

# MODULATING EFFECT OF MYCOAD<sup>®</sup> ON PERFORMANCE, MUCOSAL AND SYSTEMIC IMMUNITY IN CHICKEN AFLATOXICOSIS

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**Abstract:** To determine the aflatoxin effect on performance, and humoral and mucosal immunity, 240 one-day old chicks were divided into 4 equal groups. Treatment groups include Group 1: chickens that received a standard diet based on corn and soy as negative control, Group 2: chickens fed with a basal diet containing 3 ppm aflatoxin as positive control, Group 3: chickens fed with 0.25% Mycoad<sup>®</sup> in basal diet, and Group 4: chickens fed with diet containing 0.25% Mycoad<sup>®</sup> plus 3 ppm aflatoxin. All chickens continuously received diets from hatching until 28 days old. Growth indices, such as weight gain, feed consumption and food conversion rate, were determined weekly. At 28 days, all chickens were sacrificed. After blood sampling, serum was prepared to measure serum IgG titer against Newcastle disease vaccine using the HI method. Moreover, the heads were collected for nasal-tracheal lavage for assaying IgA against infectious bronchitis vaccine in the mucosa of the respiratory tract. The measurement of mucosal IgA was carried out using the ELISA method with specific goat anti-chicken IgA. That results indicated that chickens that received aflatoxin demonstrated lower growth indices, and fewer serum IgG and mucosal IgA titers than others did, while performance and immune responses in chickens that received Mycoad<sup>®</sup> plus aflatoxin were significantly higher than chickens fed with aflatoxin alone. Overall, it seems that aflatoxin can affect mucosal immunity in the upper respiratory tract as well as performance and humoral immune responses. Supplementation of Mycoad<sup>®</sup> to diet contaminated with aflatoxin can reduce the adverse effects of aflatoxin on performance, as well as mucosal and systemic immune responses.

**Key words:** aflatoxin; chicken; immunity; Mycoad<sup>®</sup>

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## Introduction

Aflatoxins (AFs) are secondary metabolites of various *Aspergillus* species, e.g. *Aspergillus flavus* and *Aspergillus parasiticus*. Chemically, aflatoxins are furanocoumarin compounds, and the most important ones are B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, and G<sub>2</sub> (1). Among the various types of aflatoxins, aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) is most commonly encountered, and it is also considered to have higher toxicity than other

aflatoxins (2). Aflatoxins commonly contaminate a wide variety of tropical and subtropical food/feedstuffs (3). Poultry can be exposed to high concentrations of aflatoxin via feedstuffs which lead to large economic losses (4). The toxicity of aflatoxins (aflatoxicosis) in poultry is characterized by mortality, listlessness, anorexia, reduced growth rates, negative feed conversions, fatty liver, reduced egg production, poor pigmentation, and increased susceptibility to other diseases (5, 6, 7, 8). Aflatoxin is known to have strong hepatotoxic and carcinogenic effects (9). A practical approach to detoxification is the utilization of sorbents in the

diet that adsorb aflatoxin in the gastrointestinal tract of poultry, thereby reducing bioavailability and toxicity (7, 8).

Since the discovery of aflatoxins, the negative effects of them on animal health have been an active area of research. Based on this, research during the last five decades has well elucidated the negative effects of aflatoxins on animal performance and immunity (2). Aflatoxin can be a primary immunosuppressive agent (4) that can influence the efficacy of immune response in poultry. Most studies in this field focused on the effect of this mycotoxin on the humoral and cellular immune system. To date, there has been no scientific research on the effect of aflatoxin on mucosal immunity in chickens. In this research, the mucosal respiratory immune response against infectious bronchitis (IB) vaccine in chickens affected by experimental aflatoxicosis was analyzed. Moreover, the level of mucosal Immunoglobulin A (IgA) against IB vaccine was determined in chickens fed with aflatoxin plus one standard mycotoxin adsorbent (Mycoad®) for decreasing the reverse effects of aflatoxins on mucosal immunity.

## Materials and methods

### *Production and assaying of aflatoxin*

Aflatoxin provided by *Aspergillus parasiticus* (PTCC: 1850) belongs to Iranian Scientific and Industrial Research. Aflatoxin was produced according to the Shotwell method (10) on maize with a few modifications. The aflatoxin was assessed with the HPLC method utilizing reverse-phase C18 column (250×4.6 mm, 5 µm), equipped with fluorescence detector set at 370 (excitation) and 440 (emission). The mobile phase was deionized water/methanol/acetonitrile (60:20:20) with a flow rate of 0.8 ml/min and an injection volume of 20 µL.

### *Experimental design*

A total of 240 one-day-old broiler chicks (Ross strain) were randomly divided into four groups with three replicates of 20 chicks in each separated pen during the 28-day experiment. A basal diet based on corn-soybean was balanced in accordance with the recommendation by NRC (11).

Treatment groups include Group A: chickens fed a basal diet, Group B: chickens fed 3 ppm aflatoxin in a basal diet, Group C: chickens fed a basal diet containing 0.25% Mycoad® and Group D: chickens received a basal diet containing 3 ppm aflatoxin and 0.25% Mycoad®.

In this study, the basal diets were tested for contamination by aflatoxins, fumonisins, and zearalenone. The diets were not contaminated by any mycotoxins. The maize containing aflatoxin was added to the basal diet to increase the concentration of aflatoxin in experimental diet to 3 ppm. In groups that did not receive aflatoxin, the same amount of uncontaminated maize (without aflatoxin) was added to the basal diet. All treatment groups received experimental diets throughout the growing period from hatching until 28 days old. The diet formula is presented in Table 1. Feed and water were supplied *ad libitum* to all groups, and 24-hour light was utilized throughout the experiment. All chickens were vaccinated against Newcastle disease (ND) (at 8 and 18 days old), IB (at 1 and 10 days old), and infectious bursal disease (IBD) (at 14 days old) with B1, LaSota, H120, and D78 vaccines. Data on body weight, body weight gain, feed intake, and feed conversion ratio were recorded at weekly intervals. Moreover, cumulative data were assessed.

### *Mycoad®*

Mycoad® is a commercial broad spectrum mycotoxin adsorbent for all types of feed formulated as hydrated sodium and calcium aluminosilicate (HSCAS) by Special Nutrients Inc., USA.

### *Mucosal and systemic immunoglobulin assay:*

Blood samples from all chickens at 28 days of age were collected for measurement of serum immunoglobulin G (IgG) titer against ND vaccine by conventional haemagglutinin inhibition (HI) test (12). Thereafter, all the chickens were slaughtered, and samples of the trachea and nasal mucosa were separated immediately. The mucosal surface of the trachea and nasal mucosa were washed with 1 ml of phosphate buffer saline (PBS) (pH = 7.4) containing 0.1% of bovine serum albumin (BSA) three times. Immediately after washing, the liquid extract was centrifuged,

**Table 1:** Composition of the experimental diets for broiler chicks

Ingredients %	0-18 (days old)	19-28 (days old)
Corn grain	53.00	58.50
Soybean meal	39.00	33.65
Vegetable Oil	4.00	4.00
DCP	1.35	1.18
Oyster shells	1.45	1.50
Methionine D-L	0.25	0.23
Vitamin E	0.10	0.10
Edible NaCl	0.25	0.24
Vitamin Premix*	0.30	0.30
Mineral Premix*	0.30	0.30
Calculated nutrient content		
ME (Kcal/Kg)	3005	3070
CP (%)	22.82	19.90
Ca (%)	0.95	0.92
Available Phosphorus (%)	0.45	0.41
Lysine (%)	1.24	1.15
Na (%)	0.16	0.15
Methionine+Cystine (%)	0.95	0.88

\*Supplied Per Kilogram of Feed: 8.000 IU of Vitamin A, 2000 IU Vitamin D3, 50 mg Vitamin E, 1.5 µg Vitamin B1, 2.2 mg B, 6.5 mg Vitamin K, 7 mg Vitamin B2, 2 mg Vitamin B1, 40 mg nicotinic acid, 160 µg vitamin Biothine, 12 mg Calcium pantothenate, 1 mg Folic acid 30 mg Fe, 70 mg Mn, 100 µg Se, 40 mg Zn, 6 mg Cu, 1.14 mg I.

**Table 2:** The growth indices in chickens at end of fourth week

Indices	Negative control	Chickens that received aflatoxin	Chickens that received Mycoad®	Chickens that received aflatoxin and Mycoad®
Feed Intake	1568±20 <sup>bc</sup>	1321±15 <sup>a</sup>	1635±30 <sup>b</sup>	1508±25 <sup>c</sup>
Weight Gain	1131±35 <sup>b</sup>	782±18 <sup>a</sup>	1230±43 <sup>b</sup>	1093±25 <sup>b</sup>
Feed Conversion Ratio	1.38±0.04 <sup>b</sup>	1.68±0.03 <sup>a</sup>	1.33±0.06 <sup>b</sup>	1.37±0.02 <sup>b</sup>

\*Data presented as Mean±SD.

<sup>a,b</sup>Different letters in each row represent the existence of significant differences between groups (P <0.05).

**Table 3:** The systemic and mucosal immune responses in different groups

Indices	Negative control	Chickens that received aflatoxin	Chickens that received Mycoad®	Chickens that received aflatoxin and Mycoad®
Serum IgG titer against ND vaccine	4.85±0.39 <sup>a</sup>	2.95±0.21 <sup>b</sup>	5.22±0.32 <sup>a</sup>	4.45±0.35 <sup>a</sup>
Mucosal IgA titer against IB vaccine	4.92±1.65 <sup>a</sup>	3.15±1.68 <sup>b</sup>	5.57±1.30 <sup>a</sup>	4.61±1.24 <sup>a</sup>

\* Data presented as Mean±SD.

<sup>a,b</sup>Different letters in each row represent significant differences between groups (P <0.05).

and the supernatant was collected (13, 14, 15). To measure the level of specific IgA against IB vaccine in trachea and nasal mucosa, the lavage samples were tested with a commercial ELISA kit (Synbiotic co.). Based on the lack of commercial kits for measuring the IgA, commercial IBV ELISA kit was utilized, but the conjugated HRP goat anti-chicken IgG was replaced with conjugated HRP goat anti-chicken IgA. The goat anti-chicken IgA was conjugated to horseradish peroxidase purchased separately (Bethyl Laboratories, Cat. no. A 30-103P).

For the determination of IgA titer in mucosal respiratory lavage, the mean optical density (OD) of the negative control was calculated, and three times the standard deviations were added to the OD of the negative control. Thereafter, the OD of the negative control and positive-negative threshold was utilized for the calculation of titers (16, 17). In this study, the IgA titer in each sample was calculated utilizing the KPL software program according to negative control and positive-negative threshold. The IgA titers were expressed based on log 2.

### *Statistical Analysis*

All data were analyzed using the one-way ANOVA method by SAS software (18). Significant differences among treatment groups were recognized at  $P < 0.05$  by post-hoc Tukey test.

## **Results**

### *Evaluation of Growth Parameters*

The statistical comparison of weight gain, feed intake, and feed conversion ratio at the end of the fourth week showed that chickens that received aflatoxin had the lowest weight gain and feed intake and the highest feed conversion ratio ( $P < 0.05$ ). The weight gain and feed conversion ratio in the other groups did not show significant differences. In terms of feed intake, chickens that received Mycoad<sup>®</sup> had a higher feed intake than the chickens that received aflatoxin, and aflatoxin plus Mycoad<sup>®</sup>. There was a significant difference in feed intake between chickens fed with Mycoad<sup>®</sup> and chickens that received aflatoxin, or aflatoxin plus Mycoad<sup>®</sup> ( $P < 0.05$ ) but with the negative control, no significant difference was observed (Table 2).

### *Evaluation of the humoral immune response against ND vaccine*

The results of the HI test demonstrated that the IgG titer against ND vaccine in chickens that received aflatoxin was significantly less than other treatment groups ( $P < 0.05$ ). There were no significant differences between other groups for serum IgG titer against ND vaccine (Table 3).

### *Evaluation of the mucosal immune response against IB vaccine*

There were significant differences in the mucosal respiratory IgA titer against IB vaccine in the group fed with aflatoxin alone in comparison with other groups ( $P < 0.05$ ), while there was no significant difference between other groups (Table 3).

## **Discussion**

Growth parameters, such as weight gain, feed intake and feed conversion ratio in the chickens that received 3 ppm aflatoxin, were significantly less than other with experimental groups. Review of previous studies indicates that 2.5 ppm and higher amount of aflatoxin can significantly decrease growth and food conversion efficiency. For instance, Huff et al. (19) observed that the addition of 2.5 ppm aflatoxin reduced weight gain. Moreover, Harvey et al. (20) indicated that the addition of 3.5 ppm of aflatoxin in food can decrease feed intake and weight gain. The results of the current study indicate that the addition of Mycoad<sup>®</sup> to the contaminated feed at 3 ppm level of aflatoxin can completely inhibit the reduction of weight gain, feed intake and feed conversion efficiency. The comparison of growth parameters in control chickens and chickens that received aflatoxin plus Mycoad<sup>®</sup> revealed that, although Mycoad<sup>®</sup> could not increase growth parameters in chickens that received aflatoxin, it could improve growth indices and prevent the loss of growth parameters caused by aflatoxin. Previous studies have indicated that the presence of silicate in the composition of Mycoad<sup>®</sup> as an aluminosilicate compound can tightly bind with available aflatoxin in the gastrointestinal tract and markedly decrease the bioavailability and toxicity of aflatoxin (21, 8).

In this study, the reduction in mucosal IgA titers against infectious bronchitis vaccine and serum

IgG against ND vaccine in chickens receiving aflatoxin may be due to the effects of aflatoxin on the immune system. However, suppression of immune system caused by aflatoxin in poultry has already been reported (4), but there is no report on the effects of aflatoxin on mucosal immunity.

Infectious bronchitis virus is a major poultry pathogen that is endemic worldwide and leads to serious economic losses in the poultry industry. The mucosal immunity in respiratory tract acts as a first line of defense against IB virus infection. Some studies have indicated the significant relationship between the level of mucosal respiratory IgA and resistance against IB virus infection (22, 15). Therefore, it seems that measuring the level of mucosal respiratory IgA can be a suitable alternative for monitoring the protection level against IBV. Some reports have indicated that aflatoxin can increase the sensitivity of birds to viruses (4) and cause adverse effects on the production of serum antibodies against ND virus, pasteurellosis, and IBD (23). Moreover, there is some evidence of aflatoxin's effect on the histopathologic features of the thymus, spleen and bursa of Fabricius in chickens. Thymic aplasia, splenic atrophy, and lymphoid depletion in bursa can show the effect of aflatoxin on the cellular immune response in chickens (24). Ibrahim et al. (25) showed that both percentages and means of phagocytic activities were significantly reduced in chicks fed with 2.5 ppm aflatoxin. Moreover, the leucopenia in chickens following aflatoxin toxicity at a level of 3 ppm can demonstrate the cellular immunosuppression of aflatoxin (26). The effect of aflatoxin on interferon, complement and serum proteins (27), subsequent liver damage, and inhibition of protein synthesis (8) are the possible causes of immunosuppression. It seems the effect of aflatoxin on the immune system is influenced by the dose and duration of utilizing contaminated diet with aflatoxin. For instance, Pasha et al. (28) revealed that a low level of aflatoxin (100 ppb) has no effect on the size of the bursa of Fabricius. Moreover, Kouwenhoven (29) investigated the systemic antibody production following vaccination against Newcastle disease in chickens fed with aflatoxin. In this study, 0.2-0.5 ppm of aflatoxin B1 could not change the systemic immune response to the ND, *Salmonella pullorum* and *Pasturella multocida* but higher dose (0.6-10ppm) could suppress systemic antibody response to *Salmonella* and sheep RBCs. However,

Tessari et al. (30) reported that systemic antibody production following immunization with ND vaccine was affected by 0.2 ppm aflatoxin in the diet. The result of the current study is in line with the results of Ibrahim et al. (25) who clarify that 2.5 ppm aflatoxin has negative effect on the formation of ND antibodies. Nevertheless, the effects observed in the present study may be due to high dietary aflatoxin contamination. Considering the significant difference between the groups receiving the Mycoad® in addition to aflatoxin with aflatoxin alone, it seems that Mycoad® with its binding effect reduces the gastrointestinal absorption of aflatoxin and prevents the side effects of aflatoxin on the upper respiratory mucosal immune system.

The overall results of this study showed that 3 ppm aflatoxin can decrease growth parameters, humoral and mucosal immune responses. However, Mycoad® based on the dose and duration of use as examined in recent studies could prevent inhibitory effects of aflatoxin on the growth parameters, mucosal and humoral immunity system in broiler chickens. Therefore, the control of mycotoxins as immunosuppressive agents by using a commercial mycotoxin binder is valuable for providing better records in performance and immune responses.

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## VPLIV DODATKA MYCOAD® NA PRIRAST IN IMUNSKO ODPORNOST PIŠČANCEV Z AFLATOKSIKOZO

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**Povzetek:** Da bi preučili vpliv aflatoksina na prirast in imunski odziv (sistemski in v sluznicah) piščancev, smo 240 enodnevnih piščancev razdelili na 4 enake skupine in jih od izvalitve do 28. dneva starosti hranili s 4 različnimi krmami. Skupina 1 je predstavljala negativno kontrolo (piščanci, krmljeni s standardno krmo na osnovi koruze in soje), skupina 2 je bila pozitivna kontrola (piščanci, krmljeni s standardno krmo, ki je vsebovala 3 ppm aflatoksina), v skupini 3 so bili piščanci, krmljeni s standardno krmo z dodatkom 0,25 % Mycoad®-a, v skupini 4 pa piščanci, krmljeni s krmo, ki je vsebovala 3 ppm aflatoksina in 0,25 % Mycoad®-a plus. Tedensko smo določali različne pokazatelje rasti, kot so povečanje telesne mase, poraba krme in njen izkoristek. Po 28 dneh smo vse živali žrtvovali. Z uporabo metode HI smo v serumu določili titer protiteles IgG proti virusu bolezni Newcastle ter v izpirku nosu in sapnika določili titer protiteles IgG proti cepivu kužnega bronhitisa. Izmerili smo tudi raven sluzničnih protiteles IgA z metodo ELISA s specifičnimi kozjimi protitelesi proti piščančjim protitelesom IgA. Rezultati so pokazali, da imajo piščanci, ki so prejeli aflatoksin, nižje indekse rasti in nižje titre tako serumskih protiteles IgG kot tudi sluzničnih protiteles IgA. Medtem pa sta bila prirast in imunski odziv pri piščancih, ki so prejeli poleg aflatoksina tudi Mycoad® plus, bistveno višja kot pri piščancih, krmljenih samo z aflatoksinom. Naši rezultati kažejo, da vsebnost aflatoksina v krmi piščancev vpliva na lokalni imunski odziv sluznic zgornjih dihalnih poti in na učinkovitost sistemskega imunskega odziva. Dodatek Mycoad® h krmi, onesnaženi z aflatoksini, lahko zmanjša neželene učinke aflatoksina na uspešnost cepljenj pri piščancih.

**Ključne besede:** aflatoksin; piščanci; imunost; Mycoad®