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# Effects of three *Bacillus* species on hatchability, growth performance and serum biochemistry in Japanese quails fed diet contaminated with Aflatoxin B1

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**ABSTRACT.** In total, 240 one-day-old Japanese quails (*Coturnix Coturnix Japonica*) allocated at random to 6 treatments with 4 replicates and 10 birds in each. Treatments used were: 1) Negative control (without any additives or AFB1); 2) Positive control (basal diet + 2.5 ppm AFB1; 2); 3) TA008 (positive control + 10<sup>8</sup> cfu/ml *Bacillus. megaterium* TA008); 4) TA049 (positive control + 10<sup>8</sup> cfu mL<sup>-1</sup> *Bacillus. subtilis* TA049); 5) TA010 (positive control+ 10<sup>8</sup> cfu mL<sup>-1</sup> *Brevibacillus brevis* TA010) and 6) P (positive control + 2.5 g kg<sup>-1</sup> Polysorb® in feed). Hatchability and embryonic mortality were significantly influenced by additives and AFB1 ( $p < 0.05$ ). Birds fed TA008 improved 12 % hatchability and reduced 10 % embryonic mortality in compared to positive control ( $p < 0.05$ ). Weight gain and feed conversion ratio did not affected by treatments ( $p > 0.05$ ). Feed intake was significantly improved in birds feeding by TA008 at 0-21 days ( $p < 0.05$ ). There were significant differences on relative weights of carcass, gizzard and proventriculus among treatments ( $p < 0.05$ ). Serum total protein, albumin, cholesterol, glucose, HDL, globulin and uric acid were significantly affected by treatments ( $p < 0.05$ ). These results showed that the inclusion of *Bacillus megaterium* as potential probiotic into contaminated diets could improve the adverse effects of AFB1 in Japanese quails.

**Keywords:** *Bacillus. sp.*; Aflatoxin B1; japanese quail; hatchability; productive performance.

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## Introduction

Mycotoxins are low weight molecules which produced as secondary metabolites by fungi or molds. They can result acute problems for human and animal safety. It is suggested that temperature and humidity are mainly factors included in mycotoxin production (Bryden, 2012). Many feedstuffs, such as cereals, seeds, fruits and so on, are more susceptible to mycotoxin contamination (Wu, Liao, He, Feng, et al., 2015; Wu, Liao, He, Ren, et al., 2015). Economically, these components usually caused losses every year such as loss of human and animal life, reduced poultry productions, loss of cereals and feedstuffs and etc (Mohamed, 2011). Among mycotoxins, aflatoxins B1, B2, G1 and G2 are more important mycotoxins in feeds and foods. Aflatoxins lead to serious effects on reduced growth performance, impaired hepatic tissue and disordered metabolic disease in human and animal (Oğuz et al., 2003). Also, AFB- contaminated feeds were considered as the most toxic carcinogenic potency for human (Abbès et al., 2016). Aflatoxin B1 is the most toxic one known as a group 1 carcinogenic substance by the International Agency for Research on Cancer (IARC, 2002). The technical literature involves several reports aiming to reduce the AFB (Oueslati et al., 2012); however, most of these methods have not been very effective because of the loss of product nutrition, low organoleptic qualities, disadvantages of health effects, and expensive equipment (Lee, Chen & Liu, 2017). Therefore, there is a great demand for a new practical way to reduce or inactivate the harmful effects of AFB1 (Farzaneh et al., 2012). One of the practical methods is applying microorganisms, as demonstrated by previous researches (Fan et al., 2015). According to the literature, some species such as *Lactobacillus*, *Bacillus*, *Bifidobacterium*, *Aspergillus*, and *Saccharomyces* are widely used for the AFB1- degrading ability

(Yu et al., 2015). *Bacillus* spp. was accepted by human or animals as the direct fed microbial (DFM) (Petchkongkaew et al., 2008) and AFB1 adsorption (Petchkongkaew et al., 2008). It is reported that *Pseudomonas aeruginosa* N17-1 could degrade AFB1, AFB2 and AFM1 in nutrient broth medium respectively 82.8, 46.8 and 31.9 % at 37°C after 72 hour (Sangare et al., 2014). It is showed that some *Bacillus*. sp such as *Bacillus subtilis* (Farzaneh et al., 2012) and *Bacillus licheniformis* (Petchkongkaew et al., 2008) were reduced adverse effects of AFB1. *Bacillus subtilis* ANSB060, isolated from gut fish, was able to degrading AFB1, AFG1 and AFM1 respectively 81.5, 60 and 80 % (Gao et al., 2011). The protective effects of *Bacillus subtilis* ANSB060 were documented in contaminated diets with AFB1 in broilers and layers (Fan et al., 2015). Thus, in light of these arguments, this study was evaluated aflatoxin B1 detoxification of three probiotics isolates including *Bacillus megaterium* TA008, *Bacillus subtilis* TA049 and *Brevibacillus brevis* TA010 on hatchability, growth performance, inner and lymphoid organs and serum biochemistry. These bacteria isolated previously from honey samples (Razmgah, Mojgani, & Torshizi, 2016).

## Material and Methods

### Aflatoxin production

This research was applied *Aspergillus parasiticus* PTCC-5286 culture for aflatoxin production (provided from the Iranian Research Organization for Science and Technology) It was used for the fermentation of rice grains. The content of AFB1 was measured by the method of Shotwell et al. (1966). The contaminated rice powder was incorporated into the basal diet to providing 2.5 ppm AFB1.

### Husbandry, diets, and experimental design

A total of 240 one-day-old Japanese quails (*Coturnix Coturnix Japonica*) were randomly assigned into a 6 treatment groups with 4 replicates and 10 birds in each. The experimental groups were followed as: 1) Positive control (basal diet + 2.5 ppm AFB1; 2) Negative control (without any additives or AFB1); 3) TA008 (basal diet+ 2.5 ppm AFB1 +  $10^8$  cfu mL<sup>-1</sup> *B. megaterium* TA008; 4) TA049 (basal diet + 2.5 ppm AFB1 +  $10^8$  cfu mL<sup>-1</sup> *B. subtilis* TA049) ; 5) TA010 (basal diet + 2.5 ppm AFB1 +  $10^8$  cfu mL<sup>-1</sup> *B. laterosporus* TA010) and 6) P (basal diet + 2.5 ppm AFB1 + 2.5 g kg<sup>-1</sup> commercial toxin binder Polysorb® in feed). All three *Bacilli* were added to drinking water. A standard corn-soy bean meal diet was formulated to meet nutrient requirements of Japanese quails (*Coturnix Coturnix Japonica*) National Research Council (NRC, 1994) (Table 1). Table 2 showed the composition of Polysorb.

**Table 1.** Nutrient content of ingredients in the experimental diet.

Nutrients	%
Fat	7
Fiber	4
Methionine	0.46
Lysine	1.2
Methionine + Cysteine	1.09
Calcium	1.03
Available phosphorous	0.7
Metabolizable Energy (Kcal kg <sup>-1</sup> )	2900
Crude protein	20

**Table 2.** The characterization of Polysorb.

Index	Level
Protein %	Maximum 25
Beta-glucan %	Minimum 0.9
Carbohydrate %	< 45
Fat (ether extract) %	Maximum 0.12
Ash %	17-25
Humid	Maximum 0.8
pH	5-7
Density (g L <sup>-1</sup> )	500
Heavy metals (ppm)	<10

### Performance parameters

Body weight and feed consumption of each replicate were measured weekly. Feed conversion ratio was calculated for a replicate. At 42 day of age, two birds were randomly selected per replicate and slaughtered by cervical dislocation. Then, the relative inner organs and the lymphoid organs weights were determined.

### Blood parameters

At the 42 days of age, two birds were randomly selected per replicate (n=3), then 1 ml of blood was collected from wing vein. The samples were cooled on ice and immediately centrifuged at 3,500 × rpm for 15 min. Plasma was separated and stored at -20°C for analysis. The total protein, albumin, glucose, cholesterol, triglyceride, HDL, LDL, uric acid, and globulin content were determined using the clinical chemistry analyzer (commercial kit, Bionic Co, Tehran, Iran).

## Results

### Hatchability and embryonic mortality

Table 3 showed that experimental groups were significantly influenced by additives and AFB1 ( $p < 0.05$ ). In this research birds fed *Bacillus megaterium* + 2.5 ppm AFB1 improved 12 % hatchability and reduced 10 % embryonic mortality in compared to positive control group ( $p < 0.05$ ). In addition, hatchability was improved in TA008, TA049 and Polysorb groups respectively 9%, 10% and 1% rather than that positive control group.

**Table 3.** The effects of additives and AFB1 on hatchability and embryonic death of Japanese quail.

Experimental groups	Hatchability %	Embryonic death %
TA 008	58.00 <sup>b</sup>	8.00 <sup>d</sup>
TA 049	54.00 <sup>d</sup>	11.00 <sup>c</sup>
TA 010	55.00 <sup>c</sup>	11.00 <sup>c</sup>
Polysorb	47.00 <sup>e</sup>	14.00 <sup>b</sup>
Positive control	46.00 <sup>f</sup>	18.00 <sup>a</sup>
Negative control	60.00 <sup>a</sup>	8.00 <sup>d</sup>
SEM	0.00	0.00
P value	<0.0001	<0.0001

Means with common superscripts in same column are not significantly different ( $p < 0.05$ ). SEM: Standard error means.

### Growth performance

The effects of *Bacillus* probiotics and Polysorb on growth performance of Japanese quail fed diets contaminated with AFB1 are shown in table 4. There were no significant differences on WG and FCR among treatments at 0-21 and 21-42 days of age ( $p > 0.05$ ). Birds fed dietary without any additives or AFB1 had significantly increased FI compared to those fed TA008 and Polysorb groups at 0-21 days of ages ( $p < 0.05$ ). Although, performance parameters were not significantly affected by treatments, birds fed probiotics ameliorated performance parameters in all experimental period (0-42 days).

**Table 4.** Effects of additives and AFB1 on productive performance of Japanese quail.

Treatments	Body weight gain (g)		Feed intake (g)		Feed conversion ratio	
	0-21 day	21-42 day	0-21 day	21-42 day	0-21 day	21-42 day
TA008	100.00	112.5	138.75 <sup>c</sup>	365.00	4.76	11.19
TA049	90.60	92.44	149.33 <sup>abc</sup>	375.25	4.97	15.80
TA010	87.90	110.00	166.56 <sup>ab</sup>	422.50	5.64	12.86
Polysorb	85.00	96.25	146.02 <sup>ab</sup>	382.50	5.15	12.52
PC	90.00	70.75	158.83 <sup>abc</sup>	372.50	5.01	14.94
NC	90.00	91.25	171.50 <sup>a</sup>	442.50	5.39	12.23
SEM	4.3	11.3	7.00	36.3	0.2	1.3
P value	0.292	0.173	0.030	0.598	0.149	0.175

Means with common superscripts in same column are not significantly different ( $p < 0.05$ ). SEM: Standard error means.

### Relative inner and lymphoid organs weight

The effects of additives and AFB1 on relative inner and lymphoid organs weights are shown respectively in tables 5 and 6. The relative weights of proventriculus and gizzard, abdominal fat, duodenum, jejunum and

ileum were not affected by treatments ( $p > 0.05$ ). The relative weight of bursa of fabricius in birds fed 2.5 ppm AFB1 +  $10^8$  cfu mL<sup>-1</sup> *B. megaterium* was significantly increased in comparison with TA049 group ( $p < 0.05$ ). Birds fed 2.5 ppm AFB1 +  $10^8$  cfu mL<sup>-1</sup> *B. megaterium* had significantly decreased relative liver weight rather than positive control and TA010 groups ( $p < 0.05$ ). Inclusion of *B. megaterium* TA008 and *B. subtilis* TA049 and Polysorb to the contaminated diets did improved relative spleen weight ( $p < 0.05$ ).

**Table 5.** Effects of additives and AFB1 on inner organs weights of Japanese quail.

Treatments	Proventriculus and Gizzard (g 100 g <sup>-1</sup> BW)	Fat (g 100 g <sup>-1</sup> BW)	Duodenum (g 100g <sup>-1</sup> BW)	Jejunum (g 100g <sup>-1</sup> BW)	Ilium (g 100g <sup>-1</sup> BW)
TA 008	2.49	0.385	1.08	1.08	0.837
TA 049	2.43	0.684	1.27	1.38	0.888
TA 010	2.40	0.385	1.20	1.14	0.957
Polysorb	2.58	0.658	1.22	1.13	0.868
PC	2.85	0.743	1.22	1.17	0.811
NC	2.89	0.498	1.25	1.29	0.984
SEM	0.012	0.10	0.05	0.08	0.10
P value	0.015	0.066	0.371	0.224	0.860

Means with common superscripts in same column are not significantly different ( $p < 0.05$ ). SEM: Standard error means; PC: Positive control; NC: Negative control.

**Table 6.** Effects of additives and AFB1 on lymphoid organs weights of Japanese quail.

Treatments	Bursa fabricius (g 100 g <sup>-1</sup> body weight)	Liver (g 100 g <sup>-1</sup> body weight)	Spleen (g 100 g <sup>-1</sup> body weight)
TA 008	0.12 <sup>a</sup>	2.93 <sup>c</sup>	0.045 <sup>b</sup>
TA 049	0.064 <sup>b</sup>	3.24 <sup>bc</sup>	0.041 <sup>b</sup>
TA 010	0.092 <sup>ab</sup>	3.70 <sup>ab</sup>	0.061 <sup>ab</sup>
Polysorb	0.086 <sup>ab</sup>	3.46 <sup>abc</sup>	0.048 <sup>b</sup>
PC	0.065 <sup>b</sup>	4.06 <sup>a</sup>	0.082 <sup>a</sup>
NC	0.092 <sup>ab</sup>	3.41 <sup>abc</sup>	0.057 <sup>ab</sup>
SEM	0.010	0.200	0.007
P value	0.034	0.0123	0.0192

Means with common superscripts in same column are not significantly different ( $p < 0.05$ ). SEM: Standard error means; PC: Positive control; NC: Negative control.

### Serum biochemistry

Serum albumin increased significantly in treatment inclusion 2.5 ppm AFB1 compared to others ( $p < 0.05$ ). Positive and negative control groups had higher cholesterol serum rather than other groups ( $p < 0.05$ ). Birds fed 2.5 ppm AFB1 +  $10^8$  cfu mL<sup>-1</sup> *B. megaterium* TA008 had significantly increased serum glucose in compared to Polysorb and negative control groups ( $p < 0.05$ ). Adding of *B. megaterium* TA008 and *B. subtilis* TA049 to the contaminated diets were improved HDL level rather than others ( $p < 0.05$ ). Triglyceride and LDL serum were not affected by any additives or AFB1 ( $p > 0.05$ ). Total protein serum was significantly increased in TA008 and TA049 groups in compared to Polysorb and positive control groups ( $p < 0.05$ ). Uric acid serum was similar in TA008, TA010 and negative control groups ( $p > 0.05$ ) and it was significantly decreased in comparison to others ( $p < 0.05$ ). Japanese quails fed 2.5 ppm AFB1 + Polysorb had the lowest value of globulin serum ( $p < 0.05$ ) which did not significant difference with TA010 ( $p > 0.05$ ).

**Table 7.** Effects of additives and AFB1 on blood biochemical parameters of Japanese quail.

Treatments	Albumin (g dL <sup>-1</sup> )	Cholesterol (mg dL <sup>-1</sup> )	Glucose (mg dL <sup>-1</sup> )	HDL (mg dL <sup>-1</sup> )	Triglyceride (mg dL <sup>-1</sup> )	Total protein (g dL <sup>-1</sup> )	Acid uric (mg dL <sup>-1</sup> )	Globulin (g dL <sup>-1</sup> )	LDL (mg dL <sup>-1</sup> )
TA 008	2.05 <sup>a</sup>	186.75 <sup>b</sup>	214.14 <sup>a</sup>	45.25 <sup>a</sup>	186.89	4.62 <sup>a</sup>	5.38 <sup>c</sup>	2.37 <sup>ab</sup>	101.33
TA 049	2.08 <sup>a</sup>	196.42 <sup>ab</sup>	201.40 <sup>ab</sup>	43.46 <sup>ab</sup>	234.93	4.69 <sup>a</sup>	6.11 <sup>ab</sup>	2.60 <sup>a</sup>	105.96
TA 010	2.04 <sup>a</sup>	187.98 <sup>b</sup>	202.23 <sup>ab</sup>	40.01 <sup>c</sup>	230.21	4.30 <sup>ab</sup>	5.61 <sup>c</sup>	2.25 <sup>bc</sup>	109.09
Polysorb	2.16 <sup>a</sup>	186.75 <sup>b</sup>	192.17 <sup>bc</sup>	41.75 <sup>bc</sup>	227.29	4.15 <sup>b</sup>	6.40 <sup>a</sup>	1.98 <sup>c</sup>	99.53
PC	1.81 <sup>b</sup>	219.07 <sup>a</sup>	193.72 <sup>abc</sup>	40.63 <sup>bc</sup>	204.14	4.17 <sup>b</sup>	6.52 <sup>a</sup>	2.36 <sup>ab</sup>	126.53
NC	2.11 <sup>a</sup>	219.07 <sup>a</sup>	177.87 <sup>c</sup>	39.37 <sup>c</sup>	235.55	4.44 <sup>ab</sup>	5.83 <sup>bc</sup>	2.33 <sup>ab</sup>	104.36
SEM	0.07	7.60	6.74	1.10	15.80	1.00	0.14	1.00	6.66
P value	0.004	0.0045	0.015	0.002	0.191	0.020	<0.0001	0.012	0.083

Means with common superscripts in same column are not significantly different ( $p < 0.05$ ). SEM: Standard error means; PC: Positive control; NC: Negative control.

## Discussion

### Hatchability and embryonic mortality

It was reported that the adverse effects of AFB1 on embryo appeared in early life embryonic (Hassan et al., 2012). In many cases, researchers indicated that tetratogenic characteristics of mycotoxins could reduce hatchability by causing mandibular hypoplasia, softness and abnormality of the maxillary bone which impaired the final development of embryo (Hassan et al., 2012). One research demonstrated that breeder quails fed 4-6 ppb AFB have shown markedly decreased hatchability after 3 weeks (Howarth & Wyatt, 1976). Decreasing hatchability is due to quickly AFB1 transferred to eggs. Also, aflatoxin was found in albumin and yolk egg. Furthermore, contaminated diets with aflatoxin impaired protein, carbohydrate and lipid metabolism in breeders which directly influenced hatchability (Howarth & Wyatt., 1976). It was documented that glucomanans by increasing sperm density, improving fertility and hatchability reduced adverse effects of aflatoxin B1 in poultry (Madrigal-Santillan et al., 2007).

### Growth performance

There is a general agreement that dietary aflatoxins reduce WG, FI, and increase FCR that leads to losses economical greats (Monson et al, 2015) In this research, the supplementation of *Bacillus Megaterium* (TA008) results in a high efficiency in broilers by decreasing feed intake and feed conversion ratio. In farming, this improvement can be increased feed efficiency and profitability. Agboola et al. (2015) showed that the adding of probiotic in AFB1-contaminated diets was significantly improved body weight gain without changed in FCR in broiler chicken at 0-10 day. In contrary with our finding, Bagherzadeh Kasmani and Mehri (2015) have reported that inclusion of probiotic in contaminated diets with aflatoxin was recovered feed intake and body weight gain. Also these researchers demonstrated that FCR did not affect by experimental groups in the first three weeks of experiment which in accordance with current study. Furthermore, some studies showed the dose-effect relationship between AFB1 level and performance of broilers (Dersjant-Li, 2003). Recently, it was reported that broiler performance was improved at low doses of AFB1 while decreased at high doses of AFB1 (Diaz, Calabrese, & Blain, 2008). This phenomenon is known as hormesis. In general, the better responses of Japanese quails can be related to their maturation at the end of growth phase. Accordingly, bird's reactions are duo to immune system evolution and proper expression of genes at the late grower (Reemers et al., 2010). It is reported that animal performance did not affected by aflatoxin which inconsistent with our findings (Magnoli et al., 2011). In accordance with our finding, many researchers have reported that additives including organic acids, probiotics and growth promoting antibiotics had no effects on WG and FCR (Panda et al., 2000; Vele et al., 2004). Little effects of these additives can be attributed to environmental conditions. Birds are less affected by additives when they are feeding well and raring in good conditions. It suggested was that there were no effects of additives including organic acids and probiotics on bird's performance (Hossain, Begum, & Kim, 2015). One research has been reported that adding of 105- 108 cfu kg<sup>-1</sup> probiotic (inclusion bacillus) into diets was improved body weight gain which inconsistent with current study (Zhang, Cho, & Kim, 2013).

### Relative inner and lymphoid organs weight

In this study inclusion of bacillus megaterium (TA008) into diets was numerically decreased abdominal fat which can be related to its function on process of body fat formation. Kabir et al. (2004) have suggested that adding of 2 g probiotic per 1 liter water of broilers could increase the efficiency of breast and tight muscles in compared to control group. In accordance with current study, it was found that the present of oligofructose in dietary decreased abdominal fat.

In current study, birds received bacillus megaterium (TA008) had highest bursa of fabricius weight. Also, liver and spleen weights were affected by treatments. Liver is the main organ for accumulation of aflatoxins and its poisoning signs were included increased fat penetration and finally increased weight. Aflatoxins increased liver, kidney, spleen and pancreas weights while decreased bursa of fabricius weight. In this study, increased relative liver weight of Japanese quail feeding by aflatoxin can be related to fat penetration in liver that in accordance with Denli et al. (2009) researches. In conflict with this finding, Magnoli et al. (2011) have been demonstrated that there were no effects of low doses of aflatoxin on inner organs.

### Serum biochemistry

In this study, the highest and lowest of total serum protein was observed in birds feeding by *Bacillus megaterium* (TA008) and positive control respectively. Total serum protein and albumin play a major role in the biosynthesis of most plasma proteins. Therefore, plasma proteins can usually be used to diagnose liver disorders and diseases. Aflatoxins prevented protein synthesis (Robens & Richard, 1992) and thus, changes in total serum protein and albumin can be known as aflatoxin index (Shi et al., 2006). Results indicate that, adding of *Bacillus megaterium* into contaminated diet improve and restore the decreased of total serum protein and albumin in compared to positive control. Japanese quails feeding by *Brevibacillus laterosporus* have been shown improves in serum biochemistry in diets contaminated with AFB1 (Bagherzadeh Kasmani et al., 2012). A negative effect of aflatoxin on serum glucose concentration was ameliorated by aluminosilicates, cell wall yeast and probiotic bacteria such as *Bacilli* (Bagherzadeh Kasmani et al., 2012; Bovo et al., 2015). In accordance with these results, Santurio (1999), were observed an decreased in serum cholesterol and glucose and increased in serum triglyceride in birds feeding by aflatoxin. Denli et al. (2009) have reported that inclusion of aflatoxin to the diet was reduced triglyceride and cholesterol in compared to control. In this study, birds fed 2.5 mg kg<sup>-1</sup> AFB1 was significantly increased cholesterol in compared to others. Although triglyceride did not affected by treatments, birds fed *Bacillus megaterium* had it's lowest which in agreement with Denli et al. (2009) results. One research showed that broiler breeder fed 3 mg kg<sup>-1</sup> aflatoxin had significantly decreased total serum protein and albumin. In this study, LDL was no significantly increased in birds receiving by aflatoxin. It is suggested that a decreased of liver enzymes can be reason for an increased LDL that it is a protect mechanism for fat exporting from liver to tissues. Uric acid is a final product of nitrogen metabolism. One applying of probiotics is decreased of serum NH<sub>3</sub>. Some probiotics species decreased serum cholesterol by different ways. Bacterial probiotics could ferment non digestive carbohydrates and their convert to shorts fatty acids in small intestine. This process by inhibiting cholesterol synthesis in liver decreased serum lipids. Several researches have demonstrated the effects of probiotics on reducing blood sugar and delaying hyperglycemia has been reported in diabetic mice. Increased serum uric acid levels occurred in birds with renal failure due to decreased renal excretion of uric acid (Tung et al., 1973). Santurio (1999) reported that adding of aflatoxin into diets were significantly increased blood uric acid levels. It was found that feeding by aflatoxin was significantly decreased serum total protein, albumin, globulin, and phosphorous (Chen, Horn, & Applegate, 2014).

### Conclusion

In conclusion, our experiment showed that the presence of 2.5 mg kg<sup>-1</sup> of aflatoxin B1 in diets could decrease hatchability and growth performance, change relative inner organs weight and serum biochemical properties in Japanese quails. *Bacillus megaterium* (TA008) as a probiotic could be able to ameliorate the adverse effects of AFB1 on hatchability, performance, serum biochemistry properties and relative inner organs weight.

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