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RESEARCH ARTICLE

Reduction of Aflatoxin B1 Residues in Meat and Organs of Broiler Chickens by Lactic Acid Bacteria

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A B S T R A C T

Aflatoxin B1 (AFB1) contamination of animal feed and food including meat and dairy products, even at very low, ppb, levels can adversely affect human and animal health. This study assessed the effect of yogurt whey addition at 5% to the drinking water of broiler chickens fed AFB1-contaminated diets on the resulting AFB1 residues in organs (liver, kidney), and meat (breast, leg). Groups that received whey in their drinking water showed reductions in AFB1 of 55.46% (1.19 to 0.43 ng/g), 37.68% (0.69 to 0.53 ng/g), and 59.35% (28.81 to 11.71 ng/g) in leg and breast, respectively, at the end of week 6. The concentration of AFB1 in liver, kidney, and gizzard was reduced by 60.68 % (1.88 to 0.74 ng/g), 60.64% (2.06 to 0.81 ng/g), and 52.73% (1.10 to 0.52 ng/g), respectively, at week 6 compared to those without whey addition. It is suggested that aflatoxin B1 was biodegraded by the lactic acid bacteria (LAB) in yogurt whey, which included *Lactobacillus bulgaricus*, *Streptococcus thermophilus* and *Lactobacillus lactis* when whey was added to poultry drinking water.

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INTRODUCTION

Aflatoxins are toxic fungal metabolites discovered the 1960's can be produced by *Aspergillus* species; *Aspergillus parasiticum*, *Aspergillus flavus* and to a lower extent by *Aspergillus nomius*. Mycotoxins particularly aflatoxins and their metabolites can be found in grain-based feeds, meat, dairy products and eggs, and considered carcinogenic for animals and human (Anonymous, 2004; Zahoor-ul-Hassan *et al*., 2010; Khan *et al.*, 2013; Naseem *et al*., 2018a). The presence of aflatoxins in foods generally associated with many diseases so considered as an important potential health risk factor for the community (Eshelli *et al*., 2015; Majeed *et al*., 2018). Aflatoxin B1 (AFB1) is considered as one of the most potent toxins in animal farms particularly poultry and other monogastric animals that depends on level of AFB1 concentrations and individual exposure time (Bailey *et al*., 2006; Bovo *et al*., 2014; Naseem *et al*., 2018b). Biological reduction of mycotoxins in foods by bacteria, yeast and fungi has been examined and it has been found that strains of microorganisms are capable of degrading mycotoxins through enzymatic action (Patharajan *et al*., 2010; Reddy *et al*., 2010; Elsanhoty *et al*., 2014; Ahmadzadeh *et al*., 2015; Loi *et al*., 2016; Sangi *et al*., 2018). Aflatoxin B1 degradation by beneficial bacteria such as lactic acid bacteria (LAB) that found effective in reduction the toxins from contaminated products (Zinedine *et al*., 2005). Legislation limits of mycotoxin residue are controlled in most countries, with maximum limit of 10 ng/g for total aflatoxins (AFB1, AFB2, AFG1 and AFG2) and 5 ng/g for AFB1 (Van Egmond, 1995). Therefore, in response to the periodic contamination of poultry products by aflatoxins, this study was conducted to evaluate the use of LAB present in yogurt whey, when given to broilers in their drinking water on AFB1 levels in poultry tissue.

MATERIALS AND METHODS

One hundred Hubbard chicks of 1 day-old purchased from a commercial local market and divided into 5 groups

of 20 birds each with equal mean body weight. Regular feed free of AFB1 was provided to Group I, which was considered as the control group. Groups 2, 3, 4 and 5 fed diets contaminated with four levels of AFB1 (T1: 384.5, 360.72, 350.20 and T2: 128.90, 118.62, 117.31, 110.62 AFB1 ng /g feed) and drinking water mixed with yogurt whey at 5 % during the first three weeks. Feeds were provided for chicks for 6 weeks all the time without restriction. The diets constituents providing during the experiment is shown in Table1. AFB1 was determined in broiler flesh at starting zero time and at weeks 3 to 6 in a representative sample from five randomly sacrifice selected birds.

Chemical and reagent: Sodium sulphate, sodium chloride aflatoxin AFB1 standards (98%), acetonitrile, acetone, methanol was from Sigma (St Louis, MO, USA). The stock solution of AFB1 standard were prepared in acetonitrile (AOAC International, 2000). The extracted AFB1 was cleaned and purified from impurities and interferences by passing the extracted solution through SPE-CN column (Varian Inc., Palo Alto, CA, USA), and aflatoxin immunoaffinity columns (IAC) from r-biopharm (Darmstadt, Germany).

Table 1: Broiler experimental diet composition used in the study[1,2,3].

Ingredients	Starter %	Finisher %
Corn	58.5	67.05
Soybean meal	35.65	26.00
Vegetable Oil	1.69	3.00
Limestone	1.84	1.68
DCP	1.00	1.02
Salt	0.41	0.42
DL-Methionine	0.20	0.20
L-Lysine	0.11	0.13
Coccidiostat	0.10	0.00
Vitamin Premix	0.10	0.10
Mineral Premix	0.10	0.10
Choline chloride	0.10	0.10
Antioxidant	0.10	0.10
Antifungal	0.10	0.10
Analysis: Metabolizable energy (kcal/kg of dry matter)	2978.00	3156.00
CP (%)	22.30	18.20
NPP (%)	0.45	0.40
Ca (%)	1.03	0.95
Na (%)	0.18	0.18

[1]Each kg of vitamin premix contained 2.4×10^6 IU vitamin A; 3.2×10^5 vitamin D3 ; 5.6×10^3 mg vitamin E, 640 mg vitamin K3; 500 mg vitamin B1; 1120 mg vitamin B2, 3200 mg niacin; 1600 mg Ca-D-pantothenate; 800 mg vitamin C, 2.4 mg vitamin B12, 160 mg folic acid; 7.2 mg D-biotin; 8000 mg vitamin C; 20000 mg choline chloride. [2]Each kg of mineral premix contained 8×10^4 mg manganese; 6×10^4 mg iron; 6×10^4 mg zinc; 200 mg cobalt; 100 mg iodine; 150 mg selenium. [3]DCP, dicalcium phosphate; CP, crude protein; NPP, non-phytate phosphorous.

Whey preparation, isolation and confirmation of LAB: Whey samples used were collected from commercial yogurt after its separation from solids by passage through sterile cotton cloth (Labneh). Whey samples were held at 4ºC prior to use in the drinking water. To analyze for LAB, samples of whey were taken and serially diluted in 0.85% NaCl solution to 10^{-6}, and then 1 mL from each serial dilution was inoculated in 9 ml MRS broth (De Man *et al*., 1960) and 1 mL pour-plated in 10 mL MRS agar. The plates and the broth tubes were incubated for 48 hr at 37°C. Bacterial colonies from the plates were picked individually and streaked on new MRS agar plates to have single separate colonies. Each of the isolates was selected and identified by the API 50CHL identification kit (BioMérieux, SA, Marcy l'Etoile, France).

Test protocol: A 2 g of homogenized powdered feed sample was combined with 10 mL methanol: water (70:30) mixture and shaken (700 rpm) for 50 min using an IKA shaker (IKA, Hamburg, Germany). A 100 µL of the eluate was mixed with 600 µL phosphate buffer, (pH 7.2) and the AFB1 was analyzed by HPLC. Herzallah (2009) methods was followed in determining AFB1 in chicken muscles and organ samples. Where, 50 g of flesh were blended with 100 mL mixture of acetone and water (1:1) for 10 min and filtered through "fast filtering" Whatman No.1 filter paper. An aliquot of the filtrate mixed with hexane and 5% NaCL (1:1) were shaken at 1200 rpm for 10 min using a mechanical shaker (IKA). The top hexane layer was discarded and the AFB1 extracted with chloroform (3 x 50 mL), dried over anhydrous sodium sulfate and evaporated using an IKA rotary evaporator, the residues redissolved in 1 mL phosphate buffer and cleaned by passage through an IAC column. The eluted sample evaporated under nitrogen, dissolved in methanol and analyzed for AFB1 using HPLC.

HPLC determination: Analysis of AFB1 was performed using a Waters HPLC (Waters Corp., Milford, MA, USA) at wavelengths of 365 nm (excitation) and 425 nm (emission). The Thermo LC-Si column (250 x 4.6 mm) at 40ºC and a flow rate of 2.0 mL/min and a mobile phase of a mixture of toluene, ethyl acetate, ethanol and formic acid (90:5:2.5:2.5, v/v/v/v) was used in AFB1 determination.

Recovery of aflatoxins: Calibration curve of AFB1 constructed over concentrations of 0.05-to 24 ng/mL was linear, with a correlation coefficient of 0.999 and a coefficient of variation (CV) of 1.32%, with 0.05 µg/kg detection limit (Herzallah, 2009).

Statistical analysis: The AFB1 residues data were reduced using ANOVA and general linear models (GLM) procedure in PC-SAS® version 9.0 (SAS Institute, 2000) with a significance level of $P<0.05$.

RESULTS

Analysis of yogurt whey for LAB revealed the presence of three organisms, *Lactobacillus bulgaricus* plus *Streptococcus thermophilus* which are yogurt starter cultures and *Lactobacillus lactis* which is naturally present in milk. The results found in this study for AFB1 in broiler chicken kidney, gizzard, leg (drumstick and thigh), liver and feed are presented in Tables 2 and 3. The residue levels of AFB1 in poultry muscles (breast, leg) or organs (liver, kidney) were dose dependent; as the AFB1 concentration in feed increased, the residual level in muscles or organs increased ($P<0.05$) compared to the untreated control with <0.05 µg/kg. Broiler chicken kidneys were found to contain more AFB1 at week 6 (2.06 ng/kg) than a week 3 or 4 (1.18 and 1.28 ng/g,

respectively), as shown in Table 3. The liver of untreated broiler chickens at week 6 was shown to have substantially higher AFB1 concentrations (1.88 ng/kg) than a week 3 (1.23 ng/g). In contrast, the use of yogurt whey in broiler chicken drinking water caused significant reductions in AFB1 levels from 1.19 to 0.53 µg/kg in leg muscles (T2) at week 6 (Table 2), and residual levels in kidneys were reduced from 2.06 µg/kg to 0.81 µg/kg during the same period (Table 3).

DISCUSSION

An almost exactly 3-fold greater amount of AFB1 was added to the feed in treatments T1 than T2; and although tissue levels of AFB1 were higher in TI, differences were in general <2-fold greater. No AFB1 was detected (<0.05 ng/g) in the tissues of all birds when sampled at the initiation of the tests, and during the 6 weeks of the trials in the untreated control broilers. At 384.5 ng/g in the feed (T1), AFB1was found to increase in all tissues during the 6 weeks of the trial, with the largest accumulation in the kidneys, followed in order by the liver, leg, gizzard and breast. Among the organs, AFB1 accumulation in the liver was significantly greater than in the gizzard. These results agreed with AFB1 residue data reported for liver following ingestion of AFB1-contaminated diets (Zaghini *et al*., 2005; Pasha *et al*., 2007). The results were also in agreement with data reported by Denli *et al*. (2009**)** who found that AFB1 was detected in the liver of chickens fed 0.2 ng AFB1/g feed, and this affected the performance of broiler chickens by increasing liver weights and by causing a reduction of animal weight (Al-Shawabkeh *et al*., 2009).

Muscle tissue accumulation of AFB1 continued slowly throughout the trials with significantly greater levels present in the leg than the breast meat in both trials. Greater accumulation was found at higher levels of feed contamination. These results were also in agreement with those of Denli *et al*. (2009) who found that AFB1 residues were not detected in breast muscles, although a method with an AFB1 detection limit of 0.1 ng/g was used. Residue levels also increased as the time of administration and level used increased. For example, in T2, chicken legs at week 3 contained 0.51 ng/g AFB1, while at week 6 the level was 1.19 ng/g AFB1. The increase in AFB1 in organs and muscles of broiler chickens was significant (P<0.05) between chicken parts (organs or muscles) and within treatments at week 5 and 6 for both treatments (Table 2 and 3). The results were consistent with those found by Pasha *et al.* (2007) where feeding a diet of 2.1 µg /g for 35 days caused a residue level of less than 3 ppb in gizzard, liver, and kidneys of broiler chickens. In addition, with data reported by Zaghini *et al*. (2005) who found in chickens fed 2.1 mg AFB1 a residual level between 1.9 to 4.1 ng/g in liver. This could have been due to the physiological stress of chicken organs at the levels of AFB1 used. It is also possible that absorption at these levels by the gastrointestinal tract was incomplete, and thus unabsorbed AFB1 was excreted. As rearing time progressed, concentrations of <0.05, 28.70 and 37.6 ng/g were found in T1 and <0.05, 8.30 and 28.81 ng/g were found at weeks 0, 3 and 6 respectively, in T2. The results were in consistent with those reported by Zaghini *et al*. (2005) who found that the use of mannan-oligosaccharides increased adsorption of AFB1 to the polysaccharides and decreased the residue level in poultry muscles. Whey addition to the broiler chicken drinking water produced significant reduction in accumulation of AFB1 in broiler meat and organs. The decrease by whey addition at week 6 was 43% and 37% in breast tissues of

Table 2: Aflatoxin B1 (ng/g) in flesh of broiler chickens fed an AFB1-contaminated diet and drinking water mixed with yogurt whey[1,2]

Group	Treatment 1					Treatment 2				
Chicken age (wk) sample description	G1	G2	G3	G4	G5	G1	G2	G3	G4	G5
	0	3	4	5	6	0	3	4	5	6
	AFB1(ng/g)									
Feed	<0.05	384.53[a] (±3.11)	360.72[a] (±3.20)	345.2[a] (±3.51)	350.20[a] (2.82)	<0.05	128.90[a] (±2.03)	118.62[a] (±2.11)	117.31[a] (±2.07)	110.62[a] (±2.11)
Breast	<0.05	0.51[d] (±0.10)	0.73[c] (±0.10)	0.81[c] (±0.11)	0.93[c] (±0.19)	<0.05	0.38[c] (±0.02)	0.45[c] (±0.06)	0.71[c] (±1.10)	0.69[c] (±0.06)
Legs (drumstick + thigh)	<0.05	0.89[b] (±0.11)	0.89[b] (±0.10)	1.58[b] (±0.11)	1.64[b] (±0.11)	<0.05	0.51[b] (±0.06)	0.63[b] (±0.10)	1.17[b] (±0.11)	1.19[b] (±0.13)
	Treatment 1+ Whey (lactic acid bacteria)					Treatment 2 + Whey (Lactic acid bacteria)				
Breast	<0.05	0.31[e] (±0.11)	0.53[d] (±0.09)	0.38[d] (±0.14)	0.53[e] (±0.13)	<0.05	0.26[d] (±0.02)	0.43[d] (±0.08)	0.52[e]±0.06	0.43[e] (±0.06)
Legs (drumstick + thigh)	<0.05	0.56[c] (±0.10)	0.59[e] (±0.13)	0.83[c] (±0.10)	0.81[d] (±0.11)	<0.05	0.28[d] (±0.06)	0.32[e] (±0.07)	0.68[d] (±0.11)	0.53[d] (±0.11)

[1]Aflatoxin detection limit by HPLC was 0.05 ng/g. [2]Values are means of three readings±SD. [3]Different superscript letters within columns indicate values are significantly different at P<0.05.

Table 3: Aflatoxin B1 residues (ng/g) in organs of broiler chickens fed AFB1-contaminated diets and drinking water mixed with yogurt whey [1,2]

Group	Treatment 1				
Chicken Age/wk Sample Description	G1	G2	G3	G4	G5
	0	3	4	5	6
	AFB1(ng/g)				
Kidney	<0.05	1.18[a] (±0.06)	1.28[ab] (±0.08)	1.68[a] (±0.15)	2.06[a] (±0.09)
Liver	<0.05	1.23[a] (±0.09)	1.34[a] (±0.13)	1.62[a] (±0.12)	1.88[ab] (±0.14)
Gizzard	<0.05	0.83[b] (±0.10)	0.98[b] (±0.12)	1.01[b] (±0.10)	1.10[b] (±0.10)
	Treatment 1+Whey (lactic acid bacteria)				
Kidney	<0.05	0.38[e] (±0.12)	0.68[c] (±0.10)	0.36[d] (±0.11)	0.81[c] (±0.11)
Liver	<0.05	0.62[c] (±0.13)	0.54[de] (±0.17)	0.65[c] (±0.13)	0.74[cd] (±0.11)
Gizzard	<0.05	0.43[d] (±0.10)	0.48[d] (±0.12)	0.39[d] (±0.16)	0.52[d] (±0.08)

[1]Aflatoxin detection limit by HPLC was 0.05 ng/g. [2] Values are means of three readings±SD. [3]Different superscript letters within columns indicate values are significantly different at P<0.05.

T1 and T2, respectively. Kidney and liver AFB1 concentrations were significantly ($P<0.05$) lower and were reduced from 2.06 ng/g to 0.81 ng/g by whey addition. The results were like those reported by Oluwafemi and Da-Silva (2009) where a reduction of more than 30% of residual aflatoxin was removed by *Lactobacillus* species in contaminated maize and with Huang *et al*. (2017) who found that *L. plantarum* C88 reduce the toxicity of AFB1 toxicity through regulating AFB1 metabolism.

Conclusions: This study demonstrated that yogurt whey (which contained live LAB) in drinking water was able to significantly reduce AFB1 to low levels in meat and organ tissues of broiler chickens. Reduction of aflatoxin AFB1 accumulation in broiler chicken organs or tissues most likely occurred because of direct biodegradation of AFB1 by LAB in the gastrointestinal tract. One could speculate that some reduction in tissue AFB1 concentrations might occur in response to lowered intestinal levels caused by the LAB simply through concentration gradient normalization. The experimental results have shown that yogurt whey supplementation of the water portion of poultry diets can prevent the accumulation of AFB1 in animal tissues from contaminated feed to a significant extent.

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Authors contribution: MAR: Running part of the analysis and providing some standards. NIA: Running part of the analysis. SMH: Writing the article and running the analysis. AR: Helped in reading the manuscript. AF: Provided the working place and standards. RH: Proof reading and reviewing the manuscript.

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