzyme activities were measured in triplicate. All of the procedures were performed on ice bath with distilled water. The analyses were performed at the Laboratory of Enzymology and Digestive

Physiology of the Institute of Biological Sciences of the Federal University of Goiás, according to the methodologies of the Doles® kits.

Statistical analyses

The statistical analyses were performed using SISVAR software (Ferreira, 2007). The data pertaining to the hematological and blood biochemistry parameters blood, digestive organ biometry, and pancreas enzyme activities, protein content, and absolute weight were subjected to analysis of variance and the F-test at 5% probability level.

RESULTS AND DISCUSSION

The average minimum and maximum temperatures of the experimental house were 25.7°C and 33.2°C, respectively, when broilers were 1-7 days of age (phase 1), and 25.6°C and 31.8°C, respectively when broilers were 8-21 days of age (phase 2). Internal air relative humidity values varied between 31 and 67% in phase 1 and between 31 and 75% in phase 2. The recorded temperature and humidity values were within the normal range for this season and for the region of Ceres, GO, Brazil, where the experiments were conducted.

Hematological and Blood Biochemistry Parameters

The supplementation of the starter feed with amylase produced by *Aspergillus awamori* did not influence the hematological (Table 2) and blood biochemistry (Table 3) parameters measured in 21-d-old broilers (phase 2). We did not find any studies in literature evaluating the effect of the dietary inclusion of amylase on broiler blood parameters. However, Ahmad *et al.* (2013), evaluating the dietary addition of xylanase, did not find any effect of the on the hematological and serum biochemical parameters of 28-d-old Ross broilers.

The total leukocyte counts determined in this study were higher than those reported by Macari & Luquetti (2002), but similar to those found by Borsa (2009) and

Table 2 – Hematological parameters of 21-d-old broilers fed diets with or without amylase produced by *Aspergillus awamori*.

Parameters	Treatments		CV (%)	p-value
	Control	Feed + enzyme		
Erythrocytes (x10^6 cel.mm^{-3})	1.86 ± 0.10	1.81 ± 0.14	8.42	0.4251
Hematocrit (%)	27.7 ± 0.93	26.2 ± 1.33	7.80	0.1173
Hemoglobin (g.dL^{-1})	9.33 ± 0.24	8.89 ± 0.57	8.07	0.1677
Total leukocytes (x10^3 cel.mm^{-3})	9.09 ± 1.76	8.91 ± 1.90	29.33	0.8665
Thrombocytes (x10^3.mm^{-3})	28.3 ± 3.51	31.0 ± 2.23	12.79	0.1011
PCV (%)[1]	27.67 ± 1.33	26.25 ± 0.93	4.26	0.0585
MCV (fL)[2]	148.69 ± 7.12	145.17 ± 5.76	4.41	0.3684
MCH (pg)[3]	50.13 ± 2.08	49.15 ± 1.74	3.86	0.3993
MCHC (%)[4]	33.72 ± 0.41	33.87 ± 0.44	1.27	0.5701

[1]Packed Cell Volume, [2]Mean Corpuscular Volume, [3]Mean Corpuscular Hemoglobin (pg), [4]Mean Corpuscular Hemoglobin Concentration.

Table 3 – Blood biochemistry parameters of 21-d-old broilers fed diets with or without amylase produced by *Aspergillus awamori*.

Parameters	Treatments		CV (%)	p-value
	Control	Feed + Enzyme		
Uric acid (mg.dL^{-1})	8.28 ± 1.01	8.52 ± 1.14	16.86	0.6894
Albumin (g.dL^{-1})	0.78 ± 0.14	0.74 ± 0.16	23.46	0.5927
ALT (U.L^{-1})[1]	3.42 ± 0.92	3.00 ± 1.41	73.57	0.6709
AST (U.L^{-1})[2]	237.8 ± 91.28	209.7 ± 72.15	38.34	0.4324
Calcium (mg.dL^{-1})	5.11 ± 0.71	4.92 ± 0.33	11.47	0.4256
Chloride (mg.dL^{-1})	102.2 ± 8.74	101.3 ± 4.18	12.28	0.8596
Cholesterol (mg.dL^{-1})	81.7 ± 12.91	78.2 ± 12.21	26.70	0.6929
Creatinine (mg.dL^{-1})	0.34 ± 0.03	0.32 ± 0.03	21.41	0.4795
Phosphorus (mg.dL^{-1})	4.24 ± 0.76	4.26 ± 0.79	25.52	0.9719
Magnesium (mg.dL^{-1})	3.48 ± 0.30	3.09 ± 0.25	19.80	0.1555
Serum Protein (g.dL^{-1})	2.78 ± 0.49	2.73 ± 0.23	18.77	0.7818
Triglycerides (mg.dL^{-1})	32.0 ± 6.55	37.0 ± 8.88	39.55	0.3819
α-amylase (U.dL^{-1})	271.72 ± 150.04	378.2 ± 99.57	52.08	0.1418

[1] Alanine aminotransferase, [2] Aspartate aminotransferase.

lower than those obtained by Cardoso *et al.* (2009), Egbunike *et al.* (2009) and Raghavan *et al.* (2012). It is should be noted that the amount of these cells can vary according to sex, age, as well as conditions of stress and disease (Macari & Luquetti, 2002). The obtained erythrocyte counts, and hematocrit and hemoglobin values are consistent with those observed by Borsa (2009) and Cardoso *et al.* (2009).

The serum biochemical parameters of 28-d-old Ross broilers fed diets with the addition of xylanase or were studied by Ahmad *et al.* (2013), did not observe any differences on the analyzed parameters, as shown in the present study.

The mean protein values found in the present study were similar to, and cholesterol and triglyceride levels were lower than, those obtained by Abudabos (2012) is 42-d-old male Cobb500 broilers fed diets with the inclusion of α-amylase, protease, pectinase, amyloglucosidase, cellulase, phytase and cells of *Aspergillus awamori*.

The serum calcium, cholesterol, albumin, creatinine, and ALT levels found in the present study were lower than those reported by Obikaonu *et al.* (2012), whereas those for chloride and AST were higher. The phosphorus and protein levels were similar.

Minafra *et al.* (2010) found similar blood calcium, phosphorus, and chloride levels in 21-d-old broilers fed a diet supplemented with α-amylase. However, the serum α-amylase activity level was approximately 50% higher than that in the present study. The serum AST and chlorine levels previously found by Minafra *et al.* (2009) in 21-d-old broilers fed different protein levels were similar, whereas ALT and magnesium levels were higher relative to the present study.

The hematological and biochemical parameter values observed in broiler studies, independently of strain, sex, feed, environmental conditions, management, site of blood collection, type of laboratory kit and equipment, are highly variable; however, the values obtained in the present study are within the normal range for this species. Thus, the results of this study contribute for the determination of reference values for hematological and blood biochemistry parameters for broilers under Brazilian conditions.

Biometry of the digestive organs

Dietary supplementation of amylase from *Aspergillus awamori* did not affect the biometric parameters of the digestive tract of broilers during the pre-starter (Table 4) or starter (Table 5) phases.

Evaluating the effect of diet supplementation with amylase produced by *Cryptococcus flavus* and *Aspergillus niger* on the biometry of the digestive organs of broilers, Minafra (2007) did not find any

Table 4 – Relative weights (%) of the liver, pancreas, intestines, gizzard, and proventriculus and length (cm) of the intestines of 7-d-old broilers fed diets with or without amylase produced by *Aspergillus awamori*.

Parameters	Treatments		CV (%)	p-value
	Control	Feed + Enzyme		
Liver	3.83 ± 0.44	3.96 ± 0.38	10.48	0.5933
Pancreas	0.48 ± 0.09	0.47 ± 0.12	22.38	0.8523
Small Intestine	2.37 ± 0.22	2.39 ± 0.26	10.03	0.9831
Large Intestine	6.75 ± 2.59	6.63 ± 2.55	38.41	0.9380
Gizzard	6.52 ± 0.51	7.01 ± 0.65	8.55	0.5277
Proventriculus	1.31 ± 0.26	1.35 ± 0.09	14.67	0.7403
Small Intestine (cm)	94.57 ± 7.5	91.77 ± 3.75	5.57	0.3925
Large Intestine (cm)	11.07± 1.87	11.42 ± 0.54	11.26	0.6521

Table 5 – Relative weights (%) of the liver, pancreas, intestines, gizzard, and proventriculus and GIT length (cm) of 21-d-old broilers fed diets with or without amylase produced by *Aspergillus awamori*.

Parameters	Treatments		CV (%)	p-value
	Control	Feed + Enzyme		
Liver	2.52 ± 0.27	2.60 ± 0.35	12.29	0.6632
Pancreas	0.29 ± 0.09	0.32 ± 0.05	24.25	0.4328
Small Intestine	4.99 ± 0.49	5.08 ± 0.27	7.91	0.7197
Large Intestine	0.97 ± 0.17	1.02 ± 0.23	20.56	0.7020
Gizzard	3.47 ± 0.46	3.54 ± 0.44	12.85	0.7932
Proventriculus	0.58 ± 0.07	0.70 ± 0.14	17.12	0.0870
Small Intestine (cm)	160.33 ± 13.20	156.0 ± 9.51	7.40	0.5495
Large Intestine (cm)	20.00 ± 2.28	21.5 ± 1.22	10.72	0.2956

differences in GIT length or in the relative weights of the esophagus and crop, proventriculus and gizzard, the small and large intestines, or the liver of 7-d-old broilers, as in the present study. However, during this rearing phase, that author observed lower pancreas relative weight of in the birds fed amylase, which does not agree with the result obtained in the present study. The results of present study are consistent with the findings of Marques (2007), who did not observe any differences in GIT size or in the relative weights of the pancreas, esophagus and crop, proventriculus + gizzard, or large intestine in broilers fed an enzyme complex or not during the pre-starter phase.

Minafra (2007) did not observe any effect of the dietary inclusion of amylase on the GIT length or the on relative weights of the esophagus + crop, proventriculus + gizzard, or small or large intestines of 21-d-old broilers. However, the relative weights of the liver and pancreas decreased in the broilers fed feeds supplemented with the enzyme. According to that author, when exogenous amylase is fed, the pancreas reduces its endogenous production of this enzyme and, consequently, its function, consequently reducing its size.

Marques (2007) observed that the dietary supplementation with enzymes did not affect GIT size or the relative weights of the proventriculus + gizzard or the small intestine of starter broilers. However, birds that were not fed the enzymes presented higher large intestine and lower esophagus + crop relative weights, respectively, as well as 10% heavier livers compared with those fed the enzymes. Evaluating diets with sweet potato flour in partial substitution for corn and with the inclusion or not of an enzyme complex (amylase, phytase, protease, xylanase, β-glucanase, cellulase and pectinase), Nunes *et al.* (2011) did not find any differences in the intestinal biometry of 28-d-old broilers. Nascimento (2011) evaluated the effect of an enzyme complex supplement, consisting of phytase and carbohydrolases, and dietary sodium levels on the intestinal morphometrics of 18-d-old male Cobb chickens, and did not very any effect of enzyme addition on stomach relative weight, small intestine relative weight and length, or large intestine and liver relative weights.

Enzyme activities, protein content, and absolute weight of the pancreas

The addition of amylase obtained from *Aspergillus awamori* to the pre-starter feed (Exp. 1) had no influence enzyme activities, protein content, or the absolute weight of the pancreas of 7-d-old broilers. In the starter phase (Exp. 2), enzyme supplementation reduced pancreatic amylase activity and increased pancreatic protein content of 21-d-old broilers (Table 6).

The reduction in pancreatic amylase activity during the starter phase of broiler production was attributed to the addition of this enzyme to a corn and soybean meal-based feed, because its endogenous synthesis was reduced (Zanella, 1998). Sakomura *et al.* (2004) investigated the effect of broiler age on pancreatic digestive enzyme production and observed that the activities of amylase and lipase increased with broiler age, as did the allometric growth of the pancreas. The results of the present study are consistent with those findings.

Although values different from those in the present study were reported by Minafra (2007), this author also found that the addition of enzymes to the pre-starter and starter broiler diets significantly reduced pancreas amylase activity on days 7 (5.23%) and 21 (17.45%) of the rearing period.

Table 6 – Enzyme activities, protein content, and absolute weight of the pancreas of broilers fed pre-starter and starter diets with or without amylase produced by *Aspergillus awamori*.

Parameters	Treatments		CV (%)	p-value
	Control	Feed + Enzyme		
Pre-starter phase				
Amylase ($U.mg^{-1}.10^{-3}$)	319.846	256.798	45.47	0.4243
Lipase ($U.mg^{-1}.10^{-3}$)	14.544	16.178	34.50	0.6049
Protein ($mg.mL^{-1}$)	2.808	2.573	27.56	0.5953
Absolute weight (g)	0.67	0.68	13.32	0.7387
Starter phase				
Amylase ($U.mg^{-1}.10^{-3}$)	388.38A	156.59B	54.70	0.0226
Lipase ($U.mg^{-1}.10^{-3}$)	47.58	28.07	46.93	0.0861
Protein ($mg.mL^{-1}$)	1.90A	3.31B	36.58	0.0282
Absolute weight (g)	2.35	2.54	27.05	0.6456

Means followed by different letters in the same row differ by the F-test at 5%.

The results the present study are in agreement with Marques (2007) when adding an enzyme complex to a broiler feed. The author did not observe any differences in the amylase activity or the absolute weight of the pancreas during the pre-starter and starter phases. However, the reported average values were higher than those found in the present study. This may be related to the lower weight of the pancreas determined in the present study.

CONCLUSIONS

The supplementation of pre-starter and starter feeds with amylase produced by *Aspergillus awamori* is not justified, considering the amylase activity and pancreatic protein concentration results obtained in 7- and 21-d-old broilers.

The hematological and blood biochemistry parameters of 21-d-old broilers obtained in the present study can be used as reference for the scientific community, in view of the limited information available for broilers reared under Brazilian conditions.

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