Effects of feeding corn naturally contaminated with AFB_1 and AFB_2 on performance and aflatoxin residues in broilers

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ABSTRACT: AFB_1 is the most abundant aflatoxin in food and animal feed, generally occurring along with low levels of other aflatoxins. In this experiment, broilers were administered corn that was naturally contaminated with AFB_1 and AFB_2. We found that the broilers were more sensitive during the starter period and had a poor performance during the grower period. In addition, the broilers showed a significant reduction in feed intake when consuming material contaminated with 134.0 and 23.6 μg/kg of AFB_1 and AFB_2, respectively. Our results also demonstrate that villus height and the ratio of villus height to crypt depth significantly decreased when the broilers were fed daily with diets contaminated with AFB_1 and AFB_2. Furthermore, AFB_1 residues in livers and breast muscles were determined by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) at levels of 0.137 and 0.016 μg/kg, respectively. The AFM_1 residue was also detected in livers at a level of 0.051 μg/kg but it was not detectable in breast muscles.

Keywords: mycotoxin; influence; poultry; HPLC-MS/MS

Mycotoxins are secondary toxic metabolites of filamentous fungi, which are harmful to human and animal health thus causing significant financial losses to animal industries (Hussein and Brasel, 2001; Wu and Munkvold, 2008; Zhang and Caupert, 2012). Aflatoxins (AF) naturally occur in moldy grains and are a group of metabolites produced by some strains of *Aspergillus flavus* and *Aspergillus parasiticus*. The primary aflatoxins of concern in feedstuffs are aflatoxins B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), and G_2 (AFG_2) (Monbaliu et al., 2010). The order of toxicity is $AFB_1 > AFG_1 > AFB_2 > AFG_2$. The legal limits of AFB_1 and AFT in total for several agricultural products have been established by the European Union (2–12 μg/kg for AFB_1, 4–15 μg/kg for aflatoxins total (B1 + G1 + B2 + G2), and 0.05 μg/kg for AFM_1 in milk), but the value of aflatoxins in other animal products was not recommended (European Commission, 2010).

AFB_1 is transformed into toxic metabolites primarily in the liver, and the metabolite of AFB_1-8, 9-expoxide can form covalent bonds with DNA, RNA, and proteins, resulting in a potent carcinogenic effect (Swenson et al., 1977; Nomura et al.,

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2011). AFB_2 is converted to AFB_1 in the livers of both rats and ducks, and the rates of these reactions appear to correlate with the toxic potential of AFB_2 in these species (Wogan et al., 1971). AFM_1 is the toxic metabolite of AFB_1, and AFM_2 is the hydroxylated form of AFB_2 (Wolzak et al., 1985; Bintvihok et al., 2003; Hussain et al., 2010, Bianco et al., 2012). AFM_1 and AFM_2 are commonly associated with milk and other edible animal products.

AFB_1 contaminants occur more frequently at higher concentrations than the other AF metabolites (Fandohan et al., 2005), and AFB_1 toxicity in poultry has been well elucidated (Martinez-de-Anda et al., 2010). Previous studies have shown that AFB_1 has a range of negative effects on poultry health, including poor performance (Oguz and Kurtoglu, 2000; Magnoli et al., 2011), liver and kidney lesions (Ortatatli et al., 2005), immunity damage (Marin et al., 2002), and hematology problems (Zhao et al., 2010). Moreover, the tissue deposition and residue of AFB_1 in poultry has also been determined (Oliveira et al., 2000, 2003). However, AFB_1 usually occurs in feedstuff along with low levels of other aflatoxins, and the toxicity observed in poultry is complex and diverse (Ghahri et al., 2010; Yunus et al., 2011).

AFB_1 is commonly found along with AFB_2 in corn in the southwest of China (Placinta et al., 1999), but few studies have explored the performance of broilers and the level of aflatoxin residues in the tissues of broilers administered with naturally occurring AFB_1 and AFB_2 grains. In this study, we explored the growth performance and nutrient digestibility of broilers and determined the aflatoxin residue levels in broilers fed with corn that was naturally contaminated with AFB_1 and AFB_2.

MATERIAL AND METHODS

Animals and management

A total of 1200 one-day-old COBB500 male broilers were weighed and randomly allocated into 5 treatment groups. Each treatment group had 8 replicates of 30 broilers. All the ingredients in the 5 treatment diets, except for the corn, were the same. The basal control diet was formulated and compounded to meet the nutritional requirements of commercial broilers (National Research Council, 1994) during the starter and grower periods, presented in Table 1. The basal control diet was not contaminated with AFB_1 and AFB_2. The control group (I) was fed the basal control diet. The second (II), third (III), fourth (IV), and fifth groups (V) were given feed in which the ratio of naturally contaminated corn as a substitute for normal corn was 25, 50, 75, and 100%, respectively. The study

Table 1. Composition and nutrient levels of the basal diets (%)

Items	Day 1–21	Day 22–42
Ingredient		
Corn	60.00	63.00
Soybean meal (46%)	31.20	28.17
Corn gluten meal	3.00	2.50
Soybean oil	1.14	2.27
Calcium carbonate	0.95	0.88
Calcium hydrophosphate	1.85	1.60
L-Lysine hydrochloride	0.08	0.02
DL-Methionine	0.16	0.09
Salt	0.50	0.40
Choline chloride	0.15	0.10
Vitamin and mineral premix	0.33[a]	0.32[b]
Rice hull	0.64	0.65
Total	100.00	100.00
Nutrient levels		
Crude protein	20.83	19.37
ME (MJ/kg)	12.15	12.55
Calcium	0.97	0.87
Available phosphorus	0.44	0.39
Lysine	1.11	0.97
Methionine	0.48	0.39
Methionine and cysteine	0.86	0.72
Threonine	0.78	0.74

[a]provided per kg of diet: vitamin A 12 000 IU, cholecalciferol 3000 IU, vitamin E 7.5 IU, vitamin K_3 1.5 mg, thiamin 0.6 mg, riboflavin 4.8 mg, pyridoxine 1.8 mg, vitamin B12 9 µg, folic acid 150 µg, niacin 10.5 mg, calcium pantothenic acid 7.5 mg, iron 100 mg, copper 8 mg, manganese 120 mg, zinc 100 mg, selenium 0.3 mg, iodine 0.7 mg

[b]provided per kg of diet: vitamin A 8000 IU, cholecalciferol 2000 IU, vitamin E 5 IU, vitamin K_3 1 mg, thiamin 0.4 mg, riboflavin 3.2 mg, pyridoxine 1.2 mg, vitamin B_{12} 6 µg, folic acid 100 µg, niacin 7 mg, calcium pantothenic acid 5 mg, iron 80 mg, copper 8 mg, manganese 100 mg, zinc 80 mg, selenium 0.3 mg, iodine 0.7 mg

protocol was conducted in accordance with the animal husbandry laws of the P.R.China.

The broilers were fed in cage pens for 42 days and maintained on a 24-h continuous light schedule. The initial temperature of 33 ± 1°C was gradually reduced by 2°C per week to reach 21°C after 35 days, and this temperature was maintained for the rest of the experiment. Feed and water were provided *ad libitum*.

Analysis of dietary mycotoxins

The corn was ground in a Restch ZM100 mill using a 0.75 mm sieve. Mycotoxins were extracted from 25 g of a milled sample with 50 ml of water for deoxynivalenol (DON), acetonitrile and water (90 : 10, v/v) solution for zearalenone (ZEA), methanol and water (70 : 30, v/v) solution for AF, and methanol and water (80 : 20, v/v) solution for T-2 toxin, ochratoxin A (OTA), and fumonisin B_1 (FB_1). The afore-mentioned solutions were shaken vigorously in a sealed flask for 40 min. The extract was filtered by gravity, and then 10 ml of the filtrate was mixed with 40 ml of PBS. The mixture was centrifuged at 8000 *g* for 15 min. Then, 15 ml of the supernatant was subjected to a purification process, which was carried out with an AflaStar^{TM} Immunoaffinity columns (Romer Labs Diagnostic GmbH, Tulln, Austria). After the sample had passed through the column, the column was washed with 10 ml of PBS and 10 ml of water and then it was eluted with 2 ml of methanol into a glass vial, which was maintained at 4°C until analysis.

The mycotoxins were quantified via high-performance liquid chromatography equipped with a fluorescence detector Agilent 1100 (Agilent Technologies Inc., Santa Clara, USA). Separation was achieved on a SB-C_{18} column (4.6 × 250 mm, 5 µm) (Agilent Technologies Inc., Santa Clara, USA). The mobile phases were mixtures of methanol and water (45 : 55, v/v) solution for AF, acetonitrile and water (12 : 88, v/v) solution for DON, acetonitrile and water (80 : 20, v/v) solution for T-2 toxin, acetonitrile and water and methanol (46 : 46 : 8, v/v/v) solution for ZEA, methanol and aqueous 1% acetic acid (50 : 50, v/v) solution for OTA, and methanol and water acidified with 0.1 mol/l phosphoric acid (pH = 3.3) (50 : 50, v/v) solution for OTA. The flow rate was 1 ml/min, and the injection volume was 50 µl.

A post-column photochemical derivatization was used to enhance the mycotoxin response using a PHRED photochemical reactor (AURA Industries Inc., New York, USA). The wavelengths of excitation and emission were fixed at 365 and 440 nm for AF, 330 and 460 nm for OTA, 330 and 440 nm for FB_1, and 381 and 470 nm for T-2 toxin, respectively. However, the presence of ZEA and DON was monitored at 274 and 220 nm with the fluorescence detector, respectively. The above mycotoxins were analyzed by the Test Center for Feed Quality Supervision and Inspection (Chengdu) of the Ministry of Agriculture, with detection limits (in µg/kg) of 2 for AFB_1, 0.8 for AFB_2, 2.5 for AFG_1, 1.5 for AFG_2, 100 for T-2 toxin, 300 for DON, 100 for ZEN, 30 for OTA, and 200 for FB_1 (Administration of Quality and Technology Supervision of Sichuan Province, 2010).

Body weight and feed consumption

Broilers were weighed individually, and feed consumption for each pen was measured on days 22 and 43 during the 6-week experiment. The cumulative weight gain and feed consumption were determined, and the weekly and cumulative gain : feed ratios were calculated.

Digestibility experiment

The excreta collection method was used to explore nutrient digestibility. The experiment consisted of a 2-day adaptation period and 4-day collection period. At day 43, one broiler per pen was randomly separated from the group and kept in isolation. Feed and water were provided *ad libitum*. Excreta were collected every 6 h and stored at –20°C. At the end of the collection period, the feed was collected and weighed.

Feed and excreta samples were subsequently analyzed for dry matter, organic matter, crude protein, calcium, and phosphorus according to the AOAC Standard Methods (AOAC, 1980). Moreover, the gross energy was determined by adiabatic bomb calorimetry according to instructions provided by the manufacturer (Parr Instrument Company, Moline, USA).

Intestinal morphology

On days 22 and 43, histomorphological samples were taken from the duodenum, jejunum, and

ileum. The samples were fixed in 10% formalin. Then, the fixed tissues were washed, dehydrated, trimmed, and embedded in paraffin. Sections of 5 µm were sliced, mounted on slides, and then stained with hematoxylin and eosin for histomorphological analysis. For each section, after taking a picture (4 × 10) with an Olympus CX31-32C02 optical microscope (Olympus Optical Co., Ltd., Tokyo, Japan), Image-Pro-Plus 6.0 was used to measure 8 of the typical villi from crypt mouth to villus tip and 8 of the associated crypts from crypt mouth to base. The ratio of villus height to crypt depth was calculated.

Sample preparation for HPLC-MS/MS

Eight samples of liver and breast muscle were collected from groups I, III, and V. The samples were homogenized with IKA ULTRA-TURRAX T 10 disperser (Sigma-Aldrich Corporation, St. Louis, USA). 10 g of the tissue sample was weighed and mixed with 1 ml of 20% aqueous citric acid and 2 g diatomaceous earth. The mixture was sonicated with 20 ml of dichloromethane for 40 min. After centrifugation at 11 000 *g* for 10 min, 10 ml of the supernatant was evaporated in a vacuum (Tavcar-Kalcher et al., 2007). The dry medium residue was dissolved with 3 ml of methanol and mixed with 9 ml of PBS solution. A total of 4 ml of the solution was applied to the AflaStar™ R Immunoaffinity columns (Romer Labs Diagnostic GmbH, Tulln, Austria), eluted with 3 ml of methanol, and dried by nitrogen. Then, 0.5 ml of methanol and 0.1% formic acid (30 : 70, v/v) was added and filtered through a 0.22-µm filter into a 2-ml autosampler vial.

Quantification of AFB_1 and AFM_1 residues in tissues with HPLC-MS/MS

An Agilent 1290 infinity series HPLC system with an SB-C18 column (2.1 × 50 mm) (Agilent Technologies Inc., Santa Clara, USA) was used. The column oven was set at 30°C. The gradient elution used two solutions: solution A was methanol and solution B was 0.1% formic acid in water. The gradient elution program was as follows: solution A was changed from 30% at 0 min to 90% at 5 min and from 90% at 5 min to 30% at 9 min. A flow rate of 0.3 ml/min was used.

Mass spectral analysis was performed using an Agilent 6460 Triple Quad LC/MS (Agilent Technologies Inc., Santa Clara, USA) fitted to an electrospray probe operating in the positive mode (ESI^+). The potential set on the capillary was 4 kV. The nitrogen-gas flow rates for nebulization and desolvation were 10 l/min and 35 psi, respectively. The pressure of the collision gas of nitrogen was 0.2 MPa. The signal acquisition was performed in Multiple Reaction Monitoring Mode (MRM). Precursor ions for AFB_1 and AFM_1 were m/z = 313 and 329, respectively. The product ions for the target compounds were m/z = 269, 241 for AFB_1 and 273, 259 for AFM_1. The quantitation ion transitions were m/z = 313, 241 for AFB_1 and 329, 273 for AFM_1.

Standard solutions of AFB_1 and AFM_1 were purchased from Sigma-Aldrich Corporation (St. Louis, USA). Because single point calibration was used, three concentrations of the standard solution of AFB_1 and AFM_1 in the liver were prepared at 0.04, 0.08, and 0.16 µg/l by diluting with methanol and 0.1% formic acid (30 : 70, v/v). Moreover, blank broiler tissues used for method validation were first analyzed, and no aflatoxin residue was detected. Then, 10-g portions of tissue homogenate were fortified to produce samples (n = 5) containing 0.01, 0.02, and 0.05 µg/kg of AFB_1 and AFM_1. The limit of detection (LOD) and limit of quantification (LOQ) were considered to be the concentrations in tissue samples that produced signal-to-noise (S/N) ratios of 3 and 10, respectively.

Statistical analysis

Data were analyzed by One-Way ANOVA using SPSS software, Version 11.0. When the ANOVA showed significance, Duncan's significant-difference test was applied. Data of AFB_1 and AFM_1 residues were analyzed using Independent Samples t-Tests. All statements of differences were based on a significance of $P < 0.05$.

RESULTS AND DISCUSSION

Dietary mycotoxin concentrations

The corn used in this study was mainly contaminated with AFB_1 and AFB_2. The AFB_1 contents in diets were 16.3–82.4 µg/kg in the starter period and

Table 2. Concentrations of mycotoxins in diet and corn (μg/kg)

Diet	I	II	III	IV	V	Control corn	Contaminated corn
Day 1–21							
AFB_1	nd	16.3	36.9	45.6	82.4	nd	149.6
AFB_2	nd	3.2	6.4	7.9	14.2	nd	24.2
Day 22–42							
AFB_1	nd	34.3	69.3	95.2	134.0	nd	229.0
AFB_2	nd	6.2	12.1	17.0	23.6	nd	37.8

nd = not detectable

34.3–134 μg/kg in the grower period (Table 2). The AFB_2 concentrations in diets were 3.2–14.2 and 6.2–23.6 μg/kg in the starter and grower periods, respectively. The contents of other mycotoxins, including AFG_1, AFG_2, DON, ZEA, OTA, T-2 toxin, and FB_1 were below the limit of detection.

Body weight and feed consumption

Compared with control, the body weight gain and feed consumption of broilers, which were fed diets contaminated with more than 36.9 and 6.4 μg/kg of AFB_1 and AFB_2, significantly decreased ($P < 0.05$) from days 1 to 21. The feed : gain ratio significantly increased ($P < 0.05$) when the broilers were fed diets contaminated with 82.4 and 14.2 μg/kg of AFB_1 and AFB_2 (Table 3). During days 22 to 42, only broilers that consumed diets contaminated with 134 and 23.6 μg/kg of AFB_1 and AFB_2 showed a significant reduction in feed intake. We also found that feed consumption significantly decreased over the full period for groups IV and V ($P < 0.05$), and body weight gain significantly decreased over the full period for group V ($P < 0.05$).

When the broilers were administered feed contaminated with AFB_1 and AFB_2, our results prove that some economically significant effects occurred

Table 3. Effect of corn naturally contaminated with AFB_1 and AFB_2 on performance of broilers

Diet	I	II	III	IV	V	*P*
Body weight (BW)						
Day 21	828.7 ± 7.5^{a}	831.1 ± 22.1^{a}	807.8 ± 12.0^{b}	788.7 ± 15.4^{c}	749.4 ± 17.4^{d}	< 0.001
Day 42	2434.8 ± 120.0^{ab}	2417.9 ± 113.8^{ab}	2469.7 ± 102.7^{a}	2349.6 ± 75.0^{bc}	2301.3 ± 69.3^{c}	0.011
Body weight gain (BWG)						
Day 1–21	788.2 ± 7.5^{a}	790.6 ± 22.1^{a}	767.3 ± 12.0^{b}	748.2 ± 15.4^{c}	708.9 ± 17.4^{d}	< 0.001
Day 22–42	1606.0 ± 115.8	1586.8 ± 110.8	1661.9 ± 105.5	1561.0 ± 73.5	1551.9 ± 58.8	0.177
Day 1–42	2394.2 ± 120.2^{ab}	2377.4 ± 113.8^{ab}	2429.2 ± 102.7^{a}	2309.1 ± 75.0^{bc}	2260.8 ± 69.3^{c}	0.011
Feed consumption (FC)						
Day 1–21	1134.7 ± 13.8^{a}	1134.4 ± 24.7^{a}	1109.1 ± 11.7^{b}	1091.0 ± 14.0^{b}	1050.7 ± 31.8^{c}	< 0.001
Day 22–42	3414.2 ± 205.9^{ab}	3345.0 ± 223.7^{abc}	3456.0 ± 98.3^{a}	3250.4 ± 176.8^{bc}	3221.1 ± 129.5^{c}	0.044
Day 1–42	4377.7 ± 201.0^{a}	4312.1 ± 210.1^{ab}	4416.7 ± 108.1^{a}	4179.0 ± 151.5^{bc}	4131.6 ± 148.6^{c}	0.006
FC/BWG						
Day 1–21	1.44 ± 0.02^{bc}	1.44 ± 0.02^{c}	1.45 ± 0.02^{bc}	1.46 ± 0.02^{b}	1.48 ± 0.01^{a}	< 0.001
Day 22–42	2.13 ± 0.08	2.11 ± 0.07	2.09 ± 0.09	2.08 ± 0.09	2.07 ± 0.06	0.647
Day 1–42	1.83 ± 0.04	1.82 ± 0.03	1.82 ± 0.05	1.81 ± 0.05	1.83 ± 0.03	0.891

$^{a–d}$different lowercases in the same row mean significant difference between treatments ($P < 0.05$)
data are mean (g) ± SD ($n = 8$)

in the broilers, including reduced body weight gain, feed consumption, and feed conversion rate. Aravind et al. (2003) reported that grains naturally contaminated with mycotoxins (AF 168 μg/kg, OAT 8.4 μg/kg, ZEN 54 μg/kg, T-2 toxin 32 μg/kg) significantly resulted in poor performance. Although Magnoli et al. (2011) found that diets containing 50 μg/kg AFB_1 had no effect on the performance of broilers from 28 to 46 days of age, our results prove that broilers were more sensitive to aflatoxins during the starter period, while broilers administered AFB_1- and AFB_2-contaminated diets showed a poor performance during the grower period.

Nutrient digestibility

Our results prove that corn naturally contaminated with AFB_1 and AFB_2 had no effect on the apparent digestibility of calcium and phosphorus ($P > 0.05$). Our results are in agreement with the results of Kermanshahi et al. (2007) in broilers and Applegate et al. (2009) in laying hens. However, there was a trend that showed a decrease in the apparent digestibility of dry matter, organic matter, crude protein, and gross energy of the broilers feed diets when contaminated with AFB_1 and AFB_2 ($P > 0.05$). We found that dry matter, organic matter, crude protein, and gross energy decreased from 72.5, 75.1, 51.5, and 75.6% to 69.4, 71.8, 42.9, and 72.8%, respectively. Han et al. (2008) have reported that the 20- and 40-μg/kg AFB_1 diets reduced the apparent digestibility of crude protein but had no effect on the apparent digestibility of ether extract, ash, calcium or total phosphorus.

Intestinal morphology

Tables 4 and 5 show the results of intestinal morphology. During the period from day 1 to day 21 of age, corn naturally contaminated with AFB_1 and AFB_2 significantly decreased the villus height in the duodenum ($P < 0.05$). The ratio of villus height to crypt depth in the duodenum decreased with increasing content of contaminated corn in the diets, and the same trend was observed in the jejunum and ileum. During the period from day 22 to day 42 of age, the villus height in the duodenum decreased with increasing content of contaminated corn in the diets, and the trend for crypt depth was just the opposite. Moreover, we found that the ratio of villus height to crypt depth in the duodenum showed a significant decrease ($P < 0.05$) when the broilers consumed a diet contaminated with more than 95.2 and 17.0 μg/kg of AFB_1 and AFB_2, respectively. The same trend was found in the jejunum and ileum results.

Increasing of the villus height would suggest an increased surface area capable of greater absorption of available nutrients. The ratio of villus

Table 4. Effect of corn naturally contaminated with AFB_1 and AFB_2 on intestinal morphology of broilers on day 21

Diet	I	II	III	IV	V	*P*
Duodenum (μm)						
Villus height	1479.5 ± 140.4^{a}	1202.4 ± 54.3^{b}	1263.4 ± 151.7^{b}	1210.9 ± 134.3^{b}	996.7 ± 145.7^{c}	< 0.001
Crypt depth	214.6 ± 23.8	182.4 ± 44.9	219.4 ± 36.7	207.7 ± 16.9	211.9 ± 12.8	0.32
V/C	6.96 ± 0.97^{a}	6.98 ± 2.06^{a}	5.86 ± 1.05^{ab}	5.85 ± 0.65^{ab}	4.73 ± 0.89^{b}	0.037
Jejunum (μm)						
Villus height	1172.3 ± 150.6	1057.3 ± 222.8	961.1 ± 70.4	1033.0 ± 146.6	989.6 ± 203.1	0.273
Crypt depth	213.4 ± 54.7	251.2 ± 43.0	288.0 ± 13.7	257.8 ± 56.9	242.2 ± 40.0	0.091
V/C	5.74 ± 1.37^{a}	4.32 ± 1.23^{b}	3.35 ± 0.36^{b}	4.12 ± 0.79^{b}	4.14 ± 0.90^{b}	0.008
Ileum (μm)						
Villus height	877.8 ± 112.1	895.3 ± 139.1	825.9 ± 99.7	793.0 ± 36.9	749.9 ± 21.5	0.069
Crypt depth	259.6 ± 34.7	222.3 ± 30.2	248.2 ± 48.5	281.3 ± 74.0	296.3 ± 57.6	0.115
V/C	3.43 ± 0.62^{ab}	4.09 ± 0.80^{a}	3.45 ± 0.91^{ab}	2.98 ± 0.72^{b}	2.61 ± 0.45^{b}	0.013

$^{a–c}$different lowercases in the same row mean significant difference between treatments ($P < 0.05$)

V/C = villus height/crypt depth

data are mean ± SD ($n = 8$)

Table 5. Effect of corn naturally contaminated with AFB_1 and AFB_2 on intestinal morphology of broilers on day 42

Diet	I	II	III	IV	V	*P*
Duodenum (μm)						
Villus height	1445.5 ± 175.9^{ab}	1481.1 ± 111.4^{a}	1540.7 ± 123.1^{a}	1359.1 ± 190.7^{ab}	1270.9 ± 89.0^{b}	0.047
Crypt depth	182.4 ± 20.7^{b}	237.2 ± 34.3^{a}	225.1 ± 22.9^{a}	259.9 ± 48.2^{a}	230.6 ± 23.3^{a}	0.007
V/C	7.45 ± 1.62^{a}	6.40 ± 1.30^{ab}	6.86 ± 0.22^{ab}	5.35 ± 1.01^{b}	5.58 ± 0.91^{b}	0.029
Jejunum (μm)						
Villus height	1238.0 ± 204.4	995.7 ± 171.1	1119.3 ± 206.1	1083.8 ± 199.6	1002.2 ± 129.1	0.190
Crypt depth	1 81.4 ± 26.3	185.8 ± 46.5	211.9 ± 51.0	219.0 ± 55.2	216.4 ± 43.2	0.487
V/C	6.87 ± 1.04^{a}	5.53 ± 1.25^{b}	5.41 ± 0.98^{b}	4.64 ± 0.67^{b}	4.77 ± 1.11^{b}	0.007
Ileum (μm)						
Villus height	785.5 ± 104.7	780.3 ± 109.1	800.3 ± 130.6	701.8 ± 236.4	609.6 ± 53.0	0.158
Crypt depth	136.7 ± 20.0	156.7 ± 29.9	181.9 ± 43.9	176.1 ± 56.3	142.1 ± 17.4	0.200
V/C	5.67 ± 0.86^{a}	5.22 ± 1.09^{ab}	4.56 ± 1.11^{bc}	4.02 ± 0.52^{c}	4.32 ± 0.46^{bc}	0.016

$^{a–c}$different lowercases in the same row mean significant difference between treatments ($P < 0.05$)

V/C = villus height/crypt depth

data are mean ± SD ($n = 8$)

height to crypt depth might reflect differences in the digestion and absorption of the small intestines (Caspary, 1992). A previous study has demonstrated that grains naturally contaminated with DON significantly reduced the height, width, and surface of villus in the duodenum and jejunum of broilers (Girish and Smith, 2008). However, just few reports in the literature regarding the effects of AFB_1 and AFB_2 on the intestinal morphology have been published. Our results demonstrate that the villus height and the ratio of villus height to crypt depth significantly decreased when the broilers were fed daily with diets contaminated with AFB_1 and AFB_2. We also found that the mixture of AFB_1 and AFB_2 mainly affected the morphology of the duodenum, indicating that long-term exposure to AFB_1 and AFB_2 would stimulate the proximal gastrointestinal tract, affect the characteristics of the intestine morphology, and alter the absorption of nutrients.

Method performance for HPLC-MS/MS

The techniques currently used to detect and quantify AFB_1 and AFM_1 include ELISA (Reddy et al., 2009; Yang et al., 2009), HPLC (Chen et al., 2005; Meucci et al., 2010), and HPLC-MS/MS (Ediage et al., 2011; Van Pamel et al., 2011). The HPLC-MS/MS-based method was selected in this work because AFB_1 and AFM_1 were detected in trace analyses. The peak profile of the AFB_1 and AFM_1 standard was similar to the profile of AFB_1 and AFM_1 from the liver sample (data not shown).

In our studies, the recoveries of AFB_1 and AFM_1 from fortified samples were 70.8–85.7% and 68.9–83.0% over the concentration range of 0.01 to 0.05 μg/kg. For each calibration point, the values of the relative standard deviation (RSD) ranged from 1.9 to 10.5%. The LOD for AFB_1 and AFM_1 in tissue samples, defined as the concentrations that produced the S/N ratio of 3, was 0.005 μg/kg. The LOQ for AFB_1 and AFM_1, defined as the concentration that produced the S/N ratio of 10, was 0.02 μg/kg in tissue samples. The quantification and detection limits (0.02 and 0.005 μg/kg) of our analytical technique were similar to the results of Magnoli et al. (2011), who detected AFB_1 residues in livers by the HPLC-MS/MS method.

AFB_1 and AFM_1 residues in tissues

The levels of AFB_1 and AFM_1 in the livers from group V were significantly higher than those of the livers from group III (Table 6). The levels of AFB_1 residue in the livers from groups III and V (0.049 and 0.137 μg/kg, respectively) were higher than the levels in breast muscles. In a previous study, high levels of AFB_1 residues in livers (3.51–6.97 μg/kg) and breast muscles (1.63–3.27 μg/kg) were detected when broilers were given feed containing 1.6–6.4 mg/kg AFB_1 (Hussain et al., 2010). Residue

Table 6. Concentrations of AFB_1 and AFM_1 residues in tissues of broilers (μg/kg)

Diet		Control	III	V	*P*
AFB_1	liver	nd	0.049 ± 0.025[a]	0.137 ± 0.037[b]	0.001
	muscle	nd	0.015 ± 0.003	0.016 ± 0.007	0.671
AFM_1	liver	nd	0.033 ± 0.006[a]	0.051 ± 0.009[b]	0.004
	muscle	nd	nd	nd	

[a,b]different lowercases in the same row mean significant difference between treatments ($P < 0.05$)
nd = not detectable
data are mean ± SD ($n = 8$)

levels of AFB_1 and AFM_1 were also detected in the kidneys and thighs of both male broilers and hens that were fed a diet contaminated with 50 μg/kg of AFB_1 (Micco et al., 1988). Moreover, Bintvihok and Kositcharoenkul (2006) found AFB_1 residues at levels of 0.13 and 0.02 μg/kg and the AFM_1 residues at levels of 0.32 and 0.08 μg/kg, respectively in the livers and muscles of broilers receiving feed contaminated with 100 μg/kg of AFB_1. Meanwhile, in hens that were administered a diet containing 3 mg/kg AFB_1, the ratios of AFB_1 in the feed to the residual level in the egg yolk and albumen were 4615 and 3846, respectively (Bintvihok et al., 2002). In our study, the concentrations of AFM_1 residue in the livers from groups III and V were 0.033 and 0.051 μg/kg, respectively. However, the levels of AFM_1 residue in breast muscle were not detectable.

In conclusion, our results indicate that corn naturally contaminated with AFB_1 and AFB_2 decreased growth performance and altered intestine morphology in broilers. The residues of AFB_1 and AFM_1 were detected in the edible tissues of broilers, although the animal feedstuff contained low levels of AFB_1 and AFB_2.

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