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Evaluation of a *Bacillus*-Based Direct-Fed Microbial on Aflatoxin B1 Toxic Effects, Performance, Immunologic Status, and Serum Biochemical Parameters in Broiler Chickens

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SUMMARY. The aim of the present study was to evaluate the effect of a commercial *Bacillus* direct-fed microbial (DFM) on aflatoxin B1 toxic effects, performance, and biochemical and immunologic parameters in broiler chickens. Ninety 1-day-old Cobb 500 male broiler chicks were raised in floor pens for a period of 21 days. Chicks were neck-tagged, individually weighed, and randomly allocated to one of three groups: Negative control (basal feed), aflatoxin B1 (basal feed + 2 ppm AFB1), and DFM (basal feed + 2 ppm AFB1 + *Bacillus* direct-fed microbial). Each group had three replicates of 10 chickens ($n = 30$/group). Body weight and body weight gain were calculated weekly, while feed intake and feed conversion ratio were determined when broilers were 21 days old. On day 21, all chickens were bled, gastrointestinal samples were collected, and spleen and bursa of Fabricius were weighed. This study confirmed that 2 ppm of AFB1 causes severe detrimental effects on performance, biochemical parameters, and immunologic parameters, generating hepatic lesions in broiler chickens ($P < 0.05$). However, it was also observed that DFM supplementation provided beneficial effects that might help to improve gut barrier function, anti-inflammatory and antioxidant activities, as well as humoral and cellular immunomodulation. The results of the present study suggest that this *Bacillus*-DFM added at a concentration of 10^6 spores/gram of feed can be used to counteract the negative effects that occur when birds consume diets contaminated with AFB1, showing beneficial effects on performance parameters, relative organ weights, hepatic lesions, immune response, and serum biochemical variables. The addition of this *Bacillus*-DFM might mitigate and decrease aflatoxicosis problems in the poultry industry, improving food security, alleviating public health problems, and providing economic benefits. Future studies are needed to fully elucidate the specific mechanisms by which this *Bacillus*-DFM counteracts the toxic effects of aflatoxin B1.

RESUMEN. Evaluación de un producto comercial adicionado en el alimento elaborado con *Bacillus* sobre los efectos tóxicos de la aflatoxina B1, el rendimiento productivo, el estado inmunológico y los parámetros bioquímicos en suero de pollos de engorde.

El objetivo del presente estudio fue evaluar el efecto de un producto comercial de *Bacillus* adicionado al alimento (DFM) sobre los efectos tóxicos de la aflatoxina B1, el rendimiento productivo, así como en los parámetros bioquímicos e inmunológicos en pollos de engorde. Noventa pollitos de engorde machos Cobb 500 de un día de edad fueron criados en corrales en piso por un período de 21 días. Los pollos se etiquetaron en el cuello, se pesaron individualmente y se asignaron al azar en uno de tres grupos: control negativo (alimentación basal); aflatoxina B1 (alimentación basal + 2 ppm de AFB1) y DFM (alimentación basal + 2 ppm de AFB1 + producto comercial de *Bacillus*). Cada grupo tenía tres réplicas de 10 pollos (n = 30/grupo). El peso corporal (BW) y la ganancia de peso corporal (BWG) se calcularon semanalmente, mientras que la ingesta de alimento (FI) y la conversión alimentaria (FCR) se determinaron cuando los pollos tenían 21 días de edad. Al día 21 de edad, todos los pollos se sangraron, se recolectaron muestras gastrointestinales y se pesaron el bazo y la bolsa de Fabricio. Este estudio confirmó que 2 ppm de aflatoxina B1 causan efectos detrimentales graves sobre los parámetros productivos, bioquímicos e inmunológicos, generando lesiones hepáticas en pollos de engorde ($P < 0.05$). Sin embargo, también se observó que la suplementación con el producto comercial de *Bacillus* proporcionó efectos benéficos que podrían ayudar a mejorar la función de la barrera intestinal, las actividades antiinflamatorias y antioxidantes, así como la inmunomodulación humoral y celular. Los resultados del presente estudio sugieren que este producto comercial de *Bacillus* agregado a una concentración de 10^6 esporas/gramo de alimento puede usarse para contrarrestar los efectos negativos que se producen cuando las aves consumen dietas contaminadas con aflatoxina B1, mostrando efectos beneficiosos en los parámetros productivos, peso relativo de órganos, lesiones hepáticas, respuesta inmune y variables bioquímicas séricas. La adición de este *Bacillus* podría mitigar y disminuir los problemas de aflatoxicosis en la industria avícola, mejorando la seguridad alimentaria, los problemas de salud pública y los beneficios económicos. Se requieren estudios futuros para dilucidar completamente los mecanismos específicos por los cuales este producto comercial con *Bacillus* contrarresta los efectos tóxicos de la aflatoxina B1.

Key words: aflatoxin B1, *Bacillus,* broilers, direct-fed microbial, performance

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Abbreviations: AF = aflatoxins; AFB1 = aflatoxin B1; ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; BUN = blood urea nitrogen; BW = body weight; BWG = body weight gain; CBH = cutaneous basophil hypersensitivity; CRP = C reactive protein; DFM = direct-fed microbial; FCR = feed conversion ratio; FI = feed intake; GGT = gamma-glutamyltransferase; IACUC = institutional animal care and use committee; IB = infectious bronchitis; IgA = immunoglobulin A; NC = negative control; ND = Newcastle disease; PHA = phytohemagglutinin; PPARα = proliferator activated receptor α; SEM = standard error of the mean; SOD = superoxide dismutase

Mycotoxins are low-molecular-weight compounds produced as secondary metabolites by filamentous fungi contaminating crops in the field or in warehouses under certain environmental conditions of temperature and humidity. Among mycotoxins, aflatoxins (AF) are the best known and most intensively researched worldwide because of their significant impact on health and trade (1). In terms of toxicity and occurrence, the most critical AF is aflatoxin B1 (AFB1) (2).

Poultry species are highly sensitive to the toxic effects of AFB1, even exposure levels to this mycotoxin as low as 0.1 ppm can severely damage poultry health (3), due to a wide range of metabolic changes that are associated with liver damage, reduced digestive enzyme activities, and immunosuppression (4), affecting the profitability of the productive system, resulting in substantial annual economic losses to producers (5).

Hence, control of AFB1 for the poultry industry is critical, and many methods to reduce its toxic effects have been proposed, beginning with the attempt to minimize its production through good agricultural practices during both preharvest and postharvest periods, including cultivating practices in fields, harvest, transport, and storage conditions (6). However, prevention is not always possible, and other decontaminating or detoxifying methods have been used as an alternative to diminish AFB1 toxic effects, which can be physical, chemical, or biologic treatments of contaminated feed or grains, such as physical separation, gamma irradiation, or methods using ammonia, ozone, hydrogen peroxide, or some acids and alkalis (7,8). Nevertheless, many of these methods are not currently available because they are impractical to apply on a large scale in a cost-effective manner or because they are potentially unsafe. One of the most prominent approaches to prevent aflatoxicosis in livestock is the addition of adsorbents as feed additives so that these compounds impede the intestinal absorption of AFB1 by binding it and reducing its toxic effects (9). Even though many adsorbent materials have been tested and recognized for their ability to bind AFB1 successfully (10,11), there is a risk they can also absorb some vitamins and minerals when they are used in animal feed, causing nutritional problems in animals. Clay materials also may be contaminated with toxic components, like heavy metals or dioxins, which can be released in the intestine and accumulate in animal organs (12,13,14).

Such limitations and inconveniences have motivated research on biologic methods for AFB1 degradation (1,15,16). There are several microorganisms and their enzymes that are effective in preventing and controlling the toxic effects of AFB1 (17,18,19). Probiotics are some of the most studied microorganisms, due largely to their generally regarded as safe (GRAS) character, and because they have proved to have several potential applications against AFB1 both *in vitro* and *in vivo* (20,21). *Bacillus* spp. are microorganisms that act as probiotics for humans or animals (22), and when administered in adequate amounts they confer a health benefit to the host directly or indirectly through the maintenance of the microbial balance in their digestive tract. Previously, our laboratory has screened and identified *Bacillus* spp. isolates as direct-fed microbial (DFM) candidates (23,24). This study aimed to evaluate the protective effect of previously selected *Bacillus* spp. provided as a DFM candidate on performance, biochemical parameters, and immunologic parameters of broiler chickens fed with a 2 ppm AFB1 contaminated diet.

MATERIALS AND METHODS

Animal source, diets, and experimental design. Ninety 1-day-old male Cobb 500 broiler chicks (Cobb-Vantress Inc., Arkansas) were raised in floor pens for 21 days. Chicks were neck-tagged, individually weighed, and randomly allocated to one of three groups: Negative control (basal feed), AFB1 (basal feed + 2 ppm AFB1), and DFM (basal feed + 2 ppm AFB1 + DFM). Each group had three replicates of 10 chickens (n = 30/group). Nonmedicated mash corn-soybean–based broiler starter diet was formulated to approximate the nutritional requirements of broiler chickens as recommended by the National Research Council (25) and adjusted to breeder's recommendations (26) (Table 1). AFB1 was added to the diets and mixed thoroughly to reach the specified concentration. The *Bacillus*-DFM candidate was added to the experimental diet and mixed thoroughly to the specified concentration. Birds had *ad libitum* access to water and feed. All animal handling procedures complied with the Institutional Animal Care and Use Committee (IACUC) at the University of Arkansas, Fayetteville. Specifically, the IACUC approved this study under protocol No. 15006.

Aflatoxin production. AFB1 was provided by Dr. George E. Rottinghaus, Veterinary Medical Diagnostic Laboratory, University of Missouri, Columbia, MO. AFB1 was produced through the fermentation of rice, using *Aspergillus parasiticus* Northern Regional Research Laboratory 2999 from Agriculture Research Service culture collection, U.S. Department of Agriculture, and the aflatoxin content was measured by spectrophotometric analysis. The aflatoxin classification within the rice powder consisted of 74.62% AFB1, 22.38% AFG1, 2.48% AFB2, and 0.49% AFG2. Diets containing AFB1 were analyzed, and the presence of parent AF was confirmed by high-performance liquid chromatography with a fluorescence detection method by using a Romer Derivatization Unit (Romer Labs Inc., Washington, MO).

***Bacillus* direct-fed microbial candidate.** Previous research conducted in our laboratory focused on the isolation of several *Bacillus* spp. from environmental and poultry sources as previously described (27). In the present study, the DFM candidate is a *Bacillus* spore culture consisting of three isolates combined in equal amounts (1:1:1): two *Bacillus amyloliquefaciens* and one *Bacillus subtilis*, which were previously identified by sequence analysis of 16S rRNA. Their evaluation, selection, isolation, characterization, and sporulation procedures are described in previous publications (23,24). The DFM was added into the feed to obtain the experimental diet with a final concentration of 10^6 spores/g feed.

Performance parameters. In the experiment, pen replicates were used as experimental units for growth performance parameters. Chickens were individually weighed each week to obtain the pen body weight (BW), and body weight gain (BWG) per pen was recorded at weekly intervals for 21 days. The feed intake (FI) and feed conversion ratio (FCR) were determined when broilers were 21 days old.

Table 1. Ingredient composition and nutrient content of the basal starter diet used in the study.

Ingredients and analysis	Amount (g/kg)
Ingredient	
Corn	574.5
Soybean meal	346.6
Poultry oil	34.5
Dicalcium phosphate	18.6
Calcium carbonate	9.9
Salt	3.8
DL-Methionine	3.3
L-Lysine HCl	3.1
Threonine	1.2
Choline chloride 60%	2
Vitamin premix[A]	1
Mineral premix[B]	1
Antioxidant[C]	0.5
Calculated analysis	
Metabolizable energy (MJ/kg)	12.7
Crude protein (g/kg)	221.5

[A]Vitamin premix supplied per kilogram of diet: retinol, 6 mg; cholecalciferol, 150 μg; DL-α-tocopherol, 67.5 mg; menadione, 9 mg; thiamine, 3 mg; riboflavin, 12 mg; pantothenic acid, 18 mg; niacin, 60 mg; pyridoxine, 5 mg; folic acid, 2 mg; biotin, 0.3 mg; cyanocobalamin, 0.4 mg.

[B]Mineral premix supplied per kilogram of diet: Mn, 120 mg; Zn, 100 mg; Fe, 120 mg; Cu, 10–15 mg; I, 0.7 mg; Se, 0.2 mg; and Co, 0.2 mg.

[C]Ethoxyquin.

Relative organ weight. On day 21, all chickens were euthanatized by CO_2 inhalation. The liver, spleen, bursa of Fabricius, and intestine of 15 birds (five broilers from each replicate) from each group were removed, cleaned of adherent tissues, rinsed with 0.9% saline solution, and preserved at 4 C until weighed. The weight of these organs was measured and expressed as relative organ weight: Relative weight = (Organ weight)/(Final body weight) × 100. Bursa/spleen weight ratio was also calculated.

Evaluation of aflatoxin B1 on histologic lesions in liver tissue. Hepatocellular degeneration, as well as lymphoid and heterophilic infiltration, were evaluated in the liver of the experimental birds. The livers from 12 birds from each group (four chickens from each replicate) were removed on day 21. Livers were cleaned of adherent tissues, rinsed with 0.9% saline solution, and fixed in 10% neutral buffered formalin. Once fixed, a transversal section of the middle part of the left hepatic lobule was processed routinely, dehydrated in increasing alcohol concentrations, and embedded in paraffin. The 5-μm-thick tissue sections were cut from the paraffin-embedded tissues, stained with hematoxylin and eosin, and mounted with coverslips for the histopathology analysis.

Hepatocellular degeneration was scored as follows. Score: 0 = normal or absence of cellular swelling; 0.5 = minimal vacuolar degeneration and/or fat deposition; 1 = mild vacuolar degeneration and/or fat deposition; 2 = moderate vacuolar degeneration and/or fat deposition; 3 = marked vacuolar degeneration and/or fat deposition; and 4 = severe vacuolar degeneration and/or fat deposition. The score was obtained by evaluating five fields with a magnification of 20× per tissue cut. The lesion score was obtained when the lesion covered 10 mm^2 or more of the tissue cut. The median, mode, and variance of the 60 scores were calculated per treatment (five fields for 12 tissue cuts).

Quantification of inflammatory cells (lymphocytes and heterophils) was obtained using an adapted methodology previously described (28). In a general field from the upper left end of the tissue cut, with a 5× magnification, an area of 3.4 mm^2 was evaluated. The total number of perivascular areas and clusters of inflammatory cells for each tissue cut were counted. Those fields with less than four perivascular areas or inflammatory cell clusters were excluded. To quantify the number of layers in the perivascular area, the largest radius containing a significant number of perivascular layers next to the center of the polygon in the vein was used. In lymphoid clusters, inflammatory cells were quantified by counting the number of cell layers considering a radius resulting from the largest diameter of the cluster. The number of layers was multiplied by the number of clusters, and the average was obtained. The lesion score was assigned by counting the number of cell layers, from the center of the cell cluster or the space of the perivascular area, toward the perimeter of the cluster where the greatest number of cell layers were present. The lesion score was used as follows. Score: 0 = 0 cell layers per cell cluster (normal); 0.5 = 1–11 cell layers per cell cluster (minimal inflammatory infiltrate); 1 = 12–24 cell layers per cell cluster (mild inflammatory infiltrate); 2 = 25–50 cell layers per cell cluster (moderate inflammatory infiltrate); 3 = 51–100 cell layers per cell cluster (marked inflammatory infiltrate); 4 = more than 100 cell layers per cell cluster (severe inflammatory infiltrate). The median, mode, and variance of the total of the 60 scores were calculated (five fields for 12 tissue cuts).

Serum biochemical analysis. After CO_2 asphyxiation at the end of the study, 12 birds from each treatment were randomly selected (four broilers from each replicate) and bled from the femoral vein before necropsy. Blood was centrifuged at 1118 × *g* at 4 C for 15 min, and serum was separated and preserved at −20 C until submitted for biochemical analysis. Serum concentrations of albumin, total protein, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), blood urea nitrogen (BUN), cholesterol, triglycerides, glucose, creatinine, uric acid, phosphorus, magnesium, and iron were determined using a Corning clinical chemistry analyzer (Chiron Corporation, San Jose, CA). Furthermore, superoxide dismutase (SOD) activity, mucin, citrulline, and C reactive protein (CRP) levels were determined in serum samples using commercial assay kits following the manufacturer's instructions. For SOD an assay kit (Cayman chemical company, Ann Arbor, MI, Catalog No. 706002) was used to determine three types of SOD (Cu/Zn, Mn, and FeSOD), with an optimal dilution of the samples of 1:5. ELISA kits employing double antibody sandwich technique were used for quantitative determination of mucin, citrulline, and CRP serum concentrations (MyBioSource, San Diego, CA, Catalog No. MBS2505849, MBS2601045, and MBS261842, respectively) using undiluted serum samples for mucin and citrulline and an optimal dilution of 1:20 for CRP. All samples were measured at 450 nm using an ELISA plate reader (Synergy HT, multi-mode microplate reader, BioTek Instruments, Inc., Winooski, VT).

Intestinal immunoglobulin A (IgA) levels. Total IgA quantitation from gut rinse samples was determined as previously described (29). At day 21, an intestinal segment from 12 birds (four broilers from each replicate) per group was removed. A section of 5 cm from Meckel diverticulum through the ileocecal junction was taken, rinsed, and extruded three times with 5 mL 0.9% saline solution, then the rinse was collected in a tube and centrifuged at 1610 × *g* at 4 C for 10 min. The supernatant was retrieved and stored at −20 C until tested. An indirect ELISA was performed to quantify total IgA. The commercial chicken IgA ELISA quantitation set (Bethyl Laboratories Inc., Montgomery, TX, Catalog No. E30-103) was used according to the manufacturer's instructions; 96-well plates (Nunc MaxiSorp, Thermo Fisher Scientific, Rochester, NY, Catalog No. 439454) were used. Samples were measured at 450 nm using an ELISA plate reader (Synergy HT, multi-mode microplate reader, BioTek Instruments, Inc., Winooski, VT). Chicken IgA concentration obtained was multiplied by the dilution factor in determining the amount of chicken IgA in the undiluted samples. Optimum dilution for total IgA quantitation in gut rinse samples was 1:100.

Evaluation of humoral immunity: antibody production against Newcastle disease virus. The first day of age, before the chicks were

Table 2. Evaluation of body weight (BW), body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR) in broiler chickens consuming a corn-soybean based diet contaminated with Aflatoxin B_1 (2 ppm) supplemented with or without DFM.[AB]

Parameter	NC	AFB1	DFM	SEM[C]	*P*
BW, g/broiler					
Day 0	46.23 ± 0.68a	47.92 ± 0.72a	48.12 ± 0.74a	0.4174	0.1275
Day 7	133.29 ± 4.64a	129.92 ± 2.78a	137.02 ± 4.19a	2.2763	0.4502
Day 14	320.92 ± 17.53a	272.06 ± 8.54b	318.42 ± 14.65a	8.4215	0.0263
Day 21	640.10 ± 31.51a	474.81 ± 15.57b	571.60 ± 25.47a	16.2361	0.0001
BWG, g/broiler					
Days 0–7	87.06 ± 4.24a	82.00 ± 2.71a	88.90 ± 4.15a	2.1705	0.4103
Days 7–14	187.63 ± 13.82a	142.13 ± 7.06b	181.40 ± 11.38a	6.7337	0.0097
Days 14–21	319.17 ± 16.08a	202.75 ± 9.77c	253.17 ± 14.89b	9.5832	<0.0001
Days 0–21	593.87 ± 31.21a	426.88 ± 15.66c	523.48 ± 25.42b	16.2105	0.0001
FI, g/broiler					
Days 0–21	750.55 ± 17.23a	775.93 ± 3.51a	731.97 ± 82.35a	25.1292	0.8193
FCR					
Days 0–21	1.27 ± 0.06b	1.82 ± 0.06a	1.40 ± 0.06b	0.0875	0.0016

[A]Data are expressed as mean ± SE from 30 chickens (three replicates with 10 chicks each pen).
[B]Different lowercase letters a–c indicate significant differences within rows at $P < 0.05$.
[C]SEM = Standard error of the mean.

grouped, blood samples were taken, and the maternal antibody titers against Newcastle disease virus were determined using a commercial ELISA kit (AffiniTech, LTD, Bentonville, AR, Catalog No. NDV-0200). Then, all chicks were vaccinated with Newcastle disease (ND)–infectious bronchitis (IB) vaccine (B1 ND strain plus Mass & Ark IB serotypes, Live Virus CEO, Merial, Athens, GA) by ocular administration. On day 14, broilers were vaccinated ocularly again with the same vaccine. On day 21, 12 birds (four broilers from each replicate) per treatment were randomly selected, humanely slaughtered by CO_2 inhalation, and bled from the femoral vein before necropsy. Blood was centrifuged at 1118 × *g* at 4 C for 15 min, and serum was separated and preserved at −20 C until used. The antibody titers against ND were determined using the same commercial ELISA kit used at the beginning of the study to determine maternal antibodies.

Evaluation of cellular immunity: skin response to phytohemagglutinin (PHA). The phytohemagglutinin-induced cutaneous basophil hypersensitivity (CBH) response in the interdigital skin of chickens was used to evaluate the cellular immune activity. On day 18, 12 birds from each treatment (four broilers per replicate) were randomly selected and injected intradermally in the interdigital skin between the third and fourth digits of the right foot with 0.1 mL of PHA-M (Gibco, Grand Island, NY, Catalog No. 10576015). The CBH response was evaluated by determining the thickness of the interdigital skin at the injection site with a digital caliper before injection and 12 and 24 hr after the injection. The CBH response was calculated by CBH response (mm) = (thickness 12 and 24 hr postinjection, right foot) − (thickness preinjection, right foot).

Statistical analysis. Data from performance, relative organs weight, serum biochemical analysis, and intestinal IgA levels, as well as humoral and cellular immunity evaluations, were subjected to ANOVA as a complete randomized design using the general linear models procedure of SAS. Data are expressed as mean ± standard error, and significant differences among means were determined by using the Duncan multiple range test at $P < 0.05$ (30). Data from lesion scores of liver histopathologic analysis are expressed as median (mode; variance), and differences among median values of the groups were analyzed with the Mann-Whitney *U*-test with a level of significance set at $P < 0.05$.

RESULTS

Table 2 shows apparent differences in BW from day 14 of the study, when the BW of the AFB1 group began to be lower compared with the negative control (NC) and DFM groups. At day 21, the BW of the NC and DFM groups was higher than the AFB1 group. Differences in BWG began to be observed from the second week (days 7–14), with higher values for NC and DFM groups. The last week of the experiment (days 14–21), the BWG value of DFM group remained higher than the AFB1 group, but lower than the NC group. At the end of the experiment, the NC and DFM groups obtained higher BWG values compared with the AFB1 group. None of the groups showed changes in feed consumption throughout the experiment. AFB1 group had the highest value of FCR in comparison with the NC and DFM groups, while there was no difference between the NC and DFM groups.

As shown in Table 3, there was no difference in the weight of livers from birds given the DFM compared with the NC group, but it was lower compared with the AFB1 group. No significant differences were observed in the weight of the intestines from the three groups. The relative weight of spleen increased only in birds from the AFB1 group, while those treated with DFM remained similar to the NC group. Regarding the relative weight of bursa of Fabricius, the NC group showed higher values compared with the AFB1 group, while the DFM group showed no difference with any of the other groups. The AFB1 group had the lowest bursa/spleen weight ratio value, meanwhile the DFM group was similar to the NC group.

The histologic analysis of the liver revealed a higher level of hepatic injury in the AFB1 group, compared with the NC and DFM groups, as shown in Table 4. In Fig. 1B, an extensive vacuolar degeneration with a score 3 of hepatocellular degeneration is observed in the AFB1 group, compared with a score of 0.5 from the NC group (Fig. 1A). The histologic findings in the liver samples from the AFB1 group also revealed a change in disposition and proliferation of cells in bile ducts near the liver portal space or among the hepatocytes, with the presence of focal necrosis and inflammatory cell infiltrate (Fig. 2). Both hepatocellular degeneration and lymphoid infiltration were diminished when DFM was added to the diet, since birds from this group showed lower lesion scores than those in the AFB1 group.

The SOD activity in NC and DFM groups was similar, and higher than the AFB1 group. Regarding the intestinal integrity

Table 3. Relative weight of liver, spleen, intestine, and bursa of Fabricius in broiler chickens consuming aflatoxin B_1 (2 ppm) contaminated feed during 21 days, supplemented with or without DFM.[AB]

	Relative weight (g)				
	NC	AFB1	DFM	SEM	*P*
Liver	3.301 ± 0.103b	4.427 ± 0.166a	3.516 ± 0.141b	0.1078	<0.0001
Intestine	9.804 ± 0.507a	10.211 ± 0.409a	9.781 ± 0.525a	0.2741	0.7796
Spleen	0.121 ± 0.008b	0.168 ± 0.012a	0.118 ± 0.006b	0.0062	0.0006
Bursa of Fabricius	0.286 ± 0.021a	0.226 ± 0.011b	0.260 ± 0.016ab	0.0100	0.0454
Bursa/spleen ratio	2.380 ± 0.113a	1.435 ± 0.125b	2.259 ± 0.140a	0.0953	<0.0001

[A]Data are expressed as mean ± SE from 15 chickens (three replicates with five chicks each pen).
[B]Different lowercase letters a–c indicate significant differences within rows at $P < 0.05$.

markers mucin and citrulline, no statistical differences were observed among groups. For the CRP levels, the NC group had lower serum concentration compared with the AFB1 group, while the DFM group was in between the other groups.

Antibody titer against Newcastle disease and intestinal IgA levels were measured as immunologic parameters (Table 5). In both measurements, the NC group showed higher levels than the AFB1 group. The DFM group showed higher antibody titer levels than those of group AFB1 and similar to the NC group, but regarding the intestinal IgA levels, this group only showed numerically higher levels than the AFB1 group, suggesting that DFM inclusion into the diet of broilers helps to preserve both systemic and local humoral immune response. Interestingly, the same effect was observed for the cellular immune response, evaluated with the CBH response, which was improved with the use of the DFM, as shown in Table 6. Results revealed a decrease in CBH response in birds from AFB1 group at 12 and 24 hr after the PHA-M injection when compared with the NC group. The group treated with DFM remained without difference when compared with the NC group at 12 and 48 hr after the injection and showed a higher CBH response than the AFB1 group.

Finally, the effect of the DFM against 2 ppm AFB1 on serum biochemical variables is shown in Table 7. AFB1 inclusion in feed induced significant changes in most serum biochemical parameters of the control group, except for ALP, AST, BUN, and uric acid. A decrease in serum levels of albumin, total proteins, cholesterol, triglycerides, glucose, creatinine, and minerals like inorganic phosphorus, magnesium, and iron was observed in the AFB1 group compared with the NC group. The DFM treatment helped to diminish the adverse effects of AFB1 maintaining higher serum concentrations of albumin, total proteins, cholesterol, triglycerides, and glucose in comparison with the AFB1 group. Although for some parameters, such as albumin, cholesterol, and glucose, the DFM group did not achieve similar serum levels to the NC group, it showed higher levels than the AFB1 group. Regarding the minerals, the treatment with DFM aided to maintain magnesium levels higher than the AFB1 group, while inorganic phosphorus and iron were only numerically improved. On the other hand, DFM treatment did not help to maintain creatinine serum values similar to the NC group, remaining similar to AFB1 group. An increase in the serum levels of ALT and GGT was also shown in the AFB1 group in comparison with NC and DFM groups.

DISCUSSION

There are many reports of the detrimental effects of AFB1 on performance parameters, in fact, this is the most economically significant effect of aflatoxicosis in poultry (31,32). The decrease in performance parameters results from alterations in protein and lipid utilization mechanisms, as well as the inhibitory effect of AFB1 on protein synthesis and lipogenesis, which deteriorates the digestive and metabolic efficiency of the birds and, thus, affects their growth and general health (32,33). The addition of the *Bacillus*-DFM to the diet containing AFB1 improved performance, which might occur primarily via nutrient and enzymatic digestion, since *Bacillus* strains can produce certain essential nutrients and extracellular enzymes, as well as by providing necessary growth factors to promote host growth (32,34). Furthermore, *Bacillus* strains have demonstrated the capacity to sporulate and to resist different biochemical conditions of the gastrointestinal tract of poultry, secreting active substances that could degrade AFB1 and thus reduce its absorption (35,36). Previous studies in our laboratory showed that this *Bacillus*-DFM can produce a variable set of enzymes (24), which could be able to act on the lactone or difuran ring in the AFB1 molecule, reducing its toxicity (37,38).

The enlargement of the liver and its weight increase are attributed to an increase in lipid content, which produces a friable and fatty liver (39,40). These results are supported by the histopathologic observations, in which vacuolar degeneration and severe fat deposition in the liver of chickens from AFB1 group were observed (Fig. 1), as results of the impaired lipid transport rather than increased lipid biosynthesis (41). In the AFB1 group, severe inflammatory cell infiltrates, mainly composed of lymphocytes and

Table 4. Hepatocellular degeneration and inflammatory cells infiltration in livers from broiler chickens consuming AFB1 (2 ppm) contaminated feed during 21 days supplemented with or without DFM.[AB]

	NC	AFB1	DFM
Hepatocellular degeneration	2.50 (2.00; 1.37)b	3.50 (3.00; 1.07)a	1.00 (1.00; 0.27)c
Lymphoid infiltration	1.00 (1.00; 0.40)b	3.50 (4.00; 1.37)a	1.00 (1.00; 0.40)b
Heterophilic infiltration	0.50 (1.00; 0.30)b	1.50 (1.00; 3.87)a	1.00 (1.00; 1.37)ab

[A]Data are expressed as median (mode; variance) from 12 chickens (three replicates with four chicks each pen).
[B]Different lowercase letters a–c indicate significant differences within rows at $P < 0.05$, according Mann-Whitney *U*-test.

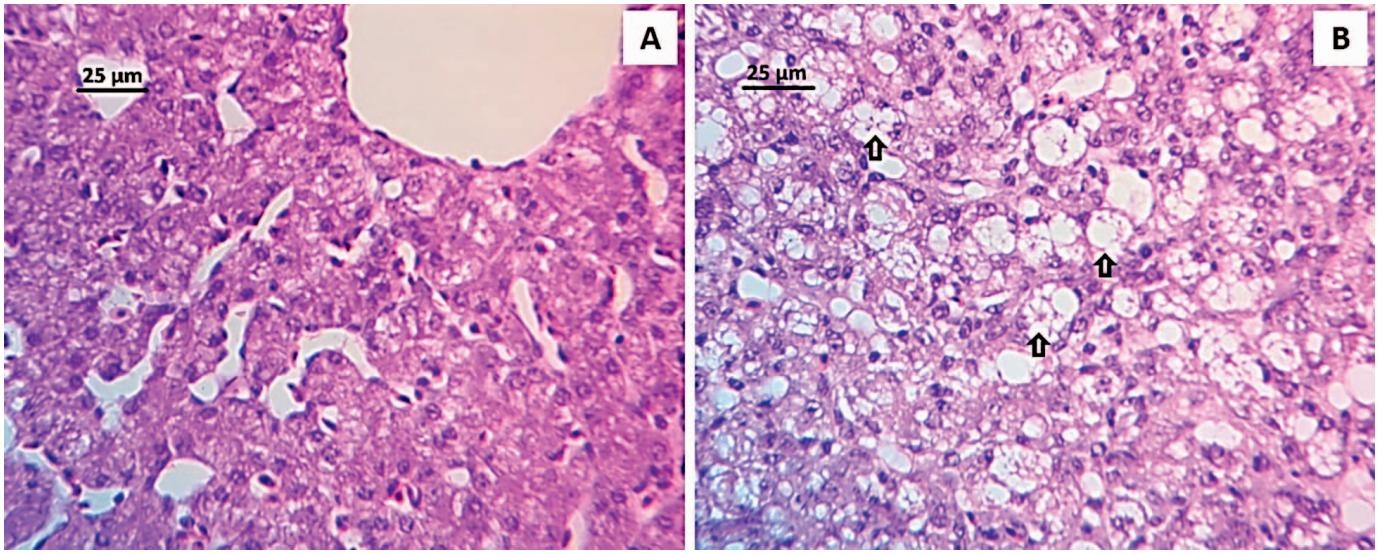

Fig. 1. Liver histopathology showing the different values of the lesion score used to evaluate hepatocellular degeneration. (A) Hepatocellular degeneration score of 0.5: liver section from a bird of NC group showing a scarce number of intracytoplasmic vacuoles. (B) Hepatocellular degeneration score of 3.0: liver section from a bird of AFB1 group, after 21 days receiving a diet with 2 ppm of AFB1, showing an increase of the number of intracytoplasmic vacuoles (arrows). Stain: Hematoxylin and eosin.

heterophils, as a mechanism to respond to degenerate vacuolated hepatocytes was also observed (42). The maintenance of liver weight and the lower severity of the histopathologic lesions in the DFM group was probably due to a modification in the synthesis, transport, and accumulation of lipids in the liver, since it has been shown that dietary supplementation with *Bacillus* strains may influence the pathway of lipid metabolism through promotion and/or suppression of serum lipid metabolites (43,44). The bursa of Fabricius in the AFB1 group showed a decreased relative weight, which might be caused by necrosis or cell depletion of this lymphoid organ, since a lower number of mitotic cells during aflatoxicosis has been shown, as well as lymphofollicular depletion and cell cycle arrest in the bursal cells of broilers (45,46). On the other hand, the increased relative weight of the spleen might be a compensatory mechanism for the

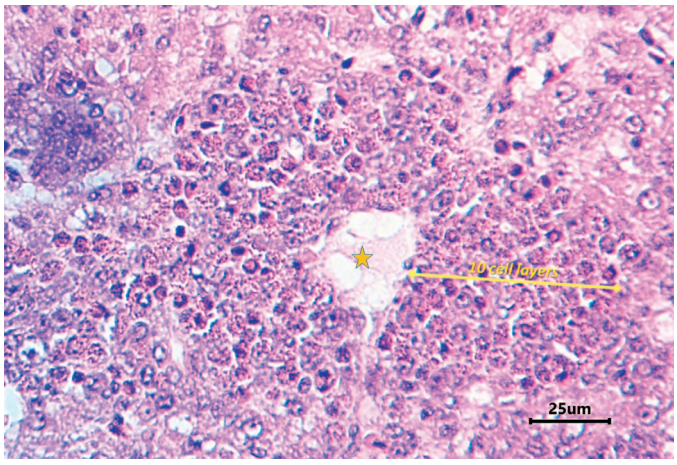

Fig. 2. Inflammatory cell layers (heterophiles) around hepatic perivascular spaces in a liver section from a bird of AFB1 group, after 21 days receiving a diet with 2 ppm of AFB1. The yellow indicates the central vein and the yellow line the counted inflammatory cell layers contained in the radius with the greatest number of cell layers, considering the center of the perivascular area as the origin of the radius.

decreased weight and activity of the bursa of Fabricius (46,47), added to the congestion of the red pulp of this organ, which results in a possibly impaired humoral and cellular immune function (48). The bursa/spleen weight ratio confirmed this effect, since lower values in the AFB1 group may reflect the greater weight of the spleen due to the increased migration of the lymphocyte subpopulations and their proliferation, as well as the diminished weight of the bursa caused by its atrophy (49,50). Dietary supplementation with this *Bacillus*-DFM showed a higher bursa/spleen ratio because the bursa preserved most of its integrity and the spleen did not receive migrating lymphocyte subpopulations; thus, this ratio can be used as a field indicator of the immune status, better than the relative weight of the bursa alone. It has been demonstrated that an essential mechanism of probiotic action is the stimulation of the immune system (51), and it could also be hypothesized that these *Bacillus* strains may successfully bind or degrade AFB1 in the gastrointestinal tract of the broilers (52), preventing its absorption and, therefore, improving the relative weight of these lymphoid tissues, which can also explain the positive effects observed in the ability to produce both systemic (against ND) and local antibodies (IgA).

Concerning the relative weights of the intestine, there was no difference among any of the experimental groups, suggesting that it is a dynamic organ that can adapt to a chronic AFB1 exposure as has been previously demonstrated (53). This can also explain the results of serum mucin and citrulline, two reliable biomarkers of the intestinal barrier health and function (54,55), which were not significantly modified in any of the experimental groups.

Since SOD is a fundamental enzyme part of the antioxidant defense systems, its activity was determined to investigate the effect of the DFM against the intracellular reactive oxygen species generated by AFB1. Data from this study show that SOD activity was decreased in chickens treated with AFB1, which confirms that there is a downregulation of SOD gene expression, as previously described (56). SOD activity was higher in chickens fed the diet supplemented with the DFM treatment, suggesting that this *Bacillus*-DFM counteracts the oxidative damage caused by AFB1

Table 5. Effect of DFM on serum levels of superoxide dismutase (SOD), mucin, citrulline and C-reactive protein (CRP), and the antibody titers against Newcastle disease (ND) and intestinal IgA levels in broiler chickens consuming a corn-soybean based diet contaminated with Aflatoxin B1 (2 ppm) during 21 days.[AB]

	SOD (U/mL)	Mucin (pg/mL)	Citrulline (nmol/mL)	CRP (ng/mL)	ND titer	Intestinal IgA (μg/mL)
NC	13.14 ± 0.18a	53.78 ± 10.18a	18.40 ± 2.64a	23.89 ± 1.17b	1327.40 ± 99.70a	42.47 ± 7.46a
AFB1	10.25 ± 0.73b	50.81 ± 8.24a	13.90 ± 1.97a	33.42 ± 4.43a	846.50 ± 29.03b	25.47 ± 2.38b
DFM	12.24 ± 0.36a	54.18 ± 6.04a	13.62 ± 3.24a	24.97 ± 2.54ab	1144.70 ± 93.06a	33.73 ± 3.06ab
SEM	0.3434	4.4983	1.5394	1.8910	58.0181	2.9942
P	0.0006	0.9507	0.3754	0.0731	0.0010	0.0629

[A]Each value represents the mean ± SE from 12 chickens (three replicates with four chicks in each pen).
[B]Different lowercase letters a–c indicate significant differences within columns at $P < 0.05$.

either by its absorption ability or antioxidant capacity. Dietary probiotics, *Bacillus* strains specifically, are beneficial in improving the adverse influence of oxidative stress and promoting the activities of antioxidant enzymes, thus helping in the oxidation resistance, scavenging hydroxyl radical, and increasing antioxidant capacity (57,58).

Antibody response to ND vaccine and total intestinal IgA concentration were lower in birds from AFB1 group as a result of immunodepression (59), which could be due to the capacity of AFB1 to inhibit RNA polymerase. This inhibition results in a decrease in protein synthesis in general, but particularly immunoglobulins; furthermore, there is increased lysosomal digestion of immunoglobulins, with severe depletion and degeneration of lymphocytes in the bursal follicles and impairment of cytokine formation by lymphocytes (60,61).

The DFM maintained the ability of the bird to produce antibodies against ND at the same level as those chickens not receiving AFB1 and showed a trend to produce a higher intestinal IgA level when compared with the AFB1 group. It has been reported that *Bacillus*-based DFM modulate humoral and cellular immune responses in broilers by increasing the cells of lymphoid organs and, thus, activating the immune response of the spleen and thymus (34). Consistent with these results, several studies have reported that DFM enhanced broiler humoral response, possibly by increasing the frequency of circulating immunoglobulin-secreting cells (62,63), which may be related to activation and maturation of epithelial immune cells, as well as an increased activity and number of T and B cells, leading to active proliferation, local induction of specific cytokines, and increased synthesis of immunoglobulins (64).

Similarly, feeding birds with 2 ppm AFB1 leads to a significant decrease in cellular immunity, as shown in the results of the CBH test. Our results show that chickens from AFB1 group had the lowest response to CBH at 12 and 24 hr after the PHA injection, which is known to be a result of the inhibition of the chemotactic ability of leukocytes and the phagocytic ability of heterophils in chicks (65). Interestingly, birds from the DFM group showed a similar CBH response to the NC group at 12 and 24 hr after PHA injection, which indicates that this DFM could improve cellular immune function. This could be related to the possible effect of probiotic bacteria to activate dendritic cells in Peyer patches, stimulating the mucosa circulating pool of T-lymphocytes (66), or the well-reported capacity of *B. subtilis* strains to significantly upregulate proinflammatory cytokines required for the initiation and regulation of cellular immunity through the differentiation of naive T cells into Th-1 cells (67,68).

Table 6. Effect of DFM on cutaneous basophil hypersensitivity response (CBH) induced by PHA-M in broiler chickens at 21 days of age consuming a corn-soybean based diet contaminated with Aflatoxin B1 (2 ppm) during 21 days.[AB]

Treatment	12 hr (mm)	24 hr (mm)
NC	0.692 ± 0.052a	0.788 ± 0.072a
AFB1	0.486 ± 0.041b	0.577 ± 0.057b
DFM	0.639 ± 0.053a	0.779 ± 0.059a
SEM	0.0312	0.0389
P	0.0153	0.0376

[A]Each value represents the mean ± SE from 12 chickens (three replicates with four chicks each pen).
[B]Different lowercase letters a–c indicate significant difference within columns at $P < 0.05$.

In the case of blood biochemical parameters, our results showed an apparent decrease in total proteins and albumin serum levels in birds from AFB1 group, when compared with NC group. Since the consumption of AFB1 causes liver damage, protein synthesis is compromised, resulting in decreased levels of serum proteins, including albumin. The decrease in the synthesis of proteins could be due to the formation of adducts, since it has been reported that AFB1 can form DNA or RNA adducts that disturb transcription and translation in gene expression, it can also form lysine adducts resulting in proteins degradation or excretion, or by selective inhibition of RNA polymerase II, impairing messenger RNA synthesis (69,70).

Another indicator of liver damage caused by AFB1 is the alteration in serum levels of hepatic enzymes (71). In the present study, birds from AFB1 group showed increased serum activity of ALT and GGT enzymes, and there was also an apparent rise in ALP activity in comparison with birds from NC group. These enzymes are located in the cytoplasm and mitochondria of hepatocytes, so when the structural integrity of the liver is damaged and the cell membrane permeability or hepatocyte necrosis increases, there is a leakage of these enzymes from the cytosol to the bloodstream (72). Furthermore, these changes are also observed in cases of obstruction or damage in the biliary system, either within the liver or in the larger bile channels outside the liver (73). On the contrary, there were no significant differences in AST serum levels when birds were fed with AFB1, which is similar to observations previously made (74). This could be expected to occur, since AST is also present in the cytoplasm and mitochondria of tissues such as skeletal and cardiac muscles (75). The intake of probiotics has been recommended for their hepatoprotective effect and their ability to reduce the disturbance in liver enzymes activities (76). In liver health, the main benefits of probiotics might occur through preventing the production and uptake of lipopolysaccharides in the gut and therefore reducing levels of low-grade inflammation (77).

Table 7. Effect of DFM on serum biochemical parameters in broiler chickens consuming a corn-soybean based diet contaminated with Aflatoxin B1 (2 ppm) during 21 days.[AB]

	NC	AFB1	DFM	SEM[C]	*P*
Albumin (g/dL)	1.12 ± 0.03a	0.61 ± 0.07c	0.78 ± 0.05b	0.0488	<0.0001
Total proteins (g/dL)	2.09 ± 0.06a	1.60 ± 0.18b	1.99 ± 0.11a	0.0805	0.0256
ALP (U/L)	278.10 ± 19.96a	344.50 ± 28.76a	267.80 ± 29.79a	16.0744	0.1043
ALT (U/L)	1.78 ± 0.22b	3.69 ± 0.49a	2.32 ± 0.46b	0.2703	0.0074
AST (U/L)	201.81 ± 7.72a	200.28 ± 14.48a	208.34 ± 9.61a	6.1510	0.8597
GGT (U/L)	12.70 ± 0.68b	15.30 ± 0.76a	12.00 ± 0.70b	0.4779	0.0075
BUN (mg/dL)	2.89 ± 0.07a	2.47 ± 0.15a	2.55 ± 0.19a	0.0881	0.1162
Cholesterol (mg/dL)	116.20 ± 4.43a	65.70 ± 7.76c	88.20 ± 8.59b	5.5329	0.0001
Triglycerides (mg/dL)	136.10 ± 11.61a	70.00 ± 6.64b	108.70 ± 14.42a	8.0853	0.0013
Glucose (mg/dL)	422.00 ± 18.09a	287.50 ± 12.60c	329.20 ± 7.33b	12.8366	<0.0001
Creatinine (mg/dL)	0.27 ± 0.01a	0.21 ± 0.01b	0.22 ± 0.01b	0.0088	0.0067
Uric acid (mg/dL)	11.54 ± 0.73a	13.61 ± 0.89a	12.85 ± 0.60a	0.4472	0.1622
P (mg/dL)	9.14 ± 0.23a	7.36 ± 0.41b	8.22 ± 0.36ab	0.2328	0.0040
Mg (mEq/L)	3.79 ± 0.14a	2.72 ± 0.10c	3.14 ± 0.10b	0.1043	<0.0001
Fe (μg/dL)	130.30 ± 5.21a	95.40 ± 8.94b	112.50 ± 5.01ab	4.5471	0.0038

[A]Data are expressed as mean ± SE from 12 chickens (three replicates with four chicks each pen).
[B]Different lowercase letters a–c indicate significant difference within rows at $P < 0.05$, according to Duncan multiple range tests.
[C]Standard error of the mean.

Previous reports have also shown that AFB1 consumption causes alterations in lipid metabolism (78,79), which might be a consequence of the impaired liver metabolism following hepatocellular damage, leading to reduced biosynthesis of cholesterol and triglycerides, concomitant with an inhibition of mobilization and transport of these lipids to peripheral tissue and resulting in accumulation of these lipids in the liver (39,80). Furthermore, recent studies have shown that AFB1 can downregulate the liver peroxisome proliferator activated receptor α (PPARα), a nuclear receptor protein that is a primary regulator of lipid and glucose homeostasis. This downregulation can lead to an increase of the expression and activity of a lipolysis enzyme, lipoprotein lipase, and therefore promotes the clearance of triglyceride-rich lipoproteins as well as circulating triglyceride levels (81). Interestingly, cholesterol and triglyceride serum levels in the DFM group were higher than in the AFB1 group, but they could not reach similar levels to those of the NC group. This is because probiotic supplementation significantly reduces the cholesterol and triglycerides serum levels in broiler chickens by affecting lipid metabolism at the level of absorption, mobilization, and recirculation (82). Several mechanisms have been reported through which probiotic microorganisms cause this hypolipemic effect. Some of these microorganisms could use the cholesterol present in the gastrointestinal tract for their metabolism, thus reducing the amount absorbed. Other microorganisms reduce the cholesterol in the blood by deconjugating bile salts in the intestine, thereby preventing them from acting as precursors in cholesterol synthesis. Moreover, it has also been proved that probiotic microorganisms inhibit or decrease the activity of enzymes such as acetyl-CoA carboxylase and hydroxymethylglutaryl-coenzyme A, enzymes related with the esterification of fatty acids to triglycerides and cholesterol synthesis pathways, reducing the storage of these lipids in serum and liver (44,83). This effect could also explain the lower liver relative weight observed in the DFM group when compared with the AFB1 group, since the lower intestinal absorption of lipids or their higher catabolism avoids the lipid redistribution from serum to the liver.

Regarding the glucose and creatinine serum levels, both were decreased when birds were fed with AFB1, which is supported by previous reports (84,85). Lower serum levels of glucose might be a consequence of the reduced activity of enzymes involved in carbohydrate catabolism and liver dystrophy associated with glycogenolysis and gluconeogenesis, but also of the downregulation of PPARα (4,81). Even though the glucose serum level of birds from the DFM group could not reach a similar level to the NC group, there was a significant increase compared with the AFB1 group. The pro-absorptive effects of DFM may cause this effect through stimulation of trans- and para-cellular diffusional absorption of glucose, due to the increased crypt cell production rates, the higher number of small pores in the villi, increased activity of the brush-border membrane enzymes, increased mucosal flux of glucose, and a marked stimulation of sodium-dependent D-glucose uptake into brush-border membrane vesicles with a corresponding increase of the sodium D-glucose cotransporter-1 throughout all the different intestinal section regions (86,87).

On the other hand, lower serum creatinine level in the AFB1 group may indicate renal toxicity and severe hepatic disease (81,84). The decreased creatinine serum level found in the AFB1 group is the result of a lower muscle mass gain associated with liver problems caused by this mycotoxin, since creatinine is a metabolite resulting from the degradation of muscle phosphocreatine, and its level decreases when there is reduced utilization of phosphocreatine for lower muscle contractions (88). Creatinine serum level of DFM group was not comparable to the NC group, meaning that this *Bacillus*-DFM could not decrease or prevent the renal injury caused by AFB1.

Finally, we analyzed some serum mineral levels such as P, Mg, and Fe, whose values from birds consuming 2 ppm AFB1 were lower than NC, which is similar to previous reports indicating that during aflatoxicosis a severe alteration in the mineral metabolism or absorption is produced, mainly due to hepatic and renal lesions (89,90,91). Decreased P and Mg levels may be the result of an imbalance between intestinal absorption and renal excretion with additional regulation by the adrenal, thyroid, and parathyroid glands (90,92); meanwhile, Fe alterations are probably related to inflammation of the liver and perturbed protein metabolism (93). Although dietary inclusion of this DFM could not wholly ameliorate

the decreased serum mineral levels in the birds that consumed AFB1, there was a marked increase in their serum levels. This might be attributed to the favorable environment in the intestinal tract caused by the *Bacillus* spp. consumption, since it has been shown that probiotic strains could decrease surface pH in the duodenum, jejunum, ileum, and cecum, creating a better environment for the absorption of minerals (93,94).

CONCLUSIONS

Results from this study confirm that 2 ppm of AFB1 caused severe toxic effects on performance, biochemical parameters, immunologic parameters, and hepatic injury in broiler chickens. Currently there are different and effective methods to counteract the adverse effects caused by contamination with AFB1; however, DFM supplementation in the diet provides extra beneficial effects, such as the regulation in intestinal flora equilibration and enterotoxigenic bacteria, barrier function, anti-inflammatory, and antioxidant activities, as well as their humoral and cellular immunomodulation. The results of the present study suggest that this *Bacillus*-DFM added at a concentration of 10^6 spores/gram of feed can be used to counteract the adverse effects occurring when broiler chickens consume diets contaminated with AFB1 levels well above those commonly found in the feed, showing beneficial effects on performance parameters, relative organ weight, hepatic lesions, immune response, and serum biochemical variables. The addition of this *Bacillus*-DFM might mitigate and decrease aflatoxicosis problems in the poultry industry, improving food security, alleviating public health problems, and providing economic benefits. However, the specific mechanism by which this *Bacillus*-DFM counteracts the toxic effects of AFB1 in broiler chickens is already unclear, and we are currently working in some studies to explore this question.

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