# Compound mycotoxin detoxifier alleviating aflatoxin B₁ toxic effects on broiler growth performance, organ damage and gut microbiota

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**ABSTRACT** The aim of this study was to evaluate the effects of compound mycotoxin detoxifier (**CMD**) on alleviating the toxic effect of aflatoxin B₁ (**AFB₁**) for broiler growth performance. One-kilogram CMD consists of 667 g aflatoxin B₁-degrading enzyme (**ADE**, 1,467 U/g), 200 g montmorillonite and 133 g compound probiotics (**CP**). The feeding experiment was divided into 2 stages (1−21 d and 22−42 d). In the early stage, a total of 300 one-day-old Ross broilers were randomly divided into 6 groups, 5 replications for each group, 10 broilers (half male and half female) in each replication. In the later feeding stage, about 240 twenty-two-day-old Ross broilers were randomly divided into 6 groups, 8 replications for each group, 5 broilers in each replication. Group A: basal diet; group B: basal diet with 40 μg/kg AFB₁; group C: basal diet with 1 g/kg CMD; groups D, E, and F: basal diet with 40 μg/kg AFB₁ plus 0.5, 1.0 and 1.5 g/kg CMD, respectively. The results indicated that AFB₁ significantly decreased average daily gain (**ADG**), protein metabolic rate, organ index of thymus, bursa of Fabricius **(BF)**, superoxide dismutase (**SOD**), glutathione peroxidase (**GSH-PX**) and catalase activities in serum, and increased AFB₁ residues in serum and liver ($P < 0.05$). Hematoxylin-Eosin (**HE**) staining analysis of jejunum, liver and kidney showed that AFB₁ caused the main pathological changes with different degrees of inflammatory cell infiltration. However, CMD additions could alleviate the negative effects of AFB₁ on the above parameters. The gut microbiota analysis indicated that AFB₁ could significantly increase the abundances of *Staphylococcus-xylosu, Esherichia-coli-g-Escherichia-Shigella*, and decrease *Lactobacillus-aviarius* abundance ($P < 0.05$), but which were adjusted to almost the same levels as the control group by CMD addition. The correlative analysis showed that *Lactobacillus-aviarius* abundance was positively correlated with ADG, SOD and BF ($P < 0.05$), whereas *Staphylococcus-xylosus* abundance was positively correlated with AFB₁ residues in serum and liver ($P < 0.05$). In conclusion, CMD could keep gut microbiota stable, alleviate histological lesions, increase growth performance, and reduce mycotoxin toxicity. The optimal CMD addition should be 1 g/kg in AFB₁-contaminated broilers diet.

**Key words:** detoxifier, aflatoxin B₁, broiler, organ damage, gut microbiota

## INTRODUCTION

Aflatoxins (**AF**) B₁, B₂, G₁, and G₂ are produced by *Aspergillus flavus* and *Aspergillus parasiticus*, among which aflatoxin B₁ (**AFB₁**) holds the highest toxicity (Liu et al., 2020). AFB₁ is well known for its teratogenic, carcinogenic, and immunosuppressive effects on human

and animals, consumption of AFB₁-contaminated feeds by poultry may decrease feed intake and growth performance as well as increase intestinal damage and mortality (Fan et al., 2021). Liver is the target organ for AFB₁ to induce liver injury involving inflammation, oxidative stress and DNA damage caused by metabolic enzymes (Murcia and Diaz, 2021). The previous study has shown that in the state of inflammation and stress, muscle protein synthesis will be decreased, and energy will be mobilized to support immunity, resulting in poor growth and immunosuppression (Zheng et al., 2021).

The damage of AFB₁ to broilers is multifaceted and comprehensive. The damage degree of AFB₁ to the organism has a linear relationship with AFB₁ content in

chicken diet. When dietary $AFB_1$ concentration was less than 100 $\mu$g/kg, it was mainly manifested as growth retardation, low nutrient digestibility and disturbed intestinal microbiota (Ortatatli et al., 2005); when dietary $AFB_1$ concentration was above 100 $\mu$g/kg, microscopic anatomical defect of internal organization began to occur except for the low growth performance (Hernández-Ramírez et al., 2020). Visceral organs (liver and kidney) presented the substantial damage when dietary $AFB_1$ concentration was higher than 1,000 $\mu$g/kg (Śliżewska et al., 2019). However, it is difficult to determine the quantization relationship because it is affected by many factors such as broiler breeds, $AFB_1$ resources and feeding managements (Bryden, 2001). The investigation report of animal feed pollution in China from 2018 to 2020 showed that the detective rate of $AFB_1$ was 99.6%, and the maximal concentration was 54 $\mu$g/kg (Zhao et al., 2021a). According to $AFB_1$ threshold in broiler diets (10−20 $\mu$g/kg) in China and Europe, growth retardation will be induced by more than 10 $\mu$g/kg $AFB_1$, which will restrict the healthy development in broiler industry (Zhao et al., 2021a).

Physical adsorbent such as montmorillonite has been reported to absorb $AFB_1$ to alleviate its toxic effects (Mao et al., 2022). Biological methods such as microorganisms and enzymes have good applications in the degradation of mycotoxin. Microorganisms including *Lactobacillus, Yeast* and *Bacillus* can degrade mycotoxin by adsorption and fermentation degradation (Liu et al., 2020). Some enzymes such as laccase and aflatoxin-degrading enzyme can degrade mycotoxin effectively (Liu et al., 2021). In addition, the combination of different antidotes (bentonite, yeast cell wall and glucose biopolysaccharides) has been proven to have synergistic effect for attenuating the negative effects of $AFB_1$ on production performance, immunological function and intestinal health in broilers and laying hens (Zhao et al., 2021b; Lai et al., 2022). A published report has confirmed $AFB_1$-degrading efficacy in broilers by combining probiotics and aflatoxin $B_1$-degrading enzyme (**ADE**) (Chang et al., 2020). However, few data have been reported about the compound mycotoxin detoxifier (**CMD**) which contained ADE, montmorillonite, and compound probiotics (**CP**) application in alleviating $AFB_1$ toxic effects on broilers performance, histopathology, and gut microbiota.

Our preliminary results showed that a triple-action compound mycotoxin detoxifier (**CMD**) could effectively degrade $AFB_1$ *in vitro*, which contained $AFB_1$-degrading enzyme, montmorillonite and CP consisting of *Lactobacillus casein, Bacillus subtilis, Candida utilis* and *Enterococcus faecalis* (Guo et al., 2021a); the further research indicated that CMD without montmorillonite could alleviate cell cytotoxicity and inflammation induced by $AFB_1$ exposure through suppressing the activations of nuclear factor kappa B p65 (**NF-$\kappa$B**), inducible nitric oxide synthase (**iNOS**), nucleotide-binding oligomerization domain containing 1 (**NOD1**) and toll like receptor 2 (**TLR2**) pathways in chicken embryo primary intestinal epithelium, liver and kidney cells

(Guo et al., 2021b). The aim of this research was to investigate the effects of CMD on growth performance, gut microbiota, and tissue injury of broilers in high-$AFB_1$-level (40 $\mu$g/kg) diet.

## MATERIALS AND METHODS

### Materials Preparation

The moldy corn containing 72.14 $\mu$g/kg $AFB_1$, 85.71 $\mu$g/kg zearalenone (**ZEA**) and 147.22 $\mu$g/kg deoxynivalenol (**DON**) was purchased from the market in Henan Province, China. The mycotoxin contents were determined by ELISA method (R-Biopharm, Darmstadt, Germany) according to manufacturer's standard instructions. According to the calculation of dietary formulation for broilers, all the normal corn meals in the basal diet were replaced by the moldy corn meals to make the diets with high-level $AFB_1$, in which the dietary $AFB_1$ concentration was determined as 40 $\mu$g/kg. Montmorillonite was provided by Henan Delin Biological Product Co., Ltd. Xinxiang, China.

*Aspergillus oryzae, Lactobacillus casein, Bacillus subtilis, Candida utilis*, and *Enterococcus faecalis* were purchased from China General Microbiological Culture Collection Center (**CGMCC**). *Bacillus subtilis* was inoculated in LB medium (g/L): peptone 10 g, yeast extract 5 g, NaCl 10 g, pH 7.0, and cultured in a rotary shaker with 180 rounds per min (**rpm**) at 37°C for 24 h. *Lactobacillus casein* and *Enterococcus faecalis* were inoculated in MRS medium (g/L): peptone 10 g, yeast extract 10 g, glucose 20 g, Tween 80 1 mL, $K_2HPO_4$ 2 g, sodium acetate 5 g, sodium citrate 2 g, $MgSO_4$ 0.2 g, $MnSO_4$ 0.05 g, pH 6.20 to 6.60, cultured statically at 37°C for 24 h. *Candida utilis* was inoculated in YPD medium (g/L): yeast extract 10 g, peptone 20 g, glucose 20 g, cultured at 30°C for 24 h in 180 rpm shaker. After incubation, 4 species of microbes were placed statically for 2 h, and then the supernatant was removed. Skimmed milk powder, trehalose dihydrate, sodium glutamate and silica were added and mixed for freeze-drying. The microbial counts were expressed as colony forming units per gram (CFU)/g.

$AFB_1$-degrading enzyme (**ADE**) was prepared from *Aspergillus oryzae*. The *A. oryzae* incubation was prepared as follows: *A. oryzae* spores were scraped off from the incubating plate with sterilized normal saline, and its concentration was adjusted to 1 × $10^8$ spores/mL. The solid-state medium formula was as the following(w/w): the ratio of wheat bran, corn meal and soybean meal were 7:1:2, 15 g sample was taken, mixed with 9 mL distilled water, put in a 250 mL triangle bottle, autoclaved at 121°C for 30 min, and then cooled to room temperature. The medium was inoculated with 2 mL of the above spore fluid, incubated at 30°C for 5 d, and then dried. The activity of $AFB_1$-degrading enzyme was 1,467 U/g. Enzyme activity was defined as the following: the amount of enzyme that could degrade 1 ng $AFB_1$ per min at pH 8.0 and 37°C was defined as one unit (Guo et al., 2021a). One kilogram of CMD consisted of

667 g ADE, 200 g montmorillonite and 133 g CP in which the visible counts of *Bacillus subtilis, Lactobacillus casein, Enterococcus faecalis* and *Candida utilis* were $1.0 \times 10^8$, $1.0 \times 10^8$, $1.0 \times 10^{10}$, and $1.0 \times 10^8$ CFU/g, respectively.

## Animals and Managements

The feeding experiment was divided into 2 stages (1 −21 d and 22−42 d). In the early stage, a total of 300 one-day-old Ross broilers were randomly divided into 6 groups, 5 replications for each group, 10 broilers (half male and half female) in each replication. In the later feeding stage, about 240 twenty-two-day-old Ross broilers were randomly divided into 6 groups, 8 replications for each group, 5 broilers in each replication. All animals used in this experiment were managed according to the guidelines of Animal Care and Use Ethics Committee in Henan Agricultural University (SKLAB-B-2010-003-01). All husbandry practices and euthanasia were performed with full consideration of animal welfare. The broilers were fed in the multi-layer cages with 24-h light and natural ventilation. The mesh diets and water were given to the birds ad libitum. The immune process was conducted with bivalent vaccine at the age of 7 d and Newcastle vaccine at the age of 21 d. The feed compositions and nutrient levels in the earlier and later stage of broilers are presented in Tables 1 and 2, respectively. The feeding experiment was designed as follows:

Group A: Basal diet containing 4.31 $\mu$g/kg $AFB_1$
Group B: Basal diet with 42.15 $\mu$g/kg $AFB_1$
Group C: Group A added with 1 g/kg CMD
Group D: Group B added with 0.5 g/kg CMD
Group E: Group B added with 1.0 g/kg CMD
Group F: Group B added with 1.5 g/kg CMD

(The proportion of moldy corn meal substituting for normal corn meal in diets was 57% in groups B, D, E, and F).

## Determinations of Nutrient Metabolic Rates

At the 17 to 19th d and 37 to 39th d during feeding experiments, metabolic experiment was performed using total excreta collection method. The plastic sheet was placed under the cages in 5 replications in each group to collect the excreta in both stages, which was collected for 3 d by changing the plastic sheets every day. Fresh excreta was collected without containing dander, feather, and feed, then mixed and added with 10% sulfuric acid to fix nitrogen, frozen at -20°C in refrigerator every day. The excreta samples of each replication from 3-d collections were mixed, dried and ground. Crude protein, crude fat, calcium and phosphorus contents in diet and excreta were determined with Kjeldahl, ether extract, potassium permanganate ($KMnO_4$) and ammonium molybdate ($(NH_4)_6Mo_7O_{24}$), respectively (Caldas et al., 2018). The calculation of apparent nutrient metabolic rate was made as follows: Nutrient apparent metabolic rate

**Table 1.** Feed compositions and nutrient levels in the earlier stage of broilers (%).

| Groups | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| Corn meal | 58.50 | 1.50 | 58.50 | 1.50 | 1.50 | 1.50 |
| Soybean meal | 33.00 | 33.00 | 33.00 | 33.00 | 33.00 | 33.00 |
| Moldy corn meal | 0.00 | 57.00 | 0.00 | 57.00 | 57.00 | 57.00 |
| Fish meal | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 |
| Soybean oil | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 |
| $CaCO_3$ | 1.40 | 1.40 | 1.40 | 1.40 | 1.40 | 1.40 |
| Dicalcium phosphate | 1.40 | 1.40 | 1.40 | 1.40 | 1.40 | 1.40 |
| Methionine | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 |
| Salt | 0.30 | 0.30 | 0.30 | 0.30 | 0.30 | 0.30 |
| Premix | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 |
| Choline chloride | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 |
| CMD | 0 | 0 | 0.10 | 0.05 | 0.10 | 0.15 |
| Total | 100 | 100 | 100 | 100 | 100 | 100 |
| ME(MJ/kg) | 12.43 | 12.43 | 12.4 | 12.43 | 12.43 | 12.43 |
| CP | 20.51 | 20.48 | 20.50 | 20.51 | 20.49 | 20.48 |
| Fat | 3.61 | 3.62 | 3.65 | 3.62 | 3.61 | 3.62 |
| Ca | 0.98 | 0.97 | 0.99 | 0.99 | 0.97 | 0.98 |
| TP | 0.64 | 0.65 | 0.66 | 0.64 | 0.65 | 0.66 |
| AP | 0.46 | 0.46 | 0.46 | 0.46 | 0.46 | 0.46 |

Note: Premix of vitamins and minerals provided per kilogram of diet: VA 12 000 IU; $VD_3$ 3,000 IU; VE 20 IU; $VK_3$ 1.0 mg; $VB_1$ 2.0 mg; $VB_6$ 3.5 mg; $VB_{12}$ 0.01 mg; Cu (as copper sulfate) 8 mg; Fe (as ferrous sulfate) 100 mg; Mn (as manganese sulfate) 80 mg; Zn (as zinc oxide) 60 mg; I (as calcium iodate) 0.45 mg; Se (as sodium selenite) 0.35 mg; Biotin 0.15 mg; Folic acid 1.25 mg; $VB_2$ (riboflavin) 6 mg; Nicotinic acid 35 mg; Calcium pantothenate 10 mg. The contents of crude protein (CP), fat, calcium, (Ca), and total phosphorus (TP) were determined, and the others were calculated. One kilogram of CMD consisted of 667 g aflatoxin $B_1$-degrading enzyme (ADE), 200 g montmorillonite and 133 g compound probiotics (CP) in which the visible counts of *Bacillus subtilis, Lactobacillus casein, Enterococcus faecalis*, and *Candida utilis* were $1.0 \times 10^8$, $1.0 \times 10^8$, $1.0 \times 10^{10}$ and $1.0 \times 10^8$ CFU/g, respectively.

**Table 2.** Feed compositions and nutrient levels in the later stage of broilers (%).

| Groups | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| Corn meal | 65.50 | 8.50 | 65.50 | 8.50 | 8.50 | 8.50 |
| Soybean meal | 27.00 | 27.00 | 27.00 | 27.00 | 27.00 | 27.00 |
| Moldy corn meal | 0.00 | 57.00 | 0.00 | 57.00 | 57.00 | 57.00 |
| Fish meal | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| Soybean oil | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 |
| $CaCO_3$ | 1.30 | 1.30 | 1.30 | 1.30 | 1.30 | 1.30 |
| Dicalcium phosphate | 1.40 | 1.40 | 1.40 | 1.40 | 1.40 | 1.40 |
| Methionine | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 |
| Salt | 0.30 | 0.30 | 0.30 | 0.30 | 0.30 | 0.30 |
| Premix | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 |
| Choline chloride | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 |
| CMD | 0.00 | 0.00 | 0.10 | 0.05 | 0.10 | 0.15 |
| Total | 100 | 100 | 100 | 100 | 100 | 100 |
| ME, MJ/kg | 12.66 | 12.66 | 12.66 | 12.66 | 12.66 | 12.66 |
| CP | 17.90 | 17.91 | 17.92 | 17.90 | 17.92 | 17.91 |
| Fat | 5.93 | 5.92 | 5.91 | 5.91 | 5.93 | 5.91 |
| Ca | 0.89 | 0.88 | 0.89 | 0.89 | 0.88 | 0.90 |
| TP | 0.60 | 0.59 | 0.61 | 0.59 | 0.60 | 0.61 |
| AP | 0.42 | 0.42 | 0.42 | 0.42 | 0.42 | 0.42 |

Note: Premix of vitamins and minerals provided per kilogram of diet: VA 12 000 IU; $VD_3$ 3,000 IU; VE 20 IU; $VK_3$ 1.0 mg; $VB_1$ 2.0 mg; $VB_6$ 3.5 mg; $VB_{12}$ 0.01 mg; Cu (as copper sulfate) 8 mg; Fe (as ferrous sulfate) 100 mg; Mn (as manganese sulfate) 80 mg; Zn (as zinc oxide) 60 mg; I (as calcium iodate) 0.45 mg; Se (as sodium selenite) 0.35 mg; Biotin 0.15 mg; Folic acid 1.25 mg; $VB_2$ (riboflavin) 6 mg; Nicotinic acid 35 mg; Calcium pantothenate 10 mg. The contents of crude protein (CP), fat, calcium, (Ca), and total phosphorus (TP) were determined, and the others were calculated. One kilogram of CMD consisted of 667 g aflatoxin $B_1$-degrading enzyme (ADE), 200 g montmorillonite and 133 g compound probiotics (CP) in which the visible counts of *Bacillus subtilis, Lactobacillus casein, Enterococcus faecalis*, and *Candida utilis* were $1.0 \times 10^8$, $1.0 \times 10^8$, $1.0 \times 10^{10}$, and $1.0 \times 10^8$ CFU/g, respectively.

(%) = 100 × (nutrient content in diet - nutrient content in excreta)/nutrient content in diet.

### Determination of Antioxidant Indexes in Serum

At the end of feeding experiment, 3 male broilers with average body weight from groups A, B, C, and F were selected to be sacrificed, respectively. The blood samples, organs and jejunal contents were collected for further analysis.

The superoxide dismutase (**SOD**), catalase (**CAT**), glutathione peroxidase (**GSH-PX**), total antioxidant capacity (**T-AOC**), and malondialdehyde (**MDA**) contents in serum were detected as antioxidant indexes. SOD activity was analyzed by monitoring inhibition rate of reducing nitroblue tetrazolium (**NBT**). GSH-PX, CAT, and MDA were determined according to the former method (Shi et al., 2006). T-AOC determination was based on measuring the fluorescence decay of R-Phycoerythrin induced by the peroxyl radicals obtained with thermal decomposition of 2,2'-azobis[2-methylpropionamidine] dihydrochloride (Zuo et al., 2013).

### Organ Index and AFB$_1$ Residues Analyses and Hematoxylin−Eosin (HE) Staining

Three samples of liver, kidney, thymus, spleen and bursa of Fabricius in groups A, B, C, and F were selected for the assays of organ indexes. The calculation of organ index was made as follows: Organ index = organ weight / live weight; intestinal length index = intestinal length / live weight.

The tissue samples of small intestine, liver, kidney, and muscle were prepared for AFB$_1$ residue detection as the following: 5 g tissues were added with 25 mL 70% methanol (v/v), homogenized by an Ultra Turrax (T10, IKA Instrument Company, Staufen, Germany) at 10,000 rpm for 2 min. The following procedure was conducted (Ghali et al., 2008).

The same parts of jejunum, liver and kidney were taken, cut at 2 cm$^3$, cleaned with physiological saline, and dried with filter paper. The samples were fixed in 10% formalin, dehydrated in ethanol, embedded in paraffin, cut at thickness of 0.6 $\mu$m, stained with Hematoxylin and Eosin, trans-parented with xylene, encased in neutral resin and then observed with a microscope.

### Intestinal Microbiota Analysis

Three samples of jejunal contents were selected for microbiota analysis in groups A, B, C and F. The total genomic DNA of each sample was extracted by DNA Kit (Omega Biotek, Norcross, GA) according to manufacturer's instructions. The V3 and V4 variable regions of the bacterial 16S rRNA genes were amplified by the universal primer pairs: 338F (5′-ACTCCTACGGGAGG-CAGCA-3′) and 806R (5′-GGACTACHVGGGTWTC TAAT-3′). Reaction conditions consisted of an initial 95° C for 3 min followed by 27 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 45 s, and a final extension of 72°C for 10 min. PCR reactions were conducted in 20 $\mu$L reaction mixtures containing 10 ng template DNA, 0.8 $\mu$L each primer (5 $\mu$M), 2 $\mu$L 2.5 mM dNTPs, 0.4 $\mu$L FastPfu polymerase, and 4 $\mu$L 5 × FastPfu buffer. The PCR products were extracted with 2% agarose gels and purified using the AxyPrep DNA Extraction Kit (Axygen Biosciences, CA), and then quantified using QuantiFluor-ST (Promega Corporation, Madison, WI). The purified amplifications were pooled in equimolar and paired-end sequenced on an Illumina platform (Biomarker Technology Co., Ltd., Beijing, China).

### Bioinformatics Analysis

The high-quality tags were clustered into the operational taxonomic units (**OTUs**) using UPARSE (version 7.1, http://drive5.com/uparse/) with a similarity threshold of 0.97. The phylogenetic affiliation of each 16S rRNA gene sequence was analyzed using the RDP Classifier (https://rdp.cme.msu.edu/) at 0.80 confidence level. The coverage percentage was calculated by Good's method (1953).

### Statistical Analyses

Experimental data were sorted out by Excel and the experimental data expressed as means and standard errors (means ± SE). The data were analyzed using the ANOVA procedures and Duncan's multiple comparisons of the SPSS software (SPSS17.0). Differences were considered statistically significance at $P < 0.05$.

### RESULTS

### Effects of CMD on Alleviating AFB$_1$ Damage for Production Performance of Broilers

Table 3 indicated that ADG in group B was significantly lower than that in groups A and C in the first feeding stage ($P < 0.05$). The F/G in group D was the highest and that in group B was the lowest among the 6 groups ($P < 0.05$). The mortality was 2% and 4% in groups B and D, respectively.

In the second feeding stage, ADG in group B was significantly lower than that in group F ($P < 0.05$); with the addition of CMD increasing, ADG was gradually increased but without significant difference ($P > 0.05$). The mortality was 5% and 2.5% in group B and D, respectively. Based on the above comprehensive analysis, more than 1.0 g/kg CMD addition in broiler diet with 40 $\mu$g/kg AFB$_l$ could remove AFB$_l$ hazards completely.

### Effects of AFB$_1$ and CMD on Nutrient Metabolic Rate of Broilers

Table 4 indicated that crude protein metabolic rate in group B was significantly lower than that in the other groups in both feeding stages except for group D in the

**Table 3.** Effects of AFB$_1$ and CMD on production performance of broilers.

| Stages | Items | Group A | Group B | Group C | Group D | Group E | Group F |
|---|---|---|---|---|---|---|---|
| 1−21 d | | | | | | | |
| | IBW, g | 50.31 ± 0.57 | 50.12 ± 0.61 | 50.02 ± 0.48 | 50.14 ± 0.61 | 50.09 ± 0.53 | 50.13 ± 0.48 |
| | FBW, g | 922.02 ± 30.98 | 842.45 ± 68.76 | 980.17 ± 66.05 | 825.18 ± 68.15 | 847.17 ± 59.33 | 890.25 ± 43.91 |
| | ADFI, g | 61.56 ± 5.42[a] | 51.89 ± 2.35[b] | 62.15 ± 2.51[a] | 59.31 ± 2.63[a] | 58.83 ± 4.87 [a] | 59.21 ± 3.57[a] |
| | ADG, g | 41.51 ± 1.26[ab] | 37.73 ± 3.03[c] | 44.43 ± 1.18[a] | 36.77 ± 1.89[bc] | 37.94 ± 2.38[bc] | 39.97 ± 0.8[bc] |
| | F/G | 1.48 ± 0.10[b] | 1.37 ± 0.22 [c] | 1.39 ± 0.04[c] | 1.61 ± 0.09[a] | 1.55 ± 0.12 [b] | 1.48 ± 0.09[b] |
| | Mortality, % | 0 | 2 | 0 | 4 | 0 | 0 |
| 22−42 d | | | | | | | |
| | IBW, g | 910.37 ± 13.15 | 909.18 ± 34.37 | 911.18 ± 29.65 | 908.35 ± 38.73 | 909.18 ± 34.37 | 909.18 ± 34.37 |
| | FBW, g | 2,207.11 ± 46.29 [ab] | 2,081.26 ± 59.68 [b] | 2,263.12 ± 63.57 [a] | 2,131.71 ± 47.65 [ab] | 2,167.35 ± 57.56 [ab] | 2,186.27 ± 47.16 [ab] |
| | ADFI, g | 118.29 ± 11.14 | 122.63 ± 6.76 | 115.14 ± 7.99 | 112.24 ± 11.6 | 107.12 ± 11.2 | 108.94 ± 10.72 |
| | ADG, g | 60.71 ± 6.61[ab] | 55.37 ± 5.68[b] | 64.61 ± 6.7[ab] | 58.33 ± 2.85[ab] | 60.03 ± 7.14[ab] | 60.77 ± 2.41[a] |
| | F/G | 1.95 ± 0.12[ab] | 2.31 ± 0.27[a] | 1.78 ± 0.18[b] | 1.92 ± 0.21[ab] | 1.78 ± 0.15[b] | 1.79 ± 0.16[b] |
| | Mortality, % | 0 | 5 | 0 | 2.5 | 0 | 0 |

Abbreviations: ADFI, average daily feed intake; ADG, average daily gain; FBW, final body weight; F/G, ADFI/ADG; IBW, initial body weight.
Group A: Basal diet; Group B: Basal diet with 42.15 $\mu$g/kg AFB$_1$; Group C: Basal diet + 1 g/kg CMD; Group D: Group B + 0.5 g/kg CMD; Group E: Group B + 1.0 g/kg CMD; Group F: Group B + 1.5 g/kg CMD. In the same row, significant differences at $P < 0.05$ levels are indicated by the different lowercase letters (a, b, and c), whereas insignificant differences at $P > 0.05$ levels are indicated by the same lowercase letters or without letters.

**Table 4.** Effects of AFB$_1$ and CMD on nutrient metabolic rate of broilers (%).

| Stages | Items | Group A | Group B | Group C | Group D | Group E | Group F |
|---|---|---|---|---|---|---|---|
| 1−21 d | Crude protein | 58.79 ± 1.06[ab] | 50.71 ± 1.89[c] | 60.31 ± 1.27[a] | 53.23 ± 1.08[c] | 57.81 ± 0.67[ab] | 57.04 ± 1.79[b] |
| | Crude fat | 78.87 ± 1.41 | 78.99 ± 1.51 | 78.49 ± 1.33 | 79.37 ± 0.84 | 79.93 ± 0.87 | 80.33 ± 1.71 |
| | Calcium | 35.63 ± 1.39 | 34.88 ± 1.73 | 36.37 ± 1.53 | 34.28 ± 1.18 | 36.03 ± 0.74 | 36.29 ± 0.98 |
| | Phosphorus | 42.49 ± 0.60 | 41.78 ± 0.59 | 43.11 ± 1.40 | 42.20 ± 0.73 | 41.96 ± 0.68 | 42.36 ± 0.52 |
| 22−42 d | Crude protein | 55.07 ± 1.56[a] | 51.06 ± 1.86[b] | 54.61 ± 3.04[a] | 55.61 ± 1.99[a] | 56.13 ± 1.28[a] | 57.10 ± 1.57[a] |
| | Crude fat | 74.06 ± 1.47 | 73.14 ± 1.78 | 74.80 ± 2.96 | 72.78 ± 1.83 | 73.11 ± 1.28 | 74.12 ± 1.60 |
| | Calcium | 32.46 ± 1.00 | 31.05 ± 0.74 | 32.40 ± 0.64 | 32.96 ± 1.46 | 31.09 ± 1.02 | 31.13 ± 1.13 |
| | Phosphorus | 39.71 ± 1.13 | 38.63 ± 0.7 | 39.66 ± 1.29 | 38.47 ± 0.75 | 38.91 ± 1.07 | 40.28 ± 1.44 |

Note: Group A: Basal diet; Group B: Basal diet with 42.15 $\mu$g/kg AFB$_1$; Group C: Basal diet + 1.0 g/kg CMD; Group D: Group B + 0.5 g/kg CMD; Group E: Group B + 1.0 g/kg CMD; Group F: Group B + 1.5 g/kg CMD. In the same row, significant differences at $P < 0.05$ levels are indicated by the different lowercase letters (a, b, and c), whereas insignificant differences at $P > 0.05$ levels are indicated by the same lowercase letters or without letters.

**Table 5.** Effects of AFB$_1$ and CMD on relative organ weights of broilers (%).

| Items | Group A | Group B | Group C | Group F |
|---|---|---|---|---|
| Jejunum | 3.03 ± 0.19[b] | 2.80 ± 0.28[b] | 3.54 ± 029[a] | 3.02 ± 0.19[b] |
| Liver | 2.36 ± 0.37 | 2.31 ± 0.30 | 2.27 ± 0.14 | 2.52 ± 0.26 |
| Kindy | 0.36 ± 0.02 | 0.30 ± 0.08 | 0.34 ± 0.03 | 0.38 ± 0.05 |
| Spleen | 0.23 ± 0.05 | 0.20 ± 0.02 | 0.24 ± 0.05 | 0.22 ± 0.02 |
| Bursa of Fabricius | 0.20 ± 0.03[a] | 0.14 ± 0.03[b] | 0.20 ± 0.03[a] | 0.19 ± 0.01[a] |
| Thymus | 0.22 ± 0.06[a] | 0.16 ± 0.01[b] | 0.21 ± 0.03[a] | 0.20 ± 0.02[a] |

Note: Group A: Basal diet; Group B: Basal diet with 42.15 $\mu$g/kg AFB$_1$; Group C: Basal diet + 1.0 g/kg CMD; Group F: Group B + 1.5 g/kg CMD. In the same row, significant differences at $P < 0.05$ levels are indicated by the different lowercase letters (a and b), whereas insignificant differences at $P > 0.05$ levels are indicated by the same lowercase letters or without letters.

first stage ($P < 0.05$); indicating that addition of CMD could improve nutrient metabolic rates of broilers challenged with AFB$_1$.

## Effects of AFB$_1$ and CMD on Relative Organ Indexes of Broilers

Table 5 showed that organ indexes such as jejunum, thymus, and bursa of Fabricius in group B were significantly lower than that in other groups ($P < 0.05$).

## Effects of AFB$_1$ and CMD on AFB$_1$ Residues in 42 d Broilers

Table 6 showed that AFB$_1$ residues in serum, jejunum, liver, and kidney tissues in group B were

significantly higher than that in group A and C ($P < 0.05$), indicating that AFB$_1$ residues were increased by adding AFB$_1$ in broiler diets. AFB$_1$ residues in serum, jejunum, liver, and kidney tissues in group F were significantly lower than that in group B ($P < 0.05$), indicating that CMD could effectively reduce AFB$_1$ residues in tissues.

## Effects of AFB$_1$ and CMD on Antioxidant Index of Broilers

Table 7 showed that the catalase, SOD and GSH-PX activities in group B were significantly lower ($P < 0.05$), and the MDA was significantly higher than that in other groups ($P < 0.05$). However, these indexes in group F were significantly improved ($P < 0.05$). Meanwhile,

**Table 6.** AFB$_1$ residues in serum, jejunum, liver, and kidney ($\mu$g/kg).

| Group | Diet | Serum | Jejunum | Liver | Kidney |
|---|---|---|---|---|---|
| A | 4.36 ± 0.10[B,a] | ND | 1.05 ± 0.07[C,b] | 0.92 ± 0.04[C,b] | 0.52 ± 0.03[C,c] |
| B | 42.3 ± 1.24[A,a] | 0.72 ± 0.05[A,d] | 7.31 ± 0.22[A,b] | 5.75 ± 0.14[A,c] | 5.16 ± 0.28[A,c] |
| C | 4.35 ± 0.01[B] | ND | ND | ND | ND |
| F | 42.18 ± 0.89[A,a] | 0.21 ± 0.07[B,e] | 5.35 ± 0.31[B,b] | 3.28 ± 0.33[B,c] | 1.12 ± 0.31[B,d] |

Note: Group A: Basal diet; Group B: Basal diet with 42.15 $\mu$g/kg AFB$_1$; Group C: Basal diet + 1.0 g/kg CMD; Group F: Group B + 1.5 g/kg CMD. In the same column, significant differences at $P < 0.05$ levels are indicated by the different capital letters (A, B, and C), whereas insignificant differences at $P > 0.05$ levels are indicated by the same capital letters. In the same row, significant differences at $P < 0.05$ levels are indicated by the different lowercase letters (a, b, and c), whereas insignificant differences at $P > 0.05$ levels are indicated by the same lowercase letters. ND: no detected.

**Table 7.** Effects of AFB$_1$ and CMD on serum antioxidant indexes of broilers.

| Items | Group A | Group B | Group C | Group F |
|---|---|---|---|---|
| Total antioxidant, mmol/mL | 0.48 ± 0.04 | 0.41 ± 0.02 | 0.47 ± 0.04 | 0.45 ± 0.08 |
| Catalase, U/mL | 8.89 ± 0.29[a] | 4.39 ± 0.23[b] | 8.87 ± 0.69[a] | 8.76 ± 0.57[a] |
| SOD, U/mL | 42.08 ± 4.95[b] | 10.98 ± 0.38[c] | 51.49 ± 0.27[a] | 55.86 ± 6.15[a] |
| MDA, nmol/mL | 3.41 ± 0.13[c] | 4.92 ± 0.05[a] | 3.44 ± 0.02[c] | 3.72 ± 0.08[b] |
| GSH-PX, $\mu$mol/mL | 510.43 ± 6.85[b] | 496.96 ± 19.57[b] | 579.57 ± 33.91[a] | 511.3 ± 2.61[b] |

Abbreviations: CAT, catalase; GSH-PX, glutathione peroxidase; MDA, malondialdehyde; SOD, superoxide dismutase.
Note: Group A: Basal diet; Group B: Basal diet with 42.15 $\mu$g/kg AFB$_1$; Group C: Basal diet + 1.0 g/kg CMD; Group F: Group B + 1.5 g/kg CMD. In the same row, significant differences at $P < 0.05$ levels are indicated by the different lowercase letters (a, b, and c), whereas insignificant differences at $P > 0.05$ levels are indicated by the same lowercase letters or without letters.

SOD and GSH-PX activities in group C were significantly higher than that in group A, inferring that CMD could improve the antioxidant level.

## Effects of AFB$_1$ and CMD on Microstructure of Jejunum, Liver, and Kidney in Broilers

*Effects of AFB$_1$ and CMD on microstructure of jejunum.* Figure 1 showed that villi in group A were normal in shape with clear boundary, but typical villi rupture and lymphocyte infiltration were observed in group B. The structure of villi in group C was normal with clear boundary and villi rupture was still observed in group F.

Table 8 showed that villus height in group B was significantly lower than that in group A and C ($P < 0.05$), but that there was no significant difference between group B and F ($P > 0.05$), suggesting that the AFB$_1$-induced reduction in villus height was irreversible. The crypt depth in group B was significantly higher than that in other groups, which lead to V/C value in group B to be significantly lower than that in other groups ($P < 0.05$).

*Effects of AFB$_1$ and CMD on microstructure of liver in broilers.* Figure 2 showed that hepatocytes were normal without necrosis, arranged in order, and the hepatic cellular nuclei were big and round, even cytoplasm in group A and C. However, hepatocytes were yellowish brown with enlarged gaps, and macrophagocytes and mechanocytes were found in group B, indicating that steatosis and inflammatory response caused by AFB$_1$ were increased in liver. The enlarged gaps of liver cells and reduced inflammatory cells were observed in group F, inferring that the CMD was able to protect liver from damage caused by AFB$_1$.

*Effects of AFB$_1$ and CMD on microstructure of kidney in broilers.* Figure 3 showed the renal tissue micrographs of 4 groups. The glomerular morphology was complete with clear boundary, and the diameter of glomerular was about 50 microns in group A and C. The inflammatory cell infiltration and freed out nuclear cells with a part of glomerular structure destroyed were obviously found in group B. The phenomenon of inflammatory cell infiltration and glomerular structure destruction in group F were significantly decreased, compared to group B, inferring that the CMD was effective in protecting the kidney from damage caused by AFB$_1$.

## Effects of AFB$_1$ and CMD on Jejunal Microflora in Broilers

*Effects of AFB$_1$ and CMD on the microbial community in broiler jejunum at species level.* Figure 4 showed that the relative abundances of the top 5 species from high to low were *Lactobacillus-aviarius, Staphylococcus-xylosus, Lactobacillus-agilis, Esherichia-coli-g-Escherichia-Shigella,* and *Lactobacillus-salivarius.* The relative abundance of Staphylococcus-xylosus in group B was higher than that in other groups ($P < 0.05$), whereas the relative abundances of *Staphylococcus-xylosu* and *Esherichia-coli-g-Escherichia-Shigella* in group F were significantly decreased compared to group B ($P < 0.05$). The relative abundance of *Lactobacillus-aviarius* in group C was higher than that in other 3 groups ($P < 0.05$), whereas the group B was the lowest ($P < 0.05$). The results showed that CMD was effective in maintaining the balance of a healthy microflora.

*Correlation analysis between intestinal microbiota at species level and other indexes.* In order to evaluate the correlation between jejunal microbiota (top 5 at the
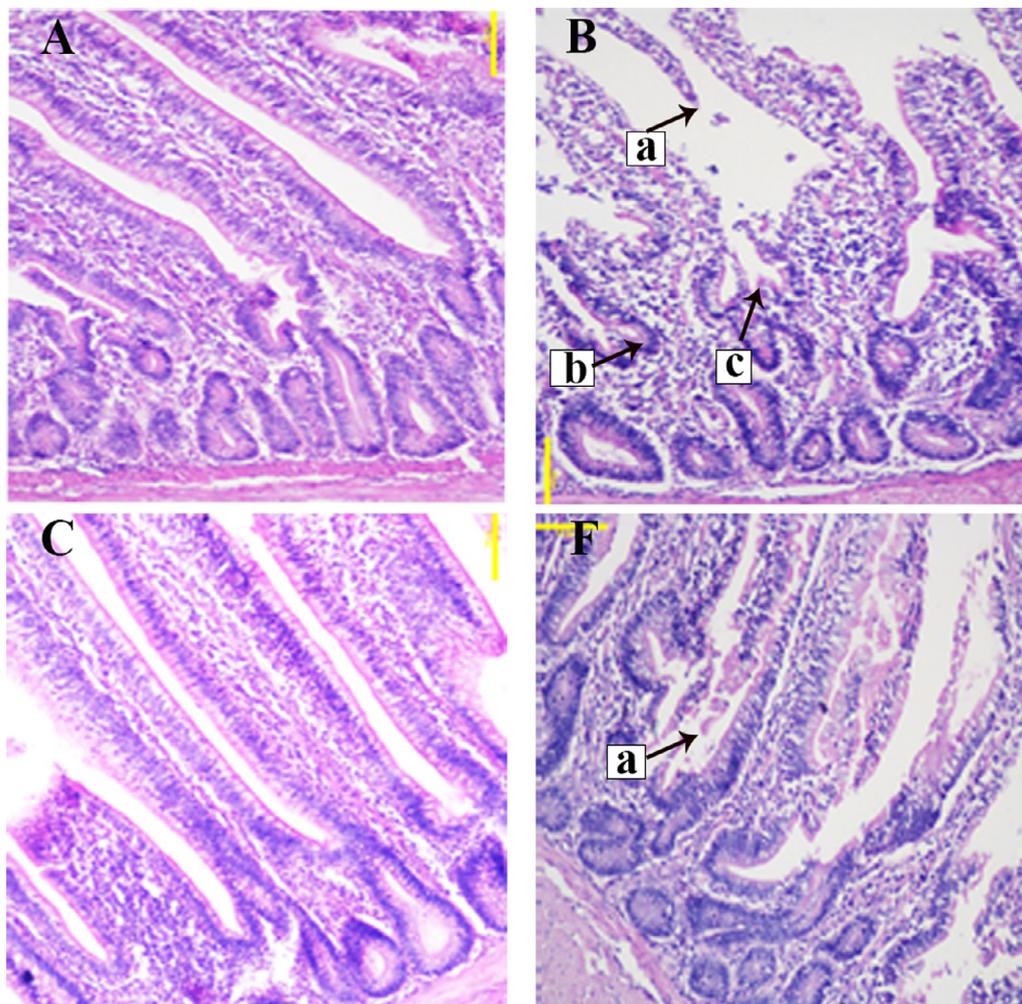
**Figure 1.** The micrograph of jejunum microstructure (HE 200×). Group A: Basal diet; Group B: Basal diet with 42.15 $\mu$g/kg AFB$_1$; Group C: Basal diet + 1.0 g/kg CMD; Group F: Group B + 1.5 g/kg CMD. (A) Rupture of intestinal villi; (B) the increased eosinophile granulocyte and monocytes; (C) the increased mucosal hyperplasia and crypt depth.

species level) and other indexes such as mycotoxin residues, serum oxidation indexes and BF, a Pearson's correlation matrix was performed by calculating the Pearson's correlation coefficients (Figure 5). This research showed that jejunal *Lactobacillus aviarius* abundance was positively correlated with ADG, BF and SOD ($P < 0.05$), and negatively correlated with AFB$_1$ residue in serum or AFB$_1$ and MDA in liver ($P < 0.05$). However, the *Staphylococcus-xylosus* and *Esherichia-coli-g-Escherichia-Shigella* abundances showed the

opposite correlation with the above indexes, illustrating that *Lactobacillus aviarius* played an important role in promoting broiler growth and decreasing AFB$_1$ residues, but *Staphylococcus-xylosus* and *Esherichia-coli-g-Escherichia-Shigella* had negative effects on broilers health.

## DISCUSSION

Compared with 10 to 20, 500, and 3,000 $\mu$g/kg for AFB$_1$, ZEA, and DON thresholds in broiler diets in China, only AFB$_1$ content (40 $\mu$g/kg) exceeds the threshold in this study. Therefore, the harm caused by moldy corn to broilers will be attributed to AFB$_1$. Poultry is more sensitive to AFB$_1$ than the other kinds of animals, and AFB$_1$ can cause liver and gastrointestinal damages, increase feed conversion rate as well as reduce production performance (Gao et al., 2022). The mechanism of AFB$_1$-induced injury in broilers is due to liver phase I metabolic enzyme (cytochromes P450, **CYP450**) in the mitochondrial to form AFB$_1$-exo-8,9-epoxide (**AFBO**), which can bind to DNA to inhibiting DNA replication and protein expression, resulting in the damage and cancelation of hepatocytes (Fan et al.,

**Table 8.** Effects of AFB$_1$ and CMD on villus height and crypt depth of jejunum in broilers.

| Groups | Villus height, $\mu$m | Crypt depth, $\mu$m | Villus height / Crypt depth |
|---|---|---|---|
| A | 0.89 ± 0.11[a] | 0.17 ± 0.01[b] | 5.05 ± 0.67[b] |
| B | 0.47 ± 0.02[b] | 0.19 ± 0.01[a] | 2.47 ± 0.10[c] |
| C | 0.98 ± 0.06[a] | 0.14 ± 0.01[c] | 7.22 ± 0.56[a] |
| F | 0.55 ± 0.02[b] | 0.10 ± 0.01[c] | 5.59 ± 0.41[b] |

Note: Group A: Basal diet; Group B: Basal diet with 42.15 $\mu$g/kg AFB$_1$; Group C: Basal diet + 1.0 g/kg CMD; Group F: Group B + 1.5 g/kg CMD. In the same column, significant differences at $P < 0.05$ levels are indicated by the different lowercase letters (a, b, and c), whereas insignificant differences at $P > 0.05$ levels are indicated by the same lowercase letters.
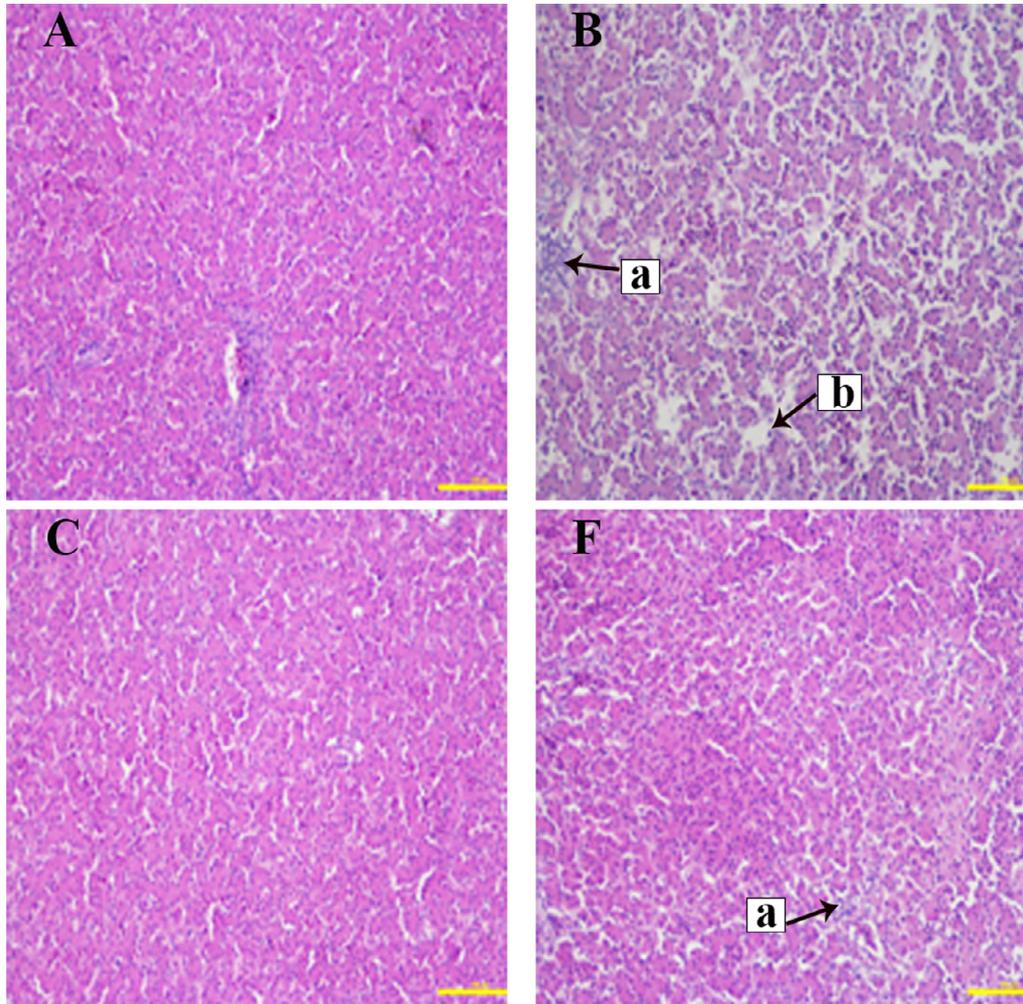
**Figure 2.** The micrograph of liver microstructure (HE 200×). Group A: Basal diet; Group B: Basal diet with 40 $\mu$g/kg AFB$_1$; Group C: Basal diet + 1.0 g/kg CMD; Group F: Group B + 1.5 g/kg CMD. (A) The increased eosinophile granulocyte and monocytes; (B) lipid vacuoles.

2021). It was reported that genetic factors, environmental factors and physiological factors determined the toxicity degrees of mycotoxins to animals by affecting the absorption, metabolism, biotransformation and excretion of mycotoxins in animals; and the degrees of toxicity included functional and microscopic defects as well as death (Bryden, 2001).

The high residual level of AFB$_1$ in organs will irritate the cells to cause tissue damage continuously. It was reported that AFB$_1$ was absorbed from the gastrointestinal tract and extensively penetrated various tissues, AFB$_1$ residue in animal edible issues and products was also a potential threat to human health (Caceres et al., 2020). In addition, small intestine is the physical barrier which usually first contacts with AFB$_1$ (Pinton and Oswald, 2014), this is the reason why AFB$_1$ residues in intestinal tissues were significantly higher than that in liver and kidney tissues. However, kidney as reabsorption and excretory organ is usually neglected, about 20% to 25% of the total amount of circulating toxins can reach the kidney; therefore, high levels of AFB$_1$ residues also exist in renal tissues (Yu et al., 2015). Probiotics in CMD were composed of *Lactobacillus casein, Bacillus subtilis, Candida utilis* and *Enterococcus faecalis* in this experiment. It was reported that *Lactobacillus* could degrade AFB$_1$ by the adsorption of peptidoglycan in cell wall, and this adsorption was reversible (Zhang et al., 2021). *Bacillus subtilis* can degrade AFB$_1$ by fermentation, and its degradation essentially is an enzymatic reaction caused by oxidoreductase biosynthesized by bacteriocin to destroy the structure of AFB$_1$ (Mao et al., 2020; Wan et al., 2021). *Lactobacillus casein* incubating components (teichoic acids on the peptidoglycan layer and $\beta$-D-glucan) have been shown to exhibit AFB$_1$ removal ability and neutralize the adverse effects of toxins on rat body weight and gut (Liew et al., 2018). Montmorillonite can alleviate the toxic harm by inhibiting the absorption of aflatoxin. The interlayer of montmorillonite could absorb AFB$_1$ by its medicinal system and the planarity of AFB$_1$ ring. It has been proven that the main mechanism in adsorption process is the chemisorptive mechanism with high enthalpy (Phillips et al., 2019). It was reported that the broiler contaminated diets with 1 mg/kg AFB$_1$ was supplemented with compound probiotics of *Lactobacillus* and yeast for 35 d, AFB$_1$ residue levels were decreased from 8.9 to 3.7$\mu$g/kg in liver, from 7.9 to 2.5 $\mu$g/kg in kidney, respectively (Śliżewska et al., 2019).

Blood antioxidant indexes induced by AFB$_1$ belong to the range of biochemical defects in this study. Liver
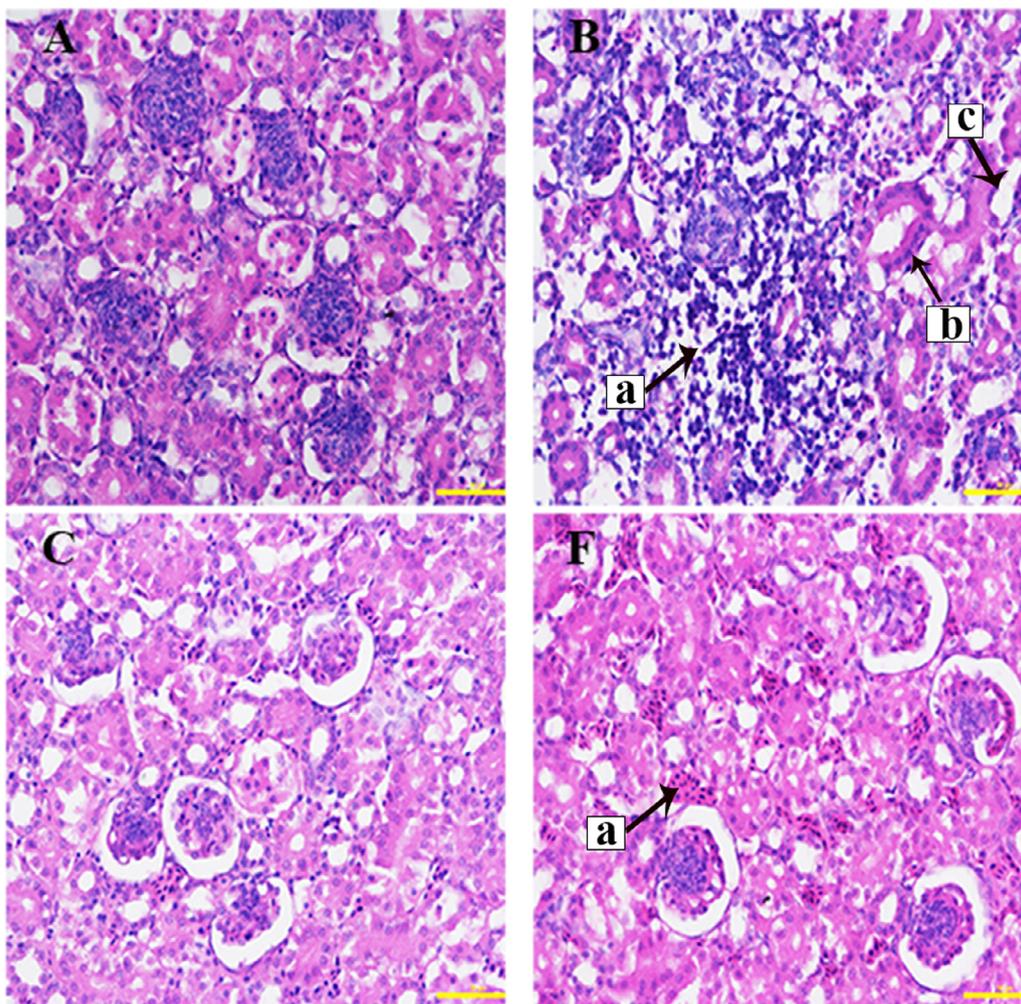
**Figure 3.** The micrograph of kidney microstructure (HE 400×). Group A: Basal diet; Group B: Basal diet with 40 $\mu$g/kg AFB$_1$; Group C: Basal diet + 1.0 g/kg CMD; Group F: Group B + 1.5 g/kg CMD. (A) Diffuse infiltration of inflammatory cells; (B) glomerular basement membrane thickening and stromal cells increasing; (C) renal tubule edema.

injury induced by AFB$_1$ is characterized by abnormal metabolism of nutrients, which was directly expressed as the decreases of serum total protein, triglyceride and glucose (Ates and Ortatatli, 2021). A published report showed that adding 1,000 $\mu$g/kg AFB$_1$ to broiler diet significantly decreased the concentrations of serum total
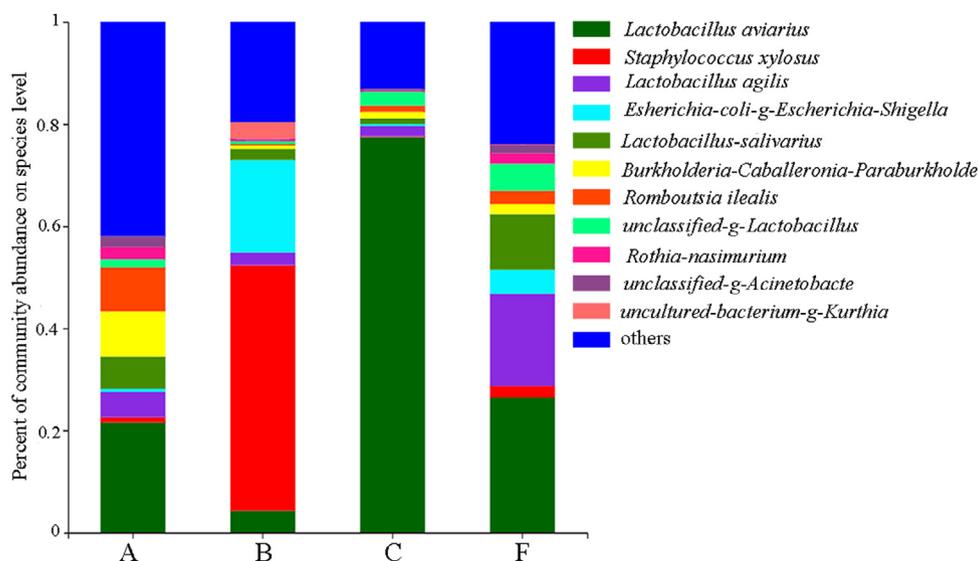


**Figure 4.** Composition of microbiota at species level in jejunal contents of broilers. Group A: Basal diet; Group B: Basal diet with 42.15 $\mu$g/kg AFB$_1$; Group C: Basal diet + 1.0 g/kg CMD; Group F: Group B + 1.5 g/kg CMD.
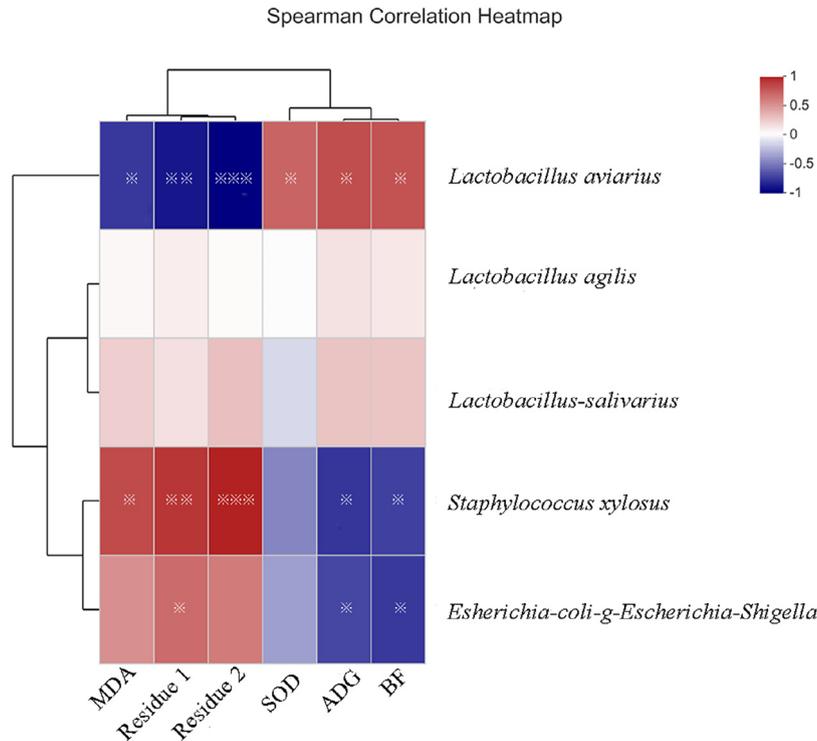
**Figure 5.** Correlation analysis of jejunal microflora with growth performance and AFB$_1$ residues for broilers. Abbreviations: ADG, average daily gain; BF, bursa of Fabricius; MDA, malondialdehyde; Residue 1, AFB$_1$ residue in serum; Residue 2, AFB$_1$ residue in liver; SOD, superoxide dismutase. * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$.

protein and triglyceride, and increased the activity of alkaline phosphatase and high-density lipoprotein content (Rashidi et al., 2020). AFB$_1$ can also reduce the filtration capacity of glomerulus, which leads to the decreases of serum calcium, phosphorus and glucose concentrations, and the increases of serum urea, creatinine, and ammonia (Yu et al., 2015). However, only the levels of serum total protein and aspartate aminotransferase were decreased significantly in this study, indicating that protein metabolism seems to be more easily affected by AFB$_1$ than sugar and lipid metabolism.

Meanwhile, AFB$_1$-induced lipid oxidation begins by disrupting cell membrane integrity through stimulating phospholipid A2, and then oxidation of polyunsaturated fatty acids induced formation of reactive oxygen species to reduce antioxidant activity, thus further aggravating liver damage (Wan et al., 2021). The previous research showed that many hepatic enzyme gene expressions including CYP450 A1, 1A2, 2A6 and 3A4 involved in conversion of AFB$_1$ to the highly reactive epoxide intermediate AFB$_1$-8,9-epoxide (**AFBO**) were improved to decrease serum SOD, catalase and GSH-PX activity as well as increase serum MDA content (Sun et al., 2015). Probiotics supplementation can significantly improve the antioxidant indexes, mainly because it can improve the oxidation level by down-regulating CYP1A1 and CYP2H1 genes expressions, up-regulating GPX gene expression and increasing SOD enzyme activity (Ramadan et al., 2018; Salem et al., 2018) The other research showed that SOD was significantly decreased, and MDA was significantly increased by feeding 100 $\mu$g/kg AFB$_1$ in boiler diet for 42 d, whereas the negative

effects were significantly decreased by adding 3 g/kg montmorillonite in broiler diet (Shi et al., 2006). Because low-level addition of montmorillonite has little effectiveness for AFB$_1$ adsorption, and high-level addition of montmorillonite has some side effects including adsorbing nutrients and decreasing nutrient concentrations, the study on the optimal-level addition of montmorillonite becomes important. In this experiment, the addition amount of montmorillonite was only 0.3 g/kg to have the good AFB$_1$−removal effect, which may be related to the synergistic effect of montmorillonite, compound probiotics and AFB$_1$-degrading enzyme.

The damages of AFB$_1$ to intestinal tract mainly present barrier function loss and inflammatory reaction, lymphocyte, or monocyte infiltration as well as mucosal hyperplasia and vacuolar degeneration, and even decrease intestinal villus height and increase intestinal crypt depth (Hernández-Ramírez et al., 2020). Liver is the primary organ attacked by AFB$_1$ which can cause many microscopic damages including high-level eosinophile granulocyte and monocytes (Rashidi et al., 2020), lipid vacuoles (Magnoli et al., 2017), inflammatory cell proliferation and infiltration, the edema and hepatocytes degeneration (Perali et al., 2020). Kidney is also the main organ attacked by AFB$_1$. The renal toxicity of dietary AFB$_1$ in broilers presented the increased glomerular basement membrane thickening and stromal cells, glomerular enlargement, tubular epithelial cell cytoplasmic vacoulation, renal glomerulus collapse and structural damage (Ortatatli, et al., 2005). This research showed that there was no change of glomerular structure in kidney by HE staining; however, mainly inflammatory cell

infiltration and significant microscopic anatomical defects were observed in intestinal and liver tissues. The degree of tissue damage may be due to the sensitivity of the different tissues to $AFB_1$ or the different contents of $AFB_1$ residues in the different tissues.

Probiotics have been proved to reduce $AFB_1$-induced microscopic damage to intestinal, liver and kidney tissues. It was reported that supplementation of *Lactobacillus* and *Saccharomyces cerevisiae* could produce significant protective effect against liver and kidney microscopic damages in broilers fed with 5 mg/kg $AFB_1$ (Śliżewska et al., 2019). According to the results of this experiment, the damage of $AFB_1$ to intestinal tissue appearance of villus rupture was the greatest, and CMD had the good protective effect on $AFB_1$-induced liver injury. In the previous experiment, the mechanism of CMD on regulating $AFB_1$-induced inflammatory response in chicken embryo primary intestinal epithelium, liver and kidney cells had been studied (Guo et al., 2021b), in agreement with this study.

This result showed that the relative weights of thymus and bursa of Fabricius were decreased by $AFB_1$ addition; however, CMD addition could restore the above results. When $AFB_1$ enters blood circulation through intestinal epithelial cells, it will enter the lymphatic circulation to cause damages of immune organs such as thymus (Guan et al., 2019), spleen (Li et al., 2019), bursa of Fabricius (Guo et al., 2021c), further cause atrophy and weight loss of these immune organs. $AFB_1$ damaged the spleen tissue of poultry by activating the NF-$\kappa$B signaling pathway, which directly presented atrophy and immunosuppression of spleen tissue (Wan et al., 2022). Similar, $AFB_1$ could cause apoptosis of bursa of Fabricius cells by up-regulating Bax, Caspase-3 and p53 protein expressions, down-regulating Bcl-2 protein expression, and improving genes expressions involved in catalase and glutathione peroxidase, which finally lead to bursa of Fabricius atrophy (Rajput et al., 2019).

The previous studies have shown the following reasons for the reduction of nutrient metabolic rates of broilers induced by $AFB_1$: 1) mycotoxin-contaminated corn had low nutrient contents (Dänicke et al., 2007); 2) decreased digestive enzyme activity such as trypsin and amylase in the intestinal lumen (Magnoli et al., 2017); 3) liver damage led to low nutrient metabolic rate (Chang et al., 2020). $AFB_1$-contaminated feed may cause broilers refuse diet which may be related to neurochemical and physical damage of salivary glands and oral cavity (Brake et al., 2000). The previous report showed that $AFB_1$ could significantly reduce the utilization of protein, decrease feed intake and growth performance (Chen et al., 2016), which is consistent with this study. Several beneficial microorganisms and bentonite have been used for reducing toxic effects on animal production. Holanda and Kim evaluated the effect of a mycotoxin-detoxifying additive compositing of clay, yeast cell wall, plant extracts and antioxidants on alleviating deoxynivalenol-induced toxicity in newly weaned pigs, which showed that the compound agent significantly improved piglet production performance and reduced oxidative stress (Holanda and Kim, 2020). Therefore, the combined materials may be the effective method to alleviate $AFB_1$ toxicity.

The results showed that the abundance of *Lactobacillus-aviarius* was positively correlated with ADG and SOD. It was reported that *Lactobacillus-aviarius* and *Lactobacillus-salivarius* were the dominant bacteria in the small intestine of broilers (Gong et al., 2007). The previous report showed that $AFB_1$ increased potentially pathogenic bacteria and reduced beneficial bacteria abundance in rat feces, and the microbiota composition was normalized by oral *Lactobacillus casein* (Huang et al., 2021). Another study showed that *Lactobacillus* had the ability to produce bacteriocin and organic acids, which could inhibit pathogens and inflammatory reaction, thus increasing body weight and improving immunity (Yan et al., 2016). Gut microbial metabolite could also affect health through gut-liver interaction (Saeedi et al., 2020) The high abundance of *Lactobacillus* was used as an indicator of healthy intestine and positive correlated with growth of broilers (Torok et al., 2011), in agreement with this study. In addition, there is a negative correlation between the abundance of *Lactobacillus-aviarius* and $AFB_1$ residue in serum and liver, maybe due to the ability of *Lactobacillus* to degrade $AFB_1$.

*Esherichia-coli-g-Escherichia-Shigella* as a kind of pathogenic bacterium could cause diarrhea and intestinal inflammation in human and broilers (Gong et al., 2019; Liu et al., 2020). In general, the optimal microbiota influences gastrointestinal development, prevents colonization of pathogens, and modulates the gut-associated systemic immunity. It can be inferred that CMD addition is able to decrease $AFB_1$ residues and increase broiler growth by increasing the proliferations of beneficial bacteria such as *Lactobacillus* species and decreasing the proliferations of detrimental bacteria such as *E. coli* and *Staphylococcus*.

The results showed that the growth performance and antioxidant capacity in boilers were significantly reduced, and $AFB_1$ residues and micro-pathological changes of intestines, liver and kidney were increased when the broilers were fed with corn contaminated by $AFB_1$ for 42 d. However, the CMD composed of compound probiotics, ADE and montmorillonite could effectively alleviate mycotoxin negative effects on the above parameters. The combination of physical and biological detoxification provides a new strategy for alleviating $AFB_1$ toxicity through $AFB_1$ degradation and absorption to reduce inflammation and improve the antioxidant level of broilers.

## DISCLOSURES

# ACKNOWLEDGMENTS

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