Preventive Veterinary Medicine 168 (2019) 9–18

Contents lists available at ScienceDirect

Preventive Veterinary Medicine

journal homepage: www.elsevier.com/locate/prevetmed

# Effect of dietary supplementation of mannanoligosaccharides on hepatic gene expressions and humoral and cellular immune responses in aflatoxin-contaminated broiler chicks

E. Jahanian[a,*], A.H. Mahdavi[a], S. Asgary[b], R. Jahanian[a], M.H. Tajadini[c]

[a] Department of Animal Sciences, College of Agriculture, Isfahan University of Technology, Isfahan 84156-83111, Iran
[b] Isfahan Cardiovascular Research Center, Cardiovascular Research Institute, Isfahan University of Medical Sciences, Isfahan, Iran
[c] Applied Physiology Research Center, Isfahan University of Medical Sciences, Isfahan, Iran

ARTICLE INFO

*Keywords:*
Broiler chicks
Mannanoligosaccharides
Aflatoxin challenge
Cellular immunity
Hepatic gene expression

ABSTRACT

The present study was conducted to investigate the effects of dietary supplementation of mannanoligosaccharides (MOS) on expression of hepatic immunological genes and immune responses in aflatoxin-contaminated broiler chicks. A total of 336 seven-day-old Ross broiler chicks were randomly allotted to 7 experimental treatments with 4 replicates and 12 birds per replicate. Experimental treatments consisted of 2 aflatoxin levels (0.5 and 2 ppm) and 3 supplemental MOS levels (0, 1 and 2 g/kg) as a 2 × 3 factorial arrangement in comparison with a control group (unchallenged group). The chicks were challenged with a mix of aflatoxins during 7–28 d of age. Results showed that aflatoxin challenge resulted in the lower antibody titers against infectious bronchitis (IBV) and bursal (IBD) diseases viruses. In addition, aflatoxin-contaminated birds had a lower ($P < 0.0001$) lymphocyte percentage and a decline in ($P < 0.01$) interleukin-2 (IL-2) mRNA abundance. Likewise, heterophil proportion, heterophil to lymphocyte ratio and gene expressions of hepatic interleukin-6 (IL-6) and C reactive protein (CRP) were raised ($P < 0.001$) by increasing dietary aflatoxin level. Dietary inclusion of MOS increased ($P < 0.05$) antibody titers against IBV, IBD and Newcastle disease virus. Lymphocyte proportion and hepatic IL-2 gene expression were greater ($P < 0.0001$) in MOS-supplemented birds. Furthermore, supplemental MOS decreased hepatic IL-6 and CRP abundances. Additionally, inclusion of 2 g/kg MOS resulted in the upregulation ($P < 0.01$) of hepatic IL-2 gene expression in birds contaminated with 0.5 ppm aflatoxin. The present results indicate that supplemental MOS could improve cellular immunity via the upregulation of hepatic IL-2 gene expression in birds challenged with aflatoxins.

## 1. Introduction

Approximately 25% of the world food crops are contaminated by fungi specially *Aspergillus parasiticus* and *Aspergillus flavus* according to the United Nations Food and Agriculture Organization (FAO, 2001) and the World Health Organization (WHO, 2000). Mycotoxins manifested wide series of biological activities including mutagenic, carcinogenic, hepatotoxic, and also immunosuppressive (Bennett and Klich, 2003; Pestka et al., 2004). Whitlow and Hagler (2002) suggested that mycotoxins act through 4 proposed mechanisms: 1) decreased feed intake, 2) depressed nutrient absorption and changed metabolism, 3) immunoinhibitory effects, and also 4) altered endocrinological status.

The immune system is vital defensive mechanism against invading organisms and foreign substances such as mycotoxins (Sharma, 1993). Exposure to mycotoxins manifests clinical signs including diarrhea, vomiting, and hemorrhage (Canady et al., 2001). Interestingly, tissues with higher protein turnover including immune organs, liver and small intestine have been found to be negatively affected by mycotoxins challenges (Feinberg and Mclaughlin, 1989). The effect of mycotoxins on immune system is either suppressive or stimulator depending on the time, duration and dose of exposure (Pestka, 2008). Aflatoxins, especially aflatoxin B1, are well known to be the most mutagenic and carcinogenic agents amongst mycotoxins (Hussein and Brasel, 2001; Bennett and Klich, 2003) and to negatively affect animals, livestock and humans health and production (Chen et al., 2008). Aflatoxins have been frequently demonstrated to exert detrimental impacts on immunity via

*Abbreviations:* MOS, mannanoligosaccharide; CRP, C reactive protein; IL2, Interleukin 2; IL6, Interleukin 6; NDV, Newcastle disease virus; IBD, infectious bursal disease; IBV, infectious bronchitis virus; OD, optical density

* Corresponding author at: Elaheh Jahanian, Department of Animal Sciences, College of Agriculture, Isfahan University of Technology, Isfahan 84156-83111, Iran.
*E-mail address:* elaheh.jahanian@gmail.com (E. Jahanian).

https://doi.org/10.1016/j.prevetmed.2019.04.005
Received 5 September 2018; Received in revised form 16 March 2019; Accepted 13 April 2019

regression of bursa of Fabricius and lowering serum immunoglobulin in ducks (Ortatatli and Oguz, 2001; Chen et al., 2014). Hence, long exposure to aflatoxin B1 elevates susceptibility to infections and the outbreaks of diseases (Thaxton et al., 1974). However, other researchers (Cheng et al., 2001; Chaytor et al., 2011) showed that the relative weights of lymphoid organs and the leukocyte counts were unaffected in animal and poultry challenged with aflatoxin. Thereby, much attention has been focused on finding efficient strategies to prevent or to reduce the immunotoxic effects derived from aflatoxins.

Several inorganic adsorbents were previously applied to control the toxic effects of mycotoxins (Santin et al., 2002a; Thieu et al., 2008). Currently, one of the most interesting adsorbents, which have attracted much attention, is organic adsorbents such as mannanoligosaccharides (MOS) and β-glucan derived from yeast cell wall (Khan et al., 2017). Yeast and yeast cell wall components ameliorate the deleterious effects of mycotoxins (Huwig et al., 2001). The cell wall components (especially β-glucans) play the strongest role in mycotoxins adsorbent capability (Yiannikouris et al., 2004). Glucomannan derived from *Saccharomyces cerevisiae* sequestrates the toxins and prevents them from being absorbed in gastrointestinal tract of animals as previously proposed by Jouany et al. (2005). The proposed action mechanism of β-glucans to adsorb aflatoxin is that they contribute to formation of hydrogen bonds with aflatoxin molecule through the presence of hydroxyl, lactone and ketone groups (Yiannikouris et al., 2006). Interestingly, it seems supplemental MOS reduced the impact of aflatoxicosis. Besides the adsorbent capacity, yeast cell wall is known to be immunostimulant because of the active components including chitin, mannan and glucan (Li and Gatlin, 2003; Rodriguez et al., 2003). Shashidhara and Devegowda (2003) exhibited that improvement in antibody titer against infectious bursal disease is evident after feeding MOS to broiler breeders.

Although the immunotoxic effects arising from aflatoxins have been frequently evidenced by several studies, little information is available regarding to the effects of dietary inclusion of MOS on hepatic gene expressions and humoral and cellular immune responses in aflatoxin-challenged birds. The present study, therefore, was conducted to investigate the influence of supplemental MOS on expression of immunity-related genes in aflatoxicated broiler chicks.

## 2. Material and methods

### 2.1. Experimental design and dietary treatments

The present study was conducted in the Poultry Research Station of Isfahan University of Technology and all protocols were approved by Isfahan University of Technology Animal Care and Use Committee. Three hundred and thirty six Ross 308 broiler chicks of seven-day-old were randomly assigned into 7 experimental treatments with 4 replicates of 12 birds each. Experimental treatments consisted of a control group (unchallenged group) and a 2 × 3 factorial arrangement of treatments including 2 aflatoxin levels (0.5 and 2 ppm) and 3 levels of supplemental MOS (0, 1 and 2 g/kg). Chicks were contaminated with a mix of aflatoxins from 7 to 28 d of age. The experimental diets were formulated to meet all of the nutritional requirements of broiler chicks according to Ross Broiler Management Manual (2009). Light was on continuously for the first week, and a 23 L: 1 D lighting schedule was performed during remaining trial period. Feed and water were offered *ad libitum*. Temperature was set on 33 °C during the first week and then reduced by 3 °C/week to 24 °C.

### 2.2. Aflatoxin preparation and measurement

*Aspergillus parasiticus* PTCC-5286 was obtained from the Iranian Research Organization for Science and Technology to produce aflatoxins. *Aspergillus parasiticus* was cultured on sterile potato dextrose agar and then incubated at 28 °C for 5 days. After providing uniform fungus spore suspension, the number of spores per mL of distilled water was counted using Hemocytometer. Amount of 150 g of rice with 150 mL of water was thoroughly mixed in a flask and autoclaved to produce a high quantity of fungus. Afterwards, 7–7.5 × $10^6$ spores/mL of suspension were inoculated in flask and then incubated at 28 °C for 5 days.

**Table 1**
Final aflatoxins concentrations in aflatoxicated rice powder.

| Aflatoxins | Concentrations (μg/kg) |
|---|---|
| B1 | 17760 |
| B2 | 1500 |
| G1 | 3180 |
| G2 | 120 |
| Total aflatoxins | 22560 |

The inoculated rice powder was used to measure the concentration of produced aflatoxins using high performance liquid chromatography (LC-10, Shimadzu, Japan) according to the standard procedure (Method 994.08) of AOAC (2000) and method described by Buttinger (2010). Table 1 shows the aflatoxins concentration in rice powder. The contaminated rice powder was used to achieve the final concentrations of 0.5 and 2 ppm aflatoxins in diets.

### 2.3. Measurement of relative weights of lymphoid organs

On d 28 of age, 2 birds per replicate were randomly selected and sacrificed to measure lymphoid organs weights using a sensitive digital scale. The relative weights of these organs were expressed as percentages of live body weight as described by Rasouli and Jahanian (2015).

### 2.4. Differential leukocyte counts

Two randomly selected birds from each pen replicate were bled into the heparin-containing tubes at 28 and 42 d of age. Differential counts of leukocytes were performed by screening the Gimsa-stained slides. The differential subpopulations of leukocytes were counted and the heterophil: lymphocyte ratio was computed according to the method described by Rasouli and Jahanian (2015).

### 2.5. Immunological responses

To determine immunological responses against infectious bronchitis virus (IBV), infectious bursal (IBD), and Newcastle (NDV) diseases viruses, chicks were orally vaccinated against these viruses at 13, 18, and 18 d of age, respectively. Then, 2 randomly-selected birds from each pen were bled and serum samples were collected at d 7 after each vaccination. Antibody titer against NDV was measured by Hemagglutination inhibition test using commercially available V-form ELISA plates as described by Jahanian (2009). Antibody titers against IBV and IBD were measured using commercial ELISA kits (IDEXX Corb, Portland, ME, USA). The kits included both negative and positive control samples. An automated IBM computerized reader was applied as described by Snyder et al. (1984). Two readings/samples were obtained for each serum dilution and mean antibody titers $\log_{10}$ were computed.

### 2.6. RNA extraction

At 28 d of age, 2 birds from each pen were randomly selected and sacrificed to take individual liver samples. Then, liver samples were immediately placed on liquid nitrogen. Total RNA was extracted from individual liver tissues using the YTA kit (Yekta Tajhiz Azma, IRAN; Cat No: YT9065), according to the manufacturer's instructions. Liver samples removed from −80 °C and placed on dry ice. After that, a 25 to 30 mg aliquot of each sample was weighed, placed into a 2-mL

microcentrifuge tube containing RB buffer, kept on dry ice for 1 h until homogenization using MS-100 TOMY homogenizer (TOMY digital biology, Tokyo, Japan) at 4500 rpm for 1 min. Following extraction, RNA was eluted by rinsing the column membrane twice with 25 μL of RNase-free water. Total RNA concentration was determined at optical density (OD) 260 (NanoDrop-1000, Thermo Fisher Scientific, Waltham, MA), and RNA purity was confirmed by assessing the ratio of OD 260 to OD 280. The purified RNA samples were kept at −80 °C until they were used.

### 2.7. cDNA preparing

Total RNA was diluted to 0.2 μL in nuclease-free water. Reverse transcription was employed using the high capacity cDNA reverse transcription kit (Reverta-L, Amplisens, Moscow, Russia) according to the manufacturer's manual and the cDNA was stored at −20 °C.

### 2.8. Primer designing

Primers were chosen from the conserved part of the coding regions of different immunological genes [interleukin-2 (IL-2), interleukin-6 (IL-6), C reactive protein (CRP)] and the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Then primers were designed using Beacon Designer Software with an annealing temperature of 60 °C and amplification size of less than 250 bp and synthesized by Bioneer (Daejeon, Korea) (Table 2).

### 2.9. Quantitative real-time PCR (qRT-PCR)

Quantitative real-time PCR (qRT-PCR) was performed using real-time PCR System (Applied Biosystems, Step One). Then, 2 μL of the cDNA was added to each well of a 48-well plate. Next, 20 μL of real time PCR master mix containing 10 μL of Fast SYBR Green Master Mix (Applied Biosystems), 1 μL of mixed forward and reverse primers, and 10 μL of sterile nuclease-free water per reaction were added to each well for a final volume of 23 μL. During the PCR reaction, samples were exposed to an initial denaturation phase at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. Gene expressions for IL-2, IL-6, and CRP were analyzed by using GAPDH as an endogenous control. Each gene was measured in triplicate and the formation of single PCR products was verified using melting curves.

For the quantitative PCR, the following cycle threshold (Ct) equations were used: ΔCt = Ct (gene of interest) - Ct (housekeeping gene); ΔΔCt = ΔCt (sample) -ΔCt (control); and relative quantity = $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

### 2.10. Statistical analysis

All data were subjected to ANOVA using the GLM procedures of SAS software (SAS Institute, 1999) as a 2 × 3 factorial arrangement of treatments including 2 levels of aflatoxin contamination and 3 MOS levels as the main effects and respective interactions (N = 48 individual birds). The following model was applied for analysis of all the traits. $Y_{ijk} = \mu + A_i + Bj + AB_{ij} + e_{ijk}$, where $Y_{ijk}$ (as dependent variables) = observed value for a particular trait, μ = overall mean, $A_i$ = effect of the ith level of aflatoxin (independent variable), B*j* = effect of the jth level of MOS (independent variable), $AB_{ij}$ = the respective interaction of aflatoxin and MOS levels (independent variable), and $e_{ijk}$ = random error associated with the ijkth recording. The dependent variables consisted of the relative lymphoid organ weights, humoral and cellular immunity, as well as hepatic gene expressions. Treatment means were compared by least significant difference (LSD) tests at $P < 0.05$ statistical level. The single degree of freedom contrast comparison was made amongst control and aflatoxicated groups to investigate the effects of aflatoxin contamination on studied parameters (N = 56 individual birds). In equation form, $L = c_1\bar{X}_1 + c_2\bar{X}_2$, where L is the weighted sum of group means, the $c_i$ coefficients represent the assigned weights of the means and $\bar{X}_i$ represents the group means. To evaluate model fit, a Shapiro-Wilk test for residual was generated to check for normality (Razali and Wah, 2011). When the residual was not normal, the log transformation of data was applied (Feng et al., 2014).

**Table 3**
Effects of dietary mannanoligosaccharides (MOS) supplementation on the relative weights (%) of lymphoid organs of aflatoxin-contaminated broiler chicks at 28 d of age (g/kg live body weigh).

| Aflatoxin levels (ppm) | MOS levels (g/kg) | Spleen | Bursa of Fabricius |
|---|---|---|---|
| Control[1] | | 0.12 | 0.27 |
| 0.5 | 0 | 0.09 | 0.18 |
| | 1 | 0.10 | 0.21 |
| | 2 | 0.11 | 0.22 |
| 2 | 0 | 0.08 | 0.17 |
| | 1 | 0.10 | 0.20 |
| | 2 | 0.11 | 0.21 |
| Aflatoxin levels | | | |
| 0.5 | | 0.10 | 0.20 |
| 2 | | 0.09 | 0.19 |
| MOS levels | | | |
| 0 | | 0.08[b] | 0.17[b] |
| 1 | | 0.10[a] | 0.20[a] |
| 2 | | 0.11[a] | 0.22[a] |
| SEM[2] | | 0.005 | 0.008 |
| *P*-value | | | |
| Aflatoxin levels | | 0.350 | 0.212 |
| MOS levels | | 0.005 | 0.001 |
| Aflatoxin × MOS | | 0.657 | 0.931 |
| Normality | | 0.956 | 0.944 |
| *Contrast* | | | |
| Control vs. aflatoxin groups[3] | | 0.001 | 0.0001 |

[a−b]Means with no common superscript within each column are significantly ($P < 0.05$) different.
[1] Control group is unchallenged birds receiving neither aflatoxins nor MOS.
[2] SEM: standard error of the mean (N = 48 individual birds).
[3] N in contrast comparison is 56 individual birds.

## 3. Results

### 3.1. Relative weights of lymphoid organs

Effect of dietary inclusion of different levels of MOS on relative weights of lymphoid organs of broilers contaminated with aflatoxin is summarized in Table 3. Aflatoxicated chicks had the smaller spleen ($P < 0.001$) and bursa ($P < 0.0001$) as compared with those of control birds (control vs. aflatoxins). However, the relative weights of

**Table 2**
Primer sequences (5′→3′) used in real-time PCR.

| Name | Symbol | Forward primer | Reverse primer | Product size (bp) |
|---|---|---|---|---|
| Interleukin-2 | IL-2 | TGCAGTGTTACCTGGGAGAA | CTTGCATTCACTTCCGGTGT | 135 |
| Interleukin-6 | IL-6 | GACTCGTCCGGAGAGGTTG | CGCACACGGTGAACTTCTT | 128 |
| C reactive protein | CRP | CGGCCCAGGAAGACCTCTACAG | CGCAGGCACACGGTGAAGTT | 136 |
| Glyceraldehyde-3 phosphate dehydrogenase | GAPDH | GGTGGTGCTAAGCGTGTTAT | ACCTCTGTCATCTCTCCACA | 128 |

**Table 4**
Effects of dietary mannanoligosaccharides (MOS) supplementation on antibody titers of aflatoxin-contaminated broiler chicks.

| Aflatoxin levels (ppm) | MOS levels (g/kg) | NDV[2] ($log_2$) | IBV[2] ($log_{10}$) | IBD[2] ($log_{10}$) |
|---|---|---|---|---|
| Control[1] | | 4.67 | 5.67 | 4.67 |
| 0.5 | 0 | 2.33 | 3.00 | 3.33 |
| | 1 | 3.67 | 3.67 | 3.67 |
| | 2 | 3.67 | 4.00 | 4.33 |
| 2 | 0 | 1.67 | 2.00 | 1.67 |
| | 1 | 3.00 | 2.67 | 3.00 |
| | 2 | 3.33 | 3.67 | 3.67 |
| Aflatoxin levels | | | | |
| 0.5 | | 3.00 | 3.56 | 3.78[a] |
| 2 | | 2.89 | 2.78 | 2.78[b] |
| MOS levels | | | | |
| 0 | | 2.00[b] | 2.50[b] | 2.50[b] |
| 1 | | 3.17[a] | 3.17[ab] | 3.33[ab] |
| 2 | | 3.67[a] | 3.83[a] | 4.00[a] |
| SEM[3] | | 0.441 | 0.408 | 0.333 |
| *P*-value | | | | |
| Aflatoxin levels | | 0.494 | 0.066 | 0.008 |
| MOS levels | | 0.020 | 0.047 | 0.007 |
| Aflatoxin × MOS | | 0.619 | 0.723 | 0.357 |
| Normality | | 0.218 | 0.225 | 0.191 |
| *Contrast* | | | | |
| Control vs. aflatoxin groups[4] | | 0.002 | 0.006 | 0.002 |

[a−b]Means with no common superscript within each column are significantly ($P < 0.05$) different.
[1] Control group is unchallenged birds receiving neither aflatoxins nor MOS.
[2] NDV: Newcastle disease virus; IBV: infectious bronchitis virus; IBD: infectious bursal disease.
[3] SEM: standard error of the mean (N = 48 individual birds).
[4] N in contrast comparison is 56 individual birds.

spleen and bursa of Fabricius weren't affected by dietary aflatoxin contamination levels at 28 d-aged chickens (0.5 ppm vs. 2 ppm).

Dietary MOS supplementation by at least 1 g/kg resulted in the enhanced relative weights of spleen ($P < 0.01$) and bursa of Fabricius ($P < 0.001$) at 28 d of age. However, the interactions between aflatoxins levels and MOS levels regarding the relative weights of lymphoid organs were not significant. The lowest weights of lymphoid organs were elicited in birds contaminated with 2 ppm aflatoxin.

### 3.2. Immunological responses

According to Table 4, antibody titers against NDV, IBD, and IBV were significantly ($P < 0.01$) lower in broilers exposed to aflatoxin compared to control birds (control vs. aflatoxins). Antibody titer against NDV wasn't affected by increasing aflatoxin level in diets (0.5 ppm vs. 2 ppm). On the other hand, an increase in aflatoxin level led to lower antibody titers against IBD ($P < 0.01$) and IBV, marginally ($P = 0.066$).

An increase in supplemental MOS level raised antibody titers against NDV ($P < 0.05$), IBD ($P < 0.01$) and IBV ($P < 0.05$). The interactions between afaltoxins levels and MOS levels on antibody titers against different viral antigens were not significant. So, feeding 2 g/kg MOS in each aflatoxins level caused the numerical increases in antibody titers in chicks.

### 3.3. Differential leukocyte count

As shown in Tables 5 and 6, the lymphocyte percentage was decreased ($P < 0.0001$) in aflatoxicated chicks at both 28 and 42 d of age, while heterophils proportion was increased ($P < 0.0001$). Consequently, heterophil to lymphocyte ratio was markedly ($P < 0.0001$) increased as the result of dietary aflatoxin contamination. However, other leukocyte subpopulations weren't affected by aflatoxin challenge (0.5 ppm vs. 2 ppm).

Dietary administration of MOS (especially 2 g/kg) resulted in an increase ($P < 0.0001$) in lymphocyte proportion and a decrease ($P < 0.001$) in heterophil proportion; subsequently, it decreased heterophil to lymphocyte ratio ($P < 0.0001$) at both d 28 and 42 of age. Dietary MOS supplementation modulated the lymphocyte and heterophil proportions in birds exposed to 0.5 ppm aflatoxin at 28 d of age, resulting in the significant ($P < 0.05$) aflatoxin × MOS interaction. However, inclusion of different levels of MOS in aflatoxin-contaminated diets didn't counteract differential leukocyte count at 42 d of age.

**Table 5**
Effects of dietary mannanoligosaccharides (MOS) supplementation on differential leukocyte percentages (%) of aflatoxin-contaminated broiler chicks at d 28 of age.

| Aflatoxin levels (ppm) | MOS levels (g/kg) | Lymphocyte | Monocyte | Eosinophil | Heterophil | H: L[2] ratio |
|---|---|---|---|---|---|---|
| Control[1] | | 74.33 | 3.00 | 0.67 | 22.00 | 0.30 |
| 0.5 | 0 | 68.33[ab] | 4.00 | 1.33 | 26.33[b] | 0.39[b] |
| | 1 | 70.33[a] | 4.67 | 1.00 | 24.00[b] | 0.34[b] |
| | 2 | 73.50[a] | 5.50 | 1.00 | 20.00[b] | 0.27[b] |
| 2 | 0 | 56.00[c] | 3.00 | 0.33 | 40.67[a] | 0.73[a] |
| | 1 | 64.00[b] | 6.33 | 0.67 | 29.00[b] | 0.45[b] |
| | 2 | 69.33[a] | 3.67 | 0.67 | 26.33[b] | 0.38[b] |
| Aflatoxin levels | | | | | | |
| 0.5 | | 70.38[a] | 4.63 | 1.13 | 23.88[b] | 0.34[b] |
| 2 | | 63.11[b] | 4.33 | 0.56 | 32.00[a] | 0.52[a] |
| MOS levels | | | | | | |
| 0 | | 62.17[c] | 3.50 | 0.83 | 33.50[a] | 0.56[a] |
| 1 | | 67.17[b] | 5.50 | 0.83 | 26.50[b] | 0.40[b] |
| 2 | | 71.00[a] | 4.40 | 0.80 | 23.80[b] | 0.34[b] |
| SEM[3] | | 0.885 | 0.805 | 0.537 | 1.447 | 0.030 |
| *P*-value | | | | | | |
| Aflatoxin levels | | 0.0001 | 0.632 | 0.314 | 0.0001 | 0.0001 |
| MOS levels | | 0.0001 | 0.144 | 1.00 | 0.0004 | 0.0001 |
| Aflatoxin × MOS | | 0.007 | 0.210 | 0.833 | 0.042 | 0.009 |
| Normality | | 0.274 | 0.198 | 0.399 | 0.105 | 0.116 |
| *Contrast* | | | | | | |
| Control vs. aflatoxin groups[4] | | 0.0001 | 0.702 | 0.501 | 0.0001 | 0.0002 |

[a−c] Means with no common superscript within each column are significantly ($P < 0.05$) different.
[1] Control group is unchallenged birds receiving neither aflatoxins nor MOS.
[2] H: L ratio: heterophil to lymphocyte ratio.
[3] SEM: standard error of the mean (N = 48 individual birds).
[4] N in contrast comparison is 56 individual birds.

**Table 6**
Effects of dietary mannanoligosaccharides (MOS) supplementation on differential leukocyte percentages (%) of aflatoxin-contaminated broiler chicks at d 42 of age.

| Aflatoxin levels (ppm) | MOS levels (g/kg) | Lymphocyte | Monocyte | Eosinophil | Heterophil | H: L[2] ratio |
|---|---|---|---|---|---|---|
| Control | | 74.50 | 3.75 | 1.00 | 20.75 | 0.28 |
| 0.5 | 0 | 71.75 | 2.50 | 0.25 | 25.50 | 0.36 |
| | 1 | 71.00 | 3.67 | 2.00 | 23.33 | 0.33 |
| | 2 | 74.00 | 2.00 | 1.50 | 22.50 | 0.30 |
| 2 | 0 | 67.00 | 3.00 | 1.25 | 28.75 | 0.43 |
| | 1 | 68.75 | 5.25 | 0.50 | 25.50 | 0.37 |
| | 2 | 71.50 | 3.00 | 1.50 | 24.00 | 0.34 |
| Aflatoxin levels | | | | | | |
| 0.5 | | 72.00[a] | 2.78 | 1.11 | 24.11 | 0.34 |
| 2 | | 69.08[b] | 3.75 | 1.08 | 26.08 | 0.38 |
| MOS levels | | | | | | |
| 0 | | 69.38[b] | 2.75 | 0.75 | 27.13[a] | 0.39[a] |
| 1 | | 69.71[b] | 4.57 | 1.14 | 24.57[ab] | 0.35[ab] |
| 2 | | 72.33[a] | 2.67 | 1.50 | 23.50[b] | 0.33[b] |
| SEM[3] | | 1.099 | 0.935 | 0.483 | 1.378 | 0.024 |
| *P*-value | | | | | | |
| Aflatoxin levels | | 0.006 | 0.242 | 0.701 | 0.084 | 0.036 |
| MOS levels | | 0.038 | 0.151 | 0.371 | 0.054 | 0.044 |
| Aflatoxin × MOS | | 0.503 | 0.858 | 0.075 | 0.843 | 0.710 |
| Normality | | 0.379 | 0.425 | 0.508 | 0.401 | 0.062 |
| *Contrast* | | | | | | |
| Control vs. aflatoxin groups[4] | | 0.009 | 0.282 | 0.224 | 0.006 | 0.006 |

a-b Means with no common superscript within each column are significantly (P < 0.05) different.
1Control group is unchallenged birds receiving neither aflatoxins nor MOS.
[2] H: L ratio: heterophil to lymphocyte ratio.
[3] SEM: standard error of the mean (N = 48 individual birds).
[4] N in contrast comparison is 56 individual birds.

### 3.4. Expression of hepatic immunological genes

According to Fig. 1, hepatic IL-2 gene expression was markedly (P < 0.01) downregulated in birds contaminated with the highest level of aflatoxin (2 ppm). In contrast, the relative abundances of mRNA of IL-6 (P < 0.0001) and CRP (P < 0.001) genes were increased in aflatoxicated birds (Figs. 2 and 3), with the greatest abundances assigned to the birds contaminated with 2 ppm aflatoxin (control vs. aflatoxins).

Hepatic IL-2 gene expression was noticeably (P < 0.001) upregulated by supplemental MOS especially at the level of 2 g/kg (Fig. 4). However, relative abundances of hepatic IL-6 (P < 0.0001) and CRP (P < 0.01) mRNA were suppressed by dietary inclusion of 2 g of MOS/kg (Figs. 5 and 6).

Notably, the interaction between experimental factors showed that dietary supplementation of MOS at the level of 2 g/kg raised (P < 0.01) the abundance of hepatic IL-2 mRNA in birds exposed to 0.5 ppm aflatoxin (Fig. 7), whereas the lowest abundance of IL-2 mRNA was found in broilers challenged with aflatoxin without supplemental MOS (Fig. 7; aflatoxins × MOS). On the other hand, the alterations in hepatic IL-6 and CRP gene expressions in aflatoxin-contaminated chicks weren't ameliorated by dietary inclusion of MOS (Figs. 8 and 9).

## 4. Discussion

Mycotoxins, are known to be the causal agents, negatively affect lymphocyte proliferation; in turn, depress immunological responses (Stec et al., 2008). Impaired immunity has been shown to cause lower resistance to infectious diseases and bacterial contamination (Rauber et al., 2013). As noted, the relative weights of lymphoid organs were affected by aflatoxin contamination of diets in the present study. Similar results were obtained by Quist et al. (2000) and Chowdhury and Smith (2007), who noted that the relative weight of spleen was lower in turkey poults receiving aflatoxicated diets; consequently, antibody titers were depressed in these birds. As previously demonstrated, mycotoxins suppress immunological responses through not only altering the structural lymphoid organ including degeneration of follicle epithelium, destruction of thymus cortex (Celik et al., 2000), but also changing their functions (Al-Anati and Petzinger, 2006). Of course,

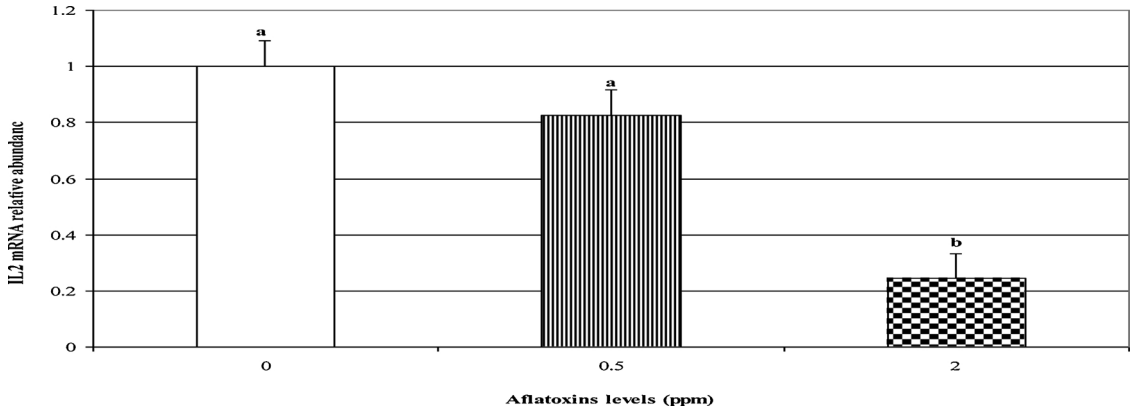


**Fig. 1.** Effect of aflatoxin challenge on hepatic interleukin-2 (IL-2) gene expression in broiler chicks at d 28 of age.
[a−b] Means with no common superscript are significantly (P < 0.05) different (N in contrast comparison is 56 individual birds).

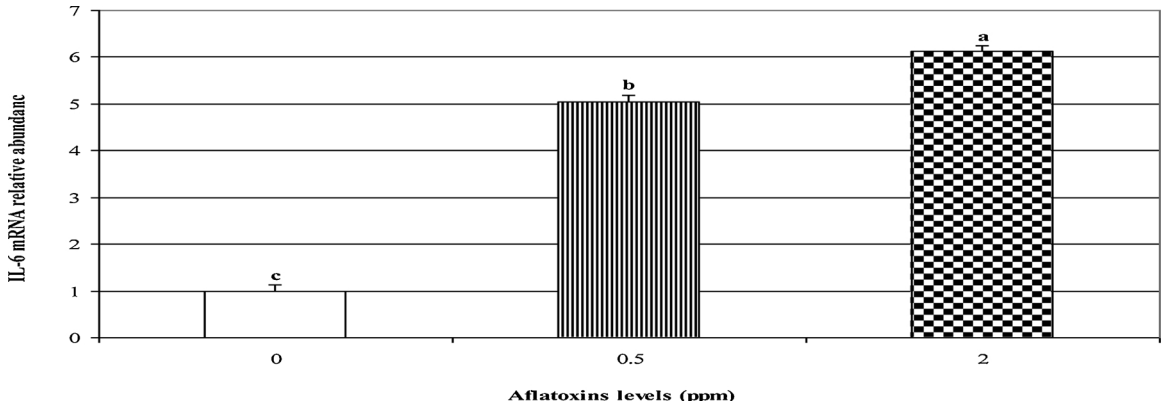


**Fig. 2.** Effect of aflatoxin challenge on hepatic interleukin-6 (IL-6) gene expression in broiler chicks at d 28 of age.
[a−b] Means with no common superscript are significantly (P < 0.05) different (N in contrast comparison is 56 individual birds).

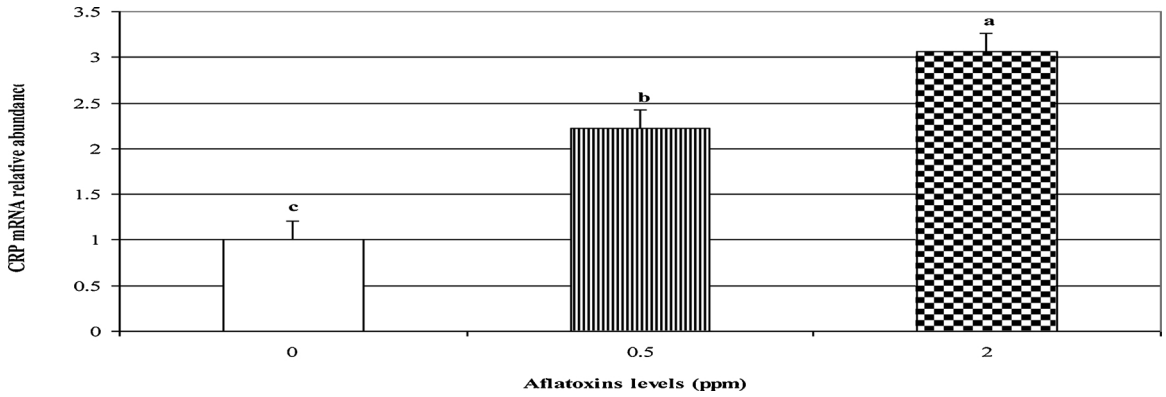


**Fig. 3.** Effect of aflatoxin challenge on hepatic C reactive protein (CRP) gene expression in broiler chicks at d 28 of age.
[a−b] Means with no common superscript are significantly (P < 0.05) different (N in contrast comparison is 56 individual birds).

Kamalavenkatesh et al. (2005) reported that the susceptibility of lymphoid organs to mycotoxins might be resulted from the induction of lysosomes and hydrolytic enzyme activities. Therefore, it is probable that the smaller lymphoid organs were derived from their degenerations. Contradictory results were obtained by several researches regarding the effects of mycotoxins on the relative weights of lymphoid organs. For example, some studies reported that the relative weights of lymphoid organs were decreased (Ortatatli and Oguz, 2001; Wang et al., 2009), while others observed that they were increased (Hegazy and Adachi, 2000; Tessari et al., 2006) or remained unaffected (Cheng et al., 2001; Dänicke et al., 2003; Chowdhury and Smith, 2007).

Dietary MOS supplementation at the level of 2 g/kg increased the relative weights of spleen and bursa of Fabricius in broiler chicks when compared to unsupplemented chicks. Mannanoligosaccharides are considered to serve as the immunomodulator agents in laying hens (Cotter et al., 2000). It seems that MOS play this activity via not only stimulating mucosal and humoral immunity (Kogan and Kocher, 2007), but also fortifying cell-mediated immunity and their cellular proliferations especially in lymphoid organs (Memis and Sakrak, 2007). Furthermore, the immunomodulatory activity of MOS might be mediated by its ability to stimulate cytokine production by macrophages (Majtan et al., 2005). Because of the presence of high mannose level, MOS bind to macrophage reception

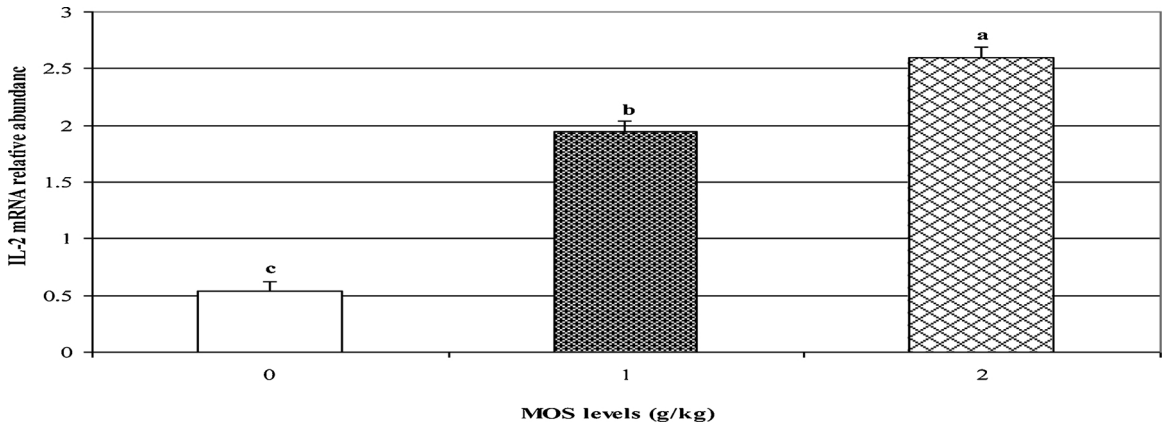


**Fig. 4.** Effect of dietary mannanoligosaccharides (MOS) supplementation on hepatic interleukin-2 (IL-2) gene expression in broiler chicks at d 28 of age.
[a−c] Means with no common superscript are significantly (P < 0.05) different (N= 48 individual birds).

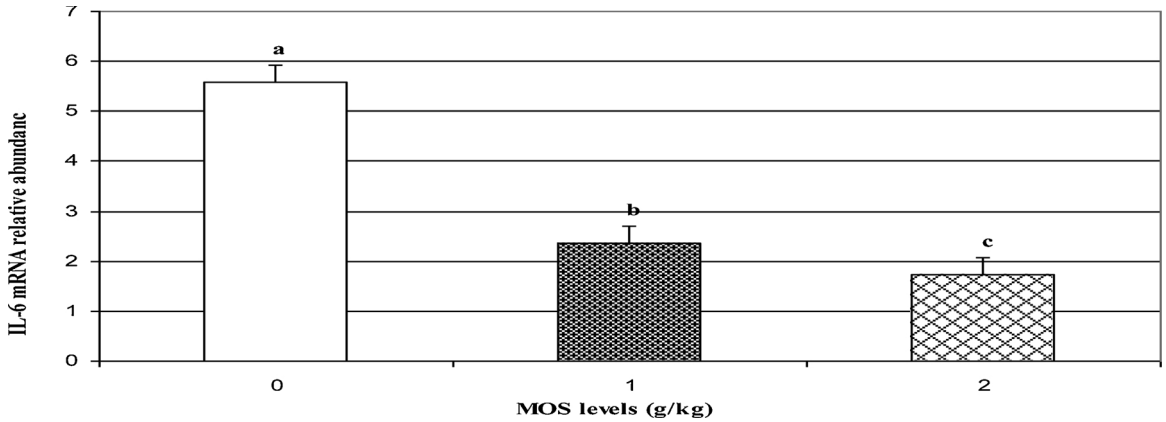


**Fig. 5.** Effect of dietary mannanoligosaccharides (MOS) supplementation on hepatic interleukin-6 (IL-6) gene expression in broiler chicks at d 28 of age.
[a−c] Means with no common superscript are significantly (P < 0.05) different (N = 48 individual birds).

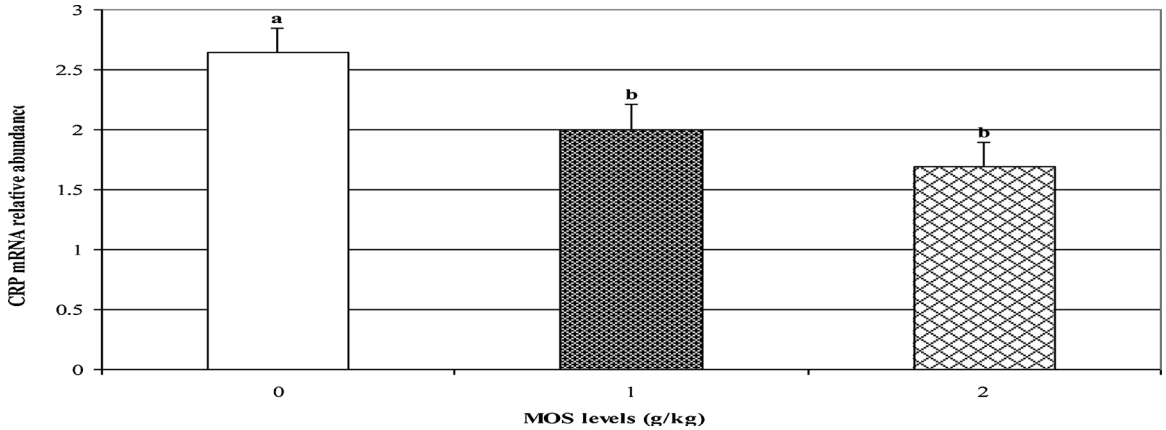


**Fig. 6.** Effect of dietary mannanoligosaccharides (MOS) supplementation on hepatic C reactive protein (CRP) gene expression in broiler chicks at d 28 of age.
[a−b] Means with no common superscript are significantly (P < 0.05) different (N = 48 individual birds).

sites (found in glycoproteins of epithelial surface), resulting in the stimulation of cascade reaction and activating macrophages and cytokines secretion (Memis and Sakrak, 2007). Our results are in line with Sadeghi et al. (2013), who found an increase in relative spleen weight in *Salmonella enteritidis*-challenged broilers after feeding MOS. However, Mohamed et al. (2008) reported that the relative weights of spleen and bursa of Fabricius weren't different in MOS-supplemented broiler chicks as compared with control birds.

Notably, dietary administration of MOS couldn't return relative weights of lymphoid organs of intoxicated birds to normal status. Nevertheless, the greatest weights of lymphoid organs were obtained in aflatoxicated birds fed on diets supplemented with 2 g/kg MOS. Similar to our results, Ghahri et al. (2010) and Nemati et al. (2015) found no changes in relative weights of lymphoid organs in birds supplemented with glucomannan accompanying with mycotoxins, whereas Girish and Devegowda (2006) showed that regression of the relative weights of thymus and bursa of Fabricius were ameliorated by supplemental glucomannan in aflatoxin-contaminated diet.

Although an increase in aflatoxins level had no influence on antibody production titer against NDV, it suppressed antibody titers against IBD and

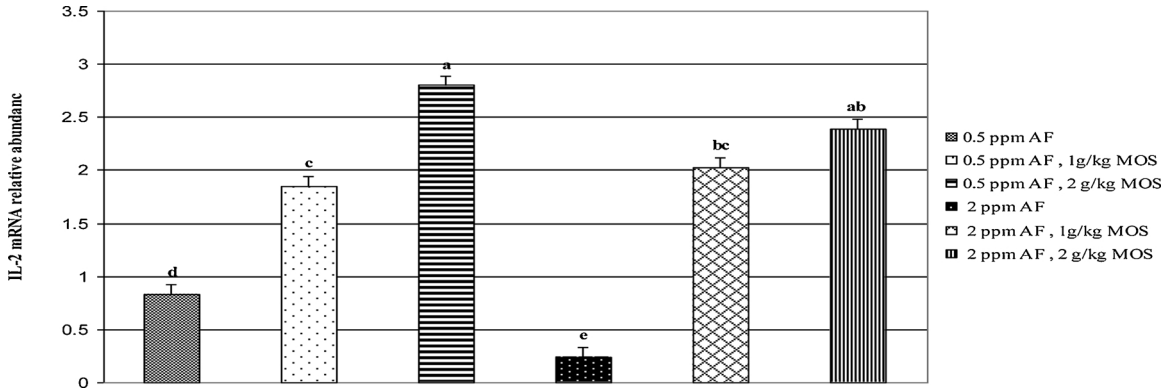


**Fig. 7.** Effect of dietary mannanoligosaccharides (MOS) supplementation on hepatic interleukin-2 (IL-2) gene expression in broilers exposed to aflatoxin (AF) at d 28 of age.
[a−e] Means with no common superscript are significantly (P < 0.05) different (N = 48 individual birds; *P*-value for normality is 0.078).

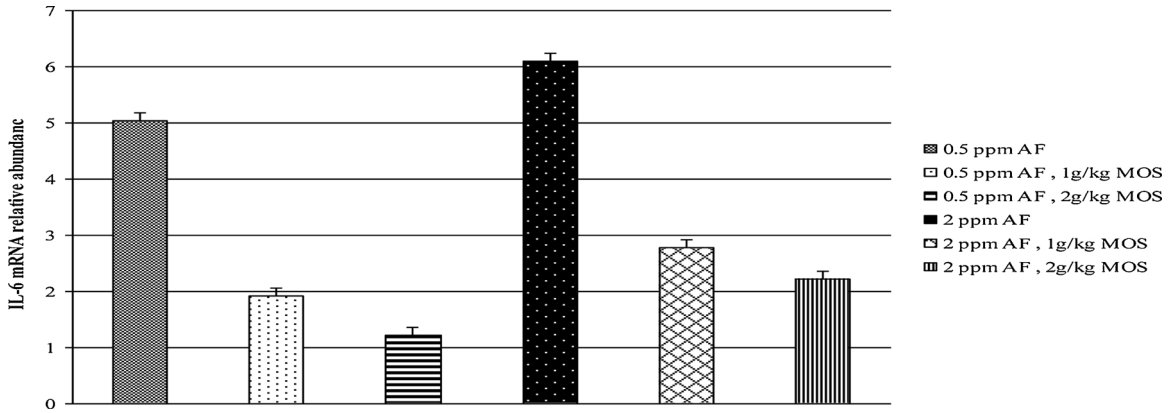


**Fig. 8.** Effect of dietary mannanoligosaccharides (MOS) supplementation on hepatic interleukin-6 (IL-6) gene expression in broilers exposed to aflatoxin (AF) at d 28 of age (N = 48 individual birds; *P*-value for normality is 0.068).

IBV (Table 4). The decreased lymphoid organ weights might be accounted for the depressed antibody titers in aflatoxicated chicks. Furthermore, the inhibition of protein synthesis, especially immunoglobulins G and A, might be responsible for immunoinhibitory activity of aflatoxins (Santin et al., 2002b; Pierron et al., 2016). On the other hand, the toxic effects of aflatoxins on lymphocytes are considered to be liable to the inhibition of antibody production and simultaneously antibody half-life depression (Azzam and Gabal, 1997). Several previous studies (Tessari et al., 2006; Girish and Smith, 2008; Ghahri et al., 2010) have found an immunoinhibitory activity of mycotoxins, as determined by suppressing immunoglobulin and antibody productions against NDV, IBD, and IBV. Contrary to our findings, Swamy et al. (2002) and Chowdhury et al. (2005) found that mycotoxin exposure had no effect on antibody titers against IBD in male chicks and ducks. These contradictory results are depending on mycotoxin level and type, duration and physiological status of animal or some other herd factors (Pestka, 2008).

An increase in aflatoxin level caused a decline in lymphocyte percentage and an increase in heterophil proportion, resulting in increased heterophil to lymphocyte ratio. Antigens induce T cells to produce cytokines; consequently, inhibition of T cells adversely affects the chemotactic ability of leukocyte and inhibits cell-mediated immune responses (Girish and Smith, 2008). Mycotoxins interfere with proliferation and differentiation of cells participating in immunomediated activities (Oswald et al., 2005). Additionally, it was demonstrated that mycotoxins not only caused lymphocytopenia and monocytopenia as well as structural and functional alterations (Rafai et al., 2000), but also they reduced lymphocyte viability (Oh et al., 2012). Girish and Smith (2008) suggested that the suppressed cell-mediated immunity seen in aflatoxin-fed birds is probably because of suppression of helper and cytotoxic T cells activity. In contrast with mentioned reports, some researchers (Deshmukh et al., 2005; Wu et al., 2015) found that the lymphocyte percentage was increased as a result of mycotoxin challenge.

As noted in Tables 5 and 6, the other leukocyte proportions weren't affected by aflatoxin contamination of diets in both 28 and 42 d of age. Consistent with our findings, Tuzcu et al. (2010) and Chaytor et al. (2011) reported that the percentages of monocytes, eosinophils and basophils weren't affected by the incremental levels of aflatoxin in mice and pigs, respectively. However, Shahrzad et al. (2014) noticed that feeding diets containing aflatoxin B1 and ochratoxin A resulted in lower monocytes proportion in quail chicks.

Dietary inclusion of MOS particularly at the level of 2 g/kg resulted in a rise in lymphocyte proportion and a decrease in heterophil proportion and heterophil to lymphocyte ratio at 28 and 42 d of age. Reinforced to our findings, Sadeghi et al. (2013) observed that supplemental MOS decreased heterophil to lymphocyte ratio in *Salmonella enteritidis*-challenged chicks. However, Basmacioglu et al. (2005) observed that dietary supplementation of 1 g/kg esterified glucomannan had no marked effect on lymphocyte percentage compared with control chicks.

Dietary MOS supplementation alleviated the depressed lymphocyte percentage in aflatoxin-challenged birds at 28 d of age; in turn, it decreased heterophil to lymphocyte ratio resulting in the significant aflatoxins × MOS interaction. This might be related to the adsorbent capacity of MOS (Yiannikouris et al., 2004). Basmacioglu et al. (2005) found that dietary supplementation of 1 g/kg esterified glucomannan decreased heterophil and lymphocyte percentages in aflatoxicated broiler chicks. Our results are inconsistent to those of Chowdhury et al. (2005), who found no significant difference in terms of total leukocyte

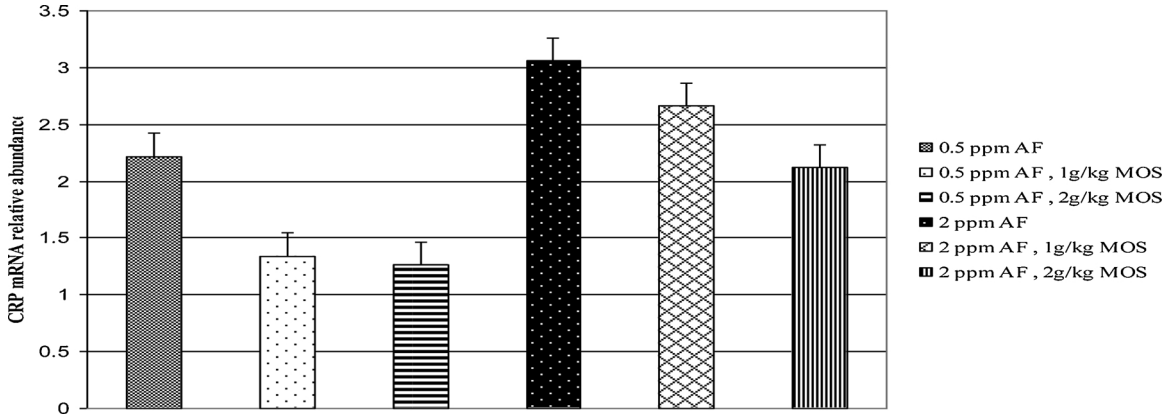


**Fig. 9.** Effect of dietary mannanoligosaccharides (MOS) supplementation on hepatic C reactive protein (CRP) gene expression in broilers exposed to aflatoxin (AF) at d 28 of age (N = 48 individual birds; *P*-value for normality is 0.092).

and lymphocyte counts in hens supplemented with glucomannan in the presence of high levels of *Fusarium* mycotoxins compared with unsupplemented hens.

As shown in Fig. 1, the relative abundance of IL-2 mRNA in liver was decreased as a result of the incremental levels of aflatoxins. It was known that IL-2 induces T cell growth and its cytotoxic activity (Yarru et al., 2009). Iiterlekin-2 is produced by T lymphocytes; consequently, it modulates the B and T-lymphocytes functions (Dinarello, 2000; Girish and Smith, 2008). Dugyala and Sharma (1996) reported that challenging with 0.7 ppm aflatoxin B1 resulted in the suppressed IL-2 gene expression in male mice. Similarly, Girish and Smith (2008) showed that dietary contamination with fumonisin B1 downregulated proinflammatory cytokines including IL-1 and IL-2 in broiler chicks. Furthermore, Marin et al. (2007) revealed that challenging with fumonisin B1 reduced the IL-2 production in swine. Xue et al. (2010) observed that feeding diet containing 0.5 ppm T-2 and 0.25 ppm ochratoxin A depressed IL-2 and interferon-γ mRNA expressions in spleen of broiler chicks, led to the lower serum concentrations of these cytokines.

Our results are in disagreement with those of Zhou et al. (1998), who revealed that the gene expressions of IL-2 and tumor necrosis factor-α (TNF-α) were upregulated in mice fed sub-chronic levels of deoxynivalenol for 4 weeks. Similarly, Bhandari and Sharma (2002) noticed that 2.5 mg fumonisin B1 treatment upregulated the expression of pro-inflammatory cytokines (especially IL-2) in livers of mice.

The incremental levels of aflatoxins upregulated the hepatic IL-6 and CRP mRNA gene expressions (Figs. 2 and 3). The raised abundance of hepatic CRP mRNA as the consequence of aflatoxin challenge is related and correlated with increasing circulating IL-6 (Ridker et al., 2000). Interleukins, group of cytokines, are vital components of the immune system. They act the physiological roles in the inflammation (Tayal and Kalra, 2007). An imbalance between cytokines generation led to various pathological disorders (Tayal and Kalra, 2007). It was demonstrated that IL-6 is a proinflammatory cytokine (Yarru et al., 2009) and acts as an essential helper factor for IgA secretion (Pestka, 2003). Dinarello (2000) stated that cytokines are the major regulators of host responses to infection, immune responses and inflammation. In agreement with the present findings, Dugyala and Sharma (1996) and Meissonnier et al. (2008) reported that aflatoxin B1 caused the induction of IL-6 gene expression in rats and pigs, respectively. Similarly, Bhandari and Sharma (2002) and Sharma et al. (2006) observed that fumonisin B1 resulted in the upregulated expression of IL-6 and IL-12 in liver of mice. An increase in IL-6 level might be arisen from acute phase response to inflammatory processes caused by aflatoxins (Chaytor et al., 2011; Li et al., 2014). Li et al. (2014) observed that aflatoxicated birds displayed the greater expressions of IL-6 and TNF-α genes compared to control group. In contrast, Han et al. (1999) noticed the downregulation of IL-6 and IL-10 gene expressions in broilers subjected to aflatoxin.

As shown, dietary inclusion of MOS by at least 1 g/kg resulted in the upregulation of IL-2 gene, and downregulated IL-6 and CRP gene expressions compared with control birds. In this regard, β-glucans were known to increase cytokine (TNF-α, IL-6 and IL-2) release in vitro (Chen et al., 2003). Moreover, Vetvicka and Vetvickova (2009) found that glucan administration exerts the ameliorative effects on immunosuppression caused by mercury. As seen in Fig. 7, dietary supplementation of MOS at the level of 2 g/kg upregulated hepatic IL-2 gene expression in birds challenged with 0.5 ppm aflatoxins. This might be attributed to adsorbent potential of MOS, resulting in the decreased toxic effects of aflatoxins on liver.

## 5. Conclusion

Taken together, aflatoxin contamination of diet not only depressed humoral and cellular immune responses in broiler chicks, but also it diminished T and B cells growth and differentiation at the gene expression level (mainly through downregulation of IL-2 gene expression). On the other hand, MOS supplementation of diet could improve immunological responses in aflatoxin-challenged birds, probably by fortifying hepatic IL-2 gene expression.

## References


Al-Anati, L., Petzinger, E., 2006. Immunotoxic activity of ochratoxin A. J. Vet. Pharmacol. Ther. 29, 79–90.

AOAC, 2000. 17th edition. Aflatoxins in Corn, Almonds, Brazil Nuts, Peanuts, and Pistachio Nuts, Multifunctional Column (Mycosep) Method. Natural Toxins-Chapter 49. Official Methods of Analysis of AOAC International, vol. II AOAC International, Gaithersburg, Maryland, USA.

Azzam, A.H., Gabal, M.A., 1997. Interaction of aflatoxin in the feed and immunization against selected infectious diseases in poultry. I. Infectious bursal disease. Avian Pathol. 26, 317–325.

Basmacioglu, H., Oguz, H., Ergul, M., Col, R., Birdane, Y.O., 2005. Effect of dietary esterified glucomannan on performance, serum biochemistry and hematology in broilers exposed to aflatoxin. Czech J. Anim. Sci. 50, 31–39.

Bennett, J.W., Klich, M., 2003. Mycotoxins. Clin. Microbiol. Rev. 16, 497–516.

Bhandari, N., Sharma, R.P., 2002. Fumonisin B1-induced alterations in cytokine expression and apoptosis signaling genes in mouse liver and kidney after an acute exposure. Toxicology 172, 81–92.

Buttinger, G., 2010. Aflatoxin measurements: how did HPLC methods evolve in the last 20 years? Food Addit. Contam. 27, 1266–1272.

Canady, R.A., Coker, R.D., Egan, S.K., 2001. T-2 And HT-2 Toxins. in: Joint FAO/WHO Expert Committee on Food Additives (JECFA) (eds) Safety Evaluation of Certain Mycotoxins in Food. International Program on Chemical Safety (IPCS). WHO Food Addit. Ser., Geneva, pp. 557–597 47.

Celik, I., Oguz, H., Demet, O., Donmez, H.H., Boydak, M., Sur, E., 2000. Efficacy of polyvinylpolypyrrolidone in reducing the immunotoxicity of aflatoxin in growing broilers. Br. Poult. Sci. 41, 430–439.

Chaytor, A.C., See, M.T., Hansen, J.A., de Souza, A.L., Middleton, T.F., Kim, S.W., 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.

Chen, H.L., Li, D.F., Chang, B.Y., Gong, L.M., Piao, X.S., Yi, G.F., Zhang, J.X., 2003. Effects of lentinan on broiler splenocyte proliferation, interleukin-2 production, and signal transduction. Poult. Sci. 82, 760–766.

Chen, F., Ma, Y., Xue, C., Ma, J., Xie, Q., Wang, G., Bi, Y., Cao, Y., 2008. The combination of deoxynivalenol and zearalenone at permitted feed concentrations causes serious physiological effects in young pigs. J. Vet. Sci. 9, 39–44.

Chen, X., Horn, N., Cotter, P.F., Applegate, T.J., 2014. Growth, serum biochemistry, complement activity, and liver gene expression responses of Pekin ducklings to graded levels of cultured aflatoxin B1. Poult. Sci. 93, 2028–2036.

Cheng, Y.H., Shen, T.F., Pang, V.F., Cheng, B.J., 2001. Effects of aflatoxin and carotenioids on growth performance and immune response in mule duckling. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 128, 19–26.

Chowdhury, S.R., Smith, T.K., 2007. Effects of feed-borne *Fusarium* mycotoxins on performance, plasma chemistry and hepatic fractional protein synthesis rates of turkeys. Can. J. Anim. Sci. 87, 543–551.

Chowdhury, S.R., Smith, T.K., Boermans, H.J., Sefton, A.E., Downey, R., Woodward, B., 2005. Effects of feeding blends of grains naturally contaminated with *Fusarium* mycotoxins on performance, metabolism, hematology, and immunocompetence of ducklings. Poult. Sci. 84, 1179–1185.

Cotter, P.F., Malzone, A., Paluch, B., Lilburn, M.S., Sefton, A.E., 2000. Modulation of humoral immunity in commercial laying hens by prebiotic. Poult. Sci. 79, 38.

Dänicke, S., Matthes, S., Halle, I., Ueberschär, K.H., Döll, S., Valenta, H., 2003. Effects of graded levels of *Fusarium* toxin-contaminated wheat and of a detoxifying agent in broiler diets on performance, nutrient digestibility and blood chemical parameters. Br. Poult. Sci. 44, 113–126.

Deshmukh, S., Asrani, R.K., Jindal, N., Ledoux, D.R., Rottinghaus, G.E., Sharma, M., Singh, S.P., 2005. Effects of *Fusarium moniliforme* culture material containing known levels of fumonisin B1 on progress of *Salmonella gallinarum* infection in Japanese quail: clinical signs and hematologic studies. Avian Dis. 49, 274–280.

Dinarello, C.A., 2000. Proinflammatory cytokines. Chest 118, 503–508.

Dugyala, R.R., Sharma, R.P., 1996. The effect of aflatoxin B1 on cytokine mRNA and corresponding protein levels in peritoneal macrophages and splenic lymphocytes. Int. J. Immunopharmacol. 18, 599–608.

FAO, 2001. Safety evaluation of certain mycotoxins in food. Prepared by the Fifty-Sixth Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA). FAO Food and Nutrition Paper No. 74.

Feinberg, B., Mclaughlin, C.S., 1989. Biochemical mechanism of action of trichothecene mycotoxins. In: Beasley, V.R. (Ed.), Trichothecene Mycotoxicosis: Pathophysiologic Effects. CRC Press, Boca Raton, FL, USA, pp. 27–35.

Feng, C., Wang, H., Lu, N., Chen, T., He, H., Lu, Y., Tu, X.M., 2014. Log-transformation and its implications for data analysis. Shanghai Arch. Psychiatry 26, 105–109.

Ghahri, H., Habibian, R., Abdollah Fam, M., 2010. Effect of sodium bentonite, mannan oligosaccharide and humate on performance and serum biochemical parameters during aflatoxicosis in broiler chickens. Global Vet. 5, 129–134.

Girish, C.K., Devegowda, G., 2006. Efficacy of glucomannan-containing yeast product (Mycosorb®) and hydrated sodium calcium aluminosilicate in preventing the individual and combined toxicity of aflatoxin and t-2 toxin in commercial broilers. Asian-Aust. J. Anim. Sci. 19, 877–883.

Girish, C.K., Smith, T.K., 2008. Impact of feed-borne mycotoxins on avian cell-mediated and humoral immune responses. World Mycotoxin J. 1, 105–121.

Han, S.Y., Young, J.J., Yea, S.S., Yang, K., 1999. Suppression of the interleukin-2 gene

expression by aflatoxin B1 is mediated through the down-regulation of the NF-AT and AP-1 transcription factors. Toxicol. Lett. 108, 1–10.
Hegazy, S.M., Adachi, Y., 2000. Comparison of the effects of dietary selenium, zinc, and selenium and zinc supplementation on growth and immune response between chick groups that were inoculated with *Salmonella* and aflatoxin or *Salmonella*. Poult. Sci. 79, 331–335.
Hussein, H.S., Brasel, J.M., 2001. Toxicity, metabolism, and impact of mycotoxins on humans and animals. Toxicology 167, 101–134.
Huwig, A., Freimund, S., Kappeli, O., Dutler, H., 2001. Mycotoxin detoxification of animal feed by different absorbents. Toxicol. Lett. 122, 179–188.
Jahanian, R., 2009. Immunological responses as affected by dietary protein and arginine concentrations in starting broiler chicks. Poult. Sci. 88, 1818–1824.
Jouany, J.P., Yiannikouris, A., Bertin, G., 2005. The chemical bonds between mycotoxins and cell wall components of *Saccharomyces cerevisiae* have been identified. Arch. Zootechnica 8, 26–50.
Kamalavenkatesh, P., Vairamuthu, S., Balachandran, C., Manohar, B.M., Raj, G.D., 2005. Immunopathological effect of the mycotoxins cyclopiazonic acid and T-2 toxin on broiler chicken. Mycopathologia 159, 273–279.
Khan, A., Aalim, M.M., Khan, M.Z., Saleemi, M.K., He, C., Khatoon, A., Gul, S.T., 2017. Amelioration of Immunosuppressive Effects of Aflatoxin and Ochratoxin A in White Leghorn Layers with Distillery Yeast Sludge. Toxin Rev. 36, 275–281.
Kogan, G., Kocher, A., 2007. Role of yeast cell wall polysaccharides in pig nutrition and health protection. Livest. Sci. 109, 161–165.
Li, P., Gatlin, D.M., 2003. Evaluation of brewers yeast (*Saccharomyces cerevisiae*) as a feed supplement for hybrid bass (*Morone chrysops* 3 M. *saxatilis*). Aquaculture 219, 681–692.
Li, Y., Ma, Q.G., Zhao, L.H., Wei, H., Duan, G.X., Zhang, J.Y., 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.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2 (-delta delta C (T)) method. Methods 25, 402–408.
Majtan, J., Kogan, G., Kovacova, E., Bilikova, K., Simuth, J., 2005. Stimulation of TNF-α release by fungal cell wall polysaccharides. Zeitschrift fur Naturforschung 60, 921–926.
Marin, D.E., Gouze, M.E., Taranu, I., Oswald, I.P., 2007. Fumonisin B1 alters cell cycle progression and interleukin-2 synthesis in swine peripheral blood mononuclear cells. Mol. Nutr. Food Res. 51, 1406–1412.
Meissonnier, G.M., Pinton, P., Laffitte, J., Cossalter, A.M., Gong, Y.Y., Wild, C.P., Bertin, G., Galtier, P., Oswald, I.P., 2008. Immunotoxicity of aflatoxin B1: Impairment of the cell-mediated response to vaccine antigen and modulation of cytokine expression. Toxicol. Appl. Pharmacol. 231, 142–149.
Memis, L., Sakrak, O., 2007. β-Glucan attenuates inflammatory cytokine release and prevents acute lung injury in an experimental model of sepsis. Shock 27, 397–401.
Mohamed, M.A., Hassan, H.M.A., El-Barkouky, E.M.A., 2008. Effect of mannan oligosaccharide on performance and carcass characteristics of broiler chicks. J. Agric. Social Sci. 4, 13–17.
Nemati, Z., Karimi, A., Besharati, M., 2015. Effects of aflatoxin B1 and yeast cell wall supplementation on the growth performance of broilers. International Conference on Innovations in Chemical and Agricultural Engineering (ICICAE'2015) Feb. 8-9.
Oh, S.Y., Boermans, H.J., Swamy, H.V.L.N., Sharma, B.S., Karrow, N.A., 2012. Immunotoxicity of *Penicillium* mycotoxins on viability and proliferation of bovine macrophage cell line (BOMACs). Open Myco. J. 6, 11–16.
Ortatatli, M., Oguz, H., 2001. Ameliorative effects of dietary clinoptilolite on pathological changes in broiler chickens during aflatoxicosis. Res. Vet. Sci. 71, 59–66.
Oswald, I.P., Marin, D.E., Bouhet, S., Pinton, P., Taranu, I., Accensi, F., 2005. Immunotoxicological risk of mycotoxins for domestic animals. Food Addit. Contam. 22, 354–360.
Pestka, J.J., 2003. Deoxynivalenol-induced IgA production and IgA nephropathy-aberrant mucosal immune response with systemic repercussions. Toxicol. Lett. 140-141, 287–295.
Pestka, J.J., 2008. Mechanisms of deoxynivalenol-induced gene expression and apoptosis. Food Addit. Contam. 25, 1128–1140.
Pestka, J.J., Zhou, H.R., Moon, Y., Chung, Y.J., 2004. Cellular and molecular mechanisms for immune modulation by deoxynivalenol and other trichothecenes: unraveling a paradox. Toxicol. Lett. 153, 61–73.
Pierron, A., Alassane-Kpembi, I., Oswald, I.P., 2016. Impact of mycotoxin on immune response and consequences for pig health. Anim. Nutr. Feed Technol. 2, 63–68.
Quist, C.F., Bounous, D.I., Kilburn, J.V., Nettles, V.F., Wyatt, R.D., 2000. The effect of dietary aflatoxin on wild turkey poults. J. Wildl. Dis. 36, 436–444.
Rafai, P., Pettersson, H., Bata, A., Papp, Z., Glavits, R., Tuboly, S., Vanyi, A., Soos, P., 2000. Effect of dietary T-2 fusariotoxin concentrations on the health and production of white pekin duck broilers. Poult. Sci. 79, 1548–1556.
Rasouli, E., Jahanian, R., 2015. Improved performance and immunological responses as the result of dietary genistein supplementation of broiler chicks. Animal 9, 1473–1480.
Rauber, R.H., Oliveira, M.S., Mallmann, A.O., Dilkin, P., Mallmann, C.A., Giacomini, L.Z., Nascimento, V.P., 2013. Effects of fumonisin B1 on selected biological responses and performance of broiler chickens. Pesqui. Vet. Bras. 33, 1081–1086.
Razali, N.M., Wah, Y.B., 2011. Power comparisons of shapiro-wilk, Kolmogorov-Smirnov, lilliefors and anderson-darling tests. J. Stat. Model. Anal. 2, 21–33.
Ridker, P.M., Hennekens, C.H., Buring, J.E., Rifai, N., 2000. C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women. N. Eng. J. Med. 342, 836–843.
Rodriguez, A., Cuesta, A., Ortuno, J., Esteban, M.A., Meseguer, J., 2003. Immunostimulant properties of a cell wall-modified whole *Saccharomyces cerevisiae* strain administered by diet to sea bream (*Sparus aurata* L.). Vet. Immunol. Immunopathol. 96, 183–192.
Ross Broiler Management Manual, 2009. Aviagene Incorporated. Huntsville, AL, USA. .
Sadeghi, A.A., Mohammadi, A., Shawrang, P., Aminafshar, M., 2013. Immune responses to dietary inclusion of prebiotic-based mannan-oligosaccharide and β-glucan in broiler chicks challenged with Salmonella enteritidis. Turk. J. Vet. Anim. Sci. 37, 206–213.
Santin, E., Maiorka, A., Krabbe, E.L., Paulillo, A.C., Alessi, A.C., 2002a. Effect of hydrated sodium calcium aluminosilicate on the prevention of the toxic effects of ochratoxin. J. Appl. Poult. Res. 11, 22–28.
Santin, E., Paulillo, A.C., Maiorka, P.C., Alessi, A.C., Krabbe, E.L., Maiorka, A., 2002b. The effects of ochratoxin/aluminosilicate interaction on the tissues and humoral immune response of broilers. Avian Pathol. 31, 73–79.
SAS, 1999. SAS Statistics User's Guide. Statistical Analytical System, 5th rev. ed. SAS Institute Inc., Cary, NC, USA.
Shahrzad, M., Khan, M.U.R., Aslam, A., Younus, M., Khan, H.M., Anjum, A.A., Masood, S., 2014. Diminution of ochratoxin A and aflatoxin B1 toxicity on hematology and serum biochemistry of Japanese quail (*Coturnix Japonica*). J. Anim. Plant Sci. 24, 1032–1038.
Sharma, R.P., 1993. Immunotoxicity of aflatoxins. J. Dairy Sci. 76, 892–897.
Sharma, N., Quanren, H., Sharma, R.P., 2006. Amelioration of fumonisin B1 hepatotoxicity in mice by depletion of T cells with anti-Thy-1.2. Toxicology 223, 191–201.
Shashidhara, R.G., Devegowda, G., 2003. Effect of dietary mannan oligosaccharide on broiler breeder production traits and immunity. Poult. Sci. 82, 1319–1325.
Snyder, D.B., Marquardt, W.W., Mallinson, E.T., Savage, P.K., Allen, D.C., 1984. Rapid serological profiling by enzyme-linked immunosorbent assay. III. Simultaneous measurements of antibody titers to infectious bronchitis virus, infectious bursal disease and Newcastle disease viruses in a single serum dilution. Avian Dis. 28, 12–24.
Stec, J., Rachubik, J., Szczotka, M., Kuźmak, J., 2008. The effects of the Penicillium mycotoxins: citrinin, ochratoxin A, and patulin on in vitro proliferation of bovine lymphocytes. Bull. Vet. Inst. Pulawy 52, 163–167.
Swamy, H.V.L.N., Smith, T.K., Cotter, P.F., Boermans, H.J., Sefton, A.E., 2002. Effects of feeding blends of grains naturally contaminated with *Fusarium* mycotoxins on production and metabolism in broilers. Poult. Sci. 81, 966–975.
Tayal, V., Kalra, B.S., 2007. Cytokines and anti-cytokines as therapeutics-an update. Eur. J. Pharmacol. 579, 1–12.
Tessari, E.N., Oliveria, C.A., Cardoso, A.L., Ledoux, D.R., Rottinghaus, G.E., 2006. Effects of aflatoxin B1 on body weight, antibody titers and histology of broiler chicks. Br. Poult. Sci. 47, 357–364.
Thaxton, J.P., Tung, H.T., Hamilton, P.B., 1974. Immunosuppression in chickens by aflatoxin. Poult. Sci. 53, 721–725.
Thieu, N.Q., Ogle, B., Pettersson, H., 2008. Efficacy of bentonite clay in ameliorating aflatoxicosis in piglets fed aflatoxin contaminated diets. Trop. Anim. Health Prod. 40, 649–656.
Tuzcu, M., Sur, E., Celik, I., Oznurlu, Y., Ciftic, M.K., 2010. Effects of aflatoxin on the proportions of peripheral blood leukocytes and alpha-naphtyl acetate esterase (ANAE) positive lymphocytes in the mouse. Kafkas Üniversitesi Veteriner Fakültesi Dergisi 16, 337–341.
Vetvicka, V., Vetvickova, J., 2009. Effects of glucan on immunosuppressive actions of mercury. J. Med. Food 12, 1098–1104.
Wang, G.H., Xue, C.Y., Chen, F., Ma, Y.L., Zhang, X.B., Bi, Y.Z., Cao, Y.C., 2009. Effects of combinations of ochratoxin A and T-2 toxin on immune function of yellow-feathered broiler chickens. Poult. Sci. 88, 504–510.
Whitlow, L.W., Hagler, W.M., 2002. Mycotoxins in feeds. Feedstuffs 74, 1–10.
WHO, 2000. Hazardous Chemicals in Humans and Environmental Health: International Programme on Chemical Safety. World Health Organisation, Geneva, Switzerland, pp. 7–9. http://whqlibdoc.who.int/hq/2000/WHO_PCS_00.1.pdf.
Wu, L., Liao, P., He, L., Ren, W., Yin, J., Duan, J., Li, T., 2015. Growth performance, serum biochemical profile, jejunal morphology, and the expression of nutrients transporter genes in deoxynivalenol (DON)-challenged growing pigs. BMC Vet. Res. 11, 144–153.
Xue, C.Y., Wang, G.H., Chen, F., Zhang, X.B., Bi, Y.Z., Cao, Y.C., 2010. Immunopathological effects of ochratoxin A and T-2 toxin combination on broilers. Poult. Sci. 89, 1162–1166.
Yarru, L.P., Settivari, R.S., Antoniou, E., Ledoux, D.R., Rottinghaust, G.E., 2009. Toxicological and gene expression analysis of the impact of aflatoxin B1 on hepatic function of male broiler chicks. Poult. Sci. 88, 360–371.
Yiannikouris, A., Francois, J., Poughon, L., Dussap, C.G., Bertin, G., Jeminet, G., Jouany, J.P., 2004. Adsorption of zearalenone by β-D-glucans in the *Saccharomyces cerevisiae* cell wall. J. Food Prot. 67, 1195–1200.
Yiannikouris, A., Andre, G., Poughon, L., Francois, J., Dussap, C.G., Jeminet, G., Bertin, G., Jouany, J.P., 2006. Chemical and conformational study of the interactions involved in mycotoxin complexation with beta-D-glucans. Biomacromolecules 7, 1147–1155.
Zhou, H.R., Yan, D., Pestka, J.J., 1998. Induction of cytokine gene expression in mice after repeated and subchronic oral exposure to vomitoxin (deoxynivalenol): Differential toxin-induced hyporesponsiveness and recovery. Toxicol. Appl. Pharmacol. 151, 347–358.