



Effect of Adding a Toxin Binder to the Aflatoxin-Infected Diet on Growth Performance, Intestinal Morphology, Immune Responses, and Liver Pathological Changes of Broilers

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ABSTRACT

This study evaluated the effects of adding a toxin binder (TB) to the aflatoxin-infected diet on broilers' performances. 875 day-old broilers were used based on a completely randomized design with 5 treatments: 1) Negative control (NC): Basal diet without aflatoxin; 2) Positive control (PC): Basal diet + 0.75 (mg/kg) of aflatoxin B1; 3, 4, and 5) PC + 1, 2, and 3 (g/kg) of TB. The TB did not affect the average daily weight gain (ADWG) and feed conversion ratio (FCR), but the addition of TB decreased average daily feed intake (ADFI) compared to NC ($p < 0.05$). In the starter phase, TB addition decreased the ADWG and ADFI ($p < 0.05$). During the grower phase, the ADFI decreased with increasing different levels of TB ($p < 0.05$). Birds that received TB had lower ADFI and FCR than those in the NC group in the finisher phase ($p < 0.05$). Adding TB to the diet increased the *Lactobacillus* count compared to the PC group and the *Salmonella* count in the ileum ($p < 0.05$). The results revealed that the villus height to crypt depth ratio and the villus width increased with TB addition compared to NC and PC ($p < 0.05$). The broilers' liver color was lighter and less yellow when 2 (g/kg) of TB were added to the diet compared to other treatments ($p < 0.05$). As a result, dietary TB improved performance, increased *Lactobacillus* count, and modified liver pathology with the addition of 2 (g/kg) of TB to the diet. However, no significant positive impact was observed on immune responses.

INTRODUCTION

Accessibility to good quality feed is the primary challenge for sustainable commercial poultry production at constant prices (Iyayi, 2008). Moreover, some studies have shown that feeding birds with poor-quality feedstuffs can adversely affect the gut system by exposing it to pathogens and other contaminants (Agboola *et al.*, 2015). The intestinal ecosystem, where feed digestion and host defense take place, is constantly exposed to pathogens (El-Miniawy *et al.*, 2014; Marin and Taranu, 2015). They can harm the normal microflora and intestinal epithelium, reducing villus height in the small intestine, and nutrient digestion and absorption (Pelicano *et al.*, 2005). Feed contamination with toxins, particularly mycotoxins, is one of the most significant issues related to broiler nutrition (Manafi & Khosravinia, 2012). Mycotoxins, produced by fungi, are secondary metabolites that can contaminate poultry diets and have adverse effects on broiler performance. The maximum permissible concentration of mycotoxins in the feed for poultry, including broilers, laying hens, and turkeys, is established at 0.02 ppm (20 ppb). Exceeding this concentration in the feed can result in detrimental effects and subsequent weight loss (Filazi *et al.*, 2017).

In order to overcome these adverse effects, the addition of toxin binders (TB) to animal feeds has been significantly increased in recent



times (Kolosova & Stroka, 2012; Agboola *et al.*, 2015). Various studies have shown that the amount of toxin absorption in the digestive tract is reduced when TB is added to the diet, leading to an improvement in nutrient utilization and broiler growth performance (Garcia *et al.*, 2003; Garcia *et al.*, 2010; Pappas *et al.*, 2014; Agboola *et al.*, 2015). Appropriate nutritional strategies can help reduce the adverse effects of mycotoxins on the growth performance and immunity of poultry. One of these nutritional approaches is the addition of TB to the diet to reduce the harmful effects of mycotoxins and convert them into non-toxic metabolites (Huwig *et al.*, 2001; Boudergue *et al.*, 2009; Pappas *et al.*, 2014). Aflatoxin B1 (AFB1) is an ordinary mycotoxin generated by *Aspergillus flavus* and *Aspergillus parasiticus*, causing primarily hepatotoxicity, and secondarily nephrotoxicity in poultry (Patil *et al.*, 2014). Aflatoxins are considered a potential human carcinogen. They can cause immunosuppression due to lesions in the thymus and bursa of Fabricius, making birds susceptible to other infections such as colibacillosis and chronic respiratory diseases (Anilkumar *et al.*, 2003). Aflatoxins are also responsible for a poor immune response to vaccines.

Nazarizadeh and Pourreza (2019) concluded that the addition of commercial TB to AFB1-containing diets reduced the adverse effects of AFB1, offering a potential solution to the aflatoxicosis problem in young broilers. Tavangar *et al.* (2021) also found that phytobiotic supplements and TB improved growth performance and intestinal morphology in broilers facing AFB1 challenges. Also, Eraslan *et al.* (2005) demonstrated that TB could mitigate aflatoxin-induced losses (1 mg/kg) in 45-day-old broilers. The TB is derived from yeast cell walls and can trap mycotoxins (Banlunara *et al.*, 2005). Additionally, TB has been shown to increase the antioxidant activity through ionic, hydrogen bonding, and hydrophobic mechanisms (Huwig *et al.*, 2001). Gao *et al.* (2008) expressed that adding yeast to broiler diets significantly increased calcium and phosphorus digestibility, improved enteric mucosal morphology, and potentially reduced aflatoxin's adverse effects by increasing microbial populations. Mesgar *et al.* (2022) documented that supplementing aflatoxin-exposed chickens' diets with a TB improved the infectious bronchitis virus (IBV) titer, consequently bolstering the avian immune system.

It is asserted that the addition of TB containing bentonite-montmorillonite and sepiolite effectively prevents the digestive absorption of feed-borne mycotoxins, thereby ensuring animal health and food

safety (Duarte & Smith, 2005). Consequently, the present investigation aimed to assess the efficacy of varying levels of TB in an aflatoxin-contaminated diet concerning growth performance.

MATERIALS AND METHODS

Mycotoxin Proliferation

The dosage of aflatoxin was 0.750 mg/kg of feed. The AFB1 was produced by naturally cultivating *Aspergillus flavus* in healthy corn for a month. To accelerate the growth of *Aspergillus flavus* fungi, 20% (w/w) water was added to the corn every day to increase its moisture content. Also, the corn was contaminated with AFB1 and subsequently analyzed for aflatoxin levels using Thin Layer Chromatography (TLC). The results indicated that the AFB1 level in corn with *Aspergillus flavus* was 0.75 (mg/kg). In this study, AFB1-contaminated diets were created by substituting the ratio of corn in the PC diet with aflatoxin-contaminated corn. All other mycotoxins were below the detection limit and were thus considered negligible.

Toxin Binder

In the present study, the commercial TB (Sepehr Makian Fartak Ltd, Iran) used included a yeast wall concentration of 25% (autolyzed yeast), activated carbon, calcium propionate, aluminosilicate, vitamin E, organic selenium, antioxidant, and *Silybum marianum* seed extract (silymarin).

Birds and Experimental Diets

The current research was conducted between April 8 and May 21, 2023, at the educational research farm of Isfahan University of Technology, Isfahan, Iran. A total of 875 one-day-old Ross 308 broilers (initial body weight = 42.5 ± 1 g) were randomly distributed into 35 pens (25 birds/pen). Five dietary treatments were randomly assigned to 7 duplicates per treatment, divided into three growth phases (starter; 0 to 10 days, grower; 11 to 24 days, and finisher; 25 to 40 days).

Experimental diets were as follows: 1) Negative control (NC): Basal diet without aflatoxin, 2) Positive control (PC): Basal diet + 0.75 (mg/kg) of AFB1, 3) PC + 1 (g/kg) of TB, 4) PC + 2 (g/kg) of TB, and 5) PC + 3 (g/kg) of TB. Feed and water were provided *ad libitum* throughout the experimental period. The Ross 308 management guide was followed during the trial, with the implementation of the recommended management practices (Ross 308 Management Guide, 2018). The



nutrient requirements for the starter, grower, and finisher phases of the broilers (Table 1) were formulated according to the nutrition specifications recommended for Ross 308 broilers.

Table 1 – Ingredients and nutrient composition of the basal diet (%).

Item	Starter	Grower	Finisher
Corn	49.56	55.11	60.53
Soybean meal (CP: 42%)	39.80	34.60	28.60
Corn gluten meal (CP: 60%)	4.00	3.30	4.00
Soybean oil	2.40	3.10	3.40
Limestone	0.78	0.75	0.60
Di-calcium phosphate	1.83	1.57	1.37
NaHCO ₃	0.23	0.26	0.27
Salt	0.20	0.18	0.18
Vitamin premix ¹	0.25	0.25	0.25
Mineral premix ²	0.25	0.25	0.25
L-Lysine-HCL	0.25	0.23	0.23
DL-Methionine	0.31	0.28	0.24
L-Threonine	0.06	0.04	0.0
Choline chloride	0.06	0.06	0.06
Phytase enzyme	0.01	0.01	0.01
Multi-enzyme	0.01	0.01	0.01
Calculated Analysis			
Metabolizable energy (kcal/kg)	3000	3100	3150
Crude protein	23.00	20.90	18.82
Dig. Lysine	1.28	1.15	1.02
Dig. Methionine	0.64	0.58	0.54
Dig. Methionine + Cysteine	0.95	0.87	0.80
Dig. Threonine	0.86	0.77	0.68
Calcium	0.96	0.87	0.78
Available phosphorus	0.48	0.43	0.39

NaHCO₃: Sodium bicarbonate

¹ Supplied per kg of diet: 12000 IU vitamin A, 5000 IU vitamin D3, 80 IU vitamin E, 3.2 mg vitamin K, 3.2 mg vitamin B1, 8.6 mg vitamin B2, 65 mg niacin, 20 mg pantothenic acid, 4.3 mg vitamin B6, 0.22 mg biotin, 2.2 mg folic acid, and 0.017 mg vitamin B12.

² Supplied per kg of diet: 16 mg copper, 1.25 mg iodine, 20 mg iron, 120 mg manganese, 0.3 mg selenium, and 110 mg zinc.

Growth Performance

At the end of each experimental phase, the weight, feed intake, and mortality of all birds were recorded per pen and categorized by treatment. Using these data, the average daily weight gain (ADWG), average daily feed intake (ADFI), and feed conversion ratio (FCR) were calculated.

Carcass Characteristics

At 40 days of age, one bird per replicate was randomly selected, weighed, and slaughtered through

carbon dioxide asphyxiation. The weight of the heart, gizzard, liver, bursa of Fabricius, thymus, and spleen was determined using a digital scale with a precision of 0.01 g, and expressed as a percentage of the live body weight.

Antibody Response against Sheep Red Blood Cells

The humoral response to sheep red blood cells (SRBC) was estimated at 23 and 30 days of age. First, 1 cc of blood was taken to assess the immune response at 30 and 37 days. After being transferred to the laboratory, their serum was isolated and the antibody titer was measured using a hemagglutination assay. The serum was first inactivated by heating the fresh serum in a water bath at 56 °C for 30 minutes. Then, 25 µL of phosphate-buffered saline (PBS) were added to the wells of 96-well plates with a v-shaped bottom. Afterward, 25 µL of serum were added to the first well of each row, mixed, and the plates were incubated at 37 °C for 30 minutes. A serial dilution of the serum samples was performed across successive rows. After adding 25 µL of a 2% SRBC suspension to each well, the plates were incubated for 30 minutes. A hemagglutination assay was conducted to determine the total antibody titers to SRBC in the serum from birds after incubation at 37 °C. The IgG and IgM antibody titers were determined using the same method as the total titers, except that 0.01 molar mercaptoethanol in PBS was used instead of PBS alone. To determine the level of IgG antibody, 25 µL of this solution was added to each well, and the subsequent steps were performed following the procedure mentioned above. The IgM antibody level was determined by calculating the difference between the total antibody and the IgG response (Eghbaldost-Jadid *et al.*, 2021).

Microflora and pH of the ileum

One broiler per replicate was selected, weighed, and killed at 40 days of age. Fresh digesta samples (1 g) were taken from the ileum (Meckel's diverticulum up to 1 cm near the ileocecal junction), collected, and gently placed in sterile sampling tubes. The samples were transferred on ice to a laboratory to count the bacterial population. The digesta sample (1 g) was serially diluted to 10⁻³ to measure *Escherichia coli* using xylose lysine deoxycholate (XLD) agar plates incubated at 37 °C for 24 hours. The *Lactobacillus* populations were evaluated using Man, Rogosa, and Sharpe (MRS) agar (1.10660.500). The samples of ileum culture were determined by serial dilution (10⁻³) and incubation at



37 °C under anaerobic conditions for 48 hours. Brilliant green agar was used to culture *Salmonella*, which was incubated for 24 hours at 37 °C and then counted. The logarithm (base 10) of colony-forming units per gram (Log10CFU/g) of digesta sample was calculated for the population of *Escherichia coli*, *Lactobacillus*, and *Salmonella* (Heidari *et al.*, 2018).

At the end of the study, the pH of the ileum contents (from Meckel's diverticulum to the ileocecal junction) was measured using a digital pH meter (Testo 205 - Germany) as indicated by Teuchert (2014). The mean values of two measurements taken in each bird were used for further evaluation.

Jejunal Morphology

On day 40, a one-centimeter section of the midpoint of the jejunum from one bird per replicate was sampled as described by Eftekhari *et al.* (2017). After removing the gut digesta, sections were isolated for morphological assessments. The sections were washed with distilled water to prevent tissue loss. Subsequently, the samples were fixed in formalin (10% neutral-buffered formalin solution) for about 24 hours. A 0.5 ± 0.05 µm thick segment was processed (cut with a microtome, Sakura SRM 200, Tokyo, Japan), embedded in paraffin, stained with eosin blue, and 10 mm-diameter parts were assessed for their villus height, crypt depth, villus width, and muscle thickness using a light microscope. Two cross-sections from each bird were observed at 40× magnification (Olympus CX31, Tokyo, Japan), as detailed by Eftekhari *et al.* (2017), and the average of at least 10 measurements taken from cross-sectional samples were used for further analysis. The villus surface area was calculated using the formula $\pi(VW) \times (VL)$, where VW indicates villus width and VL indicates villus length (Sakamoto *et al.*, 2000).

Liver Color Index and Pathological Parameters

The left hepatic lobe was viewed at a resolution of 14 megapixels in an image processing box under the same light conditions for all samples. One method for evaluating color parameters is the image processing method, i.e., L* (lightness/brightness), a* (redness/greenness), and b* (yellowness/blueness). The images were examined in Photoshop software to estimate color parameters (Amjadi *et al.*, 2019).

At 40 days of age, 7 birds per treatment were randomly chosen and slaughtered. The liver portion was extracted after a detailed dissection of the carcass. One centimeter of the liver tissue was fixed in 10% buffered formalin. Then, the tissues underwent dehydration with increasing condensations of ethyl alcohol (50%, 75%, and 98%), cleared in xylene, and embedded in paraffin blocks (Garcia *et al.*, 2010). Microtome sections (5 µm thick) were stained with hematoxylin and eosin and evaluated under a light microscope (Olympus CX31, Tokyo, Japan). The samples were then graded based on the severity of liver lesions from +1 to +3, where +1 indicated mild pathological changes, +2 indicated moderate pathological changes, and +3 indicated hyper-pathological changes (Kiernan, 2015).

Statistical Analysis

All data were analyzed in accordance with a completely randomized design arrangement using a generalized linear model (GLM) (SAS 9.4). The normality and variance homogeneity test of data were conducted using analysis of variance (ANOVA). In addition, treatment sums of squares for AFTB1 content of the diets were divided into linear (Lin) and quadratic (Quad) responses to increasing levels of TB in the diet (0, 1, 2, and 3 g/kg). The Tukey test and orthogonal contrast were used to detect differences among treatment means, and the differences were considered significant at $p < 0.05$. A trend was defined as a P-value between 0.05 and 0.10 ($0.05 < p \leq 0.10$).

RESULTS AND DISCUSSION

Growth Performance

The TB had no significant effect on the ADWG and FCR, but it significantly reduced ADFI compared to NC in the starter phase (Table 2). Additionally, an orthogonal contrast between TB and NC revealed that both ADWG and ADFI significantly decreased with an increase in the TB level. Another orthogonal contrast between TB and NC showed that ADFI decreased by increasing TB as much as 1 to 2 (g/kg) of the diet in the grower phase. In the finisher phase, chickens that received TB exhibited significantly lower ADFI and FCR ($P < 0.05$) compared to the NC treatment. Also, throughout the entire experimental period, the inclusion of TB did not significantly impact the ADWG; however, it resulted in a notable reduction in the FCR ($P < 0.05$).



Table 2 – Effects of dietary treatments on growth performance of broilers subjected to aflatoxin B1.

Treatments ¹	Starter (1-10 d)			Grower (11-24 d)			Finisher (25-40 d)			Whole period (0-40 d)		
	ADWG (g/b/d)	ADFI (g/b/d)	FCR	ADWG (g/b/d)	ADFI (g/b/d)	FCR	ADWG (g/b/d)	ADFI (g/b/d)	FCR	ADWG (g/b/d)	ADFI (g/b/d)	FCR
NC	19.96	24.03 ^a	1.20	83.24	118.45	1.42	85.10	169.34 ^a	1.99 ^a	66.03	110.96 ^a	1.68 ^a
PC	19.26	22.89 ^b	1.19	82.12	117.51	1.43	87.76	167.17 ^a	1.91 ^{ab}	66.46	109.54 ^b	1.64 ^{ab}
1 g/kg TB	19.13	22.69 ^b	1.19	81.01	115.19	1.43	86.86	154.96 ^b	1.79 ^{bc}	65.70	104.09 ^b	1.58 ^b
2 g/kg TB	19.09	22.61 ^b	1.19	81.51	115.10	1.41	83.99	148.39 ^b	1.77 ^{bc}	64.79	101.58 ^b	1.56 ^b
3 g/kg TB	19.36	22.64 ^b	1.17	83.24	116.77	1.40	87.92	151.19 ^b	1.71 ^c	66.94	103.22 ^b	1.54 ^b
SEM ² TB	0.276	0.190	0.015	1.622	1.039	0.028	1.215	3.582	0.053	1.801	2.70	0.02
p-value³												
Treatment	0.238	0.001	0.589	0.826	0.145	0.943	0.139	0.001	0.006	0.317	0.001	0.008
NC vs. TB	0.025	0.001	0.178	0.503	0.036	0.768	0.484	0.001	0.001	0.752	0.001	0.001
PC vs. TB	0.150	0.230	0.530	0.915	0.150	0.564	0.378	0.150	0.073	0.700	0.001	0.008
Lin	0.809	0.306	0.270	0.611	0.839	0.621	0.735	0.299	0.515	0.963	0.330	0.387
Quad	0.399	0.526	0.574	0.404	0.247	0.717	0.062	0.801	0.339	0.067	0.620	0.335

ADWG: Average daily weight gain, ADFI: Average daily feed intake, FCR: Feed conversion ratio, NC: Negative control, PC: Positive control, TB: Toxin binder.

¹Negative control, Positive control + 0.75 (mg/kg) of aflatoxin, Positive control +1, 2, and 3 (g/kg) of TB.

²Standard error of mean.

³Treatment: Overall effects of treatments; Lin: linear effects of increasing the inclusion levels of TB; Quad: quadratic effects of increasing the inclusion levels of TB.

^{ab}Means within the same column with different letters are statistically significant ($p < 0.05$).

Similar to our results (PC treatment containing 0.75 (mg/kg) of AFB1 at the starter phase), Nazarizadeh & Pourreza (2019) observed that the ADFI of growing broilers decreased due to the presence of AFB1 (0, 2, and 4 ppb). Nalle *et al.* (2019) demonstrated that dietary aflatoxin levels (including 10 ppb) and TB (including 10 ppb) showed no correlation with broilers' feed intake, FCR, and body weight gain during 7- and 14-day trials. In contrast to our study, Wang *et al.* (2005) observed improved growth performance in broilers when using a toxin adsorbent in corn-contaminated feed. Additionally, Agboola *et al.* (2015) reported that the addition of TB, rather than stimulants for antibiotic development, increased body weight gain in broilers.

In the present experiment, TB improved FCR and ADFI in the diet containing AFB1, possibly by prolonging feed retention time in the broiler's intestine during the finisher phase. TB can increase the digestibility of certain nutrients by exposing them to enzymatic effects for an extended period. The present study hypothesized that grains contaminated with AFB1, influenced by different agronomic conditions, might negatively affect nutrient digestibility, livestock performance, and intestinal health in broilers. Some detrimental effects of feeding low-quality grains could be mitigated by adding a TB, leading to improved nutrient digestibility, gastrointestinal health, and function.

Poultry are fed with various combinations of feedstuffs, which are produced under different circumstances. Toxicogenic fulmination caused by

aflatoxins depends on the amount ingested and the duration of exposure, as well as on livestock species, lifetime, and nutritional status (Valchev *et al.*, 2020). Aflatoxins are the primary dangerous toxins in diets, troubling poultry farmers. Aflatoxins are considered particularly toxic because they are rapidly absorbed in the intestine (Whitlow *et al.*, 2002), and the symptoms of aflatoxicosis in birds are well-defined (Wan *et al.*, 2013). The metabolism of this combination in liver tissue causes the production of toxic substances, leading to liver damage and inhibition of protein synthesis (Yunus *et al.*, 2011). Several researchers have reported similar findings of elevated liver enzyme activities due to aflatoxicosis (Wade & Sapkota, 2017; Ulaiwi, 2018; Zabiulla *et al.*, 2021). AFB1 is also believed to cause malabsorption of macronutrients, leading to digestive issues. Additionally, it is believed that the presence of AFB1 reduces the activity of digestive enzymes (Devegowda & Murthy, 2005).

The effects of aflatoxin on diet yield are not always consistent due to various diet combinations, particularly different protein levels and sources or various tryptophan accumulations in the diet (Khanipour *et al.*, 2019). These factors have been shown to alter protein mechanism and animal response to aflatoxin in birds or to enhance aflatoxin metabolism. Furthermore, the type of toxin used in the experiment ("naturally occurring" vs. "purified" form) can impact the outcomes. Negative impacts on animal performance in trials tests can be attributed to various factors, such as



the poultry strain used, the accumulation of mycotoxins in the diet, and the studies conducted on the subject. The compromised growth performance outcomes may be linked to gut physiological alterations due to toxins. Moreover, the digestive enzyme activities, including maltase and sucrose, were impacted when poultry were fed with the mycotoxin (aflatoxin) in their diet (Applegate *et al.*, 2009; Chen *et al.*, 2016).

Carcass Characteristics

The relative weight of visceral organs is presented in Table 3. The data showed an increased relative weight of the bursa of Fabricius by adding 3 (g/kg) of TB ($p < 0.01$); however, adding TB to the diet containing AFB1 had no significant effect on the relative weight of other organs. The relative weight of the spleen was significantly lower in the PC treatment as compared to the NC ($p < 0.05$). In addition, based on the contrast response, increasing the TB level in the diet had a quadratic effect on the relative weights of the gizzard ($p < 0.05$).

Table 3 – Effects of dietary treatments on the relative weights of organs (carcass characteristics; %BW) of broilers subjected to aflatoxin B1.

Treatments ¹	Bursa of Fabricius	Thymus	Spleen	Liver	Gizzard	Heart
NC	0.09 ^b	0.36	0.14 ^a	2.16	1.42	0.32
PC	0.07 ^b	0.34	0.11 ^b	2.16	1.28	0.31
1 (g/kg) TB	0.07 ^b	0.31	0.12 ^{ab}	1.99	1.49	0.36
2 (g/kg) TB	0.07 ^b	0.34	0.13 ^{ab}	2.28	1.37	0.34
3 (g/kg) TB	0.13 ^a	0.31	0.12 ^{ab}	2.01	1.36	0.33
SEM ²	0.008	0.028	0.007	0.099	0.072	0.019
p-value³						
Treatments	0.001	0.605	0.056	0.186	0.324	0.506
NC vs. TB	0.702	0.202	0.157	0.472	0.921	0.300
PC vs. TB	0.386	0.462	0.039	0.464	0.112	0.195
Lin	0.412	0.613	0.329	0.544	0.664	0.677
Quad	0.734	0.875	0.065	0.937	0.024	0.074

NC: Negative control, PC: Positive control, TB: Toxin binder.

¹Negative control, Positive control + 0.75 (mg/kg) of aflatoxin, Positive control +1, 2, and 3 (g/kg) of TB.

²Standard error of mean.

³Treatments: Overall effects of treatments; Lin: linear effects of increasing the inclusion levels of TB; Quad: quadratic effects of increasing the inclusion levels of TB.

^{ab}Means within the same column with different letters are statistically significant ($p < 0.05$).

Valchev *et al.* (2020) reported that the relative weight of the liver, kidney, spleen, heart, pancreas, proventriculus, and gizzard significantly increased in aflatoxin-fed broilers (0.5 mg/kg of diet). Furthermore,

they reported that the concurrent utilization of mycotoxin NG with AFB1 decreased the relative weight of the thymus and bursa of Fabricius. Similar to this study, Khalique *et al.* (2015) showed that the weight of the bursa of Fabricius significantly increased with the addition of TB. Spleen weight is shown to be a susceptible indicator of immunotoxicological effects (immune stimulation or depletion), stress, and physiological perturbations. Valchev *et al.* (2020) indicated that the relative weight of the spleen increased in the group fed diets contaminated with AFB1, described as a compensatory mechanism in decreased practical activity, lower bursa of Fabricius, and thymus weights (Nabi *et al.*, 2018).

According to the study by Sakhare *et al.* (2007), the decline in the weight of immune-competent organs is probably due to necrosis and lower density of lymphoid cells. Furthermore, studies have indicated that mycotoxins can impact the immune system through the atrophy of the lymphoid organs, lymphocyte depletion, and enlargement of the kidney and liver (Riahi *et al.*, 2021). Consequently, the increase in the relative weight of lymphoid organs, coupled with the addition of TB supplement to the diet, demonstrates that TB has the potential to avert immune system deterioration in cases of mycotoxin-contaminated feed (2016).

Antibody Response against Sheep Red Blood Cells

The data associated with fed aflatoxin and TB on antibody production titer against SRBC in broilers are shown in Table 4. The results of the current study revealed that the treatments had no significant effect on the immunity parameters of broilers. An increase in IgG was observed when 3 (g/kg) of TB was added to the diet compared to other treatments ($p < 0.05$). Also, according to the contrast response, increasing the level of TB in the diet linearly increased the IgG titer and quadratically increased the total titer in the secondary response ($p < 0.05$).

The highest concentrations of the immunoglobulins against SRBC were reported on day 28 in the NC, PC + 1 (g/kg) of TB, and PC + 3 (g/kg) of TB groups, with no significant difference observed between them. These results are consistent with Sarrami *et al.* (2019) findings, showing that the highest levels of IgG were achieved by adding 1 and 3 (g/kg) of TB to the diet. The highest immunoglobulin responses to SRBC at 35 days of age among the TB-treated treatments were



related to PC + 2 (g/kg) of TB and PC + 1 (g/kg) of TB, while the highest IgG levels were linked to the NC and 1 (g/kg) of TB groups. However, there was no significant difference in IgM levels between the two periods. In the PC group, the lowest antibody titer and IgG against SRBC were recorded (Sarrami *et al.*, 2019).

Table 4 – Effects of dietary treatments on the response to sheep red blood cells and immunoglobulin titer in broilers subjected to aflatoxin B1.

Treatments ¹	Primary Response (30 d)			Secondary Response (37 d)		
	Total	IgG	IgA	Total	IgG	IgA
NC	2.00	1.00	1.00	4.20	1.83 ^b	2.20
PC	2.14	1.14	1.00	4.67	1.43 ^b	2.86
1 (g/kg) TB	2.14	0.86	1.28	4.33	1.71 ^b	2.50
2 (g/kg) TB	2.00	0.86	1.14	3.83	1.83 ^b	2.00
3 (g/kg) TB	2.00	1.14	0.86	5.86	3.28 ^a	2.57
SEM ²	0.168	0.129	0.159	0.606	0.365	0.391
p-value³						
Treatments	0.929	0.329	0.402	0.162	0.012	0.597
NC vs. TB	0.819	0.767	0.673	0.539	0.401	0.747
PC vs. TB	0.648	0.265	0.648	0.931	0.101	0.297
Lin	0.495	1.000	0.473	0.242	0.002	0.443
Quad	1.000	0.569	0.115	0.040	0.139	0.306

NC: Negative control, PC: Positive control, TB: Toxin binder.

¹Negative control, Positive control + 0.75 (mg/kg) of aflatoxin, Positive control +1, 2, and 3 (g/kg) of TB.

²Standard error of mean.

³Treatments: Overall effects of treatments; Lin: linear effects of increasing the inclusion levels of TB; Quad: quadratic effects of increasing the inclusion levels of TB.

^{ab}Means within the same column with different letters are statistically significant ($p < 0.05$).

Manafi *et al.* (2014) reported that the addition of aflatoxin to the diet influenced antibody titer, and the harmful effects of aflatoxin in chicks at 42 days of age could be significantly altered by the addition of TB. Another study reported that using a fungus TB may improve broilers' immunity when they are fed with mycotoxins (Pasha *et al.*, 2007). Some researchers have reported that the cell-mediated immunity and particular antibody production in response to SRBC (Verma *et al.*, 2004), infectious bronchitis and bursal disease virus, and Newcastle disease (ND) might be suppressed by aflatoxins from natural or artificially contaminated feed at 200 ppb to 2.5 mg/kg of diet (Bagherzadeh-Kasmani *et al.*, 2012).

Reduced levels of IgA and IgG weaken the immune system in aflatoxin-infected broilers (Grasman, 2010). In agreement with the current experiment, Abed *et al.* (2018) indicated that *Saccharomyces cerevisiae* increased the antibody titer in diets infected

with aflatoxin. Our study has shown that adding *Saccharomyces cerevisiae* yeast wall to TB could result in elevated antibody titers in birds.

The immunosuppressive effect of aflatoxins has been observed to be dependent on their ability to prevent protein synthesis, including IgG and IgA, as reported by Rajput *et al.* (2017). Consequently, the incidence of infections in broilers was reduced, which may have contributed to our results. Thus, in challenging situations, it can be expected that the existence of fungus binders may modify this mechanism. However, it is believed that the immune system's susceptibility to suppression by toxins is due to the vulnerability of cells that continuously proliferate and differentiate into immune-mediated activities, and a multifaceted chain of communication is established between cellular and humoral components. Toxins that inhibit immune function can ultimately lead to decreased persistence of infections, reactivation of severe contaminations, and reduced efficacy of vaccines and medicines (Dvorska & Surai, 2001).

Microflora and pH of the Ileum

Microflora count of ileum digesta and ileum pH data are indicated in Table 5. Treatments had a significant effect on the *Lactobacillus* and *Salmonella* bacteria counts in the ileum. Similar to NC, the TB supplementation increased the *Lactobacillus* counts compared to PC ($p < 0.01$). The *Salmonella* population in the ileum was found to be higher in PC and TB treatments than in NC ($p < 0.05$). However, TB addition resulted in a numerical reduction of the *Salmonella* population compared to the PC. Moreover, based on the contrast response associated with TB, the increasing TB levels in the diet led to a linear decrease in the *Escherichia Coli* population ($p < 0.05$). The experimental diets did not affect the *Escherichia coli* counts and ileum pH.

Clarke *et al.* (2018) reported that feeding pigs with a low-quality wheat diet including 2 (g/kg) of TB resulted in the enhancement of *Lactobacillus* and a numerical decline of *Enterobacteriaceae*, recognizing the interest of adding TB to toxin-contaminated cereal grains, particularly in the absence of feed growth stimulants. *Lactobacillus* species were chosen as indicators of beneficial bacteria, while *Enterobacteriaceae* species were chosen based on their possible association with gastrointestinal imbalance (Nyachoti *et al.*, 2006). The harmful effects of aflatoxin on broilers include an incrementing number of gram-negative bacteria and a



decline in the population of gut *Lactobacillus* (Galarza-Seeber *et al.*, 2016). The results showed a significant decrease in *Lactobacillus* count in the PC group compared to TB and NC. So, these results indicated a notable improvement in *Lactobacillus* count by using TB-treated groups in the diet containing AFB1. *Lactobacillus* bacteria can protect broilers against the harmful effects of aflatoxicosis (El-Hack *et al.*, 2018).

Table 5 – Effects of dietary treatments on ileum microbial population and ileum pH of broilers subjected to aflatoxin B1 (Log10 CFU/g).

Treatments ¹	Ileum pH	Ileum Bacteria		
		<i>Lactobacillus</i>	<i>Salmonella</i>	<i>Escherichia Coli</i>
NC	6.24	7.21 ^a	5.76 ^b	6.59
PC	6.24	6.66 ^b	6.48 ^a	6.63
1 (g/kg) TB	6.14	7.27 ^a	6.28 ^a	6.63
2 (g/kg) TB	6.35	7.43 ^a	6.20 ^a	6.54
3 (g/kg) TB	6.31	7.29 ^a	6.09 ^{ab}	6.49
SEM ²	0.164	0.087	0.143	0.121
p-value³				
Treatments	0.929	0.001	0.029	0.856
NC vs. TB	0.906	0.274	0.021	0.782
PC vs. TB	0.942	0.001	0.094	0.458
Lin	0.605	0.615	0.093	0.002
Quad	0.853	0.124	0.536	0.203

NC: Negative control, PC: Positive control, TB: Toxin binder.

¹Negative control, Positive control + 0.75 (mg/kg) of aflatoxin, Positive control +1, 2, and 3 (g/kg) of TB.

²Standard error of mean.

³Treatments: Overall effects of treatments; Lin: linear effects of increasing the inclusion levels of TB; Quad: quadratic effects of increasing the inclusion levels of TB.

^{ab}Means within the same column with different letters are statistically significant ($p < 0.05$).

It is hypothesized that an imbalance between beneficial and harmful bacteria may be present in the gut of non-pressure broilers. If a balance of bacteria is maintained, the broilers may exhibit optimal performance, but when subjected to stress and pressure, such as aflatoxin challenges, the useful bacteria tend to decline, while harmful bacteria may proliferate. This imbalance can result in clinical symptoms (e.g., diarrhea) or subclinical issues (e.g., reduced performance and inefficient digestion). Protective microbial populations are naturally established in the gut, but they can be influenced by various environmental and dietary factors (Lutful-Kabir, 2009). Therefore, managing these conditions may help re-establish beneficial protective bacteria.

Jejunal Morphology

The jejunal morphology data of broilers are presented in Table 6 and Figure 1. The results of the orthogonal contrast between NC and TB showed a significant decrease in the effect of the villus height to crypt depth ratio at 42 days of age ($p < 0.05$). The use of TB in the diet containing AFB1 increased the villus width in broilers compared to NC and PC ($P = 0.05$).

Table 6 – Effects of dietary treatments on the intestinal morphology of the jejunum of broilers subjected to aflatoxin B1.

Treatments ¹	Villus Height (μm)	Crypt Depth (μm)	VH:CD ²	Villus Width (μm)	Villus Surface Area (mm ²)	Muscle Thickness (μm)
NC	948.16	79.58	12.02	87.65 ^b	0.26	245.26
PC	831.45	80.82	10.63	89.63 ^b	0.23	274.24
1 (g/kg) TB	872.17	81.69	10.67	93.26 ^{ab}	0.26	277.95
2 (g/kg) TB	825.38	77.23	10.67	102.18 ^a	0.27	242.91
3 (g/kg) TB	818.92	78.99	10.61	95.33 ^{ab}	0.24	224.43
SEM ³	45.927	5.125	0.605	3.480	0.019	18.183
p-value⁴						
Treatments	0.265	0.977	0.409	0.053	0.753	0.213
NC vs. TB	0.051	0.957	0.037	0.043	0.799	0.891
PC vs. TB	0.897	0.804	0.978	0.045	0.300	0.236
Lin	0.710	0.635	0.987	0.059	0.627	0.027
Quad	0.643	0.935	0.645	0.074	0.284	0.535

NC: Negative control, PC: Positive control, TB: Toxin binder, VH: Villus height, CD: Crypt depth.

¹Negative control, Positive control + 0.75 (mg/kg) of aflatoxin, Positive control +1, 2, and 3 (g/kg) of TB.

²VH: CD: Villus height to crypt depth ratio.

³Standard error of mean.

⁴Treatments: Overall effects of treatments; Lin: linear effects of increasing the inclusion levels of TB; Quad: quadratic effects of increasing the inclusion levels of TB.

^{ab}Means within the same column with different letters are statistically significant ($p < 0.05$).

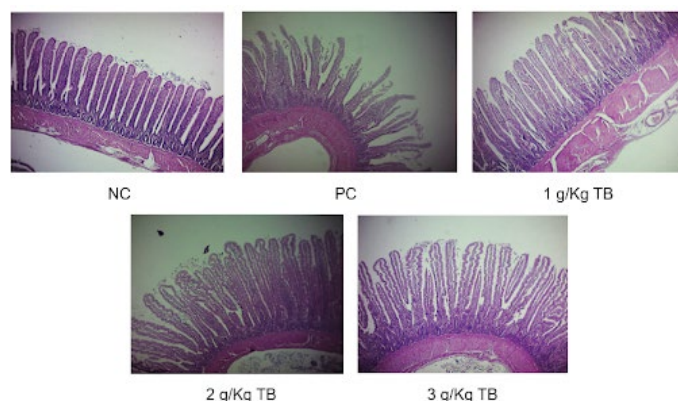


Figure 1 – Effect of toxin binder on the morphological changes of the jejunum in broilers.



The stability and health of the gastrointestinal tract, particularly the gut, are crucial for the growth of useful bacteria in the intestine and, eventually, the efficiency of broilers. In some experiments investigating the influence of diet on intestinal health, the morphological characteristics of the intestine are used as the primary method for examining its condition (Eftekhari *et al.*, 2017). Research has shown that the development of gut morphology is the result of using fungal TB in broilers. Also, the absorption of toxic compounds in the mucosal wall of the digestive system was reduced by fungus TB (Agboola *et al.*, 2015). The addition of deoxynivalenol (a challenging factor like aflatoxins) to diets may cause a decline in villus height in the gut, gradually decreasing the gut's relative density (weight : length) (Yunus *et al.*, 2012). These researchers reported that adding a high level of deoxynivalenol to the diet significantly decreased the short circuit of the jejunal epithelium, reflecting the function of gut epithelial transport per area unit.

The length of the jejunum was increased linearly in week 4 due to the elevated dietary level of deoxynivalenol, which also improved macronutrient retention (Yunus *et al.*, 2012). Lee *et al.* (2018) demonstrated that gut morphology was not affected by any of the tested levels of fumonisin, AFB1 (0 or 10 ppm), or TB (0 or 0.2%). Removing pathogenic factors which may otherwise hinder growth from the gut can optimize the digestion, absorption, and metabolism of nutrients. This, in turn, can improve enteric morphology. Commercial TBs appear to inhibit the absorption of AFB1 in the gut and help enhance the jejunal morphology.

Liver Color Indices

The liver color test results are reported in Table 7. The liver color of broilers was lighter (L^* values, $p < 0.05$) and less yellow (b^* values, $p < 0.01$) when they were fed with a diet containing 2 (g/kg) of TB and aflatoxin compared to the liver color of broilers in the exposed groups. An orthogonal contrast between TB and NC indicated a significantly reduced effect on L^* value (lightness) ($p < 0.05$). Moreover, another orthogonal contrast demonstrated a significantly reduced effect on b^* value (yellowness) ($p < 0.01$) between TB and PC. In addition, based on the contrast response, increasing the level of dietary TB caused a linear decrease in the redness/greenness (a^* values, $p < 0.05$) of the liver color. Also, increasing the TB level in the diet had a quadratic effect on the liver color ($p < 0.05$).

Table 7 – Effects of dietary treatments on liver color in broilers subjected to aflatoxin B1.

Treatments ¹	Liver Color ²		
	L	a	b
NC	23.20 ^a	23.13	19.87 ^a
PC	20.34 ^{ab}	23.61	21.00 ^a
1 (g/kg) TB	19.94 ^{ab}	21.24	17.64 ^a
2 (g/kg) TB	17.64 ^b	21.22	12.67 ^b
3 (g/kg) TB	21.27 ^a	21.80	17.40 ^a
SEM ³	1.068	0.866	1.341
p-value⁴			
Treatments	0.028	0.264	0.003
NC vs. TB	0.026	0.149	0.054
PC vs. TB	0.389	0.057	0.008
Lin	0.450	0.038	0.069
Quad	0.048	0.030	0.001

NC: Negative control, PC: Positive control, TB: Toxin binder,

¹Negative control, Positive control + 0.75 (mg/kg) of aflatoxin, Positive control +1, 2, and 3 (g/kg) of TB.

² L^* value: Lightness; a^* value: Redness; b^* value: Yellowness.

³Standard error of mean.

⁴Treatments: Overall effects of treatments; Lin: linear effects of increasing the inclusion levels of TB; Quad: quadratic effects of increasing the inclusion levels of TB.

ab Means within the same column with different letters are statistically significant ($p < 0.05$).

According to a study by Saleemi *et al.* (2020), the color, size, and consistency of broilers' livers were not impacted when they were fed with a diet contaminated with AFB1 and supplemented with 2 (g/kg) of TB. Aflatoxicosis happens in birds worldwide. One of the indirect effects of aflatoxicosis is an increased number of liver condemnations due to heightened susceptibility to infectious diseases in many species. One of the main toxins is AFB1, which primarily affects the liver. AFB1 is a carcinogen, and severe aflatoxicosis causes neoplasia in many organs - like the liver - in most species (Wang *et al.*, 2008). Mycotoxins are quickly metabolized in the body and excreted in urine or feces. Residues are transient in the liver and kidney, and in lower concentrations relative to the exposure dose. Prolonged exposure to toxins can result in changes in the color and size of the liver and kidneys, serving as a visible indicator of feed contamination at the time of slaughter (Hoerr *et al.*, 2020).

Liver Pathological Parameters

The data related to the liver pathological changes are shown in Table 8 and Figure 2. The percentage of liver necrosis was 14% when using 1 and 2 (g/kg) of



TB, and 28% when using 3 (g/kg) of TB. Evaluated hepatitis in the liver due to aflatoxin infection showed that all birds indicated this complication in the control treatment and with 2 and 3 (g/kg) of TB. The level of liver fat degeneration was lower in the diet containing TB than in the PC treatment. Previous studies have shown that the antioxidant system was impaired by aflatoxins, which could induce hepatic lipid peroxidation (Shi *et al.*, 2006; Gowda *et al.*, 2008; Hou *et al.*, 2008). Some researchers showed that lower aflatoxin levels (30 to 200 µg/kg) induced hepatic structural extension, hepatocytic vacuolation, fatty degeneration, bile-duct hyperplasia, and necrosis through microscopic investigation, which was similar to the findings of the present study (Ortatatli *et al.*, 2005; El-lakany *et al.*, 2011).

AFB1 and AFB2 caused pathological damage to the hepatocytes in the 100% infected groups.

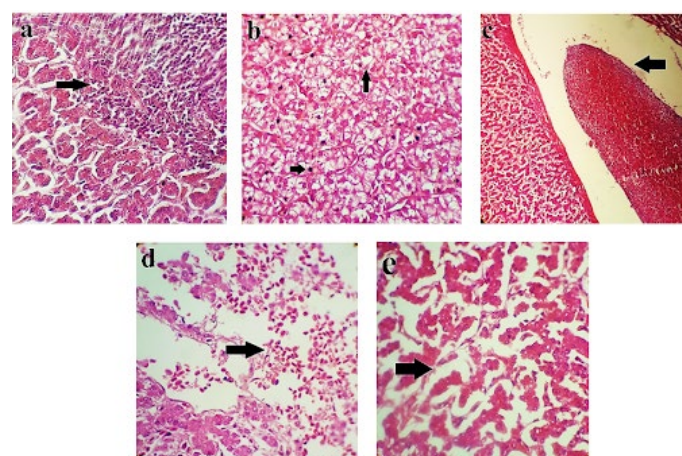


Figure 1 – Photomicrography of the histological section of the liver.

^a Hepatitis with immune cell infiltration (arrow) and cloudy swelling of hepatocytes; ^b Fat degeneration and vacuolar changes with necrosis; ^c Congestion; ^d Hemorrhages; ^e Normal structure change and Remac separation.

Table 8 – Effects of dietary treatments on liver pathological parameters of broilers subjected to aflatoxin B1.

	Percentage of Birds Showing Lesions of Various Scores (%)				
	NC ¹	PC	TB (g/kg)		
			1	2	3
Necrosis	0	0	14	14	28
Hepatitis	67	100	86	100	100
Fat degeneration	50	100	57	57	86
Congestion ²	67	100	100	100	100
Hemorrhages	0	0	0	0	28
Swelling of hepatocytes	67	100	86	86	100
Remac separation	0	14	14	14	14
Histopathological Lesion Scores ³					
Necrosis	-	-	+1	+1	+1
Hepatitis	+1	+2	+1	+2	+1
Fat degeneration	+1	+2	+2	+2	+1
Congestion	+1	+2	+1	+2	+1
Hemorrhages	-	-	-	-	+1
Swelling of hepatocytes	+1	+1	+1	+1	+1
Remac separation	-	+1	+1	+1	+1

¹Negative control, Positive control + 0.75 (mg/kg) of aflatoxin, Positive control +1, 2, and 3 (g/kg) of TB.

²Congestion is a localized increase of blood in a particular tissue.

³Scores +1: Mild pathological change; Scores +2: Moderate pathological change; Scores +3: Hyper-pathological change.

Liver congestion was observed in all birds in the PC and TB groups. Liver hemorrhages occurred in only 28% of birds fed with 3 (g/kg) of TB. The percentages of hepatocyte swelling were approximately 67%, 100%, 86%, 86%, and 100% when birds were fed with NC, PC, and 1, 2, and 3 (g/kg) of TB, respectively. Remac separation was around 14%, except for NC. As reported by Yunus *et al.* (2012), variations in the ultrastructure of hepatocytes showed that corn naturally infected by

Valchev *et al.* (2016) reported that turkeys treated with 0.2 (mg/kg) of AFB1 showed congestion in their livers, characterized by powerful dilation of capillaries with activation of the endothelium, pericapillary edema, and granular degradation of the cytoplasm of hepatocytes. They also showed that birds treated with 0.4 (mg/kg) of AFB1 had severely impaired liver structure. Apart from the congestive events in blood vessels and round cell proliferation, necrobiotic areas and vacuolar degeneration were present in many hepatocytes (Valchev *et al.*, 2016). Furthermore, these authors reported that turkeys treated with both 0.2 (mg/kg) of AFB1 and 0.5 (g/kg) of mycotoxin NG exhibited significantly milder liver dystrophic changes compared to birds treated with only 0.2 (mg/kg) of AFB1. These changes included generalized vascular hyperemia and milder vacuolar degeneration of hepatocytes. Hepatotoxic effects of AFB1 were shown in several animal species: ducklings (Cheng *et al.*, 2001); broilers (Kana *et al.*, 2014; Valchev *et al.*, 2014); quails (Eraslan *et al.*, 2004); rats (Liu *et al.*, 2001); pigs (Shi *et al.*, 2005); and rabbits (Hanafi *et al.*, 2010). AFB1 and its metabolites, such as AFB1 -8, 9- epoxide, can combine with DNA, leading to the destruction of hepatocytes and subsequent impairment of liver function (Rushing and Selim, 2019).

Through observation of pathological modifications, we found that a diet contaminated with AFB1 damaged the hepatic tissue. We assumed that feeding aflatoxins



would lead to hepatitis, fat degeneration, congestion, and hemorrhages, causing swelling of hepatocytes.

CONCLUSION

Based on the obtained data, it was concluded that feeding AFB1-contaminated diets (0.6 mg of AFB1/kg) to broilers from 1 to 42 days of age had negative impacts on growth and immunity. A TB supplement efficiently mitigates the adverse impacts of mycotoxins by binding to them, inhibiting their absorption, and facilitating their excretion. Consequently, it supports bird performance improvement and strengthens the immune system of the birds. Hence, the current study recommends the utilization of a TB supplement to counteract the adverse impacts of mycotoxins, particularly in cases where the feed is contaminated with these toxins.

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AUTHOR CONTRIBUTIONS

M. Aami Azghadi and M. Sedghi: Experiment manager, article editor; Z. Sarrami: investigation, and statistical analyst; M. V. Kimiaetalab: Article editor; R. Ghasemi: writing original draft preparation; I. Mohammadi: formal analysis. All authors have read and agreed to the published version of the manuscript.

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DATA AVAILABILITY STATEMENT

Data will be available upon request.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ANIMAL WELFARE STATEMENT

The authors confirm that they have adhered to the animal welfare statement in this manuscript,

and that all of the EU standards for the protection of animals and/or feed legislation have been met. The only exception was for stock density; in this case, the final body weight was set to be less than 30 kg/m², which was lower than that mentioned in the Council Directive 2007/43/EC of June 28, 2007. We also confirm that we have followed the animal welfare guide, as adopted by FASS (2010). All animal care and experimental procedures were approved by the Animal Policy and Welfare Committee of Isfahan University of Technology. Also, this study followed the ARRIVE guidelines.

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