

Amylase and xylanase supplementation in broiler diets containing protease and phytase: growth performance and intestinal health

Bárbara Colcetta¹ , Tânia Luiza Kohler¹ , Mayara Marisa Tesche Câmara¹ , Giovanna de Souza Silva¹ , Débora Piotrowski¹ , Ana Caroline Hammes¹ , Cristine Kaufmann¹ , Nilton Rohloff Junior¹ , Ricardo Vianna Nunes¹ , Cristiane Regina do Amaral Duarte² , Cinthia Eyng^{1*} 

¹ Universidade Estadual do Oeste do Paraná, Departamento de Zootecnia, Marechal Cândido Rondon, PR, Brasil.

² Universidade do Estado de Mato Grosso, Departamento de Ciências Biológicas, Tangará da Serra, MT, Brasil.

*Corresponding author:
cinthiaeyng@hotmail.com

Received: May 13, 2025

Accepted: February 18, 2026

How to cite: Colcetta, B.; Kohler, T. L.; Câmara, M. M. T.; Silva, G. S.; Piotrowski, D.; Hammes, A. C.; Kaufmann, C.; Rohloff Junior, N.; Nunes, R. V.; Duarte, C. R. A. and Eyng, C. 2026. Amylase and xylanase supplementation in broiler diets containing protease and phytase: growth performance and intestinal health. *Revista Brasileira de Zootecnia* 55:e20250087. <https://doi.org/10.37496/rbz5520250087>

Editors:

Ines Andretta
Catarina Stefanello

Copyright: This is an open access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.



ABSTRACT - This study evaluated the effects of xylanase combined with increasing amylase levels in broiler diets containing protease and phytase on performance, intestinal histomorphology, cecal short-chain fatty acid profile, serum interleukin (IL) levels, and economic viability over a 28-d period. A completely randomized experimental design was adopted in a 2 × 3 + 1 factorial arrangement (100 or 200 U kg⁻¹ amylase × 0, 1,000 and 2,000 U kg⁻¹ xylanase + positive control (PC), without enzymes), with seven replicates of 20 birds. Diets with amylase and xylanase also included 2,500 U kg⁻¹ protease, whereas all diets had 500 U kg⁻¹ phytase. Enzyme matrix values accounted for 100 kcal of metabolizable energy and 6% of crude protein and digestible amino acids. At 14 d, broilers receiving xylanase levels presented better feed conversion ratio (FCR) compared with those fed diets without xylanase (P = 0.014). However, regardless of the enzyme supplementation level, FCR was worse compared with PC (P = 0.0001). Diets containing 100 U kg⁻¹ amylase resulted in better histopathology scores (I See Inside®). No difference (P>0.05) was observed between PC and enzyme-supplemented groups for intestinal morphometric parameters, concentrations of acetic, butyric and isovaleric acids, or serum levels of IL-6, IL-10 and IL-16 at 28 d. Feed cost/t of broilers fed 100 U kg⁻¹ of amylase or 1,000 U kg⁻¹ of xylanase was 3.15% and 3.24% lower, respectively, than PC. Although the inclusion of amylase and xylanase in reduced-energy and protein diets containing protease and phytase did not improve the broiler performance and did not match the PC group, it maintained gut health and immunological parameters. The use of 100 U kg⁻¹ of amylase and 1,000 U kg⁻¹ of xylanase enabled an average 3.25% reduction in production cost.

Keywords: cytokine, energy reduction, exogenous enzyme, histological lesion, short-chain fatty acid

1. Introduction

Broiler diets are primarily composed of cereal grains such as corn, soybean meal, and wheat. However, these ingredients contain non-starch polysaccharides (NSPs), which act as antinutritional factors due

to the absence of endogenous enzymes capable of degrading their complex carbohydrate structures. NSPs impair nutrient digestibility and gut health, especially when included at high levels or provided through less-refined byproducts such as wheat bran.

NSPs are classified as soluble and insoluble. Soluble NSPs – such as β -glucans and arabinoxylans – are prevalent in wheat grain and wheat bran. These polysaccharides increase digesta viscosity (Mendis et al., 2016; Kim et al., 2022), creating favorable conditions for microbial fermentation of undigested nutrients, which can disrupt gut integrity (Wang et al., 2021) and compromise performance (Józefiak et al., 2007). Insoluble NSPs, including cellulose, lignin, and some hemicellulose fractions, are commonly found in corn and deactivated soybeans. These components encapsulate starch and protein within plant cell walls, limiting their accessibility to digestive enzymes and reducing nutrient availability (Nguyen et al., 2021).

Given the NSP profile of these ingredients, exogenous enzymes are widely used to improve nutrient utilization. Xylanase can hydrolyze cell walls rich in soluble fibers, releasing starch, lipids, and proteins and thereby improving the accessibility of endogenous enzymes (Nelson and Cox, 2014; Saleh et al., 2025). The hydrolysis of β -glucans and arabinoxylans also produces xylooligosaccharides (XOs), which are fermented by gut bacteria into short-chain fatty acids (SCFAs), improving gut health and reducing inflammatory conditions (Baker et al., 2021).

The inclusion of amylase also benefits broiler performance by acting on undigested starch. The digestibility of this component varies according to the grain type and the degree of processing. The rate of nutrient utilization is influenced by physiological limitations of broilers, such as age, the site of digestion, and the high feed intake of broilers, which provides a greater substrate for digestion (Cowieson et al., 2019; Stefanello et al., 2019).

In this context, we hypothesized that the inclusion of corn, soybeans, and wheat in broiler diets, despite their high digestibility, can negatively affect gut health and growth performance. Furthermore, we proposed that the dietary inclusion of amylase and xylanase, in combination with protease and phytase, could mitigate these effects, support broiler physiological responses, and enable the use of reduced dietary energy and protein levels. Therefore, the research question addressed in this study was whether broilers fed diets supplemented with exogenous enzymes formulated with reduced energy and protein levels would differ in performance, intestinal health, immune parameters, and economic viability when compared with broilers fed a nutritionally adequate control diet. The null hypothesis (H_0) was that broilers fed enzyme-supplemented, reduced-nutrient diets would not differ from those fed the control diet in growth performance, intestinal histomorphology, cecal SCFA profile, serum interleukins levels, or production cost over a 28-d period.

2. Material and methods

2.1. Local and Animal Ethics Committee

The experiment was conducted at the Poultry Research Center of Universidade Estadual do Oeste do Paraná - Unioeste, Marechal Cândido Rondon, Paraná, Brazil. The trial was conducted in accordance with ethical principles in animal experimentation, with protocol approved by the Ethics Committee for the Use of Animals in Research of the University (protocol n° 23/2022).

2.2. Housing, birds and treatments

The experiment was conducted in an experimental aviary, where birds were housed in 49 boxes, each measuring 1.96 m² and equipped with a tubular feeder, a nipple drinker, and a concrete floor covered with pine shavings previously used for six flocks. The experimental period was divided into two phases, according to the feeding program adopted, the starter phase (1-14 d) and the grower phase (15-28 d). During the first three days, the temperature was maintained near 33 °C and was gradually decreased to 23 °C at the end of the experiment. When necessary, cooling and air renewal were carried

out by exhaust fans and evaporative cooling pads. The lighting program provided 24 h (natural plus artificial) until day 3, 1 h of darkness between days 4 and 7, and 6 h of darkness from days 8 to 28, controlled by an automatic panel (model SMAAI 4).

A total of 980 one-day-old male Cobb 500® broiler chickens, with an initial average weight of 46 ± 0.40 g, were distributed in a completely randomized design in a $2 \times 3 + 1$ factorial scheme, with six combinations of two amylase levels and three xylanase levels and one positive control, totaling seven treatments, with seven replicates and 20 birds per experimental unit (EU). Diets were formulated based on the nutritional requirements of the lineage.

2.3. Experimental diets

Birds received water and feed *ad libitum* over the 28-d period. The experimental diets (Table 1) were formulated based on the nutritional requirements of the strain for the starter and grower phases and were provided in pelleted form. Pelletizing was performed in a Chavantes® pelletizer (7.5-15 CV model) with a capacity of 300 kg/h, equipped with a circular matrix with 5.0 mm diameter holes, operating at a speed of 15.0 m/sec, and a conditioner with a capacity of 100 kg/h. Maximum steam injection was applied in the conditioning process, with 1.0 kg/cm^3 of pressure, addition of steam in the conditioner, and a temperature of 80-90 °C. After the pelleting process, the feed underwent a drying/cooling process to reach an average temperature of 37 °C. The nutritional composition of the experimental feeds was determined by near-infrared reflectance spectrophotometry technique (NIRS) (Model FT-NIR TANGO, Brucker, Safat, Kuwait) (Table 2).

The experimental diets consisted of combinations of 100 or 200 U kg⁻¹ of amylase with 0, 1,000, or 2,000 U kg⁻¹ of xylanase, corresponding to 100 g and 200 g of amylase product and 0, 100 g and 200 g of xylanase product per tonne of feed, respectively, and one positive control (without enzyme supplementation). The inclusion of enzyme associations and doses in the diets was performed by replacing the inert material (kaolin). Diets supplemented with amylase and xylanase also contained 2,500 U kg⁻¹ of protease (125 g of product per tonne of feed). The nutritional matrix was valued at 100 kcal of metabolizable energy and 6% of crude protein (CP) and digestible amino acids (lysine, methionine + cystine, threonine, and tryptophan), reflecting the expected nutrient release from protease and amylase. In contrast, xylanase was added on top of the matrix-reduced diets, without assigning a matrix value, to isolate its additive effects and potential interactions with amylase. All diets also contained 500 U kg⁻¹ of phytase (100 g of product per tonne of feed), and its nutritional matrix value was considered equivalent to 0.12% calcium and available phosphorus. The recovery of protease and xylanase activities from the diets in the starter phase was analyzed by a commercial laboratory (Toledo, Paraná, Brazil) (Table 3). The methodology for amylase recovery was still under development; therefore, it was not possible to quantify this enzyme in mashed and pelleted feeds.

The xylanase consisted of an endo-1,4-β-xylanase produced via fermentation of *Trichoderma longibrachiatum* (GIM 3.534), with an activity of 10,000 xylanase units per gram (U g⁻¹). One xylanase unit corresponds to the amount of enzyme required to release 1 micromole of reducing sugar from a xylan solution (5 mg/mL) at pH 5.5 and 37 °C.

The amylase consisted of an α-amylase produced via fermentation of *Bacillus subtilis* (ACCC 11088), with an activity of 1,000 amylase units per gram (U g⁻¹). One unit of α-amylase activity corresponds to the amount of enzyme required to hydrolyze 1 g of soluble starch within 1 h at pH 6.0 and 60 °C.

The phytase consisted of a 6-phytase produced via fermentation of *Aspergillus niger* (ACCC 30557), with an activity of 5,000 phytase units per gram (U g⁻¹). One phytase unit corresponds to the amount of enzyme required to release 1 micromole of inorganic phosphorus per min from a sodium phytate solution (mmol/L) at pH 5.0 and 37 °C.

The protease was produced via fermentation of *Aspergillus niger* (ACCC 33326) and *Bacillus subtilis* (GIM 1286), with an activity of 20,000 protease units per gram (U g⁻¹). One unit of protease activity corresponds to the amount of enzyme required to release 1 microgram of tyrosine per min from casein at 40 °C and pH 3.0 and 7.2.

Table 1 - Composition and calculated levels of experimental diets for initial (1 to 14 d) and grower (15 to 28 d) phases

Item	Initial		Grower	
	PC	ENZ	PC	ENZ
Ingredient (kg t⁻¹)				
Corn	368.55	437.93	370.46	435.95
Soybean meal (46%)	288.00	245.52	209.52	170.25
Wheat grain	150.00	150.00	175.00	175.00
Whole deactivated soybean	80.00	80.00	100.00	100.00
Wheat bran	50.00	50.00	75.00	75.00
Refined soybean oil	28.93	2.18	39.49	13.43
Dicalcium phosphate	10.32	10.32	7.71	7.71
Limestone (36%)	9.24	9.24	8.87	8.87
NaCl	4.287	4.292	3.835	3.839
L-Lysine HCl (78%)	2.746	2.949	3.141	3.325
DL-Methionine (98%)	3.294	2.975	2.977	2.680
L-Threonine (98%)	1.259	1.216	1.319	1.276
Choline chloride (60%)	0.500	0.500	0.350	0.350
Vitamin premix ¹	0.250	0.250	0.200	0.200
Mineral premix ²	0.500	0.500	0.500	0.500
Mycotoxins adsorbent ³	1.000	1.000	1.000	1.000
Anticoccidial additive ⁴	0.500	0.500	-	-
Filler (kaolin) ⁵	0.625	0.625	0.625	0.625
Calculated composition				
Metabolizable energy (kcal kg ⁻¹)	3,000	2,900	3,100	3,000
Available protein (%)	22.00	20.66	20.00	18.80
Calcium (%)	0.743	0.734	0.645	0.672
Available phosphorus (%)	0.450	0.443	0.400	0.389
Sodium (%)	0.200	0.200	0.180	0.180
Chloride (%)	0.361	0.361	0.338	0.338
Digestible lysine (%)	1.200	1.127	1.100	1.034
Digestible methionine (%)	0.596	0.560	0.540	0.510
Digestible cystine (%)	0.292	0.274	0.274	0.255
Digestible met+cys (%)	0.888	0.834	0.814	0.765
Digestible threonine (%)	0.792	0.742	0.726	0.682
Digestible tryptophan (%)	0.249	0.230	0.224	0.205

PC - Positive control - feed formulated according to the nutritional recommendations; ENZ - PC reducing 100 kcal, 6% of crude protein and amino acids (lysine, met+cys, threonine and tryptophan) and adding enzymes.

¹ Composition of starter vitamin premix (1 – 14 d), levels per kilogram of feed: vitamin A, 10,000 IU kg⁻¹; vitamin D3, 2,500 IU kg⁻¹; vitamin E, 20 IU kg⁻¹; vitamin K3, 2 mg kg⁻¹; vitamin B1, 2 mg kg⁻¹; vitamin B2, 5 mg kg⁻¹; vitamin B6, 3 mg kg⁻¹; vitamin B12, 12 mg kg⁻¹; niacin, 35 mg kg⁻¹; pantothenic acid, 12 mg kg⁻¹; folic acid, 1 mg kg⁻¹; biotin, 50 mcg kg⁻¹. Composition of grower vitamin premix (15 – 28 d), levels per kilogram of feed: vitamin A, 8,000 IU kg⁻¹; vitamin D3, 2,000 IU kg⁻¹; vitamin E, 16 IU kg⁻¹; vitamin K3, 1.60 mg kg⁻¹; vitamin B1, 1.60 mg kg⁻¹; vitamin B2, 4 mg kg⁻¹; vitamin B6, 2.40 mg kg⁻¹; vitamin B12, 9.60 mg kg⁻¹; niacin, 28 mg kg⁻¹; pantothenic acid, 9.60 mg kg⁻¹; folic acid, 0.80 mg kg⁻¹; biotin, 40 mcg kg⁻¹.

² Composition of mineral premix (1 – 28 d), levels per kilogram of feed: manganese, 70 mg kg⁻¹; zinc, 60 mg kg⁻¹; iron, 50 mg kg⁻¹; copper, 8 mg kg⁻¹; iodine, 0.80 mg kg⁻¹; selenium, 0.30 mg kg⁻¹.

³ Bentonite.

⁴ Anticoccidial additive: Nicarbazin+Narasin (80%/80%), levels per kilogram of feed: 80 mg kg⁻¹.

⁵ The inclusion of enzyme associations and doses on diets was done replacing the filler (kaolin), providing levels of amylase (100 and 200 U kg⁻¹) and xylanase (1,000 and 2,000 U kg⁻¹).

All diets contained 500 U kg⁻¹ of phytase, and its nutrition valorization of calcium and phosphorus was 0.12%. Treatments containing amylase and xylanase contained 2,500 U kg⁻¹ of protease.

2.4. Samples collection and analysis performed

At 28 d, one bird/EU was selected, weighed, and euthanized by cervical dislocation. Fragments of the jejunum were collected for intestinal morphometry analysis by light microscopy and I See Inside® (ISI) score index. The cecal content was stored in a sterile tube (50 mL) and immediately frozen at -20 °C for subsequent analysis of the SCFA profile. Blood samples were collected via brachial puncture into sterile vacuum tubes and immediately centrifuged, with the serum being separated and frozen to determine the serum cytokine concentrations.

Table 2 - Composition analyzed by NIR spectrophotometer of experimental diets in the starter (1 to 14 d) and grower (15 to 28 d) phases

Treatment (U kg ⁻¹)	Moisture (%)	Crude protein (%)	Ash (%)	Ether extract (%)	Crude fiber (%)
Starter phase					
Positive control	13.50	21.93	4.44	6.58	3.22
100 U amylase + 0 U xylanase	13.14	20.74	4.46	4.28	3.16
100 U amylase + 1,000 U xylanase	12.95	20.23	4.45	4.78	3.17
100 U amylase + 2,000 U xylanase	12.94	21.24	4.48	4.62	3.15
200 U amylase + 0 U xylanase	12.52	20.79	4.06	4.63	2.84
200 U amylase + 1,000 U xylanase	12.50	20.66	4.39	4.83	3.01
200 U amylase + 2,000 U xylanase	12.13	20.67	4.47	4.68	3.02
Grower phase					
Positive control	13.89	19.70	4.14	7.75	2.97
100 U amylase + 0 U xylanase	13.24	18.84	3.89	5.68	2.94
100 U amylase + 1,000 U xylanase	13.00	19.37	3.78	5.67	2.72
100 U amylase + 2,000 U xylanase	13.15	18.81	3.75	6.10	3.24
200 U amylase + 0 U xylanase	12.67	18.61	3.85	5.92	3.29
200 U amylase + 1,000 U xylanase	12.08	18.92	4.02	6.05	3.19
200 U amylase + 2,000 U xylanase	12.25	18.80	4.17	5.85	3.28

Positive control (PC) - feed formulated according to the nutritional recommendations.

Treatments containing enzymes had a reduction of 100 kcal and 6% of crude protein and amino acids (lysine, methionine, met + cys, threonine and tryptophan).

Table 3 - Declared and analyzed activity (U kg⁻¹) of protease and xylanase of experimental diets (starter phase)

Treatment/Feed*	Protease		Xylanase	
	Mashed	Pelleted	Mashed	Pelleted
100 U amylase + 0 U xylanase	3180	3460	N/A	N/A
100 U amylase + 1,000 U xylanase	3780	3640	1321	402
100 U amylase + 2,000 U xylanase	3540	3770	2951	1614
200 U amylase + 0 U xylanase	3440	3580	N/A	N/A
200 U amylase + 1,000 U xylanase	3580	3060	1603	292
200 U amylase + 2,000 U xylanase	3440	3710	2391	623

N/A - not analyzed.

* Enzyme recoveries were performed before (mash feed) and after pellet processing (pelletized feed).

2.5. Growth performance

To evaluate growth performance, birds and feed were weighed at the beginning of the experimental period and at 14 and 28 d to determine average feed intake (AFI), weight gain (WG), and feed conversion ratio (FCR). Mortality was recorded daily for subsequent performance correction, as recommended by Sakomura and Rostagno (2016).

2.6. Intestinal morphometry

To analyze the morphometry of the intestinal mucosa, villus height, crypt depth, villus height to crypt depth ratio, and absorption area were determined. Two-centimeter cross-sectional histological fragments of the jejunum were collected immediately proximal to Meckel's diverticulum, washed with saline solution, fixed in buffered formalin solution (10%) for 24 h, and then dehydrated in a series of increasing concentrations of alcohol, diaphanized in xylene, and embedded in paraffin (Luna, 1968).

After semi-serial microtomy (7- μ m sections), the sections were stained using the hematoxylin and eosin technique. Digital images were obtained using a light microscope (4x objective), and morphometric analyses were performed using the Image-Pro Plus 4.1 imaging system from Media Cybernetics. For each slide, the length and width of 20 villi and the depth and width of 20 crypts were measured. These morphometric measurements were used to calculate the absorption surface area of the intestinal mucosa according to Kisielinski et al. (2002). The villus height to crypt depth ratio was calculated by dividing the villus height by the crypt depth.

2.7. I See Inside® score

The I See Inside methodology is an index based on a numerical alteration score applied to intestinal histological analysis, as described by Kraieski et al. (2017). The variables evaluated included lamina propria thickness, epithelial thickness, enterocyte proliferation, inflammatory cell infiltration in the epithelium, inflammatory cell infiltration in the lamina propria, increase of goblet cells, congestion, and the presence of *Eimeria* oocysts.

An impact factor (IF) is defined for each alteration observed in the analyses, according to the degree of reduction of the organ functionality. The IF ranges from 1 to 3, with 3 being the most impactful for the organ's function. Furthermore, the extent of each lesion (intensity) or the observed frequency relative to the unaffected organs in each organ/tissue per animal was evaluated. The score ranged from 0 to 3: score 0 (absence of lesion or frequency), score 1 (alteration of up to 25% of the area or frequency observed), score 2 (alteration of 25 to 50% of the area or frequency observed) and score 3 (extent alteration of more than 50% of the area or frequency observed). To calculate the final ISI index value, the impact factor (IF) of each alteration was multiplied by its respective score number, and the results of all alterations were summed. The sum of all previously mentioned parameters resulted in the total ISI value for each bird (each bird was considered a replicate for statistical analysis).

2.8. Short-chain fatty acids

The determination of SCFAs was performed according to Del Valle et al. (2018). The concentrations of acetic, butyric, isobutyric, propionic, valeric, and isovaleric acids in cecal samples were determined by gas chromatography using a Shimadzu® GC-2010 Plus chromatograph equipped with an AOC-20i automatic injector, a Stabilwax-DA™ capillary column (30 m, 0.25 mm ID, 0.25 μ m df, Restek®), and a flame ionization detector (FID). Two grams of cecal content were diluted in NaOH and subsequently centrifuged at 3,000 rpm for 5 min. Then, 1 mL of the supernatant was transferred to a microtube, to which 1 M orthophosphoric acid (p.a., Merck®) and a fortified mixture of free volatile acids (Supelco®) were added.

An aliquot of 1 μ L from each sample was injected with a split ratio of 40:1, using helium as the carrier gas at a linear speed of 42 $\text{cm}\cdot\text{s}^{-1}$, with the separation of the analytes achieved in a chromatographic run of 11.5 min. The injector and detector temperatures were set at 250 °C and 300 °C, respectively, with an initial column temperature of 40 °C. The column temperature ramp began with a gradient from 40 °C to 120 °C at a rate of 40 °C min^{-1} , followed by a gradient from 120 °C to 180 °C at 10 °C min^{-1} , and finally from 180 °C to 240 °C at 120 °C min^{-1} , maintaining the final temperature of 240 °C for an additional 3 min.

For the quantification of analytes, a calibration method was performed using dilutions of the WSFA-2 standard (Supelco®) and glacial acetic acid (Sigma-Aldrich®), analyzed under the conditions described above. The determination and integration of peaks were performed using the GCsolution software v. 2.42.00 (Shimadzu®). The results were expressed in mmol kg^{-1} .

2.9. Serum concentration of cytokines

The serum concentration of cytokines (IL-6, IL-10, and IL-16) was measured using the Milliplex commercial kit specific for chicken (Catalog # GCYT1-16K; MilliporeSigma Co., Burlington, MA),

following the manufacturer's instructions. The thawed serum samples were diluted (1:4) with 25 µL of appropriate matrix solutions, standard solutions, and control solutions provided in the commercial kit. The samples were then incubated and agitated for 16–18 h at 2–8 °C. After reaching room temperature, 25 µL of the detection antibody preparation was added. The samples were incubated and agitated on a plate shaker for 1 h at room temperature. Subsequently, 150 µL of Sheath Fluid PLUS solution was added, and the samples were agitated for an additional 5 min. Cytokine quantification was performed using the Luminex xMAP® technology (Luminex Corp., Austin, TX), which employs magnetic microspheres conjugated with specific antibodies for each detectable target.

2.10. Economic analysis

The economic analysis was conducted by considering the cost/t of feed (US\$/t) for each feeding program by animal phase, average weight, and average feed consumption per phase according to Bellaver et al. (1985). The feed cost/t of broiler produced was calculated based on the feed cost, using ingredient prices from April 2025.

2.11. Statistical analysis

All statistical analyses were performed using SAS® Version OnDemand (2021) program (SAS Inst. Inc., Cary, NC, USA), considering a significant level of 5%. Data were initially tested for normality (Shapiro-Wilk test), homogeneity of variances (Levene's test), and outlier removal using the PROC UNIVARIATE procedure. For normally distributed data, an analysis of variance (ANOVA) was conducted to evaluate the isolated and interactive effects of amylase and xylanase levels. Comparisons among amylase levels were performed using an F-test, while comparisons among xylanase levels were performed by Tukey's test. The enzyme treatments were compared to the positive control by Dunnett's test. All procedures were conducted using the PROC GLM procedure.

For ISI variables, which did not exhibit normal distribution, non-parametric tests were applied using the PROC NPAR1WAY procedure. The evaluation of the isolated effect of the enzyme combination and the comparison with the positive control were performed using the Kruskal-Wallis test. When significant effects were observed, xylanase levels were compared using Dunn's test. To assess the interaction between treatments, the Friedman test was employed.

The following overall model was used:

$$Y_{ij} = \mu + A_i + B_j + AB_{ij} + e_{ij},$$

in which Y_{ij} = value observed in the experimental unit that received the combination of factor A ($i = 1, 2, \dots, I$) with the factor B ($j = 1, 2, \dots, J$); μ = overall mean of the experiment; A_i = fixed effect of factor A; B_j = fixed effect of factor B; AB_{ij} = fixed effect of the interaction between factor i and factor j ; and e_{ij} = random error in factor i and level j .

3. Results

No interaction ($P > 0.05$) was observed between amylase and xylanase supplementation in either of the evaluated phases for performance parameters. Considering the isolated effects, a difference was observed among the xylanase levels during the starter phase, in which birds receiving diets containing 1,000 U kg⁻¹ of xylanase presented better feed conversion ratio (FCR; $P = 0.014$) compared to the group without xylanase supplementation (Table 4). When comparing the enzyme inclusion treatments with the PC, regardless of the level of amylase ($P < 0.001$) or xylanase ($P < 0.001$), a worse FCR was observed for the starter and grower phases, respectively (Table 4).

There was no interaction between amylase and xylanase levels, nor isolated effect for the intestinal morphometric parameters ($P > 0.05$), demonstrating that villus height, crypt depth, villus height to crypt depth ratio, and absorption surface area were similar among treatments (Table 5).

Table 4 - Performance of broilers fed diets containing different doses of amylase and xylanase in the starter (1-14 d) and grower (15-28 d) phases

Treatment	1-14 d			1-28 d		
	WG (g)	AFI (g)	FCR	WG (g)	AFI (g)	FCR
Positive control	451	551	1.216	1863	2547	1.376
Amylase (U kg ⁻¹)						
100	449	559	1.246*	1823	2577	1.414*
200	454	566	1.246*	1822	2576	1.416*
Xylanase (U kg ⁻¹)						
0	447	562	1.255a*	1814	2570	1.419*
1,000	458	568	1.241b*	1835	2588	1.411*
2,000	450	559	1.244ab*	1818	2571	1.415*
SEM	15.60	19.87	0.01	54.55	63.47	0.02
CV (%)	3.46	3.54	1.07	2.98	2.47	1.22
P-value						
Amylase	0.357	0.250	0.395	0.879	0.915	0.710
Xylanase	0.157	0.535	0.014	0.558	0.704	0.448
Amylase vs xylanase	0.250	0.649	0.119	0.386	0.481	0.410
Dunnett amylase	0.613	0.188	<0.001	0.198	0.555	<0.001
Dunnett xylanase	0.331	0.362	<0.001	0.222	0.593	<0.001

WG - weight gain; AFI - average feed intake; FCR - feed conversion ratio; CV - coefficient of variation; SEM - standard error of the mean (n = 7 per treatment).

PC - Positive control feed formulated according to the nutritional recommendations, without adding exogenous enzymes. Treatments containing enzymes had a reduction of 100 kcal and 6% of crude protein and amino acids (lysine, methionine, met + cys, threonine and tryptophan). All diets contained 500 U kg⁻¹ of phytase, and its nutrition valorization of calcium and phosphorus was 0.12%. Treatments containing amylase and xylanase contained 2,500 U kg⁻¹ of protease.

Means with different letters in the same column indicate differences (P<0.05) by Tukey's test.

* Mean different from the positive control by the Dunnett's test.

Table 5 - Morphometric results of the jejunal segment of broiler chickens fed diets containing different doses of amylase and xylanase at 28 d

Treatment	VH (μm)	CD (μm)	VCR	AA (μm)
Positive control	729.59	83.44	9.35	15.39
Amylase (U kg ⁻¹)				
100	722.27	75.01	10.33	13.95
200	694.38	74.09	10.24	13.53
Xylanase (U kg ⁻¹)				
0	725.84	73.12	10.63	14.39
1,000	707.88	76.67	10.01	14.15
2,000	691.26	73.87	10.21	12.68
SEM	83.79	13.52	1.80	2.33
CV (%)	11.78	17.84	17.75	16.69
P-value				
Amylase	0.288	0.825	0.880	0.557
Xylanase	0.557	0.762	0.631	0.110
Amylase vs xylanase	0.422	0.350	0.089	0.470
Dunnett amylase	0.466	0.277	0.531	0.200
Dunnett xylanase	0.674	0.388	0.483	0.059

VH - villus height; CD - crypt depth; VCR - villus/crypt ratio; AA - absorption surface area; CV - coefficient of variation; SEM - standard error of the mean (n = 7 per treatment).

PC - Positive control feed formulated according to the nutritional recommendations, without adding exogenous enzymes. Treatments containing enzymes had a reduction of 100 kcal and 6% of crude protein and amino acids (lysine, methionine, met + cys, threonine and tryptophan). All diets contained 500 U kg⁻¹ of phytase, and its nutrition valorization of calcium and phosphorus was 0.12%. Treatments containing amylase and xylanase contained 2,500 U kg⁻¹ of protease.

Interactions were observed between amylase and xylanase levels for the parameters of lamina propria thickness (LPT) ($P = 0.009$), epithelial thickness (ET) ($P = 0.020$), enterocyte proliferation (EP) ($P = 0.021$), inflammatory infiltration in the epithelium (IIE) ($P < 0.001$), inflammatory infiltration in the lamina propria (IILP) ($P = 0.022$), and total ISI score ($P = 0.014$; Table 6). Unfolding the interaction values, the treatment receiving 100 U kg^{-1} of amylase without xylanase had lower values of LPT ($P = 0.019$), IIE ($P < 0.001$), and IILP ($P < 0.001$) compared to the group that received $2,000 \text{ U kg}^{-1}$ of xylanase and the same level of amylase. The treatment of $1,000 \text{ U kg}^{-1}$ of xylanase with 100 U kg^{-1} of amylase provided a lower ET value ($P = 0.021$) compared to the group that did not receive xylanase; however, the value was similar when supplemented with 200 U kg^{-1} of amylase ($P = 0.002$). No xylanase with 200 U kg^{-1} of amylase resulted in lower EP values ($P = 0.034$). Evaluating the amylase levels with each xylanase level, the lowest dose of amylase with $2,000 \text{ U kg}^{-1}$ of xylanase resulted in lower ($P = 0.002$) and higher ($P = 0.001$) values of ET and IILP, respectively. The treatment with the lowest doses of xylanase and amylase had the lowest IIE values ($P = 0.026$). The lowest total ISI score was observed in the treatments of 0 and $1,000 \text{ U kg}^{-1}$ of xylanase with 100 U kg^{-1} of amylase ($P = 0.009$). Comparing the levels of amylase inclusion with each level of xylanase supplementation, the lowest value ($P = 0.021$) for this parameter was observed with the highest levels of each enzyme ($2,000 \text{ U kg}^{-1}$ of xylanase with 200 U kg^{-1} of amylase; Table 7).

Considering the isolated effects, the higher amylase dose decreased the presence of oocysts (PO) value ($P < 0.001$). However, the inclusion of 100 U kg^{-1} amylase resulted in a higher PO value ($P < 0.001$) than that observed in the PC group. Regardless of amylase level, higher values of lamina propria thickness (LPT) ($P = 0.002$), epithelial thickness (ET) ($P < 0.001$), goblet cells (GC) ($P = 0.001$), and total ISI score ($P < 0.001$) were observed in treatments containing this enzyme. The inclusion of $1,000$ and $2,000 \text{ U kg}^{-1}$ xylanase increased LPT ($P < 0.001$) and inflammatory cell infiltration in the lamina propria (IILP) ($P = 0.032$). The highest xylanase dose increased enterocyte proliferation (EP) ($P = 0.010$) and inflammatory cell infiltration in the epithelium (IIE) ($P < 0.001$). Regardless of xylanase level, higher values of ET ($P < 0.001$), GC ($P = 0.001$), PO ($P = 0.001$), and total ISI score ($P = 0.0001$) were observed.

Table 6 - Histological evaluations using the I See Inside (ISI®) method of the jejunal segment of broiler chickens fed diets containing different doses of amylase and xylanase at 28 d

Treatment	LPT	ET	EP	IIE	IILP	GC	CONG	PO	Total
Positive control	1.99	1.20	1.01	1.63	2.70	2.09	0.13	0.00	10.74
Amylase (U kg^{-1})									
100	2.24*	1.36*	1.05	1.62	2.90	2.33*	0.05*	0.71*	12.26*
200	2.24*	1.41*	1.05	1.67	2.81	2.34*	0.02*	0.06	11.59*
Xylanase (U kg^{-1})									
0	2.13	1.39*	1.02	1.60	2.70	2.35*	0.03*	0.31*	11.53*
1,000	2.29*	1.32*	1.05	1.58	2.95*	2.29*	0.05*	0.25*	11.77*
2,000	2.31*	1.44*	1.08**	1.74*	2.91*	2.38*	0.02*	0.60*	12.48*
SEM	0.02	0.01	0.01	0.01	0.03	0.02	0.01	0.04	0.09
CV (%)	34.23	35.18	23.04	29.93	39.14	34.18	68.57	74.11	25.78
P-value									
Amylase	0.973	0.136	0.880	0.154	0.182	0.932	0.107	<0.001	0.062
Xylanase	0.009	0.020	0.021	<0.001	0.018	0.341	0.389	0.302	0.014
Amylase vs xylanase	0.009	0.020	0.021	<0.001	0.022	0.337	0.339	0.265	0.014
Dunnett amylase	0.002	<0.001	0.243	0.334	0.262	0.001	0.001	<0.001	<0.001
Dunnett xylanase	<0.001	<0.001	0.010	<0.001	0.032	0.001	0.004	<0.001	<0.001

LPT - lamina propria thickness; ET - epithelial thickness; EP - enterocytes proliferation; IIE - inflammatory cell infiltration in the epithelium; IILP - inflammatory cell infiltration in the lamina propria; GC - goblet cells; CONG - congestion; PO - presence of oocysts; SEM - standard error of the mean ($n = 7$ per treatment).

PC - Positive control feed formulated according to the nutritional recommendations, without adding exogenous enzymes. Treatments containing enzymes had a reduction of 100 kcal and 6% of crude protein and amino acids (lysine, methionine, met + cys, threonine and tryptophan). All diets contained 500 U kg^{-1} of phytase, and its nutrition valorization of calcium and phosphorus was 0.12%. Treatments containing amylase and xylanase contained $2,500 \text{ U kg}^{-1}$ of protease.

* Mean different from the positive control by the Dunn's test.

Table 7 - Unfolding the interaction between amylase and xylanase levels on I See Inside scores in the jejunum of broiler chickens fed diets containing different doses of amylase and xylanase at 28 d

Xylanase (U kg ⁻¹)	LPT			ET			EP			IIE			IILP			Total		
	100	200	P-value	100	200	P-value	100	200	P-value	100	200	P-value	100	200	P-value	100	200	P-value
	Amylase (U kg ⁻¹)																	
0	2.10b	2.16	0.523	1.44a	1.34b	0.067	1.04	1.01b	0.281	1.56b	1.64	0.180	2.54bB	2.86A	0.031	11.59b	11.47	0.991
1,000	2.26ab	2.31	0.556	1.29b	1.36b	0.202	1.04	1.06a	0.436	1.51bB	1.65A	0.026	3.01a	2.89	0.229	11.98b	11.56	0.380
2,000	2.37a	2.26	0.266	1.34abB	1.53aA	0.002	1.08	1.08a	1.000	1.78a	1.71	0.189	3.13aA	2.69B	0.001	13.21aA	11.75B	0.021
P-value	0.019	0.186		0.021	0.002		0.255	0.034		<0.001	0.451		<0.001	0.284		0.009	0.587	

LPT - lamina propria thickness; ET - enterocytes proliferation; IIE - inflammatory cell infiltration in the epithelium; IILP - inflammatory cell infiltration in the lamina propria.
Means with different lowercase letters in the same column or uppercase letters in the same row indicate differences (P<0.05) by the Friedman test.

Lower congestion values were observed regardless of amylase ($P = 0.001$) or xylanase level ($P = 0.004$) (Table 6).

No interaction or isolated effects ($P > 0.05$) were observed for the cecal SCFA profile (Table 8) or serum interleukin concentrations (Table 9).

Table 8 - Concentration of short-chain fatty acids (SCFA) (mmol kg^{-1}) in the cecal content of broiler chickens fed diets containing different doses of amylase and xylanase at 28 d

Treatment	Acetic	Butyric	Isovaleric
Positive control	29.85	5.13	2.31
Amylase (U kg^{-1})			
100	32.38	5.52	2.54
200	29.82	4.43	2.50
Xylanase (U kg^{-1})			
0	29.57	4.86	2.59
1,000	30.30	5.21	2.59
2,000	33.43	4.83	2.43
SEM	8.03	1.80	0.57
CV (%)	25.97	35.63	22.92
P-value			
Amylase	0.288	0.102	0.817
Xylanase	0.382	0.863	0.801
Amylase vs xylanase	0.230	0.219	0.358
Dunnett amylase	0.548	0.183	0.589
Dunnett xylanase	0.586	0.875	0.663

CV - coefficient of variation; SEM - standard error of the mean ($n = 7$ per treatment).

PC - Positive control feed formulated according to the nutritional recommendations, without adding exogenous enzymes. Treatments containing enzymes had a reduction of 100 kcal and 6% of crude protein and amino acids (lysine, methionine, met + cys, threonine and tryptophan). All diets contained 500 U kg^{-1} of phytase, and its nutrition valorization of calcium and phosphorus was 0.12%. Treatments containing amylase and xylanase contained 2,500 U kg^{-1} of protease.

Isobutyric, propanoic and valeric acids were not considered because they were below the limit of quantification.

Table 9 - Concentration of serum interleukins (pg mL^{-1}) in broiler chickens fed diets containing different doses of amylase and xylanase at 28 d

Treatment	IL-6	IL-10	IL-16
Positive control	54.32	21.14	150.55
Amylase (U kg^{-1})			
100	41.81	20.62	146.93
200	41.95	19.95	148.65
Xylanase (U kg^{-1})			
0	40.16	23.27	150.85
1,000	45.85	17.44	147.77
2,000	39.69	19.71	144.18
SEM	8.19	8.57	8.76
CV (%)	5.53	42.03	5.92
P-value			
Amylase	0.967	0.851	0.601
Xylanase	0.468	0.220	0.111
Amylase vs xylanase	0.350	0.596	0.084
Dunnett amylase	0.617	0.943	0.641
Dunnett xylanase	0.288	0.345	0.227

IL-6 - interleukin-6; IL-10 - interleukin-10; IL-16 - interleukin-16; CV - coefficient of variation; SEM - standard error of the mean ($n = 7$ per treatment).

PC - Positive control feed formulated according to the nutritional recommendations, without adding exogenous enzymes. Treatments containing enzymes had a reduction of 100 kcal and 6% of crude protein and amino acids (lysine, methionine, met + cys, threonine and tryptophan). All diets contained 500 U kg^{-1} of phytase, and its nutrition valorization of calcium and phosphorus was 0.12%. Treatments containing amylase and xylanase contained 2,500 U kg^{-1} of protease.

No interaction ($P > 0.05$) was observed between amylase and xylanase supplementation for the economic analysis (Table 10). Considering the isolated effects, the feed cost per ton of broiler produced was lower for diets containing 100 U kg⁻¹ amylase than for the PC group ($P = 0.028$). The inclusion of 1,000 U kg⁻¹ xylanase in the diet also reduced feed cost, although the effect was only marginally significant ($P = 0.063$).

Table 10 - Feed cost/t of broilers produced with diets containing doses of amylase and xylanase at 28 d

Treatment	Feed cost (US\$/t broiler)
Positive control	598.79
Amylase (U kg ⁻¹)	
100	579.91*
200	584.25
Xylanase (U kg ⁻¹)	
0	584.89
1,000	578.78*
2,000	581.77
SEM	15.72
CV (%)	2.70
P-value	
Amylase	0.383
Xylanase	0.701
Amylase vs xylanase	0.521
Dunnnett amylase	0.028
Dunnnett xylanase	0.063

CV - coefficient of variation; SEM - standard error of the mean (n = 7 per treatment).

PC - Positive control feed formulated according to the nutritional recommendations, without adding exogenous enzymes. Treatments containing enzymes had a reduction of 100 kcal and 6% of crude protein and amino acids (lysine, methionine, met + cys, threonine and tryptophan). All diets contained 500 U kg⁻¹ of phytase, and its nutrition valorization of calcium and phosphorus was 0.12%. Treatments containing amylase and xylanase contained 2,500 U kg⁻¹ of protease.

* Mean different from the positive control by the Dunnnett's test.

The cost of feed per ton of chicken was calculated by the cost of feed based on the prices of ingredients for the month of April 2025.

4. Discussion

Broilers tend to increase feed intake to compensate for energy and nutrient deficits resulting from enzyme matrix valorization, which can reduce the feed efficiency (Dessimoni et al., 2019). In the present study, FCR was negatively affected by lowering metabolizable energy and amino acid diet levels. The response to enzyme supplementation depends on several factors, including the energy and nutritional reduction of the diet, bird age, the quality of the diet, the presence of antinutritional factors, and the ingredient and diet processing (Olukosi and Bedford, 2019).

One way to improve feed efficiency in poultry is to process feed, such as by pelleting, which can modify carbohydrate structures and improve nutrient digestibility. Pelleted diets increase feed intake in broilers because they facilitate feed consumption and reduce feed wastage (Abu et al., 2023). Massuquetto et al. (2019) reported that broilers fed pelleted diets show greater feed intake than those fed mash diets.

Although thermal processing can improve feed utilization, this process can reduce enzyme activity due to the high temperatures used in the process, thereby reducing enzyme stability and efficiency (Pucci et al., 2010). Lamp et al. (2015) observed lower performance for broilers that were fed pelleted diets containing exogenous β -glucanase. The authors found a reduction of approximately 50% in β -glucanase activity in the pelleted diet. The thermal processing of the diet caused lower enzyme activity, which reduced the digestibility of the diet and, consequently, the broiler's growth performance.

The enzyme recovery analysis performed on the feed in our study demonstrated that thermal processing affected the enzymatic activity of xylanase. These results suggest that the inferior performance of broilers fed diets containing xylanase, when compared with the PC group, was affected by the low activity of the enzyme in the diets after pelleting. However, an improvement in FCR was observed during the initial phase in broilers supplemented with 1,000 U kg⁻¹ of xylanase compared with those not receiving the enzyme. Therefore, although nutrient digestibility was not evaluated, the improved FCR observed indicates a potential response to xylanase supplementation, even under reduced enzymatic activity.

A limitation of our study is the absence of a negative control diet without enzyme supplementation formulated with the same nutrient levels as the enzyme-supplemented treatments. Therefore, the results observed in the enzyme-supplemented groups should be interpreted as the combined effect of nutrient reduction and enzyme inclusion, rather than as an isolated enzymatic response.

In addition, the PC diet differed in formulation from the enzyme-supplemented diets, particularly regarding oil inclusion. Dietary oil levels are known to affect pellet quality, palatability, digestion, absorption, and rate of passage through the gastrointestinal tract (Shoaib et al., 2023). Thus, part of the differences observed between the PC and the remaining treatments in performance, and ISI scores may be partially associated with these formulation differences and not exclusively with enzyme supplementation.

Exogenous enzymes such as xylanase, protease, and amylase exert complementary modes of action in broiler diets by improving nutrient availability and supporting gut health. Xylanase hydrolyzes arabinoxylans in cereal cell walls, reducing digesta viscosity and releasing encapsulated nutrients, which may enhance the access of endogenous enzymes to starch and protein fractions and improve feed utilization (Saleh et al., 2024; Saleh et al., 2025). Protease supplementation contributes to improved protein hydrolysis and amino acid availability, reducing the presence of undigested nitrogenous compounds in the intestinal lumen that are associated with excessive fermentation and impairment of intestinal integrity (Saleh et al., 2025). Amylase acts on residual or slowly digestible starch fractions, potentially improving starch utilization when endogenous amylase secretion is limited, such as during early life stages (Cowieson et al., 2019; Stefanello et al., 2019). These mechanisms may influence the gut health, which can be reflected in morphometric characteristics such as villus height, crypt depth, and absorptive surface area.

Villus height, crypt depth, and absorption area are key indicators of nutrient absorption capacity and intestinal health of broilers. A higher villus height to crypt depth ratio and greater absorptive surface indicate increased potential for nutrient absorption (Alqhtani et al., 2022). However, nutrient utilization depends on multiple factors, including endogenous enzyme activity, intestinal integrity, microbiota composition, transporter gene expression, diet composition, and the age of the bird (Ravindran and Abdollahi, 2021). In our study, enzyme supplementation did not significantly alter the intestinal morphometric parameters compared with the positive control, indicating that energy and protein reduction did not impair the morphometry of the jejunum of broilers at 28 d.

Buclaw (2016) states that the need for greater villus height to increase absorption area requires faster epithelial growth, which requires more energy and nutrients for cell proliferation. The greater need for cell maintenance and renewal may occur when there are stressors, such as those coming from the diet, including the greater demand for nutrients for digestion, toxins, antinutritional factors and pathogens that cause lesions and desquamation in the intestinal epithelium (Kogut et al., 2018). High levels of CP and antinutritional factors, such as NSPs, are associated with lesions in the intestinal epithelium of broilers due to low digestibility and excessive fermentation in the intestine.

A previous study reported that diets with reduced crude protein (CP) levels without protease supplementation impair broiler performance due to amino acid imbalances, particularly threonine deficiency (Cardinal et al., 2019). With protease supplementation, it is possible to improve the amino acid balance through enhanced dietary protein digestibility. Consequently, these authors observed improvements in intestinal health and broiler performance in birds fed diets with reduced CP levels

supplemented with protease compared with broilers receiving reduced-CP diets without enzyme supplementation. In the present study, diets containing amylase and xylanase had a 6% reduction in CP and amino acid levels but showed morphometric results similar to those observed in broilers from the positive control group. Protease supplementation reduces the inclusion of protein ingredients in the formulation, such as soybean meal, which may contain antinutritional factors. Therefore, this reduction may decrease the presence of harmful compounds in the digesta, thereby contributing to the maintenance of intestinal epithelial integrity.

Lower ISI scores are indicative of better intestinal health. Dietary stressors that alter the intestinal mucosa, as well as tissue renewal resulting from epithelial damage and desquamation, can increase nutrient requirements (Parsaie et al., 2007), since the demand for energy and nutrients for intestinal maintenance is greater than that required by other organs. The intestinal mucosa contains a vast surface area of absorptive epithelial cells (Choct, 2009), and evidence suggests that increased thickness of the lamina propria and mucosa is associated with the action of stressors. Conversely, reductions in this thickness may facilitate nutrient transport and utilization, thereby contributing to the maintenance of intestinal health (Miles et al., 2006; Cardinal et al., 2019). In the present study, lamina propria thickness (LPT) and epithelial thickness (ET) values were higher in treatments containing enzymes, indicating greater difficulty in nutrient transport and utilization, which may explain the lower performance observed relative to the positive control (PC) group.

In general, 100 U kg⁻¹ of amylase provided better health parameters when compared with treatments containing xylanase supplementation. However, this benefit was not reflected in a better ISI score or in the performance of the animals when compared to the PC group. Although improvements were found in some ISI score parameters when considering the interaction among amylase and xylanase, evaluating the parameters in isolation it was observed that, in general, the lowest scores were found in animals that received the diet without the enzyme supplementation, indicating that enzyme supplementation under reduced-nutrient conditions was not sufficient to fully match the performance and ISI scores of the positive control.

We observed no differences in SCFA concentrations among the treatments. Xylanase contributes to the release of nutrients through hydrolysis of the cereal cell wall, degrading the xylose units and releasing XOs. The formation of SCFAs depends on the amount of substrate contained in the ingredients, and the amount of NSPs varies among different cereals and may vary within them (Ward, 2021). According to Šimić et al. (2023), diets with wheat bran enhance the concentration of SCFAs (valeric and propionic acid) in the cecal content of broilers at 35 d compared to the group that received a diet without the ingredient.

The experimental diets in our study contained a high proportion of ingredients with high levels of soluble NSPs. Therefore, it is possible to infer that the amount of substrate (arabinoxylans) present in the diets was similar and was not markedly affected by the action of the xylanase. The results found for SCFAs corroborate those obtained in morphometry. Xylooligosaccharides (XOs) act as a prebiotic substrate or as signaling molecules for specific groups of bacteria that produce SCFAs, such as butyric acid, which acts as the main source of energy for colonocytes and helps maintain the integrity of intestinal epithelial cells (Lopetuso et al., 2013). We observed no difference in the SCFA profile, therefore, there were no further incentives to increase the intestinal morphometrics.

Isobutyric, propanoic, and valeric acids were below the quantification limit in our analysis, indicating that these substances are rapidly absorbed and used by the broiler. The digesta flow through the intestinal tract is dynamic, and reverse peristalsis in chickens can result in variability in the local concentrations of the different SCFAs, making their quantification difficult (González-Ortiz et al., 2020). Therefore, a possible explanation for the variability in SCFA measurements could be attributed to their volatile concentrations, which depend on the production and absorption rates at the exact time of measurement (Šimić et al., 2023).

Both IL-6 and IL-16 are cytokines with pro-inflammatory characteristics (Saleh and Al-Zghoul, 2019), whereas IL-10 is a regulatory cytokine that suppresses the activity of these cytokines (He et al., 2011).

A lower inflammatory state results in lower serum concentrations of IL-6 and IL-16 and, consequently, lower serum concentrations of IL-10. Under these conditions, birds do not need to divert nutrients toward the production of regulatory interleukins and can allocate them more efficiently to productive functions (Lu et al., 2014). According to Cardinal et al. (2019), excess nutrients in the intestinal lumen can promote excessive fermentation and increase pathogen proliferation, leading to epithelial alterations, intestinal permeability (leaky gut), and the activation of inflammatory responses (Dal Pont et al., 2020). In the present study, no differences were observed in serum concentrations of IL-6, IL-10, or IL-16, suggesting that the NSP levels in the diets were not sufficient to induce a systemic inflammatory response associated with intestinal infection. Although broilers receiving enzyme-supplemented diets showed lower growth performance and ISI results compared with the positive control (PC) group, these treatments did not trigger an inflammatory condition.

The addition of enzymes to the broiler diets aims mainly to reduce feed costs through the contribution of nutrients that the enzyme can release, maintaining the nutritional requirements demanded by the animal in their respective production stages (Pasquali et al., 2017). Therefore, from an economic perspective, enzyme supplementation associated with the reduction in dietary energy and amino acid levels in the present study may be economically viable. The inclusion of 100 U kg⁻¹ of amylase reduced production cost by US\$ 18.88/t of chicken produced, representing a cost reduction of 3.15% in relation to the control group. With the inclusion of 1,000 U kg⁻¹ of xylanase resulted in a greater reduction in production costs, decreasing costs by US\$ 20.01/t of chicken produced, which represented a reduction of 3.34% compared with the control diet.

The inclusion of amylase and xylanase in reduced energy and protein diets was economically viable despite lower growth performance compared with the positive control. Although the weight of the chicken produced was lower, with a nutritional value of 100 kcal of energy and 6% of proteins and amino acids, the production cost of the chicken was lower.

This study presents a novel approach by evaluating the inclusion of xylanase in diets with reduced energy and protein levels combined with increasing amylase levels in diets already supplemented with protease and phytase under commercial-like pelleting conditions. While prior studies have examined individual enzymes or fixed combinations, few have addressed the layered effect of these four enzymes on both gut health and economic performance in nutrient-restricted conditions. These findings offer practical insights for optimizing enzyme strategies in modern broiler nutrition.

5. Conclusions

The inclusion of amylase and xylanase in diets with reduced energy and protein levels did not improve the growth performance of broilers at 28 d compared to broilers fed a diet with regular nutrient levels. Furthermore, the enzyme supplementation did not alter gut health or immunological parameters. The analysis of feed costs suggests that the use of 100 U kg⁻¹ of amylase and 1,000 U kg⁻¹ of xylanase enables an average reduction of 3.25% in the production cost/t of broilers produced.

Data availability

The entire dataset supporting the results of this study was published in the article itself.

Author contributions

Conceptualization: Nunes, R. V. and Eyng, C. **Formal analysis:** Colcetta, B.; Câmara, M. M. T.; Silva, G. S.; Piotrowski, D.; Hammes, A. C.; Kaufmann, C. and Rohloff Junior, N. **Investigation:** Colcetta, B.; Câmara, M. M. T. and Rohloff Junior, N. **Methodology:** Kohler, T. L.; Duarte, C. R. A. and Eyng, C. **Supervision:** Nunes, R. V. **Writing – original draft:** Colcetta, B. **Writing – review & editing:** Kohler, T. L.; Duarte, C. R. A. and Eyng, C.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

The authors gratefully acknowledge the financial support provided by the Brazilian research agencies Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES; Finance code 001) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), which partially funded this study.

References

- Abu, M. H.; Alabi, O. J. and Jiya, E. Z. 2023. Pellet versus mash: Assessing the impact of feed forms on growth performance, nutrient digestibility, carcass characteristics and health of broiler chickens – A review. *Nigerian Journal of Animal Science and Technology* 6:52-64.
- Alqhtani, A. H.; Al Sulaiman, A. R.; Alharthi, A. S. and Abudabos, A. M. 2022. Effect of exogenous enzymes cocktail on performance, carcass traits, biochemical metabolites, intestinal morphology, and nutrient digestibility of broilers fed normal and low-energy corn-soybean diets. *Animals* 12:1094. <https://doi.org/10.3390/ani12091094>
- Baker, J. T.; Duarte, M. E.; Holanda, D. M. and Kim, S. W. 2021. Friend or foe? Impacts of dietary xylans, xylooligosaccharides, and xylanases on intestinal health and growth performance of monogastric animals. *Animals* 11:609. <https://doi.org/10.3390/ani11030609>
- Bellaver, C.; Fialho, E. T.; Protas, J. F. S. and Gomes, P. C. 1985. Radícula de malte na alimentação de suínos em crescimento e terminação. *Pesquisa Agropecuária Brasileira* 20:969-974.
- Bucław, M. 2016. The use of inulin in poultry feeding: a review. *Journal of Animal Physiology and Animal Nutrition* 100:1015-1022. <https://doi.org/10.1111/jpn.12484>
- Cardinal, K. M.; Moraes, M. L.; Andretta, I.; Schirmann, G. D.; Belote, B. L.; Barrios, M. A.; Santin, E. and Ribeiro, A. M. L. 2019. Growth performance and intestinal health of broilers fed a standard or low-protein diet with the addition of a protease. *Revista Brasileira de Zootecnia* 48:e20180232. <https://doi.org/10.1590/rbz4820180232>
- Choct, M. 2009. Managing gut health through nutrition. *British Poultry Science* 50:9-15. <https://doi.org/10.1080/00071660802538632>
- Cowieson, A. J.; Vieira, S. L. and Stefanello, C. 2019. Exogenous microbial amylase in the diets of poultry: what do we know? *Journal of Applied Poultry Research* 28:556-565. <https://doi.org/10.3382/japr/pfy044>
- Dal Pont, G. D.; Farmell, M.; Farmell, Y. and Kogut, M. H. 2020. Dietary factors as triggers of low-grade chronic intestinal inflammation in poultry. *Microorganisms* 8:139. <https://doi.org/10.3390/microorganisms8010139>
- Del Valle, T. A.; Zenatti, T. F.; Antonio, G.; Campana, M.; Gandra, J. R.; Zilio, E. M. C.; Mattos, L. F. A. and Moraes, J. G. P. 2018. Effect of chitosan on the preservation quality of sugarcane silage. *Grass and Forage Science* 73:630-638. <https://doi.org/10.1111/gfs.12356>
- Dessimoni, G. V.; Sakomura, N. K.; Donato, D. C. Z.; Soares, L.; Sarcinelli, M. F.; Malheiros, E. B. and Dalólio, F. S. 2019. Growth performance and carcass yield of broiler chickens in response to carbohydrases and its association with phytase. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia* 71:983-989. <https://doi.org/10.1590/1678-4162-10345>
- González-Ortiz, G.; Olukosi, O. A.; Jurgens, G.; Apajalahti, J. and Bedford, M. R. 2020. Short-chain fatty acids and ceca microbiota profiles in broilers and turkeys in response to diets supplemented with phytase at varying concentrations, with or without xylanase. *Poultry Science* 99:2068-2077. <https://doi.org/10.1016/j.psj.2019.11.051>
- He, H.; Genovese, K. J. and Kogut, M. H. Modulation of chicken macrophage effector function by T_H1/T_H2 cytokines. *Cytokine* 53:363-369. <https://doi.org/10.1016/j.cyto.2010.12.009>
- Józefiak, D.; Rutkowski, A.; Jensen, B. B. and Engberg, R. M. 2007. Effects of dietary inclusion of triticale, rye and wheat and xylanase supplementation on growth performance of broiler chickens and fermentation in the gastrointestinal tract. *Animal Feed Science and Technology* 132:79-93. <https://doi.org/10.1016/j.anifeeds.2006.03.011>
- Kim, E.; Morgan, N. K.; Moss, A. F.; Li, L.; Ader, P. and Choct, M. 2022. The flow of non-starch polysaccharides along the gastrointestinal tract of broiler chickens fed either a wheat- or maize-based diet. *Animal Nutrition* 9:138-142. <https://doi.org/10.1016/j.aninu.2021.11.004>
- Kisielinski, K.; Willis, S.; Prescher, A.; Klosterhalfen, B. and Schumpelick, V. 2002. A simple new method to calculate small intestine absorptive surface in the rat. *Clinical and Experimental Medicine* 2:131-135. <https://doi.org/10.1007/s102380200018>

- Kogut, M. H.; Genovese, K. J.; Swaggerty, C. L.; He, H. and Broom, L. 2018. Inflammatory phenotypes in the intestine of poultry: not all inflammation is created equal. *Poultry Science* 97:2339-2346. <https://doi.org/10.3382/ps/pey087>
- Kraieski, A. L.; Hayashi, R. M.; Sanches, A.; Almeida, G. C. and Santin, E. 2017. Effect of aflatoxin experimental ingestion and *Eimeria* vaccine challenges on intestinal histopathology and immune cellular dynamic of broilers: applying an Intestinal Health Index. *Poultry Science* 96:1078-1087. <https://doi.org/10.3382/ps/pew397>
- Lamp, A. E.; Evans, A. M. and Moritz, J. S. 2015. The effects of pelleting and glucanase supplementation in hulled barley based diets on feed manufacture, broiler performance, and digesta viscosity. *Journal of Applied Poultry Research* 24:295-303. <https://doi.org/10.3382/japr/pfv028>
- Lopetuso, L. R.; Scaldaferrri, F.; Petito, V. and Gasbarrini, A. 2013. Commensal Clostridia: leading players in the maintenance of gut homeostasis. *Gut Pathogens* 5:23. <https://doi.org/10.1186/1757-4749-5-23>
- Lu, H.; Adedokun, S. A.; Adeola, L. and Ajuwon, K. M. 2014. Anti-inflammatory effects of non-antibiotic alternatives in coccidia challenged broiler chickens. *The Journal of Poultry Science* 51:14-21. <https://doi.org/10.2141/jpsa.0120176>
- Luna, L. G. 1968. Manual of histologic staining methods of the Armed Forces Institute of Pathology. 3rd ed. Mc Graw-Hill, New York, NY.
- Massuquetto, A.; Panisson, J. C.; Marx, F. O.; Surek, D.; Krabbe, E. L. and Maiorka, A. 2019. Effect of pelleting and different feeding programs on growth performance, carcass yield, and nutrient digestibility in broiler chickens. *Poultry Science* 98:5497-5503. <https://doi.org/10.3382/ps/pez176>
- Mendis, M.; Leclerc, E. and Simsek, S. 2016. Arabinoxylans, gut microbiota and immunity. *Carbohydrate Polymers* 139:159-166. <https://doi.org/10.1016/j.carbpol.2015.11.068>
- Miles, R. D.; Butcher, G. D.; Henry, P. R. and Littell, R. C. 2006. Effect of antibiotic growth promoters on broiler performance, intestinal growth parameters, and quantitative morphology. *Poultry Science* 85:476-485. <https://doi.org/10.1093/ps/85.3.476>
- Nelson, D. L. and Cox, M. M. 2014. Princípios de bioquímica de Lehninger. 6.ed. Artmed, Porto Alegre.
- Nguyen, H. T.; Bedford, M. R. and Morgan, N. K. 2021. Importance of considering non-starch polysaccharide content of poultry diets. *World's Poultry Science Journal* 77:619-637. <https://doi.org/10.1080/00439339.2021.1921669>
- Olukosi, O. A. and Bedford, M. R. 2019. Comparative effects of wheat varieties and xylanase supplementation on growth performance, nutrient utilization, net energy, and whole-body energy and nutrient partitioning in broilers at different ages. *Poultry Science* 98:2179-2188. <https://doi.org/10.3382/ps/pey582>
- Parsaie, S.; Shariatmadari, F.; Zamiri, M. J. and Khajeh, K. 2007. Influence of wheat-based diets supplemented with xylanase, bile acid and antibiotics on performance, digestive tract measurements and gut morphology of broilers compared with a maize-based diet. *British Poultry Science* 48:594-600. <https://doi.org/10.1080/00071660701615788>
- Pasquali, G. A. M.; Oliveira, R. F.; Aiello, P. A. B.; Polycarpo, G. V.; Crivellari, R. and Cruz-Polycarpo, V. C. 2017. Performance and economic viability of broiler chicken fed diets with multienzyme complexes. *Acta Scientiarum. Animal Sciences* 39:91-96. <https://doi.org/10.4025/actascianimsci.v39i1.32765>
- Pucci, L. E. A.; Rodrigues, P. B.; Freitas, R. T. F.; Fialho, E. T.; Nascimento, G. A. J. and Alvarenga, R. R. 2010. Efeito do processamento, suplementação enzimática e nível nutricional da ração para frangos de corte no período de 22 a 42 dias de idade. *Ciência e Agrotecnologia* 34:1557-1565. <https://doi.org/10.1590/S1413-70542010000600029>
- Ravindran, V. and Abdollahi, M. R. 2021. Nutrition and digestive physiology of the broiler chick: state of the art and outlook. *Animals* 11:2795. <https://doi.org/10.3390/ani11102795>
- Sakomura, N. K. and Rostagno, H. S. 2016. Métodos de pesquisa em nutrição de monogástricos. 2.ed. Funep, Jaboticabal.
- Saleh, K. M. M. and Al-Zghoul, M. B. 2019. Effect of acute heat stress on the mRNA levels of cytokines in broiler chickens subjected to embryonic thermal manipulation. *Animals* 9:499. <https://doi.org/10.3390/ani9080499>
- Saleh, A. A.; El-Naggar, K.; El Sayed El Bayoumi, M.; Amber, K.; Kamal, M. A.; Alhotan, R. A.; Hussein, E. O.; Galik, B. and Kirrella, A. A. 2025. Effects of supplementing low-energy and/or protein diets with xylanase and protease on broiler chicken growth performance, nutritional utilization, blood biochemical parameters, and gene expression associated with growth. *Cogent Food & Agriculture* 11:2520959. <https://doi.org/10.1080/23311932.2025.2520959>
- Saleh, A. A.; Mousa, A.; Amber, K.; Badwi, N.; Shoukry, M.; Khairy, M.; Nusairat, B.; Odetallah, N.; Amy Lin, Y.-M. and Selim, S. 2024. Effect of endo-1,4-beta-xylanase supplementation to low-energy diets on performance, blood constituents, nutrient digestibility, and gene expressions related growth of broiler chickens. *Journal of Animal Physiology and Animal Nutrition* 108:99-110. <https://doi.org/10.1111/jpn.13870>
- Shoaib, M.; Bhatti, S. A.; Ashraf, S.; Hamid, M. M. A.; Najam-us-Sahar; Javed, M. M.; Amir, S.; Aslam, N.; Roobi, A.; Iqbal, H. H.; Asif, M. A.; Nazir, U. and Saif-ur-Rehman, M. 2023. Fat digestion and metabolism: effect of different fat sources and fat mobilisers in broilers' diet on growth performance and physiological parameters – a review. *Annals of Animal Science* 23:641-661. <https://doi.org/10.2478/aoas-2022-0083>
- Šimić, A.; González-Ortiz, G.; Mansbridge, S. C.; Rose, S. P.; Bedford, M. R.; Yovchev, D. and Pirgozliev, V. R. 2023. Broiler chicken response to xylanase and fermentable xylooligosaccharide supplementation. *Poultry Science* 102:103000. <https://doi.org/10.1016/j.psj.2023.103000>

- Stefanello, C.; Vieira, S. L.; Soster, P.; Santos, B. M.; Dalmoro, Y. K.; Favero, A. and Cowieson, A. J. 2019. Utilization of corn-based diets supplemented with an exogenous α -amylase for broilers. *Poultry Science* 98:5862-5869. <https://doi.org/10.3382/ps/pez290>
- Wang, J.; Liu, S.; Ma, J. and Piao, X. 2021. Changes in growth performance and ileal microbiota composition by xylanase supplementation in broilers fed wheat-based diets. *Frontiers in Microbiology* 12:706396. <https://doi.org/10.3389/fmicb.2021.706396>
- Ward, N. E. 2021. Debranching enzymes in corn/soybean meal-based poultry feeds: a review. *Poultry Science* 100:765-775. <https://doi.org/10.1016/j.psj.2020.10.074>