Probiotics and Antimicrobial Proteins (2020) 12:994–1001
https://doi.org/10.1007/s12602-019-09606-w

# Protective Effects of *Bacillus subtilis* ANSB060, *Bacillus subtilis* ANSB01G, and *Devosia* sp. ANSB714-Based Mycotoxin Biodegradation Agent on Mice Fed with Naturally moldy Diets

Yongpeng Guo[1] · Xueting Huo[1] · Lihua Zhao[2] · Qiugang Ma[1] · Jianyun Zhang[1] · Cheng Ji[1] · Lihong Zhao[1]

Published online: 12 November 2019


**Abstract**
Mycotoxins are toxic secondary metabolites produced by toxigenic fungi that commonly contaminate agricultural crops. The purpose of the current study was to evaluate whether *Bacillus subtilis* ANSB060, *Bacillus subtilis* ANSB01G, and *Devosia* sp. ANSB714-based mycotoxin biodegradation agent (MBA) could alleviate the negative effects of naturally moldy diet containing aflatoxin (AF), zearalenone (ZEN), and deoxynivalenol (DON) on growth performance, serum immune function, and antioxidant capacity as well as tissue residues in mice. A total of 54 mice were randomly divided into three dietary treatments: basal diet (CON), multi-mycotoxins contaminated diet (MCD) containing AF, ZEN and DON and multi-mycotoxins contaminated diet plus MBA at a dose of 1.0 g $kg^{-1}$ feed (MCD + MBA). Mice fed with moldy diet showed a significant decrease in body weight gain ($p < 0.05$), whereas the relative weight of the liver, spleen and uterus were remarkably increased ($p < 0.05$). Serum IgA and IgM contents were significantly decreased in MCD treatment compared with that in CON treatment ($p < 0.05$). In contrast, serum interleukin-1β (IL-1β), interleukin-2 (IL-2), and interleukin-6 (IL-6) concentrations were significantly promoted in mice fed with moldy diet ($p < 0.05$). Besides, the exposure to mycotoxins caused marked down-regulation of serum superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities in mice ($p < 0.05$). The addition of MBA effectively counteracted these toxic effects of moldy diet on mice. And DON residues in kidneys of mice consuming moldy diet were eliminated by the supplementation with MBA. Taken together, *Bacillus subtilis* ANSB060, *Bacillus subtilis* ANSB01G, and *Devosia* sp. ANSB714-based mycotoxin biodegradation agent has great potential use as a microbial additive to counteract mycotoxins contamination in food and feed.

**Keywords** Aflatoxin · Zearalenone · Deoxynivalenol · Biodegradation · Mice

## Introduction

Mycotoxins are a chemically diverse group of toxic secondary metabolites produced by mycotoxigenic fungi, primarily belonging to *Aspergillus*, *Fusarium*, *Penicillium*, *Claviceps*, and *Alternaria* [1]. Of approximately 400 mycotoxins identified up to now, aflatoxin $B_1$ ($AFB_1$), zearalenone (ZEN), and deoxynivalenol (DON) have received particular concern because they cause enormous economic loses in animal husbandry and pose a significant threat to human health. Among aflatoxins, $AFB_1$ is the most toxic and well known for its genotoxic, carcinogenic, teratogenic and immunosuppressive characteristics [2]. $AFB_1$ has been classified as Group I human carcinogen by the International Agency for Research on Cancer (IARC) [3]. Both DON and ZEN are *Fusarium* mycotoxins. Animal exposure to DON is usually associated with gastroenteritis, with clinical signs including feed refusal, vomiting, diarrhea, and gastrointestinal hemorrhage [4]. Immune system is also a central target of DON. Notably, chronic dietary exposure to DON promotes the expression of cytokines, chemokines, and inflammatory genes with concurrent immune stimulation, whereas high-dose exposure induces leukocyte apoptosis and impairment of macrophage/neutrophil function with concomitant immune suppression [5]. ZEN is an estrogenic toxin that can competitively bind to estrogen receptors, consequently leading to precocious puberty, infertility, abortion, and other reproductive problems [6].

Yongpeng Guo and Xueting Huo contributed equally to this work.

✉ Lihong Zhao
zhaolihongcau@cau.edu.cn

[1] College of Animal Science and Technology, State Key Laboratory of Animal Nutrition, China Agricultural University, 100193 Beijing, People's Republic of China

[2] College of Animal Science and Technology, National Engineering Laboratory for Animal Breeding, China Agricultural University, 100193 Beijing, People's Republic of China

Mycotoxins are frequently found in food and feed, and the point of contamination can be in the field before harvest, post-harvest, during processing, storage, and feeding despite the most strenuous efforts of prevention [7]. Commonly contaminated agricultural commodities are maize, wheat, barley, peanuts, forage, rice, fruits, and their processed products. On a global scale, it is estimated that about 25% of the world's crops are affected by mycotoxins annually [8]. In China, a recent survey showed that $AFB_1$, ZEN, and DON contaminated 0.6%, 10.8%, and 38.2% of complete pig feeds, respectively, between 2016 and 2017, at levels exceeding the Chinese regulatory limits [9]. Often multi-mycotoxins occur in a contaminated substrate since a certain strain of mold may produce more than one mycotoxin, or several species of fungi can be present simultaneously, producing different kinds of mycotoxins [10, 11]. As example, Streit et al. [12] reported the presence of 7 to 69 mycotoxins in all of 83 naturally contaminated samples of feed and feed raw materials. The co-exposure to the mycotoxin combinations may lead to enhanced toxicity by the possible additive or synergic effect [13, 14].

In response to the risk of exposure to mycotoxins from food and feeds, diverse remediation approaches have been proposed to mitigate mycotoxin contamination in agricultural commodities. These strategies are often categorized into physical, chemical, and biological principles. Physical removal of highly contaminated grains may be achieved by cooking, roasting, cleaning, and milling [15]. Another physical removal approach is the application of mycotoxin binders. Although the incorporation of mycotoxin binders is economically feasible and there are a variety of commercially available mycotoxin binders as feed additives, the efficacy of these adsorbents towards different classes of mycotoxins is variable, with most commercial adsorbents being poorly able to bind DON [16, 17]. Furthermore, some may have adverse nutritional effects related to unspecific binding of minerals and vitamins exist [18]. The chemical strategies include the use of acids, bases, hydrogen peroxide, and the use of ozonation [19]. However, the application of chemical methods is limited due to the potential toxicity of chemical residues and their negative effects on the palatability and nutritive quality of food and feed [17, 18]. In contrast, biological degradation approaches, which comprise microbial and enzymatic transformation of mycotoxins into nontoxic or less toxic metabolites, are generally more specific, efficient, and environmentally friendly [19]. In our previous works, we isolated three bacterial strains *Bacillus subtilis* ANSB060 [20], *Bacillus subtilis* ANSB01G [21], and *Devosia* sp. ANSB714 [22], which could degrade 81.5 % of $AFB_1$, 85 % of ZEN, and 97.3 % of DON, respectively, in liquid culture. Besides, the addition of *Bacillus subtilis* ANSB060 to aflatoxins contaminated diet could reduce aflatoxins residues in the liver of broilers [23]. *Bacillus subtilis* ANSB01G was shown to be capable of effectively alleviating ZEN toxicosis in gilts [24], and DON-induced negative effects in pigs could be counteracted by dietary supplementation with *Devosia* sp. ANSB714 [25]. In the current study, we are aiming at evaluating the efficacy of mycotoxin biodegradation agent (combination of *B. subtilis* ANSB060, *B. subtilis* ANSB01G, and *Devosia* sp. ANSB714) in counteracting the adverse effects of naturally moldy diet contaminated with multiple mycotoxins on mice.

## Materials and Methods

### Chemicals and Microbial Feed Additive

Aflatoxins ($AFB_1$, $AFB_2$, $AFG_1$, and $AFG_2$), zearalenone (ZEN), deoxynivalenol (DON), and ochratoxin A (OTA) standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of analytical grade or higher and purchased from Sinopharm Chemical Reagent Beijing Co., Ltd (Beijing, China). The mycotoxin biodegradation agent (MBA) is a multiple bacterial strain mix in powder obtained from Henan Trillion-Zhongyuan Bio-Sin Co., Ltd (Zhengzhou, China), which contains *B. subtilis* ANSB060 ($5 \times 10^8$ CFU $g^{-1}$), *B. subtilis* ANSB01G ($7.5 \times 10^8$ CFU $g^{-1}$), and *Devosia* sp. ANSB714 ($7.5 \times 10^8$ CFU $g^{-1}$).

### Animals and Diets

BALB/c female mice (35 d) were purchased from Vital River Laboratory Animal Technology Co., Ltd. in Beijing. All the mice were housed in transparent polypropylene cages in animal care room artificially conditioned at temperature 24 ± 1 °C and humidity 50 ± 5 %, and on a 12-h light-dark cycle. After 1 week of acclimation, all the mice were divided into three groups (six cages per group and three mice per cage) as follows: basal diet (CON), multi-mycotoxins contaminated diet (MCD), and multi-mycotoxins contaminated diet plus MBA at dose of 1.0 g $kg^{-1}$ of feed (MCD + MBA). The contents of mycotoxins including $AFB_1$, $AFB_2$, $AFG_1$, $AFG_2$, DON, ZEN, and OTA in feed ingredients and formulated diets were measured with high performance liquid chromatography (HPLC) as described by Li et al. [26]. Ingredient composition and nutrient contents of CON and MCD diet were shown in Table 1. All mice were fed ad libitum for 4 weeks. Body weight gain and feed intake were recorded in the experimental period.

### Sample Collection and Serum Biochemistry Analyses

At the end of the 28-day feeding trial, all mice were fasted overnight. Samples of blood were obtained from orbital venous plexus. After centrifugation at 2000× g for 15 min, serum was separated and stored at −80 °C for further analysis. Mice were euthanized by cervical dislocation. The liver, heart, spleen, uterus, and kidneys were dissected out immediately, rinsed in chilled saline

**Table 1** Ingredient composition and nutrient contents of control and moldy diet

| Item | CON[1] diet | MCD[2] diet |
|---|---|---|
| Ingredients, % | | |
| Normal diet[3] | 52 | 52 |
| Normal corncob | 29 | 22 |
| Moldy corncob | 0 | 7 |
| Normal peanut meal | 17 | 8 |
| Moldy peanut meal | 0 | 9 |
| Pig oil | 2 | 2 |
| Chemical composition | | |
| Protein (%) | 18.59 | 18.59 |
| Fat (%) | 4.42 | 4.42 |
| Fiber (%) | 12.17 | 12.17 |
| Ash (%) | 3.38 | 3.38 |
| Carbohydrates (%) | 38.74 | 38.74 |
| Total energy (MJ/kg) | 17.23 | 17.23 |
| $AFB_1$[4] (μg/kg) | 2.02 | 23.57 |
| $AFB_2$[4](μg/kg) | 1.53 | 6.56 |
| $AFG_1$(μg/kg) | ND[5] | ND |
| $AFG_2$(μg/kg) | ND | ND |
| ZEN[4] (mg/kg) | 0.09 | 0.66 |
| DON[4] (mg/kg) | 0.12 | 2.63 |
| OTA(μg/kg) | ND | ND |

[1] CON, control diet. [2] MCD, mycotoxins contaminated diet. [3] Provided by Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). [4] Mycotoxins in pellet diets were determined by HPLC. [5] Not detected

solution, dried on a filter paper, and weighed separately to calculate the organ index. The liver and kidney samples were stored at −80 °C for further examination.

The levels of serum immunoglobulin A (IgA), immunoglobulin G (IgG), immunoglobulin M (IgM), total superoxide dismutase (T-SOD), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The measurements were performed following the manufacturer's instructions. Interleukin-1β (IL-1β), interleukin-2 (IL-2), interleukin-6 (IL-6), and necrosis factor α (TNF-α) were measured by R-911 automatic radioimmunoassay counter (China University of Technology Industrial Co., Hefei, China).

## Determination of Mycotoxin Residues in Tissues

Analysis of $AFB_1$ residues in liver was carried out according to the method provided by Fan et al. [23] with some modifications. Briefly, the ground-defrosted liver samples (2.5 g) were added with 0.5 g of NaCl and blended in 10 mL of methanol/water (80/20, V/V) for 3 min on a vortex shaker. After centrifugation at 5000× g for 10 min, the supernatant was transferred and filtered through a paper filter. An aliquot of 3 mL of filtrate was diluted with 12 mL of PBS containing 0.1 % Tween-20 and subjected to an immune-affinity column. Following washes with 10 mL of PBS and 10 mL of double distilled water, respectively, $AFB_1$ was eluted with 1 mL of methanol. The eluent was evaporated to dryness at 40 °C under a stream of nitrogen. The residues were dissolved in 100 μL of mobile phase before HPLC analysis. HPLC system was equipped with a post-column photochemical derivation and a fluorescence monitor. The wavelengths for excitation and emission were 360 nm and 440 nm, respectively. The separation of $AFB_1$ was achieved by a Diamonsil® C18 reverse phase column (5 μm, 4.6 × 150 mm). The mobile phase was methanol-water (45:55) with the flow rate of 1 mL $min^{-1}$, and the injection volume was 20 μL.

Zearalenone residues in the liver were analyzed using the method of Duca et al. [27] with a few modifications. A quantity of 2.5 g of ground-defrosted liver samples were blended in 10 mL of buffer solution of acetic acid-ammonium acetate (pH 4.8). The solution was incubated at 37 °C for 15 h with 80 μL of a solution of glucuronidase/arylsulfatase with pH 4.0 adjusted with glacial acetic acid. Subsequently, the mixture was extracted with 10 mL of acetonitrile and 200 μL of NaOH solution (1 M) while being stirred at 200 rpm for 60 min. After centrifugation at 5000× g for 10 min, the supernatant (10 mL) was collected and mixed with 40 mL of buffer phosphate solution, pH 7.4. Then, the solution was transferred and filtered through a glass fiber filter paper. The filtrate (40 mL) was loaded onto an immune-affinity column, followed by washes with 10 mL of PBS and 10 mL of double distilled water, respectively. The retained ZEN was eluted with 1 mL of methanol. And the eluent was evaporated to dryness at 40 °C under a stream of nitrogen. The residues were dissolved in 100 μL of mobile phase and subjected to HPLC analysis. HPLC was performed with a fluorescence monitor at 274 nm for excitation and 440 nm for emission and acetonitrile-methanol-water (46:46:8) as the mobile phase at the flow rate of 1 mL $min^{-1}$. The separation of ZEN was achieved by a Diamonsil® C18 reverse phase column (5 μm, 4.6 × 150 mm).The injection volume was 20 μL.

DON residues in kidney were determined using the method described by Zhao et al. [22] with a slight modification. Specifically, 1 g of freeze-dried kidney samples were placed in 10 mL of eppendorf tube containing 5 mL of sodium acetate buffer (pH 5.5). Afterwards, 100 μL of β-glucuronidase (85, 000 U $mL^{-1}$) was added in the tube. Following enzymatic hydrolysis at 37 °C for 12 h, the samples were extracted with a mixture of acetonitrile and water and then centrifuged at 5000× g for 30 min. An aliquot of 8 mL of PBS containing 0.1 % Tween-20 was added to 2 mL of the collected extract and vortex agitated for 30 s. After centrifugation at 5000× g for 10 min, the supernatant (8 mL) was passed through an immune-affinity column at a flow rate of 1 mL $min^{-1}$ by

gravity and subsequently washed with 10 mL of PBS and 10 mL of double distilled water, respectively. The eluent was evaporated to dryness at 40 °C under a stream of nitrogen. The residues were dissolved in 100 μL of mobile phase and applied to HPLC analysis. HPLC was performed with a UV detector set at 218 nm and methanol-water (10:90) as the mobile phase at the flow rate of 1 mL $min^{-1}$. The separation of DON was achieved by a Diamonsil® C18 reverse phase column (5 μm, 4.6 × 150 mm). The injection volume was 20 μL.

### Statistical Analysis

All data obtained in this experiment were analyzed using SAS software (Version 9; SAS Institute, Inc., Cary, NC, USA) by performing one-way analysis of variance (ANOVA), followed by Duncan's multiple range test as a post hoc comparison. All statements of statistical significance were based on a probability of $p < 0.05$.

## Results

### Growth Performance and Relative Organ Weight

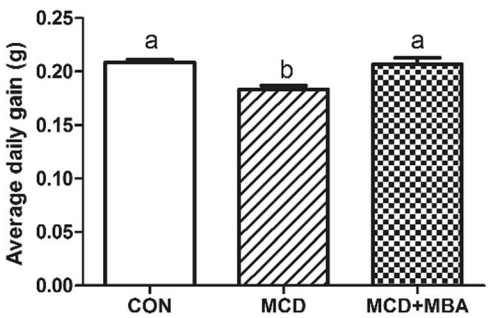
The effects of MBA on the body weight gain and feed intake of mice fed with multi-mycotoxins contaminated diet are shown in Fig 1. The average body weight gain of mice in the MCD group (0.18 g) were significantly decreased ($p < 0.05$), whereas no difference was observed between the CON and MCD + MBA groups (both 0.21 g). There was no difference on average daily feed intake of mice among the three treatments ($p > 0.05$). As shown in Table 2, there were no differences in the relative weight of the heart and kidney among the three groups ($p > 0.05$). However, mycotoxins contaminated diet resulted in higher relative weight of the liver, spleen, and uterus compared with the control diet ($p < 0.05$). Supplementing MBA into naturally moldy diet counteracted the effect of mycotoxins on the relative weight of visceral organs, resulting in no significant differences between the CON and MCD + MBA groups ($p > 0.05$).

### Serum Immunoglobulins and Cytokines Concentrations

The effects of MBA on serum immunoglobulins and cytokines concentrations of mice fed with multi-mycotoxins contaminated diet are summarized in Table 3. The level of serum IgG content in MCD treatment was not different with that in CON treatment ($p > 0.05$). However, the serum IgA and IgM levels were significantly reduced ($p < 0.05$) in mice fed diet contaminated with mycotoxins when compared with mice fed control diet. The addition of MCD with mycotoxin biodegradation agent improved serum IgA and IgM levels, and the values in the MCD + MBA treatment were not significantly different with that in mice fed a basal diet ($p > 0.05$). Regarding the serum cytokines, the concentrations of IL-1β, IL-2, and IL-6 were significantly increased ($p < 0.05$) in the MCD group compared to the control, but no statistical difference in the content of serum TNF-α was found among the three treatments ($p > 0.05$). Adding mycotoxin biodegradation agent into multi-mycotoxins contaminated diet significantly decreased the serum IL-1β, IL-2, and IL-6 levels ($p < 0.05$) compared with that in mice fed with multi-mycotoxins contaminated diet. Besides, there were no significant differences on the levels of serum IL-1β and IL-2 between the MCD + MBA treatment and CON treatment ($p > 0.05$), while the value of serum IL-6 in MCD + MBA treatment was higher($p < 0.05$) than that in CON treatment.

### Serum Antioxidant Parameters

The effects of MBA on the serum antioxidant parameters of mice fed with multi-mycotoxins contaminated diet are shown in Fig 2. There was no significant difference in the level of serum MDA content among the three treatments ($p > 0.05$). Serum GSH-$P_X$ (754.31 U $mL^{-1}$) and SOD (71.46 U $mL^{-1}$) activities in MCD treatment were significantly lower ($p <$



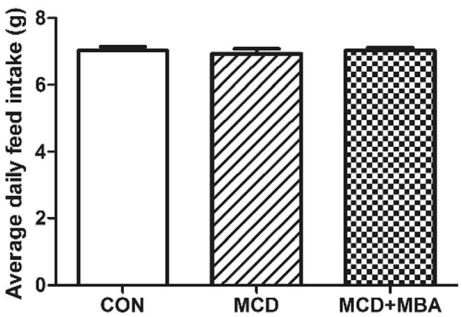




**Fig. 1** Effects of MBA on average daily gain and average daily feed intake of mice fed with muli-mycotoxins contaminated diet. CON = control diet; MCD = naturally contaminated diet with aflatoxin $B_1$, zearalenone, and deoxynivalenol; MCD + MBA = naturally contaminated diet with aflatoxin $B_1$, zearalenone, and deoxynivalenol plus 1.0 g MBA $kg^{-1}$ diet. Columns with different letters differ significantly ($p < 0.05$)

**Table 2** Effects of MBA on the relative organ weight of mice fed with muli-mycotoxins contaminated diet.

| Treatment | Organ weight(g/100 g body weight) | | | | |
|---|---|---|---|---|---|
| | Heart | Liver | Spleen | Kidney | Uterus |
| CON | 0.58 ± 0.05 | 4.27 ± 0.14$^{b}$ | 0.38 ± 0.03$^{b}$ | 1.20 ± 0.08 | 0.35 ± 0.03$^{b}$ |
| MCD | 0.59 ± 0.03 | 5.09 ± 0.18$^{a}$ | 0.47 ± 0.02$^{a}$ | 1.09 ± 0.07 | 0.51 ± 0.02$^{a}$ |
| MCD + MBA | 0.55 ± 0.03 | 4.07 ± 0.20$^{b}$ | 0.36 ± 0.02$^{b}$ | 1.16 ± 0.08 | 0.37 ± 0.02$^{b}$ |

Results are expressed as mean ± standard error of six replicates. Means within a column with different letters differ significantly ($p < 0.05$). CON = control diet; MCD naturally contaminated diet with aflatoxin $B_1$, zearalenone, and deoxynivalenol; MCD + MBA = naturally contaminated diet plus 1.0 g MBA $kg^{-1}$ diet

0.05) than that (867.69 and 88.05 U $mL^{-1}$) in CON, whereas no difference was found between the MCD + MBA and CON groups ($p > 0.05$).

### Mycotoxin Residues in Tissues

Neither $AFB_1$ nor ZEN was determined in liver samples of mice exposed to multi-mycotoxins in this study. DON residue in kidney samples of mice fed with contaminated diet was 10.35 ± 1.38 ng $g^{-1}$. Moreover, when supplemented with MBA, there were no detectable DON residues in the kidneys of mice consuming moldy diet.

## Discussion

In the last few years, academics have focused on the occurrence, toxicity, and detoxification of a single mycotoxin. However, more often multi-mycotoxins occur in a naturally contaminated substrate, which has been a great concern since the health risk from this multi-mycotoxins exposure is not well-known [13]. In China, co-contamination with $AFB_1$, ZEN, and DON was commonly found in feed and feed ingredients [9, 25].The consumption of mycotoxins by animals has been shown to decrease growth performance, cause organ damage, disrupt immune function, and induce oxidative stress [28–31], resulting in huge economic losses for producers. Moreover, previous studies indicated that the combination of multi-mycotoxins in diet led to further adverse effects on animals than when consumed alone [32–34]. Due to the structural diversity of mycotoxins, the current available mycotoxin-detoxifying agents aiming at a specific mycotoxin hardly meet the need of protecting animals from the negative effects of multi-mycotoxins exposure. In this situation, a novel mycotoxin biodegradation agent MBA, which could simultaneously detoxify $AFB_1$, ZEN, and DON, was developed and applied as feed additive to alleviate mycotoxicosis in mice.

The reduction of body weight gain is an important manifestation of mycotoxicosis in animals. Weaver et al. [34] showed that 4.8 mg $kg^{-1}$ DON and 0.3 mg $kg^{-1}$ ZEN in feed caused a 33 % reduction in average daily gain in pigs. In another report, piglets fed diet contaminated with *Fusarium* mycotoxins (1.1 mg $kg^{-1}$ DON and 0.5 mg $kg^{-1}$ ZEN in feed) had a 41 % decrease in average daily gain after the feeding trial for 25 days [29]. Additionally, Chaytor et al. [28] found that body weight gain of pigs was reduced by 16 % after exposure to 120 μg $kg^{-1}$ AF and 600 μg $kg^{-1}$ DON in feed for 33 days. Consistent with these reports, the body weight gain of mice fed the contaminated diet was decreased by 12 % after the feeding trial for 28 days. However, the presence of MBA in the multi-mycotoxins contaminated diet effectively counteracted the adverse effects of mycotoxins on body weight gain in mice. Mycotoxicosis in animals can also cause alterations in visceral organ weights, including the enlargement of the liver and spleen. In the present study, the relative weights of the liver, spleen, and uterus were significantly increased in mice fed multi-mycotoxins contaminated diet. As a result of incorporation of MBA in the contaminated diet, the relative weight of the liver, spleen, and uterus returned to the normal proportions observed in the CON treatment. The liver

**Table 3** Effects of MBA on the serum immunoglobulins and cytokines concentrations of mice of mice fed with muli-mycotoxins contaminated diet

| Treatment | IgA(g/L) | IgG(g/L) | IgM(mg/L) | IL-1β (pg/mL) | IL-2 (pg/mL) | IL-6 (pg/mL) | TNF-α (pg/mL) |
|---|---|---|---|---|---|---|---|
| CON | 1.56 ± 0.03$^{a}$ | 2.64 ± 0.03 | 366.00 ± 5.49$^{a}$ | 18.96 ± 0.46$^{b}$ | 20.48 ± 0.26$^{b}$ | 109.39 ± 1.83$^{c}$ | 49.97 ± 0.60 |
| MCD | 1.45 ± 0.03$^{b}$ | 2.66 ± 0.04 | 346.33 ± 2.69$^{b}$ | 23.01 ± 0.55$^{a}$ | 25.60 ± 0.65$^{a}$ | 134.14 ± 1.36$^{a}$ | 51.65 ± 1.18 |
| MCD + MBA | 1.55 ± 0.04$^{a}$ | 2.70 ± 0.05 | 354.83 ± 5.24$^{ab}$ | 20.02 ± 0.58$^{b}$ | 21.40 ± 0.82$^{b}$ | 116.12 ± 2.56$^{b}$ | 50.28 ± 1.30 |

Results are expressed as mean ± standard error of six replicates. Means within a column with different letters differ significantly ($p < 0.05$). CON = control diet; MCD = naturally contaminated diet with aflatoxin $B_1$, zearalenone, and deoxynivalenol; MCD + MBA = naturally contaminated diet plus 1.0 g MBA $kg^{-1}$ diet

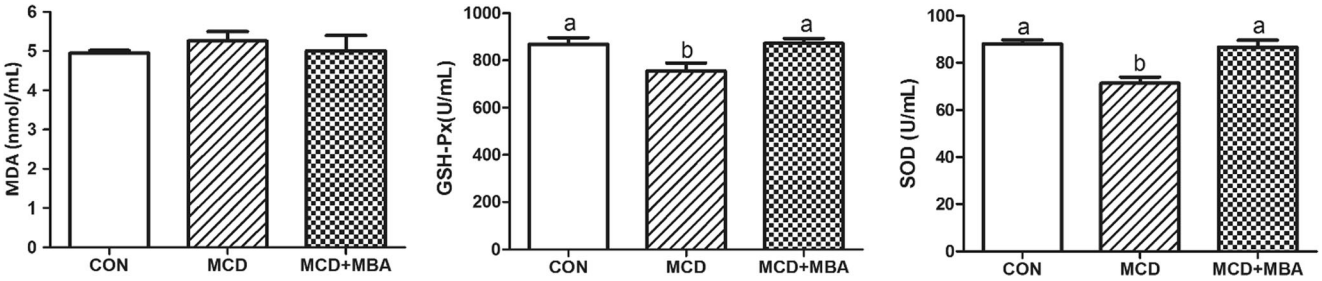



**Fig. 2** Effects of MBA on the serum antioxidant parameters of mice fed with muli-mycotoxins contaminated diet. CON = control diet; MCD = naturally contaminated diet with aflatoxin $B_1$, zearalenone, and deoxynivalenol; MCD + MBA = naturally contaminated diet with aflatoxin $B_1$, zearalenone, and deoxynivalenol plus 1.0 g MBA $kg^{-1}$ diet. Columns with different letters differ significantly ($p < 0.05$)

is the main site for mycotoxins metabolism and a major target organ for mycotoxicosis. In the report of Weaver et al. [30], pigs challenged with AF and DON (0.15 mg $kg^{-1}$ and 1.1 mg $kg^{-1}$ in feed, respectively) resulted in remarkable increase in relative liver weights. Another research indicated that relative uterus weight was significantly increased in prepubertal gilts following exposure to 3.9 mg $kg^{-1}$ DON and 0.42 mg $kg^{-1}$ ZEN in feed [35]. Conversely, Shi et al. [29] did not observe any significant change in relative organ weights of the liver, spleen, and reproductive organs in immature gilts exposed to ZEN and DON (0.6 mg $kg^{-1}$ and 0.8 mg $kg^{-1}$ in feed, respectively). In another study, Chaytor et al. [28] found that pigs fed diets containing a combined contamination of DON and AF did not result in alteration of relative weights of visceral organs. These contrasting results may be attributed to the variation in animal species and age, health condition, and levels of mycotoxins as well as exposure time.

There is a lack of information on the combined impacts of multiple mycotoxins on animal immune function. The decrease in serum IgA and IgM levels in mice co-administrated with multiple mycotoxins was contradictory to the previous reports showing that individual and combined treatment with $AFB_1$ and DON had no effect on serum Ig subclasses production [28, 36, 37]. However, a recent report found a significant increase in serum IgA and IgG in gilts fed diet contaminated with ZEN and DON [29]. On the other hand, Yin et al. [38] reported that maternal exposure to high levels of ZEN (2.77 mg $kg^{-1}$ in feed) decreased serum IgA and IgG levels in pregnant sows and their offspring. Thus, more detailed studies are needed to clarify the mode of action of individual and combined mycotoxins on the humoral immune system. Nevertheless, the present study suggested that the addition of MBA in the diet naturally contaminated with multiple mycotoxins effectively alleviated the mycotoxins-mediated impairment of serum immunoglobulins production. There is accumulating evidence suggesting that mycotoxins can induce inflammatory reaction. In the current study, up-regulations of serum pro-inflammatory cytokines IL-1β, IL-2, and IL-6 were observed in mice following combined dietary exposure to $AFB_1$, ZEN, and DON. Previous investigation on mice also demonstrated that DON exposure led to elevation of serum IL-1β, IL-2, IL-6, and TNF-α [22]. Besides, Pistol et al. [39] reported that ZEN could induce the expression of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α, and IFN-γ) in the spleen of pigs. In addition, Li et al. [37] also found an increase of mRNA expression of pro-inflammatory cytokines (IL-6, TNF-α, and IFN-γ) in the spleen of broiler chickens fed $AFB_1$ contaminated diet. These findings suggested that dietary exposure to mycotoxins triggered inflammatory responses, which may further induce cell apoptosis, immune dysfunction, and organ damage. In the present study, the addition of MBA in the contaminated diet effectively reduced inflammation, resulting in serum cytokines levels similar to those in mice in control treatment.

Researches have indicated that oxidative stress plays critical roles in the cytotoxic mechanism of mycotoxins. Oxidative stress can be defined as the misbalance between the generation of intracellular oxidant species and antioxidant capacity, which subsequently causes damage to all major classes of cellular macromolecules, such as DNA, proteins, and lipids. As the direct product of lipid peroxidation, MDA is often seen as an indicator of oxidative stress in the organism. SOD and GSH-Px are major components of the antioxidant system and participate in reactive oxygen species (ROS) scavenging. SOD are a class of enzymes that catalyze the dismutation of superoxide ($O_2^-$) into hydrogen peroxide ($H_2O_2$), representing an important antioxidant defense in all cells exposed to oxygen, while GSH-Px catalyzes the conversion of hydrogen peroxide into water and molecular oxygen. In the current study, serum SOD and GSH-Px activities showed significant reduction in mice treated with mycotoxins, whereas MDA content was not altered. Similarly, lipid peroxidation was not affected in pigs following combined dietary exposure to 4.8 mg $kg^{-1}$ DON and 0.3 mg $kg^{-1}$ ZEN in feed [34]. In addition, consumption of 1.1 mg $kg^{-1}$ DON and 0.5 mg $kg^{-1}$ ZEN in feed by piglets did not alter MDA level in the liver, but the hepatic SOD activity was significantly decreased [31]. In the report of Zhang et al. [40], ducks fed moldy maize naturally contaminated with $AFB_1$ (22 μg $kg^{-1}$) had reduced SOD and GSH-Px activities and increased MDA level in the liver. Taking literatures and the present results together, it can be speculated that SOD activity is a sensitive indicator for mycotoxins exposure,

and the decrease of SOD activity may trigger lipid peroxidation, resulting in elevation of MDA content. The supplementation of contaminated diet with MBA promoted serum SOD and GSH-Px activities, which indicated that MBA could overcome the combined toxicity of $AFB_1$, ZEN, and DON on antioxidant system, by means of detoxifying these mycotoxins in digestive tract.

Apart from a direct exposure of humans to mycotoxins through contaminated cereals, a potential exposure route might be from edible tissues and products of animals consuming mycotoxins. In the current study, there were no detectable $AFB_1$ or ZEN residues in the liver of mice fed contaminated diet, while DON was retained in the kidneys at the concentration of $10.35 \pm 1.38$ ng $g^{-1}$. It has been well documented that the kidney is the major tissue for DON retention, and the concentrations of DON decrease from kidney to serum and liver of pigs exposed to DON [25, 41, 42]. Consistent with our previous reports [22, 25], the addition of MBA containing *Devosia* sp. ANSB714 eliminated DON residues in kidneys of mice fed DON-contaminated diet, suggesting that *Devosia* sp. ANSB714 could reduce DON absorption in digestive tract.

In conclusion, the results presented in this study indicated that consumption of diet contaminated simultaneously with $AFB_1$, ZEN, and DON led to depression of body weight gain, immune function, and antioxidant capacity of mice. The adding of mycotoxin biodegradation agent into multi-mycotoxin-contaminated diet could effectively alleviate mycotoxicosis in mice. Thus, MBA has a great application potential in livestock production.

**Funding information** This study was supported by National Natural Science Foundation of China (grant number 31772637) and National Key Research and Development Program of China (No. 2018YFD0500600).

**Compliance with ethical standards** The experiment was performed according to the guidelines for the care and use of laboratory animals established by the National Research Council and was approved by the Animal welfare Committee of China Agricultural University(ethical approval code: AW17109102-2-1).

**Conflict of Interest** The authors declare that they have no conflict of interest.

## References

1. Steyn PS (1995) Mycotoxins, general view, chemistry and structure. Toxicol Lett 82-83:843–851
2. Mishra HN, Das C (2003) A review on biological control and metabolism of aflatoxin. Crit Rev Food Sci Nutr 43:245–264
3. Ostry V, Malir F, Toman J, Grosse Y (2017) Mycotoxins as human carcinogens-the IARC monographs classification. Mycotoxin Res 33:65–73
4. Sobrova P, Adam V, Vasatkova A, Beklova M, Zeman L, Kizek R (2010) Deoxynivalenol and its toxicity. Interdiscip Toxicol 3:94–99
5. Pestka JJ, Zhou HR, Moon Y, Chung YJ (2004) Cellular and molecular mechanisms for immune modulation by deoxynivalenol and other trichothecenes: unraveling a paradox. Toxicol Lett 153:61–73
6. Zinedine A, Soriano JM, Molto JC, Manes J (2007) Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: an oestrogenic mycotoxin. Food Chem Toxicol 45:1–18
7. Edite Bezerra da Rocha M, Freire FCO, Erlan Feitosa Maia F, Izabel Florindo Guedes M, Rondina D (2014) Mycotoxins and their effects on human and animal health. Food Control 36:159–165
8. Council for Agricultural Science and Technology (1980) Mycotoxins, economics and health risks, Report No.116, Ames.
9. Ma R, Zhang L, Liu M, Su Y, Xie W, Zhang N, Dai J, Wang Y, Rajput SA, Qi D, Karrow NA, Sun L (2018) Individual and combined occurrence of mycotoxins in feed ingredients and complete feeds in China. Toxins 10:113
10. Li X, Li P, Zhang Q, Li R, Zhang W, Zhang Z, Ding X, Tang X (2013) Multi-component immunochromatographic assay for simultaneous detection of aflatoxin $B_1$, ochratoxin A and zearalenone in agro-food. Biosens Bioelectron 49:426–432
11. Pereira VL, Fernandes JO, Cunha SC (2014) Mycotoxins in cereals and related foodstuffs: a review on occurrence and recent methods of analysis. Trends Food Sci Technol 36:96–136
12. Streit E, Schwab C, Sulyok M, Naehrer K, Krska R, Schatzmayr G (2013) Multi-mycotoxin screening reveals the occurrence of 139 different secondary metabolites in feed and feed ingredients. Toxins 5:504–523
13. Smith MC, Madec S, Coton E, Hymery N (2016) Natural co-occurrence of mycotoxins in foods and feeds and their in vitro combined toxicological effects. Toxins 8:94
14. Speijers GJ, Speijers MH (2004) Combined toxic effects of mycotoxins. Toxicol Lett 153:91–98
15. Halász A, Lásztity R, Abonyi T, Bata Á (2009) Decontamination of mycotoxin-containing food and feed by biodegradation. Food Rev Int 25:284–298
16. Hahn I, Kunz-Vekiru E, Twaruzek M, Grajewski J, Krska R, Berthiller F (2015) Aerobic and anaerobic in vitro testing of feed additives claiming to detoxify deoxynivalenol and zearalenone. Food Addit Contam Part A 32:922–933
17. Zhu Y, Hassan YI, Lepp D, Shao S, Zhou T (2017) Strategies and methodologies for developing microbial detoxification systems to mitigate mycotoxins. Toxins 9:130
18. Vanhoutte I, Audenaert K, De Gelder L (2016) Biodegradation of mycotoxins: tales from known and unexplored worlds. Front Microbiol 7:561
19. Loi M, Fanelli F, Liuzzi VC, Logrieco AF, Mule G (2017) Mycotoxin biotransformation by native and commercial enzymes: present and future perspectives. Toxins 9:111
20. Gao X, Ma Q, Zhao L, Lei Y, Shan Y, Ji C (2011) Isolation of *Bacillus subtilis*: screening for aflatoxins $B_1$, $M_1$, and $G_1$ detoxification. Eur Food Res Technol 232:957–962
21. Lei Y, Zhao L, Ma Q, Zhang J, Zhou T, Gao C, Ji C (2014) Degradation of zearalenone in swine feed and feed ingredients by *Bacillus subtilis* ANSB01G. World Mycotoxin J 7:143–151
22. Zhao L, Li X, Ji C, Rong X, Liu S, Zhang J, Ma Q (2016) Protective effect of *Devosia* sp. ANSB714 on growth performance, serum chemistry, immunity function and residues in kidneys of mice exposed to deoxynivalenol. Food Chem Toxicol 92:143–149
23. Fan Y, Zhao L, Ma Q, Li X, Shi H, Zhou T, Zhang J, Ji C (2013) Effects of *Bacillus subtilis* ANSB060 on growth performance, meat quality and aflatoxin residues in broilers fed moldy peanut meal naturally contaminated with aflatoxins. Food Chem Toxicol 59: 748–753
24. Zhao L, Lei Y, Bao Y, Jia R, Ma Q, Zhang J, Chen J, Ji C (2015) Ameliorative effects of *Bacillus subtilis* ANSB01G on zearalenone

toxicosis in pre-pubertal female gilts. Food Addit Contam Part A 32:617–625
25. Li X, Guo Y, Zhao L, Fan Y, Ji C, Zhang J, Ma Q (2018) Protective effects of *Devosia* sp. ANSB714 on growth performance, immunity function, antioxidant capacity and tissue residues in growing-finishing pigs fed with deoxynivalenol contaminated diets. Food Chem Toxicol 121:246–251
26. Li X, Zhao L, Fan Y, Jia Y, Sun L, Ma S, Ji C, Ma Q, Zhang J (2014) Occurrence of mycotoxins in feed ingredients and complete feeds obtained from the Beijing region of China. J Anim Sci Biotechnol 5:1–8
27. Duca RC, Bravin F, Delaforge M, Vladescu L, Badea IA, Criste RD (2009) Development of a new HPLC method used for determination of zearalenone and its metabolites in broiler samples. Influence of zearalenone on the nutritional properties of broiler meat. J Agric Food Chem 57:10497–10504
28. Chaytor AC, See MT, Hansen JA, de Souza AL, Middleton TF, Kim SW (2011) Effects of chronic exposure of diets with reduced concentrations of aflatoxin and deoxynivalenol on growth and immune status of pigs. J Anim Sci 89:124–135
29. Shi D, Zhou J, Zhao L, Rong X, Fan Y, Hamid H, Li W, Ji C, Ma Q (2018) Alleviation of mycotoxin biodegradation agent on zearalenone and deoxynivalenol toxicosis in immature gilts. J Anim Sci Biotechnol 9:42
30. Weaver AC, See MT, Hansen JA, Kim YB, De Souza AL, Middleton TF, Kim SW (2013) The use of feed additives to reduce the effects of aflatoxin and deoxynivalenol on pig growth, organ health and immune status during chronic exposure. Toxins 5:1261–1281
31. Zheng W, Ji X, Zhang Q, Du W, Wei Q, Yao W (2018) Hydrogen-rich water and lactulose protect against growth suppression and oxidative stress in female piglets fed *Fusarium* toxins contaminated diets. Toxins 10:228
32. Gerez JR, Pinton P, Callu P, Grosjean F, Oswald IP, Bracarense AP (2015) Deoxynivalenol alone or in combination with nivalenol and zearalenone induce systemic histological changes in pigs. Exp Toxicol Pathol 67:89–98
33. Jia R, Ma Q, Fan Y, Ji C, Zhang J, Liu T, Zhao L (2016) The toxic effects of combined aflatoxins and zearalenone in naturally contaminated diets on laying performance, egg quality and mycotoxins residues in eggs of layers and the protective effect of *Bacillus subtilis* biodegradation product. Food Chem Toxicol 90:142–150
34. Weaver AC, See MT, Kim SW (2014) Protective effect of two yeast based feed additives on pigs chronically exposed to deoxynivalenol and zearalenone. Toxins 6:3336–3353
35. Doll S, Danicke S, Schnurrbusch U (2003) The effect of increasing concentrations of *Fusarium* toxins in the diets for piglets on histological parameters of the uterus. Mycotoxin Res 19:73–76
36. Choi BK, Jeong SH, Cho JH, Shin HS, Son SW, Yeo YK, Kang HG (2013) Effects of oral deoxynivalenol exposure on immune-related parameters in lymphoid organs and serum of mice vaccinated with porcine parvovirus vaccine. Mycotoxin Res 29:185–192
37. Li Y, Ma Q, Zhao L, Wei H, Duan G, Zhang J, Ji C (2014) Effects of lipoic acid on immune function, the antioxidant defense system, and inflammation-related genes expression of broiler chickens fed aflatoxin contaminated diets. Int J Mol Sci 15:5649–5662
38. Yin S, Meng Q, Zhang B, Shi B, Shan A, Li Z (2015) Alleviation of zearalenone toxicity by modified halloysite nanotubes in the immune response of swine. Food Addit Contam Part A 32:87–99
39. Pistol GC, Braicu C, Motiu M, Gras MA, Marin DE, Stancu M, Calin L, Israel-Roming F, Berindan-Neagoe I, Taranu I (2015) Zearalenone mycotoxin affects immune mediators, MAPK signalling molecules, nuclear receptors and genome-wide gene expression in pig spleen. PLoS One 10:e0127503
40. Zhang L, Ma Q, Ma S, Zhang J, Jia R, Ji C, Zhao L (2016) Ameliorating Effects of *Bacillus subtilis* ANSB060 on growth performance, antioxidant functions, and aflatoxin residues in ducks fed diets contaminated with aflatoxins. Toxins 9:1
41. Doll S, Danicke S, Valenta H (2008) Residues of deoxynivalenol (DON) in pig tissue after feeding mash or pellet diets containing low concentrations. Mol Nutr Food Res 52:727–734
42. Goyarts T, Danicke S, Valenta H, Ueberschar KH (2007) Carry-over of *Fusarium* toxins (deoxynivalenol and zearalenone) from naturally contaminated wheat to pigs. Food Addit Contam 24: 369–380