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# Phytase and xylanase in diets with nutritional adjustments and their effects on serum biochemistry, morphometry and intestinal health of broilers

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**Abstract:** To examine the association between phytase and xylanase in diets with nutritional adjustments on intestinal morphometry, serum biochemistry and microbiology of broilers, 250 broilers were evaluated in a completely randomized design with five treatments and five replicates. The following treatments were tested: positive control diet - without phytase or xylanase; negative control diet - with an energy reduction of 100 kcal/kg, without phytase or xylanase; and three diets containing xylanase and phytase and energy reductions of 50, 100 and 150 Kcal/kg. For all energy-reduced diets, the nutritional matrix of phytase with phosphorus (0.15%), calcium (0.165%) and sodium (0.035%) was considered. An effect of the association between enzyme inclusion and metabolizable energy reduction in the diets was observed only by contrast analysis, for villus height. Intestinal health was not changed. Only the serum phosphorus concentration was altered by the treatments at the different evaluated ages. The association of phytase (500 FTU/kg) with xylanase (16000 BXU/kg) in diets with reductions of up to 150 Kcal/kg metabolizable energy, 0.15% digestible P, 0.165% Ca and 0.035% Na does not alter the intestinal morphometry, serum biochemistry or microbiology of broilers.

**Key words:** Exogenous enzyme, intestinal mucosa, non-starch polysaccharide, serum phosphorus, total bacterial count.

## INTRODUCTION

In recent years, broiler performance has been enhanced by maximizing the metabolizability of corn- and soybean-meal based diets with the use of highly specialized exogenous enzymes, particularly phytase and xylanase. These enzymes act on phytates and non-starch polysaccharides (NSPs), respectively (Vaz et al. 2013, Schramm et al. 2017), two of the main antinutritional factors present in grains (Rostagno et al. 2017).

The observed improvement in broiler performance is related to improved utilization of the nutrients and energy released by those enzymes (Masey-o Neill et al. 2014, Ghayour-Najafabadi et al. 2018). The breakdown of phytate molecules releases phosphorus (P), minerals, proteins, amino acids and starch. Xylanases, in turn provide more energy to the bird by acting on carbohydrate hydrolysis in the plant cell wall and releasing starch, lipids and proteins (Cowieson 2005), which are encapsulated within the insoluble cellular matrix or nutrients that

have been incorporated by the viscosity of NSPs during their passage through the gut (Carré 2004).

However, the better broiler performance achieved with the use of enzymes is related not only to improved nutrient utilization, but also to mucosal integrity and intestinal microbiota balance (Woyengo et al. 2011). The morphofunctional properties of the digestive system can be modulated by feeding, improving the intestinal health of these birds. Associating intestinal health data with the serum biochemical profile can provide relevant information about the metabolism of broilers and elucidate the effect of exogenous enzymes added to diets on their health, especially when the nutritional matrix of each enzyme is considered in formulations.

Studies have suggested positive effects on microbiota balance and morphology when xylanase was used in broiler diets (Nian et al. 2011, Liu & Kim 2017). The xylooligosaccharides produced by exo-xylanases and/or β-xylosidases can act as prebiotics (Manning & Gibson 2004, Barreto et al. 2015) capable of stimulating the growth and activity of beneficial microorganisms in the digestive system of broilers (Lafond et al. 2011, Masey-o Neill et al. 2014, Liu & Kim 2017) and short-chain fatty acid production (Kiarie et al. 2014).

The use of phytase alone, on the other hand, may not have effects on the intestinal mucosa (Pirgozliev et al. 2008), but the association between phytase and xylanase may have an additive effect, since the efficiency of phytase added to the diet can be improved by the access to the substrate released by hydrolysis of the NSPs. Similarly, phytase insufficiency may reduce the action of carbohydrases on nutrients that may be bound to the phytate molecule (Olukosi et al. 2007, Schramm et al. 2017).

However, when using exogenous enzymes, one must maintain an ideal ratio between nutrients to prevent the occurrence of antagonisms and enzymatic action from being silenced. High levels of calcium (Ca) and sodium (Na) can interact with other minerals (iron, magnesium and zinc) and endogenous amino acids, respectively, making them unavailable for absorption and thus masking the effect of phytase (Qian et al. 1997, Cowieson et al. 2011).

On these bases, this study examines the association between phytase and xylanase in diets with nutritional adjustments on the intestinal morphometry, serum biochemistry and microbiology of broilers.

## MATERIALS AND METHODS

The study was developed at the Experimental Laboratory of Poultry Science at the Faculty of Veterinary Medicine and Animal Science, Federal University of Mato Grosso do Sul. The procedures employed in this study were approved by the Ethics Committee on Animal Use (CEUA\UFMS approval no. 737/2015).

A total of 250 one-day-old male broilers of the Cobb 500 line were distributed in a completely randomized design with five treatments and five replicates of 10 broilers per experimental unit. The broilers were standardized by weight (± 10% of the average weight of the experimental unit) so that all experimental units had similar weights.

Broilers were housed in twenty-five 2.5-$m^2$ cages in a conventional shed with fiber-cement roofing and earthen floor. Wood shavings (untreated wood chips) were used as bedding material; the bed was considered new, as it had not been used previously. Cages were equipped with brood heaters, trough feeders and automatic poultry drinkers. Feed and water

were provided *ad libitum*. The lighting program adopted was 24 h of light (natural + artificial) daily.

Thermal conditions in the poultry house were monitored using a temperature and humidity sensor at 07h00 and 17h00. The following mean values were obtained: average temperature: 24.5°C, minimum temperature - 20.8 ± 0.7°C, maximum temperature: 28.3 ± 0.9°C; air relative humidity: 66% ± 7%.

The experimental treatments consisted of a positive control diet fully meeting the nutritional requirements, without phytase or xylanase; a negative control diet with a metabolizable energy (ME) reduction of 100 Kcal/kg, without phytase or xylanase; and diets with ME reductions of 50, 100 and 150 Kcal/kg, with phytase and xylanase. For all energy-reduced diets, the nutritional matrix of phytase with reductions of 0.15% P, 0165% Ca and 0.035% Na was considered.

The phytase used in the experiment was derived from the microorganism *Escherichia coli*, while xylanase was derived from *Tricoderma reesei*. Their respective inclusion levels were fixed at 75 g/t (500 FTU/kg) and 100 g/t (16000 BXU/kg). Both enzymes were acquired from commercial enterprises.

The experimental diets were based on corn and soybean meal and were formulated so as to meet the nutritional requirements of broilers as proposed by Rostagno et al. (2011) for each development stage, namely, pre-starter (1 to 7 days; Table I), starter (8 to 21 days; Table II), grower (22 to 33 days; Table III) and pre-slaughter - (34 to 42 days; Table IV), except for ME, available P, Ca and Na.

At 21 days of age, five broilers were selected per treatment based on the mean body weight of the experimental unit, under a margin of ±10%. These were feed-deprived for 6 h and subsequently desensitized by cervical dislocation and slaughtered by complete bleeding between the occipital and atlas bones.

After slaughter, laparotomy was performed and samples approximately three centimeters long were collected from segments of the small intestine (jejunum and ileum), considering the jejunum from the distal portion of the duodenal loop to Meckel's diverticulum (vitelline diverticulum) and the ileum as the portion anterior to the cecum.

The intestine samples were washed with saline solution to remove the intestinal contents, fixed in 10% formaldehyde solution and subsequently dehydrated in increasing alcohol concentrations, cleared in xylol and paraffin-embedded according to the methodology described by Beçak & Paulete (1976). Longitudinal and semiserial histological sections with 7-µm thickness were obtained and subsequently stained by the Hematoxylin-Eosin method.

Image capturing was achieved by morphometry using a microscope (Leica DMi8). The images were captured with a 4x objective and analyzed using a computerized imaging system (LAS X). Small intestine morphometry was evaluated by measuring villus length, crypt depth and villus:crypt ratio of each replicate per segment and calculating the average of these values.

Intestinal microbiology analysis was performed every seven days from the first day of age until the end of the experiment. Sampling was performed in a pool consisting of one broiler from each replicate (five broilers per treatment). Samples were collected by cloacal swabbing and packed in flasks containing 0.1% peptone water.

For quantitative analysis, total mesophilic aerobic bacteria were counted by the pour-plate count agar technique (PCA). For this purpose, serial dilutions of the swabs were carried out.

**Table I. Centesimal and calculated compositions of experimental diets for broilers in the pre-starter phase (1 to 7 days).**

| Ingredient % | Positive control | Negative control | Reduction of metabolizable energy (Kcal/kg), with phytase and xylanase | | |
|---|---|---|---|---|---|
| | | | 50 | 100 | 150 |
| Corn, 7.88% | 54.05 | 54.05 | 54.05 | 54.05 | 54.05 |
| Soybean meal, 46% | 38.82 | 38.82 | 38.82 | 38.82 | 38.82 |
| Soybean oil | 2.57 | 1.42 | 1.99 | 1.42 | 0.85 |
| Dicalcium phosphate | 1.90 | 1.09 | 1.09 | 1.09 | 1.09 |
| Limestone | 0.91 | 1.00 | 1.00 | 1.00 | 1.00 |
| Salt | 0.51 | 0.42 | 0.42 | 0.42 | 0.42 |
| Mineral and vitamin supplement[1] | 0.40 | 0.40 | 0.40 | 0.40 | 0.40 |
| DL-methionine | 0.36 | 0.36 | 0.36 | 0.36 | 0.36 |
| L-lysine HCl | 0.27 | 0.27 | 0.27 | 0.27 | 0.27 |
| L-threonine | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 |
| Inert | 0.10 | 2.05 | 1.48 | 2.05 | 2.62 |
| Xylanase | 0.00 | 0.00 | 0.01 | 0.01 | 0.01 |
| Phytase | 0.00 | 0.00 | 0.0075 | 0.0075 | 0.0075 |
| TOTAL | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| **Calculated values** | | | | | |
| Metabolizable energy (Kcal/kg) | 2960 | 2860 | 2910 | 2860 | 2810 |
| Crude protein (%) | 22.71 | 22.71 | 22.71 | 22,71 | 22.71 |
| Dig. arginine (%) | 1.43 | 1.43 | 1.43 | 1.43 | 1.43 |
| Dig. lysine (%) | 1.32 | 1.32 | 1.32 | 1.32 | 1.32 |
| Dig. methionine + cystine (%) | 0.95 | 0.95 | 0.95 | 0.95 | 0.95 |
| Dig. threonine (%) | 0.86 | 0.86 | 0.86 | 0.86 | 0.86 |
| Dig. tryptophan (%) | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 |
| Calcium (%) | 0.92 | 0.75 | 0.75 | 0.75 | 0.75 |
| Potassium (%) | 0.87 | 0.87 | 0.87 | 0.87 | 0.87 |
| Av. phosphorus (%) | 0.47 | 0.32 | 0.32 | 0.32 | 0.32 |
| Chlorine (%) | 0.35 | 0.30 | 0.30 | 0.30 | 0.30 |
| Sodium (%) | 0.22 | 0.18 | 0.18 | 0.18 | 0.18 |

**[1]Provides per kg of diet: 65.25 g choline; 2,750,000 IU vitamin A; 500,000 IU vitamin D3; 4,000 IU vitamin E; 375 mg vitamin K3; 300 mg vitamin B1; 1,125 mg vitamin B2; 500 mg vitamin B6; 4,000 mcg vitamin B12; 8,750 mg niacin; 2,300 mg pantothenic acid; 100 mg folic acid; 15 mg biotin; 7,500 mg iron; 2,250 mg copper; 15 g manganese; 15 g zinc; 250 mg iodine; 62.5 mg selenium; 2,500 mg avilamycin; 10 g nicarbazin; 3,750 mg semduramicin.**

These were then dispensed into sterile plates (100 µL), on which 10 mL of molten PCA agar was poured. Plates were kept in an oven at 37°C for 24 h.

The number of colony-forming units (CFU/mL) was obtained after counting and averaging the colony-containing plates. Qualitative analysis was also performed to identify the main genus/species of enteric bacteria. This step was carried out by culturing an aliquot of the liquid containing the swabs in MacConkey agar.

After incubation at 37°C for 24 h, the samples were identified by biochemical reactions in specific media for enterobacterium identification. All microbiological analyses were performed according to the techniques recommended by Winn-Júnior et al. (2008).

For the evaluation of the serum biochemical profile, blood was collected from two broilers per experimental unit at 21 and 42 days of age, after a neck section performed during slaughter. The blood was placed in identified tubes which were then centrifuged at 6000 rpm for 10 min to obtain the serum. Concentrations of Ca (mg/dL), P (mg/dL), total cholesterol and triglycerides were analyzed using Cobas 111® commercial kits based on the principles of spectrophotometry.

Data from the analysis of intestinal morphometry and serum biochemical profile were subjected to analysis of variance, and the means were compared by Tukey's test ($P < 0.05$) and also by orthogonal contrasts, by the Scheffe test ($P < 0.05$). The analyses were carried out using SAS statistical program (University version). The following contrasts were tested: C1 - diet with reductions of ME (100 Kcal/kg), P, Ca and sodium, with phytase and xylanase × diet with reductions of ME (100 Kcal/kg), P, Ca and Na, without phytase or xylanase, indicating the effect of enzyme supplementation on the ME reduction of 100 Kcal/kg; C2 - positive control diet × all other treatments, which indicates the effect of the reduction of ME, P, Ca and Na in relation to the positive control diet; C3 - positive control diet × treatments with reductions of 50, 100 and 150 Kcal/kg of ME and P, Ca and Na, containing phytase and xylanase, indicating the effect of ME reduction, nutritional adjustments and enzyme supplementation in relation to positive control diet.

Descriptive statistics were used for the quantitative and qualitative microbiological analyses.

**Table II. Centesimal and calculated compositions of experimental diets for broilers in the starter phase (8 to 21 days).**

| Ingredient % | Positive control | Negative control | Reduction of metabolizable energy (Kcal/kg), with phytase and xylanase | | |
|---|---|---|---|---|---|
| | | | 50 | 100 | 150 |
| Corn, 7.88% | | 58.16 | 58.16 | 58.16 | 58.16 |
| Soybean meal, 46% | 34.75 | 34.75 | 34.75 | 34.75 | 34.75 |
| Soybean oil | 2.62 | 1.48 | 2.05 | 1.48 | 0.91 |
| Dicalcium phosphate | 1.49 | 0.67 | 0.67 | 0.67 | 0.67 |
| Limestone | 0.91 | 1.01 | 1.01 | 1.01 | 1.01 |
| Salt | 0.50 | 0.39 | 0.39 | 0.39 | 0.39 |
| Mineral and vitamin supplement[1] | 0.40 | 0.40 | 0.40 | 0.40 | 0.40 |
| DL-methionine | 0.31 | 0.31 | 0.31 | 0.31 | 0.31 |
| L-lysine HCl | 0.27 | 0.27 | 0.27 | 0.27 | 0.27 |
| L-threonine | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 |
| Inert | 0.52 | 2.47 | 1.88 | 2.45 | 3.02 |
| Xylanase | 0.00 | 0.00 | 0.01 | 0.01 | 0.01 |
| Phytase | 0.00 | 0.00 | 0.0075 | 0.0075 | 0.0075 |
| TOTAL | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| **Calculated values** | | | | | |
| Metabolizable energy (Kcal/kg) | 3050 | 2950 | 3000 | 2950 | 2900 |
| Crude protein (%) | 21.20 | 21.20 | 21.20 | 21.20 | 21.20 |
| Dig. arginine (%) | 1.31 | 1.31 | 1.31 | 1.31 | 1.31 |
| Dig. lysine (%) | 1.22 | 1.22 | 1.22 | 1.22 | 1.22 |
| Dig. methionine + cystine (%) | 0.89 | 0.89 | 0.89 | 0.89 | 0.89 |
| Dig. threonine (%) | 0.79 | 0.79 | 0.79 | 0.79 | 0.79 |
| Dig. tryptophan (%) | 0.24 | 0.24 | 0.24 | 0.24 | 0.24 |
| Calcium (%) | 0.84 | 0.68 | 0.68 | 0.68 | 0.68 |
| Potassium (%) | 0.81 | 0.81 | 0.81 | 0.81 | 0.81 |
| Av. phosphorus (%) | 0.40 | 0.25 | 0.25 | 0.25 | 0.25 |
| Chlorine (%) | 0.33 | 0.27 | 0.27 | 0.27 | 0.27 |
| Sodium (%) | 0.21 | 0.17 | 0.17 | 0.17 | 0.17 |

**[1]Provides per kg of diet: 65.25 g choline; 2,750,000 IU vitamin A; 500,000 IU vitamin D3; 4,000 IU vitamin E; 375 mg vitamin K3; 300 mg Vitamin B1; 1,125 mg vitamin B2; 500 mg vitamin B6; 4,000 mcg vitamin B12; 8.750 mg niacin; 2,300 mg pantothenic acid; 100 mg folic acid; 15 mg biotin; 7,500 mg iron; 2,250 mg copper; 15 g manganese; 15 g zinc; 250 mg iodine; 62.5 mg selenium; 2,500 mg avilamycin; 10 g nicarbazin; 3,750 mg semduramicin.**

**Table III. Centesimal and calculated compositions of experimental diets for broilers in the grower phase (22 to 33 days).**

| Ingredient % | Positive control | Negative control | Reduction of metabolizable energy (Kcal/kg), with phytase and xylanase | | |
|---|---|---|---|---|---|
| | | | 50 | 100 | 150 |
| Corn, 7.88% | 60.44 | 60.44 | 60.44 | 60.44 | 60.44 |
| Soybean meal, 46% | 31.63 | 31.63 | 31.63 | 31.63 | 31.63 |
| Soybean oil | 4.17 | 3.03 | 3.60 | 3.03 | 2.46 |
| Dicalcium phosphate | 1.34 | 0.53 | 0.53 | 0.53 | 0.53 |
| Limestone | 0.89 | 0.98 | 0.98 | 0.98 | 0.98 |
| Salt | 0.46 | 0.37 | 0.37 | 0.37 | 0.37 |
| Mineral and vitamin supplement[1] | 0.30 | 0.30 | 0.30 | 0.30 | 0.30 |
| DL-methionine | 0.30 | 0.30 | 0.30 | 0.30 | 0.30 |
| L-lysine HCl | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 |
| L-threonine | 0.07 | 0.07 | 0.07 | 0.07 | 0.07 |
| Inert | 0.15 | 2.10 | 1.53 | 2.10 | 2.67 |
| Xylanase | 0.00 | 0.01 | 0.01 | 0.01 | 0.01 |
| Phytase | 0.00 | 0.0075 | 0.0075 | 0.0075 | 0.0075 |
| TOTAL | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| **Calculated values** | | | | | |
| Metabolizable energy (Kcal/kg) | 3150 | 3050 | 3100 | 3050 | 3000 |
| Crude protein (%) | 19.82 | 19.82 | 19.82 | 19.82 | 19.82 |
| Dig. arginine (%) | 1.22 | 1.22 | 1.22 | 1.22 | 1.22 |
| Dig. lysine (%) | 1.13 | 1.13 | 1.13 | 1.13 | 1.13 |
| Dig. methionine + cystine (%) | 0.83 | 0.83 | 0.83 | 0.83 | 0.83 |
| Dig. threonine (%) | 0.73 | 0.73 | 0.73 | 0.73 | 0.73 |
| Dig. tryptophan (%) | 0.21 | 0.21 | 0.21 | 0.21 | 0.21 |
| Calcium (%) | 0.76 | 0.59 | 0.59 | 0.59 | 0.59 |
| Potassium (%) | 0.75 | 0.75 | 0.75 | 0.75 | 0.75 |
| Av. phosphorus (%) | 0.35 | 0.20 | 0.20 | 0.20 | 0.20 |
| Chlorine (%) | 0.32 | 0.27 | 0.27 | 0.27 | 0.27 |
| Sodium (%) | 0.20 | 0.16 | 0.16 | 0.16 | 0.16 |

**[1]Provides per kg of diet: 65.25 g choline; 2,750,000 IU vitamin A; 500,000 IU vitamin D3; 4,000 IU vitamin E; 375 mg vitamin K3; 300 mg vitamin B1; 1,125 mg vitamin B2; 500 mg vitamin B6; 4,000 mcg vitamin B12; 8,750 mg niacin; 2,300 mg pantothenic acid; 100 mg folic acid; 15 mg biotin; 7,500 mg iron; 2,250 mg copper; 15 g manganese; 15 g zinc; 250 mg iodine; 62.5 mg selenium; 2,500 mg avilamycin; 10 g nicarbazin; 3,750 mg semduramicin.**

**Table IV. Centesimal and calculated compositions of experimental diets for broilers in the pre-slaughter phase (34 to 42 days).**

| Ingredient % | Positive control | Negative control | Reduction of metabolizable energy (Kcal/kg), with phytase and xylanase | | |
|---|---|---|---|---|---|
| | | | 50 | 100 | 150 |
| Corn, 7.88% | 63.30 | 63.30 | 63.30 | 63.30 | 63.30 |
| Soybean meal, 46% | 28.96 | 28.96 | 28.96 | 28.96 | 28.96 |
| Soybean oil | 4.36 | 3.21 | 3.78 | 3.21 | 2.64 |
| Dicalcium phosphate | 1.12 | 0.31 | 0.31 | 0.31 | 0.31 |
| Limestone | 0.79 | 0.88 | 0.88 | 0.88 | 0.88 |
| Salt | 0.44 | 0.36 | 0.36 | 0.36 | 0.36 |
| Mineral and vitamin supplement[1] | 0.30 | 0.30 | 0.30 | 0.30 | 0.30 |
| DL-methionine | 0.26 | 0.26 | 0.26 | 0.26 | 0.26 |
| L-lysine HCl | 0.24 | 0.24 | 0.24 | 0.24 | 0.24 |
| L-threonine | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 |
| Inert | 0.15 | 2.11 | 1.54 | 2.11 | 2.68 |
| Xylanase | 0.00 | 0.00 | 0.01 | 0.01 | 0.01 |
| Phytase | 0.00 | 0.00 | 0.0075 | 0.0075 | 0.0075 |
| TOTAL | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| **Calculated values** | | | | | |
| Metabolizable energy (Kcal/kg) | 3200 | 3100 | 3150 | 3100 | 3050 |
| Crude protein (%) | 18.78 | 18.78 | 18.78 | 18.78 | 18.78 |
| Dig. arginine (%) | 1.14 | 1.14 | 1.14 | 1.14 | 1.14 |
| Dig. lysine (%) | 1.06 | 1.06 | 1.06 | 1.06 | 1.06 |
| Dig. methionine + cystine (%) | 0.77 | 0.77 | 0.77 | 0.77 | 0.77 |
| Dig. threonine (%) | 0.69 | 0.69 | 0.69 | 0.69 | 0.69 |
| Dig. tryptophan (%) | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 |
| Calcium (%) | 0.66 | 0.50 | 0.50 | 0.50 | 0.50 |
| Potassium (%) | 0.71 | 0.71 | 0.71 | 0.71 | 0.71 |
| Av. phosphorus (%) | 0.31 | 0.16 | 0.16 | 0.16 | 0.16 |
| Chlorine (%) | 0.32 | 0.26 | 0.26 | 0.26 | 0.26 |
| Sodium (%) | 0.19 | 0.16 | 0.16 | 0.16 | 0.16 |

**[1]Provides per kg of diet: 1,104 mg pantothenic acid; 4.5 mg biotin; 3,000 mg copper; 43.48 g choline; 10 g iron; 333.33 mg iodine; 20 g manganese; 1,500 mg niacin; 60 mg selenium; 900,000 IU vitamin A; 90 mg vitamin B1; 900 mcg vitamin B12; 300 mg vitamin B2; 120 mg vitamin B6; 150,000 IU vitamin D3; 1,500 IU vitamin E; 150 mg vitamin K3; 20 g zinc.**

## RESULTS AND DISCUSSION

The morphometry of the jejunum and ileum of the broilers at 21 days of age was not influenced (P>0.05) by the evaluated treatments, according to the means test. However, by contrast C1, phytase and xylanase addition reduced (P<0.05) ileal villus length in the animals fed diets with a reduction of 100 Kcal/kg of ME and adjusted for the levels of P, Ca and Na (Table V).

A similar result was reported by Wu et al. (2004), who supplemented xylanase (1,000 BXU/kg) and phytase (500 FTU/kg) in wheat-containing diets and observed a reduction in the size of the duodenal villi of broiler chickens at 21 days of age. The authors reported that this result is unexpected and difficult to explain, as also noted in this study.

The absence of treatment effects on the other variables is possibly related to the high metabolizability of corn and soybean meal, since highly digestible diets probably cause fewer lesions in the intestinal mucosa. Thus, it is possible to reduce the energy destined for the protein turnover of the intestinal epithelium and, consequently, increase the net energy destined for lean meat deposition, ultimately resulting in increased weight gain and improved feed conversion.

The intestinal villi are responsible for the absorption of nutrients in the intestinal lumen and are constantly renewed by cells that migrate from the crypt (Leser & MØlbak, 2009). Intestinal inflammation, which can be either due to ingredients that are aggressive to the mucosa—as in the case of NSPs—or a dense population

**Table V. Intestinal morphometry of 21-day-old broilers fed diets with reductions in metabolizable energy (MER), P, Ca and Na and supplemented with phytase and xylanase (PX).**

| MER (Kcal/kg)* | PX | Jejunum | | | Ileum | | |
|---|---|---|---|---|---|---|---|
| | | Villus height | Crypt depth | Villus:crypt | Villus height | Crypt depth | Villus:crypt |
| | | --------- µm -------- | | | --------- µm --------- | | |
| 0 | Without | 821.56 | 125.39 | 6.71 | 760.02 | 131.42 | 5.88 |
| 100 | Without | 726.09 | 99.79 | 7.31 | 787.23 | 146.07 | 5.63 |
| 50 | With | 764.21 | 111.03 | 7.27 | 819.37 | 105.09 | 7.89 |
| 100 | With | 816.05 | 109.94 | 7.76 | 618.13 | 118.65 | 5.24 |
| 150 | With | 706.82 | 91.34 | 7.78 | 723.85 | 155.80 | 4.85 |
| Mean | | 763.84 | 106.21 | 7.43 | 737.14 | 134.77 | 5.68 |
| CV (%)[1] | | 12.21 | 21.41 | 21.79 | 14.66 | 22.21 | 21.07 |
| P-value | | 0.306 | 0.336 | 0.889 | 0.173 | 0.261 | 0.099 |
| | | Orthogonal contrast[2**] | | | | | |
| C1 | | 0.169 | 0.515 | 0.687 | 0.034 | 0.192 | 0.634 |
| C2 | | 0.255 | 0.132 | 0.423 | 0.692 | 0.999 | 0.975 |
| C3 | | 0.330 | 0.157 | 0.393 | 0.521 | 0.773 | 0.870 |

[1]CV = coefficient of variation; [2]P<0.05 is significant by the Scheffe test;
*Reduction of 0 Kcal/kg - positive control; Reduction of 100 Kcal/kg, without enzymes - negative control;
**C1: diet with a ME reduction of 100 Kcal/kg, with phytase and xylanase × diet with a ME reduction 100 Kcal/kg, without phytase or xylanase; C2: positive control diet × all other treatments; C3: positive control diet × diets with ME reductions of 50, 100 and 150 Kcal and adjustments for P, Ca and Na, containing phytase and xylanase.

of pathogenic microorganisms, reduces the amount of enterocytes in the apical extremity of the villi and crypt hyperplasia. As a result, the villus:crypt ratio decreases, which is an undesirable effect (Wu et al. 2004, Domeneghini et al. 2006, Leser & MØlbak 2009).

Pirgozliev et al. (2008) supplemented diets containing corn, soybean meal and gluten for 21-day-old broilers with phytase (250, 500 and 2500 FTU/kg) and did not observe alterations in the height or thickness of ileal villi. The same was reported by Fernandes et al. (2017), who evaluated the effect of addition of 50 g/t of an enzyme complex (xylanase, amylase and protease) in starter diets (21 days) with or without energy reduction (2970 and 2820 Kcal/kg, respectively). Those authors did not observe changes in villus height or crypt depth in the duodenum and jejunum of broilers.

The treatments did not change the number of bacteria in the gastrointestinal tract of the broilers. This is a positive fact, because it does not compromise the process of intestinal colonization, an event necessary to ensure a healthy digestive system (Table VI).

In the first day of life of the broilers, no colonies were observed in any of the treatment groups. This result was already expected, since the number of microorganisms in the gastrointestinal tract during embryonic phase is practically zero (Van der Wielen et al. 2002). Moreover, the short time between birth and the analyses did not allow for colonization, since the broilers had not yet been fed and were not in contact with the bed.

In the first day of life, the presence of an Enterobacterium species (*Serratia rubidae*) was only identified in the group fed the diet containing phytase and xylanase with a ME reduction of 150 Kcal/kg and adjusted for P, Ca and Na.

However, this species is commonly found in the gastrointestinal tract of chickens. The fact that total bacterial count revealed no colony formation but enterobacteria were identified for this treatment is due to the culture medium used for each analysis. The qualitative technique used to identify gram-negative bacteria is performed on MacConkey agar, with a high concentration of bile salts that provides a rapid growth of enterobacteria, possibly stimulating the growth of the species found.

From the 7th day, gram-negative bacteria of the species *Escherichia coli*, *Klebsella ozaenae* and *Proteus mirabilis* were detected in broilers fed the diets with ME reductions of up to 100 Kcal/kg; and of *Escherichia coli*, *Klebsiella ozaenae* and *Enterobacter agglomerans* in broilers fed the diet with a ME reduction of 150 Kcal/kg associated with phytase and xylanase.

Despite belonging to the family Enterobacteriaceae and being potentially pathogenic, mainly in immunosuppressed animals (Nian et al. 2011, Liu & Kim 2017) when housed in regions outside the intestine, these bacteria are commonly found in broilers' microbiota (Praxedes et al. 2012). This may explain their presence in the gastrointestinal tract of the broilers in this study, regardless of the treatment.

The species of enterobacteria found in this experiment are naturally present in large amounts in organic matter, soil, water and even on the internal and external surface of *Alphitobius diaperinus*, commonly known as darkling beetle (Segabinazi et al. 2005), one of the main plagues of poultry farming.

Presence of *Actinomyces viscosus* was only detected in the treatment constituted by the ME reduction of 150 Kcal/kg plus enzymes on the 7th day, in one bird. *Actinomyces viscosus* is an actinobacterium of human prevalence (Figueroa-Gordon et al. 2009) that is not common in the

**Table VI.** Total bacterial count (TBC - CFU/mL x10$^4$) and intestinal bacteria found in broilers fed diets with reductions in energy, P, Ca and Na and supplemented with phytase and xilanase.

| Broiler age (days) | Reduction of metabolizable energy (Kcal/kg) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 - Positive control | | 100 - Negative control | | 50 - with phytase and xylanase | | 100 - with phytase and xylanase | | 150 - with phytase and xylanase | |
| | TBC | Bacterium | TBC | Bacterium | TBC | Bacterium | TBC | Bacterium | TBC | Bacterium |
| 1 | Negative | - | Negative | - | Negative | - | Negative | - | Negative | *S. rubidae* |
| | | | | | | | | | | |
| 7 | 8.57 | *E. coli*<br>*K. ozaenae* | 0.50 | *E. coli*<br>*K. ozaenae* | 4.10 | *E. coli*<br>*K. ozaenae* | 2.17 | *E. coli*<br>*K. ozaenae* | 1.50 | *E. coli*<br>*K. ozaenae*<br>*A. viscosus* |
| | | | | | | | | | | |
| 14 | 18.00 | *E. coli* | 12.15 | *E. coli*<br>*P. mirabilis* | 7.50 | *E. coli* | 8.00 | *E. coli* | 4.00 | *E. coli*<br>*E. agglomerans* |
| | | | | | | | | | | |
| 21 | 9.83 | *E. coli*<br>*K. ozaenae*<br>*P. mirabilis* | 5.63 | *E. coli*<br>*K. ozaenae* | 1.30 | *E. coli*<br>*K. ozaenae* | 14.07 | *E. coli*<br>*P. mirabilis* | 4.80 | *E. coli*<br>*K. ozaenae* |
| | | | | | | | | | | |
| 28 | 18.90 | *E. coli* | 46.40 | *E. coli* | 8.20 | *E. coli* | 109.60 | *E. coli*<br>*P. mirabilis* | 3.20 | *E. coli* |
| | | | | | | | | | | |
| 35 | 13.77 | *E. coli*<br>*P. mirabilis* | 22.63 | *E. coli*<br>*P. mirabilis* | 11.03 | *E. coli* | 6.50 | *E. coli* | 3.60 | *E. coli* |
| | | | | | | | | | | |
| 42 | 12.33 | *E. coli*<br>*P. mirabilis* | 17.97 | *E. coli* | 137.90 | *E. coli*<br>*P. mirabilis* | 240.33 | *E. coli*<br>*P. mirabilis* | 55.50 | *E. coli* |

gastrointestinal tract of broilers. Because it is dispersed in the air, this actinobacterium might have been inhaled by the broiler and stopped in its gastrointestinal tract via the respiratory system and eliminated. As the broilers were healthy and subjected to proper sanitary management, no symptoms or proliferation of this bacterial species were detected.

Gao et al. (2008) evaluated diets containing wheat associated with or without an enzyme complex (1 g/t; xylanase - 1218 U/g, glucanase - 63 U/g, cellulose - 40 U and pectinase - 61 U/g) and did not find significant changes in lactobacilli and coliform counts in the cecum of 21-day-old chickens. The same findings were reported by Leite et al. (2012), who evaluated an enzyme complex composed of amylase, pectinase, betaglucanase, cellulase, protease and phytase, in the proportion of 200 g/t, in diets containing sorghum, millet and soybean meal for broilers at 14 and 28 days of age.

The higher bacterial count observed at the end of the rearing period (42 days) may be related to the continuous contact of the broilers with the bed containing excreta and traces of feed that fell from the feeders and accumulated throughout the experimental period. However, the proper management of drinkers allowed for low bed moisture and low proliferation of pathogenic microorganisms.

For the serum biochemical levels of chickens at 21 and 42 days of age (Table VII), only the phosphorus concentration was altered ($P < 0.05$) at both ages.

At 21 days of age, broilers fed the negative control diet presented the lowest ($P<0.05$) concentration of serum phosphorus. The serum concentrations of the birds fed diets with ME reductions of 100 and 150 Kcal/kg were intermediate, as they did not differ statistically from the positive and negative control treatments. These results reflected the concentration of P absorbed and available for use in the broiler metabolism and showed that enzyme supplementation, especially phytase, was effective in the breakdown of phytate molecules by means of sequential dephosphorylation reactions, producing smaller molecules of myo-inositol phosphate esters and inorganic phosphors suitable for absorption into the intestinal lumen (Bedford & Partridge 2010).

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At 42 days of age, regardless of the level, the energy-reduced diets supplemented with phytase and xylanase resulted in similar serum P levels ($P>0.05$) compared to positive control diet. The lowest ($P<0.05$) P concentration was also observed in the blood serum of broilers fed the negative control diet.

At both ages, the concentration of P present in the blood serum was within the range considered normal for broilers (5 to 7 mg/dL) (Vieites et al. 2011, Manangi et al. 2018), except for the birds from the negative control treatment. The low serum P concentration observed in the broilers fed the negative control diet indicated that they were under hypophosphatemia due to

the low P intake, and it was probably fostered by the reduction of ME.

Phosphorus-deficient broilers present a reduction in feed intake due to loss of appetite. As a consequence, their performance is impaired, which is due mainly to disturbances in energy metabolism caused by the insufficient amount of P to form adenosine triphosphate (ATP) (Moe 2008).

By C1 contrast analysis, only in the final rearing period did the serum P concentration of broilers fed diets with a ME reduction of 100 Kcal/kg and adjusted for P, Ca and Na levels increase ($P<0.05$) when phytase and xylanase were added to the formulation. However, by contrast C2, the reduction of energy, P, Ca and Na in the diets reduced ($P<0.05$) the serum phosphorus level at both ages. This shows that nutrient reduction can only be done when associated with enzyme supplementation, a fact underpinned by the absence of differences in C3 contrast.

Lelis et al. (2010) tested a diet containing 2955 Kcal/kg of ME supplemented with phytase (500 UFT/kg), taking into account its nutritional matrix (0.36% crude protein, 0.1% calcium, 0.13% phosphorus, 45 Kcal ME and 0.010% digestible lysine) for broilers at 25 days, and reported an improvement of 29.47% in the ileal metabolizability coefficient of P and 39.42% more P retained in relation to the control treatment. This means a larger amount of phosphorus was absorbed and sent to the bloodstream to be readily used, either for bone deposition or for maintenance of plasma levels.

Only at 21 days of age and by C1 contrast did the cholesterol of broilers fed the diet with a ME reduction of 100 Kcal/kg containing phytase and xylanase declined (by 45.60 mg/dL) ($P<0.05$)

**Table VII. Serum biochemical profile of broilers fed diets with reductions in metabolizable energy (MER), P, Ca and Na and supplemented with phytase and xylanase (PX).**

| MER Kcal/kg* | PX | 21 days** | | | | 42 days** | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | P | Ca | TC | TRG | P | Ca | TC | TRG |
| | | ----------mg/dL---------- | | | | ----------mg/dL---------- | | | |
| 0 | Without | 5.44a | 7.53 | 89.00 | 20.37 | 6.16a | 8.54 | 90.40 | 16.32 |
| 100 | Without | 2.38b | 7.64 | 119.60 | 31.40 | 1.92b | 6.39 | 83.80 | 24.17 |
| 50 | With | 5.64a | 7.88 | 111.80 | 32.94 | 5.34a | 7.96 | 78.20 | 12.82 |
| 100 | With | 2.68ab | 7.27 | 74.00 | 30.10 | 6.31a | 7.34 | 97.80 | 20.97 |
| 150 | With | 4.23ab | 7.94 | 118.00 | 33.26 | 5.33a | 8.29 | 88.60 | 19.03 |
| Mean | | 4.07 | 7.65 | 102.64 | 29.61 | 5.01 | 7.70 | 87.76 | 18.66 |
| CV (%)[1] | | 38.90 | 33.19 | 29.84 | 38.87 | 27.72 | 21.87 | 24.94 | 50.67 |
| P-value[2] | | 0.009 | 0.993 | 0.108 | 0.398 | 0.001 | 0.301 | 0.692 | 0.405 |
| | | | Orthogonal contrast[3]*** | | | | | | |
| C1 | | 0.774 | 0.820 | 0.029 | 0.860 | <0.001 | 0.381 | 0.324 | 0.598 |
| C2 | | 0.043 | 0.905 | 0.279 | 0.058 | 0.052 | 0.230 | 0.766 | 0.543 |
| C3 | | 0.139 | 0.900 | 0.437 | 0.062 | 0.492 | 0.448 | 0.848 | 0.795 |

*Reduction of 0 Kcal/kg - positive control; Reduction of 100 Kcal/kg, without enzymes - negative control;
**Ca: calcium; P: phosphorus; TC: total cholesterol; TRG: triglycerides;
*** C1: diet with a ME reduction 100 Kcal/kg, with phytase and xylanase × diet with a ME reduction 100 Kcal/kg, without phytase or xylanase; C2: positive control diet × all other treatments; C3: positive control diet × diets with ME reductions of 50, 100 and 150 Kcal and adjustment for P, Ca and Na, containing phytase and xylanase.
[1]CV = coefficient of variation; [2] Means followed by distinct letters in the column differ by Tukey's test ($P<0.05$); [3]$P<0.05$ is significant by the Scheffe test.

compared to those fed diets containing the same ME reduction level and without enzymes. The cholesterol levels found in the different treatments are close to the normal range of –125 to 200 mg/dL of blood (Kaneko et al. 2008, Santos et al. 2014).

Calcium concentration was not influenced (P>0.05) by the treatments evaluated at both ages. It was considered normal (5 to 10 mg/dL) for broilers fed corn- and soybean meal-based diets (Minafra et al. 2010, Vieites et al. 2011, Santos et al. 2014).

## CONCLUSION

The association of phytase (500 FTU/kg) with xylanase (16000 BXU/kg) in diets with ME reductions of 50, 100 and 150 Kcal/kg, 0.15% digestible P, 0.165% Ca and 0.035% Na does not alter the intestinal morphometry, serum biochemistry or microbiology of broilers.

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