Effects of compound probiotics and aflatoxin-degradation enzyme on alleviating aflatoxin-induced cytotoxicity in chicken embryo primary intestinal epithelium, liver and kidney cells

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Abstract

Aflatoxin B$_1$ (AFB$_1$) is one of the most dangerous mycotoxins for humans and animals. This study aimed to investigate the effects of compound probiotics (CP), CP supernatant (CPS), AFB$_1$-degradation enzyme (ADE) on chicken embryo primary intestinal epithelium, liver and kidney cell viabilities, and to determine the functions of CP + ADE (CPADE) or CPS + ADE (CPSADE) for alleviating cytotoxicity induced by AFB$_1$. The results showed that AFB$_1$ decreased cell viabilities in dose-dependent and time-dependent manner. The optimal AFB$_1$ concentrations and reactive time for establishing cell damage models were 200 µg/L AFB$_1$ and 12 h for intestinal epithelium cells, 40 µg/L and 12 h for liver and kidney cells. Cell viabilities reached 231.58% ($p < 0.05$) for intestinal epithelium cells with CP addition, 105.29% and 115.84% ($p < 0.05$) for kidney and liver cells with CPS additions. The further results showed that intestinal epithelium, liver and kidney cell viabilities were significantly decreased to 87.12%, 88.7% and 84.19% ($p < 0.05$) when the cells were exposed to AFB$_1$; however, they were increased to 93.49% by CPADE addition, 102.33% and 94.71% by CPSADE additions ($p < 0.05$). The relative mRNA abundances of IL-6, IL-8, TNF-α, iNOS, NF-κB, NOD1 (except liver cell) and TLR2 in three kinds of primary cells were significantly down-regulated by CPADE or CPSADE addition, compared with single AFB$_1$ group ($p < 0.05$), indicating that CPADE or CPSADE addition could alleviate cell cytotoxicity and inflammation induced by AFB$_1$ exposure through suppressing the activations of NF-κB, NOD1 and TLR2 pathways.

Introduction

Mycotoxins are toxigenic fungal secondary metabolites that mainly produced by *Aspergillus*, *Penicillium* and *Fusarium* to have great threat to human and animal health worldwide. The Food and Agriculture Organization (FAO) showed that approximately 25% of worldwide agricultural raw materials were contaminated with mycotoxins, leading to health problems and enormous economic losses (FAO 2013). So far, at least 400 kinds of mycotoxins such as aflatoxins, zearalenone, deoxynivalenol, fumonisin, patulin, T-2 toxin and ochratoxins have been identified (Cimbalo et al. 2020). Aflatoxins are the most known mycotoxins and have more than 20 types including aflatoxin B$_1$ (AFB$_1$), B$_2$, G$_1$, G$_2$ and M$_1$, among them AFB$_1$ is the most toxic mycotoxin due to its high frequency of contamination in various cereals such as nuts, corn and rice (Negash 2018). AFB$_1$ is able to cause poor feed efficacy, hepatotoxic, carcinogenic, teratogenic, immunosuppressive and other devastating effects on humans and animals (Meissonnier et al. 2008; Trebak et al. 2015; Zhang et al. 2016). Therefore, it is classified as group 1 carcinogen by the International Agency for Research on Cancer (IARC 2012).

Poultry is more sensitive to AFB$_1$ than the other kinds of animals. Digestion of AFB$_1$-contaminated feeds and its residues in poultry products will cause potential health hazard for humans and poultry (Peng et al. 2014). It was known that moldy food contains large amounts of AFB$_1$, especially in moldy peanuts and cereals. In poultry, AFB$_1$ can severely affect the immune system, thus leading to immunosuppression (Liu et al. 2016). AFB$_1$ can also cause apoptosis, gross and histopathological lesions in different organs, especially in liver, kidney, muscles and bursa of fabricius (Chen et al. 2014; Peng et al. 2014). It was also reported that AFB$_1$ intoxication can increase mortality, liver and kidney pathology, and decrease bodyweight and feed intake for broilers (Saleemi et al. 2019). Therefore, it is necessary to develop effective detoxification strategies to increase AFB$_1$ degradation and alleviate AFB$_1$-induced inflammatory and immunosuppression in chickens.

Up to date, several strategies have been reported to degrade AFB$_1$ including physical, chemical and biological methods. The physical detoxification methods (absorption, heating and irradiation) and the chemical detoxification
methods (ammonization, solvent extraction and oxidation) have many limitations such as nutritional losses, expensive equipment and low efficiency (Gregorio et al. 2014; Arzandeh and Jinap 2015; Zhu et al. 2016). It was found that the biological method was more effective to degrade mycotoxins (Das et al. 2014; Melvin et al. 2014; Fernández et al. 2015). Many species of microbes such as bacteria, molds and yeasts have demonstrated the capability to biodegrade AFB\textsubscript{1}, due to the metabolic transformation of AFB\textsubscript{1} by microbes or their adsorption ability for mycotoxins. It was reported that addition of lactic acid bacteria and \textit{S. cerevisiae} to AFB\textsubscript{1}-contaminated diet could reduce AFB\textsubscript{1} residues and prevent degenerative changes in the liver and kidney of broilers (Śliżewska et al. 2019). In addition, \textit{Aspergillus oryzae} have been reported to be able to degrade AFB\textsubscript{1} (Alberts et al. 2009). Another report also showed that the cooperation of compound probiotics (CP) and AFB\textsubscript{1}-degradation enzyme (ADE) could degrade AFB\textsubscript{1} effectively (Zuo et al. 2013; Huang et al. 2019).

It was reported that liver and kidney are the primary target organs of AFB\textsubscript{1} (Gholami-Ahangaran et al. 2016; Pérez-Acosta et al. 2016). In addition, the small intestine is the physical barrier which usually first contacts and absorbs AFB\textsubscript{1}, thus affecting intestinal health (Pinton and Oswald 2014). However, the optimal strategies for alleviating the negative effects of AFB\textsubscript{1} on intestine, liver and kidney of chickens have not been reported. Therefore, the aim of this study was to investigate the toxic effects of AFB\textsubscript{1} on chicken embryo primary intestinal, liver and kidney cells, and explore the efficacy of combination between CP and ADE to alleviate AFB\textsubscript{1}-induced toxicity and inflammatory for chickens.

Materials And Methods

Chemicals and AFB\textsubscript{1} preparation

Phosphate-buffered saline (PBS), 0.25% pancreatin with ethylenediaminetetraacetic acid (EDTA), collagenase (C8140, 246 U/mg), neutral protease (D6430, 0.5 U/mg), penicillin-streptomycin and thiazolyl blue tetrazolium bromide (MTT) were purchased from Beijing Solarbio Biotechnology Co., Ltd. Beijing, China. Collagenase and protease were dissolved in PBS to make 3000 U/mL and 0.5 U/mL, respectively. Percoll separation solution was diluted with PBS to 50%. Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12 at 1/1), M199 medium and fetal bovine serum (FBS) were purchased from Biological Industries (Kibbutz Beit-Haemek, Israel). Aflatoxin B\textsubscript{1} was purchased from Sigma-Aldrich (St. Louis, MO, U.S.), dissolved in 50% methanol to make 8 \(\mu\)g/mL AFB\textsubscript{1} concentration as the stock solution, filtered with 0.22 \(\mu\)m membrane high-flow filter (Sartorius Stedim Biotech Gmbh, Goettingen, Germany), and stored at 4°C for the following experiment.

Probiotics and AFB\textsubscript{1}-degrading enzyme preparation

Based on the previous research in our laboratory, four species of microorganisms with high AFB\textsubscript{1}-degrading abilities including \textit{Bacillus subtilis} (\textit{B. subtilis}, CGMCC1.0504), \textit{Enterococcus faecalis} (\textit{E. faecalis}, CGMCC1.2135), \textit{Candida utilis} (\textit{C. utilis}, CGMCC2.0615) and \textit{Lactobacillus casein} (\textit{L. casein}, CGMCC1.2884) were selected, which were obtained from China General Microbiological Culture Collection Center (CGMCC), Beijing, China. The microbes were incubated to more than \(1.0 \times 10^9\) CFU/mL according to the published protocols (Huang et al. 2018). After centrifugation at 4°C and 12000\(\times\)g for 10 min, the microbes and supernatants were collected, respectively. The supernatants were filtered through 0.22 \(\mu\)m membrane to remove the microbes, and then diluted to the final concentrations required by experiment design with cell media for subsequent experiments. The microbes were also adjusted to the different concentrations with cell media. Based on the previous results obtained with response
surface regression design in our laboratory in vitro, the optimal final counts of *B. subtilis, L. casein, E. faecalis* and *C. utilis* for AFB1-degradation were $1.0 \times 10^5$, $1.0 \times 10^5$, $1.0 \times 10^7$ and $1.0 \times 10^5$ CFU/mL to make the basal compound probiotics (CP). In order to measure the effects of different CP concentrations on cell viability or alleviating AFB1-induced cytotoxicity, the final counts of *B. subtilis, L. casein, E. faecalis* and *C. utilis* in CP were further designed as $1.0 \times 10^2$, $1.0 \times 10^2$, $1.0 \times 10^4$ and $1.0 \times 10^2$ CFU/mL to make CP1; $1.0 \times 10^3$, $1.0 \times 10^3$, $1.0 \times 10^5$ and $1.0 \times 10^3$ CFU/mL to make CP2; $1.0 \times 10^4$, $1.0 \times 10^4$, $1.0 \times 10^6$ and $1.0 \times 10^4$ CFU/mL to make CP3; $1.0 \times 10^5$, $1.0 \times 10^5$, $1.0 \times 10^7$ and $1.0 \times 10^5$ CFU/mL to make CP4; $1.0 \times 10^6$, $1.0 \times 10^6$, $1.0 \times 10^8$ and $1.0 \times 10^6$ CFU/mL to make CP5, respectively. Their corresponding supernatants were combined together to make CPS1, CPS2, CPS3, CPS4 and CPS5.

The AFB1-degradating enzyme was extracted from solid-state fermentation of *Aspergillus oryzae (A. oryzae, CGMCC3.4437)* according to the previous protocol (Huang et al. 2019). The crude enzyme solution of 10% AFB1-degrading enzyme was diluted with cell medium and stored at 4°C for further use. The AFB1-degrading enzyme activity in 10% crude enzyme solution was determined to be 51 U/mL according to the previous protocol (Gao et al. 2011).

**Primary chicken embryo intestinal epithelium, liver and kidney cell preparation**

The 14-day-old fertilized chicken eggs were purchased from Kaifeng Breeding Chicken Co., Ltd. Kaifeng, China, which were cleaned by 75% alcohol, placed in a vertical-flow clean bench ultra-clean, and handled with ultraviolet irradiation for 20 min. The air chamber of embryo was carefully broken with the tweezers, the chicken was taken out and quickly decapitated, followed by taking out small intestine, liver and kidney tissues, and rinsed in PBS containing 1% penicillin (10000 U/mL)-streptomycin (10 mg/mL) (Beijing Solarbio Biotechnology Co., Ltd. Beijing, China).

The mesentery of the small intestine was carefully exfoliated in PBS solution, cut into 1 mm size, put into 5 mL centrifuge tube, and washed with PBS until the supernatant was clear. After removing the washing solution, 1 mL 0.25% pancreatin was added to digest the tissues at 37 °C for 10 min with shaking once every 2 min. The tissues were centrifuged at 1000 r/min for 5 min to remove supernatant, and then 2 mL DMEM/F12 medium supplemented with 10% FBS and 1% penicillin-streptomycin were added. The filtrate was collected using 200-mesh sieve, and the cells were cultured in a 5% CO2 incubator at 37°C for 2 h. The supernatant was removed after centrifuged with 1000 r/min for 10 min, the cells were adjusted to $5.0 \times 10^5$ cells/mL with DMEM/F12 supplemented with 2.5% FBS and 1% penicillin + streptomycin. 0.2 mL or 2 mL cells were put in a 96-well or 12-well culture plate, and cultured at 37°C in a 5% CO2 incubator. The incubating cell medium was replaced every 2 d.

Liver cells were prepared as above and modified as following: 1 mL collagen protease and 1 mL neutral protease were added to digest the tissues at 37°C for 30 min with shaking once every 3 min. Then 2 mL M199 medium supplemented with 10% FBS and 1% penicillin and streptomycin were added. After shaking up and down, the filtrates were collected with a 200-mesh sieve, centrifuged with 1000 r/min for 10 min to remove the supematant. 1.5 mL M199 medium supplemented with 10% FBS and 1% penicillin and streptomycin were added to the centrifuge tube, and then 3 mL 50% percoll separation solution were added and mixed well, centrifuged for 15 min at 3000 r/min. After centrifugation the upper layer was removed, and the middle layer was taken out and put into a new centrifuge tube; then equivalent volume M199 medium was added to the new centrifuge tube, centrifuged for 10 min at 1000 r/min. At last the liver cells were resuspended with M199 medium supplemented with 10% FBS and 1%
penicillin and streptomycin, adjusted and cultured as above. Kidney cells were prepared with the same protocol as liver cells, modified by using DMEM/F12 medium to replace M199 medium.

**Cell viability assay and experimental design**

Three kinds of primary cells were seeded into 96-well plates. Cell viability was measured by MTT assay every 2 d (Fotakis and Timbrell 2005). The growth curves of three kinds of cells were plotted with time as the abscissa and absorbance value as the ordinate. The following experiments were carried out in the logarithmic phase of cells. The experimental designs were as follows:

1. **Effect of different AFB$_1$ concentrations on cell damage:** Three kinds of cells were seeded into 96-well plates with a density of $5.0 \times 10^5$ cells/mL, cultured to their logarithmic phases, followed by removing the culture medium and washing twice with PBS, and subsequently incubated with different concentrations of AFB$_1$ for 6, 12, 24 and 48 h, respectively. AFB$_1$ concentrations were 0, 40, 80, 120, 160 and 200 µg/L for intestinal epithelium cells; 0, 10, 20, 40 and 80 µg/L for the liver and kidney cells. AFB$_1$ was diluted with the corresponding cell media without serum and antibiotics.

2. **Effect of CP or CPS on cell viability:** The cells were prepared as above. CP and CPS were diluted with the corresponding cell media without serum and antibiotics. The cells were incubated with the different concentrations of CP or CPS for 12, 24 and 48 h, respectively.

3. **Effect of ADE on cell viability:** ADE was diluted with the cell medium without serum and antibiotics to make the final concentrations at 0, 0.0001%, 0.001%, 0.01%, 0.1% and 1%, which was incubated with cells for 6, 12, 24 and 48 h, respectively.

4. **The functions of CPADE and CPSADE for alleviating cytotoxicity:** The cell culture was 12 h. The detail design was listed in Table 1. The previous report in our laboratory showed that CPADE and CPSADE were more effective than CP, CPS and ADE for degrading AFB$_1$ (Huang et al. 2018); therefore, CP, CPS and ADE were not considered for alleviating cytotoxicity induced by AFB$_1$ in this study.

At the end of above cell incubations, each well was added with 10 µL 5 mg/mL MTT and incubated for 4 h. Then the cell supernatants were removed and 150 µL DMSO was added to each well. Thereafter, the plates were shaken for 10 min at room temperature. The absorbances (A) were determined at 490 nm wavelength with a reference wavelength of 630 nm by an ELx 800 microplate reader (BIO-TEK Instruments Inc., Winooski, VT, USA). The cell viability (%) = $(A_{490nm} - A_{630nm}$ value in the experimental groups) / $(A_{490nm} - A_{630nm}$ in the control groups) × 100%.

**Table 1 The experimental designs for CPADE or CPSADE to alleviate primary cell damages induced by AFB$_1$**

| Primary cells       | Control          | AFB$_1$   | CPADE or CPSADE | CPADE or CPSADE + AFB$_1$ |
|---------------------|------------------|-----------|------------------|--------------------------|
| Intestinal epithelium cells | DMEM/F12  | 200 µg/L | CP2 0.001%ADE | CP2 0.001%ADE + 200 µg/L AFB$_1$ |
| Liver cells         | M199            | 40 µg/L  | CPS4 0.001%ADE | CPS4 0.001%ADE + 40 µg/L AFB$_1$ |
| Kidney cells        | DMEM/F12        | 40 µg/L  | CPS3 0.001%ADE | CPS3 0.001%ADE + 40 µg/L AFB$_1$ |

**Reverse transcription PCR and quantitative real-time PCR**
The primary intestinal epithelium, liver and kidney cells were seeded with a density of $5.0 \times 10^5$ cells/mL in 12-well culture plates and allowed to adhere for 24 h, respectively. After four treatments (Control, AFB$_1$, CPADE or CPSADE, CPADE or CPASDE + AFB$_1$) for three kinds of primary cells for 12 h respectively, total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) according to the standard manufacturer's instructions, and then dissolved in 50 µL RNase-free water, stored at -80°C. The quality and concentration of RNA samples were measured by NanoDrop ND-1000 Spectrophotometer (Nano-Drop Technologies, Wilmington, DE, U.S.). Approximately 1 µg total RNA from each sample was reversely transcribed into cDNA by TB GREEN kit (TaKaRa, Dalian, China). Quantitative RT-PCR was performed with CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). All the primers used in this study were listed in Table 2. The β-actin was used as a house-keeping gene, and the relative gene abundances in chicken embryo primary intestinal epithelium, liver and kidney cells were analyzed using the $2^{\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Table 2. Primer sequences of some genes for quantitative RT-PCR

| Gene      | Accession number | Primer sequence (5′-3′) |
|-----------|------------------|-------------------------|
| β-actin   | LO8165           | F: GAGAAATTGTGCGTGACATCA  |
|           |                  | R: CCTGAACCTCTCATGCGCA   |
| IL-6      | AJ309540         | F: CAAGGTGACGAGGAGGAGAC  |
|           |                  | R: TGCCGAGGAGGATTCTT    |
| IL-8      | AJ009800         | F: ATGAACGGCAAGCTTGAGCTG |
|           |                  | R: TCCAAGCACACTCCTTCATCC |
| iNOS      | U46504           | F: CAGCTGATTTGGGTGAT     |
|           |                  | R: TTTCTTTGGCCTAGGGTC    |
| NF-κBp65  | NM_205129        | F: GTGTGAGAGAAAGGACTG    |
|           |                  | R: GGCACGGTTTGATAGATG    |
| TNF-α     | NM_204267        | F: GAGCTGTGACTTGGCTG    |
|           |                  | R: AAGCAACAACCACATGCA    |
| NOD1      | JX465487         | F: AGCACGTGCTCATGTGCC    |
|           |                  | R: TGAGGTTGGTAAGGTCTGCT  |
| TLR2      | NM_001161650     | F: GGGCTCAGGCAAAAATC    |
|           |                  | R: AGCAGGGTTTCTACGTCCA   |

Statistical analysis

All experimental data were presented as means ± standard deviations. The data were analyzed using one-way analysis of variance (ANOVA) by the Duncan method with SPSS 20.0 software (Sishu Software, Shanghai Co., Ltd. Shanghai, China). All graphs were generated using GraphPad Prism 8. Differences were considered as statistically significance at $p < 0.05$.

Results

The growth curves of primary intestinal epithelium, liver and kidney cells of chicken embryo

Figure 1 demonstrated that the logarithmic growth phases of intestinal epithelium, liver and kidney cells appeared during the incubation periods of 8-12, 6-12 and 6-12 d, and reached the logarithmic peak on the 10 th, 12 th and 6 th d, respectively ($p < 0.05$).

Effects of AFB$_1$ on the viabilities of primary intestinal epithelium, liver and kidney cells

Table 3 showed that AFB$_1$ decreased cell viability in dose-dependent and time-dependent manners. The higher AFB$_1$ concentrations and longer incubation time caused more serious damages for three kinds of cells. AFB$_1$ had
insignificant effect on intestinal epithelium cell viability when its concentration was below 80 μg/L within 48 h incubation ($p > 0.05$); however, it was significantly influenced when AFB$_1$ concentration were more than 80 μg/L ($p < 0.05$), especially under the condition that the incubation time was 48 h. Liver and kidney cells of chicken embryo were more sensitive to AFB$_1$ than intestinal epithelium cells. They were significantly influenced by 80 μg/L AFB$_1$ within 6 h incubation, 40 μg/L AFB$_1$ within 12 h incubation, 20 μg/L AFB$_1$ within 24 h incubation, 10 μg/L AFB$_1$ within 48 h incubation ($p < 0.05$), compared with the control group. After considering the above results, AFB$_1$ concentrations and reaction time were confirmed as 200 μg/L and 12 h for intestinal epithelium cells, 40 μg/L AFB$_1$ and 12 h for liver and kidney cells in the subsequent experiments.

Table 3 Effects of different AFB$_1$ concentrations and incubation time on primary cell viability (%)

| Time (h) | AFB$_1$ concentrations (μg/L) |
|---------|-------------------------------|
|         | Intestinal epithelium cells   | Liver cells                     | Kidney cells       |
|         | 0                             | 40                             | 80                 | 120                            | 160                            | 200                             |
| 6       | 100.00±2.94$^a$               | 105.88±5.88$^a$                | 102.94±8.82$^a$    | 91.18±8.82$^{ab}$               | 94.12±5.88$^{ab}$               | 88.24±8.82$^b$                |
|         | 100.00±0.29$^a$               | 100.00±11.43$^a$               | 102.86±17.14$^a$   | 97.14±8.57$^a$                 | 100.00±8.57$^a$                 | 85.71±7.14$^b$                |
| 12      | 100.00±14.63$^a$              | 102.44±2.44$^a$                | 100.00±2.44$^a$    | 100.00±2.44$^a$                | 97.56±4.88$^a$                 | 85.37±7.32$^b$                |
|         | 100.00±5.13$^a$               | 100.00±5.13$^a$                | 94.87±7.69$^{ab}$  | 82.01±5.88$^{ab}$               | 85.71±7.14$^b$                 | 48.72±5.13$^c$                |
| 24      | 100.00±4.88$^{ab}$            | 109.76±4.88$^a$                | 112.2±4.88$^a$     | 100.00±7.32$^{ab}$              | 85.71±4.76$^b$                 | 71.53±3.61$^c$                |
|         | 100.00±9.52$^a$               | 109.52±9.52$^a$                | 104.76±7.14$^a$    | 80.95±7.14$^b$                 | 73.68±3.29$^c$                 | 56.25±3.13$^c$                |
| 48      | 100.00±2.13$^a$               | 89.66±5.31$^b$                 | 79.12±4.54$^c$     | 74.17±3.68$^c$                 | 73.68±3.29$^c$                 | 47.69±4.62$^d$                |
|         | 100.00±0.23$^a$               | 68.18±4.55$^b$                 | 72.73±9.09$^b$     | 79.55±4.55$^b$                 | 75.00±4.55$^b$                 | 48.72±5.13$^c$                |

Data were expressed as mean ± SD (n = 8). The different lowercase letters in the same row indicate significant difference from each other ($p < 0.05$), while the same lowercase letters in the same row indicate insignificant difference from each other ($p > 0.05$).

Table 4 showed that different concentrations of CP and CPS had different effects on three kinds of cell viabilities. The relative cell viabilities reached 231.58%, 163.33% and 138.32% ($p < 0.05$) for intestinal epithelium, liver and kidney cells at CP2 levels for 12 h incubation, respectively; which reached 136.13% and 115.84% ($p < 0.05$) at CPS4 levels after 12 h incubation for intestinal epithelium and liver cells, 105.29% ($p < 0.05$) at CPS3 levels after 12 h incubation for kidney cells. According to the above results, the optimal incubation time was selected as 12 h in the
subsequent experiment. In general, the liver and kidney cells can't directly contact with microbes; therefore, CPS was selected in the subsequent experiments for liver and kidney cell incubations.

Table 4 Effects of different CP or CPS concentrations and incubation time on primary cell viabilities (%)

| Time (h) | CP1 or CPS1 | CP2 or CPS2 | CP3 or CPS3 | CP4 or CPS4 | CP5 or CPS5 |
|----------|-------------|-------------|-------------|-------------|-------------|
| Intestinal epithelium cells | | | | | |
| CP | 12 | 198.25±10.53b | 231.58±5.26c | 157.89±5.26a | 145.61±7.02a | 207.02±3.51b |
| | 24 | 130.23±9.30a | 120.93±11.16ab | 90.70±8.10d | 109.3±7.67c | 109.30±6.60bc |
| | 48 | 84.78±8.70a | 80.43±4.35a | 60.87±3.04b | 47.83±4.35c | 50.00±3.52c |
| CPS | 12 | 116.85±5.01bc | 113.88±4.87c | 105.39±1.52d | 136.13±1.59a | 122.24±4.24b |
| | 24 | 104.00±3.50d | 132.00±11.4c | 157.00±2.80a | 116.00±3.70b | 113.92±5.80b |
| | 48 | 103.00±3.27a | 102.00±4.87a | 104.00±3.89a | 99.00±2.91a | 104.00±5.17a |
| Liver cells | | | | | |
| CP | 12 | 141.67±0.08b | 163.33±0.6a | 130.00±0.33b | 110.00±2.60c | 103.33±1.70c |
| | 24 | 127.50±0.10a | 102.50±1.25c | 87.50±1.20e | 95.00±1.60d | 112.50±1.13b |
| | 48 | 68.09±0.40b | 59.57±1.50c | 59.57±2.67c | 78.72±0.88a | 80.85±1.29a |
| CPS | 12 | 99.87±1.89b | 99.76±0.88b | 102.41±1.57b | 115.84±3.74a | 114.07±0.72a |
| | 24 | 100.10±1.26b | 102.34±1.26b | 101.79±2.19b | 117.25±1.99a | 114.96±6.46a |
| | 48 | 99.12±0.76b | 97.37±1.89b | 96.01±2.93b | 99.00±2.91a | 102.94±2.24a |
| Kidney cells | | | | | |
| CP | 12 | 124.30±4.67b | 138.32±1.87a | 106.54±1.87c | 123.36±10.28b | 118.69±4.67b |
| | 24 | 120.00±7.62B | 130.05±2.86a | 72.38±21.90c | 53.33±4.76d | 56.19±6.67d |
| | 48 | 67.24±3.45a | 51.72±1.72b | 54.31±19.83b | 29.31±8.62c | 33.62±4.31c |
| CPS | 12 | 101.37±1.18b | 99.50±2.26b | 105.29±1.34a | 97.56±3.67b | 89.25±1.28c |
| | 24 | 100.58±2.12b | 102.25±2.14b | 111.30±0.94a | 100.41±2.97b | 77.78±2.07c |
| | 48 | 100.28±1.33B | 103.65±2.43b | 106.72±5.81a | 90.00±2.05c | 48.26±2.66d |

Data were expressed as mean ± SD (n = 8). The different lowercase letters in the same row indicate significant difference from each other (p < 0.05), while the same lowercase letters in the same row indicate insignificant difference from each other (p > 0.05). CP: compound probiotics; CPS: cell-free compound probiotics supernatant.

**Effects of ADE on viability of three kinds of primary cells**

Figure 2 showed that the relative viabilities of three kinds of cells were significantly decreased (p < 0.05) when ADE concentrations were between 0.01% and 1%; however, the cell viabilities were significantly increased when ADE concentrations were between 0.001% and 0.0001% (p < 0.05). Therefore, the optimal ADE content was selected as 0.001% in the subsequent experiment.

**Effects of CPADE or CPSADE on alleviating viabilities of three primary cells induced by AFB$_1$**

Figure 3 showed that the relative viabilities of intestinal epithelium, liver and kidney cells induced by AFB$_1$ were significantly decreased to 87.12%, 88.7% and 84.19% (p < 0.05), whereas CPADE or CPSADE addition significantly increased the cell viabilities to 93.49%, 102.33% and 94.71% (p < 0.05), respectively.

**Effects of CPDE or CPSADE on mRNA abundances of some genes in the three kinds of primary cells induced by AFB$_1$**

Figure 4 indicated that AFB$_1$ exposures during intestinal epithelium, liver and kidney cell incubations could up-regulate the mRNA abundances of some genes such as IL-6, IL-8, TNF-α (except for liver), NF-κBp65, iNOS, NOD1.
(except for liver) and TLR2 ($p < 0.05$); however, CPADE or CPSADE addition could retrieve the above results. It could be concluded that CPADE or CPSADE addition was able to alleviate cell inflammation induced by AFB$_1$ through positively regulating some signal pathways.

**Discussions**

Aflatoxin is a ubiquitous dietary contaminant all over the world, which leads to low feed intake, low efficiency, and substantial economic losses to producers (Tedesco et al. 2004). Aflatoxin B$_1$ is frequently detected in stored cereals such as corn, wheat and other feedstuffs or diets to cause liver damage and immunity inhibition of domestic animals (Kraieski et al. 2016; Yuan et al. 2016). AFB$_1$ residual in domestic animals may be harmful to human and public health. Liver is the main target organ of AFB$_1$, but AFB$_1$ is also detected in kidney and intestinal tract of animals. Therefore, it is necessary to find an effective and safe method to alleviate AFB$_1$ for animal and human. Nowadays, probiotics have been widely used to degrade mycotoxins. It was reported that Bacillus subtilis could germinate in the intestinal tract, and reduce AFB$_1$ absorption and residues in the internal organs of broilers (Salem et al. 2018). The compound probiotics of B. subtilis, L. casei and C. utilis were reported to increase production performance, alleviate histological lesions, degrade mycotoxins and decrease mycotoxin residues in broilers (Chang et al. 2020). In order to increase the efficiency of alleviating AFB$_1$-induced cell damage, the compound probiotics was combined with AFB$_1$-degrading enzyme in this study.

This result showed that the viabilities of three kinds of primary cells were decreased with increasing AFB$_1$ concentrations and incubation time, suggesting that both of them are the main factors for determining the extent of AFB$_1$ toxicity. In general, liver and kidney cells are more sensitive than intestinal cells, which may be related to different effects of different AFB$_1$ concentrations in the various cell types and organs (Zain 2011). AFB$_1$ can be metabolized to high reactive metabolites by cytochrome P450 enzyme system in liver cell, resulting in formation of AFB$_1$-DNA adducts to cause carcinogenesis and mutations (Valeria et al. 2020; Owumi et al. 2020). The kidney cell could be directly damaged by AFB$_1$ through increasing cell apoptosis and death (Li et al. 2019). For the intestinal epithelium cells, AFB$_1$ damage was mainly presented from barrier function loss and inflammatory response (Hernández-Ramírez et al. 2019). Because intestinal epithelium cells usually contact with AFB$_1$ directly, the long-term adaptation makes them be insensitive to AFB$_1$ than liver and kidney cells. The addition of compound probiotics and mycotoxin-degrading enzyme could contribute to cell proliferations and alleviate the toxicity induced by AFB$_1$, which may be from mycotoxin biodegradation (Huang et al. 2018). It was found that the different concentrations of CP or CPS at different reaction times had different effects on the viabilities of three kinds of cells; therefore, the optimal CP or CPS concentrations and reaction time were selected for improving viabilities of different cell types. It was also indicated that CP was more effective than CPS for increasing cell viabilities, maybe due to the interaction between primary cells and microbes.

The previous researches have indicated that lactic acid bacteria can synthesize a wide variety of polysaccharides during their growth process (Round et al. 2011; Poole et al. 2018). These polysaccharides can be classified into two kinds, one kind can be tightly linked to the cell surface forming the capsular polysaccharides, which are loosely attached to the extracellular surface, or secreted to the environment as exopolysaccharides (Castro-Bravo et al. 2018). Capsular polysaccharide adhesion to intestinal epithelial cells is believed to help probiotic bacteria to transiently colonize and persist on epithelial cells for decreasing the colonization of intestinal pathogens (Castro-Bravo et al. 2018). Another kind is called extracellular polysaccharides, which can modulate intestinal immunity and reduce the secretion of proinflammatory cytokines (Laino et al. 2016). Enterococcus faecalis can directly produce
extracellular polysaccharide (Rossi et al. 2015), which may be the reason why CP is able to improve cell vitality more than CPS in this study. However, the long-term incubation of CP or CPS was harmful to cells, the reason may be due to the secondary metabolites produced by probiotics to influence cell growth.

*Aspergillus oryzae* can produce many kinds of enzymes such as protease and amylase except for AFB$_1$-degradation enzyme, which may affect cell paste and growth. The reason why high ADE concentrations could influence cell viability may be due to the high levels of enzymes existing in ADE to damage cells, so low ADE concentration is selected in this study. It was reported that supplementation of *L. bulgaricus* or *L. rhamnosus* could produce significant protective effect against AFB$_1$-induced liver damage and inflammatory response (Chen et al. 2019). Moreover, the addition of compound probiotics and mycotoxin-degradation enzyme could prevent broilers from damages induced by AFB$_1$ (Zuo et al. 2013). In this study, four kinds of compound probiotics plus AFB$_1$-degradation enzyme additions significantly increased the cell viability induced by AFB$_1$, inferring that CPDE or CPSADE could alleviate the toxicology induced by AFB$_1$ in three kinds of primary cells.

The previous studies have demonstrated that AFB$_1$ exposure can induce inflammation response in different cells and organs (Zhang et al. 2019; Wang et al. 2019; Zhao et al. 2019). Inflammation is a response against infection, illness and injury by the excessive expressions of chemokines and inflammatory cytokines such as factor-alpha (TNF-α), interleukin-6 (IL-6) and interleukin-8 (IL-8) (Barutta et al. 2015; Guo et al. 2015). TNF-α is a proinflammatory cytokine, which can stimulate various kinds of cells to produce chemokines to cause tissue damage and inflammation response (Shanmugam et al. 2016). It can be speculated that the degree of AFB$_1$-induced damage may be decreased by suppressing the overexpression of inflammatory cytokines. In this study, AFB$_1$ exposure significantly up-regulated the mRNA abundances of IL-6, IL-8 and TNF-α in the three kinds of primary cells, but CPDE or CPSADE addition significantly down-regulated their mRNA abundances in the intestinal and kidney cells except for TNF-α in liver cells, indicating that probiotic combined with ADE may directly decrease the production of TNF-α or indirectly suppress gene expressions of some pro-inflammatory cytokines such as IL-6 and IL-8 (Weninger and Andrian 2003).

NF-κB is an important nuclear transcription factor and a major regulator for anti-inflammatory. The activated NF-κB plays a vital role in inflammatory response by regulating multiple cytokines (Zhang et al. 2018). In response to the inflammation cytokines, inducible nitric oxide synthase (iNOS) can catalyze the production of NOD, which is the potent pro-inflammatory mediator (Surh et al. 2001). NOD1 is an innate immune sensor, which consists of a C-terminal leucine-rich region (LRR), central NOD and N-terminal caspase-activating domain (CARD) (Ma et al. 2020). NOD1 plays an important role in response to pathogen infection to induce activation of intracellular signaling pathway, leading to pro-inflammatory response (Caruso et al. 2014; Robertson et al. 2016). Several studies also showed that TLRs and NODs can participate in production of pro-inflammatory molecules to enhance immune responses (Van-Heel et al. 2005; Fritz et al. 2005). It was reported that NLRs, NOD1 and NOD2 have the similar domain architectures and functions, but have the different CARD domain numbers (Trindade and Chen 2020). The previous studies have confirmed that NOD1 and NOD2 can activate the classical NF-κB and MAPK pathways, which are related to cell inflammation and apoptosis (Seger and Wexler 2016).

TLRs also play the vital roles in innate immune system, the effects of different mycotoxins on gene expression of TLR2, TLR4 and TLR7 have been reported (Chen et al. 2013). It has been reported that 600 μg/kg AFB$_1$ in broiler diet can simultaneously down-regulate the expressions of TLR2, TLR4 and TLR7 genes in the intestinal tissues of broilers, and decrease the expressions of cytokines such as IFN-γ and TNF-α to reduce the innate immunity of broilers (Wang et al. 2018). However, another research showed that mixed aflatoxins B and G could up-regulate TLR2
and TLR4 transcripts (Malvandi et al. 2013), corresponding with this study, which may due to the dose-dependent effect of aflatoxins (Peng et al. 2016).

In this study, AFB₁ exposure could up-regulate NF-κBp65, iNOS, NOD1 and TLR2 mRNA abundances in intestinal, kidney and liver cells to cause to the multiple inflammatory pathway responses, in agreement with the previous reports (Yan et al. 2020); however, CPADE or CPSADE addition could down-regulate their mRNA abundances except for NOD1 and TNF-α in liver cells, indicating that CPADE or CPSADE was able to alleviate cell inflammations and damages induced by AFB₁ through suppressing the pathway activations of NF-κB, iNOS, NOD1 and TLRs.

It can be concluded that CPADE or CPSADE is able to alleviate AFB₁-induced cytotoxicity and inflammation of chicken embryo primary intestinal epithelium, liver and kidney cells by down-regulating mRNA abundances of inflammation cytokines through suppressing the activations of NF-κB, iNOS, NOD1 and TLRs signal pathways. These findings provide insights into the future development of strategies for CPADE or CPSADE to protect the primary cells from AFB₁-induced damages.

**Abbreviations**

AFB₁: Aflatoxin B₁; IL-6, Interleukin 6; IL-8, Interleukin 8; iNOS, Inducible nitric oxide synthase; NF-κBp65, Nuclear factor kappa B p65; TNF-α, Tumor necrosis factor α; NOD1, Nucleotide-binding oligomerization domain containing 1; TLR2, Toll like receptor 2; CP: compound probiotics, CPS: Cell-free compound probiotic supernatant; ADE: AFB₁-degradation enzyme; CPADE: Compound probiotics plus AFB₁-degradation enzyme; CPSADE: Cell-free compound probiotics supernatant plus AFB₁-degradation enzyme.

**Declarations**

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Not applicable.

**Authors’ Contributions**

HWG and QQY conceived and designed the experiments; HWG, CQL, XXX and XWD performed the experiments; JC and PW analyzed the data; XFH and QLW contributed reagents/materials/analysis tools. HWG drafted the manuscript. QQY reviewed and revised the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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