

Reducing liver aflatoxin M1 residues in chicks with mycofix plus 3.0[®] during aflatoxicosis

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Abstract

Three hundreds and sixty male one-day-old broilers (Cobb), were distributed to three experiments, one hundred and twenty chicks for each, in order to elucidate the efficiency of 0.05, 0.15 and 0.25% Mycofix addition, in reducing the liver aflatoxin M1 (AFM1) residual level in chicks fed diets contaminated with Aflatoxin at a rate of 2.5, 3.5 and 5 ppm. Chicks were reared for 28 days. At the end of the experiments, chicks were killed by cervical dislocation and livers were collected for determination of liver AFM1 residual level. Determination was performed using thin layer chromatography method. Results showed that the addition of 0.25% mycofix to the feeds contaminated with 2.5, 3.5 ppm were responsible for reducing liver residual AFM1 levels.

Keywords: Tramadol, analgesia, antinociception, chicks.

Available online at <http://www.vetmedmosul.org/ijvs>

تخفيض ثمالة سم الافلا M1 في أكباد الأفراخ المتسممة بسموم الافلا باستخدام مايكوفكس (3+)[®]

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الخلاصة

استخدم ثلاثمائة وستون من ذكور فروج اللحم نوع (Cobb) بعمر يوما واحدا و تم توزيعهم إلى ثلاث تجارب بواقع مائة عشرون فرخا لكل تجربة، لمعرفة مدى كفاءة اضافة 0.05، 0.15 و 0.25 % من المايكوفكس في تخفيض مستوى سم الافلا M1 في اكباد الأفراخ التي غدت بعليقه لوتت بسم الافلا وبتركيز 2.5، 3.5 و 5 جزء بالمليون. ربّيت الأفراخ لمدة 28 يوما. في نهاية التجارب، قُتِلَت الأفراخ بالإزاحة العنقية، جُمِعَت الأكباد لتحديد سم الافلا M1 باستخدام طبقة كروماتوغرافيا الرقيقة أظهرت النتائج إن إضافة 0.25 % مايكوفكس إلى العلائق الملوثة بسم الافلا عند المستويين 2.5، 3.5 جزء بالمليون كانت قد خفضت من مستوى سم الافلا M1 في الاكباد.

Introduction

Aflatoxins (AF) are class of mycotoxins produced by fungal species of the genus *Aspergillus* (*A. flavus*, *A. parasiticus* and *A. nomius*), which are included in the difurocumarocyclopentanone (1). They are potent mycotoxins that contaminate feed ingredients routinely used for poultry rations. Major forms of AF include B1, B2, G1, and G2, with AFB1 being the most common and biologically active component (2). Natural contamination of broiler feed with AF has been recently documented here in

Mosul province (Iraq) (3,4). The toxicity of AF in broiler chickens has been widely investigated by the determination of their carcinogenic, mutagenic, teratogenic (5,6) and growth inhibitory (7,8) effects, biochemical-hematological (9,10), immunological (11,12) and pathological (13,14) effects. In addition, affected birds retain residues of the aflatoxin in their tissues (15,16). These residues are highest in the liver, gizzard and kidney, but there exists a large individual bird variation in the amount of residues retained and in the duration of time required for their total clearance (17). Trucksess et al. (18) found aflatoxin residues in all

edible tissues of all hens fed 8 ppm, when scarified 7 days after exposure, with the highest levels of B1 was found in the liver and ova and M1 in kidney.

Micco et al. (19) investigated the residual levels of B1 and its metabolites (B2a, M1 kidney and Ro) in tissues and organs of male broiler chickens and laying hens after long-term administration of a diet contaminated with 50 ppb AFB1. Residual levels of aflatoxins B1, M1, and Ro were detected in liver, kidney and thigh muscles of both male broilers and hens.

Aflatoxins are of primary public health concern including aflatoxin B1, B2, G1 and G2 and an animal metabolite, aflatoxin M1, which occurs in meat, eggs and primarily in milk when lactating dairy cows are fed rations containing aflatoxin B1 (20,21). Owing to the well-known carcinogenic effects, several studies have highlighted the importance of establishing appropriate food safety management programs for aflatoxins (22). Producers, researchers and governments aim to develop effective prevention management and decontamination technologies to minimise the toxic effects of AF. Besides of the preventive management, relatively new approaches have been employed including physical, chemical and biological treatments to detoxify AF in contaminated feeds and feedstuffs (23). Since the beginning of 1990s, the adsorbent-based studies have been performed to remove AF from contaminated feed and alleviate the toxicity of AF in poultry (24). Zeolites (25), bentonites (26) and clinoptilolite (CLI), natural zeolites and a member of heulandite–stilbite group (27), were preferred because of their high binding capacities for AF and their reducing effect on AF absorption from the gastrointestinal tract. A new promising adsorbent, Mycofix, was effectively used in poultry for amelioration of ochratoxicosis and aflatoxin due to the dual mode of adsorption of mycotoxins with suitably located polar functional groups like AF by selective blend of minerals (28), and for alleviation of T-2 toxicosis (29), aflatoxin inducing coccidiosis (30). The aim of the present study was to evaluate the effectiveness of Mycofix in reducing aflatoxin metabolite M1 residues in liver of broilers fed diet contaminated with graded levels of aflatoxins.

Materials and methods

The experiments were carried out in the animal house research division and the department of veterinary public health at the college of veterinary medicine, university of Mosul.

Broilers: Three hundreds and sixty, male one-day old broilers (Cobb), obtained from local hatchary, were divided to three experiments, one hundred and twenty chicks for each, they were weighed individually, wing banded, and

housed in a heated battery cages under continuous fluorescent lighting. Feed and water were provided ad libitum. Chicks were reared in individual wire cages for 28 days and fed a standered broiler diet with 22.0 % crude protein and 2950 metabolizable energy (Kcal/kg). Diets were designed to satisfy the recommendations of the NRC (1984) (31). Mixed feeds used in all experiments were checked and confirmed to be free from aflatoxin, ochratoxin and zearalenone as determined by thin-layer chromatography (32).

Aflatoxin: Aflatoxin was prepared through inoculation of rice by *Aspergillus parasiticus* NRRL 2999 (Kindly obtained from the college of Agriculture and Forestry, University of Mosul) (33,34). Fermented rice was then autoclaved and ground. The aflatoxin content was measured by spectrophotometric analysis (Multi-purpose ultra-violet spectrophotometer/Desaga) (35), which modified by (36). The percentages of aflatoxins content in the powder were 81, 14, 4, and 1% form the types of aflatoxins B1, G1, B2, and G2, respectively. The rice powder was incorporated into the basal diet to produce the desired level of 2.5, 3.5, and 5 ppm in each of the experiments.

Mycofix plus 3.0®: Mycofix plus 3.0 is the product of Biomin® GTI GmbH. Herzogenburg, Austria. Mycofix® Plus originally contained the components: Synergistic blend of minerals, Biological constituent, Synergistic blend of minerals, Biological constituent, BBSH 797, phytogenic substances, and Phycophytic constituents (44).

Design of the experiments: Three experiments were carried out, in each experiment one hundred and sixty, one-day old, male broiler chicks were randomly assigned into eight treatments (20 birds /group, 10 birds /replicate).

The first experiment include: Control group; 0.0 mycofix or aflatoxin. Mycofix 0.05% (Biomin ®/Austria). Aflatoxin 2.5 ppm. Aflatoxin 3.5 ppm. Aflatoxin 5 ppm. Mycofix 0.05 % +Aflatoxin 2.5 ppm. Mycofix 0.05 % +Aflatoxin 3.5 ppm. Mycofix 0.05 % +Aflatoxin 5 ppm.

The second experiment was designed as the following: Control group; 0.0 mycofix or aflatoxin. Mycofix 0.15%. Aflatoxin 2.5 ppm. Aflatoxin 3.5 ppm. Aflatoxin 5 ppm. Mycofix 0.15 % +Aflatoxin 2.5 ppm. Mycofix 0.15 % +Aflatoxin 3.5 ppm. Mycofix 0.15 % +Aflatoxin 5 ppm.

The third experiment involved the following: Control group; 0.0 mycofix or aflatoxin. Mycofix 0.25%. Aflatoxin 2.5 ppm. Aflatoxin 3.5 ppm. Aflatoxin 5 ppm. Mycofix 0.25 % +Aflatoxin 2.5 ppm. Mycofix 0.25 % +Aflatoxin 3.5 ppm. Mycofix 0.25 % +Aflatoxin 5 ppm.

Chicks in all treatments were killed at the end of the experiment (28 days) by cervical dislocation; livers were collected from 20 birds of each treatment in order to get samples of 100 g liver tissues/group.

Determination of aflM1 in liver:

Determination of AFM1 in liver samples was performed according to the method adopted from official methods of analysis of the Association of Official Analytical Chemists (1982) (37). The method was briefly as follows: Liver samples were collected from 20 birds of each group and homogenized (up to four samples/group, with 100 gs of liver tissues/sample). To the homogenized samples 10 ml of 20% citric acid were added and thoroughly mixed in a flask with a heavy glass stirring rod. The stirred samples were allowed to settle about 5 minutes and then were stirred again. After second stirring 200ml of dichloromethane were added and vigorously shaken for 30 min. The mixture was filtered in an Erlenmeyer flask containing 10 gm of sodium sulphate. The filtrated materials were then evaporated to near dryness in a bath at about 45°C. Clean up of the samples were carried out by column packed with sodium sulphate, silica gel, sodium sulphate. Twenty five ml of dichloromethane were added to the concentrated residual dried liver samples. The content then swirled gently to complete residual dissolving. The dissolved content were then transferred to column, and allowed to drain freely to top of sodium sulphate Column was then washed with 25 ml glacial acetic acid: toluene (1:9), then with 25 ml of hexane and with 25 ml of acetonitrile: diethyl ether: hexane (1:3:6). The column was eluted with 60 ml of a mixture of dichloromethane and acetone (1:4). Aflatoxins were obtained after evaporation of the organic solvent in a water bath at about 45°C. Hundred µl acetonitrile: benzene (1:9) were added to dry residue and mixed vigorously for about 1 min for AFM1 determination by thin layer chromatography (Merk/Germany). Two directional thin layer chromatography analysis method was used for AFM1 determination by applying 20 µl aliquot of the sample on 20X20 TLC plates (Merck ready made plates, 25 mm thickness) with 5 and 10 µl of standard AFM1 (Sigma). Plates were positioned in direction 1, in a developing tank containing isopropanol:acetone:chloroform (8:10:82). Developing was continued until the solvent reaches the score line, then plates were removed, dried and positioned in the second direction in a second developing chamber containing water: methanol: diethyl ether (1:6:93). Plates then were removed after solvent reaches the score line, dried and examined under long-wave UV light (365 nm). Calculation of the mass fraction, w, of AFM1 was as follows:

$$W = \frac{P_s V_s V_1 V_2}{M V_2 V_4} \text{ (mg/kg)}$$

Where Ps= mass concentration of standard M1 solution (0.25mg/L).

Vs= Volume of standard spot giving fluorescence intensity equal to that of sample spot (µl).

V1=Final volume of sample extract (100µl).

V2= volume of sample extract spot (20 µl).

V3=Volume of dichloromethane employed (200 ml).

V4=Volume of filtrate record.

M=mass of sample (100g).

Statistical analysis:

All experimental data were subjected to the analysis of variance (38). Least square means were compared by Duncan's multiple range test. All statements of differences were based on significance of $P < 0.05$.

Results

Experiment 1

The effect of inclusion 0.05% Mycofix in the AF contaminated diets with 2.5, 3.5 and 5 ppm AF on their liver residual AFM1 levels is presented in Figure 1. Results showed that no AFM1 residues were detected in the liver of groups 1 and 2, when fed diets free from AF. Feed contaminated with AF at a rate of 2.5, 3.5 and 5 ppm in-groups 3, 4 and 5, was responsible for a significant ($P < 0.05$) increasing proportion of liver residual AFMI levels with each increase in AF level, and were 12.3, 17.1 and 22.1 ppb respectively. The adsorption effect of 0.05% Mycofix addition on AF contaminated diets in treatments 6, 7 and 8 was not significant through the numerical reduction of residual AFM1 in livers of chicks in these groups to 11.6, 16.4 and 21.7 ppb respectively, i.e., 6,4 and 2% respectively (Figure 1).

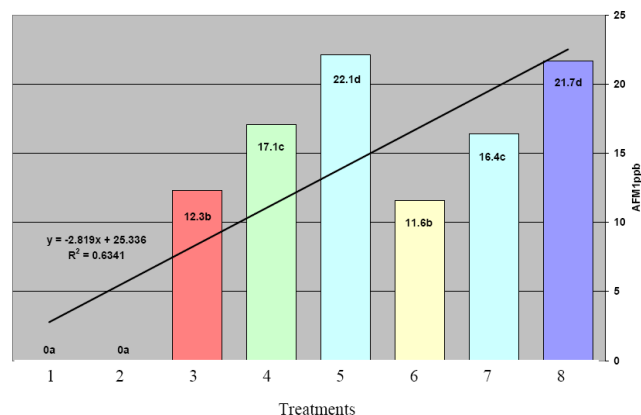


Figure 1: The effect of inclusion 0.05% Mycofix on AFM1 liver residue for chicks fed AF contaminated diets at 2.5, 3.5 and 5 ppm for 28 days of age.

abc Values in column with no common superscripts differ significantly ($P < 0.05$).

Experiment 2

The effect of inclusion 0.15% Mycofix on AFM1 liver residue for chicks fed contaminated diets at 2.5, 3.5 and 5 ppm AF for 28 days of age is presented in Figure 2. Feed contaminated with 2.5, 3.5 and 5 ppm in groups 3, 4 and 5, was responsible for an increasing proportion of liver residual AFMI levels with each increase in AF level, and were become 12.1, 17.5 and 22.9 ppb respectively. The adsorption or even absorption effect of 0.15% Mycofix addition on AF contaminated diets in treatments 6, 7 and 8 was evident through the non significant reduction of residual AFM1 in livers of chicks in these groups to 10.8, 16.1 and 21.5 ppb respectively, The percentage of this reduction was proportional with each increase in the Mycofix inclusion rate, and were 10, 8 and 6% respectively.

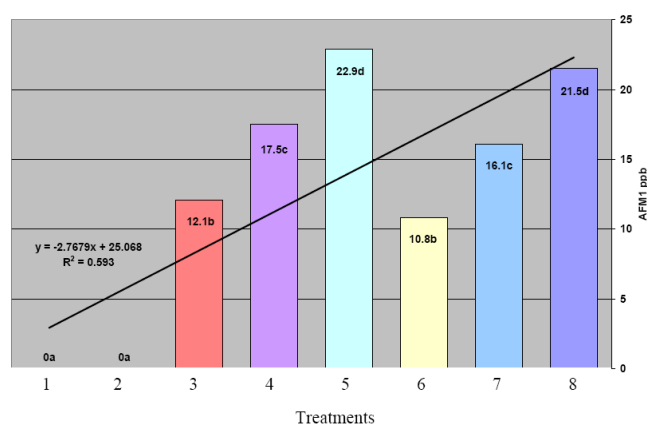


Figure 2: The effect of inclusion 0.15% Mycofix on AFM1 liver residue for chicks fed AF contaminated diets at 2.5, 3.5 and 5 ppm for 28 days of age.
 abc Values in column with no common superscripts differ significantly ($P < 0.05$).

Experiment 3

The effect of inclusion 0.25% Mycofix on AFM1 liver residue for chicks fed contaminated diets at 2.5, 3.5 and 5 ppm AF for 28 days of age is presented in Figure 3. Results showed that Feed contaminated with 2.5, 3.5 and 5 ppm AF in groups 3, 4 and 5, was responsible for an increasing proportion of liver residual AFMI levels with each increase in AF level, and were 12.4, 17.3 and 22.2 ppb respectively. The adsorption effect of 0.25% Mycofix addition on AF contaminated diets in treatments 6, 7 and 8 was evident through the significant ($P < 0.05$) reduction of residual AFM1 in livers of chicks in these groups to 7.5, 12.3 and 17.8 ppb respectively, compared with their respective groups that fed AF alone. The percentages of reduction were 40, 30 and 20% respectively.

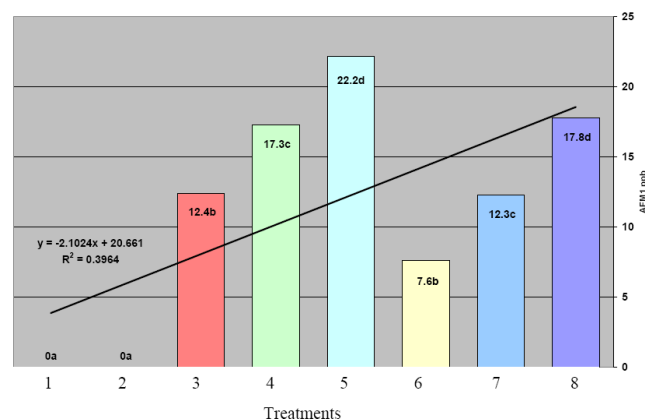


Figure 3: The effect of inclusion 0.25% Mycofix on AFM1 liver residue for chicks fed AF contaminated diets at 2.5, 3.5 and 5 ppm for 28 days of age.
 abc Values in column with no common superscripts differ significantly ($P < 0.05$).

Collectively there was an increasing trend to the adsorption of AF by mycofix with each increase in its inclusion rate from 0.05 to 0.25% to the AF levels in the three experiments. The percentages of the reduction in liver AFM1 residue were graduated from 2% when Mycofix was added at its lowest level (0.05%) to the diet contaminated with 5 ppm (experiment 1), to 65.3%, when the chicks in the experiment 3 fed a diet contaminated with 2.5 ppm and amended with 0.25% Mycofix (Figure 4). The relative AF levels in experimental feeds to the resulting liver AFM1 residual levels are presented in Tables 1, 2 and 3. From table 1 it is evident that there was very limited advantage in the addition of Mycofix at a rate of 0.05% to the diets contaminated with 2.5, 3.5 and 5 ppm in improving the relative feed: tissue AF levels, and were 6, 4 and 2% respectively. In favor of Mycofix with AF than AF alone. Some more benefit was gained with the raising Mycofix addition dose to 0.15% and its addition to the 2.5, 3.5 and 5 ppm contaminated diets on the percentages of the relative feed: tissue AF levels which were 12, 8.7 and 6.5 respectively in favor chicks fed both AF and Mycofix compared with those chicks fed AF alone (Table 2).

The best results obtained for the relative feed: tissue AF levels were those found in groups of chicks fed AF contaminated with 2.5, 3.5 and 5 ppm levels and amended with the highest Mycofix inclusion rate of 0.25%. The percentages of advantages were 65.3, 40.6 and 24.6 respectively (Table 3).

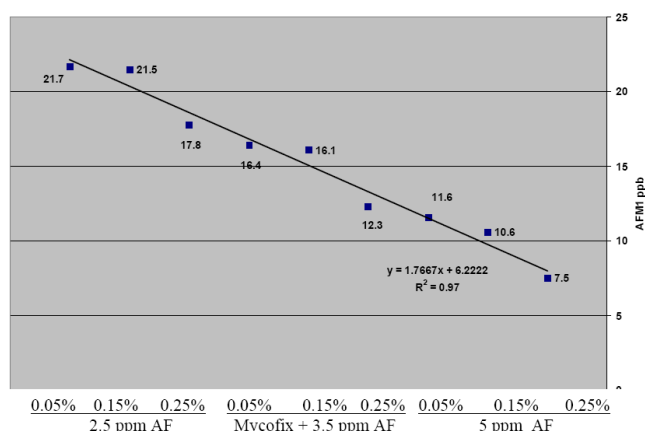


Figure 4: Increasing trend of AF adsorption with increasing Mycofix inclusion rate and decreasing AF contamination rate in experimental studies.

Table 1: Relative AF levels in feed to AFM1 residue levels in liver of chicks at 4 weeks, when Mycofix added at a rate of 0.05% to AF contaminated diets.

AF ppm	Mycofix %	Liver residual level AFM1 ppb	Conversion factor AF in Feed: AFM1 in liver ppb	Compared with AF group (%)
2,5	-	12.3	203.2	
3,5	-	17.1	204.6	
5	-	22.1	226.2	
2,5	0.05	11.6	215.5	+6.0
3,5	0.05	16.4	213.4	+4.0
5	0.05	21.7	230.4	+2.0

Table 2: Relative AF levels in feed to AFM1 residue levels in liver of chicks at 4 weeks, when Mycofix added at a rate of 0.15% to AF contaminated diets.

AF ppm	Mycofix %	Liver residual level AFM1 ppb	Conversion factor AF in feed: AFM1 in liver ppb	Compared with AF group (%)
2,5	-	12.1	206.6	
3,5	-	17.5	200	
5	-	22.9	218.3	
2,5	0.15	10.8	231.4	+12.0
3,5	0.15	16.1	217.3	+8.7
5	0.15	21.5	232.5	+6.5

Table 3: Relative AF levels in feed to AFM1 residue levels in liver of chicks at 4 weeks, when Mycofix added at a rate of 0.25% to AF contaminated diets.

AF ppm	Mycofix %	Liver residual level AFM1 ppb	Conversion factor AF in feed: AFM1 in liver ppb	Compared with AF group (%)
2,5	-	12.4	201.6	
3,5	-	17.3	202.3	
5	-	22.2	225.2	
2,5	0.25	7.5	333.3	65.3
3,5	0.25	12.3	284.5	40.6
5	0.25	17.8	280.8	24.6

The effect of dietary AF and AF with Mycofix on the residual AFM1 level in the liver with respect to action level in experimental chicks are presented in Figure 5. From figure it is clear that all residual AFM1 levels in livers of chicks fed diets contaminated with 2.5 and 3.5 ppm AF alone or those groups of chicks fed diets with these AF levels and 0.05% and 0.15% had residual AFM1 levels at or higher than the permissible action levels. Both the mentioned Mycofix addition levels of 0.05% and 0.15% to the 5 ppm AF contaminated diets were not effective in returning residual AFM1 to the acceptable action level of 20 $\mu\text{g kg}^{-1}$ or less, while this was significantly ($P < 0.05$) achieved only after feeding chicks with diet amended with 0.25% Mycofix.

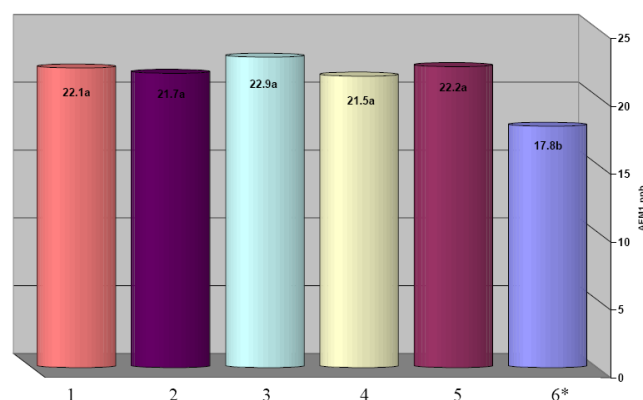


Figure 5: The permissible AFM1 levels (< 20 ppb) in livers of chicks fed AF and AF with Mycofix in three experimental studies.

*1=5 ppm AF 2=5 ppm AF + 0.05% Mycofix 3=5 ppm AF
 4=5 ppm AF+0.15% Mycofix 5=5 ppm AF 6=5 ppm AF + 0.25% Mycofix

abc Values in column with no common superscripts differ significantly ($P < 0.05$)

Discussion

Mycotoxin contamination of foods, feeds, animal derived products like eggs, milk, milk products and meats is very complicated issue, especially in the developing countries, especially those located in the tropical and sub-tropical regions with warm climates, which favor the growth of AF producing *Aspergilli* (39).

Humans in these countries including our country, Iraq, will be exposed to the AF and their metabolites if they urged to consume contaminated animal products when these animals ate feed containing aflatoxin (40). Really, we cannot eliminate mycotoxin contamination from our foodstuffs, because AF problem in foods is longstanding, unavoidable and seemingly inextricable (41,42). The recent study performed by us here in Mosul governorate show that the level of AF contamination of broilers feed estimated by Elisa test, were ranged from 22 to 2263 ppb (43). So, limiting these AF levels was strongly needed to reduce the adverse effects of AF-contaminated feed commodities on animal health, and then through the food chain, human health.

Mycofix plus product line represents specially developed additives that protect animal health by deactivation of mycotoxins taken in with respectively contaminated feed. Their sophisticated dual mode of adsorption of mycotoxins with suitably located polar functional groups like AF by selective blend of minerals (44).

Our results show that the addition of 0.05%, 0.15% and 0.25% mycofix to the feeds contaminated with 2.5, 3.5 ppm were responsible for reducing liver residual AFM1 levels to the action or permissible levels of AF in human foods of (20 ppb). Reducing liver residual AFM1 levels to that of acceptable one after increasing AF contamination feed to 5 ppb, was attained only by addition of 0.25% mycofix.

These results were in accordance within the finding of maximum tolerated levels ppb for aflatoxins in foods of many countries, during 1987 and 1996, as they were in a medium of 4 µg kg⁻¹ and a range of 0-50 ppb during 1987 in 29 different countries, and in a medium of 4 µg kg⁻¹ during 1996, but with range of 0-30 ppb in 33 countries (45). It is interesting to note that the results of this experiment confirm the facts; that only the highest inclusion rate of Mycofix (2.5 kg/ton of feed) was effective in restoring AFM1 liver residual levels below FDA action level, when AF was added to the broiler feeds in three high levels of 2.5, 3.5 and 5 ppm, as recommended by the producers, who advise to add Mycofix at a rate of 0.25% when feed is contaminated with AF with levels >300 ppb. Using low and medium Mycofix levels to the mentioned AF feed contaminated diets were unable to reduce residual AFM1 liver levels to that of the permissible food and drug administration (FDA) level of 20 ppb (47). No attempt was

tried to estimate residual AFM1 in muscle tissues, because very low levels or non detectable levels for residual AFM1 was suspected to be found in muscle. As cleared by the experiment performed by (46), who found that broilers at 30 days of age, AFM1 level was higher in liver than that in muscle, and that AFM1 was only found in liver and not in muscle till the end of 50 days experiment.

It should be stressed that the obtained low residual AFM1 levels here, are not considered as completely safe, since the only safe level is "zero" (39). Although AFM1 has been tested less extensively, it appears to be toxicologically similar to AFB1. AFM1 is considered to be a genotoxic agent, based on its activity in vitro and its structural similarity with AFB1. It is a less potent liver carcinogen, with a probable carcinogenic potency in laboratory animals within a factor of 10 of AFB1 (48). The importance of AFM1 residues in animal products could be related to the associations which exist between consumption of AF in those carriages of hepatitis B virus and the risk of liver cancer is determined by the presence in serum of the hepatitis B surface antigen (HBsAg⁺ or HBsAg⁻) (49). About 50 to 100% of liver cancer cases are estimated to be associated with persistent infection of hepatitis B (or C) virus, i.e., AF in HBsAg⁺ individuals. Thus, reduction of the intake of AF in populations with a high prevalence of HBsAg⁺ individuals will have greater impact on reducing liver cancer rates than reductions in populations with a low prevalence of HBsAg⁺ individuals (50). Hepatocellular carcinoma is the most common cancer in the world with 473,000 new cases appearing per year, with 80% of these cases appearing in developing countries. Moreover, there is substantial evidence that low-level exposure to AF may cause suppression of the immune system and increased susceptibility to disease (51). AF is also excreted in mother's milk and increases the morbidity of children with Kwashiorkor (52) and young are more sensitive than adults to AF.

So, we tried to improve feed security and food safety in order to maintain markets and protect human health by using one of the most recently promising adsorbent, an AF binder adsorbent, Mycofix plus.

It should be stressed that special consideration should be taken in that feeds and foods must be examined for their contamination with AF. Many food products are tested by the food and drug administration (FDA) in the United States, the institute of public health in Japan and many other agencies around the world regularly in the marketplace for AF, including cereals, eggs, milk, cheese, yogurt and meats (53). Additional procedures that could be used for reducing the presence of mycotoxin contamination in food is the application of hazard analysis of critical control point (HACCP) principles (46). Therefore we need to work to improve feed security and food safety in order to maintain markets and protect human health.

These practices should be employed in the more suspecting AF contamination foods and feeds in tropical and subtropical countries of high AF contamination levels, like our country Iraq.

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