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Dietary Supplementation with sodium bentonite and coumarin alleviates the toxicity of aflatoxin B_1 in rabbits

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ABSTRACT

Eighty-four male New Zealand White rabbits with average body weight 778 ± 65 g were blocked into four groups to evaluate the ability of sodium bentonite and coumarin in alleviating the toxicity of aflatoxin B1. The first group was fed on a diet without any treatment (CON), while the remaining three diets were added with aflatoxin B1 at 0.25 ppm diet. Diet fed to the third and fourth group of rabbits were further supplemented with sodium bentonite at 5 g/kg (SOB) and coumarin at 5 g/kg (COU) of the diet, respectively. Feeding aflatoxin-contaminated diet (AFL) caused necrosis of liver tissue and reduced the weight gain, average daily gain, feed conversion ratio, nutrient digestibility coefficients, and nitrogen balance of rabbits. This, in turn, was reflected as a reduction in carcass characteristics. The serum collected from rabbits fed aflatoxin-contaminated diet showed decreased levels of total protein, albumin, globulin, glucose, total cholesterol, and triglycerides, and increased concentrations of urea, creatinine, and liver enzymes. Further, aflatoxin diet increased the cecal pH, and decreased the ammonia nitrogen, total volatile fatty acids, and individual fatty acids proportion of cecal fluid. Supplementing sodium bentonite and coumarin at 5 g/kg diet reduced the negative effects of aflatoxin B1 on growth performance, digestibility of nutrients, biochemical parameters, carcass characteristics, and cecal fermentation profile. Furthermore, the coumarin-supplemented group showed better body weight gains and carcass weights compared to the rabbits fed with diets containing sodium bentonite. In conclusion, both sodium bentonite and coumarin supplementation was beneficial in ameliorating the toxicity of aflatoxin B1. Further, the increased body weight gains and better-feed conversion in coumarin-supplemented rabbits project the coumarin as a better anti-aflatoxigenic supplement.

1. Introduction

Mycotoxins are the cytotoxic and highly corrosive metabolites of fungi and are considered as common contaminants in feed materials (Jedziniak et al., 2019). It has been estimated that one-fourth of the feed ingredients produced globally is contaminated with mycotoxins (Hassan et al., 2016). The contamination of feed and forage resources with mycotoxin is still a significant problem, which needs to be addressed. The negative impacts of mycotoxins in either animals or humans have been studied recently (Barati et al., 2018; Liew and Mohd-Redzwan, 2018). Among different mycotoxins, aflatoxins are considered as most toxic fungal metabolites, which are primarily produced by *Aspergillus flavus*, *A. fumigatus*, and *A. parasiticus*.

Rabbits are studied as the most sensitive species for aflatoxin contamination (Mezes, 2008). An investigation by Hassan et al. (2016) in rabbit farms indicates that aflatoxins cause a wide range of symptoms, including anorexia, diarrhea, depression, reduced weight gains, and high mortality. The aflatoxin exposure is known to cause bile duct proliferation, fatty infiltration of liver, hepatic lesions, and suppressed immune response, thus affecting the biological functions negatively (Meissonnier et al., 2008). Employing cost-effective and practical methods for detoxification is need of the hour. Several studies have been conducted to minimize the harmful effects of aflatoxins by using adsorbents such as aluminosilicates, bentonites, and zeolites; but only limited studies were reported in rabbits.

Bentonite is an alumino-hydro-silicate material, which possesses the

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ability to bind ability with many organic substances. Supplementation of non-nutritive adsorptive materials to the diets is one of the best methods to reduce the absorption of aflatoxins from intestine (Miazzo et al., 2005). The primary mechanism of action of sodium bentonite is to form complexes with the mycotoxins, thereby reducing their absorption at intestine level. The safety perspective, along with the capability of forming complexes with mycotoxins projected the bentonite compounds as anti-mycotoxigenic feed additives in feed industry.

Aflatoxin is known to involve in B1-DNA adducts formation within the hepatocytes leading to the suppressed liver function (Rotimi et al., 2019). A recent study reported the importance of the extent of availability of Cytochrome P450 enzymes in the bioactivation of aflatoxins (Elzaki et al., 2019). Further, the detoxification of aflatoxins is facilitated by conjugation of glutathione and the procedure depends upon the activity of glutathione S-transferase activity (Karacaa et al., 2019). Coumarin has been proved to prevent aflatoxicosis by enhancing the glutathione S-transferase activity, reducing aflatoxin B1-DNA adducts formation, suppressing p450 enzyme activity, and enhancing the liver function (Tulayakul et al., 2007; Devienne et al., 2005; Ko et al., 2006). Apart from these functions, coumarins are also known to possess several beneficial properties such as anti-cancer, antibacterial, anti-thrombotic, anticoagulant, and anti-mutagenic effects (Helal et al., 2014). Previously, coumarin is used in ameliorating the negative effects of aflatoxins in pigs (Tulayakul et al., 2007), rat (Kelly et al., 2000), and fish (Shehata and Mohamed, 2012).

Despite the generally recognized as safe (GRAS) category provided to the bentonites and coumarin by European Commission (EFSA, 2010; Devreese et al., 2012), they were not approved as safe anti-mycotoxigenic additives by Food and Drug Approval (FDA). The phenomenon could be explained by the concerns over the effectiveness of bentonites and coumarin on preventing the accumulation of aflatoxin residues in muscle and other edible organs. Hence, determining the meat residues is essential to project any additive as an efficient anti-mycotoxigenic feed supplement. The present study was conducted with an objective to determine the effects of aflatoxin B1 on the growth performance, nutrient digestibility coefficients, nitrogen balance, biochemical parameters, carcass characteristics, and cecal fermentation patterns, meat residues, along with protecting effects of sodium bentonite and coumarin against the dietary aflatoxins.

2. Materials and methods

2.1. Aflatoxin quantification

Aspergillus flavus MD 341 was obtained from the Central Laboratory of Residues in Agriculture Products, Agriculture Pesticides Residues Centre, Dokki, Egypt, for production of aflatoxin B1 on liquid media containing 2% yeast extract and 20% sucrose. The aflatoxin concentration was determined by using the method of AOAC (1990). The media was sprayed with corn to obtain 450 ppm aflatoxin B1. The media was found to contain aflatoxin B1 alone.

The basal diet did not contain any detectable aflatoxin levels (below 1 μg/kg diet; ppb). Corn was obtained from a private feed mill (already contaminated with mold) and was stored at 20% moisture for two weeks to promote mold growth. The presence of aflatoxin in the corn was confirmed by thin-layer chromatography (TLC). Aflatoxin free corn was replaced with naturally contaminated corn in the formulation of the contaminated-diet treatments. The samples were randomly selected from four different portions of the whole sample. The analysis of the contaminated diet showed that it contained 435–470 ppb aflatoxin (detection limit: 1 ppb). The aflatoxin in the contaminated diet was composed of 86.11% AFB_1, 5.33% AFB_2, 6.92% AFG_1, and 1.64% AFG_2.

During the experimental period, the control and the contaminated diets were analyzed for aflatoxin and other mycotoxins. The levels of aflatoxin in the control diet were below the detection limits. Aflatoxin levels in the contaminated diet ranged from 270 to 230 ppb. The presence of other mycotoxins was not detected.

Coumarin was prepared according to method of Furniss et al. (1978), as follows: Immerse a 3 L nicked flask containing 1-L conc. H_2SO_4 in ice bath. Add a solution of 100 g (0.91 mol) of resorcinol in 134 g (1.03 mol) of ethyl aceto-acetate dropwise with continuous stirring for 2 h. Keep the reaction mixture at room temperature for about 18 h and pour it with vigorous stirring into a mixture of crushed ice and water. Collect the final crude yield after recrystallization in 95% ethanol.

2.2. Rabbits and diets

Eight four male growing New Zealand White Rabbits aged eight weeks old with an average body weight of 778.23 ± 65.0 g were blocked by weight into four equal groups (21 animals each, seven replicates of 3 rabbits each). Experimental rabbits were housed individually in galvanized metal wire cages equipped with feeding and water troughs. The four groups were fed as; control diet consisting of the basal diet with no aflatoxin or additives (CON), positive control diet consisting of a diet naturally contaminated with aflatoxin at 0.25 ppm diet (AFL), and AF diet supplemented with sodium bentonite or coumarin at 5 g/kg diet (SOB or COU). The dosage of aflatoxin and the additives were selected by analyzing the existing reports on toxic and lethal doses of aflatoxin poisoning in rabbits (Mezes, 2008; Helal et al., 2014; Amer et al., 2018).

Experimental diets were offered daily at 8.30 a.m. Feed refusals were collected daily. The recordings of body weights were collected weekly. Clean and fresh drinking water was provided *adlibitum*. The rabbits were periodically monitored for ill effects, if any, and treated immediately.

2.3. Digestibility trials

At the end of the feeding experiment, four digestibility trials were carried out for seven days, including three days for adaptation and four days for quantitative collection of feces and urine. Seven rabbits from each group were individually confined in stainless-steel metabolic cages equipped for separate collection of feces and urine. Daily amounts of feed intake, feces and urine out-put were determined and daily recorded during the collection period. Individual composite samples of dry feces and acidified urine were kept in glass bottles and stored at 4 °C for chemical analysis.

2.4. Collection of blood contents

At the end of the experiment, seven rabbits from each group were used for collection of blood samples by venipuncture. Blood samples were collected into sterile centrifuge tubes, which were later centrifuged at 5000 × *g* for 10 min. Serum was separated and kept at −20 °C until assayed for determination of serum urea, creatinine, total proteins, glucose, and the activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT).

2.5. Slaughter technique

After completion of the feeding experiment, seven representative rabbits randomly chosen from each group and allowed for overnight fasting. Later, the fasted rabbits were weighed and hand slaughtered. After complete bleeding, the drained blood was collected and weighed. Slaughtered animals were de-skinned, dressed out and the weight of hot carcass without head was recorded. Edible offals (liver, heart, spleen and kidneys), non-edible offals (head, lungs& trachea, clean empty gastro-intestinal tract (GIT), and testicles), and trimmings (fur, four legs, and blood) were separately weighed and recorded. The whole

Table 1
Ingredients and chemical composition of the experimental diets.

Ingredients	% (dry matter basis)
Berseem hay	30.0
Yellow corn grains	15.5
Barley grains	21.0
Wheat bran	11.0
Soybean meal	18.0
molasses	3.0
Dicalcium phosphate	0.7
Sodium chloride	0.3
Vitamin and mineral premix	0.3
DL-Methionine	0.2
Chemical analysis (% on DMB)	
Dry matter	90.04
Organic matter	94.52
Crude protein	16.13
Crude fiber	11.79
Ether extract	2.89
Nitrogen free extract	63.71
Calcium	0.82
Phosphorus	0.46

carcass of each rabbit was de-boned and the resultant amounts of meat and bone were separately weighed and recorded. De-boned meat of each rabbit was minced, oven-dried for 72 h, and weighed to determine body water content. The dry meat was finely ground to determine protein, fat and ash proportions.

2.6. Chemical analysis

Chemical composition of feeds and feces were determined for dry matter (DM), crude protein (CP), crude fiber (CF), ether extract (EE), and ash according to the standard methods of AOAC (2005). Nitrogen free extract (NFE) was calculated by difference. Urinary nitrogen (UN) was determined by the micro-kjeldahl method. The chemical composition of the de-boned meat was determined according to the methods of AOAC (2005). Chemical composition of the basal diet is provided in Table 1.

2.7. Samples and **analysis** of cecal digesta

At the end of the trial, seven rabbits from each group were slaughtered by severing the jugular vein and cecal contents were squeezed out into beakers. Immediately, the cecal contents were strained through two layers of sterile gauze and the resultant strained liquors were used for measuring pH values by electronic digital pH meter. Thereafter, the contents were centrifuged at 7000 × *g* for 12 min. The supernatant fluid was divided into two parts. One part was acidified with 0.2 M hydrochloric acid solution (one ml-ml^{-1} sample) to be used for determination of ammonia nitrogen (NH_3–N) concentration while the other was treated with a solution of 5% orthophosphoric acid (v/v) plus 1% mercuric chloride (w/v) (0.1 ml-ml^{-1} sample) for determination of total volatile fatty acids (TVFA) concentrations and individual VFAs proportions. Cecal NH_3–N concentrations were measured by spectrophotometry according to Chaney and Marbach (1962). Total VFAs concentrations were measured by steam distillation as per the procedure of Eadie et al. (1967). The molar proportions of VFAs were analyzed using high performance liquid chromatography (HPLC; Model Water 600; UV detector, Millipore Crop.) according to the method of Mathew et al. (1997).

2.8. Analysis of AFB1 residues in liver

Seven liver samples from each treatment were preserved at −20 °C for analyzing the residue of AFB1. Analysis of AFB1 residues was performed according to Tavcar-Kalcher et al. (2007). The ground sample was mixed thoroughly with an aqueous solution of citric acid and diatomaceous earth. The mixture was extracted with dichloromethane. The filtered extract was dried, filtered again, and an aliquot was evaporated to dryness. Later, the residue was dissolved in methanol and mixed with buffer and applied into an immunoaffinity column. Finally, the Aflatoxin B1 was evaluated from the column and the concentration of AFB1 in the final solution was determined by an HPLC method with fluorescence detection after derivatization with bromine in the Kobra cell (R-Biopharm Rhone Ltd., Glasgow, UK).

2.9. Histopathological examination

Liver samples from rabbits fed the diets were obtained to evaluate lesions and other abnormalities. Samples were obtained from five rabbits from each group and were fixed in 10% neutral buffered formalin solution. The formalin-fixed samples were dehydrated in graded alcohol and embedded in paraffin. Sections of 3–5 μm were obtained and stained with hematoxylin-eosin. Two sections of liver tissue from each rabbit were examined by light microscopy for lesions, if any, as per the protocols of Pandey and Chauhan (2007).

2.10. Statistical analysis

The data of body weights, feed intakes, average daily gains, and feed conversion ratio (FCR) were subjected to General Linear Model (GLM) repeated measures analysis considering the recording day as repeated measure with fixed effects of dietary treatments (D), recording day as week period (W), and the interactions among them (D × W) according to the model;

$$Y_{ijk} = \mu + D_i + W_j + (D \times W)_{ij} + A_k + e_{ijk}$$

Where Y_{ijk} is the dependent variable, μ is the overall mean, D_i the effect of dietary treatment (i = 4), Wj the effect of sampling week (j = 10), $(D \times W)_{ij}$ the interaction between dietary treatment and sampling week, A_k the animal's random effect, and e_{ijk} the residual error. The interaction effects of week of experiment and diet fed (D × W) was observed by adjusting the confidence interval as per Bonferroni correction.

The data of nutrient digestibility, blood components, carcass characteristics, and cecal fermentation patterns were subjected to General Linear Model (GLM) multivariate analysis. Post-hoc analysis was performed, wherever necessary, by using Tukey's honestly significant difference (Tukey-HSD) Test. The significances were tested for both diet (D) and source of supplement (S; sodium bentonite vs. coumarin). The *P*-value of less than 0.05 is considered as significant, while the value between 0.05 and 0.10 is considered as a non-significant trend towards significance. The entire statistical analysis was performed using SPSS (Version 23.0).

3. Results

3.1. Growth performance

The effect of supplementation of sodium bentonite and coumarin on the growth performance of rabbits is presented in Table 2. Supplementing the sodium bentonite and coumarin to aflatoxin containing diet improved ($P < 0.001$) the body weight (BW), average daily gain (ADG), feed intake (FI) and feed conversion ratio (FCR) compared to aflatoxin diet (A). Between the two supplemental sources, COU diet caused improved ($P < 0.001$) FBW, BWG, and ADG compared to SOB diets. A significant ($P < 0.001$) week × diet interaction was found on all the growth parameters measured. The weekly body weights and FCR of the rabbits fed experimental diets were presented in Figs. 1 and 2, respectively.

Table 2
Growth performance of the rabbits fed experimental diets.

Parameter	Diet				SEM	*P* Value			
	CON	AFL	SOB	COU		W	D	W × D	S
Initial BW (gm)	780.5	778.7	774.6	779.2	11.5	–	–	–	–
Final BW (gm)	2523.6^{b}	1619.2^{a}	2541.8^{c}	2794.2^{d}	19.6	–	< 0.001	–	< 0.001
BW gain (gm)	1743.1^{b}	840.5^{a}	1767.2^{b}	2015.0^{c}	16.7	0.05	< 0.001	< 0.001	< 0.001
ADG	24.9^{b}	12.0^{a}	25.2^{b}	28.8^{c}	0.24	0.05	< 0.001	< 0.001	< 0.001
FI	106.4^{b}	88.1^{a}	108.4^{b}	112.9^{b}	1.34	< 0.001	< 0.001	< 0.001	0.174
FCR	4.27^{a}	7.34^{b}	4.30^{a}	3.92^{a}	0.15	< 0.001	< 0.001	< 0.001	0.175

CON – Control, AFL – Aflatoxin, SOB – Sodium bentonite, COU – Coumarin, BW – Body weight, ADG – Average daily gain, FI – Feed intake, FCR – Feed conversion ratio, W – Week, D - Diet, W × D - Week × Day interaction, S - Source (SOB *vs.* COU).
^{abc}Rows bearing different superscripts differ significantly (n = 21).

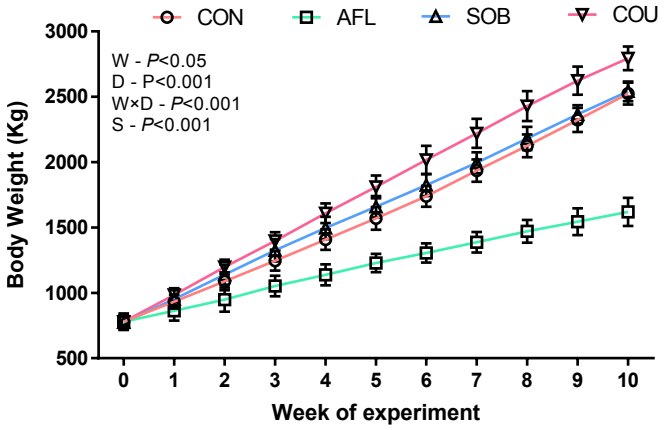

Fig. 1. Weekly body weights of the rabbits fed experimental diets. W - Week of experiment, D - Diet fed, W × D - Week × Diet interaction (n = 21).

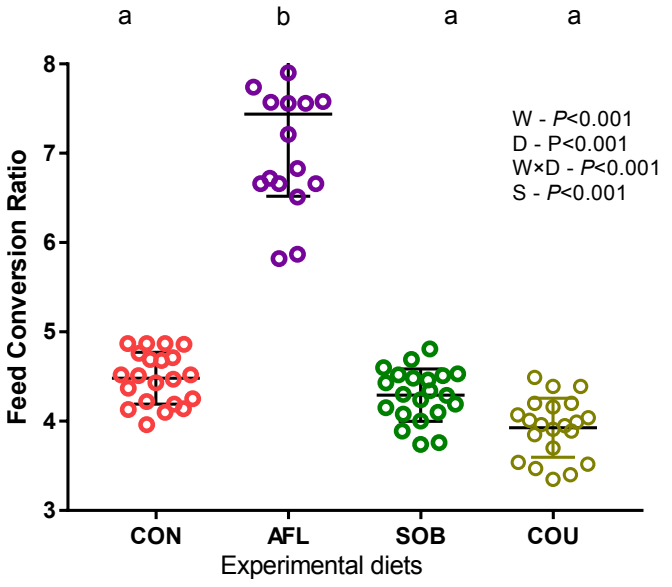

Fig. 2. Feed conversion ratio of the rabbits fed experimental diets. ^{abc}Columns bearing different superscripts differ significantly. W - Week of experiment, D - Diet fed, W × D - Week × Diet interaction (n = 21).

3.2. Nutrient digestibility and nitrogen balance

The nutrient digestibility coefficients and nitrogen (N) balance of the rabbits fed experimental diets are summarized in Table 3. Supplementing the mycotoxin binders in the rabbits' diets improved the digestibility coefficients of DM, OM, CP, EE, and CF. Further, the SOB and COU diets improved (P < 0.001) the N balance by decreasing (P < 0.05) the N excreted through urine and feces. No differences were observed between the supplemental sources for nutrient digestibility and N balance, except for fecal N, which tended (P = 0.093) to be higher in COU fed rabbits.

Table 3
Nutrient digestibility and nitrogen balance of the rabbits fed experimental diets.

Parameter	Diet				SEM	*P* value	
	CON	AFL	SOB	COU		D	S
Digestibility coefficients (%)							
Dry matter	62.4^{b}	57.5^{a}	64.0^{b}	64.5^{b}	0.77	< 0.001	0.843
Organic matter	65.7^{b}	60.6^{a}	67.1^{b}	66.6^{b}	0.77	< 0.001	0.902
Crude protein	64.9^{b}	56.3^{a}	66.6^{b}	65.2^{b}	0.63	< 0.001	0.199
Crude fiber	44.0^{b}	35.7^{a}	47.6^{c}	46.8^{c}	0.74	< 0.001	0.939
Ether extract	74.9^{b}	67.4^{a}	76.7^{c}	76.9^{c}	0.72	< 0.001	0.969
Nitrogen balance							
Nitrogen intake	3.43^{b}	2.89^{a}	3.49^{c}	3.51^{c}	0.03	< 0.001	0.843
Urinary N	0.93^{a}	1.05^{b}	0.97^{a}	0.94^{a}	0.02	0.020	0.345
Fecal N	1.19^{a}	1.26^{b}	1.14^{a}	1.21^{ab}	0.03	0.039	0.093
Excreted N	2.12^{a}	2.31^{b}	2.11^{a}	2.16^{a}	0.03	< 0.001	0.913
N balance	1.28^{b}	0.58^{a}	1.40^{bc}	1.38^{c}	0.05	< 0.001	0.980

CON – Control, AFL – Aflatoxin, SOB – Sodium bentonite, COU – Coumarin, D - Diet, S - Source (SOB vs COU).
^{abc}Rows bearing different superscripts differ significantly (n = 7).

Table 4
Serum biochemical parameters of the rabbits fed experimental diets.

Parameter	Diet				SEM	*P* Value	
	CON	AFL	SOB	COU		D	S
Total protein (g/dL)	7.53^{b}	5.61^{a}	7.75^{bc}	7.97^{c}	0.06	< 0.001	0.071
Albumin (g/dL)	4.70^{b}	3.26^{a}	4.88^{b}	5.01^{bc}	0.05	< 0.001	0.352
Globulin (g/dL)	2.83^{b}	2.35^{a}	2.87^{b}	2.97^{b}	0.05	< 0.001	0.678
Albumin/Globulin ratio	1.67^{b}	1.39^{a}	1.70^{b}	1.70^{b}	0.04	< 0.001	1.000
Glucose (mg/dL)	79.10^{b}	65.93^{a}	81.02^{b}	80.45^{b}	2.43	< 0.001	0.998
Total cholesterol (mg/dL)	95.11^{b}	78.93^{a}	92.83^{b}	92.10^{b}	2.32	< 0.001	0.996
Triglycerides (mg/dL)	85.13^{b}	74.16^{a}	83.59^{b}	83.98^{b}	2.17	0.006	0.994
Urea (mg/dL)	30.21^{ab}	35.72^{b}	29.65^{a}	29.87^{a}	1.17	0.021	0.993
Creatinine (mg/dL)	0.74^{a}	1.01^{b}	0.75^{a}	0.86^{a}	0.04	< 0.001	0.995
AST (IU/L)	23.95^{a}	32.81^{b}	23.88^{a}	23.04^{a}	0.58	< 0.001	0.761
ALT (IU/L)	14.74^{a}	19.36^{b}	14.04^{a}	14.11^{a}	0.68	< 0.001	0.998

CON – Control, AFL – Aflatoxin, SOB – Sodium bentonite, COU – Coumarin, D - Diet, S - Source (SOB vs COU).
^{abc}Rows bearing different superscripts differ significantly (n = 7).

Table 5
Carcass characteristics of the rabbits fed experimental diets.

Parameter	Diet				SEM	*P* Value	
	CON	AFL	SOB	COU		D	S
Carcass composition (gm)							
Live body weight	2444[b]	1743[a]	2464[b]	2696[c]	20.70	< 0.001	< 0.001
Slaughter weight	2315[b]	1624[a]	2324[b]	2554[c]	19.34	< 0.001	< 0.001
Carcass weight	1319[b]	832[a]	1389[c]	1528[d]	12.81	< 0.001	< 0.001
Dressing percent	53.96[b]	47.77[a]	56.39[c]	56.69[c]	0.43	< 0.001	0.969
Organ weights (gm)							
Liver	2.63[a]	4.07[b]	2.67[a]	2.70[a]	0.05	< 0.001	0.959
Kidney	0.73[a]	0.85[b]	0.74[a]	0.75[a]	0.02	< 0.001	0.998
Heart	0.32	0.34	0.32	0.32	0.01	0.117	0.968
Carcass composition (%)							
Moisture	72.61	73.80	72.68	72.62	0.01	0.104	0.979
Protein	21.98	20.83	21.92	21.90	0.09	0.130	0.996
Ether extract	4.67	4.87	4.31	4.39	0.09	0.125	0.847
Total ash	1.34	1.43	1.36	1.39	0.03	0.207	0.884

CON – Control, AFL – Aflatoxin, SOB – Sodium bentonite, COU – Coumarin, D - Diet, S - Source (SOB vs COU).
[abc]Rows bearing different superscripts differ significantly (n = 7).

3.3. Biochemical parameters

The biochemical parameters of the rabbits fed experimental diets are presented in Table 4. Rabbits fed SOB and COU diets showed higher serum profile of total protein, albumin, globulin, albumin-globulin ratio, glucose, total cholesterol, and triglycerides content compared to AF diet. Further, the serum concentrations of urea, creatinine, AST, and ALT were higher in the rabbits fed AFL diet compared to those fed on CON, SOB, and COU diets. The biochemical constituents were not altered with the source of supplement, except for total protein, which tended (P = 0.071) to increase on supplementation of coumarin in the basal diet.

3.4. Carcass characteristics

The effect of supplementing mycotoxin binders on the carcass characteristics of rabbits is presented in Table 5. Supplementing sodium bentonite and coumarin to the aflatoxin-contaminated diet improved the measured carcass characteristics viz. live body weight, slaughter weight, carcass weight, dressing percent, and the weights of liver and kidney. However, the diets did not affect (P > 0.05) the moisture, protein, ether extract, and total ash content of the carcass. The rabbits fed on coumarin-supplemented diet showed higher LBW, SW, and CW compared to those fed with diet containing sodium bentonite. The content of aflatoxin residues in the meat of rabbits fed experimental diets is shown in Fig. 3. As expected, the aflatoxin residue content was higher ($P < 0.001$) in aflatoxin-contaminated diet compared to the diets supplemented with either sodium bentonite or coumarin. Histopathological studies of the liver tissues of rabbits fed CON, AFL, SOB, and COU diets are presented in Figs. 4–7, respectively. Aflatoxin contamination severely affected the hepatic tissue by causing diffused fatty degeneration and focal necrosis (Fig. 5). No histopathological changes were observed in the rabbits fed diets containing sodium bentonite and coumarin.

3.5. Cecal fermentation patterns

The cecal fermentation patterns of the rabbits fed experimental diets are presented in Table 6. Dietary supplementation of sodium bentonite or coumarin decreased ($P < 0.001$) the cecal pH and increased the NH_3–N, TVFA, and the individual VFA fractions viz. acetate, propionate, and butyrate. No significant differences were noticed between the two supplemental sources.

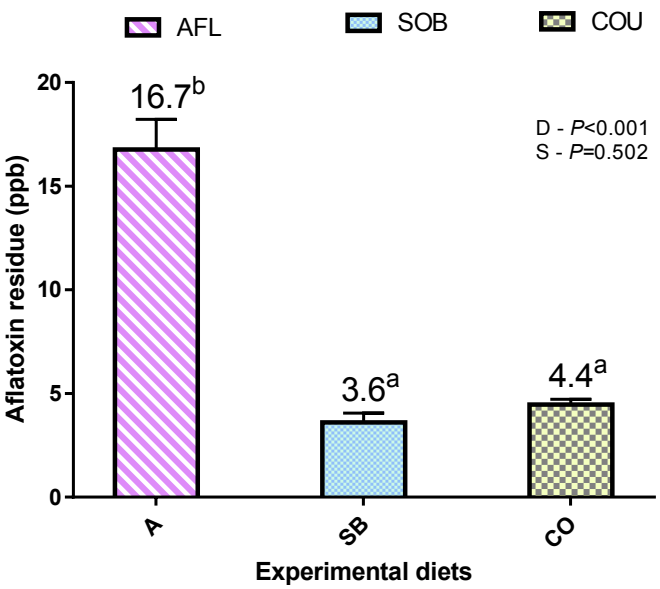

Fig. 3. Aflatoxin residues in the meat of rabbits fed experimental diets. [ab]Columns bearing different superscripts differ significantly (n = 7).

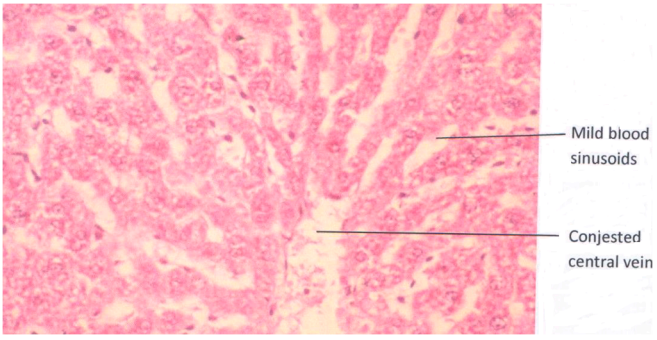

Fig. 4. Photomicrograph of control liver group shows normal hepatic architecture formed of tubules of hepatocytes separated by thin sinusoidal blood vessel.

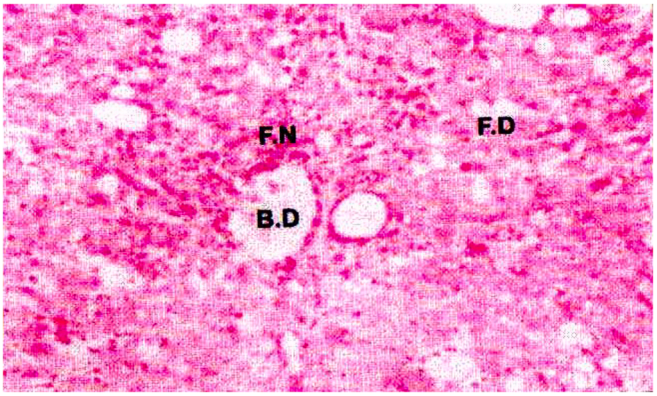

Fig. 5. Photomicrograph of liver of rabbit treated with Aflatoxin B1 showing hyperplasia of bile ducts with newly formed bile ductules (B.D), diffused fatty degeneration (F.D) and focal necrosis of hepatocytes (F.N).

4. Discussion

This study evaluated the efficacy of sodium bentonite and coumarin to ameliorate the toxic effects of AFB1 on growth patterns, nutrient digestibility coefficients, nitrogen balance, biochemical parameters, carcass characteristics, and cecal fermentation patterns of rabbits. The

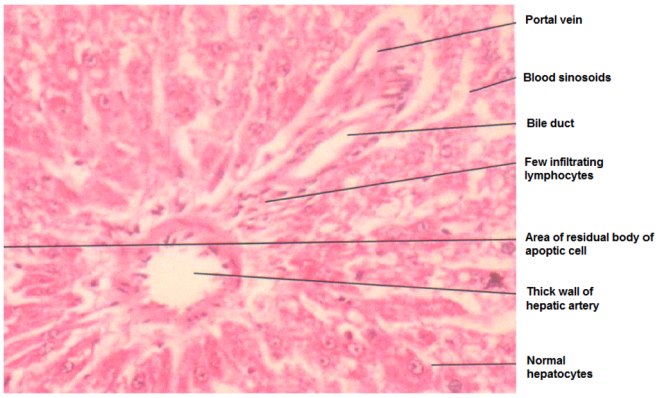

Fig. 6. Photomicrograph of liver of rabbit treated with Aflatoxin B_1 and sodium bentonite shows normal hepatic architecture formed of tubules of hepatocytes separated by thin sinusoidal blood vessel.

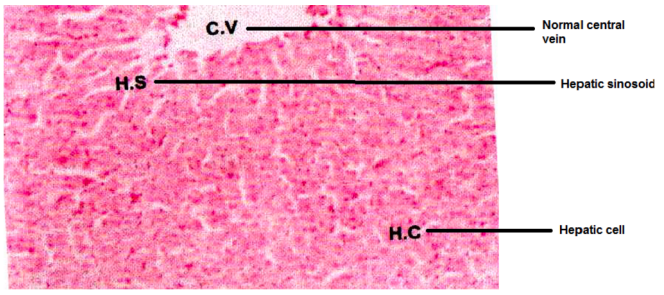

Fig. 7. Photomicrograph of liver of rabbit treated with Aflatoxin B_1 and coumarin shows normal central vein, hepatic sinusoid and hepatic cell.

Table 6
Cecal fermentation patterns of the rabbits fed experimental diets.

Parameter	Treatment				SEM	*P* Value	
	CON	AFL	SOB	COU		D	S
Cecal pH	5.70^{b}	5.90^{c}	5.51^{a}	5.50^{a}	0.02	< 0.001	0.948
NH_3–N (mmol/Lt)	19.94^{c}	13.96^{a}	16.95^{b}	16.92^{b}	0.05	< 0.001	0.989
TVFA (mmol/Lt)	69.35^{b}	45.26^{a}	73.99^{c}	73.63^{c}	0.30	< 0.001	0.359
Individual VFA (mmol/Lt)							
Acetate	55.00^{b}	36.24^{a}	53.31^{c}	53.10^{c}	0.32	< 0.001	0.972
Propionate	4.71^{b}	3.05^{a}	7.74^{c}	7.56^{c}	0.11	< 0.001	0.680
Butyrate	8.50^{b}	4.73^{a}	12.00^{c}	11.65^{c}	0.12	< 0.001	0.322
Individual VFA (%)							
Acetate	79.31^{b}	80.05^{b}	71.68^{a}	72.12^{a}	0.30	< 0.001	0.742
Propionate	6.79^{a}	6.72^{a}	10.42^{b}	10.27^{b}	0.17	< 0.001	0.945
Butyrate	12.26^{b}	10.46^{a}	16.14^{c}	15.82^{c}	0.19	< 0.001	0.693

CON – Control, AFL – Aflatoxin, SOB – Sodium bentonite, COU – Coumarin, D - Diet, S - Source (SOB vs COU).
^{abc}Rows bearing different superscripts differ significantly (n = 7).

toxic dose of aflatoxin and quantity of additives to be added depends upon the species. For instance, in dairy cows, supplementation of sodium bentonite at 20 g/kg diet ameliorated the negative effects of the aflatoxins at 5 μg/kg diet (EFSA, 2010). However, the present study revealed the sodium bentonite at 5 g/kg diet could ameliorate the negative effects of aflatoxin at 0.25 ppm. As per the literature collected, no research was ever conducted to detect the efficacy of coumarin in ameliorating the negative effects of aflatoxin in dairy cows.

4.1. Growth performance

Adding aflatoxin at 0.25 ppm diet decreased the final BW, BW gain, ADG, FI, and FCR of the rabbits. Similar results were reported elsewhere (Helal et al., 2014; Shehata, 2010; Meshreky et al., 2007). The toxic metabolites generated by liver might cause anorexia and decreased feed intake (Yunus et al., 2011). In addition, the aflatoxin-induced depression of feed intake may also be related to the impaired synthesis of nucleic acids and reduced protein synthesis (Helal et al., 2014). Detoxification of aflatoxins requires glutathione enzymes, which are composed of methionine and cysteine (Guilford and Hope, 2014). Therefore, the aflatoxin detoxification procedure may deplete the metabolic availability of methionine, thus impairing the growth of rabbits (Meshreky et al., 2007; Shehata, 2010). Further, mycotoxins are known to reduce the rate of excreta passage through the GIT, thus causing a marked decline in excreta emptying rate and FCR (Danicke et al., 2003a, 2003b).

Supplementation of SOB or COU additives at 5 g/Kg BW successfully ameliorated the negative effects caused by aflatoxin within the diet. The beneficial role of sodium bentonite in adsorbing the aflatoxins is well documented earlier (Gul et al., 2017; Pasha et al., 2007). Sodium bentonite acts as an adsorbing agent by trapping the aflatoxin molecules by ion-exchange mechanism and prevents the absorption of mycotoxins through the intestinal lumen in systemic blood (Amer et al., 2018). Coumarin enhances the activity of hepatic aldo-keto reductase and glutathione S-transferase to conjugate aflatoxin B1, thus ameliorating its toxic effects (Tulayakul et al., 2007).

Further, it was observed that the COU additive provided a better performance compared to the diet supplemented with SOB. The positive role of coumarin on the BWG, ADG, and FCR could be attributed to their therapeutic roles in growth modulation, antioxidant effects, and prevention of disease spread (Rohini and Srikumar, 2014). Besides, Coumarin supplementation is known to improve the absorption of protein and fat (Ko et al., 2006). The role of coumarin compounds in increasing the secretion of bile salts and lipolytic enzymes is well documented (Hahn, 1966). Apart from the aflatoxin ameliorating effect, the beneficial outcomes of coumarins could also be ascribed to their antimicrobial activities (Jacobson et al., 2002).

4.2. Nutrient digestibility and N balance

Feeding AFL diet reduced the nutrient digestibility coefficients and N balance, while supplementation of sodium bentonite and coumarin ameliorated their negative effects. These results are in agreement with that from Abbasi et al. (2018), who reported a decreased nutrient digestibility coefficients on feeding aflatoxin-contaminated diets. Because of the role of GIT as the main route of entry of aflatoxins through diet or bile, nutrient digestibility is the primarily affected parameter on feeding aflatoxin-contaminated diet (Bbosa et al., 2013). Aflatoxins are known to interfere with the utilization of dietary nutrients, thus decreasing the digestibility coefficients and nutritive value (Kumar et al., 2017; Feddern et al., 2013). Besides, aflatoxin-B1 damages the liver, thereby changing the gene expression of liver enzymes and decreasing the activity of digestive enzymes, subsequently affecting the intestinal morphology and function (Qu et al., 2017). Amer et al. (2018) reported a positive role of bentonite in improving the digestion coefficients of DM, EE, ash, and CF in rabbits fed aflatoxin-based diet as negative control. Further, Helal et al. (2014)Helal et al. (2014) revealed increased nutrient digestibility coefficients on feeding the aflatoxin-intoxicated rabbits with coumarin at 2.5 or 5.0 g/Kg body weight. The sodium bentonite at 5 gm/Kg diet might have adhered to mycotoxins by adsorption, thus preventing the aflatoxins' negative effects on nutrient absorption. Further, the coumarin's hepatoprotective role might have counteracted the negative effects of aflatoxins on the liver and, therefore the secretion of digestive enzymes.

4.3. Biochemical parameters

Liver is the primary target organ for aflatoxin B1. Rabbits fed with AFL diet showed a lower total protein, albumin, globulin, and albumin-globulin ratio compared to other diets. In corroboration, Nazarizadeh

and Pourreza (2019) reported a decreased albumin and total protein on feeding mycotoxin-contaminated diet in broilers. Decreased serum protein may be due to the inhibitory effect of aflatoxin B1 on the protein synthesis by degenerating the endoplasmic reticulum (Helal et al., 2014; Rotimi et al., 2017). The decreased globulin concentration in rabbits fed AFL diet reveals a lowered immune system due to the depressed humoral and cellular immunity (Ma et al., 2015). Addition of aflatoxin B1 increased the serum levels of ALT and AST enzymes. Consistently, few authors (Shehata, 2010; Helal et al., 2014; Yaman et al., 2016) reported raised serum levels of ALT and AST in rabbits fed with mycotoxin contaminated diets. Aflatoxin B1 is known to cause hepatic physical change and impaired hepatic function, thus increasing the serum levels of ALT and AST (Barati et al., 2018; He et al., 2013). Further, the increased urea and creatinine levels in the rabbits fed aflatoxin could be due to the effect of mycotoxins on impairing renal function, as indicated by Bbosa et al. (2013). Supplementation of sodium bentonite and coumarin ameliorated the negative effects of aflatoxin on serum biochemical parameters. Similarly, the Hassan et al. (2016) revealed the ameliorating effects of N-acetyl cysteine and probiotic on the increased serum levels of biochemical parameters and liver enzymes in the rabbits exposed to different aflatoxin standards such as B_1, B_2, G_1, and G_2. The beneficial effect of sodium bentonite on biochemical parameters could be attributed to their role in capturing the positively charged ions, thus ameliorating the poisoning effects of aflatoxins (Mirabdolbagi et al., 2007). Further, the hepatoprotective effects of coumarin could have prevented the negative effects of aflatoxins on various biochemical parameters, which are prone to alter with the affected liver function.

4.4. Carcass characteristics

The rabbits allotted to AFL diet showed lower live body weight, carcass weight, slaughter weight, and dressing percentage. The rabbits supplemented with coumarin provided higher live body weight, slaughter weight, and carcass weights compared to those fed with SOB diet. These results are in line with the findings of Helal et al. (2014), who reported increased slaughter weights in rabbits supplemented with coumarin at 2.5 or 5.0 Kg per body weight. Coumarins possess great importance as therapeutic medicine and are known to possess a wide range of biological functions and pharmaceutical actions (Rohini and Srikumar, 2014). Moreover, the positive effect of coumarin on carcass weight is related to the higher final body weight. According to Tulayakul et al. (2007), coumarin enhances the activity of glutathione S-transferase to conjugate aflatoxin B1, thus ameliorating its toxic effects.

Feeding aflatoxin-containing diet increased the weights of liver and kidney. The liver and kidney collected from the rabbits fed AFL diet were pale and swollen. Consistent with these results, Nazarizadeh and Pourreza (2019) revealed an increased liver weight on feeding AFB1 at 4 µg/Kg body weight. Nevertheless, the livers from the rabbits fed diets supplemented with sodium bentonite and coumarin showed no gross lesions.

4.5. Histopathology of liver tissue

Investigation of the histopathological sections of liver tissue revealed the toxic effects of aflatoxin-B1 and the mitigation potentiality of sodium bentonite and coumarin at tissue level. Marked histopathological lesions were noticed in the liver tissue of rabbits fed AFL diet. Examination of the liver tissue of rabbits fed AFL diet revealed hyperplasia of bile ducts, diffused fatty degeneration, and focal necrosis of hepatocytes. In line with our results, Amer et al. (2018) demonstrated that feeding aflatoxin-contaminated diet to rabbits causes hydropic degeneration in hepatocytes with perivascular aggregation of round cells. Further, Yaman et al. (2016)Yaman et al. (2016) found severe histopathological lesions in liver of rats fed aflatoxin at 25 µg/day. Metabolism of Aflatoxin B1 in liver produces pro-reactive free radicals as end products, which cause cell damage and lipid peroxidation (Chandra and Bishnoi, 2015). Aflatoxin B1 type is also potent enough to increase the expression of proteins such as bax and p53, which are pro-apoptotic, thus causing cell death (Brahmi et al., 2012). Besides, the focal necrosis of hepatocytes could be attributed to the exhausted glutathione stores as a result of aflatoxin metabolism (Abdel-Wahhab et al., 2010).

4.6. Cecal fermentation patterns

The mean cecal pH was highest, while the NH_3–N, TVFA, and their proportions were lowest in the rabbits fed aflatoxin-contaminated diet alone. The NH_3–N and TVFA depend upon the total microbial count of cecum, which is determined by the quantity of total roughage content. The lower NH_3–N and TVFA contents in the rabbits fed AFL diet might be because of the reduced total feed intake. The residue concentration was higher in the rabbits fed aflatoxin-containing diet while supplementation of the diet with SOB and COU reduced the residue concentrations by one-fourth levels. Hence, supplementation of mycotoxin binders to aflatoxin-contaminated diets may reduce the risk to consumers' health. The accumulation of aflatoxin residues in the liver of broilers fed with diets containing aflatoxin-B1 toxin was reported earlier (Hussain et al., 2010, 2016). The reduced aflatoxin B1 residues within the livers of coumarin-supplemented groups might be due to the higher excretion from the body and reduced aflatoxin B1-DNA adducts within the organs (Helal et al., 2014).

5. Conclusion

In conclusion, the contamination of rabbit diets with aflatoxin B1 may cause adverse effects on the overall performance. The results indicate that supplementation of sodium bentonite and coumarin at 5 g/Kg diet mitigates the toxic effects of aflatoxin B1. However, further studies have to be undertaken to observe whether the sodium bentonite or coumarin at doses lower than 5 g/Kg diet could ameliorate the negative effects of aflatoxin B1. Further, between the two anti-aflatoxigenic supplements tested, coumarin is found to be a superior one because of the better production performance and carcass characteristics of rabbits supplemented with coumarin.

Statement of animal rights

The research was performed in accordance with the ethical standard laid down in the 1996 declaration of Helsinki and its later amendments.

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