

A novel aflatoxin-binding *Bacillus* probiotic: Performance, serum biochemistry, and immunological parameters in Japanese quail

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ABSTRACT Two experiments were performed to screen bacilli isolated from quails for their aflatoxin removal potential and to assess the efficiency of their amelioration of experimental aflatoxicosis. Nonhemolytic bacilli were selected for in vitro aflatoxin B₁ (AFB₁) removal and conventional probiotic tests. The isolate with the highest scores was selected for assessment in field experiments and was identified as *Berevibacillus laterosporus* (*Bl*). In the second experiment, 125 male Japanese quails (21 d old) were divided into 5 groups with 5 replications to compare the toxin removal efficiency of *Bl* with that of a commercial toxin binder, improved Millbond-TX (IMTX). The experimental groups were as follows: Control (without any feed additive or AFB₁); AFB₁ (2.5 mg/kg); AFB₁ + *Bl* (2.5 mg/kg + 10⁸ cfu/mL); AFB₁+IMTX (2.5 mg/kg + 2.5 g/kg); and *Bl* (10⁸ cfu/mL). The greatest BW gain and slaughter and carcass weights were found in the *Bl* group and the lowest values were observed in the AFB₁ group ($P < 0.05$). Feeding AFB₁ alone to the chicks resulted in a

significant decrease in serum albumin, total protein, and glucose and cholesterol levels but a significant increase in serum uric acid, urea, creatinin and phosphorus ($P < 0.05$). Treatment of birds on AFB₁ with *Bl* restored these to their original levels ($P < 0.05$). AFB₁ + *Bl*-fed birds had serum aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, and alkaline phosphatase enzyme activity similar to control birds ($P < 0.05$). Antibody titer against Newcastle disease virus was found to be lowest in the AFB₁ group but highest in the *Bl* group ($P < 0.05$). Antibody production against sheep red blood cells was lower in the AFB₁ group compared with the AFB₁ + *Bl* group ($P < 0.05$). *Berevibacillus laterosporus* supplementation of the AFB₁ diet restored the skin response to 2,4-dinitro 1-chlorobenzene to levels comparable with control birds ($P < 0.05$). It can be concluded that selected indigenous *Bl* is a promising probiotic with AFB₁ removal potential.

Key words: Japanese quail, aflatoxicosis, *Berevibacillus laterosporus*, performance, immune system

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INTRODUCTION

Aflatoxins are a group of mycotoxins that are largely produced by the fungi *Aspergillus flavus* and *A. parasiticus* (Diaz et al., 2002). Aflatoxin B₁ (AFB₁) is the most toxic and most prevalent compound, followed by AFG₁, AFB₂, and AFG₂ with decreasing toxicity (Busby and Wogan, 1984). Aflatoxins can easily contaminate various types of crops and cause heavy economic losses; they have a wide range of biological effects on different species (Karaman et al., 2005). Significant changes in serum biochemistry parameters are generally regarded as indicative of aflatoxicosis (Basmacioglu et al., 2005). Decreased serum concentrations of total protein, albumin, cholesterol (Rosa et al., 2001), uric acid (Oğuz et

al., 2002), inorganic phosphorus, and calcium (Harvey et al., 1990) are often reported due to aflatoxin poisoning in avian and porcine species. Biochemical changes are sensitive indicators of aflatoxicosis and precede performance impairment and the emergence of clinical symptoms. Immune suppression by aflatoxin has been well documented in poultry as a consequence of vaccination failure in affected commercial flocks (Thaxton et al., 1974). Contact sensitivity to 2,4-dinitro 1-chlorobenzene (DNCB) has been shown to induce a delayed hypersensitivity reaction in chickens and used as a convenient model to investigate cellular immune response and its regulation in the chicken (Awadhiya et al., 1982; Huynh and Chubb, 1987).

Several methods have been developed to reduce aflatoxin contamination in animal feed. However, most of these are not of practical value (Diaz et al., 2002). Recently, the interest of researchers has been focused on biological methods to take advantage of their simplicity

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and affordability. The main step of these methods is the choice of microorganisms for diminishing the presence of aflatoxins in contaminated feed and food supplies (Teniola et al., 2005).

Probiotics are defined by Fuller as “live microbial food supplements which beneficially affect the host by improving its intestinal microbial balance” (Fuller, 1991). Furthermore, lactic acid bacteria are important aflatoxin reducer microorganisms, as has been emphasized recently (el-Nezami et al., 1998; Peltonen et al., 2000, 2001; Lee et al., 2003; Shahin, 2007; Hernandez-Mendoza et al., 2009). It appears that AFB₁ is bound to the surface components of probiotic bacteria (Haskard et al., 2001). The destruction of specific components of the bacterial cell wall, such as carbohydrates and proteins, reduced AFB₁ binding by *L. rhamnosus* strain GG, suggesting the importance of the cellular envelope in AFB₁ binding (Haskard et al., 2000; Hernandez-Mendoza et al., 2009). It is likely, however, that multiple mechanisms are involved in AFB₁ binding.

Members of the *Bacillus* genus are considered the most promising as probiotics for use in animal feeds because of their extraordinary extended shelf life and resistance to environmental conditions compared with more conventional probiotics based on lactic acid bacteria (Shivaramaiah et al., 2011). Wolfenden et al. (2010) screened potential *Bacillus* probiotics based on heat/ethanol treatment, hemolytic activity, and antimicrobial activity against some pathogens.

Although mycotoxin removal is well established in lactobacilli species, there has been no published report on mycotoxin removal by poultry probiotics based on bacilli. Hence, the present study was performed to assess the AFB₁ removal potential of a *Brevibacillus laterosporus* (*Bl*) probiotic in vitro and in vivo.

MATERIALS AND METHODS

Experiment 1

Isolation and Growth Conditions. The animal use protocol was approved by the institutional animal care of Tarbiat Modares University. Forty healthy Japanese quails aged 21 d that were not receiving any medical treatment and had not received antibiotics were selected. The quails had free access to water for 24 h before slaughter. Tissue samples were taken from different parts of the gastrointestinal tract (crop, jejunum, ileum, and cecca) under aseptic conditions. Homogenized samples were transferred to buffered peptone water (1:10 vol/vol). Samples were placed in a water bath at 80°C for 15 min and then plated on nutrient agar medium (Merck; Darmstadt, Germany). Plates were incubated aerobically at 37°C for 24 to 48 h (Barbosa et al., 2005; Guo et al., 2006). Any isolate with colony morphology consistent with that of the *Bacillus cereus* group (*B. cereus*, *B. mycoides*, *B. thuringensis*, and *B. anthracis*) was excluded from further investigations (Wolfenden et al., 2010).

Pathogenicity Test. Aerobic spore-forming isolates were cultured by streaking on blood agar media (Difco, Franklin Lakes, NJ) containing 5% defibrinated sheep blood and incubated for 24 h at 37°C to evaluate their possible hemolytic activity (Wolfenden et al., 2010). Only γ -hemolytic (nonhemolytic) bacteria were retained.

Aflatoxin B₁ Binding Assay. Aflatoxin B₁ (Sigma-Aldrich, Steinheim, Germany) was suspended in benzene/acetonitrile (97:3 vol/vol) to obtain a concentration of 2 mg/mL. A working solution of 5 μ g/mL of AFB₁ was prepared in PBS and the benzene/acetonitrile was evaporated by gently heating in a water bath. The cell count of an overnight culture of *Bacillus* isolates obtained in a shaking incubator (150 rpm, 37°C) was adjusted to 7th McFarland tube, equal to approximately 1 to 1.5 $\times 10^{10}$ cfu/mL. The culture was then centrifuged (3,000 $\times g$, 15 min) and the bacterial pellets were washed twice with 5 mL of Milli-Q water. Bacterial pellets were resuspended in 1.5 mL of AFB₁ working solution and incubated at 37°C for 4 h. Cells were pelleted (3,000 $\times g$, 15 min) and aliquots (1 mL) of the supernatant were collected for AFB₁ quantification (Peltonen et al., 2000). The AFB₁ concentration was estimated by ELISA (Ridascreen Aflatoxin B₁ Art. No. 1211, R-Biopharm, Darmstadt, Germany).

Selection and Identification. To choose a candidate isolate for field experiments, conventional probiotic tests were performed on the isolates that already showed superior AFB₁ removal ability. Tests included antagonistic activity, cell surface hydrophobicity, coaggregation, aggregation, enzymatic activities, low pH tolerance, bile salt tolerance, and antibiotic sensitivity (Kirby et al., 1957; Kim et al., 2007; Surachon et al., 2011). Based on the results obtained in the AFB₁-binding and conventional probiotic tests, an isolate was selected for further assessment in in vivo experiments. Identification of the selected isolate was performed using standard taxonomic descriptions from Sneath (1986) with commercially available strips (API 50CHB, API Laboratory Products Ltd., Biomerieux, France). The results were analyzed using the API Web database (<https://apiweb.biomerieux.com>) for species-level identification.

Experiment 2

Experimental Birds and Treatments. In total, 125 male Japanese quails (*Coturnix japonica*), aged 21 d, were randomly assigned to one of 5 treatment groups with 5 replicates of 5 birds each. All birds were fed a similar base diet formulated to meet or exceed the nutritional requirements of Japanese quails (NRC, 1994; Table 1). The experimental groups included the control (without any feed additive or AFB₁), AFB₁ (2.5 mg/kg), AFB₁ + *Bl* (2.5 mg/kg + 10⁸ cfu/mL), AFB₁ + IMTX (2.5 mg/kg + 2.5 g/kg) and *Bl* (10⁸ cfu/mL) groups. Supplementation was continued for 4 wk. The IMTX (Milwhite Inc., Brownsville, TX) is an inert

Table 1. Composition of the basal diets

Item	Grower (21–49 d)
Ingredient (%)	
Yellow corn	42.32
Soybean meal (44% CP)	40.20
Vegetable oil	7.48
Fish meal (65% CP)	7.30
CaCO ₃	1.21
Dicalcium phosphate	0.01
Sodium chloride	0.28
Mineral and vitamin premix ¹	0.50
DL-Methionine	0.03
Washed sand	0.67
Total	100
Calculated value ²	
ME (kcal/kg)	3,130
CP (%)	25.90
Lys (%)	1.40
Met + Cys (%)	0.81
Calcium (%)	0.86
Nonphytate phosphorus (%)	0.32

¹Supplied the following per kilogram of diet: retinyl acetate, 9,000 IU; cholecalciferol, 2,000 IU; DL- α -tocopheryl acetate, 12.5 IU; menadione sodium bisulfite, 1.76 mg; biotin, 0.12 mg; thiamine, 1.2 mg; riboflavin, 3.2 mg; calcium D-pantothenate, 6.4 mg; pyridoxine, 1.97 mg; nicotinic acid, 28 mg; cyanocobalamine, 0.01 mg; choline chloride, 320 mg; folic acid, 0.38 mg; MnSO₄·H₂O, 60 mg; FeSO₄·7H₂O, 80 mg; ZnO, 51.74 mg; CuSO₄·5H₂O, 8 mg; iodized NaCl, 0.8 mg; Na₂SeO₃, 0.2 mg.

²Calculated from NRC (1994).

montmorillonite clay-based adsorbent that is obtained from natural clay deposits (Miles and Henry, 2007).

Aflatoxin Production. Aflatoxin was produced from an *A. parasiticus* PTCC-5286 culture (obtained from the Iranian Research Organization for Science and Technology) by fermentation of rice grains and its AFB₁ content was determined by the method of Shottwell et al. (1966). The contaminated rice powder was incorporated into the base diet to provide 2.5 mg of AFB₁/kg of feed.

Performance Parameters. The birds were weighed at weekly intervals for up to 4 wk on a pen basis and the feed intake per pen was recorded at weekly intervals. The feed conversion ratio was calculated from feed intake and BW gain data. Slaughter and carcass weights were measured at the end of the experiment on d 49.

Serum Biochemical Analysis

Samples of approximately 1 mL of whole blood were drawn from 10 birds in each treatment through puncture of a wing vein on d 49. Concentrations of albumin, total protein, glucose, cholesterol, uric acid, urea, creatinine and phosphorus were determined in serum samples. The serum activities of aspartate aminotransferase (**AST**), alanine aminotransferase (**ALT**), lactate dehydrogenase (**LDH**), and alkaline phosphatase (**ALP**) were also determined. The analyses of the serum samples were performed immediately by spectrophotometric methods using commercially available kits (Parsazmun, Tehran, Iran).

Humoral Immune Response: Antibody Production Against Newcastle Disease Virus and Sheep Red Blood Cells

Vaccination against the Newcastle disease (**ND**) virus was performed on d 41 using an eye dropper (Live B1 strain; Vetrina; Zagreb, Croatia). The anti-ND titer was assessed by a hemagglutination inhibition test on sera obtained on d 48.

Birds were also injected into the breast muscle with SRBC (5% vol/vol in sterile PBS, 0.2 mL/chick) at 41 d. Blood samples were drawn 7 d after the injection. The antibody levels against SRBC were determined by hemagglutination test. Heat inactivated plasma (56°C for 30 min) was analyzed for anti-SRBC titer as previously described (Qureshi and Havenstein, 1994).

Cellular Immune Response: Skin Response to 2,4-Dinitro 1-Chlorobenzene

2,4-Dinitro 1-chlorobenzene (Merck; Darmstadt, Germany) was dissolved in a mixture of acetone and olive oil (4:1 vol/vol) to a final concentration of 1 mg/mL. On d 48, the skin of the birds was anointed with 0.1 mL of DNCB solution. An area of approximately 10 cm² without feathers on the left lateral abdomen was chosen for the challenge with DNCB. The same area on the right side was treated with the solvent alone. The skin thickness (on both sides) before and 24 h after the

Table 2. Effect of aflatoxin B₁ and feed additives on performance of Japanese quail

Group	Aflatoxin B ₁ (mg/kg)	Additive ¹	BW gain (g/bird/d)	Feed conversion ratio (g/g)	Slaughter weight (g)	Carcass weight (g)
Control	0	—	3.21 ^a	4.10 ^{ab}	259.97 ^a	166.57 ^a
<i>Bl</i> ²	0	<i>Bl</i>	3.33 ^a	3.89 ^b	266.30 ^a	171.63 ^a
AFB ₁	2.5	—	2.10 ^b	5.77 ^a	204.22 ^c	132.63 ^c
AFB ₁ + <i>Bl</i>	2.5	<i>Bl</i>	2.98 ^a	4.33 ^{ab}	256.97 ^a	169.81 ^a
AFB ₁ +IMTX ³	2.5	IMTX	2.90 ^a	4.65 ^{ab}	231.87 ^b	149.64 ^b
SEM			0.133	0.265	5.66	3.78
<i>P</i> -value			0.022	0.048	<0.001	<0.001

^{a-c}Different superscripts in each column show significant differences ($P < 0.05$).

¹Additive: *Bl* (10⁸ cfu/mL of drinking water); IMTX (2.5 g/kg of feed).

²*Bl*: *Brevibacillus laterosporus*.

³IMTX: Commercial toxin binder (Milwhite Inc., Brownsville, TX).

Table 3. Effect of aflatoxin B₁ and feed additives on some serum biochemical variables

Group	Aflatoxin B ₁ (mg/kg)	Additive ¹	Total protein (g/dL)	Albumin (g/dL)	Triglyceride (mg/dL)	Glucose (mg/dL)	Cholesterol (mg/dL)	Creatinine (mg/dL)	Uric acid (mg/dL)	Blood urea nitrogen (mg/dL)	Phosphorus (mg/dL)
Control	0	—	3.50 ^a	2.35 ^b	115.2 ^b	209.06 ^a	146.77 ^a	0.56 ^a	5.90 ^b	3.30 ^c	6.74 ^{ab}
<i>Bl</i> ²	0	<i>Bl</i>	3.70 ^a	3.36 ^a	206.1 ^a	182.61 ^b	93.22 ^c	0.54 ^b	4.80 ^b	2.90 ^d	5.92 ^b
AFB ₁	2.5	—	1.96 ^b	1.37 ^d	82.44 ^c	163.04 ^c	69.50 ^d	0.56 ^a	19.80 ^a	4.50 ^a	7.55 ^a
AFB ₁ + <i>Bl</i>	2.5	<i>Bl</i>	4.10 ^a	3.42 ^a	67.93 ^c	182.24 ^b	106.66 ^{bc}	0.54 ^b	7.90 ^b	3.80 ^b	6.09 ^b
AFB ₁ +IMTX ³	2.5	IMTX	2.60 ^b	2.25 ^c	90.07 ^b	186.59 ^b	115.05 ^b	0.54 ^b	18.05 ^a	3.90 ^b	7.11 ^{ab}
SEM			0.22	0.20	13.81	4.43	7.03	0.008	1.75	1.00	0.21
<i>P</i> -value			0.001	<0.001	<0.001	0.002	<0.001	0.004	<0.001	<0.001	0.044

^{a-d}Different superscripts show significant differences ($P < 0.05$).

¹Additive: *Bl* (10^8 cfu/mL of drinking water); IMTX (25 g/kg of feed).

²*Bl*: *Brevibacillus laterosporus*.

³IMTX: Commercial toxin binder (Milwhite Inc., Brownsville, TX).

challenge was measured to assess reactions. Differences before and after the challenge were calculated to determine the mean increase in skin thickness in each bird. For each bird, the average of 3 repeat measurements was used for analysis (Verma et al., 2004).

Statistical Analysis

The data were analyzed by a general linear model for a completely randomized experimental design using SAS software (SAS Institute Inc., 1990); the means were compared by Duncan's multiple range test ($P \leq 0.05$).

RESULTS

Experiment 1: Isolation, Hemolysis Test, AFB₁ Binding, and Identification

Three hundred fifty nonhemolytic spore-forming isolates were prescreened from the 40 quail samples. All isolates shared the typical characteristics of *Bacillus* species; that is, they were gram-positive, rod-shaped, catalase-positive, and spore-forming aerobic bacteria.

After conducting the AFB₁ removal test as well as some conventional probiotic tests consisting of acid and bile tolerance, antagonistic activity, and hydrophobicity, one isolate was selected for field experiments with growing quails. The selected isolate was able to remove 57.3% of the AFB₁ and was identified as *Brevibacillus laterosporus*.

Experiment 2

Performance. The results showed that AFB₁ has a significant effect on quail performance (Table 2). The greatest BW gain was found in the *Bl* group and the lowest in the AFB₁ group. Body weight gain in AFB₁ + *Bl* birds was not significantly different from the control group ($P > 0.05$). The feed conversion ratio was significantly affected by both AFB₁ and *Bl* ($P < 0.05$), and AFB₁ group had higher value than the other groups. The highest slaughter and carcass weights were found in the *Bl* group and vice versa for the AFB₁ group ($P < 0.05$).

Serum Biochemistry and Enzyme Activity. The effects of AFB₁, *Bl*, and IMTX on serum biochemical variables are shown in Tables 3 and 4. The serum total protein and albumin significantly reduced and serum uric acid increased in AFB₁ group compared with the AFB₁ + *Bl*, *Bl*, and control groups ($P < 0.05$). Adding IMTX to AFB₁-contaminated feed significantly increased serum uric acid ($P < 0.05$; Table 3). The highest value of serum phosphorus and the lowest amount of glucose was observed in the AFB₁ group ($P < 0.05$).

Serum AST activity in the AFB₁ group was significantly higher than that in the other groups ($P < 0.05$). Adding IMTX to the contaminated diet increased the activity of AST, LDH, and ALP enzymes compared

Table 4. Effect of aflatoxin B₁ and feed additives on selected serum enzyme activity

Group	Aflatoxin B ₁ (mg/kg)	Additive ¹ (mg/kg)	Aspartate amino-transferase (IU/L)	Alanine amino-transferase (IU/L)	Lactate dehydrogenase (IU/L)	Alkaline phosphatase (IU/L)
Control	0	—	141.59 ^b	5.95 ^b	590.00 ^c	375.34 ^c
<i>B^P</i>	0	<i>B^l</i>	130.01 ^c	6.12 ^b	576.67 ^c	361.60 ^c
AFB ₁	2.5	—	160.60 ^a	10.25 ^a	753.33 ^b	425.12 ^b
AFB ₁ + <i>B^l</i>	2.5	<i>B^l</i>	122.07 ^d	6.28 ^b	626.66 ^c	383.35 ^c
AFB ₁ +IMTX ³	2.5	IMTX	132.33 ^c	11.57 ^a	1,146.66 ^a	458.65 ^a
SEM			3.58	0.669	57.44	10.04
<i>P</i> -value			<0.001	<0.001	<0.001	<0.001

^{a-c}Different superscripts show significant differences ($P < 0.05$).

¹Additive: *B^l* (10⁸ cfu/mL of drinking water); IMTX (25 g/kg of feed).

²*B^l*: *Berevibacillus laterosporus*.

³IMTX: Commercial toxin binder (Milwhite Inc., Brownsville, TX).

with the other groups ($P < 0.05$). Supplementation of *B^l* in birds fed AFB₁ restored the elevated activity of these enzymes (Table 4).

Humoral Immune Response

Antibody production against ND on d 48 was lower in the AFB₁ group compared with the *B^l* group (Table 5). Antibody titer against SRBC on d 48 was significantly different among groups ($P < 0.05$), and the AFB₁ group had a lower titer than the AFB₁ + *B^l* group ($P < 0.05$).

Cellular Immune Response

Increases in skin thickness in response to DNCB were significantly different among groups ($P < 0.05$; Table 5). The lowest skin response was observed in the AFB₁ group and there was no significant difference between the AFB₁ + *B^l* and control groups ($P < 0.05$).

DISCUSSION

Prescreening of *Bacillus* isolates by amplification in broth before agar isolation has been reported previously (Földes et al., 2000; Wolfenden et al., 2010; Shivaramaiah et al., 2011). However, selection of bacilli from the digestive tract of poultry to determine their AFB₁ removal ability is reported here for the first time. Probiotics with high mycotoxin-binding ability have good prospects as mycotoxin-binding organisms. Practical use of binder strains is anticipated in the near future (Shetty and Jespersen, 2006). The mycotoxin binding property of probiotic bacteria is attributed to cell wall moieties (el-Nezami et al., 1998; Haskard et al., 2000; Hernandez-Mendoza et al., 2009).

Dietary inclusion of AFB₁ reduced the growth of quails and supplementation of *B^l* to birds prevented the loss in BW gain and feed conversion. *Bacillus* isolates have previously been shown to increase BW gain (Shivaramaiah et al., 2011; Wolfenden et al., 2011).

Low growth rate and poor performance are the most prevalent symptoms of aflatoxicosis in poultry and live-

stock. Failure to gain BW will lead to economic losses. The relationship between commercial performance of broilers and aflatoxin contamination of diets has been reviewed by Shane (1994). Aflatoxin effects on feed intake, BW gain, and the feed conversion ratio may be a result of anorexia, reluctance and prevention of protein synthesis, and lipogenesis (Oğuz et al., 2000; Parlat et al., 2001). The aflatoxin effects on growth performance in this study are in accordance with those in previous studies (Allameh et al., 2005; Shi et al., 2006; Pasha et al., 2007).

Serum total protein, albumin, creatinine, urea, and glucose concentrations have also been described as valuable parameters of hepatic injury and function (Mathur et al., 2001). In our study, AFB₁-contaminated feed resulted in an increase in serum creatinine, uric acid, urea, and phosphorus concentrations and a decrease in concentrations of serum albumin, total protein, glucose, and triglyceride. Madheswaran et al. (2004) also reported a decrease in serum total protein, albumin, globulin, glucose, and cholesterol when Japanese quails were fed 3 ppm of AFB₁ alone or in combination with T2 toxin for 35 d. It is worth mentioning that they did not observe any change in serum urea, uric acid, creatinine, or phosphorus. Nazar et al. (2012) observed a significant reduction in protein, albumin, and globulin concentrations in the plasma of Japanese quails fed an AFB₁-contaminated diet. The biochemical changes during aflatoxicosis could be due to the inhibition of protein synthesis in the liver along with other damage to the liver and kidney (Tung et al., 1975; Kubena et al., 1993; Shi et al., 2006). Aflatoxins produce a large number of active metabolites during a biological conversion and these bind to DNA and RNA and reduce protein production (Doerr et al., 1983). Impaired protein and albumin biosynthesis was observed in chickens fed an aflatoxin-contaminated diet (Oğuz et al., 2002; Basmacioglu et al., 2005). Supplementation of contaminated feed with *B^l* could ameliorate the aflatoxin-induced increase in uric acid and creatinine concentrations and return these to levels similar to controls. The similarity of serum metabolites in the AFB₁ + *B^l* and control groups could be the result of toxin binding by *B^l* as in

Table 5. Effect of aflatoxin B₁ and feed additives on immune response to Newcastle disease virus, sheep red blood cells, and 2,4-dinitro 1-chlorobenzene

Group	Aflatoxin B ₁ (mg/kg)	Additive ¹	Anti-NDV ² titer (log ₂)	Anti-SRBC titer (log ₂)	Increase in mean skin thickness to DNCB ³ (mm)
Control	0	—	1.75 ^{bc}	3.2 ^b	0.492 ^b
<i>Bl</i> ⁴	0	<i>Bl</i>	3.20 ^a	4.1 ^a	0.950 ^a
AFB ₁	2.5	—	1.15 ^c	2.4 ^c	0.076 ^d
AFB ₁ + <i>Bl</i>	2.5	<i>Bl</i>	1.70 ^{bc}	3.2 ^b	0.520 ^b
AFB ₁ +IMTX ⁵	2.5	IMTX	2.30 ^b	2.8 ^{bc}	0.182 ^c
SEM			0.062	0.165	0.134
<i>P</i> -value			<0.001	<0.001	<0.001

^{a-d}Different superscripts show significant differences ($P < 0.05$).

¹Additive: *Bl* (10⁸ cfu/mL of drinking water); IMTX (25 g/kg of feed).

²NDV: Newcastle disease virus.

³DNCB: dinitrochlorobenzene.

⁴*Bl*: *Brevibacillus laterosporus*.

⁵IMTX: Commercial toxin binder (Milwhite Inc., Brownsville, TX).

experiment 1 this activity was evidenced (57.3% AFB₁ removal).

Activity of serum enzymes such as ALP, ALT, AST, and LDH provides a sensitive and specific measure of hepatic function or injury (Kubena et al., 1997; Mathur et al., 2001; Abbès et al., 2006). Feeding AFB₁ alone increased AST, ALT, LDH, and ALP activity compared with the control diet. Supplementation of *Bl* in contaminated feed restored the activity of these enzymes. Surprisingly, IMTX supplementation of AFB₁-contaminated feed resulted in an increase in serum LDH and ALP activity ($P < 0.05$); this increase in ALT was numeric. In the AFB₁ + IMTX group, AST activity was decreased in comparison to the AFB₁ group ($P < 0.05$). Aravind et al. (2003) reported that broilers fed a naturally AFB₁-contaminated diet had significant increases in the activity of these enzymes at 21 d of age. Similar results were observed by others (Shi et al., 2006; Gowda et al., 2008), which suggests that mycotoxins exert a direct toxic effect on animal livers. The supplementation of *Bl* to birds fed AFB₁-contaminated feed proved to be an effective means of protecting against aflatoxicosis as judged by serum metabolites and enzyme activity.

The immunomodulatory effects of probiotics have been reported frequently (Kabir et al., 2004; Haghghi et al., 2005, 2006; Nayeypor and Hashemi, 2007; Apata, 2008; Brisbin et al., 2008). Interestingly, the immune response of the AFB₁ + *Bl* group was similar to that of the control group. Our results confirm previous reports that stated that consumption of aflatoxin-contaminated feed in broilers reduced the production of antibodies (Qureshi et al., 1998; Verma et al., 2004; Tessari et al., 2006). Weakening of the immune system may be the result of the prevention of protein synthesis, which includes a reduction in IgG and IgA, a reduced number of lymphocytes, and an effect on the bursa (Sur and Celik, 2003).

Birds of AFB₁ group had the lowest skin thickness after challenge with DNCB, which confirmed the results of earlier studies (Singh et al., 1990; Bakshi, 1991). The AFB₁-induced decrease in skin response to DNCB was

restored to normal when birds on contaminated feed were supplemented with *Bl*. Several researchers used topical DNCB to evaluate the cell mediated immunosuppressive effect of mycotoxins in broilers (Singh et al., 1990; Bakshi, 1991; Verma et al., 2004; Nazar et al., 2012). However, cell mediated immune-stimulant effect of probiotic administration via drinking water on skin response to DNCB was reported by Karimi Torshizi et al. (2010).

In conclusion, the *Bacillus* probiotic isolated in the present study proved its AFB₁-binding activity in vitro. In vivo results verified its AFB₁-binding activity in quails with regard to performance, serum biochemistry, and immune responses. The common list of probiotic selection criteria could be amended by inserting a mycotoxin-binding property, which could result in the introduction of powerful multifunctional probiotic preparations in the near future.

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