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Control of Aflatoxin Residues in Broiler Chicken Using *Saccharomyces cerevisiae* Fortified Ration

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Abstract

The current research was designed to examine the protective effect of probiotic-fortified ration against aflatoxin B1 (AFB1) toxicity and its residual level in broilers' edible tissues. Sensitive high-performance liquid chromatography coupled with a fluorescence detector (HPLC-FLD) was used for measuring the toxin. Ninety, one-day-old Cobb chicks were allocated into three equal groups (n=30) with three replicates per group. The first control group (G1) was fed a balanced basal diet only and the second group (G2) received AFB1 (2 mg/kg basal diet), while the third group (G3) received a combination of AFB1 (2 mg/kg basal diet) with *Saccharomyces cerevisiae* (SC; 1.5 g/Kg basal diet). Experimental birds were monitored for 6 weeks, their growth performance was then compared. AFB1 residue was assessed in the meat and liver sample. AFB1 resulted in a significant ($P<0.05$) reduction of growth performance parameters such as body weight and carcass yield in comparison to the control and SC supplemented groups. Moreover, AFB1 residue significantly ($P<0.05$) diminished in SC fortified group when compared with the AFB1 group. In conclusion, probiotics such as *Saccharomyces cerevisiae* could be considered as a potential feed additive and a growth promoter. Besides, its role in controlling AFB1 residue in the edible tissues of boiler chicken.

KEYWORDS
Aflatoxins, HPLC-FLD, Mycotoxins, Probiotic.

INTRODUCTION

Broiler meat is an indispensable source to the ever-growing population worldwide. The recent reports of the Food and Agriculture Organization (FAO) determined that about a quarter of the total broiler feed available in the markets is contaminated with Mycotoxins (MTs) (Chang *et al.*, 2016). Feed contamination is the main route of broiler exposure for MTs resulting in economic losses for broiler producers and food and feed processors (Bryden, 2012). MTs are of serious concern in public health and poultry manufacture due to the considerable health problems (Serra *et al.*, 2018), environmental pollution, and economic losses worldwide (Luo *et al.*, 2018).

MTs are commonly present in broiler feed as a result of secondary metabolites of various fungal species mainly Aspergillus (Bagherzadeh Kasmani *et al.*, 2012). *Aspergillus flavus* produces type B1 and B2 aflatoxins (Monem *et al.*, 2015). The subtype one and two refer to the major and minor aflatoxins (AFs), respectively. AFB1 is a group of highly toxic and carcinogenic AFs (Bryden, 2012). AFB1 has the greatest agro-economic impact due to its substantial effect on livestock life via its immense impacts on growth performance and production. Besides, the economic loss in the scientific research in a trial reduces the severity and consequent problems caused by mycotoxins (Chang *et al.*, 2016). The potential health hazards are resulting from the consumption of AFs contaminated diet by both humans and livestock. For broilers, AFs affect the birds' health by reducing bird's efficiency with poor nutrients conversion (Ortatatli and Oğuz, 2001), inducing extensive hemorrhage and hepatic failure (Azzam and Gabal, 1998, Oguz *et al.*, 2003), and lowering immune efficiency (Sridhar *et al.*, 2015). Concerning human health, AFs induce acute and chronic effects (Felizardo and Câmara, 2013; Algahtani *et al.*, 2020) including mutagenic, teratogenic, carcinogenic group I (mainly liver and kidney), and immunosuppressive effect (FAO/WHO, 2001).

Frequently, AFs exist in zones characterized by a humid and warm environmental condition which is an appropriate condition for the molds' flourishing and growth. Also, the molds can grow in a temperate climate. These environmental factors that influence the presence of AFs in feeds are more difficult to be con-

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trolled (Bryden, 2012). Therefore, such contamination of feedstuff with AFs is difficult to be avoided in poultry farms where the storage facilities may be abused.

Despite the continuous international efforts to set global rules and preventive measurements to control MTs, especially AFB1, practical measures do not been sufficiently implemented (Oguz, 2011). Therefore, when the danger of AFs contamination in feed and feedstuff cannot be controlled, their decontamination is imperatively required with effective methods. Several ways have been implemented including biological and chemical substances to detoxify aflatoxins' contaminated rations (Celik *et al*., 2001, Oguz, 2011). An approach to the problem depends on the usage of adsorbent or chelating agents to eliminate AFs and decrease their absorption or increase their clearance from the gastrointestinal tract (Savi *et al*., 2017, Moretti *et al*., 2018, Huwig *et al*., 2001). Previous studies clarified that biological degradation would be the most effective decontamination method, which could allow detoxification of AFs without resulting in a noticeable decrease in feed palatability or nutritive quality (Celik *et al*., 2001). Importantly, the successful economical detoxication technique must be able to remove all harmful residual traces of AFs without impairing the nutritional value of the diet.

In this context, the present research was conducted to determine the detoxification ability of *Saccharomyces cerevisiae* (SC) as a direct microbial feed in the degradation of AFB1 in broilers as well as its ability to overcome AFB1 adverse effect on growth performance and AFB1 residual level in meat and liver. Moreover, the study aimed to use the reliable technique developed by our lab to test the presence of AFB1 in edible tissues using HPLC-FLD analysis (Monem *et al*., 2015).

MATERIALS AND METHODS

Chemicals and materials

AFB1 standard with a purity of 99% was used for the calibration curve (Sigma, St. Louis, USA). HPLC-grade solvents were used for preparing the mobile phase (Merck, Darmstadt, Germany). Analytical grade acetone and dichloromethane were obtained from Fischer Scientific, UK. De-ionized water was obtained using Cronus Filter (UK). The probiotics SC was purchased from Biomin GmbH, Austria.

Preparation of aflatoxins

A toxigenic strain of *Aspergillus flavus* (GenBank: KP137700) was used to prepare AFs mainly AFB1. This strain was isolated from poultry feed and subculture by Animal Health Research Institute, Giza. The toxigenic A. flavus are sub-cultured for 7 days on different media: 25% glycerol nitrate agar and malt extract agar at 25°C as well as Czapek yeast extract agar at 25°C and 37°C (Magnoli *et al*., 2006). AFs were qualitatively confirmed by the presence of blue fluorescence on the plate and comparison Retention Factor (RF) value of the spot versus the value of the standard (Shotwell *et al*., 1981). Thin Layer Chromatography (TLC) was used to screen AFB1. Yellow corn was proved either free from fungal or AFs contaminations via gross examination and TLC analysis, respectively. For three successive days, the corn was autoclaved at 121°C for 15 min. After that, a 10 ml spore suspension containing 106 spores/ml was added to the autoclaved corn and the whole mixture was incubated for 21 days at 28-30°C. After 21 days, the fungal growth was killed by heating at 60°C for 24 h and the mixture was pulverized. A representative 25 g of the powder was evaluated for AFB1 content (AOAC Method, 1995). The mixture containing the desired level of AFB1 (2 mg/kg diet) was incorporated into the broilers' basal diet.

Birds' management and experimental design

Adapted 90 one-day-old Cobb chicks were purchased from Ismailia-Misr Poultry hatchery, Ismailia Company. They were housed in adjusted environmental conditions; relative humidity of 50±10% and gradual temperature of 24±2°C. Ration and water were available ad libitum. The birds were randomly separated into three groups (n= 30); G1: fed basal diet without treatment and served as control -ve group, G2: fed basal diet mixed with AFB1 (2 mg/kg) and served as control +ve group, G3: received AFB1 basal diet and SC probiotic (1.5g/Kg diet). All treatments continued for 42 consecutive days. The diet was formulated to satisfy the diet requirements according to the NRC (1994). The birds were routinely vaccinated against IB, ND, AI (H5N1), and Gumboro as illustrated in Table 1.

Tissue sampling

Birds' general health condition and growth performance were assessed. After slaughtering, the meat and liver samples were immediately dissected, cleaned from blood using 0.9% NaCl solution, and dried. Then, they were kept at -80°C until analysis.

HPLC-FLD protocol

Preparation of stock and working standards

A stock solution of AFB1 standard was prepared using HPLC grade acetonitrile following the "Association of Official Analytical Chemists method" (AOAC Method, 1995). The stock solution was diluted using methanol to get different working solution concentrations of AFB1. The standard solutions used for the HPLC experiment calibration curve.

HPLC-FLD analysis

Samples recovery was investigated using 2g of the blank meat samples spiked with 0.5 mL of AFB1 at different levels: 10, 20, and 40 µg/kg. The spiked samples were kept in dark for 30 minutes then the extraction process was started. Citric acid 20% (200 µl) was added. Then 4 ml of dichloromethane was added, and the tubes were shaken for 30 minutes. The filtered mixtures were evaporated by a nitrogen evaporator. The dried matrix was eluted with 1ml hexane then the Solid Phase Extraction step was performed. Samples elution was performed using 5ml of dichloromethane: acetone (4:1). The samples were centrifuged at 14000 rpm for 10 min then the supernatant was separated and filtered by a disc filter 0.45µm into HPLC vials.

The chromatographic separation was performed with a reversed-phase Extend-C18 Zorbax Agilent column with the following dimensions: 250 mm, 4.6 mm, and 5 µm. An optimized validated method (Monem *et al*., 2015) using Agilent Series 1050 quaternary gradient pump with autosampler, Series 1050 FLD detector, and HPLC 2D Chemstation software (Hewlett-Packard, Les Ulis, France) was used. To get optimum resolution, the column temperature was adjusted to 30°C at a flow rate of 1.0 ml/min. The extracts of meat and liver samples were analyzed using an isocratic mobile phase formed from acetonitrile/methanol/water 20:20:60, respectively. The injection volume of the samples and standard solutions was 20 µl.

Statistical analysis

Once the data were entered into the spreadsheet and analyzed, the statistical results were analyzed by variance homogeneity distribution. The data were analyzed by variance method (ANOVA) using SPSS 18.0 software. The significant differences were examined using multiple range Duncan tests to compare the means. results were presented as means ± standard error of means (SEM). The results that express probability at P <0.05 are considered statistically significant.

RESULTS AND DISCUSSION

The validation parameters including specificity, selectivity, precision (intra- and inter-day variability), stability, and robustness were performed to establish the validity of the analytical method. The calibration curve of the AFB1 standard was linear (r > 0.999) over the concentration range as shown in Figure 1A and Figure 1B. The accuracy of the analysis was determined by recovery protocol, and it was found to be 101.3167 ±2.06 at 10, 20, and 40 µg AFB1. The mean accuracy assay was 93.958. The relative standard deviation (RSD%) was found to be 0.047.

The effect of *S. cerevisiae* on mortality rate, final body weight, and carcass yield of broilers fed AFB1 contaminated diet had been presented in Table 2. The final body weight and carcass yield of the birds were significantly (P<0.05) influenced by dietary AFB1. Addition of *S. cerevisiae* to AFB1 contaminated diet of broiler chickens significantly (P<0.05) enhance the body weight and carcass yield.

The effect of *S. cerevisiae* on AFB1 residual levels in meat and liver was presented in Table 3. The AFB1 residue in both meat and liver of broilers was significantly higher in the group fed an aflatoxin-contaminated diet as shown in Figures 2 and 3. Adding *S. cerevisiae* to the AFB1 contaminated diet of broilers significantly (P<0.05) decrease the level of AFB1 residue in the edible tissues.

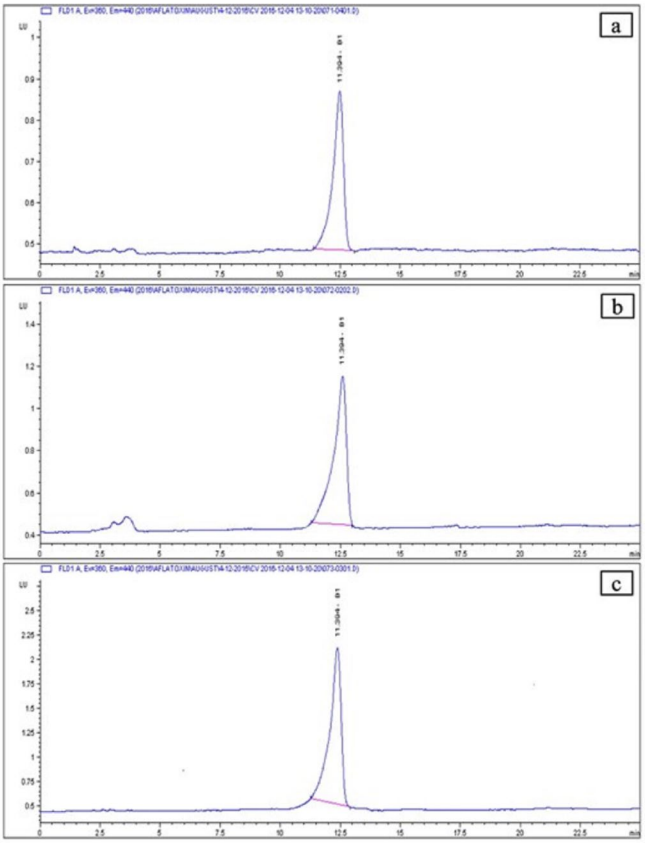

Figure 1a. Chromatogram of total aflatoxin B1 standard concentrations; 1A includes a) 0.016 ppm, b) 0.032 ppm, and c) 0.064 ppm.

Table 1. The vaccination program.

Age/day	Vaccine	Trade name	Company	Administration
6th day	ND& IB	Hitchner IB	Izo S.p.A, Italy	Eye drop
7th day	ND& IB	Inactivated ND&IB vaccine	(Intervet, Holland)	Sub/cutaneous injection
8th day	AI (H5N1)	Inactivated AI(H5N1) vaccine	(Marial, Spain)	Eye drop
14th day	Gumboro	Bursine® IBD	(Pfizer, USA)	Eye drop
20th day	ND& IB	Nobilis® clone 30 &MA5	(Intervet, Holland)	Drinking water
24th day	Gumboro	Bursine® IBD	(Pfizer, USA)	Drinking water

ND= Newcastle Disease, IB= Infectious Bronchitis, and AI (H5N1) = Avian Influenza.

Table 2. Effect of dietary Aflatoxin and *Saccharomyces cerevisiae* on mortality rate, final body weight, and carcass yield in broiler chickens.

Treatments	no.	Mortality rate (%)	Final body weight (kg)	Carcass yield (kg)
G_1 (-ve)	30	1 (10%)	1.43±0.045^b	1.06±0.019^b
G_2 (+ve)	30	17 (56.7%)	1.31±0.027^c	1.00±0.010^c
G_3 (AFs+SC)	30	1 (10%)	1.61±0.038^a	1.25±0.026^a

Value followed by different superscript letters are significantly different (P< 0.05)

Table 3. Effect of dietary *Saccharomyces cerevisiae* on Aflatoxin B1 residual level in broiler chickens' meat and liver (Mean ± SD).

Experimental groups	Experimental no.	Total sample analyzed	No. of positive samples	Aflatoxin B1 (ppm)			
				Residue value range		Mean level	
				Liver	muscle	Liver	muscle
G_1 (-ve)	30	29	0 (0%)	ND	ND	ND	ND
G_2 (+ve)	30	13	13 (100%)	0.15:0.32	0.03:0.16	0.210±0.016^a	0.142±0.007^a
G_3 (AFs+SC)	30	29	29 (100%)	0.03:0.19	0.02:0.08	0.080±0.012^b	0.040±0.005^b

Value followed by different superscript letters are significantly different (P< 0.05), ND= not detected.

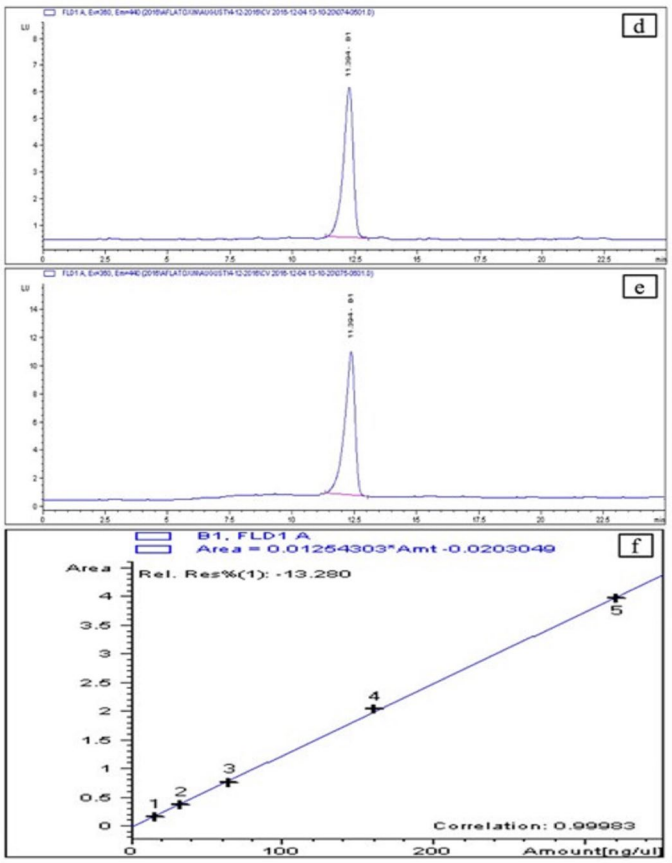

Figure 1b. Chromatogram of total aflatoxin B1 standard concentrations; 1A includes d) 0.160 ppm, e) 0.320 ppm, and f) calibration curve of total aflatoxin B1 concentrations.

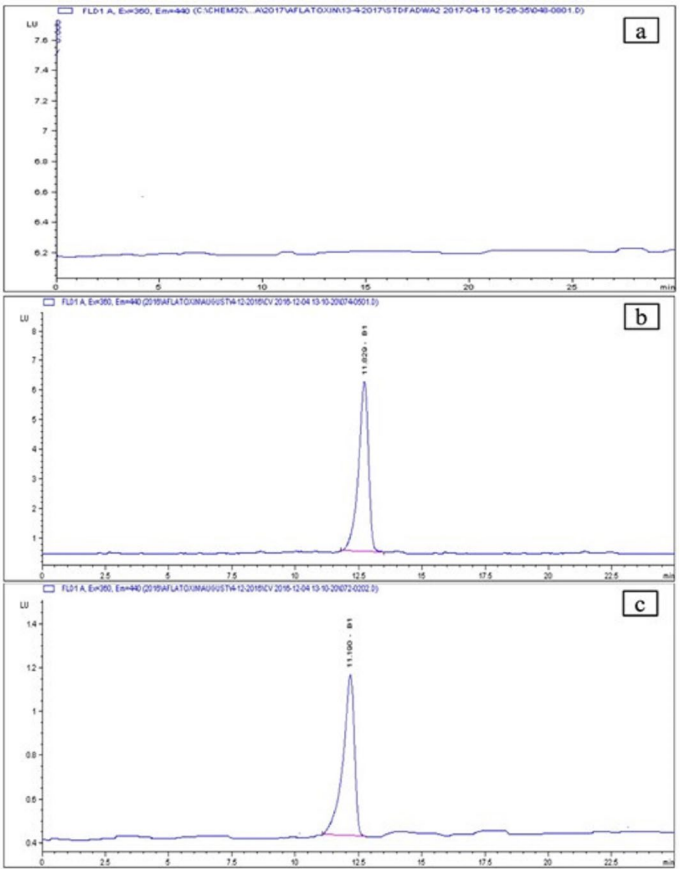

Figure 2. Effect of dietary S. cerevisiae on AFB1 residual level in broiler chicken meat. Chromatogram of a) Control group, b) Aflatoxins group, and c) S. cerevisiae group.

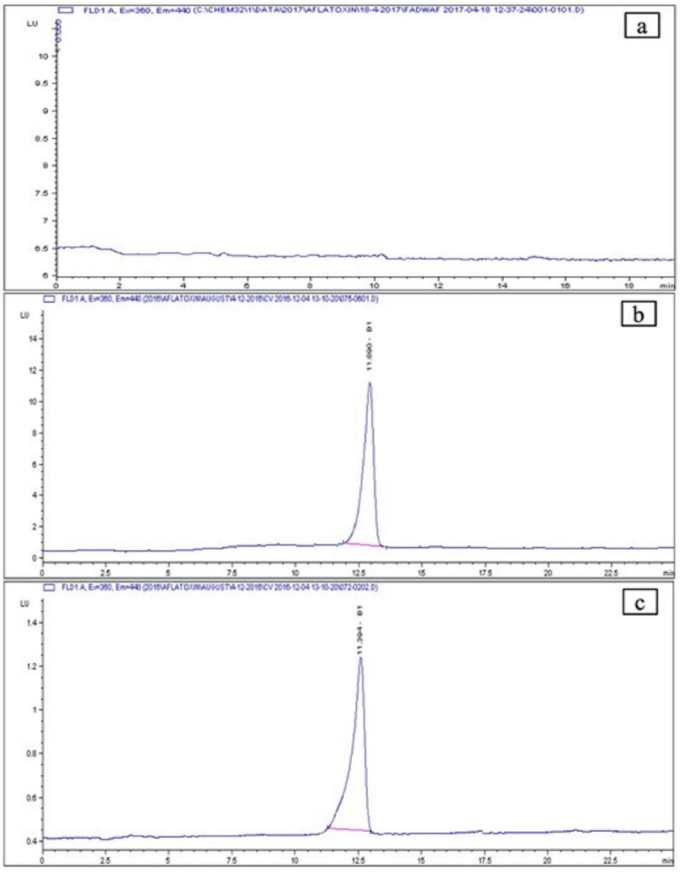

Figure 3. Effect of dietary S. cerevisiae on Aflatoxin B1 residual level in broiler chicken liver. Chromatogram of a) Control group, b) Aflatoxins group, and c) S. cerevisiae group.

Broiler meat is one of the most important food suppliers of cheap protein sources worldwide. Therefore, genetic selection of high-performance poultry traits has been performed intensively to magnify its production (Nikulin *et al*., 2017). However, MTs including AFs are of major concern in poultry production which reduces animal efficiency and feed quality. Besides, AFs may cause public health hazards and serious economic losses. Hence, prevention of mold growth and its AFs production in feed and feedstuff is imperatively critical especially when feed contamination cannot be avoided. Therefore, researchers and producers aim to improve the effective chelating and preventive materials to minimize the harmful effects of AFs.

Although the strengthening of the legislation, establishing of perfect detection methods, and setting up strict management system, the AFs residue still exists (Salim *et al*., 2018). From this aspect, the current trend in poultry production pointed to reducing the toxic effect of AFs via increasing the use of toxin-binding compounds such as aluminosilicates, zeolite (Pappas *et al*., 2014), and activated charcoal (Khadem *et al*., 2012) which lessen the absorption of AFs from the digestive tract. Nutritional control of the AFs residue by using feed additives fortified diet may have beneficial impacts and afford a simple avenue for enhancing birds' health and production (Park *et al*., 2016, Farhat-Khemakhem *et al*., 2018). Trials with these inert adsorbents have been verified to be fruitful, but with the high inclusion rate of these substances in the diet. This possibly leads to interactions with feed nutrients which are taken into the researchers' consideration (Dwyer *et al*., 1997, Rosa *et al*., 2001). Therefore, various studies proposed biological components as a superlative method for decontamination which could facilitate the elimination of AFs without affecting the feed nutritive quality and feed palatability (Bata and Lásztity, 1999). Besides, the effective detoxication process must be economical and able to exclude traces of toxin which causes harmful residues in the edible tissues (Kubena *et al*., 1998, Bailey *et al*., 1998). *S. cerevisiae* is one of the most extensively commercialized yeast species and acts as an optimum adsorbent. Moreover, *S. cerevisiae* is rich in vitamin B complex and protein contents (40-

45%) (Khadem *et al*., 2012).

In the current study, AFB1 inclusion in the broilers diet showed a decrease in growth performance, especially body weight gain but this adverse effect was ameliorated via adding *S. cerevisiae* into their diet. These results agreed with the outcomes of Motawe *et al*. (2014) who reported a significant increase in broiler chickens' performance, body weight gain and feed conversion, after adding different types of bacteria and yeast to their diet. On the contrary, Lee *et al*. (2010) reported no improvement in body weight gain by direct-fed microbial in broiler chickens' diet. Decreasing the growth performance in broiler-fed AFs could be due to the disturbance in the metabolic process and food utilization in the liver as well as loss of appetite due to the AFs' toxic effect. *S. cerevisiae* contains immune-stimulating substances such as mannan oligosaccharides and β-glucans. Consequently, this strain of yeast could be used as a growth promoter and immune stimulant (Celik *et al*., 2000; Çelýk *et al*., 2003).

The healthiness of the liver is one of the most important signs of general health performance because the liver is responsible for several chemical actions needed to survive. Besides, the liver secretes chemicals that are used by other parts of the body (Huff *et al*., 1988). Moreover, the liver can be affected by any chemical agents emitted from the intestine. As there is a boundless relation between liver healthiness and body performance. High AFB1 residue in the liver reported in the current study is indicative of improper liver function which might cause deficient performance. Devegowda *et al*. (1998) attributed the disturbance of growth performance to the depletion of liver glutathione enzyme which is used in AFs detoxification. Glutathione enzyme is composed of methionine and cysteine which are building stones in protein structure and in turn this detoxification process leads to the consumption of extra amino acids which consequently noticed in poor growth and decreased body weight.

Furthermore, probiotics such as yeast have antimicrobial properties and other health-related benefits through the maintenance of the intestinal biostructure (Wang and Gu, 2010; Zhang *et al*., 2016). Probiotics not only adsorb the mycotoxin, but also prevent some specific intestinal pathogens, produce various nutrients, improve the chicken intestinal metabolism Zarei *et al*., 2018), and enhance the general performance as well as improve the local and systemic immunity (Korver, 2012; Wang *et al*., 2017). In vitro, SC was found to bind more than 77% of added mycotoxins into the chicken diet as well as modified SC mannan-oligosaccharides derivative resulted in 95% binding of the MTs (Devegowda *et al*., 1996, Shetty and Jespersen, 2006).

Sustainable mycotoxins especially AFB1 residue in meat and edible products are a food safety concern. The result of the current research shows that exposure to AFB1 at a dose of 2 mg/kg diet for 42 days led to the accumulation of AFB1 in broiler meat at a level ranging from 93 to 167 ppb and in the liver at a level ranging from 142 to 324 ppb. The majority of the AFB1 residue was higher in the liver than in the meat. In a previous study when hybrid tilapia were exposed to 1.641 mg/kg of AFB1, the toxin residue was detected in the livers but not the musculature (Deng *et al*., 2010). These results propose that the liver plays a vital role in the metabolism, detoxification, and excretion of AFs and their metabolites. In the present study, the concentrations of AFB1 in meat and liver decreased considerably due to SC inclusion in the diet.

CONCLUSION

The present research indicates that *S. cerevisiae* as a feed additive can effectively decrease the sustainable level of AFB1. Moreover, the HPLC-FLD analysis method is a valid and reliable technique for the detection of AFB1 in edible tissues. As the risk of MTs exposure continues in developing countries owing to a lack of strict food security, quality control, poverty, and malnutrition, the researcher must be focused on the economic solution for this problem. Additionally, in vivo experiment is imperatively needed to confirm the usefulness of the biological preparation in the protection of broilers against occasional AFs contamination. SC needs to be tested for MTs binding capabilities. Future studies need to be directed toward the probiotics' binding complex stability, particularly under variable gastrointestinal conditions.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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