Effects of the inclusion of a *Bacillus* direct-fed microbial on performance parameters, bone quality, recovered gut microflora, and intestinal morphology in broilers consuming a grower diet containing corn distillers dried grains with solubles

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ABSTRACT Distillers dried grains with solubles (DDGS) have increasingly been used in poultry diets as a consequence of rising grain costs. Some, but not all, sources of DDGS have a variable compositional value, and a high inclusion of this by-product could be considered a risk factor for presentation of enteric diseases. Presently, 2 experiments were conducted using a starter corn-soybean diet (zero to 7 d) and a corn-DDGS-soybean grower diet (8 to 28 d) with or without inclusion of a *Bacillus*-direct-fed microbial (DFM). In both experiments, day-of-hatch chicks were randomly assigned to 2 different groups: control group without DFM or *Bacillus*-DFM group, containing 10^6 spores/g of feed. In each experiment, 8 pens of 20 chicks (n = 160/group) were used. Performance parameters of BW, BW gain (BWG), feed intake (FI), and feed conversion (FCR) were evaluated in each growth phase. Additionally, in experiment 2, intestinal samples were collected to determine duodenal and ileal morphology (n = 8/group), as well as the microbiota population of total lactic acid bacteria (TLAB), total Gram-negative bacteria (TGNB), and total anaerobic bacteria (TAB) on d 28 (n = 16/group). Furthermore, both tibias were evaluated for bone strength and bone composition (n = 16/group). In both experiments BW, BWG, and FCR were improved by the DFM when compared to the control group ($P < 0.05$). In experiment 2, chickens supplemented with the DFM had less TGNB in the foregut intestinal segment and higher TLAB counts in both foregut and hindgut sections ($P < 0.05$). In addition significant increases in tibia breaking strength and bone mineralization were observed in the DFM group when compared with the control. In the case of intestinal morphology, DFM dietary inclusion increased villus height (VH), villus width, villus area, muscular thickness, and the VH to crypt depth ratio (VH:CD) in both duodenum and ileum sections. Results of the present study suggest that consumption of a selected *Bacillus*-DFM producing a variable set of enzymes could contribute to enhanced performance, intestinal microbial balance, and bone quality in broiler chickens consuming a grower diet that contains corn-DDGS.

Key words: *Bacillus*-DFM, DDGS, enzymes, microbiota, bone quality

INTRODUCTION

Distillers dried grains with solubles (**DDGS**) are by-products obtained during production of biofuels as renewable energy sources. During starch fermentation from cereal grains, ethanol and CO_2 are produced; meanwhile, the remaining nutrients are concentrated in the DDGS fraction (Singh et al., 2005). Corn is the main cereal used in ethanol production in the Unite States; however, other grains such as wheat, barley, and sorghum are utilized in different regions of the world for the same purpose (Donohue and Cunningham, 2009). The growth of ethanol production has resulted in increased quantities of DDGS available to feed producers, therefore making this by-product an attractive alternative feed ingredient during elevated corn cost periods (Stein, 2007). DDGS do provide a rich source of protein, minerals, xanthophylls, and other nutrients in poultry diets; nevertheless, nutritional content should be considered before feed formulation (Wang et al., 2007a).

According to Świątkiewicz and Koreleski (2008), high-quality DDGS can be included in starter diets for broilers and turkeys in a 5 to 8% without causing a detrimental effect on performance. In the case of grower-finisher diets for broilers and turkeys or feed formulation of laying hens, a 12 to 15% dietary inclusion level can be incorporated, partially replacing in a cost-effective way, soybean meal, corn, and other feed ingredients. However, the principal limitation on use of different DDGS sources obtained from multiple ethanol plants or cereal grains as a feed component has been the high nutritional composition variability and bioavailability of nutrients, observed especially for lysine, methionine, minerals, and energy (Spiehs et al., 2002; Fastinger et al., 2006). Furthermore, Behnke (2007) reported that inclusion of 5 to 7% DDGS could have a negative impact on pellet quality, increasing the percentage of fines per feed batch. Additionally, Barekatain et al. (2013) observed under a necrotic enteritis disease challenge that incorporating 20% of sorghum DDGS may lead to more severe intestinal lesions that could be related to an accelerated proliferation of *Clostridium perfringens*; however, more studies are required to support these results involving utilization of corn as the cereal source of DDGS. The majority of the reported compositional profiles of corn DDGS (100% dry matter basis) have focused mainly on common constituents, such as crude protein (28.7 to 32.9%), crude fiber (5.4 to 10.4%), crude fat (8.8 to 12.4%), ash (3.0 to 9.8%), phosphorus (0.42 to 0.99%), lysine (0.61 to 1.06%), methionine (0.54 to 0.76%), and tryptophan (0.18 to 0.28%) (US Grains Council, 2012). Nevertheless, non-starch polysaccharides (**NSP**) make up 25 to 30% of the DDGS, with the 2 major components of the NSP being arabinoxylans and cellulose (Kim et al., 2008; Jaworski et al., 2015). Therefore, targeting the indigestible components specific to DDGS with the correct blend of supplemental exogenous enzymes and utilization of new modified dry-grind processes to optimize starch fermentation can allow a more efficient utilization of this co-product, as well as increase its percentage of inclusion in livestock diets, resulting in greater economic returns (Singh et al., 2005; Min et al., 2011).

The use of spores from selected *Bacillus* strains as direct-fed microbials (**DFM**), have been shown to prevent gastrointestinal disorders and impart numerous nutritional benefits, including the production of extracellular enzymes, such as amylase (Ibrahim et al., 2012), protease (Olajuyigbe and Ajele, 2005), lipase (Shah and Bhatt, 2011), cellulase (Hendricks et al., 1995), xylanase (Monisha et al., 2009), and phytase (Choi et al., 2001). In this regard, exogenous enzymes produced by *Bacillus* spp. may help to degrade complex antinutritional factors in poultry diets and improve nutrient absorption. Nonetheless, it is important to mention that not all *Bacillus* bacteria synthesize the same type of enzymes, therefore requiring selection and characterization of adequate isolates according to target substrates

in the diet (Latorre et al., 2015b). Previous studies published by our laboratory suggest that the dietary inclusion of a previously selected *Bacillus*-DFM based on in-vitro enzyme production profiles could contribute to enhance bone strength, reduce digesta viscosity, and improve both intestinal microbial balance and performance parameters in poultry consuming diets that contained a considerable percentage of alternative cereal grains rich in soluble NSP in comparison to corn (Latorre et al., 2014b, 2015a). Therefore, the objectives of the present study were to evaluate the inclusion of a previously selected *Bacillus*-DFM on growth performance, bone quality, intestinal microflora, and intestinal epithelial morphology of broiler chickens consuming a grower diet containing corn DDGS.

MATERIALS AND METHODS

Animal Source and Experimental Diets

In the present study, 2 independent experiments were conducted. For all experiments day-of-hatch male broiler chicks were obtained from Cobb-Vantress (Siloam Springs, AR). In both experiments, chicks were neck-tagged and randomly located to one of 16 floor pens (300 cm × 150 cm) with new pine shavings as litter in an environmentally controlled room. Temperature was maintained at 34°C for the first 5 d and was then gradually reduced until a temperature of 23°C was achieved at 21 d of age. In both trials, a mash starter corn-soybean based diet (zero to 7 d) and a mash grower diet containing 8% DDGS (8 to 28 d) were offered according to the phase of production. Prior to formulating the experimental diets, it was determined that DDGS contained: moisture 13.1%, crude protein 29.2%, crude fat 11.0%, crude fiber 8.5%, calcium 0.14%, and phosphorus 0.72%. The experimental diets were formulated to approximate the nutritional requirements of broiler chickens as recommended by the NRC (1994), and adjusted to breeder's recommendations (Cobb-Vantress Inc., 2015). No antibiotics or coccidiostats were added to the feed (Table 1). The chemical composition of the experimental diets was determined by AOAC international (2000) methods for moisture (930.15), crude protein (984.13), crude fat (920.39), calcium (968.08), and phosphorus (965.17). All animal handling procedures were in compliance with the Institutional Animal Care and Use Committee at the University of Arkansas, Fayetteville.

Experimental Design

In order to show that similar results can be achieved independently, two experiments were conducted in the present study in broiler chickens that were raised during the starter and grower production phases. In both trials, broilers chicks were randomly assigned to either a control group or a DFM candidate group fed a diet

Table 1. Ingredient composition and nutrient content of a corn-soybean starter diet and a corn-DDGS-soybean grower diet used in all experiments on as-is basis.

Item	Starter diet	Grower diet
Ingredients (%)		
Corn	57.34	56.68
Soybean meal	34.66	27.05
DDGS	-	8.00
Poultry fat	3.45	4.09
Dicalcium phosphate	1.86	1.59
Calcium carbonate[1]	0.99	1.03
Salt	0.38	0.34
DL-Methionine	0.33	0.26
L-Lysine HCl	0.31	0.32
Threonine	0.16	0.12
Vitamin premix[2]	0.20	0.20
Mineral premix[3]	0.10	0.10
Choline chloride 60%	0.20	0.20
Antioxidant[4]	0.02	0.02
Calculated analysis		
Metabolizable energy (kcal/ kg)	3,035	3,108
Crude protein (%)	22.16	20.73
Ether extract (%)	5.68	7.11
Lysine (%)	1.35	1.20
Methionine (%)	0.64	0.57
Methionine + Cystine (%)	0.99	0.91
Threonine (%)	0.92	0.82
Tryptophan (%)	0.28	0.24
Total calcium (%)	0.90	0.84
Available phosphorus (%)	0.45	0.42
Determined analysis		
Crude protein (%)	21.15	20.30
Ether extract (%)	6.05	6.78
Calcium (%)	0.94	0.90
Phosphorus (%)	0.73	0.69

[1]Inclusion of 10^6 spores/g of feed mixed with calcium carbonate.

[2]Vitamin premix supplied the following per kg: vitamin A, 20,000 IU; vitamin D3, 6,000 IU; vitamin E, 75 IU; vitamin K3, 6.0 mg; thiamine, 3.0 mg; riboflavin, 8.0 mg; pantothenic acid, 18 mg; niacin, 60 mg; pyridoxine, 5 mg; folic acid, 2 mg; biotin, 0.2 mg; cyanocobalamin, 16 μg; and ascorbic acid, 200 mg (Nutra Blend LLC, Neosho, MO).

[3]Mineral premix supplied the following per kg: manganese, 120 mg; zinc, 100 mg; iron, 120 mg; copper, 10 to 15 mg; iodine, 0.7 mg; selenium, 0.4 mg; and cobalt, 0.2 mg (Nutra Blend LLC, Neosho, MO).

[4]Ethoxyquin.

supplemented with 10^6 spores/gram of feed of a *Bacillus*-DFM previously selected based on in vitro enzyme activity (Latorre et al., 2015b). Each treatment was comprised of 8 pens of 20 chicks (n = 160/group), and for evaluation of growth performance, each replicate was used as the experimental unit. Every wk, all broilers were individually weighed and BW, BW gain (**BWG**), and feed intake (**FI**) data per pen were obtained to calculate the feed conversion ratio (**FCR**) for starter, grower, and the overall experimental periods. Additionally in experiment 2 at 28 d of age, 2 broilers per replicate (n = 16/group) were humanely killed by CO_2 asphyxiation to collect intestinal samples for determination of the recovered microbiota population of total lactic acid bacteria (**TLAB**), as well as total Gram-negative bacteria (**TGNB**) and total anaerobic bacteria (**TAB**). Additionally, intestinal sections of the duodenum and ileum were obtained to evaluate morphological parameters (n = 8/group). In the case of evaluation of bone quality parameters, both tibias from 2 birds per replicate were collected (n = 16/group).

Details about measurement procedures are described below.

Selection and Identification of Bacillus spp.

Previous research conducted in our laboratory focused on isolation of several *Bacillus* spp. from environmental and poultry sources (Shivaramaiah et al., 2011; Wolfenden et al., 2011; Menconi et al., 2013). Three *Bacillus* strains were selected as superior producers of cellulase and xylanase based on a qualitative enzyme activity evaluation (Latorre et al., 2015b). Identification and characterization of the different isolates were carried out using an API 50 CHB test kit (bioMerieux, Marcy l'Etoile, France), and individual plates of each strain also were sent for 16S rRNA sequence analysis to a specialized laboratory (Midi labs, Newark, DE). One of the 3 *Bacillus* strains was identified as *B. subtilis*, and the other 2 isolates were identified as *B. amyloliquefaciens*. Then, the selected *Bacillus* spp. strains were sporulated and mixed during the DFM preparation process before dietary inclusion.

Direct-fed Microbials (DFM) Preparation

In an effort to grow high numbers of viable spores, a modified version of a solid state (**SS**) fermentation medium developed by Zhao et al. (2008) was used in the present study. Briefly, to prepare the SS fermentation medium, ammonia broth was added to a mixture of 70% rice straw and 30% wheat bran at the rate of 40% by weight. Then, the SS fermentation medium was added to 250 mL Erlenmeyer flasks and sterilized by autoclaving for 30 min at 121°C. Each of the 3 selected *Bacillus* strains candidates was grown, individually, overnight at 37°C in test tubes containing 10 mL of tryptic soy broth (**TSB**, Becton Dickinson, Sparks, MD). After incubation, 2 mL of each candidate culture were added separately to the previously prepared SS fermentation medium flasks. The inoculated flasks were incubated for 24 h at 37°C to promote growth of the *Bacillus* spp. candidates, and incubated for another 72 h at 30°C to trigger the initiation of the sporulation process. Following this, the inoculated SS fermentation medium was removed from the Erlenmeyer flasks, placed onto Petri dishes, and dried at 60°C for 18 hours. Then, the SS fermentation medium was aseptically ground into a fine powder that contained stable *Bacillus* spores ($\sim 10^{11}$ spores/g). Spores from each isolate (1:1:1) were combined to produce the *Bacillus*-DFM candidate final product containing $\sim 3 \times 10^{11}$ spores/g. The *Bacillus*-DFM candidate was included into each experimental diet to reach a final concentration of 10^6 spores/g using a rotary mixer for 15 minutes. Samples of feed containing the DFM candidate were subjected to 100°C for 10 min to eliminate vegetative cells and validate the amount of spores per g of feed after inclusion and mixing steps. Following heat-treatment, 10-fold dilutions of

the same feed samples were plated on tryptic soy agar plates (**TSA**, Becton Dickinson, Sparks, MD), letting spores germinate to vegetative cells after incubation at 37°C for 24 h, hence representing the number of spores present per g of feed.

Enumeration of Recovered Intestinal Microflora

For determination of total recovered bacteria, intestinal sections from the duodenum to Meckel's diverticulum (foregut) and from Meckel's diverticulum to ceca (hindgut) were aseptically collected, separated into sterile bags, and homogenized. Samples were weighed and 1:4 wt/vol dilutions were made with sterile 0.9% saline. Later, 10-fold dilutions of each sample from each group were made in a sterile 96-well Bacti flat-bottom plate and then plated on different culture media for enumeration of TLAB on Man Rogosa Sharpe agar (Difco Laboratories, Detroit, MI,); TGNB on MacConkey agar (VWR, Suwanee, GA), and TAB on TSA containing sodium thioglycollate (Becton Dickinson, Sparks, MD). All plates were incubated at 37 °C for 18 h and bacterial counts were expressed in colony forming units (Log_{10} cfu/g of tissue).

Bone Parameters

Bone parameters were measured according to the methods described by Zhang and Coon (1997). Tibias from each chicken were cleaned of attached tissues. Bones from the left leg were subjected to conventional bone assays as described below and tibias from the right leg were used to determine breaking strength. The bones from the left tibia were dried at 100°C for 24 h and weighed. Then the samples were ashed in a muffle furnace (Isotemp muffle furnace, Fisher Scientific, Pittsburgh, PA) at 600°C for 24 h in crucibles, cooled in a desiccator, and weighed. From the left tibia, total calcium content was obtained by inductively coupled plasma (968.08; AOAC International, 2000) and total phosphorus content was determined by colorimetry using the molybdo-vanadate method (967.17; AOAC International, 2000). In the case of the right tibia samples, the tibial diaphysis from individual birds was cleaned of adherent tissues, the periosteum was removed, and the biomechanical strength of each bone was measured using an Instron 4502 (Norwood, MA) material testing machine with a 590 kg load cell. The bones were held in identical positions and the mid-diaphyseal diameter of the tibial mid-shaft, which was also the site of impact, was measured using a dial caliper. The maximum load at failure was determined in the tibial mid-section between epiphyses, using a 3-point flexural bend fixture with a total distance of 30 mm between the 2 lower supporting ends. The load, defined as force in kilograms per square millimeter of cross-sectional area (kg/mm^2), represents bone strength. The rate of loading was kept constant at 20 mm/min collecting 10 data points per second. The data were automatically calculated using Instron's Series IX Software (Norwood, MA).

Intestinal Morphometric Analysis

Intestinal sections were standardized: for duodenum, a 0.5 cm section was collected from the middle of the descending duodenum; and for ileum, a 0.5 cm section was collected from the mid-ileum at the Meckel's diverticulum. Duodenal and ileal sections were fixed in 10% neutral buffered formalin and embedded in paraffin, sectioned (5-mm thick), set on a glass slide, and stained with hematoxylin and eosin (**H&E**), then examined by light microscopy. Photomicrographs of random selected fields of each intestinal sample were acquired using a microscope equipped with a Leica DFC450C camera and Leica V 3.8.0. software (Leica Application Suit) and used for morphometric analysis. ImageJ 1.47v software (Rasband, 1997–2012) was used to make the measurements in the morphometric analysis of the different intestinal sections. For villus length of the duodenum and ileum, an average of 10 villi per bird were measured, with a total of 8 broilers per group. Villus height (**VH**) was measured from the tip of the villus to the top of the lamina propria. Crypt depth (**CD**) was measured from the base of the invagination between the villus upwards the region of transition between the crypt and villus (Aptekmann et al., 2001). Data from VH and CD were used to obtain the VH to CD ratio (**VH:CD**). Moreover, villus width (**VW**) was measured at the base area of each villi, and the villus surface area was calculated using the formula $(2\pi)(VW/2)(VH)$, where VW = villus width, and VH = villus height (Sakamoto et al., 2000).

Statistical Analysis

In all experiments, data were subjected to one-way ANOVA as a completely randomized design using the GLM procedure of SAS (SAS Institute, 2002). In both experiments, for the evaluation of growth performance (BW, BWG, FI, and FCR) each of the 8 replicates of 20 chickens was considered as the experimental unit, whereas data on bone quality (n = 16/group), intestinal microbiota (n = 16/group), and intestinal morphology (n = 8/group) were based on randomly selected broilers from all replicates of each group. Data are expressed as mean $\pm$ SE and a *P*-value less than 0.05 was set as the standard for significance.

RESULTS

The results of the evaluation of performance parameters (BW, BWG, FI, and FCR) in broiler chickens consuming a corn-DDGS-soybean grower diet with or without dietary inclusion of a *Bacillus*-DFM candidate of experiment 1 are summarized in Table 2. In this

Table 2. Evaluation of body weight, body weight gain, feed intake, and feed conversion ratio in broiler chickens consuming a corn-DDGS-soybean grower diet with or without dietary inclusion of a *Bacillus*-direct-fed microbial (DFM) (Experiment 1).[1]

Item	Control	*Bacillus*-DFM
Body weight (g)		
d 0	47.2 ± 0.5^a	47.5 ± 0.2^a
d 7	150.6 ± 3.2^a	148.8 ± 1.2^a
d 28	1437.0 ± 14.4^b	1484.0 ± 14.5^a
Body weight gain (g)		
d 0 to 7	103.4 ± 2.9^a	101.3 ± 1.1^a
d 8 to 28	1286.4 ± 13.3^b	1335.3 ± 14.1^a
d 0 to 28	1389.8 ± 14.3^b	1436.6 ± 14.6^a
Feed intake (g)		
d 0 to 7	177.0 ± 6.4^a	175.1 ± 7.3^a
d 8 to 28	2081.8 ± 19.8^a	2052.3 ± 20.8^a
d 0 to 28	2212.6 ± 19.9^a	2182.6 ± 19.6^a
Feed conversion ratio		
d 0 to 7	1.17 ± 0.02^a	1.18 ± 0.04^a
d 8 to 28	1.62 ± 0.01^a	1.53 ± 0.02^b
d 0 to 28	1.54 ± 0.01^a	1.47 ± 0.01^b

[1]Data are expressed as mean $\pm$ SE.
[a,b]Means in each row with different superscripts are significantly different ($P < 0.05$).

Table 3. Evaluation of body weight, body weight gain, feed intake, and feed conversion ratio in broiler chickens consuming a corn-DDGS-soybean grower diet with or without dietary inclusion of a *Bacillus*-direct-fed microbial (DFM) (Experiment 2).[1]

Item	Control	*Bacillus*-DFM
Body weight (g)		
d 0	39.7 ± 0.2^a	39.9 ± 0.3^a
d 7	115.3 ± 1.7^a	116.2 ± 1.5^a
d 28	1409.0 ± 7.9^b	1444.0 ± 12.6^a
Body weight gain (g)		
d 0 to 7	75.6 ± 1.8^a	76.3 ± 1.6^a
d 8 to 28	1294.1 ± 8.7^b	1328.6 ± 12.1^a
d 0 to 28	1369.7 ± 7.9^b	1404.8 ± 12.6^a
Feed intake (g)		
d 0 to 7	130.8 ± 3.2^a	130.5 ± 2.3^a
d 8 to 28	1879.0 ± 10.2^a	1838.0 ± 13.3^b
d 0 to 28	2010.0 ± 9.7^a	1966.6 ± 13.6^b
Feed conversion ratio		
d 0 to 7	1.13 ± 0.01^a	1.12 ± 0.02^a
d 8 to 28	1.45 ± 0.03^a	1.38 ± 0.01^b
d 0 to 28	1.43 ± 0.02^a	1.36 ± 0.01^b

[1]Data are expressed as mean $\pm$ SE.
[a,b]Means in each row with different superscripts are significantly different ($P < 0.05$).

experiment, during the starter phase (zero to 7 d), broilers consuming the diet supplemented with the DFM showed similar values in all the growth performance variables that were evaluated in comparison to the control group. On the other hand, during the grower phase (8 to 28 d) when 8% of DDGS was included in the diet, supplementation with the *Bacillus*-DFM improved BWG in 48 g and FCR in 9 points when compared to the control group ($P < 0.05$) (Table 2). Similarly, in experiment 2, inclusion of the *Bacillus*-DFM showed similar growth performance values in comparison to the control group during the starter phase (zero to 7 d); however, in the grower phase (8 to 2 8d) a significant increase in BWG of 34 g and 7 points more efficient FCR were observed in the group consuming the diet supplemented with the *Bacillus*-DFM compared to the control. Additionally in experiment 2, FI was reduced ($P < 0.05$) 41 g in the DFM group compared to the control (Table 3). At the end, in both trials, addition of the DFM improved performance parameters during the overall experimental period (zero to 28 d), showing consistency of results between experiments (Tables 2 and 3).

Table 4 shows the results of the determination of total bacterial counts recovered from the foregut and hindgut intestinal segments in 28-day-old broiler chickens from experiment 2. Chickens that received the *Bacillus*-DFM had reduced counts of TGNB and increased numbers of TLAB in the foregut intestinal section ($P < 0.05$); however, similar values were obtained for TAB between experimental groups. In the case of the hindgut section, supplementation with the DFM significantly increased the count of TLAB and reduced the TAB numbers compared to the microflora of the unsupplemented group; nevertheless, no difference was observed in TGNB between experimental treatments.

Results of the assessment of bone strength and bone composition in broiler chickens fed with a corn-DDGS-soybean grower diet with or without dietary inclusion of a *Bacillus*-DFM candidate in experiment 2 are summarized in Table 5. Bone strength and composition were measured in 28-day-old broilers, showing that supplementation with the *Bacillus*-DFM significantly improved tibial load at break and tibial breaking strength, as well as calcium and phosphorus content ($P < 0.05$) compared to chickens receiving a control grower diet. However, tibia diameter values were similar between supplemented and unsupplemented groups.

Table 6 shows the results of the intestinal morphometric analysis of duodenal and ileal sections of broiler chickens at 28 d of age from experiment 2. Significant increases in VH, VW, villus area, muscular thickness, and VH:CD ratio were shown in chickens that received the DFM in the duodenum and ileum when compared with chickens from the control group. Additionally, consumption of the DFM reduced CD in both evaluated intestinal sections in comparison to broilers fed the control grower diet ($P < 0.05$).

DISCUSSION

Distillers dried grains with solubles are by-products that can be obtained from different cereal grains during biofuel production (Świątkiewicz and Koreleski, 2008). As ethanol production has expanded in recent years, the availability of DDGS as a feedstuff for poultry diets has increased (Wang et al., 2007b). Nonetheless, to choose the correct percentage of inclusion of DDGS, it is important to know the actual nutritional composition profile of this raw material, due to commonly high nutritional variability among sources (Loar II et al., 2010). Although DDGS usually have a good nutritional value and can be included at high levels in other livestock

Table 4. Determination of total bacterial counts in the foregut and hindgut intestinal segments in broiler chickens consuming a corn-DDGS-soybean grower diet with or without dietary inclusion of a *Bacillus*-direct-fed microbial (DFM) (Experiment 2).[1,2]

Item	Foregut[3]			Hindgut[3]		
	TGNB[4]	TLAB[4]	TAB[4]	TGNB[4]	TLAB[4]	TAB[4]
Control	4.70 ± 0.18^a	5.19 ± 0.29^b	5.24 ± 0.28^a	6.76 ± 0.41^a	6.10 ± 0.42^b	7.14 ± 0.60^a
Bacillus-DFM	3.75 ± 0.17^b	6.11 ± 0.19^a	5.67 ± 0.49^a	5.89 ± 0.49^a	7.37 ± 0.04^a	6.39 ± 0.61^b

[1]Data are expressed as Log_{10} cfu/g mean $\pm$ SE.
[2]Bacteria enumeration evaluated from 28-day-old broilers, n = 16/group.
[3]Forgut: from duodenum to Meckel's diverticulum, hindgut: from Meckel's diverticulum to ceca.
[4]TGNB = Total Gram-negative bacteria recovered, TLAB = Total lactic acid bacteria recovered, TAB = Total anaerobic bacteria recovered from different intestinal sections in each experimental group.
[a,b]Means in each column with different superscripts are significantly different ($P < 0.05$).

Table 5. Assessment of bone strength and bone composition in broiler chickens fed with a corn-DDGS-soybean grower diet with or without dietary inclusion of a *Bacillus*-direct-fed microbial (DFM) (Experiment 2).[1,2]

Item	Load at break (kg)	Tibia diameter (mm)	Breaking strength (kg/mm²)	Calcium (%)	Phosphorus (%)
Control	35.85 ± 1.47^b	6.84 ± 0.21^a	5.26 ± 0.02^b	35.24 ± 0.10^b	16.60 ± 0.30^b
Bacillus-DFM	42.88 ± 2.75^a	7.14 ± 0.31^a	5.99 ± 0.01^a	39.26 ± 0.24^a	20.83 ± 0.66^a

[1]Data are expressed as mean $\pm$ SE.
[2]Bone measurements evaluated from 28-day-old broilers, n = 16/group.
[a,b]Means in each column with different superscripts are significantly different ($P < 0.05$).

Table 6. Morphometric analysis of duodenum and ileal tissue in chickens at 28 d of age (Experiment 2).[1,2]

Tissue	Control	*Bacillus*-DFM
Duodenum		
Villus height (μm)	337.20 ± 3.07^b	457.24 ± 4.66^a
Villus width (μm)	40.07 ± 0.44^b	44.43 ± 0.22^a
Crypt depth (μm)	64.07 ± 1.14^a	55.23 ± 0.44^b
Area (mm²)[3]	42.38 ± 0.52^b	63.95 ± 0.85^a
Muscular thickness (μm)	46.79 ± 0.82^b	60.42 ± 0.40^a
VH:CD[4]	5.34 ± 0.06^b	8.32 ± 0.11^a
Ileum		
Villus height (μm)	140.88 ± 3.06^b	166.90 ± 3.81^a
Villus width μm	33.91 ± 0.82^b	39.62 ± 0.62^a
Crypt depth (μm)	46.88 ± 1.64^a	38.59 ± 1.00^b
Area (mm²)[3]	15.32 ± 0.59^b	21.15 ± 0.73^a
Muscular thickness (μm)	34.86 ± 0.44^b	43.16 ± 0.64^a
VH:CD[4]	3.02 ± 0.03^b	4.55 ± 0.13^a

[1]Data are expressed as mean $\pm$ SE.
[2]Morphometric analysis evaluated from 28-day-old broilers, n = 8/group.
[3]$2\pi \times$ (villus width/2) $\times$ villus height (Sakamoto et al., 2000).
[4]VH:CD = Villus height to crypt depth ratio.
[a,b]Means in each row with different superscripts are significantly different ($P < 0.05$).

rations such as swine, 10% has traditionally been the recommended feeding limit for broiler chickens during the grower period (Stein, 2007). This upper feeding limit is linked to the high level of indigestible fiber components present in either wheat or corn DDGS (Barekatain et al., 2013). Compared with protein sources such as soybean meal, the nutritional value of DDGS is lower, due to its inferior protein quality, partly caused by the excessive pretreatment and drying conditions during the ethanol production process, and high level of non-starch polysaccharides (**NSP**) (~30%) (Martinez-Amezcua et al., 2007). Diets high

in NSP reduce effective energy and nutrient utilization in poultry and other monogastric animals due to a lack of the endogenous enzymes needed to break down the complex cell wall polysaccharides that encapsulate other nutrients (Bedford et al., 1991; Bedford and Classen, 1993; Bedford and Schulze, 1998). Hence, exogenous enzymes have been used as feed additives in poultry diets to diminish this antinutritional cage effect (Choct, 2006; Slominski, 2011). For instance, NSP-hydrolyzing enzymes may increase the accessibility of phytase to phytin, increasing phosphorus availability and absorption (Zijlstra et al., 2010). For instance, it has been well documented that inclusion of xylanase in diets containing feed ingredients with a high content of NSP significantly improves intestinal viscosity, accelerates feed passage time through the gastrointestinal tract, and enhances digestibility of dietary protein and fat sources resulting in an improvement in growth performance (Choct et al., 1995; Langhout et al., 1997). One of the principal sources of the exogenous enzymes used by biotechnology and animal feed additive companies are bacteria from the genus *Bacillus* (Monisha et al., 2009; Shah and Bhatt, 2011; Ibrahim et al., 2012). *Bacillus* are Gram-positive, rod-shaped, facultative anaerobe bacteria with a remarkable life cycle including generation of endospores in nutritionally limited environments (Cutting, 2011). *Bacillus* spp. spores' ability to resist rough environmental conditions, surviving high temperature during feed pelletization, as well as tolerating extreme pH, dehydration, high pressures, caustic chemicals, and long storage periods have made them suitable for commercialization and distribution as direct-fed microbials (Cartman et al., 2007). Furthermore, it has been shown that *Bacillus*

spores can persist and change their distribution according to the variable biochemical conditions of the gastrointestinal tract (**GIT**)of broiler chickens; therefore, supporting the hypothesis of a possible full life cycle development in the GIT, suggesting that these bacteria could be considered part of the metabolically active host microbiota (Latorre et al., 2014a). Previously, our laboratory has screened and identified different *Bacillus* spp. isolates as DFM candidates for production of different exogenous enzymes, such as cellulase, amylase, lipase, xylanase, and phytase. Moreover, we have demonstrated in vitro that the inclusion of cereal grains (wheat, rye, barley, and oats) with a higher content of soluble NSP in comparison to corn increased digesta viscosity and *C. perfringens* growth. Nevertheless, the dietary inclusion of the selected *Bacillus*-DFM candidate in non-corn-based diets significantly reduced both viscosity and *C. perfringens* proliferation when compared to control non-supplemented diets, supporting the results of the in vitro enzyme selection profile of the *Bacillus* isolates conforming the DFM (Latorre et al., 2015b). Additionally, during in vivo trials, chickens and/or turkeys fed a rye-based diet without DFM supplementation showed an increase in bacterial translocation and digesta viscosity, accompanied by reduced bone mineralization; however, these adverse effects were ameliorated by the inclusion of the previously selected *Bacillus*-DFM candidate (Latorre et al., 2014b, 2015a). In the present study, supplementation with the *Bacillus*-DFM in a grower diet containing 8% DDGS improved performance parameters, bone quality, intestinal microflora balance, and intestinal morphology, therefore supporting our previous results. Chickens that received the DFM in the grower-DDGS diet improved performance, bone quality parameters, and the surface area of absorption in both duodenal and ileal intestinal sections, and this could be related to a more efficient utilization of the diet due to the production of exogenous enzymes by the DFM. Moreover, a significant reduction in VH:CD ratio in the control group may be attributed to a higher presence of insoluble fiber in the intestinal digesta from the DDGS when compared to DFM supplemented group, therefore increasing cellular turnover as suggested by Barekatain et al. (2013). It was also interesting to observe that broilers receiving the *Bacillus*-DFM had a higher count of lactic acid bacteria in the foregut and hindgut intestinal sections. Perhaps the improvement in VH and VW was due to an elevated production of short chain fatty acids by lactic acid bacteria in the intestinal lumen (Scheppach, 1994; Hosoi et al., 2000). On the other hand, production of antimicrobial and antioxidant compounds by the *Bacillus*-DFM together with a reduction of substrates available in the intestinal lumen could diminish the bacterial population of Gram-negatives and anaerobic bacteria (Wolfenden et al., 2010), therefore decreasing the level of intestinal inflammation and enhancing epithelial integrity, nutrient absorption, and bone strength and composition. In summary, the re-

sults of this study suggest that the dietary inclusion of a previously selected *Bacillus*-DFM based on in-vitro enzyme production profiles could contribute to enhance performance and bone quality, and improve both intestinal microbial balance as well as epithelial morphology in broiler chickens consuming diets that contained a considerable percentage of DDGS. Further studies to evaluate metabolomics and microbiome analysis as well as other gut inflammation biomarkers in chickens fed with this selected *Bacillus*-DFM in different poultry diets are currently being evaluated.

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