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Distillers dried grains with soluble and enzyme inclusion in the diet effects broilers performance, intestinal health, and microbiota composition

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ABSTRACT This study tested the effect of distillers dried grains with soluble (**DDGS**) inclusion in a broiler diet, with or without supplementation of exogenous enzymes, on the microbiota composition, intestinal health, diet digestibility and performance. A total of 288 one-day-old chickens was assigned to 6 treatments (8 replicate of 6 birds each) according to a completely randomized design with a 3 × 2 factorial scheme with 3 DDGS levels (0, 7 and 14%) and 2 inclusions of exogenous enzymes (with or without a multicarbohydrase complex + phytase [**MCPC**]). The results exhibited that DDGS inclusion up to 14% did not impair broilers performance up to 28 d, however, DDGS-fed animals exhibited significant improvement with the MCPC supplementation. No effects of the enzymes in the ileal digestibility were found at 21 d. DDGS inclusion in the diet affected dry matter and gross energy digestibility. Broilers fed diets with MCPC were found to have less intestinal histological alteration thus better gut health. No effect of DDGS, enzyme or interaction of those were observed for intestinal permeability and in the serum inflammatory biomarker (calprotectin) at 7 and 28 d. The increase of DDGS percentage in the diet reduced the diversity of the ileal microbiota but increased the cecal microbiota diversity. The inclusion of DDGS showed positive effects on microbiota composition due to a reduction of Proteobacteria phylum in the ileum at 28d and a reduction in the presence of Enterococcaceae family in the ileum at 14 and 28d. The inclusion of MCPC complex might promote beneficial changes in the ileal and cecal microbiota due reduce of Proteobacteria, Bacillaceae and Enterobacteriaceae. The supplementation of xylanase, β-glucanase, arabinofuranosidase and phytase to a DDGS diet improves performance and intestinal health allowing the use of these subproduct in the poultry nutrition.

Key words: carbohydrase, corn subproduct, gut health, histological lesion, intestinal microbiota

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INTRODUCTION

Distillers dried grains and soluble (**DDGS**) are one of the byproducts of ethanol production from corn which had become a popular feed ingredient because of its availability and cost. However, DDGS have some characteristics that may impair its wide use in poultry nutrition. The composition of the corn, industrial practices, as well as dry and oil extraction process all interfere in DDGS composition and the bioavailability of nutrients, especially lysine (Spiehs et al., 2002; Fastinger et al., 2006; Almeida et al., 2013a). Thus, DDGS can have a great variability in composition that may interfere in the diet formulation. The concentration of components in the DDGS is higher when compared with base-line corn especially the amount of nonstarch polysaccharides (**NSP**) (Swiatkiewicz et al., 2016). For example, in one study, corn DDGS presented approximately 16% cellulose, 8% xylan, and 5% arabinan (based to cellulosic biomass) (Kim et al., 2008); and in another, corn DDGS had 26.5% total NSP (3.55% soluble and 23.5% insoluble NSP), 21.5% arabinoxylans and 0.32% β-glucans (Świątkiewicz and Koreleski, 2007). As nonruminant animals poorly digest NSP, the excess of fiber in the feed can reduce the overall digestibility of the diet (Bederska-

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Łojewska et al., 2017), increase gut permeability (Tellez et al., 2015), and reduce the beneficial intestinal microbiota (Langhout, 2000). NSP can be metabolized by the microbiota, but the chicken intestinal microbiota is not as efficient as other nonruminants in fiber fermentation (Józefiak et al., 2004).

Studies feeding chicken with DDGS diets have shown varied results. Some studies have shown that DDGS inclusion in the diet impaired performance and diminished feed digestibility (Campasino et al., 2015; Kim et al., 2016; Kim et al., 2018). In other studies, no difference in performance or other parameters were observed between animals fed with DDGS diet and control diets (Lumpkins et al., 2004; U.S. Grains Council, 2012), whereas some studies found benefits for cecal microbiota (Perez et al., 2011; Abudabos et al., 2017). Although, most of the studies with feeding DDGS to broilers presented negative effects, in theory, DDGS has the potential to act as a prebiotic and improve gut health. This hypothesis is supported by the fact that a meaningful fraction of dead yeast cells are present in DDGS as remnants of the yeast fermentation to produce alcohol (Shurson, 2018), and in the potential of yeast and its derivates to enhance intestinal microbiota and immunity, nutrient digestibility, and feed efficiency in livestock animals (Vohra et al., 2016). Accordingly to Rochell (2018), it is possible that many of the benefits derived from feeding concentrated yeast cell wall components such as mannan-oligosaccharides, mannan-glucans, and nucleotides are intrinsic to DDGS as well.

To minimize the negative impacts of DDGS use in nonruminant diets and achieve greater economic returns authors have suggested the use of exogenous enzymes in a DGGS-based diet (Zijlstra et al., 2010; Min et al., 2011; Opoku et al., 2015; Swiatkiewicz et al., 2016). Studies with enzymes supplementation in DDGS diets demonstrated benefits such as a reduction of the intestinal viscosity (Waititu et al., 2014), and an improvement in performance and digestibility (Barekatain et al., 2013; Swiatkiewicz et al., 2014). Although the majority of DDGS's negative effects in chickens is due to its high concentration of fiber, an enzyme strategy of choice usually is the supplementation of exogenous enzymes that target these NSPs (**NSPenz**) such as xylanase, β-glucanase, and β-mannanase (Dal Pont et al., 2022). Interestingly, the positive effects of NSPenz supplementation to DGGS-based diets is not limited to improvement of digesta viscosity and consequently improvement of diet digestibility. It had been advocated that the central role of the NSPenz is the modulation of intestinal microbiota (Aftab and Bedford, 2018). This is reasoned in the potentially production of short-chain xylans and xylo-oligosaccharides from xylanases, because this compounds can be used by *Lactobacillus* and *Bifidobacterium* species and act as prebiotic modulating the enteric microbiota (Collins et al., 2005; Thammarutwasik et al., 2009; Sun et al., 2015; Morgan et al., 2019). Thus, the supplementation of NSPenz in DDGS diets may demonstrate beneficial effects by reducing the fiber content in the chicken intestine, producing prebiotic-like compounds, and maybe unravel the potential of the yeast and yeast derivates in the DDGS to act on the intestine.

Therefore, the objective of this study was to evaluate the effect of DDGS inclusion in a broiler diet, with or without supplementation of exogenous enzymes, on the microbiota composition, intestinal health, diet digestibility and broiler performance. The hypothesis of this study was that the inclusion of DDGS in a broilers diet would impact the gut health, involving dysbiosis, intestinal barrier permeability, and inflammatory processes. In addition, we postulated that the use of exogenous enzymes in DDGS diets would reduce the negative effects of the ingredient and improve intestinal microbiota diversity and composition, and gut health via the prebiotic-like compounds formed and the fiber present in the DDGS.

MATERIAL AND METHODS

The current trial was conducted in metabolic cages at the Agricultural Research Service Facility of the United States Department of Agriculture (**ARS-USDA**), College Station, Texas, United States. The experimental protocol was in accordance with United States Department of Agriculture Animal Care and Use Committee guidelines (USDA IACUC #2019-020).

A total of 288 one-day-old chickens were assigned to 6 treatments with 8 replicates (6 birds/experimental unit, 48 cages). The treatments differed by the inclusion of DDGS in the diet fed to the birds (0, 7 and 14% inclusion of DDGS) and by the inclusion of exogenous enzymes (with or without a multicarbohydrase complex + phytase [**MCPC**]) using a completely randomized 3 × 2 (3 DDGS inclusion X 2 enzyme inclusion) factorial design.

The basal diet used for the experiment was a corn-soybean based, in the treatments with DDGS the proper adjusts were made and all diets were iso-energetic and iso-nitrogenous (Table 1). The multicarbohydrase complex used was composed of xylanase, β-glucanase, arabinofuranosidase (**ABF**), and phytase complex and it was included in a 100 g/t of feed. In the treatments with enzyme inclusion the enzyme was added in substitution to inert (Kaolin), and no formulations adjustments were made considering the enzyme matrix. For dietary formulation, 2 phases were considered to initial phase (1−14 d) and growing (15−28 d), and all the feed were formulated based on the requirements used by the North American poultry industry (Table 1). Water and feed were offered ad libitum and environmental conditions were maintained for each growing phase according to the Cobb manual recommendations (2018). Chickens were placed on metabolic battery brooder with 95.5 × 33 × 38 cm.

Enzymatic Complex

The phytase used was a bacterial 6-phytase (EC 3.1.3.26) from the species *Buttiauxella* and expressed in a fungus (*Trichoderma reesei*), with an activity of 10,000 units of phytase (FTU/kg) per gram. One

Table 1. Dietary formula and chemical composition of feed offered to broilers in the current study.

	1–14 d			15–21 d			21–28 d		
Ingredients (%)	0%	7%	14%	0%	7%	14%	0%	7%	14%
Corn[1]	53.05	48.90	44.75	54.86	50.71	46.56	56.73	52.58	48.43
Soybean meal[1]	38.96	34.97	30.99	35.14	31.15	27.16	34.89	30.90	26.92
DDGS[1]	0.00	7.00	14.00	0.00	7.00	14.00	0.00	7.00	14.00
Soybean oil	3.55	4.62	5.68	4.95	6.02	7.08	4.32	5.39	6.46
Monocalcium phosphate	1.526	1.368	1.209	1.353	1.194	1.036	1.350	1.192	1.034
Limestone	1.310	1.419	1.527	1.147	1.255	1.363	1.150	1.258	1.366
NaCl	0.527	0.530	0.533	0.506	0.509	0.512	0.506	0.509	0.512
Mineral supplement[2]	0.050	0.050	0.050	0.050	0.050	0.050	0.050	0.050	0.050
Vitamin supplement[3]	0.130	0.130	0.130	0.130	0.130	0.130	0.130	0.130	0.130
DL − methionine 99%	0.379	0.367	0.356	0.350	0.339	0.327	0.348	0.337	0.325
L - lysine 78%	0.388	0.517	0.646	0.392	0.521	0.650	0.398	0.527	0.655
L - threonine 99%	0.117	0.117	0.117	0.114	0.114	0.114	0.114	0.114	0.114
Inert (Kaolin)	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010
Celite	0.00	0.00	0.00	1.00	1.00	1.00	0.00	0.00	0.00
Chemical composition									
EMA kcal kg^{-1}	3,012	3,012	3,012	3,100	3,100	3,100	3,100	3,100	3,100
Crude Protein %	21.52	21.52	21.52	19.85	19.85	19.85	19.85	19.85	19.85
Calcium %	0.925	0.925	0.925	0.818	0.818	0.818	0.818	0.818	0.818
Av. P %	0.441	0.441	0.441	0.397	0.397	0.397	0.397	0.397	0.397
Dig. Met + Cys %	0.948	0.948	0.948	0.881	0.881	0.881	0.881	0.881	0.881
Dig. Lysine %	1.282	1.282	1.282	1.190	1.190	1.190	1.190	1.190	1.190
Dig. Threonine %	0.846	0.846	0.846	0.786	0.786	0.786	0.786	0.786	0.786

Abbreviation: Av., available; dig., digestible.
Multicarbohydrase complex + phytase were added in substitution to inert (Kaolin).
Enzymes (xylanase, β-glucanase arabinofuranosidase and phytase) were added on top of the feed formulation, with no adjust to the enzyme matrix.
[1]Crude protein: Corn 6.13%; Soybean meal 46%; DDGS 29.9% (NIRS method).
[2]Mineral supplement, per kg of diet: 50 mg iron; 10 mg copper; 65 mg manganese; 65 mg zinc; 1 mg iodine.
[3]Vitamin supplement, per kg of diet: 14,300 IU vitamin A; 5,200 IU vitamin D3; 71.5 IU vitamin E; 3.9 mg vitamin K3; 2.99 mg vitamin B1; 9.10 mg vitamin B2; 15.6 mg pantothenic acid; 5.2 mg vitamin B6; 3.25 mcg vitamin B12; 78 mg nicotinic acid; 2.6 mg folic acid; 325 mcg biotin; 390 mcg selenium.

phytase unit (FTU) is the amount of enzyme that releases 1 μmol of inorganic orthophosphate from sodium phytate substrate per min at pH 5.5 and 37°C.

The multicarbohydrase complex was added to the feed to provide a minimum of 1,250 visco units of endo-β-1,4-xylanase, 860 visco units of endo-1,3(4)-β-glucanase, and 4,600 units of ABF. One visco unit of endo-1, 4-β-xylanase or endo-1,3(4)-β-glucanase activity is defined as the amount of enzyme that is hydrolyzed by the substrate (wheat arabinoxylan and barley β-glucan, respectively); one such unit reduces solution's viscosity, resulting in a change in relative fluidity of 1 arbitrary unit per min per mL (or per gram) under the conditions of the assay (pH 5.5 and 30°C). Units of ABF refer to the amount of enzyme that releases 1 nmol of arabinose per minute from the hydrolysis of arabinoxylan wheat in defined assay conditions (pH 4 and 50°C). The recovery of phytase and xylanase from diets was performed by Laboratoire CARAT (Commentry, France) (Table 2).

Performance

Broiler chickens, furnished feed, and leftover feed were weighted on d 7, 14, 21, and 28 posthatch for calculation of body weight gain (**BWG**), average feed intake (**FI**), and feed conversion ratio (**FCR**).

Necropsies

Necropsies were performed on d 7, 14, and 28, when one chicken from each pen (8 per treatment) were randomly selected and euthanized by cervical dislocation. During the necropsy, samples of duodenum, jejunum and ileum were collected for histological analysis, and ileal and cecal content were collected for microbiota assessment.

Table 2. Analyzed activities of phytase (FTU/kg) and xylanase (visco unit/kg) in the experimental diets.

	Without enzyme			With enzyme		
	0% DDGS	7% DDGS	14% DDGS	0% DDGS	7% DDGS	14% DDGS
Phytase						
1–14 d	13	16	20	542	619	891
15–28 d	51	46	28	653	681	793
Xylanase						
1–14 d	103	73	82	>1,800	>1,800	>1,800
15–28 d	118	88	104	>1,800	>1,800	>1,800

In the treatments with enzyme inclusion the phytase was added at level of 1,000 FTU/kg and the multicarbohydrase complex (MCC) was added to the feed to provide a minimum of 1,250 visco units of endo-b-1,4-xylanase, 860 visco units of endo-1,3(4)-b-glucanase, and 4,600 ABF units of arabinofuranosidase.

Digestibility

For determination of digestibility of nutrients and metabolizable energy at 21 d, 1% of insoluble ash source (Celite; Lompoc, CA) was added to the experimental diets as an undigestible marker from d 15 to 21. After a 3d adaptation period (from 15−17 d) excreta samples were collected for 4 consecutive days (from 18−21 d). The excreta were collected twice daily (8:00 and 17:00 h) and stored at −20°C.

At first, samples were dried at 135°C following AOAC (2005) Method 930.15 "Moisture in Animal Feed" and dry matter (**DM**) was calculated (% DM = 100 - % Moisture). The acid insoluble ash was determined based on AOAC (2005) 920.08 "Sand and Silica in Plants, Gravimetric Method." Total nitrogen from the samples followed AOAC (2005) 968.06-1969 using a Fisons NA2000 Carbon Nitrogen Analyzer, then the nitrogen concentration was converted to protein (Protein = %N * 6.25). Gross energy of feed and excreta were measured with a 6200 Isoperibol Calorimeter (ParrInstrument Company, Moline, IL).

Digestibility of nutrients and apparent metabolizable energy (AME) were calculated as per equations by Sakomura and Rostagno (2016). using the indigestibility factor (**IF**):

$$IF = \frac{\%AIA\ in\ the\ diet}{\%AIA\ in\ the\ excreta}$$

Considering the IF the coefficient of digestibility (**CD**) of DM and crude protein (**CP**) were calculated as follows:

$$CD = \frac{[Nutrient\ dietary\ content - (Nutrient\ intestinal\ content \times IF)]}{(Nutrient\ dietary\ content \times 100)}$$

Histological Analysis

The intestinal samples (duodenum, jejunum, and ileum) were stored in formalin (10%) for at least 24 h, then they were dehydrated, infiltrated, and embedded in paraffin following standard histological practices. Paraffin blocks were cut in 5 μm sections added to glass slides and stained with hematoxylin and eosin. Then slides were scanned (Leica's Aperio AT2 Digital Whole Slide Scanner; Leica Biosystems Inc., Buffalo Grove, IL) and digitally analyzed by treatment-blind pathologists (Aperio ImageScope v12.4.0.5043 from Leica Biosystems).

For evaluation of intestinal tissue, 20 villi per bird were evaluated (160 villi/treatment) at 10× magnification (40× used to confirm alterations) using the scanned pictures. The "I See Inside" (**ISI**) microscopy methodology (patent INPI BR 1020150036019) was used to measure histologic alterations on the intestine, and a final intestinal health index was produced (Kraieski et al., 2017; Belote et al., 2018; Belote et al., 2019). In this methodology, a score from 0 to 3 is given to each alteration depending on the extent of the lesion: the greater the lesion, the higher the score. The score is then multiplied by an impact factor (IF, 1-3) which is based on the importance of the alteration to the function of the tissue. The final ISI index is then calculated as sum of all alteration index:

$$ISI\ index = \sum score * IF$$

The alterations evaluated, and its impact factor were: lamina propria thickness*(2), epithelial thickness*(1), enterocytes proliferation*(1), inflammatory cell infiltration in the epithelium*(1), inflammatory cell infiltration in the lamina propria*(3), goblet cells proliferation*(2), congestion*(2), and the presence of *Eimeria sp.* oocysts*(3). Thus, animals with high ISI index had a worse intestinal health. For statistical analysis each bird was considered as replicate.

Intestinal Permeability and Inflammation Biomarker Analysis

Oral administration of fluorescein isothiocyanate dextran (**FITC-d**) and quantification of its passage into blood was used in order to evaluate gut permeability (Zhang et al., 2016). At 7 and 28 d, one chicken was randomly selected from each pen (n = 8) and oral-gavage 1 mL (2.2 mg/bird) of FITC-d (100 mg, MW 4,000; Sigma-Aldrich, Canada). After 2 h, the birds were bleed. The blood was kept in ice in the dark up to centrifugation and serum collection. Right after centrifugation, the FITC-d concentration in the serum was performed according to Bortoluzzi et al. (2019). In this methodology, the higher the gut permeability, higher the blood level of FITC-d.

Assessment of serum calprotectin, a biomarker for intestinal inflammation, was performed in the same samples used for FITC-d analyses. Calprotectin was quantified with a chicken specific ELISA kit (#MBS7606348, MyBiosource Inc., San Diego, CA).

Microbiota

DNA of the ileal and cecal content was isolated with the Qiagen PowerViral Environmental RNA/DNA Isolation kit following the manufacturer recommendations (Mo Bio; Qiagen, Carlsbad, CA). Then the DNA was sent for sequencing of the V3-V4 region of the bacterial DNA at the University of Minnesota Genomics Center (Minneapolis, MN).

The V3V4 region of 16S rRNA (CCTACGGGAGGCAGCAG and GGACTACHVGGGTWTCTAAT, respectively) were amplified using the forward indexing primer (AATGATACGGCGACCACCGAGATCTACAC[i5]TCGTCGGCAGCGTC) and Reverse indexing primer (CAAGCAGAAGACGGCATACGAGAT [i7] GTCTCGTGGGCTCGG). The PCR cycling conditions were: 5 min at 95°C, for DNA denaturation, then 35 cycles of 98°C for 20s, annealing at 55°C for 15s, and extension at 72°C for 60s and at the last cycle 72°C for 5min. Final extension was at 72°C for 5 min. PCR

reactions were performed using Kapa HiFidelity HotStart Polymerase (Anachem, Dublin, Ireland).

Indexed PCR products were normalized using SequalPrep Normalization Plate Kit from ThermoFisher (Cat. A1051001). Pooled samples were concentrated to ~100 ul, and cleaned using SPRI purification. Cleaned library was quantified by Qubit and underwent fragment analysis by Agilent Tapestation. Library was diluted to 2 nM. Then, Pooled sample was denatured with NaOH, diluted to 8 pM in Illumina's HT1 buffer, spiked with 15% PhiX, and heat denatured at 96°C for 2 min immediately prior to loading. A MiSeq 600 cycle v3 kit was used to sequence the sample. The Nextera adapter sequences used for post-run trimming were: Read 1: CTGTCTCTTATACACATCTCCGAGCCCACGAGACNNNNNNNNATCTCGTATGCCGTCTTCTGCTTG; and Read 2: CTGTCTCTTATACACATCTGACGCTGCCGACGANNNNNNNNGTGTAGATCTCGGTGGTCGCCGTATCATT.

Bioinformatics

The sequences were processed and analyzed using a Quantitative Insights Into Microbial Ecology 2 (Bolyen et al., 2019) v 2019.7 pipeline. The raw sequences were uploaded to the National Center for Biotechnology Information Sequence Read Archive under the project number PRJNA836942. Briefly, the sequences were demultiplexed and the amplicon sequence variant table was created using DADA2 (Callahan et al., 2016). Prior to downstream analysis, sequences assigned as chloroplast, mitochondria, and low abundance amplicon Sequence Variants, containing less than 0.01% of the total reads in the dataset, were removed. All samples were rarefied to even sequencing depth, based on the lowest read depth of samples, to 1,148 sequences per sample.

Alpha diversity was measured with the Chao1 (richness), and Shannon diversity indices. In order to estimate the similarity or dissimilarity of the microbiota between treatments, distance matrices were calculated by weighted and unweighted UniFrac and visualized via 3D plots. Unweighted UniFrac is calculated based on taxa detection in each individual sample, and the phylogenetic distance between those taxa, showing the microbiota profile. Weighted UniFrac considers all the above but goes beyond including the abundance of each taxon in the sample. Weighted UniFrac show composition but tends to emphasize most abundant taxa.

Statistical Analysis

Data from the current experiment was analyzed according to a completely randomized design in 3 × 2 factorial arrangement, consisting of 3 DDGS inclusion (0, 7, and 14%) and 2 enzyme supplementations (yes, no), totaling 6 treatments.

Data normality was checked through Shapiro-Wilk test. Data with normal distribution (performance, digestibility, FITC-d, calprotectin) were then submitted to analysis of variance considering the factorial arrangement and means from DDGS and MCPC groups compared by Tukey and F test, respectively, at 5% probability level. Software JMP Pro 16.0.0 (SAS Institute Inc.) was used.

ISI data, alpha-diversity index and bacteria abundance, nonparametric data, were evaluated by Kruskal-Wallis test at 5% of probability using Software JMP, and for the evaluation of factors interaction (DDGS * Enzyme) data were submitted to Freedmand test using the PROC FREQ procedure of SAS OnDemand for Academics 2022 (SAS Institute Inc., Cary, NY).

RESULTS

Performance

No interaction of DDGS*Enzyme was observed for BWG and FI at 7d, but enzyme presence increased BWG and FI (Table 3). An interaction was observed for FCR wherein animals fed with 7% DDGS and MCPC presented a better FCR than the group fed with 14% DDGS plus MCPC. At 14 d, the supplementation of MCPC enhanced the BWG and FCR in animals fed with 14% DDGS diet. Multicarbohydrase complex + phytase supplementation increased the FI regardless of the diet composition.

From 1 to 21 d posthatch, the inclusion of 14% of DDGS in the feed reduced BWG compared to 7% DDGS. However, MCPC supplementation enhanced the BWG of the 14% DDGS-fed animals being similar to 0 and 7% DDGS groups. During this same period, the FCR of the 14% DDGS-fed animals exhibited significant improvement with the MCPC supplementation. Further, the enzyme complex improved the FI of birds from 1 to 21 d period.

Considering the entire experimental period (1−28 d), the addition of the enzymes increased the FI and BWG off all groups, but improved broilers the FCR only of birds fed with the 7% DDGS. Moreover, at 28 d, FCR of animals fed 14% DDGS diet plus enzyme was similar than the broilers in the 7% DDGS group.

Digestibility

No interactions between enzyme by DDGS inclusion was observed on digestibility parameters (Table 4). No effects of the enzymes in the ileal digestibility were found. DDGS inclusion in the diet effected DM and gross energy digestibility. Surprisingly, 14% of DDGS did not compromise DM and gross energy digestibility if compared with control diet (corn-soybean and 0%DDGS). The inclusion of 7% of DDGS reduced DM and gross energy digestibility when compared to control.

Histological Analysis

The ISI index score and distribution of duodenum, jejunum and ileum displayed in Figures 1−4. The Figure 5 represents the parameters analyzed in the ISI

Table 3. Performance of broilers fed diets containing levels of DDGS (0, 7, or 14%) with or without inclusion of multicarbohydrase complex + phytase.

		1–7 d			1–14 d			1–21 d			1–28 d		
DDGS (%)	Enzymes	BWG (g)	FI (g)	FCR	BWG (g)	FI (g)	FCR	BWG (g)	FI (g)	FCR	BWG (g)	FI (g)	FCR
0	No	130.0	145.3	1.119^{AB}	418.1^{AB}	517.7	1.239^{AB}	853.6^{AB}	1,133.2	1.328^{AB}	1,442.8	1,983.8	1.376^{AB}
	Yes	135.7	152.2	1.106^{AB}	445.3^{A}	549.2	1.234^{AB}	897.4^{A}	1,192.5	1.329^{AB}	1,514.8	2,048.1	1.381^{AB}
7	No	133.2	145.0	1.090^{AB}	434.8^{AB}	530.6	1.221^{AB}	887.9^{A}	1,192.1	1.343^{AB}	1,469.0	2,065.8	1.407^{A}
	Yes	132.3	148.7	1.124^{A}	430.9^{AB}	534.1	1.242^{AB}	895.4^{A}	1,177.5	1.315^{AB}	1,551.3	2,123.8	1.355^{B}
14	No	127.5	144.9	1.119^{AB}	400.5^{B}	510.0	1.274^{A}	802.3^{B}	1,111.1	1.363^{A}	1,352.0	1,958.2	1.422^{A}
	Yes	137.9	147.7	1.071^{B}	454.1^{A}	543.6	1.197^{B}	902.3^{A}	1,178.3	1.306^{B}	1,515.1	2,081.6	1.374^{AB}
DDGS (%)													
0		133	149	1.113	432	533	1.237	876	1,163	1.329	1,476	2,016	1.379
7		133	147	1.107	433	532	1.232	891	1,185	1.331	1,510	2,095	1.383
14		133	146	1.096	427	527	1.236	856	1,145	1.333	1,439	2,020	1.397
Enzymes													
No		130^{b}	145^{b}	1.110	418^{b}	519^{b}	1.245	850^{b}	1,146^{b}	1.344^{b}	1,424^{b}	2,003^{b}	1.401^{b}
Yes		135^{a}	150^{a}	1.101	443^{a}	542^{a}	1.225	899^{a}	1,183^{a}	1.317^{a}	1,528^{a}	2,085^{a}	1.371^{a}
CV (%)		4.93	3.53	2.99	5.73	4.04	2.97	5.24	4.51	2.32	6.14	6.08	2.36
P-value													
DDGS		0.998	0.367	0.332	0.799	0.648	0.914	0.075	0.117	0.872	0.074	0.143	0.217
Enzyme		0.012	0.005	0.362	0.001	0.001	0.057	0.001	0.020	0.004	0.0003	0.028	0.002
DDGS*Enzyme		0.065	0.506	0.005	0.008	0.098	0.002	0.029	0.071	0.042	0.330	0.716	0.032

Abbreviations: BWG, body weight gain; FCR, feed conversion ratio; FI, feed intake.
$^{A-B}$Means in the same row with different letters differ significantly ($P \leq 0.05$) by Tukey's test.
$^{a-b}$Means in the same row with different letters differ significantly ($P \leq 0.05$) by F test.

methodology in the intestine and Figure 6 represents some intestinal histologic alterations found in the treatments supplemented with enzyme.

At 14 d, no effect of the DDGS * enzyme interaction was observed in the duodenum and ileum. Broilers fed diets with MCPC in the diet were found to have a lower ISI score (less histological alteration thus better gut health) in the duodenum and ileum. In the jejunum, an interaction was observed between enzyme and DDGS %, where the enzyme improved the intestinal health (reduced the ISI score) of the broiler fed with 14% DDGS diet, group fed 14% DDGS plus enzymes presented a better score than the corn diet.

At 28 d, ISI score exhibited an interaction effect of DDGS and enzyme in all intestinal segments. The addition of enzyme complex in the 0% and 7% DDGS diets improved duodenum health status, although the enzyme did not improve the duodenum of animals fed with 14% DDGS. Broilers fed control diet (0% DDGS) and 7% DDGS diet with no enzyme added presented a worst duodenum health. The jejunum health was better in the animals fed with 7% DDGS regardless the supplementation of enzyme. Regarding the ileum status, the addition of enzyme improved the intestinal health of all treatments (reduced the ISI score).

Evaluating each parameter determined in the ISI methodology, we observed that the jejunum of birds fed with 14% DDGS diet presented congestion at 14 d, which was reduced by the supplementation of MCPC in the diet (data not shown). The presence of inflammatory cells in the ileal lamina propria of broilers fed with 0% DDGS diet was reduced by the addition of MCPC at 14 d. The MCPC supplementation reduced the presence of goblet cell and the epithelial thickness in the duodenum of broilers fed with 14% diet. The enzyme reduced the immature enterocyte proliferation (in duodenum, jejunum, and ileum) at 14 d. Additionally, MCPC reduced the lamina propria thickness (jejunum and ileum) and epithelial thickness (jejunum) of broilers fed with DDGS diets at 14d.

Broilers fed with 7% of DDGS exhibited a higher infiltration of immune cells in the lamina propria in duodenum at 28 d. At 28 d, chickens fed with the control diet or the 7% DDGS diet presented reduction of immature enterocytes proliferation and lamina propria thickness in the duodenum when supplemented with MCPC. Animals fed with DDGS diet exhibited an increase in lamina

Table 4. Apparent metabolizable energy and digestibility coefficients of dry matter, crude protein, and gross energy determined in broilers fed diets containing levels of DDGS (0, 7, or 14%) with or without inclusion of multicarbohydrase complex + phytase at 21 d.

DDGS (%)	Enzymes	DM (%)	CP (%)	GE (%)	AME (kcal/kg)
0	No	74.33	68.75	78.39	3,523
	Yes	72.15	69.54	76.46	3,516
7	No	73.75	70.24	77.89	3,500
	Yes	74.65	70.83	78.67	3,530
14	No	71.21	68.44	75.94	3,491
	Yes	73.85	69.35	77.98	3,504
DDGS (%)					
0		74.49^{A}	69.79	78.53^{A}	3,527
7		71.68^{B}	68.99	76.20^{B}	3,503
14		73.80^{A}	69.79	77.93^{A}	3,502
Enzymes					
No		73.41	69.51	77.58	3,513
Yes		73.24	69.54	77.53	3,508
P-value					
DDGS		0.001	0.735	0.001	0.499
Enzymes		0.756	0.974	0.908	0.808
DDGS*Enzymes		0.618	0.331	0.789	0.749

Abbreviations: AME, apparent metabolizable energy; CP, crude protein; DM, dry matter.
$^{A-B}$Means in the same row with different letters differ significantly ($P \leq 0.05$) by Tukey's test.

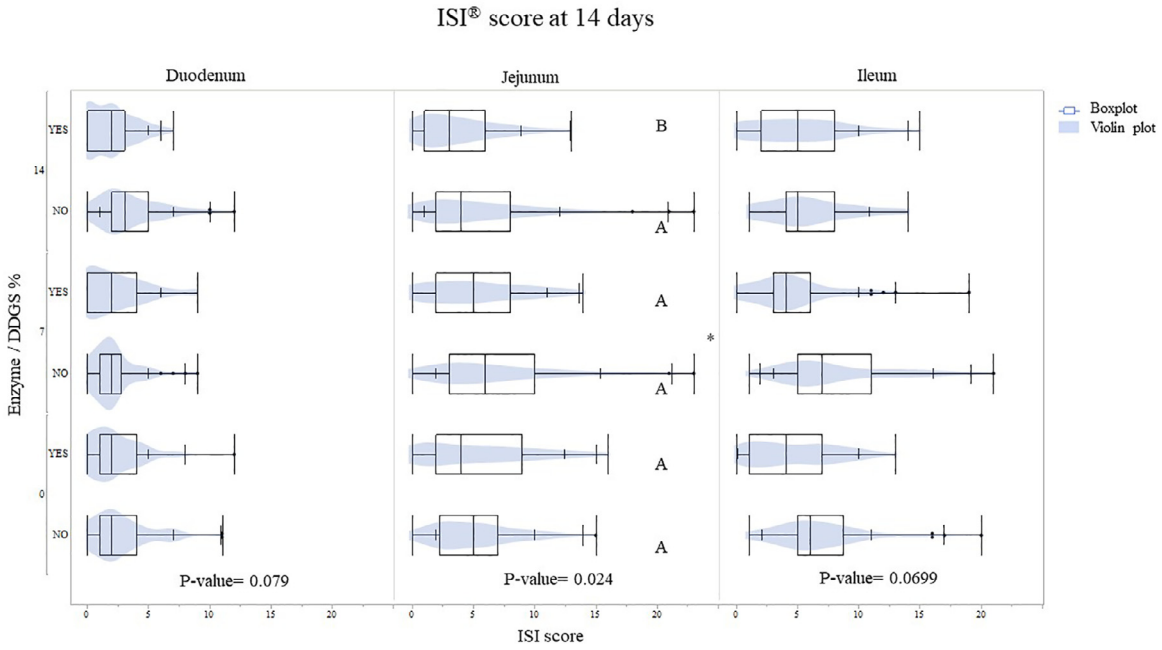

Figure 1. Distribution of ISI score (gut health parameter) of duodenum, jejunum and ileum of chickens fed diets with inclusion of DDGS (0, 7, and 14%) and multicarbohydrase complex + phytase at 14 d. Boxsplot shows the data distribution with quartiles and outliers, Violin plot shows regions of data density. Higher ISI scores represent more alterations in the histologic evaluation thus worse gut health. Abbreviations: DDGS, dried grains with soluble; ISI, I See Inside.

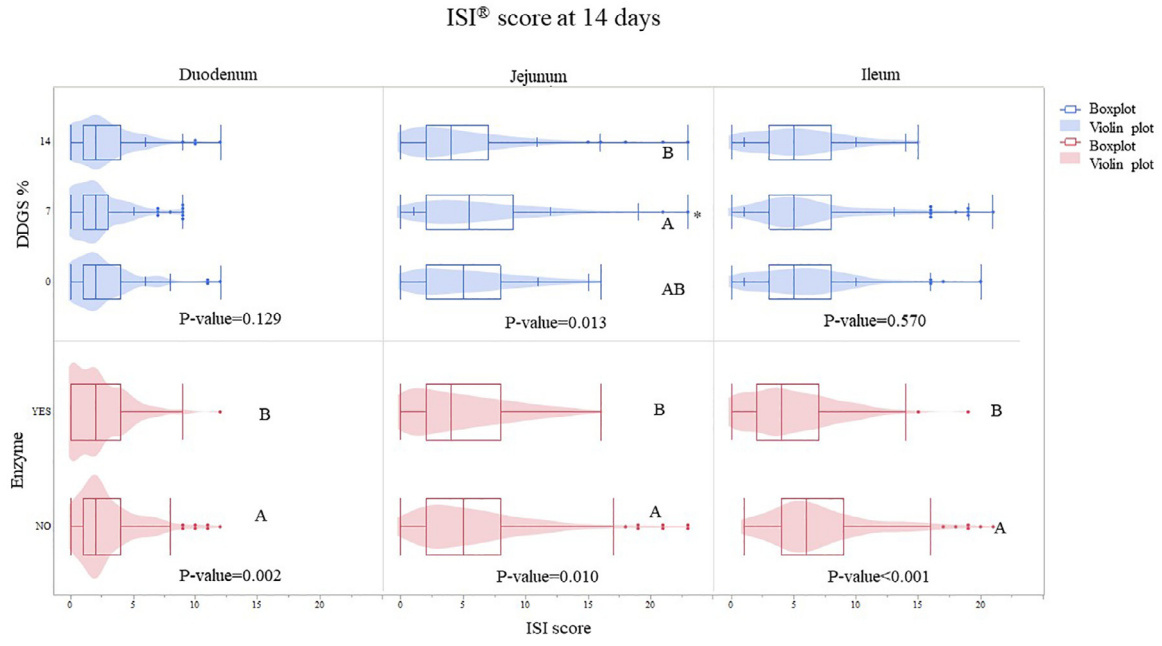

Figure 2. Distribution of ISI score (gut health parameter) of duodenum, jejunum and ileum of chickens fed diets with inclusion of DDGS and multicarbohydrase complex + phytase at 14 d by enzyme and DDGS factors. Boxsplot shows the data distribution with quartiles and outliers, Violin plot shows regions of data density. Higher ISI scores represent more alterations in the histologic evaluation thus worse gut health. Abbreviations: DDGS, dried grains with soluble; ISI, I See Inside.

propria thickness (ileum) and the increased presence of inflammatory cells in the lamina propria (jejunum and ileum) at 28 d. The MCPC supplementation reduced the infiltration of immune cells in the epithelium (in duodenum, jejunum, and ileum), the lamina propria thickness (in jejunum and ileum) and the presence of goblet cells (jejunum and ileum) at 28 d. However, enzyme supplementation increased the presence of inflammatory cells in the lamina propria in the jejunum at 28 d.

Intestinal Permeability and Inflammation Biomarker Analysis

No effect of DDGS, enzyme or interaction of those were observed for intestinal permeability, measured by FITC-d method, at 7 and 28 d (Table 5).

Additionally, no differences were observed in the inflammatory biomarker (calprotectin) at 7 and 28 d (Table 5).

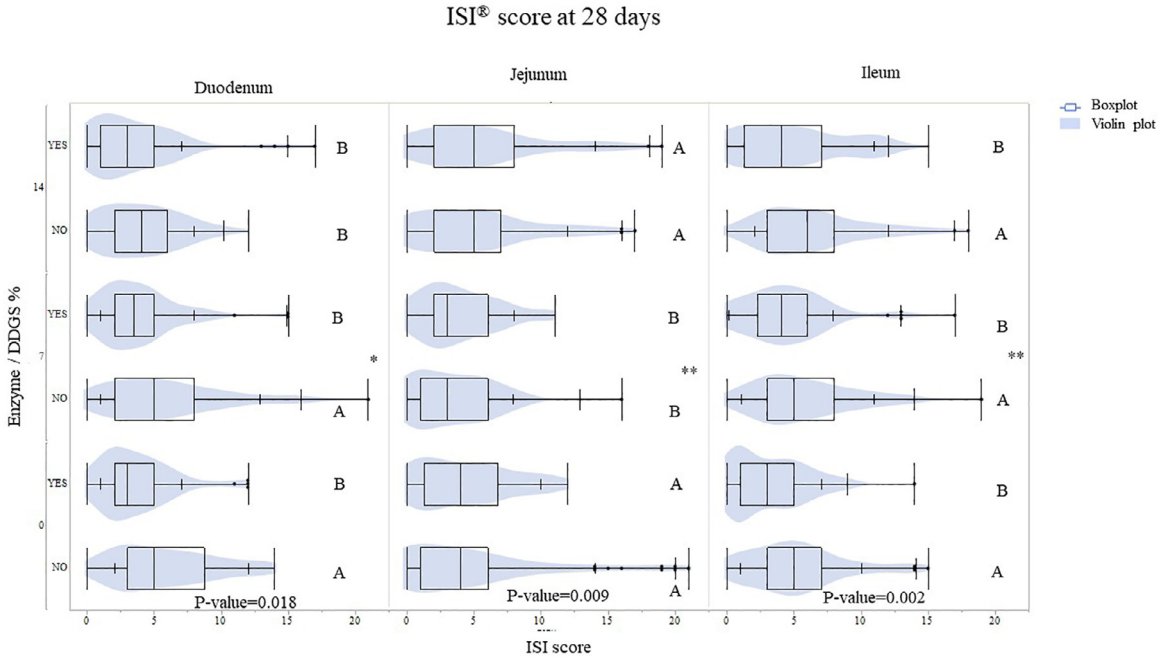

Figure 3. Distribution of ISI score (gut health parameter) of duodenum, jejunum and ileum of chickens fed diets with levels of DDGS (0, 7, and 14%) and multicarbohydrase complex + phytase at 28 d. Boxsplot shows the data distribution with quartiles and outliers, Violin plot shows regions of data density. Higher ISI scores represent more alterations in the histologic evaluation thus worse gut health. Abbreviations: DDGS, dried grains with soluble; ISI, I See Inside.

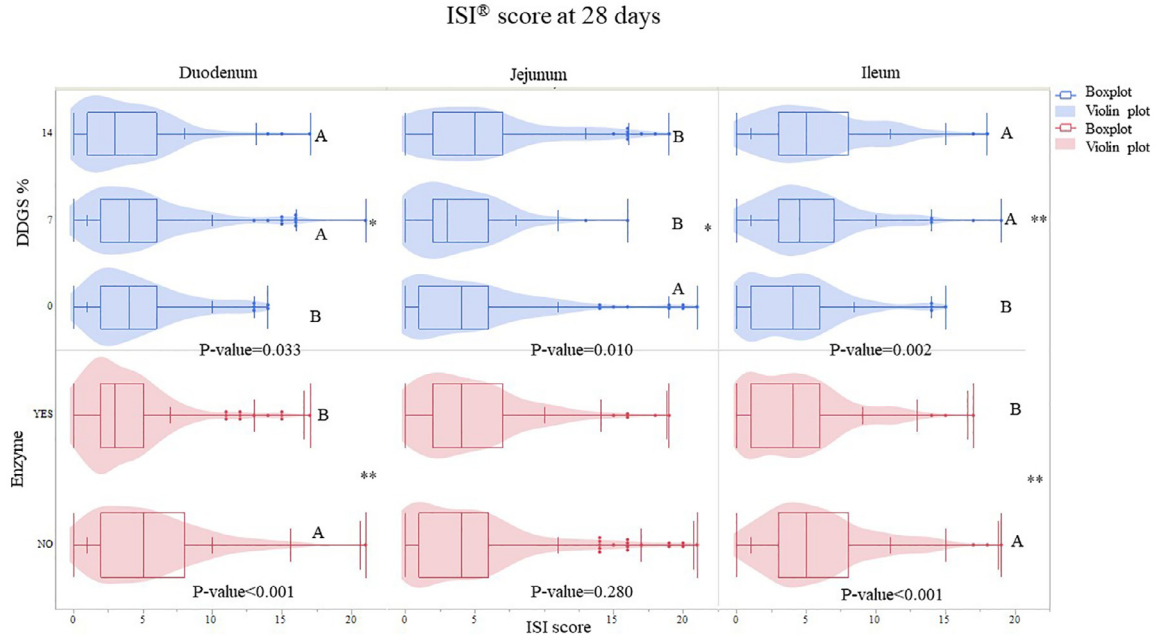

Figure 4. Distribution of ISI score (gut health parameter) of duodenum, jejunum and ileum of chickens fed diets with levels of DDGS (0, 7, and 14%) and multicarbohydrase complex + phytase at 28 d by enzyme and DDGS factors. Boxsplot shows the data distribution with quartiles and outliers, Violin plot shows regions of data density. Higher ISI scores represent more alterations in the histologic evaluation thus worse gut health. Abbreviation: DDGS, dried grains with soluble; ISI, I See Inside.

Microbiota

DDGS inclusion in the diet affected the alpha diversity (diversity within the sample) of ileal and cecal microbiota at 14 d (Figure 7) but did not influence the alpha diversity at 28 d (Supplemental data, Table 1). Chao 1 and Shannon index were positively correlated with DDGS inclusion at cecal level and negatively at ileal level. Ileal and cecal microbiota presented different responses to DDGS inclusion in the diet at 14 d. The increase of DDGS percentage in the diet reduced the alpha diversity in the ileum but increased the alpha diversity in the ceca. There was no effect of enzyme nor enzyme by DDGS interaction in the alpha diversity of the ileal and cecal microbiota at the 2 time points evaluated (Supplemental data, Table 1).

UniFrac distances of the ileal and cecal microbiotas indicated community structure variations. The

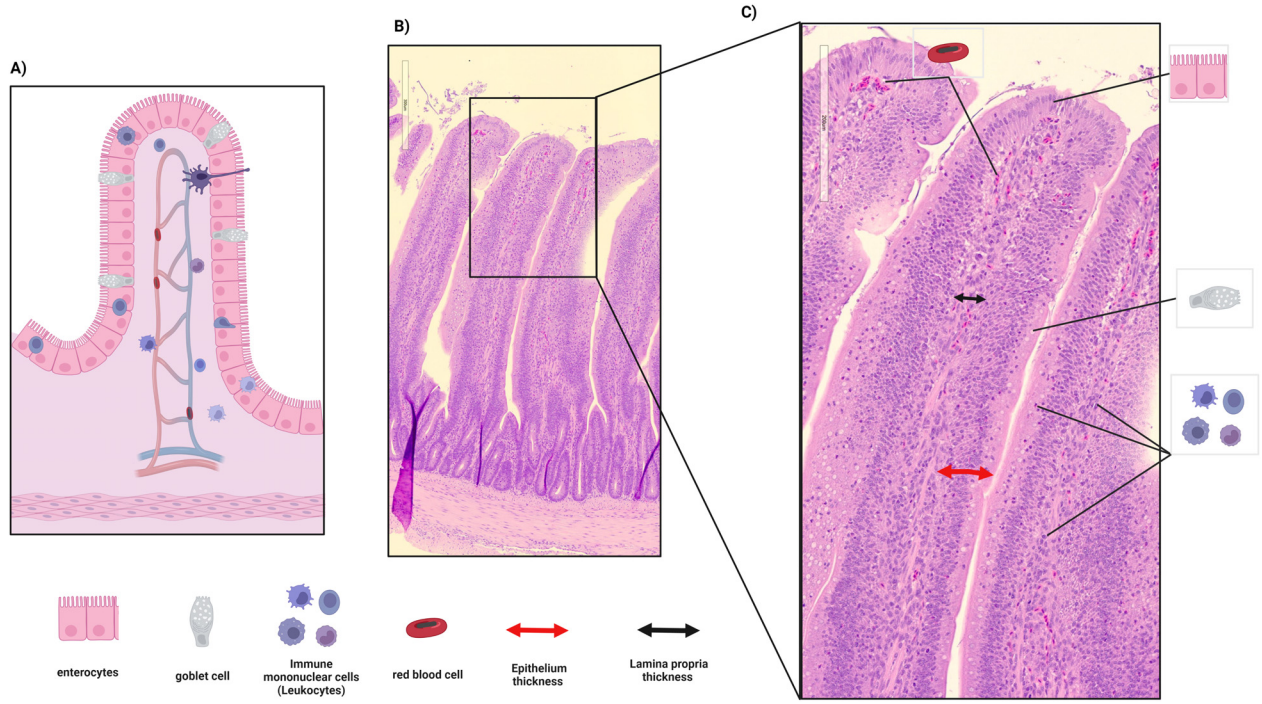

Figure 5. (A) Representation of a healthy villus showing epithelium with enterocytes, goblet cells and immune cells, and lamina propria with capillary network and immune cells; (B) Photomicrographs of hematoxylin and eosin-stained chicken jejunum (10×), villi of a broiler fed with 7% DDGS diet supplemented with enzymes at 14 d of age; (C) Zoom of a jejunum villi (20×) of a broiler fed 7% DDGS diet supplemented with enzymes at 14 d of age highlighting lamina propria thickness (black arrow), epithelium (red arrow), immune nuclear cells, goblet cells, erythrocytes (red blood cells), and enterocytes. Abbreviation: DDGS, dried grains with soluble.

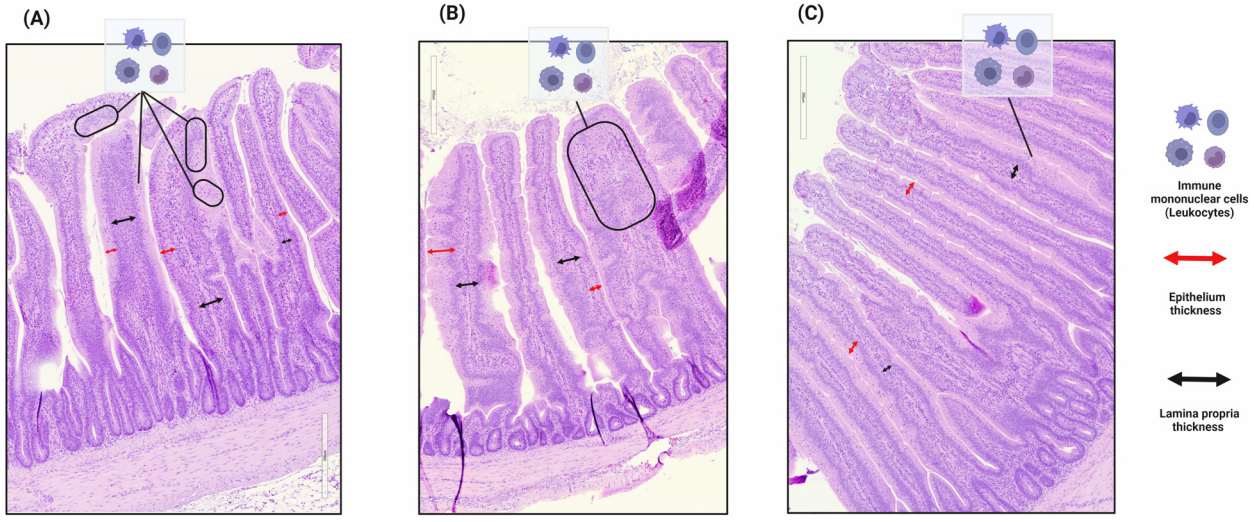

Figure 6. (A) Photomicrographs of hematoxylin and eosin-stained jejunum (10×) of a broiler fed with 7% DDGS diet supplemented with enzymes on d 14. The histological section shows an increased infiltration of immune cells in the epithelium and increased epithelium and lamina propria thickness. (B) Photomicrographs of hematoxylin and eosin-stained jejunum (10×) of broiler fed diet with 7% DDGS supplemented with enzymes on d 14. The histological section shows increased infiltration of immune cells in the lamina propria. (C) Photomicrographs of hematoxylin and eosin-stained jejunum (10×) of broiler fed diet with 14% DDGS supplemented with enzymes on d 14. Lamina propria (black arrow), epithelium (red arrow), and immune cells are highlighted. Abbreviation: DDGS, dried grains with soluble.

treatments affected cecal microbiota profile (unweighted) at 14 d, and ileal microbiota at 28 d (Supplemental data, Table 2). Pairwise comparisons were performed to identify the meaningful effects in the beta diversity (Supplemental data, Tables 3 and 4). On 14 d, the inclusion of 7 or 14% of DDGS in the diet modified the cecal microbiota profile (unweighted) compared to control diet (Figures 8A and 8B). Additionally, birds fed 14% of DDGS showed a different bacterial community structure (weighted) compared to birds fed control diet or diet with 7% inclusion of DDGS (14 d). The supplementation of enzyme to the diets with 14%, but not 0 or 7% of DDGS modified the cecal microbiota profile (unweighted). Furthermore, the supplementation of enzyme in the diet with 7% DDGS promoted a cecal microbiota profile (unweighted) similar to the birds fed control diet at 14 d. However, the supplementation of the enzyme to the diet with 14% DDGS did not promote

Table 5. Serum FITC-d and serum concentration of calprotectin of broilers fed diets with levels of DDGS (0, 7 or 14%) with or without inclusion of multicarbohydrase complex + phytase.

		Serum FITC-d (mg/mL)		Calprotectin (ng/mL)	
DDGS (%)	Enzyme	D 7	D 28	D 7	D 28
0	No	0.189	0.129	4.428	21.314
	Yes	0.184	0.138	4.177	15.931
7	No	0.182	0.140	4.351	19.666
	Yes	0.186	0.138	4.325	16.065
14	No	0.193	0.136	3.974	13.382
	Yes	0.175	0.142	3.746	12.201
DDGS (%)					
0		0.186	0.133	4.302	18.623
7		0.184	0.139	4.339	17.745
14		0.185	0.139	3.867	12.792
Enzyme					
No		0.188	0.135	4.223	18.266
Yes		0.182	0.139	4.050	14.842
P-value					
DDGS		0.969	0.522	0.291	0.121
Enzyme		0.523	0.344	0.556	0.169
DDGS*Enzyme		0.608	0.542	0.943	0.778

a similar microbiota compared to the control group. The addition of enzyme to the diets without DDGS tended ($P = 0.10$) to affect the cecal community structure (weighted) at 14 d.

At 28 d, most of the differences regarding the beta diversity were observed in ileal microbiota (Supplemental data, Table 4). The inclusion of 7% of DDGS modified the profile and the structure (unweighted and weighted indexes) of the ileal microbiota of broilers compared to animals fed control diets (Figure 8D). The supplementation of enzyme also modified the ileal microbiota in animals fed control diets (Figure 8E), but did not show the same effect in diets containing DDGS.

The bacterial phylum abundance was not affected by the different diets at 14 d, with ileal and cecal microbiota presenting predominance of Firmicutes (Table 6). The relative abundance of the main bacterial families at 14 d is described in Figure 9. There was an interaction between DDGS by enzyme in the ileal microbiota abundance, wherein the inclusion of enzyme to 7% DDGS diet increased the abundance of Lactobacillaceae ($P = 0.07$) and reduce the abundance of Lachnospiraceae in the ileum at 14d ($P = 0.091$). When analyzing the main effects, it was observed that the inclusion of DDGS in the diets reduced the presence of Enterococcaceae ($P = 0.036$) and Streptococcaceae ($P = 0.020$) (Supplemental data, Table 5). In the cecal microbiota, chickens fed diets with 14% DDGS increased the abundance of the family Clostridiaceae ($P = 0.008$) and tend to increase Erysipelotrichaceae ($P = 0.052$) and Bacillaceae ($P = 0.093$).

The relative abundance of the main bacterial families at 28 d is described in Figure 10. No interaction (DDGS × enzyme) was observed either for ileal or cecal microbiota (supplemental data). However, in the ileal microbiota at 28 d, DDGS inclusion increased Firmicutes abundance ($P = 0.012$) and reduced Proteobacteria ($P = 0.012$) (Table 6). The inclusion of 14% of DDGS in the diet reduced the abundance of Enterococcaceae bacteria ($P = 0.046$), and 7% inclusion of DDGS reduced the abundance of Ruminococcaceae and Lachnospiraceae bacteria ($P = 0.019$ and 0.041, respectively). In the cecal microbiota at 28 d, the enzyme

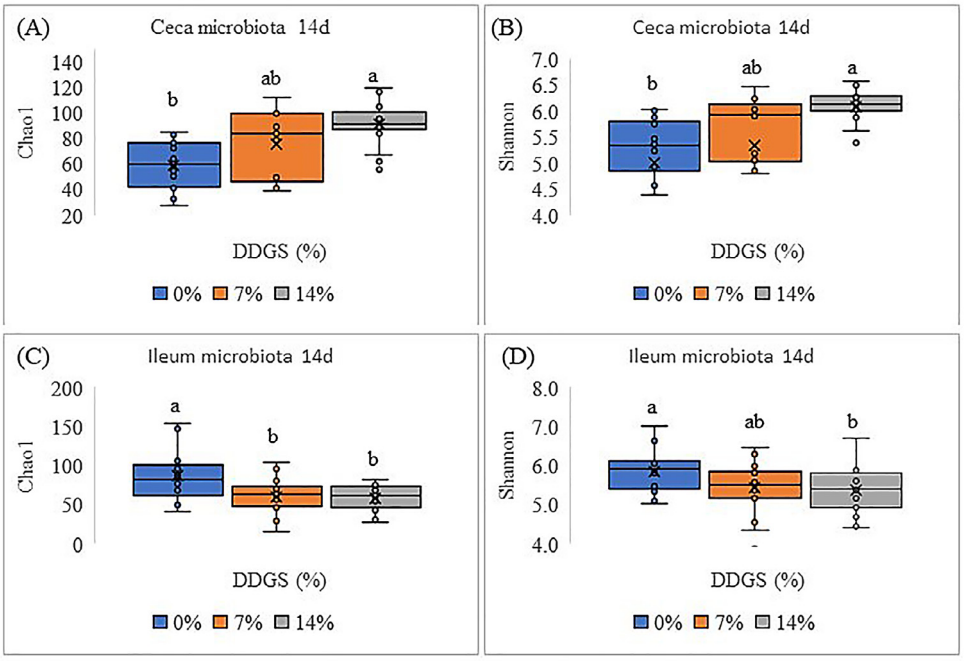

Figure 7. Alpha diversity (Chao1 and Shannon parameters) distribution of ileal and cecal microbiota of broilers fed diets with different inclusion of DDGS (0, 7, or 14%) at 14 d. (A) Chao1 of cecal microbiota of broilers fed with 0, 7, or 14% of DDGS at 14 d; (B) Shannon of cecal microbiota of broilers fed with 0, 7, or 14% of DDGS at 14 d; (C) Chao1 of ileal microbiota of broilers fed with 0, 7, or 14% of DDGS at 14 d; (D) Shannon of ileal microbiota of broilers fed with 0, 7, or 14% of DDGS at 14 d. ab: different letters show significant differences ($P < 0.05$). X: shows the mean of each group. Chao index: number of operacional taxonomic units (OTUs) comprising the microbiota. Shannon index: biodiversity based on sequences uniformity amongst operacional taxonomic units (OTUs). Abbreviation: DDGS, dried grains with soluble.

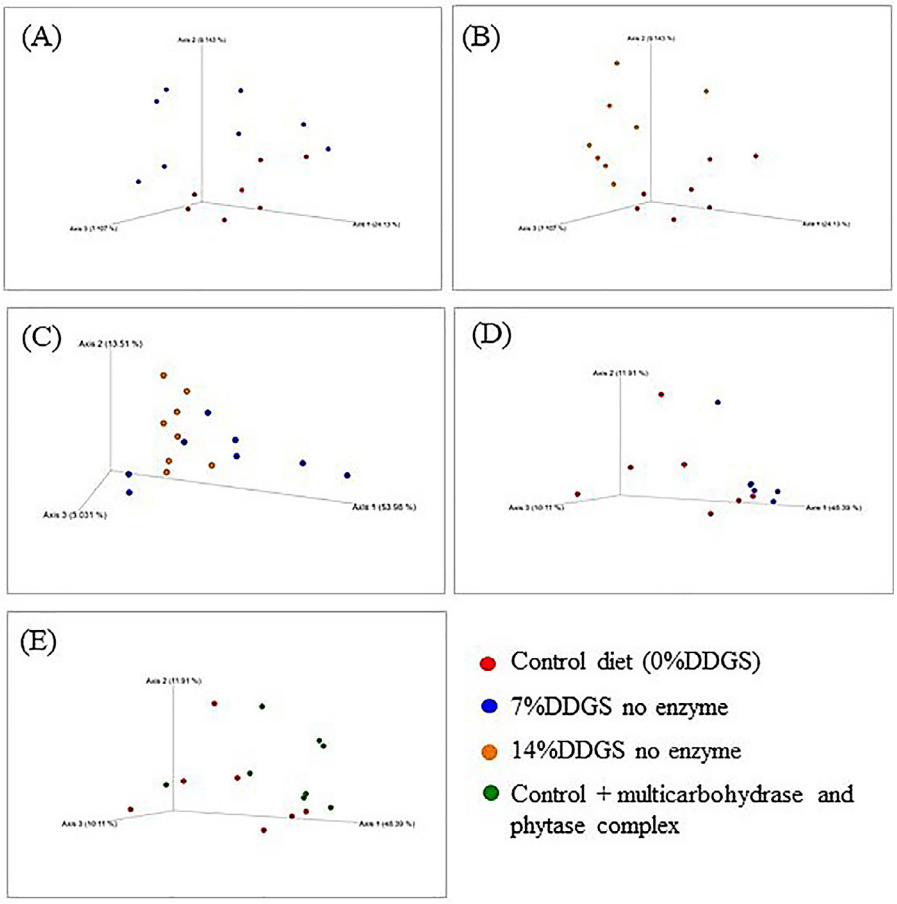

Figure 8. 3D beta diversity plot of broilers microbiota. (A) Unweighted UniFrac cecal microbiota of broilers fed with control diet (0%DDGS, red) and 7%DDGS (blue) at 14 d; (B) Unweighted UniFrac cecal microbiota of broilers fed with control diet (0%DDGS, red) and 14%DDGS (orange) at 14 d; (C) Weighted UniFrac cecal microbiota of broilers fed with 7%DDGS (blue) and 14%DDGS (orange) at 14 d; (D) Unweighted UniFrac ileal microbiota of broilers fed with control diet (0%DDGS, red) and 7%DDGS (blue) at 28 d; (E) Unweighted UniFrac ileal microbiota of broilers fed with control diet (0%DDGS, red) and control diet supplemented with enzyme (green) at 28 d. Abbreviation: DDGS, dried grains with soluble.

supplementation increased the presence of Actinobacteria ($P = 0.055$) and reduced the presence of Proteobacteria ($P = 0.055$) and tended to reduce Bacillaceae and Enterobacteriaceae abundance ($P = 0.063$ and 0.080, respectively) and to increase Coriobacteriaceae ($P = 0.055$).

DISCUSSION

Some reports in the literature have argued that DDGS reduces the broiler performance (Campasino et al., 2015) especially in young chickens (Lumpkins et al., 2004; Loar et al., 2012). However, no differences in

Table 6. Relative abundance (%) of main bacterial phylum present in the ceca and ileum of broilers fed diets with levels of DDGS (0, 7, or 14%) with or without inclusion of multicarbohydrase complex + phytase.

	14 d				28 d				
	Ileum		Ceca		Ileum		Ceca		
DDGS (%)	Firmicutes	Proteobacteria	Firmicutes	Proteobacteria	Firmicutes	Proteobacteria	Firmicutes	Proteobacteria	Actinobacteria
0	99.42	0.58	99.46	0.54	99.9^{B}	0.1^{A}	99.85	0.06	0.07
7	99.36	0.30	99.71	0.29	100.0^{A}	0.0^{B}	99.88	0.07	0.04
14	99.88	0.12	99.61	0.39	100.0^{A}	0.0^{B}	99.95	0.04	0.01
Enzyme									
No	99.40	0.40	99.50	0.50	100.00	0.00	99.90	0.1^{a}	0.0^{a}
Yes	99.70	0.30	99.60	0.40	100.00	0.00	99.90	0.0^{b}	0.1^{a}
P-value									
DDGS	0.112	0.110	0.658	0.712	0.012	0.012	0.866	0.792	0.520
Enzyme	0.804	0.794	0.795	0.953	0.339	0.323	0.888	0.080	0.055
DDGS*Enzyme	0.676	0.598	0.630	0.317	0.641	0.641	0.873	0.588	0.718

$^{A-B}$Means in the same row with different letters differ significantly ($P \leq 0.05$) by Tukey's test.
$^{a-b}$Means in the same row with different letters differ significantly ($P \leq 0.05$) by F test.

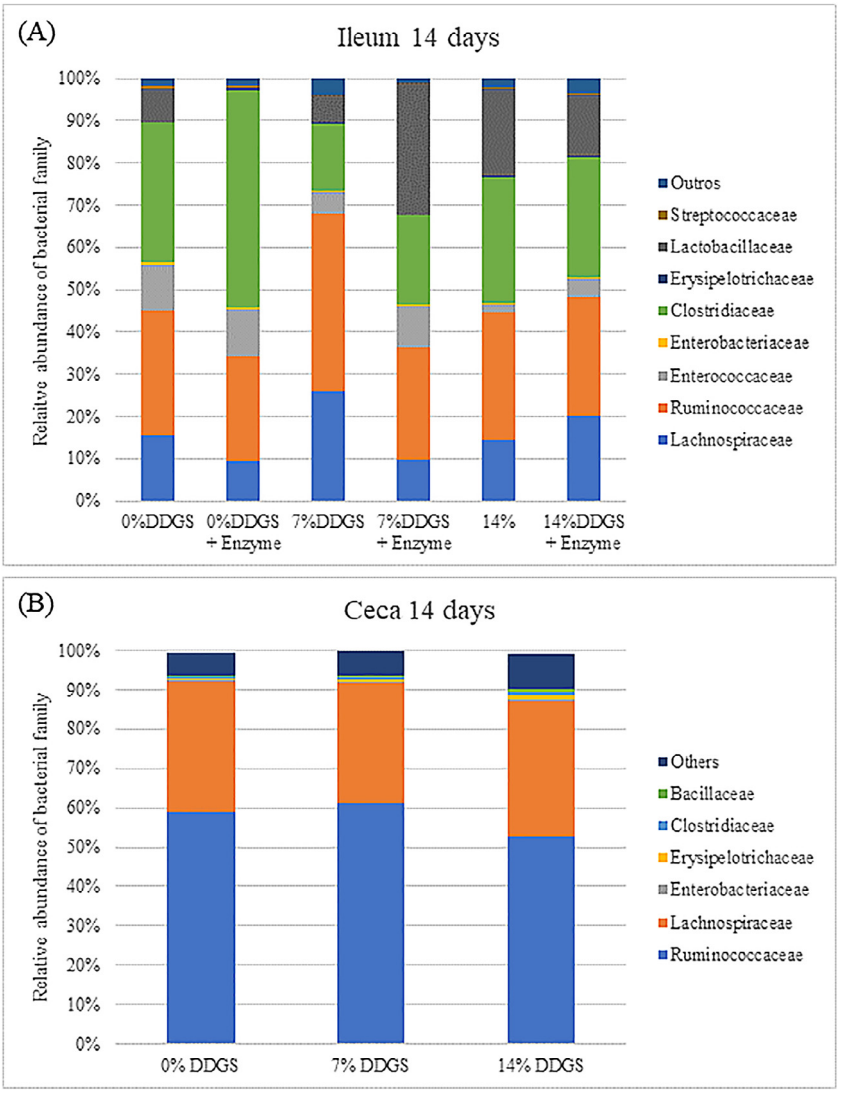

Figure 9. Relative abundance (%) of main bacterial families present in (A) ileal of broilers fed diets with different inclusion of DDGS (0, 7, or 14%) in a corn-soybean based diet with or without inclusion of multicarbohydrase complex + phytase (MCPC) at 14 d. The inclusion of MCPC in the diet with 7% DDGS tend to increase the abundance of Lactobacillaceae bacteria ($P = 0.070$) and reduce the abundance of Lachnospiraceae in the ileum at 14d ($P = 0.091$).; (B) Cecal microbiota of broilers at 14 d based on DDGS inclusion in the diets. Inclusion of DDGS in the diet tend to increase the abundance of Clostridiaceae ($P = 0.008$), 14%DDGS diet increased the abundance of Erysipelotrichaceae ($P = 0.052$) and Bacillaceae ($P = 0.093$). Abbreviation: DDGS, dried grains with soluble.

performance were observed at any level of inclusion (6, 12, or 18%) in the grower phase suggesting that beyond the starter period chicks could efficiently use higher levels of DDGS (Lumpkins et al., 2004). Also, more experiments had shown that if diets were formulated on a digestible amino acid basis, 15% of DDGS can be fed from 1 to 42 d with no adverse effects on performance or carcass composition (Wang et al., 2007). The present results also demonstrates that a diet with up to 14% of DDGS had no negative effects on broiler live performance when compared to control diet (corn-soybean meal) if formulated to have the same protein and energy levels. In the present study, only the inclusion of 14% DDGS reduced BWG at 21 d (Table 3). It is thought that the impairment of performance during the starter and grower periods might occur due to a marginal lysine deficiency, which is most limiting when birds are young (Lumpkins et al., 2004). DDGS amino acid profile is dependent on corn composition which has a low lysine content (Han and Liu, 2010). Additionally, lysine is susceptible to Maillard reactions during DDGS drying, which can reduce the concentration and digestibility of this amino acid (Almeida et al., 2013b) leading to marginal lysine deficiency. Evaluating the amino acid digestibility of broilers fed with a 20% DDGS diet, Kim et al. (2018) observed that lysine was the only amino acid that tended to have a lower digestibility. Therefore, further attention may be needed to the lysine level in the diets formulated with DDGS, especially if diets are aimed at the initial phase. Moreover, the literature shows that inclusion of DDGS at rates higher than 15% can cause an impairment of broiler performance even if added to grower and finisher diets (Wang et al., 2007; Loar et al., 2012).

Although fiber present in corn grain is not transformed to ethanol during the fermentation process, DDGS has a high percentage of insoluble fiber. Thus, the supplementation of diets with high inclusion of

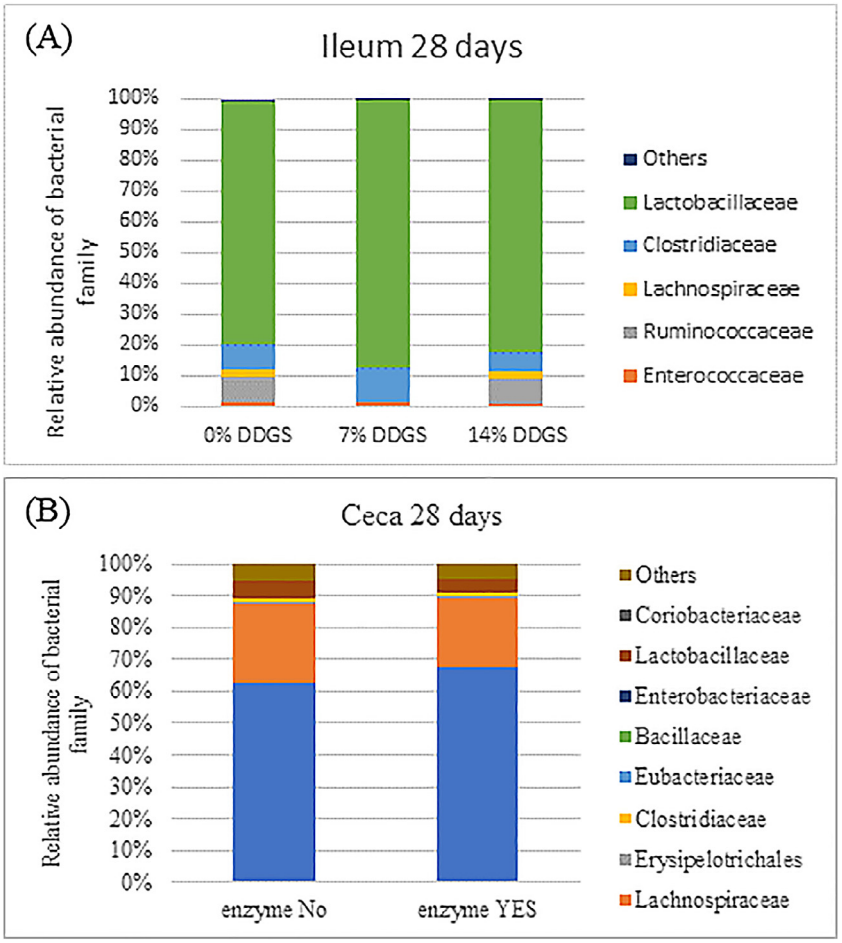

Figure 10. Relative abundance (%) of main bacterial families present in (A) Ileal microbiota of broilers fed diets with different inclusion of DDGS (0, 7, or 14%) in a corn-soybean based diet at 28 d. Inclusion of 14% of DDGS in the diet reduced the abundance of Enterococcaceae bacteria ($P = 0.046$), and the 7%DDGS inclusion reduced the abundance of Ruminococcaceae and Lachnospiraceae bacteria ($P = 0.019$ and 0.041, respectively) in the ileum at 28 d; (B) Cecal microbiota of broilers at 28 d based on multicarbohydrase complex + phytase (MCPC) inclusion in the diets. The supplementation of MCPC tends to reduce Bacillaceae and Enterobacteriaceae abundance ($P = 0.063$ and 0.080, respectively) and to increase Coriobacteriaceae ($P = 0.055$) in the ceca at 28 d. Abbreviation: DDGS, dried grains with soluble.

DDGS with NSP-hydrolysing enzymes is one method to improve diets nutritive value (Swiatkiewicz et al., 2016). In the current study, the multicarbohydrase (xylanase, β-glucanase, ABF) and phytase complex showed the most efficacy in increasing BWG and improving FCR when added to the 14% DDGS diet, at 14 and 21d. Inclusion of the enzymes in diets with 7% of DDGS showed an important FCR benefit later at 28 d. Additionally, if not considering the DDGS factor, MCPC increased the FI and BWG of chickens in all time points. Some experiments analyzing DDGS diets supplemented with enzymes did not show improvement in animal performance. For example, the inclusion of NSPase complex (xylanase, β-glucanase, and α-galactosidase) did not show an effect of the enzyme or interaction (DDGS x enzyme) on the performance (Campasino et al., 2015). Abudabos et al. (2017) tested 2 enzymatic complexes (protease + α-amylase + pectinase + phytase + glucoamylase + cellulase complex, and β-glucanase + β -xylanase) in diets with 6, 12, 18, and 24% of DDGS and did not find important effects on broiler performance as well. Although, supplementation of xylanase and phytase combination improved growth performance, digestibility of DM and phosphorus and biomechanical quality of femurs in broilers fed with a DDGS diet (Swiatkiewicz et al., 2014). In a review about the topic, Swiatkiewicz et al. (2016) observed that the efficacy of exogenous feed enzymes added to poultry DDGS diets is not consistent and depends on factors such as the age and physiological stage of the animals, the activity of the enzymes, the chemical composition of the DDGS, the ingredient inclusion in the diet, and the overall composition of the diet. In the present work, as mentioned, an effect of the enzyme in performance happened in the groups fed with DDGS diets, this result is probably related to the higher presence of enzyme substrate (fiber and phytate) in the DDGS diets compared to corn-soybean meal diet.

The inclusion of DDGS in the chicken diet usually results in a decrease of nutrient digestibility. A reduction of ileal digestible energy has been observed with 10 and 15% DDGS inclusion (Campasino et al., 2015), at 20% inclusion DDGS decreased the CP digestibility (Kim et al., 2018), and that the inclusion of DDGS linearly decreased ileal digestibility of DM, energy, and ileal digestible energy (Adeola and Zhai, 2012). In the current study, the inclusion of 7% of DDGS in the diet reduced the DM and GE digestibility than animals fed with 14% DDGS. However, providing the chickens fed with 14%

DDGS did not result in a reduction in DM or gross energy digestibility when compared to corn-soybean group (Table 4). The diminished digestibility of broilers fed high levels of DDGS diet is associated with the higher NSP concentrations in the feed that impair nutrient availability and affect the gastrointestinal tract function (Swiatkiewicz et al., 2016). One of the objectives to adding exogenous enzymes to poultry feed is to hydrolyze the fraction that chickens cannot efficiently digest, as the fiber and phytate. Nonstarch polysaccharide degrading enzymes (Liu et al., 2011; Romero et al., 2013; Campasino et al., 2015) and phytase (Olukosi et al., 2010; Deniz et al., 2013) have been shown to improve nutrients digestibility in corn DDGS diets. However, in the present work, we added a multicarbohydrase (xylanase, β-glucanase, ABF) and phytase complex to the diets and despite the positive performance effects the MCPC inclusion did not affect nutrient digestibility. These results agree with the earlier findings, when no effects of a multienzyme preparation (xylanase, β-glucanase, mannanase and phytase) was observed in the digestibility indices of broilers fed with 30% DDGS diet (Min et al., 2011). It is important emphasize that the efficacy of exogenous feed enzymes is highly dependent on the dose of substrate. Moreover, in the present experiment the improvement in performance observed with the supplementation of the enzyme may be related to the microbiota modulation and intestinal health.

The inclusion of 7 or 14% of DDGS in the broiler diet did not affect intestinal health at 14d (based on ISI score), but a longer feeding period (up to 28 d) did increase histological alteration in the duodenum and ileum. Data examining the interactions of corn DDGS and gastrointestinal health in fowl are fairly sparse (Rochell, 2018). Therefore, the present data are crucial to understanding the effects of DDGS on the gut and to evaluation how a prolonged and chronical exposure to insoluble NSP can induce morphophysiological changes in the gut. In the present study, at 28 d, a higher ISI score was observed in broilers fed DDGS diets, as a result of the increased infiltration of inflammatory cells in the lamina propria, a thicker lamina propria and epithelium.

The supplementation of MCPC reduced the ISI score, that is, reduced the intestinal histological alterations thus improving the gut health. Interestingly, at 14 d, the supplementation of MCPC in the 14% DDGS feed resulted in a better ISI score for jejunum than the corn diet. At 28 d, enzyme supplementation also improved the intestinal health. The lower ISI score in MCPC supplemented treatments resulted from a decrease in inflammatory cell infiltration into epithelium and lamina propria, consequent thinner epithelium and lamina propria, and the reduction of immature enterocyte proliferation and goblet cells. The improvements in gut health observed in the present study might be due to a reduction in insoluble fiber, and its antinutritional effects, and modulation of the intestinal microbiota. Further, insoluble fiber has some negative effects on the chicken gut, including reduction of feed digestibility (Bedford et al., 2010), a reduction of fat emulsification (Campbell et al., 1983), and the stimulation of host pattern recognition receptors that activate innate immunity. The addition of a NSP enzyme (β-mannanase) have shown to eliminate most of the immune signaling promoted by β-galactomannan in the jejunum of chickens and changed several gut integrity-related and intestinal metabolic/growth pathways (Arsenault et al., 2017). Recent findings have indicated that the central role of the NSPenz is the modulation of the gut microbiota (Aftab and Bedford, 2018). Studies have shown that xylanases might produce prebiotic-like compounds, such as short-chain xylans and xylo-oligosaccharides, that modulate the gut microbiota (Collins et al., 2005; Morgan et al., 2019). These compounds can act as a substrate for *Lactobacillus* spp. and *Bifidobacterium* spp., increasing their population and reducing pathogenic bacteria (Thammarutwasik et al., 2009; Sun et al., 2015). However, literature suggests that positive results in the gut are only observed with combination of enzymes. Studies did not show positive effects on pig's gut with supplementation of xylanase alone (Passos et al., 2015; Li et al., 2018; Taylor et al., 2018), but carbohydrase blends have shown improvement in the small intestinal barrier function, reduction of immune activation (Li et al., 2018) and increase in villus height:crypt depth ratio and reduction of mucosal macrophages (Jiang et al., 2015). The effects on intestinal health probably were due the modulation of intestinal microbiota, the reduction of the antinutritional effects of the insoluble fiber and phytate on the gut, and the reduction of immune activation promoted by fiber.

In addition to the ISI methodology, we evaluated the epithelial permeability using the FITC-d method, and intestinal inflammation by measuring the inflammatory biomarker, calprotectin in the serum. Although, we observed effect of the treatments in the intestinal ISI score, we observed no effects of the diets on the FITC-d permeability nor serum calprotectin concentration. We believe that no difference was observed because the inclusion of DDGS was not sufficient detrimental to cause an important inflammation and increase in gut permeability. Previously, our lab had observe an increase of serum calprotectin in animals submitted to a dietary models for chronic intestinal inflammation (Dal Pont et al., 2021), however in this case the broilers were fed a diet with 30% of rice bran, which probably was a stronger challenge than the 14% DDGS of the present study. Although, no significative changes in the serum calprotectin concentration, we can observe that the birds fed with MCPC diet presented a numeric reduction of calprotectin concentration at 28d, the number of samples might be higher to identify a statistical difference in calprotectin. Additionally, the calprotectin serum concentration presented an important increment with the age of the bird, as was also observed in our previous study (Dal Pont et al., 2021).

The present study demonstrated an important effect of DDGS on the intestinal microbiota diversity and

composition of broilers. The diversity within sample was affected only at 14 d by the inclusion of DDGS which indicates a more susceptible microbiota early in the chicken's life. Broilers are more sensitive to feed manipulations early in life because their digestive system is still developing (Batal and Parsons, 2002). The diversity of the ileal microbiota was reduced with the addition of DDGS to the diet, but it increased the cecal microbiota diversity. In the literature, broilers fed with 10% DDGS diets also presented higher diversity in the cecal microbiota than corn diet groups at 14 d, but no difference in the ileum diversity was observed (Perez et al., 2011). Abudabos et al. (2017) observed that DDGS concentrations were positively correlated with ceca richness index, increasing the number of detectable species in the segment at 35 d. This improvement of cecal diversity promoted by DDGS might occur because the cecal is the site where most of the fermentation takes place (Hijova and Chmelarova, 2007). Moreover, cecal digestive processes, such as the production of short chain fatty acids by bacterial fermentation, can provide up to 10% of a chicken's metabolizable energy (Józefiak et al., 2004). Furthermore, the inclusion of DDGS reduced the abundance of Proteobacteria in the ileum at 28d. The phylum Proteobacteria contain mainly opportunistic pathogens such as Escherichia, Salmonella, and Proteus (Latorre et al., 2018); thus, the inclusion of DDGS might demonstrate positive modulation to the broiler gut microbiota. Additionally, feeding broilers a diet containing DDGS reduced the presence of Enterococcaceae in the ileum at 14 and 28d. Probiotic bacteria also have been shown to be effective in reducing the Enterococcaceae family in chickens (Rodrigues et al., 2020). Thus, the present data, in agreement with previous literature, describes positive effects of DDGS to the ileal and cecal microbiota, probably caused by its insoluble fiber and yeast derivate content, which may be related with the performance of DDGS fed broilers being similar to control groups.

Studies testing the supplementation of 2 enzymatic complexes (protease + α-amylase + pectinase + phytase + glucoamylase + cellulase complex or β-glucanase + β -xylanase) in DDGS diets did not detected any influence of the enzymes on cecal microbiota composition (Abudabos et al., 2017). However, in the present study, the MCPC supplementation was efficient in reducing the differences in cecal microbiota in the chickens fed with 7% DDGS diet to the corn diet at 14d, but the additive was not enough to revert the changes caused by the 14% inclusion of DDGS. At 28 d, the inclusion of MCPC in the corn-soybean diet modulated the ileal microbiota but did not produce any differences in the diets containing DDGS. The inclusion of MCPC demonstrated some benefits for the microbiota, such as the reduction of Proteobacteria presence in the ceca at 28 d. The supplementation of MCPC to the diet containing 7% of DDGS tended to increase the abundance of Lactobacillaceae bacteria in the ileum. Abundance of *Lactobacillus*, bacteria from the Lactobacillaceae family, in the ceca have been positively associated with better feed efficiency in hens (Yan et al., 2017). Additionally, the supplementation of enzyme tended to reduce Bacillaceae and Enterobacteriaceae abundance and to increase Coriobacteriaceae in the ceca at 28d. Enterobacteriaceae consist of glucose fermenters and comprehend *Escherichia coli* and pathogenic *Salmonella enterica.* Enterobacteriaceae family is used a standard marker of gut dysbiosis (Rivera-Chávez et al., 2017), and its decline can collaborate for the succession of anaerobic microorganisms along with intestinal maturation, and fermentative metabolism (Matamoros et al., 2013). Bacillaceae are predominately anaerobic spore-forming bacilli of the genus Clostridium and the aerobic or facultatively anaerobic endospore. Thus, the inclusion of MCPC complex might promote beneficial changes in the ileal and cecal microbiota due reduce of Proteobacteria, Bacillaceae and Enterobacteriaceae. Although we observed an improvement in performance in the DDGS fed groups with the supplementation of the enzyme, we did not identify important effects of the enzyme in the microbiota of broiler fed with DDGS diet.

CONCLUSIONS

DDGS inclusion up to 14% did not impair broilers performance when compared to corn-soybean based diet up to 28 d. The supplementation of xylanase, β-glucanase, ABF and phytase improved the feed conversion of broilers fed diets with 14% of DDGS at 21 d and of broilers fed with 7% of DDGS at 28 d. Additionally, the inclusion of the enzyme complex increased the weight gain and feed consumption of broilers fed with corn-soybean based diet and diets with 7 or 14% of DDGS. The present study did not demonstrate any effects of the carbohydrase and phytase complex on digestibility; however, the enzymes improved the intestinal health of broilers. The inclusion of DDGS at 7 and 14% in the diet modulated the chicken intestinal microbiota presenting some positive effects, as the reduction of Proteobacteria presence in the ileum. Therefore, the supplementation of xylanase, β-glucanase, ABF and phytase to a DDGS diet improves performance and intestinal health allowing the use of these subproduct in the poultry nutrition.

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DISCLOSURES

The authors declare the following financial interests/ personal relationships which may be considered as potential competing interests: Cinthia Eyng reports financial support was provided by Adisseo France SAS.

SUPPLEMENTARY MATERIALS

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