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Probiotics and clay detoxifier protected growth performance and intestinal barrier of lambs fed diet contaminated with aflatoxin B_1

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ABSTRACT

Probiotics or clay detoxifier can improve the intestinal health of monogastric animals fed diets contaminated with aflatoxin B_1 (AFB_1), but little is known in ruminants. This study aimed to investigate the effect of probiotics and clay detoxifier on the growth performance, enterotoxigenic bacteria, endotoxins and intestinal barrier of lambs fed diet contaminated with AFB_1. Lambs (24) were randomly allocated into 4 groups with 6 replicates. Treatments included control, AFB_1 (100 µg/kg), probiotics (AFB_1 + probiotics @ 3×10^9 cfu/kg) and clay (AFB_1 + clay @ 4.0 g/kg of feed). The trial lasted for 35 d. Results showed that AFB_1 worsened body weight gain and feed conversion ratio, and these were recovered by probiotics and clay detoxifier supplementation. Also, AFB_1 increased cecal counts of *Clostridium perfringens*, *Salmonella*, *Escherichia coli* and gram-negative bacteria, serum endotoxin and diamine oxidase, but decreased duodenal mRNA expressions of claudin-1, IgA inducing protein, junctional adhesion molecule 2 (JAM-2), joining chain of multimeric IgA and IgM (J-chain) and occludin. Probiotics ameliorated these negative effects, but for *Clostridium perfringens* and J-chain, whereas clay detoxifier only showed beneficial effects on *Escherichia coli*, gram-negative bacteria, endotoxins, claudin-1 and JAM-2. In addition, probiotics were more protective against enterotoxigenic bacteria and enterotoxic markers than clay detoxifier. The results suggest that the probiotics are capable of restoring growth performance and protecting intestinal barrier in lambs fed diet contaminated with AFB_1.

Key words: Aflatoxin B_1, Clay detoxifier, Intestinal barrier, Lambs, Probiotics

Aflatoxins are fungal toxins that are regularly found in maize and other types of crops due to improper storage or processing, and aflatoxin B_1 (AFB_1) is most toxic. Exposure to AFB_1 is associated with stunted growth, delayed development, liver damage and liver cancer (Klingelhoefer *et al*. 2018, Liu *et al*. 2018, Salem *et al*. 2018). It is known that non-nutritive adsorbents, such as clay detoxifiers, are effective against AFB_1 contamination in animal feed industry (Mahmood *et al*. 2017), but the physical adsorption has a big problem. AFB_1 absorbed in the feces leads to a secondary pollution to the environment (Liu *et al*. 2018). So, the degradation of AFB_1 by microbiota may be an optimal measure for diminishing the contamination (Adebo *et al*. 2017).

Naturally, a low dose of AFB_1 ingested with feed can be decomposed by gastro-intestinal microbiota, particularly beneficial rumen bacteria (Upadhaya *et al*. 2009), and this is why ruminants are not as sensitive to the toxin as monogastric animals. However, with the increasing contamination of AFB_1 in animal feeds, the toxin is also threatening farm ruminants, but literature about this is very limited. Recent studies have showed that probiotics have potential against AFB_1 contamination. Redzwan *et al*. (2016) reported that fermented milk drink containing probiotic *Lactobacillus casei* strain *Shirota* reduced AFB_1-lys concentrations in humans. *Saccharomyces cerevisiae* strains retained their viability and AFB_1 binding ability under gastrointestinal conditions and improved ruminal fermentation (Dogi *et al*. 2011), but when AFB_1 at a low concentration of 5.03 µg/kg in feed commercial dried yeast product did not affect the transfer of aflatoxin from feed to milk of ewes (Battacone *et al*. 2009). The information about this in meat ruminants is unclear.

The present study aimed to investigate the effect of probiotics and clay detoxifier on the growth performance, enterotoxigenic bacteria, enterotoxic markers and intestinal mucosal barrier of lambs fed dietary aflatoxin B_1.

MATERIALS AND METHODS

Probiotic strains included *Lactobacillus acidophilus* (ACCC11073), *Lactobacillus plantarum* (CICC21863) and *Enterococcus faecium* (CICC20430), which are authorized as feed additives by the Ministry of Agriculture of China (No. 2045-2013). The probiotic strains were obtained from Hongxiang Biological Feed Laboratory at Henan University of Science and Technology (China), and combined equally

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to reach a supplementing dose of 3×10^9 colony-forming units (cfu)/kg of feed.

The AFB_1 was produced using *Aspergillus flavus* from the China General Microbiological Culture Collection Center (China) and maize substrate as described by Liu *et al*. (2018). Uncontaminated control maize was replaced by the moldy maize to yield an AFB_1 concentration of 100 µg/kg of diet. The clay detoxifier was hydrated sodium calcium aluminosilicate and was added @ 3.0 g/kg at the expense of maize in the formulation.

The nutrition levels of the total mixed ratio diet were as recommended by Feeding Standards of Meat-producing Sheep and Goats (Ministry of Agriculture of China, 2004). Water contents of all ingredients and diets were controlled under 12% and the materials stored in a cool, dry, dark and well-ventilated place. No antibiotics were used either in feed or water throughout the experiment. The compositions and nutrients of basal diet are listed in Table 1.

All the experimental procedures were approved by the Animal Ethics Committee of the Henan University of Science and Technology (Luoyang, China).

Male small-tailed Han (24) 60 days old with 11.17 kg±0.55 (mean±SD) of initial body weight were assigned to the 4 dietary treatments in a randomized block design. All lambs were housed individually in replicated pens (2 m×1.5 m) with wooden slatted floors and had free access to diets twice daily (07:00 and 19:00 h) with approximately 10% feed refusal and drinking water. The feeding trial lasted 35 days, consisting of 5 days for adaptation followed by 30 days of dietary treatment. Animals were weighed at the start and end of the feeding trial and monitored for general health twice a day.

Table 1. Ingredients and nutrient levels of basal diet

Item	Content (%)
Ingredients[1]	
Corn	42.5
Soybean meal	6.0
Wheat bran	9.0
Alfalfa meal	40.0
Limestone	0.5
Dicalcium phosphate	1.0
Titanium dioxide	0.5
Premix[2]	0.5
Chemical analysis	
Crude protein	14.98
Ether extract	2.86
Neutral detergent fibre	22.14
Acid detergent fibre	12.84
Calcium	1.00
Phosphorus	0.61
Calculated DE (MJ/kg)[3]	11.84

[1]Aflatoxin B_1 is not detectable (<2 µg/kg). [2]The premix provided the following per kg of diet: Vitamin A, 12,000 IU; Vitamin D, 2,000 IU; Vitamin E, 30 IU; Cu, 12 mg; Fe, 64 mg; Mn, 56 mg; Zn, 60 mg; I, 1.2 mg; Se, 0.4 mg; Co, 0.4 mg; NaCl, 6.4 g. [3]Calculated by Chinese Feed Database (25th edn, 2014).

At the end of the trial, 4 lambs (out of 6) per treatment were randomly selected and blood was drawn from the jugular vein of each lamb into heparinized evacuated tubes approximately 3 h after morning feeding. The sample was centrifuged at 3,000×g for 15 min to obtain the plasma for the analysis of enterotoxic markers. The lambs were euthanized by CO_2 suffocation, and dissected. Appropriately 10 g of cecal contents were collected and immediately stored at –40°C for detecting bacterial populations. Duodenum was dissected, washed with 0 to 4°C of phosphate-buffered saline, and then mucosa was collected and stored in RNAlater for gene expression analysis.

The AFB_1 contents in the samples were detected by commercial kits (Longke Fangzhou Biotech, China) with a sensitivity of 2 µg/kg. Briefly, 0, 2, 5, 10, 20 and 50 µg/l AFB_1 standard solutions were used to make the calibration curve, and all of them were included in an ELISA test kit. The concentrations of plasma endotoxin were measured using a limulus amoebocyte lysate (LAL)-based kit (LALQCL-1000, USA). Samples and standards were incubated for 10 min at 37°C with LAL and then for another 6 min with the colorimetric substrate. The internal control for recovery calculation was included in the assessment. The reaction was stopped with 25% acetic acid and then the absorbance was read at 405 nm. Diamine oxidase activity (1 ml) in serum was examined by a spectrophotometric assay. Diamine oxidase standard (D7876-250) was purchased from Sigma-Aldrich.

Each cecal content (1 g) was diluted with sterile buffered peptone water (0.1%, 9 ml, 0 to 4°C) and mixed. The suspension of each sample was serially diluted between 10^{-1} to 10^{-7} dilutions, and each diluted sample (100 µl) was subsequently spread onto duplicate selective agar plates for bacterial counting. The number of cfu was expressed as a logarithmic ($\log_{10}$) transformation per gram of intestinal digesta. Cecal bacterial populations were detected using commercial media including *Escherichia coli* (*E. coli*) chromogenic medium (HB7001), *Clostridium perfringens* (*C. perfringens*) sulfite polymixin sulphadiazine agar base (HB0256), *Salmonella* deoxycholate hydrogen sulfide lactose agar (HB4087), and Gram-negative bacteria ($Gram^-$) selection medium (HB8643). The media were purchased from Qingdao Hope Bio-Technology Co. Ltd. (China).

Total mRNA isolation, cDNA synthesis, primers synthesis and qPCR reagents for intestinal samples were carried out using commercial kits according to the manufacturer's instructions (TaKaRa Co. Ltd, China). The mRNA concentration was determined by the OD reading at 260 nm, and the purity was checked using A260/A280 ratio (1.8 to 2.0) and A260/A230 ratio (>1.5) on a NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific Inc., USA). The mRNA profiles of target genes were expressed as the relative expression to the beta-actin gene. Primer information for qPCR is listed in Table 2. The qPCR reactions were set at 10 µl with 5 µl of SYBR Green Master Mix, 1 µl of primer, 4 µl of 10 × diluted cDNA. All

Table 2. Primers for quantitative real-time PCR

Item	Accession number	Primer (5'→3')		Length (bp)
		Forward	Reverse	
Occludin	XM_018065681.1	gagccgcagcaaacctaatcac	caggcaagagtggaggcaac	225
Claudin-1	XM_005675123.3	ttcatcctggcgtttctggg	gttgcttgcagagtgctgtt	209
JAM-2	XM_018051483.1	tcccgaatcaccaacagctc	tcctctgagcatagcacacg	244
IGIP	XM_005683028.3	gcattctgtattaaatctgtgcagc	tgatattgaacaaactcaagccg	171
J-chain	XM_005681729.3	aggtcatgctcactgcacaa	agtgatagggtgcggtagt	146
GAPDH	XM_005680968.3	agccgtaacttctgtgctgt	ttcccgttctctgccttgac	234

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IGIP, IgA inducing protein; J-chain, joining chain of multimeric IgA and IgM; JAM-2, junctional adhesion molecule 2.

qPCRs were run in triplicate on the same thermal cycles (50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min) on the ABI Prism 7900 HT Fast Real-Time PCR System (Applied Biosystems Inc., USA). No amplification signal was detected in water or no-RT RNA samples.

The data were analyzed by using ANOVA program (SPSS 2012) and the means were tested for the significant difference by Tukey's b test at P<0.05.

RESULTS AND DISCUSSION

Effect of probiotics and clay detoxifier on the growth performance of lambs: Compared to the control diet, AFB_1 contamination decreased (P<0.05) feed intake (FI) and body weight gain (BWG), and deteriorated (P<0.05) feed conversion ratio (FCR) of lambs (Table 3). The findings were consistent with reports that animals fed the diet with AFB_1 contamination showed poor growth performance (Liu *et al*. 2018, Salem *et al*. 2018). Compared to the AFB_1 diet, the probiotics and clay detoxifier positively influenced (P<0.05) BWG and FCR, and there was no difference in the growth parameters between probiotics and clay detoxifier, which demonstrated that the two supplements had the same effectiveness against aflatoxins in the animal feed.

Table 3. Effect of probiotics and clay detoxifier on the growth performance of lambs

Item	Control	AFB_1	Probiotics	Clay	SEM	P value
Dietary factors						
AFB_1 (µg/kg)	<2	100	100	100		
Probiotics[1] (cfu/kg)	–	–	3×10^9	–		
Clay[2] (g/kg)	–	–	–	4.0		
Growth performance						
FI (kg/lamb)	31.42^a	28.67^b	31.28^a	30.11^{ab}	0.372	0.009
BWG (kg/lamb)	7.35^a	4.74^b	6.91^a	6.55^a	0.257	<0.001
FCR	4.32^b	6.08^a	4.59^b	4.64^b	0.175	<0.001

[abc]Means (n=6) within a row not sharing a superscript were significantly different (P<0.05). [1]Probiotics included an equal amount of *Lactobacillus acidophilus* (ACCC11073), *Lactobacillus plantarum* (CICC21863) and *Enterococcus faecium* (CICC20430). [2]Clay was hydrated sodium calcium aluminosilicate. –, component was not included. AFB_1, aflatoxin B_1; FI, feed intake; BWG, body weight gain; FCR, FI/BWG.

The application of physical detoxifier against AFB_1 in ruminants is not as common as in mono-gastric animals due to the partial removal of toxins by ruminal bacteria (Drobna *et al*. 2017). Hence literature about the effect of AFB_1 contamination on the growth of meat ruminants is very limited. In theory, absorption coupled with biodegradation of AFB_1 by microbes have more advantages than the physical absorption alone, as shown in mono-gastric animals (Abbes *et al*. 2016, Liu *et al*. 2018). However, this was not exhibited by the probiotics, and the BWG and FCR in the diets with probiotics or clay supplementation reached to the levels of control diet.

Effect of probiotics and clay detoxifier on the gut microbiota and enterotoxicity of lambs: Enterotoxigenic bacteria and enterotoxicity were also influenced by the dietary treatments (Table 4). Compared to the control diet, the diet with AFB_1 contamination increased (P<0.05) the counts of *C. perfringens, E. coli, Salmonella* and Gram–bacteria in the cecum of lambs. Inclusion of probiotics in the AFB_1 diet decreased (P<0.05) these bacteria, but only *E. coli* was lowered to the level of the control diet. Also, the clay detoxifier diet decreased (P<0.05) *E. coli* and Gram$^-$ bacteria, but did not affect *C. perfringens* and *Salmonella*. In contrast, the counts of enterotoxigenic bacteria in the probiotics diet were lower (P<0.05) than the clay detoxifier diet, indicating that the probiotics were more effective on AFB_1 detoxification.

It is known that *C. perfringens, E. coli* and *Salmonella* are gut opportunistic pathogens which can rapidly proliferate when the gut environment is deteriorated by negative factors, inadequate or unbalanced diet, and this can be improved by the supplementation of probiotics. Shakira *et al*. (2018) found that yeast increased *Lactococcus* counts and decreased *Enterococcus* and *Coliform* counts, which leads to improved gastro-intestinal tract microbial balance, and milk production and milk fat contents in lactating cows. Similarly, probiotics supplement was able to modify microflora balance by reducing *Salmonella, Shigella* and *Clostridia*, and increasing

Table 4. Effect of probiotics and clay detoxifier on the enterotoxic markers of lambs

Item	Control	AFB_1	Probiotics	Clay	SEM	P value
Dietary factors						
AFB_1 (μg/kg)	<2	100	100	100		
Probiotics[1] (cfu/kg)	–	–	3×10^9	–		
Clay[2] (g/kg)	–	–	–	4.0		
Enterotoxigenic bacteria (Log_{10} cfu/g of cecal feces)						
C. perfringens	1.52^c	2.46^{ab}	2.06^b	2.53^a	0.099	<0.001
E. coli	5.25^c	8.16^a	5.59^c	7.39^b	0.258	<0.001
Salmonella	0.54^c	1.25^a	0.82^b	1.05^a	0.053	<0.001
Gram$^-$	4.44^d	10.23^a	5.71^c	7.54^b	0.477	<0.001
Enterotoxicity						
Endotoxin (EU/ml)	0.32^c	0.53^a	0.37^c	0.47^b	0.018	<0.001
DAO (U/ml)	0.24^c	0.40^a	0.29^b	0.37^a	0.014	<0.001

^{abcd}Means within a row not sharing a superscript were significantly different (P<0.05). [1]Probiotics included an equal amount of *Lactobacillus acidophilus* (ACCC11073), *Lactobacillus plantarum* (CICC21863) and *Enterococcus faecium* (CICC20430). [2]Clay was hydrated sodium calcium aluminosilicate. –, component was not included. AFB_1, aflatoxin B_1; DAO, diamine oxidase; Gram$^-$, Gram-negative bacteria.

lactic acid bacteria and *Bifidobacteria* in goats (Apas *et al*. 2010, Maragkoudakis *et al*. 2010). However, literature on this aspect in ruminants is unavailable, which requires more studies.

The increased gut opportunistic pathogens and Gram$^-$ bacteria in the AFB_1 diet can produce more toxins including lipopolysaccharides and effector proteins that lead to enterotoxicity and immune disorders of animals. Interestingly, in the present study, the supplementation of probiotics decreased (P<0.05) the plasma contents of endotoxins and DAO (an enterotoxic marker), but clay detoxifier only reduced (P<0.05) endotoxins. Apas *et al*. (2010) reported that probiotics administration increased body weight and decreased fecal putrescine and mutagen concentration in goats. Maragkoudakis *et al*. (2010) found that probiotics did not affect the antioxidant capacity and the concentrations of IgA, IgM and IgG in goat plasma, but milk fat composition in the probiotics treatment had a significantly higher content of polyunsaturated fatty acids such as linoleic, α-linolenic and rumenic acids. Interaction of probiotics with lipopolysaccharides and bacterial effectors need to be explored.

Effect of probiotics and clay detoxifier on the mRNA expression of duodenal mucosal barrier genes: The mRNA profiles of duodenal mucosal barrier claudin-1, IgA inducing protein (IGIP), junctional adhesion molecule-2 (JAM-2), joining chain of multimeric IgA and IgM (J-chain) and occludin were down-regulated (P<0.05) by the AFB_1 diet compared to the control diet (Table 5). The inclusion of probiotics up-regulated (P<0.05) the mRNA of claudin-1, IGIP, JAM-2 and occludin. The clay detoxifier up-regulated (P<0.05) the transcriptional levels of claudin-1 and JAM-2. These findings indicate that both probiotics and clay detoxifier can partially protect intestinal mucosa against AFB_1 toxicity.

Table 5. Effect of probiotics and clay detoxifier on the mRNA expression of duodenal mucosal barrier genes of lambs

Item	Control	AFB_1	Probiotics	Clay	SEM	P value
Dietary factors						
AFB_1 (μg/kg)	<2	100	100	100		
Probiotics[1] (cfu/kg)	–	–	3×10^9	–		
Clay[2] (g/kg)	–	–	–	4.0		
Claudin-1	5.05^a	2.90^c	4.01^b	3.63^b	0.177	<0.001
IGIP	2.87^a	2.34^b	2.84^a	2.54^{ab}	0.069	0.008
JAM-2	3.03^a	2.24^b	2.98^a	2.86^a	0.074	<0.001
J-chain	6.52^a	4.83^b	5.99^{ab}	4.91^b	0.177	<0.001
Occludin	4.64^a	3.48^c	3.97^b	3.52^c	0.108	<0.001

^{abc}Means within a row not sharing a superscript were significantly different (P<0.05). [1]Probiotics included an equal amount of *Lactobacillus acidophilus* (ACCC11073), *Lactobacillus plantarum* (CICC21863) and *Enterococcus faecium* (CICC20430). [2]Clay was hydrated sodium calcium aluminosilicate. –, component was not included. AFB_1, aflatoxin B_1; IGIP, IgA inducing protein; JAM-2, junctional adhesion molecule 2; J-chain, joining chain of multimeric IgA and IgM.

Aside from the microbial barrier, the physical barrier (epithelial cells and tight junction) and immune barrier also contributed to the intestinal mucosal barrier. The claudin-1 and occludin are integral membrane protein genes and main components of tight junction strands. The IGIP stimulates relatively high levels of IgA production in a variety of tissues (Austin *et al*. 2003). JAM-2 is localized in the tight junction and acts as an adhesive ligand for immune cells. The J-chain is required for IgM and IgA to be secreted into mucosa. *Lactobacillus frumenti* up-regulated intestinal zonula occludens1, occludin, and claudin-1 in piglets (Hu *et al*. 2018). Likewise, *Lactobacillus plantarum* partially protected against the toxicity of unconjugated bilirubin and up-regulated occludin, zonula occludens, claudin-1, claudin-4, JAM-1 and F-actin by activating the protein kinase pathway in Caco-2 cells (Zhou *et al*. 2010). Whether probiotics influence the structure and function of epithelial cells in the present study needs further research.

It can be concluded that diet supplemented probiotics or clay protected the growth performance and intestinal mucosal barrier of lambs fed diet contaminated with AFB_1.

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