Mycotoxins binder supplementation alleviates aflatoxin B_1 toxic effects on the immune response and intestinal barrier function in broilers

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ABSTRACT This experiment was conducted to evaluate whether a commercial mycotoxins-binder, XL, could effectively attenuate the negative effects of Aflatoxin B_1 (**AFB_1**) on growth performance, immunological function, and intestinal health in birds. Two hundred forty 1-day-old Arbor Acres broiler chickens were randomly divided into 4 treatments using a 2 × 2 factorial randomized design with 2 levels of dietary mycotoxins binder (0 or 2g /kg) and 2 AFB_1 supplemented levels (0 or 200 μg/kg) from 0 to 42 d. Results showed that AFB_1 exposure impaired growth performance by decreasing BWG in 1−21 d and 1−42 d, decreasing FI in 1−21 d, increasing FCR in 1−21 d and 1−42 d ($P < 0.05$). Broilers fed AFB_1- contaminated diet impaired the immune function, as evident by decreasing IgA contents, Newcastle disease antibody titers in serum, and sIgA contents of jejunal mucosa at 21 d ($P < 0.05$). On the other hand, AFB_1 challenge significantly increased the gene expression of proinflammatory factors in spleen at 21 d and liver at 42 d, and significantly decreased *claudin-1* expression at 42 d and *occludin* expression at 21 d, and increased *claudin-2* at 21 d in jejunum of broiler chickens ($P < 0.05$) compared to the basal diet group. Dietary XL supplementation significantly decreased the gene expression of *IL-6* in spleen at 21 d and *IL-1β* in liver at 42 d, cytochrome P450 3A4 (***CYP3A4***) expression in liver at 21 d of broilers ($P < 0.05$) compared with the nonsupplemented birds, regardless of AFB_1 challenged or not. Inclusion of 2 g/kg XL increased serum ALB at 42 d, IgM and IgA at 42 d, Newcastle disease antibody titer level at 35 d ($P < 0.05$). Dietary XL addition enhanced intestinal barrier function by increasing the expression of *claudin-1* at 21 d and *Occludin* at 42 d ($P < 0.05$) in jejunum. Conclusively, 2 g/kg mycotoxins-binder can relieve the toxic effect of AFB_1 on broilers.

Key words: mycotoxins binder, aflatoxin B_1, broiler chickens, immunological function, intestinal barrier function

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INTRODUCTION

Aflatoxin (**AF**) is a secondary metabolite of *Aspergillus* species, which can contaminate food and agricultural products (Olarte et al. 2012; Jallow et al. 2021). As one form of AF, aflatoxin B_1 (**AFB_1**) is well known as the potent and dangerous teratogen, carcinogen classified in group 1 by IARC and immune-suppressor produced naturally by *Aspergillus flavus* (***A. flavus***) or *Aspergillus parasiticus* (***A. parasiticus***) for human and animals (Fouad et al., 2019; Jallow et al., 2021). Chicken exposed to ABF_1 is reported to depress feed intake, growth performance, immune response, damage organs, disturb the balance of gut microbiota, and increase mortality (Williams et al., 2011; Chang et al., 2020; Rashidi et al., 2020). Consequently, income of poultry producer and human health suffered hazard risk from AFB_1 (Khlangwiset et al., 2011). Organ damage induced by AF common occurs in liver (Rashidi et al., 2020), kidney (Śliżewska et al., 2019), reproductive organs (Doerr and Ottinger, 1980; Ortatatli et al., 2002), digestive tract (Feng et al., 2017; Poloni et al., 2020), pancreas (Ortatatli et al., 2002), immune organs (Peng et al., 2014; Rasouli-Hiq et al., 2017), and bones (Raju et al., 2005) in poultry species. And the degree of organ damage depends on the degree of contamination and the susceptibility of the bird species to AFB_1. Major serum biochemistries changed significantly, such as reduction of total protein, TG, albumin, Ca, P (Rashidi et al., 2020), globulin (Chen et al., 2016),

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oxidoreductase activities and concentration (Ali Rajput et al., 2017) and immunoglobulin content (Chen et al., 2014), are supposed to be indicators of aflatoxicosis. Immunosuppression induced by AFB_1 including higher skin response to mitogens (Bagherzadeh Kasmani et al., 2012), decrease of specific immunity response to Newcastle disease (**ND**), avian influenza (**AI**) viruses (Rashidi et al., 2020), sheep red blood cells (**SRBC**) (Bhatti et al., 2017), and impaired cell-mediated immunity (**CMI**) (Giambrone et al., 1985; Hoerr, 2010) in birds has been demonstrated. Intestinal health impairments from AFB_1 exposure, including mechanical, immune (Jiang et al., 2015; Liu et al., 2018b), chemical, microbial barrier (Chang et al., 2020), and also have been documented.

The broiler industry has been preceded only by the pig industry which is the largest animal husbandry, and supply the second most highly consumed meat in China (Xin et al., 2016). In order to control and degrade mycotoxins, biological, physical, and chemical methods for detoxicating have been selected in broiler production (Solis-Cruz et al., 2019; Rashidi et al., 2020). Mycotoxin binders, XL, is a kind of additive package composed of bentonite with a high content of smectite binding agent, inactive yeast cell wall fractions from a Saccharomyces cerevisiae strain, activated β-1,3/1,6-glucans (glucose biopolysaccharides). Core modes of action of major compositions for XL to detoxicate are binding mycotoxins, protecting intestine, and modulating immune system (Morales-López et al., 2009; Shannon et al., 2017; Pascual et al., 2020). A published report showed positive effects of XL on counteracting the harmful effects of mycotoxins on performance in laying hens (Zhao et al., 2021). However, whether the commercial mycotoxins-binder could effectively attenuate the toxic effects of AFB_1 when exposed to broiler chickens remains unknown. The objective of this study was to evaluate the efficacy of XL to reduce the toxicity of AFB_1 to broilers.

MATERIALS AND METHODS

Animal Ethics Statement

The broiler care and use protocol was approved by the China Agricultural University Animal Care and Use Committee, Beijing, P. R. China.

Experimental Design and Animal Management

Two hundred and forty 1-day-old Arbor Acres (**AA**) male broiler chickens were purchased from Beijing Arbor Acres Poultry Breeding Company randomly assigned to 4 treatments with 6 replicates of 10 birds each. The experiment lasted 42 d which included 2 phases (1−21 d and 22−42 d). Feed and water were given *ad libitum* for the 6-wk exposure period. The composition of the basal diet and nutrient levels are showed in Table S1.

The feeding experiment was designed as follows:

Group A: Basal diet.
Group B: Basal diet with 2 g /kg XL (Trouw Nutrition, Amersfoort, The Netherlands).
Group C: Basal diet with 200 μg/kg AFB_1 (Sigma-Aldrich, St. Louis, MO).
Group D: Basal diet with 2 g /kg XL +200 μg/kg AFB_1.

Broiler chickens were housed in wire cages and maintained under 23L:1D for this experiment after receiving continuous light for the first 24 h. The room temperature was maintained at 32°C to 34°C during the first 5 d and then gradually decreased by 2°C/wk to reach a final room temperature of 22°C to 24°C.

Measurement of Growth Performance and Organ Index

Body weight (**BW**), feed intake (**FI**), and mortality were recorded on the 0, 21, and 42 d, and average body weight gain (**BWG**), average FI, and feed conversion ratio (**FCR**) were calculated during this trial. All performance parameters were corrected according to mortality.

On the 21 and 42 d., 6 birds in each treatment (1 bird from each cage) were humanely euthanized and tissue samples were collected. Detoxification organ and immune organs of the liver, spleen, bursa, and thymus were collected and weighed. Calculate organ index as organ index = organ weight (mg)/body weight (g).

Determination of Serum Protein and Immunoglobulin Levels by ELISA

At 21 and 42 d, 6 birds in each treatment (1 bird from each cage) were selected to collect blood. An approximately 10 mL blood sample was collected from the jugular vein into a non-heparinized tube, placed at room temperature for 30 min, centrifuged at 3,000 *g* for 10 min, and the serum was separated and stored in 1.5 mL eppendorf tubes at -20°C until further analysis. According to the Elisa kit instruction, the levels of serum total protein (**TP**), albumin (**ALB**), globulin (**GLO**), IgG, IgA, and IgM were determined. Serum GLO content was calculated as the difference between TP and ALB.

The birds were vaccinated intramuscularly with inactivated Newcastle Disease vaccine at 7 d and 21 d, respectively. Six birds in each treatment at 21 d and 35 d were selected to collect Blood samples (5.0 mL). All antibodies and reference sera used in the assay were purchased from IDEXX Laboratories Inc.

Intestinal Morphology and Jejunal mucous sIgA

At 21, 42 d, one bird per replicate was randomly selected and slaughtered and the whole gastrointestinal tract was immediately exposed. Then 10-cm intestinal

segments of jejunum were sniped to scrape mucous membrane. All intestine samples were wrapped in Eppendorf tubes and stored at −80°C until further analysis. Another section of jejunum which was fixed in the 4% PFA was cut into smaller sections for preparation of histological slides. The slides were stained with hematoxylin and eosin and then captured images under a microscope (Leica, DM750). The villus height (**VH**), crypt depth (**CD**), VH to CD (**VH/CD**) ratio, and mucosal thickness were measured from stained samples by using the ImagePro Plus 6.0 software (Media Cybernetics, Bethesda, MD).

The VH was measured from the tip to the bottom of the villi, and CD as the distance between its mouth and its base. Eight well-oriented villi and crypts were randomly selected on each slide to determine VH and CD. The average value of the 6 structures per chicken was used. Mucosal thickness was measured as the distance between the mucosal epithelium and the muscular layer (Rubio et al., 2010).

The content of jejunal mucous sIgA was measured in accordance with instructions introduced by a chicken secretory immunoglobulin A (**sIgA**) ELISA Kit (Bethyl Laboratories, Inc.). The absorbance will be read at wavelength of 450 nm by a multiskan spectrum microplate spectrophotometer (Spectra Max i3x). The content of sIgA was presented as μg/mL mucous suspension.

Total RNA Extraction, Reverse Transcription and Quantitative Real-Time PCR

Total RNA was extracted from spleen, liver, and jejunum (50 mg) using Trizol reagent according to the manufacturer's instructions. The 260:280 nm absorbance ratio in an ultraviolet spectrophotometer (NanoDrop-2000, Thermo Fisher Scientific) was used to estimate the concentration and purity of the total RNA (OD_{260}/OD_{280}: 1.8−2.0). Then total RNA was stored at −80°C or synthesize cDNA stored at -20°C until next procedure. The primer sequences were presented in Table S2. The parameters of PCR reactions were 95°C for 5 min for one cycle, and then 95°C for 30 s for 40 cycles, 60°C for 30 s. Samples were run in triplicate. The qPCR reaction was 10-μL reaction volume and conducted with a 7500 Real-Time PCR system (Xue et al., 2021) . Gene expressions for *IL-1β, IL-6, CYP3A4, claudin-1, claudin-2, claudin-3*, and *occludin* were normalized against the level of housekeeping gene expression (β-actin).

Statistical Analysis

All analyses were performed by the GLM procedure SPSS 16.0 software (SPSS Inc., Chicago, IL) as a 2 × 2 factorial arrangement (2 levels of AFB1 challenge and 2 levels of XL treatment). The data were analyzed by two-way ANOVA analysis of variance with AFB1 and XL as the fixed factors. When interactive effects differed significantly, Duncan's multiple comparisons test was used to separate means. Differences were considered significant at $P < 0.05$, although probability values up to $0.05 \leq P < 0.10$ are shown in the text if data suggest a trend. The results are expressed as treatment means with their pooled SEM.

RESULTS

Growth Performance

Growth performance results (including body weight gain, feed intake, and feed conversion ratio) of broiler chickens at different growth periods are shown in Table 1. AFB_1 exposure significant decreased BWG during 1−21 d ($P < 0.001$) and 1−42 d ($P < 0.01$), decreased FI during 1−21 d ($P < 0.05$), increased FCR during 1−21 d ($P < 0.001$) and 1−42 d ($P < 0.05$) compared to the control group. Compared to nonsupplemented birds, the

Table 1. Effects of dietary AFB_1 and mycotoxins binder on growth performance of broiler.[1,2,3]

		BWG (g)			FI (g)			FCR		
Groups		1−21 d	21−42 d	1−42 d	1−21 d	21−42 d	1−42 d	1−21 d	21−42 d	1−42 d
Control		603	1,827	2,430	933	3,120	4,054	1.55	1.71	1.67
XL		628	1,809	2,437	967	3,148	4,115	1.54	1.74	1.69
AFB_1		555	1,796	2,351	91	3,083	3,995	1.64	1.72	1.70
XL+AFB_1		548	1,807	2,355	923	3,108	4,031	1.69	1.72	1.71
SEM[4]		8.2	9.4	15.3	7.6	17.0	23.1	0.015	0.007	0.006
The main effect										
AFB_1	-	628	1,818	2,433	950	3,134	4,084	1.55	1.72	1.68
	+	548	1,801	2,353	917	3,096	4,013	1.67	1.72	1.71
XL	-	579	1,811	2,390	923	3,102	4,024	1.60	1.73	1.68
	+	588	1,808	2,396	945	3,128	4,073	1.61	1.71	1.70
P-value[5]										
AFB_1		<0.001	0.413	0.008	0.024	0.282	0.132	<0.001	0.688	0.021
XL		0.362	0.862	0.849	0.109	0.463	0.298	0.261	0.207	0.163
AFB_1 × XL		0.106	0.461	0.969	0.419	0.965	0.785	0.105	0.312	0.728

[a-b]Means with different superscripts in the same column differ significantly ($P < 0.05$).
[1]Each value represents the mean values of 6 pens of 10 animals each (n = 6).
[2]Abbreviations: AFB_1, aflatoxin B_1; BWG, body weight gain; FI, feed intake; FCR, feed conversion ratio.
[3]− represents without AFB1 or XL supplementation; + represents supplemented with AFB_1 or XL.
[4]SEM, standard error of the mean.
[5]*P*-values represent the main effect of AFB1 exposure, the main effect of the dietary XL, and the interaction between AFB_1 exposure and XL treatments.

addition of XL did not significantly affect BWG, FI, and FCR. No significant differences were found in mortality rate across all stage by AFB_1 or XL addition (Table S3). Besides, there was no interaction effect ($P > 0.05$) in growth performance between AFB_1 exposure and XL supplementation on growth parameters.

Organ Index and Relative mRNA Expression of Inflammatory Cytokines and CYP3A4 in the Spleen and Liver

Relative organ weights including liver, spleen, bursa of Fabricius, and thymus from broiler were calculated in this study are shown in Table S4. Neither mycotoxins binder nor AFB_1 addition affected the organ weight of broiler chickens ($P > 0.05$). Notably, bursary of Fabricius index and thymus index in 21 d was almost more than 2 times and 4 times as weight as the corresponding organ index in 42 d, respectively; while spleen index in 21 d was about half of that in 42d.

The effects of AFB_1 contamination and mycotoxins binder addition on *IL-6* expression in spleen of broiler chickens are shown in Table 2. AFB_1-contaminated diet significantly increased *IL-6* gene expression at 21d ($P < 0.001$) and tended to increase *IL-6* at 42 d ($P = 0.052$). The addition of mycotoxins binder significantly decreased ($P < 0.001$) *IL-6* expression at 21 d. There was a significant ($P < 0.05$) interaction effect on *IL-6* expression at 21 d between AFB_1 exposure and XL supplementation.

As noted in Table 2, results revealed that AFB_1-contaminated diet increased *IL-1β* and *IL-6* expression in liver at 42 d ($P < 0.05$). Inclusion of XL to diet significantly decreased IL-6 at 42 d ($P = 0.002$), tended to decrease *IL-1β* at 42 d ($P = 0.058$), decreased *CYP3A4* expression at 21 ($P = 0.014$) and 42 d ($P = 0.007$) in liver. Moreover, there were significant interactions to *IL-1β* expression at 42 d ($P = 0.009$) rather than *IL-1β* at 21 d or *IL-6*, *CYP3A4* gene expression between AFB_1 exposure and XL treatment.

Serum Proteins and Immunoglobulins

AFB_1 exposure tended to decrease GLO at 21 d ($P < 0.1$) compared with the unchallenged birds. Chickens received XL diets showed higher content of ALB at 42 d ($P < 0.05$) compared to the nonsupplemented birds. There was significant interaction effect for ALB at 42 d ($P = 0.027$) and GLO at 21 d ($P = 0.031$) between AFB_1 exposure and XL addition (Table 3).

In addition, AFB_1 exposure significantly increased content of IgA ($P = 0.015$) as compared to the control. Compared with the nonsupplemented birds, supplementation of XL significantly increased serum IgM ($P < 0.001$) and IgA ($P = 0.005$) concentrations at 42 d, also showed an increased trend in IgA content at 21 d ($P < 0.1$), but showed no significant effects on IgG. No significant interaction effects were found on IgG, IgM, and IgA between AFB_1 exposure and XL addition (Table 4).

Antibody Response to Newcastle Disease

The effects of AFB_1 challenge and mycotoxins binder addition on serum antibody response to Newcastle Disease of broiler chickens are shown in Figure 1. No significant cooperative effect was found between AFB_1 exposure and XL addition in terms of serum antibody titer to Newcastle in broiler ($P > 0.05$). AFB_1 exposure significant decreased ($P = 0.005$) serum antibody titer to Newcastle at 21 d when compared with the nonsupplemented birds. Compared to nonsupplemented

Table 2. Effects of dietary AFB_1 and XL on relative mRNA expression of *IL-1β*, *IL-6*, and *CYP3A4* in the spleen and liver of broiler chickens.[1,2,3]

		Spleen		Liver					
		IL-6		*IL-1β*		*IL-6*		*CYP3A4*	
Groups		21 d	42 d	21 d	42 d	21 d	42 d	21 d	42 d
Control		1.00[b]	1.00	1.00	1.00[b]	1.00	1.00	1.00	1.00
XL		1.28[b]	0.54	1.21	1.40[b]	0.38	0.14	0.34	0.34
AFB_1		8.43[a]	1.24	1.09	3.52	0.67	2.30	0.67	1.01
XL+AFB_1		0.80[b]	2.38	0.86	1.24[b]	0.46	0.69	0.27	0.37
SEM[4]		0.748	0.274	0.198	0.294	0.088	0.235	0.091	0.125
The main effect									
AFB_1	-	1.14	0.77	1.10	1.20	0.69	0.53	0.69	0.65
	+	4.62	2.38	1.47	2.38	0.56	1.50	0.56	0.69
XL	-	4.71	1.12	1.55	2.34	0.83	1.71	0.83	1.00
	+	1.04	1.46	1.03	1.31	0.42	0.41	0.42	0.34
P-value[5]									
AFB_1		<0.001	0.052	0.323	0.020	0.417	0.014	0.417	0.864
XL		<0.001	0.50	0.176	0.058	0.014	0.002	0.014	0.007
AFB_1 × XL		<0.001	0.127	0.064	0.009	0.203	0.291	0.203	0.889

[a-b]Means with different superscripts in the same column differ significantly ($P < 0.05$).
[1]Each value represents the mean values of 6 pens of 10 animals each (n = 6).
[2]AFB_1, aflatoxin B_1; BWG, body weight gain; FI, feed intake; FCR, feed conversion ratio.
[3]− represents without AFB_1 or XL supplementation; + represents supplemented with AFB_1 or XL.
[4]SEM, standard error of the mean.
[5]*P*-values represent the main effect of AFB_1 exposure, the main effect of the dietary XL, and the interaction between AFB_1 exposure and XL treatments.

Table 3. Effects of dietary AFB_1 and mycotoxins binder on serum protein of broiler chickens.[1,2,3]

Items		TP (g/L)		ALB (g/L)		GLO (g/L)	
		21 d	42 d	21 d	42 d	21 d	42 d
Control		36.21	37.924	17.09	17.93[ab]	20.25[a]	18.54
XL		33.97	37.14	17.91	17.60[ab]	17.77[ab]	21.63
AFB_1		34.77	35.224	19.90	15.49[b]	12.98[b]	17.93
XL+AFB_1		36.25	37.90	20.72	22.16[a]	18.75[a]	19.17
SEM[4]		0.573	0.578	1.018	0.849	1.010	0.773
The main effect							
AFB_1	-	35.09	37.53	17.50	18.82	19.01	20.09
	+	35.51	36.56	20.31	17.77	15.60	18.55
XL	-	36.49	36.57	18.49	16.71	16.61	18.24
	+	35.11	37.52	19.32	19.88	18.21	20.40
P-value[5]							
AFB_1		0.719	0.406	0.193	0.480	0.092	0.330
XL		0.745	0.414	0.697	0.043	0.364	0.175
AFB_1 × XL		0.122	0.145	0.999	0.027	0.031	0.554

[a-b]Means with different superscripts in the same column differ significantly ($P < 0.05$).
[1]Each value represents the mean values of 6 pens of 10 animals each (n = 6).
[2]Abbreviations: ALB, albumin; GLO, globulin; TP, total protein.
[3]− represents without AFB_1 or XL supplementation; + represents supplemented with AFB_1 or XL.
[4]SEM, standard error of the mean.
[5]*P*-values represent the main effect of AFB_1 exposure, the main effect of the dietary XL, and the interaction between AFB_1 exposure and XL treatments.

Table 4. Effects of dietary AFB_1 and mycotoxins binder on serum immunoglobulins of broiler chickens.[1,2,3]

Items		IgG (g/L)		IgM (g/L)		IgA (g/L)	
		21 d	42 d	21 d	42 d	21 d	42 d
Control		3.96	4.24	1.65	1.57	2.25	2.16
XL		3.92	4.26	1.64	1.63	2.28	2.21
AFB_1		3.99	4.21	1.58	1.57	2.20	2.18
XL+AFB_1		4.10	4.22	1.64	1.64	2.24	2.25
SEM[4]		0.035	0.016	0.014	0.010	0.010	0.010
The main effect							
AFB_1	-	3.94	4.25	1.64	1.59	2.26	2.19
	+	4.04	4.21	1.61	1.61	2.22	2.21
XL	-	3.97	4.23	1.61	1.57	2.22	2.18
	+	4.00	4.24	1.64	1.63	2.26	2.22
P-value[5]							
AFB_1		0.14	0.291	0.209	0.597	0.015	0.365
XL		0.621	0.716	0.255	0.000	0.093	0.005
AFB_1 × XL		0.278	0.972	0.169	0.537	0.681	0.170

[a-b]Means with different superscripts in the same column differ significantly ($P < 0.05$).
[1]Each value represents the mean values of 6 pens of 10 animals each (n = 6).
[2]Abbreviations: IgG, immunoglobulin G; IgM, immunoglobulin M; IgA, immunoglobulin A.
[3]− represents without AFB_1 or XL supplementation; + represents supplemented with AFB_1 or XL.
[4]SEM, standard error of the mean.
[5]*P*-values represent the main effect of AFB_1 exposure, the main effect of the dietary XL, and the interaction between AFB_1 exposure and XL treatments.

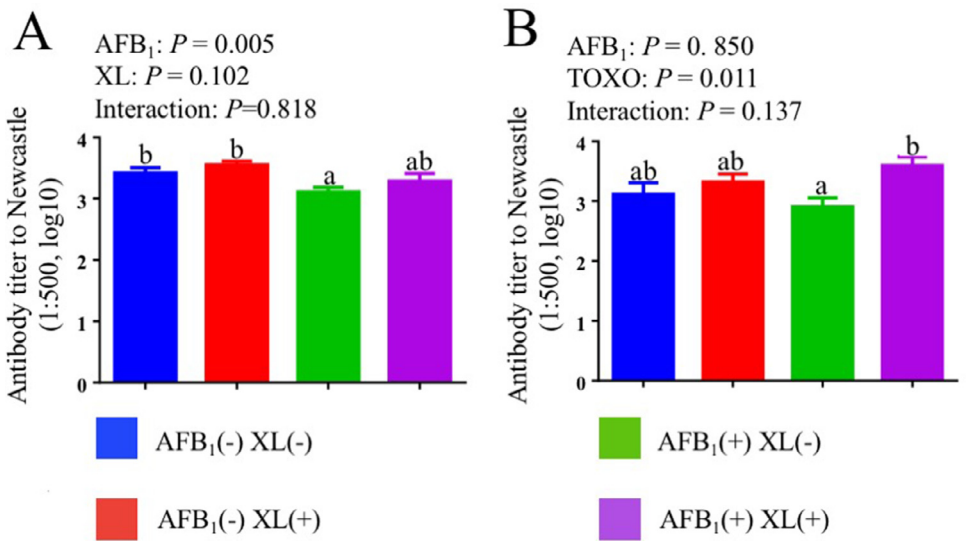

Figure 1. Effects of dietary AFB_1 and mycotoxins binder on serum antibody response to Newcastle Disease of broiler chickens at day 21 (A) and day 35 (B).[12] [a-b] Means with different superscripts in the same column differ significantly ($P < 0.05$) [1] Each value represents the mean values of 6 pens of 10 animals each (n = 6). [2] *P*-values represent the main effect of AFB_1 exposure, the main effect of the dietary XL, and the interaction between AFB_1 exposure and XL treatments.

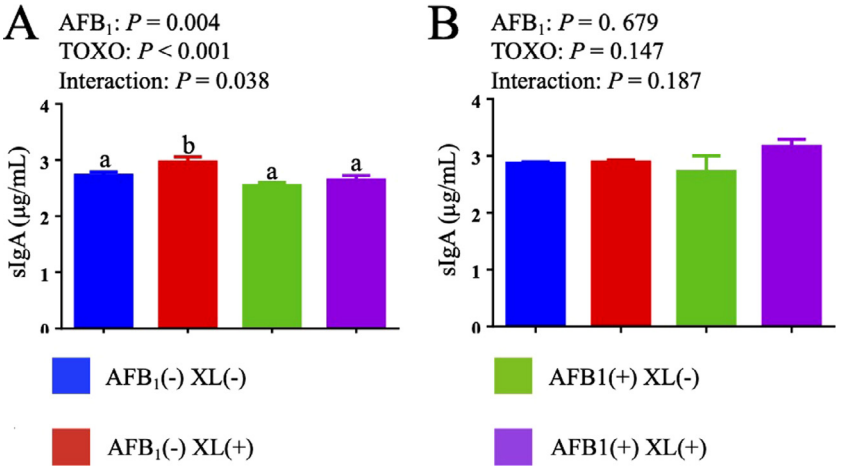

Figure 2. Effects of dietary AFB_1 and mycotoxins binder on the content of jejuna mucous sIgA of broiler chickens at 21 d (A) and 42 d (B).[123] [a-b] Means with different superscripts in the same column differ significantly ($P < 0.05$). [1] Each value represents the mean values of 6 pens of 10 animals each (n = 6). [2] sIgA = secretory immunoglobulin A. [3] *P*-values represent the main effect of AFB_1 exposure, the main effect of the dietary XL, and the interaction between AFB_1 exposure and XL treatments.

groups, the addition of XL increased antibody titer to Newcastle at 35 d ($P = 0.011$).

The Content of sIgA in Jejunal Mucous

The effects of dietary AFB_1 and mycotoxins binder addition on the content of sIgA in jejunal mucous of broiler chickens are shown in Figure 2. AFB_1 challenge significant decreased sIgA contents of jejunal mucous at 21 d ($P < 0.05$). In addition, there were significant interactions to sIgA at 21 d ($P < 0.05$) between AFB_1 exposure and XL supplementation.

mRNA Expression of Tight Junctions in Jejunum

No significant cooperative effect was observed between AFB_1 exposure and XL supplementation in terms of jejunal mucosa morphology of broiler chickens ($P > 0.05$). Neither mycotoxins binder nor AFB_1 poisoning affected villus height, crypt depth, and VH/CD in the jejunal mucosa of broiler chickens ($P > 0.05$; Table S5).

The effects of dietary AFB_1 and XL addition on tight junction gene expression in jejunum of broiler chickens are shown in Table 5. AFB_1 exposure significantly decreased *Occludin* expression ($P = 0.007$) at 21 d, and decreased *claudin-1* expression at 42 d ($P < 0.001$), while increased *claudin-2* expression at 21 d compared to contaminated groups. Compared with the non-supplemented group, the addition of XL significantly increased *claudin-1* ($P < 0.001$) at 21 d, increased *Occludin* ($P = 0.035$) expression at 42 d, decreased *claudin-2* ($P = 0.003$) gene expression at 42 d. There were significant interactions to expression of *claudin-2, claudin-3*, and *Occludin* at 42 d between AFB_1 or XL.

Table 5. Effects of dietary AFB_1 and mycotoxins binder on relative mRNA expression of tight junctions in the jejunum of broiler exposure to AFB_1.[1,2]

		Claudin-1		*Claudin-2*		*Claudin-3*		*Occludin*	
Items		21 d	42 d	21 d	42 d	21 d	42 d	21 d	42 d
Control		1.00	1.00	1.00	1.00[ab]	1.00	1.00[ab]	1.00	1.00[a]
XL		2.40	1.44	0.68	0.80[bc]	1.43	0.67[b]	1.10	1.00[a]
AFB_1		0.73	0.46	4.71	1.33[a]	1.02	0.53[b]	0.58	0.49[b]
XL+AFB_1		2.32	0.56	3.56	0.44[c]	1.38	1.28a	0.72	1.14[a]
SEM[3]		0.211	0.108	0.220	0.102	0.105	0.098	0.076	0.084
The main effect									
AFB_1	-	1.70	1.22	0.84	0.90	1.21	0.83	1.05	1.00
	+	1.53	0.51	4.13	0.89	1.20	0.90	0.65	0.82
XL	-	0.87	0.73	2.85	1.17	1.01	0.76	0.79	0.75
	+	2.36	1.00	2.12	0.62	1.40	0.97	0.91	1.07
P-value[4]									
AFB_1		0.574	<0.001	<0.001	0.930	0.941	0.675	0.007	0.211
XL		<0.001	0.091	0.203	0.003	0.071	0.220	0.372	0.035
AFB_1 × XL		0.759	0.278	0.467	0.049	0.878	0.004	0.867	0.035

[a-b]Means with different superscripts in the same column differ significantly ($P < 0.05$).
[1]Each value represents the mean values of 6 pens of 10 animals each (n = 6).
[2]– represents without AFB_1 or XL supplementation; + represents supplemented with AFB_1 or XL.
[3]SEM, standard error of the mean.
[4]*P*-values represent the main effect of AFB_1 exposure, the main effect of the dietary XL, and the interaction between AFB_1 exposure and XL treatments.

DISCUSSION

Poultry are highly sensitive to aflatoxicosis (Arafa et al., 1981; Huff et al., 1986) induced by one of the most common carcinogenic pollutants, AFB_1, in broiler feed. AFB_1 poisoning induced liver damage, serum biochemical variables imbalanced, immunity inhibition, intestinal dysfunction, and inflammation increase the susceptibility of broilers to diseases (Yuan et al., 2016; Kraieski et al., 2017; Zhang et al., 2019; Rosim et al. 2020), suppress the broiler production (Rashidi et al., 2020) and increase the morbidity and mortality of broilers, resulting in the increase of poultry breeding cost (Bintvihok and Kositcharoenkul, 2006). In accord with previous studies (Denli et al., 2009; Liu et al., 2018a), our results also showed that 200 μg/kg AFB_1 in diets significantly reduced the body weight gain and feed intake of Arbor Acres broiler chickens, increased feed conversion ratio after the first 21 days of feeding, but caused a nonsignificant mortality difference, declaring subclinical aflatoxicosis occurred to birds in early stage.

Mycotoxins binders have been reported to be the promising, effective, economical approach counteracting with contamination induced by AFB_1. In the current study, addition of 2 g/kg commercial mycotoxins binder (**XL**) containing smectite binding agent, specific glucose biopolymers and activated β-1,3/1,6-glucans showed no significant effect on growth performance. These results were inconsistent with some previous study (Liu et al., 2018a), but in accordance with others (Morales-López et al., 2009; Liu et al., 2018c). The different effects of adsorbents on production performance may be largely attributable to inclusion concentration of binders and AFB_1, varied quality, and poultry breeds (Zhao et al., 2010, 2021; Liu et al., 2018a).

The spleen, bursa of Fabricius, and thymus are important components of the immune system of poultry, and these organ indexes can indirectly evaluate the immune status of poultry. While the current results showed no significant effect of AFB_1 on immune organ index (spleen and bursa of Fabricius), which was confirmed by previous study (Gómez-Espinosa et al., 2017). Gómez-Espinosa et al. (2017) reported that 6-day-old Turkey fed with diet exposed to AFB_1 and AFB_2 for 2 weeks, results show that no significant relative spleen and bursa of Fabricius weight changes were noted, but severe depletion of lymphoid cells in the bursa of Fabricius and spleen were observed from histopathological examination, indicating sensitivity of lymphoid organs to AFB_1. This also means that 200 μg/kg AFB_1 may have caused organ damage in broilers, but the damage was not severe enough to cause organ index change. Additionally, supplementation of 2g/kg mycotoxins binders also did not affect immune organ weight change in current study. It was documented that inclusion of 3g/kg yeast cell walls improved the relative weights of Fabricius and thymus ($P < 0.01$; Zhang et al., 2012). It is speculated the dose of mycotoxins binders used in this study was not enough to cause significant change of immune organ index.

The spleen is the peripheral immune organ of poultry and can produce a variety of antibodies. It is primarily responsible for immune response and plays an anti-inflammatory and protective role. Proinflammatory cytokine *IL-6* can induce the immune response, alleviate the damage caused by toxins to the body and actively release other inflammatory mediators (Wei et al., 2012). In this study, AFB_1 increased the mRNA expression level of the proinflammatory factor *IL-6*, further confirming the results of previous studies (Long et al., 2016). Although low dose AFB_1 in this study could not change the spleen index, it may damage the spleen by increasing and induce spleen inflammation (Meissonnier et al., 2008). However, adding XL to the diet can inhibit the high expression of *IL-6* induced by AFB_1, suggesting that XL may relieve spleen injury by regulating the expression of pro-inflammatory factor genes.

Liver is the main organ for toxin metabolism in poultry, which can promote detoxification and protect the body from toxicity (Fouad and El-Senousey, 2014). AFB_1 has strong hepatotoxicity in a dose-dependent manner (Yarru et al., 2009), which can cause liver lipid metabolism imbalance, promote lipid deposition in the liver and lead to liver enlargement (Siloto et al., 2013). In addition, it can inhibit the activity of antioxidant enzymes and secretion of anti-inflammatory cytokines induces lipid peroxidation and secretion of pro-inflammatory cytokines, and finally leads to liver cell damage and necrosis (Wang et al., 2019). The results showed that the mRNA expression levels of proinflammatory cytokines *IL-1β* and *IL-6* in the liver of broilers were significantly increased compared with those in the control group, but the organ index was not significantly changed. The results were consistent with those in the spleen, suggesting that low dose AFB_1 can cause inflammatory response in the liver of broilers, but its structure and function were less damaged. mycotoxins binder was found to relieve liver inflammation by downregulating *IL-1β* and *IL-6* mRNA levels compared to control levels. In addition, mycotoxins binder can also regulate the mRNA expression level of liver *CYP3A4*, which is the isoenzyme of CYP450s, mainly exists in the liver of animals and plays a major role in activation and he metabolism of AFB_1 (Crespi et al., 1991; Shen et al., 1996). It can participate in the metabolic activation of AFB_1 to produce the ultimate carcinogenic intermediate, (AFB_1-8,9-epoxide, AFBO) which interacts with macromolecules, particularly DNA (Swenson et al., 1977), thereby destroying the cell structure, causing cell damage and apoptosis (Fasullo et al., 2017). In addition, reactive oxygen species generated during metabolism can also cause oxidative damage to cells, further exacerbating the hepatotoxicity of AFB_1 (Deng et al., 2018). The expression level of *CYP3A4* in broilers' liver was decreased after inclusion of mycotoxins binder to the diet, indicating that mycotoxins binder could inhibit the

activity of *CYP3A4*, and thus alleviate the toxic effect of AFB_1 metabolites on broilers.

Liver is the main site of protein synthesis and metabolism in the body, and concentrations of serum total protein, albumin and globulin, in part, reflect hepatic injury and function (Abdel-Wahhab et al., 1999; Mathur et al., 2001). Compared with the control group, the serum globulin content of broilers in AFB_1 group tended to drop which proved again that liver damage of broilers would be caused by AFB_1 contamination level of 200 μg/kg in the diet. Inclusion of XL increased serum ALB, indicating its possible role as immunopotentiator and liver protector.

As indicators of humoral immune responses, serum immunoglobulin such as IgG, and IgM, IgA are determined frequently. Consistent with the reduced tendency observed in serum protein contents in birds exposed to AFB_1 in this study, IgA reduction was also found, which confirmed previous reports (Chen et al., 2014). Not only in chicken, AFB_1-contaminated diets were found a significant effect on titer serum concentrations of the IgG, and IgM, IgA in mice (Long et al., 2016), pigs (Marin et al., 2002), dairy cow (Xiong et al., 2018), and human (Turner et al., 2003). While dietary supplementation with XL increased serum ALB and IgM, IgA, which in agreement with previous studies, indicating components in XL can improve immune response in chicks (Liu et al., 2018a).

Epidemiological data showed that the outbreak of Newcastle disease in broiler chickens was highly correlated with AF contamination of feed (Yunus et al., 2009). In our study, diet contaminated with 200 μg/kg AFB_1 significantly reduced serum Newcastle disease antibody titer of broilers which confirms previous reports (Bagherzadeh Kasmani et al., 2012), indicating that AFB_1 reduced the immune response of broilers Newcastle disease vaccine. In addition, it was also observed a significant reduction of the content of sIgA in jejunal mucosa in birds exposed to AFB_1, and the results of this study on humoral and mucosal immunity were consistent with previous studies (Jiang et al., 2015; Liu et al., 2017). Interestingly, the serum anti-Newcastle disease titer of broilers increased after the addition of mycotoxins binder in the diet, indicating that mycotoxins binder could relieve the suppression of AFB_1 on the immune function of broilers.

The physical barrier in small intestinal mucosa, consisting of intestinal epithelial cells, intestinal mucus, commensal bacteria, sIgA, gut-associated lymphoid tissue, refers to the total structure and function of the intestine that can prevent hazardous substances such as harmful bacteria and toxins from passing through the intestinal mucosa and entering other tissues, organs and blood circulation in the body (Barbara, 2006; Branca et al., 2019). sIgA, secreted by IgA plasma cells in the intestinal mucosa, is one of the most abundant immunoglobulins on mucosal surfaces, plays a vital role in protecting the mucosal surfaces against pathogens and maintaining homeostasis with the commensal microbiota (Salerno-Goncalves et al., 2016; He et al., 2020, Rochereau et al., 2021). Tight junctions (**TJs**) largely determine the barrier function and the baseline level of inflammation in the body, which is correlated to intestinal permeability (Suzuki, 2013). TJ permeability is partly determined by the properties of claudins (Barrett, 2020). The claudin family is a key factor in determining the characteristics of paracellular transfer pathways, forming a tightly connected skeleton with proteins such as occludin, which can maintain the barrier function of intestinal mucosa (Grenier and Applegate, 2013). Claudin-1, a key constituent of the tight junction complex, maintains the integrity of the paracellular barrier and regulates water homeostasis. Claudin-1 has been shown to be dysregulated in inflammatory bowel disease (Kucharzik et al., 2001; Weber et al., 2008). Claudin-2, known as a channel-forming TJ protein permeable to small cations and water, is distinctively upregulated in most inflammatory and infectious diseases of the intestine indicator (Rosenthal et al., 2010; Zhang et al., 2013) . Claudin-3 strongly associated with intestinal barrier disruption, is a potential marker for intestinal barrier failure induced (Lu et al., 2013; Shim et al., 2015). Occludins, together with claudins, interacts with each other on their extracellular sides to promote junction assembly (Costantini et al., 2009). Therefore, the secretion of sIgA and the mRNA profiles of *claudin-1, claudin-2, claudin-3*, and *occludin* can directly reflect the intestinal mucosal barrier function to an extent.

Our results demonstrated that AFB_1 exposure decreased sIgA and mRNA expression of *claudin-1* and *occludin*, increase *claudin-2* expression, which is consistent with previous findings (Liu et al., 2018b). Previous study reports that broilers' small intestinal mucosa could be damaged under long-term exposure and low-level of AFB_1 contamination (Kana et al., 2010), and the intestinal mucosal barrier also be damaged, following by the loss of intestinal mucosal barrier function (Chen et al., 2016). Impaired intestinal mucosal barrier will lead to increased intestinal permeability and bacterial migration (Maresca et al., 2008). However, inclusion of XL to the diet could increase the mRNA expression level of *claudin-1* and *Occludin*, decrease *claudin-2* expression in broilers jejunum, indicating that XL can maintain the integrity of the tight junction of broilers' intestinal tract, and thus enhance the function of intestinal mucosal barrier in broilers to some extent. This result displays similar effect of dietary XL on laying hens (Zhao et al., 2021).

CONCLUSIONS

In summary, broiler chickens fed with diet contaminated with 200 μg/kg AFB_1 presented poor growth performance, especially in early stage. These adverse effects of aflatoxicosis may closely associate with impaired liver, immune dysfunction, inflammation of immune organs and gut barrier induced by AFB_1. Even though the supplementation of 2 g/kg mycotoxins binder did not

significantly counteract the negative production outcome induced by AFB_1, could alleviate the liver impairment and immune suppression, enhance the intestinal mucosal barrier function and improve intestinal health by improving serum protein and immunoglobulin levels, the titer of the antibody to Newcastle disease, down-regulating the expression of *CYP3A4* in liver and pro-inflammatory factor in liver and spleen, up-regulating the expression of *claudin-1* and *occludin* and decreasing *claudin-2* gene expression and increase sIgA secretion in jejunum. However, further studies are needed to explore the protective roles of dietary mycotoxins binder supplementation on AFB_1 challenges in broilers.

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Author contributions: **Yujiao Lai, Meng sun, Bingkun Zhang and Yuming Guo** conceived and designed the experiments; Meng sun and Jiaqi Lei performed the experiments; **Yujiao Lai, Meng sun,** Jiaqi Lei and Yang he analyzed the data; Yujiao Lai and Mengsun wrote the paper, **Bingkun Zhang, Yang He, Yanming Han, Yuanyuan Wu and Dongying Bai reviewed and edited**. All authors have read and agreed to the published version of the manuscript. All authors have read and agreed to the published version of the manuscript.

Data availability statement: The datasets used and/or analyzed during the current study are publicly available.

DISCLOSURES

All authors declare that there are no conflicts of interest.

SUPPLEMENTARY MATERIALS

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