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Effect of supplemental lactic acid bacteria on growth performance, glutathione turnover and aflatoxin B_1 removal in lambs

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Abstract: This study investigated the effect of lactic acid bacteria (LAB) on growth performance, glutathione turnover, aflatoxin B_1 (AFB_1) residue and AFB_1-DNA adduct in growing lambs. Diets were a 2 × 2 factorial design, including AFB_1 at 0 or 100 μg/kg and LAB at 0 or 3 × 109 cfu/kg. Results showed that AFB_1 decreased ($P < 0.05$) feed intake (FI), body weight gain (BWG), and activities of glutathione (GSH), glutathione s-transferases (GSTs) and glutathione reductase (GR) in liver and duodenal mucosa, whereas these parameters were increased ($P < 0.05$) by supplemental LAB. AFB_1 increased and LAB decreased the feed conversion ratio ($P < 0.05$). Interactions ($P < 0.05$) were found on BWG, liver GSTs and mucosal GSH. LAB decreased ($P \leq 0.001$) AFB_1 residue in liver, kidney, plasma and faeces and AFB_1-DNA adduct in kidney, plasma and faeces. It can be concluded that LAB can partially protect against AFB_1 toxicity by facilitating glutathione turnover and reducing AFB_1 toxicity in lambs.

Keywords: aflatoxin B_1-DNA adduct; aflatoxin B_1 residue; body weight gain; feed conversion ratio; feed intake

Aflatoxin is a fungal toxin that commonly contaminates maize and other cereals during production, harvest, storage or processing. Exposure to aflatoxin is known to cause both chronic and acute hepatocellular injury in animals (Li et al. 2019; Souza et al. 2019). Aflatoxin B_1 (AFB_1) is considered the most toxic and can be metabolized by cytochrome-P450 enzymes to the reactive intermediate AFB_1-8,9 epoxide, which then binds to cell DNA and results in DNA adduct formation in the event of inadequate or deficient glutathione (GSH) activity against the toxic group (Gallagher et al. 1996; Dohnal et al. 2014). The AFB_1-8,9 epoxide is also capable of causing aflatoxicosis when it binds to proteins in the form of amino acid adducts and consequently results in hepatic cirrhosis, nutritional deficits and immunological suppression (Guengerich et al. 1996; Li et al. 2019; Rosim et al. 2019). Currently, physical adhesion to aflatoxin in feeds is commonly used to decrease aflatoxicity, while biological adsorption and degradation of AFB_1 may be a novel integrated method for aflatoxin removal. Researches have shown that lactic acid bacteria (LAB) can partially protect against AFB_1 in monogastric animals by decreasing immunotoxicity and oxidative stress, then consequently improving the growth performance (Gratz et al. 2006; Abbes et al. 2016; Liu et al. 2018a). In ruminants, administration of *Lactobacillus rhamnosus* GG reduced AFB_1 absorption in the gastrointestinal tract, increased AFB_1 excretion via the faeces and consequently alleviated the toxic effect of AFB_1 on the hepatic tissue and growth performance (Zhang et al. 2019).

GSH, a powerful antioxidant naturally produced and recycled in the body, performs a critical function in animal health through cleaning electrophilic metabolites of oxidative process (Wu et al. 2004; Novaes et al. 2013). Recent studies have shown

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that GSH activity or its pertinent enzymes could be influenced by LAB supplementation in AFB_1-induced models of rodents or broilers. Abbes et al. (2016) reported that co-treatment through LAB on AFB_1 or fumonisin B_1 suppressed DNA fragmentation levels, normalized splenic lipid peroxidation and increased GSH levels, upregulated expressions of anti-oxidation protective enzymes, and normalized mRNA levels of analysed cytokines. Liu et al. (2017) found that LAB supplementation increased the activities of GSH pathway related parameters in broilers fed an AFB_1 contaminated diet. However, there is very limited information on the effect of LAB on GSH turnover based on AFB_1 contaminated diet in ruminants.

The present study aimed to investigate the effect of LAB on the growth performance, concentration of GSH, and activities of glutathione s-transferases (GSTs) and glutathione reductase (GR), AFB_1 residue and AFB_1-DNA adduct in Chinese Hu lambs.

MATERIAL AND METHODS

Lactic acid bacteria strains and diets. The LAB strains in this study are permitted to be used in the feed additive industry in China (No. 2045-2013) and were obtained from Hongxiang Biological Feed Laboratory at Henan University of Science and Technology (Luoyang, China). The LAB strains included *Lactobacillus acidophilus* (ACCC11073), *Lactobacillus plantarum* (CICC21863) and *Enterococcus faecium* (CICC20430), which were combined at an equal amount and added at a dose of 3.0×10^9 cfu/kg of feed. AFB_1 was produced as described by Liu et al. (2018a), and modulated to 100 μg/kg of feed using non-contaminated corn meal (mesh size 2.00 mm) as a diluent at the expense of corn in the formulation.

A 2 × 2 completely randomized factorial design with two factors as AFB_1 and LAB supplementation or not was used in the present study. Four dietary treatments were: (1) control diet, without AFB_1 and LAB; (2) AFB_1 contaminated, control + AFB_1; (3) LAB supplementation, control + LAB; and (4) AFB_1 contaminated and LAB supplementation, control + AFB_1 + LAB. One batch of basal diet in the form of total mixed ration was prepared according to the description in Progress on the Sheep and Goats Industry in China (Wang et al. 2016), then the LAB and AFB_1 were added using a step by step blending method. The nutrition levels of the total mixed ration diet met the 2004 Feeding Standards of Meat-producing Sheep and Goats by the Ministry of Agriculture of China. Moisture of all ingredients and diets was controlled under 12% and the materials were stored in a cool, dry, dark and well-ventilated place. No antibiotics were used either in feed or water throughout the experiment. The basal diet composition and nutrient levels are listed in Table 1.

Animals and samples. The experimental protocol of the present study was approved by the Institutional Committee for Animal Use and Ethics of Henan University of Science and Technology. A total of 24 castrated Chinese Hu male lambs at approximately 60 days of age with initial body weight of 11.17 kg ± 0.55 (mean ± SD) were randomly assigned to four dietary treatments with six replicates and each lamb was regarded as the research unit. All lambs were housed individually in replicated pens (2 × 1.5 m) with wooden slatted floors and had free access to drinking water. The diets were offered twice daily (at 7.00 and 19.00 h) with approximately 10% extra feed to ensure free choice feeding. Feed supplied and residual feed were recorded daily. Feed intake was the difference between feed supplied and residual feed. The residual feed was mixed with the next feed ration. Body weight was measured at the beginning and end of the feeding trial to minimize the possible weighing stress. All animals were monitored for general health twice a day. The feeding trial lasted

Table 1. Ingredients and nutrient levels of basal diet[1]

Ingredients	Contents (%)	Nutrients	Contents (%)
Corn	44.4	dry matter	87.20
Wheat bran	5.0	crude protein	14.98
Soybean meal	7.0	digestible energy (MJ/kg)	11.83
Alfalfa meal	40.0	crude fat	2.81
Dicalcium phosphate	1.1	Ca	1.03
Limestone	0.5	P	0.60
Premix[2]	2.0		

[1]calculated by 2014 Chinese Feed Database, aflatoxin B_1 is not detectable, < 2 μg/kg of feed

[2]premix provided the following per kg of diets: vitamin A 12 000 IU, vitamin D 2000 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

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for 35 days with 5 days of adaptation followed by 30 days of experimental period. Total average feed intake (FI) and total average body weight gain (BWG) per lamb were calculated according to the records of daily feed intake and body weight, respectively. Feed conversion ratio (FCR) was calculated as FI divided by BWG.

At the end of the trial, 4 lambs (out of six) per treatment were randomly selected and blood was drawn from the jugular vein of each lamb into heparinized evacuated tubes in approximately 5 h after morning feeding. The sample was then centrifuged at 3000 *g* for 15 min to obtain the plasma for the analysis of AFB_1 residue and AFB_1-DNA adduct. Then, the lambs were euthanized by CO_2 suffocation and dissected. The liver and kidney were removed, and rectal faeces were collected. The duodenum was vertically dissected and rinsed with 0–4°C phosphate buffered saline to remove digesta, and then the mucosa was gently scraped using a microscope slide. Partial samples of liver, kidney and faeces were air-dried for the measurement of AFB_1 and AFB_1-DNA adduct, and the remaining liver sample and duodenal mucosa were sorted at –40°C for the analysis of GSH and related enzymes.

Chemical and biochemical analysis. The AFB_1 contents in samples were detected by commercial kits (Longke Fangzhou Biotech, China) with sensitivity of detection at 2 μg/kg. Briefly, 0, 2, 5, 10, 20 and 50 μg/l of AFB_1 standard solutions were used to make the calibration curve, and all of them were included in an ELISA test kit. The AFB_1-DNA adducts were detected using an ELISA kit purchased from Cell Bioabs, USA. The toxin contents were expressed as μg/kg on an air-dry basis (65°C) in the feed, liver, kidney, faeces and plasma, and as μg/l in the plasma samples.

Commercial kits from the Nanjing Jiancheng Bioengineering Institute (China) were used for the detection of reduced GSH (detection range from 0.3 to 147.1 mg/l), GSTs (detection range from 6.0 to 22.0 U/ml), and GR (detection range from 1.6 to 320 U/l). The units of GSH, GSTs and GR were finally calculated and expressed as mg/g, μmol/min/g and U/g, respectively. All chemical and biochemical analyses of collected samples were performed in triplicates.

Statistical analysis. Parameters for growth performance and glutathione turnover from 24 lambs (6 lambs per treatment) were analysed using a two-way ANOVA of SAS software (Version 9.4, 2013). The AFB_1 residue and AFB_1-DNA adduct in AFB_1 treatment and AFB_1 + LAB treatment from 16 lambs (4 lambs per treatment) were analysed using an independent samples *t*-test because AFB_1 was undetectable in control and LAB treatment. Also, the *t*-test was used to separate the differences between control and AFB_1 group, LAB and LAB + AFB_1 group. Differences between variables for factorial ANOVA were separated using Tukey's HSD test. Values in tables are means and standard errors of the mean (SEM).

RESULTS AND DISCUSSION

Mortality and growth performance. Clinical or subclinical aflatoxicosis in farm animals has recently resulted in millions of dollars in annual losses to producers with the frequent occurrence of AFB_1 contamination across the world. The subclinical symptom of aflatoxicosis includes depressed growth rates, increased susceptibility to disease, reduced feed utilization and other adverse effects. Growth performance result of LAB and AFB_1 in

Table 2. Effect of aflatoxin B_1 (AFB_1) and lactic acid bacteria (LAB) on the growth performance of Chinese Hu lambs (6 animals per group, 24 in total)

Item	Treatment				SEM	*P*-value		
	Control	LAB	AFB_1	AFB_1 + LAB		LAB	AFB_1	interaction
FI (kg/lamb)	26.22	26.75	25.40	26.75^B	0.145	0.002	< 0.001	0.301
BWG (kg/lamb)	6.37	7.34	3.66	5.50^B	0.340	< 0.001	0.040	0.040
FCR	4.28	3.69	7.14	4.97^B	0.325	0.002	< 0.001	0.054

BWG = body weight gain, FCR = feed conversion ratio, FI = feed intake, SEM = standard error of the means

AFB_1 added at 100 μg/kg; LAB added at 3×10^9 cfu/kg

^{B}statistically different from: $^B AFB_1$, $P < 0.01$

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growing lambs is shown in Table 2. There were no significant interactions between dietary treatments in relation to FI and FCR. For the main effect on FI and FCR, the supplementation of LAB was able to improve ($P < 0.01$) both FI and FCR in growing lambs. The presence of AFB_1 exerted a negative effect on FI and FCR ($P < 0.01$). A significant ($P < 0.05$) interaction was found on BWG in the present study. The inclusion of AFB_1 in the diet reduced ($P < 0.05$) BWG compared to control group. Supplementation of LAB to AFB_1 diet increased ($P < 0.05$) BWG compared to the treatment with AFB_1 only and it reached the same level as the control diet without AFB_1 addition. Recent studies have shown that probiotics including LAB improved the growth performance of monogastric animals exposed to AFB_1 (Gratz et al. 2006; Liu et al. 2018a; Salem et al. 2018), but this is very limited in ruminants due to their relatively lower susceptibility to toxins.

In the present study, no mortalities occurred across the treatments, which indicates that the present AFB_1 level is not a lethal dose. However, the depressed growth performance demonstrated the negative effect of subclinical symptoms caused by aflatoxicosis. Moreover, the ruminants are less sensitive to aflatoxins than monogastric animals due to the possible adsorption or decomposition of AFB_1 by ruminal microbiota. Indeed, *Lactobacillus* species isolated from the lamb stomach mucosa showed antifungal activity and aflatoxin binding ability (Gallo et al. 2015; Drobna et al. 2017).

LAB increased GSH turnover. The result of the effect of LAB and AFB_1 on liver and duodenal mucosa is presented in Table 3. Among dietary treatments main effects of two dietary factors were found on GSH and GR in liver ($P \leq 0.001$). The presence of AFB_1 decreased ($P < 0.01$) GSH and GR activities compared to the group without AFB_1. Supplementation of LAB increased ($P < 0.01$) GSH and GR activities in liver compared to the control group. For GST activity in liver, there was a significant interaction between treatments ($P = 0.046$). The presence of AFB_1 decreased ($P < 0.05$) GST activity compared to the control group and supplementation of LAB to the AFB_1-treated diet improved ($P < 0.05$) GST activity compared to the group with AFB_1 only. For GSH activity in the duodenal mucosa, there was a significant interaction between treatments ($P = 0.022$). The inclusion of AFB_1 decreased ($P < 0.05$) GST activity compared to the control group and supplementation of LAB to the AFB_1-treated diet improved ($P < 0.05$) GST activity compared to the group with AFB_1 only. Among dietary treatments main effects were found on GST and GR in the duodenal content ($P < 0.05$). The presence of AFB_1 decreased ($P < 0.01$) GST and GR activities compared to the group without AFB_1. Supplementation of LAB increased ($P < 0.01$) GST and GR activities in liver compared to the control group.

The AFB_1 toxicity to GSH or its related enzymes in monogastric animals or ruminants has been well documented (Larsson et al. 1994; Liu et al. 2017, 2018b). Importantly, in the present study, the positive effects of LAB on GSH, GSTs and GR in liver and duodenal mucosa indicate the beneficial modulation of dietary LAB on GSH turnover. However, most studies on LAB and GSH pathway

Table 3. Effect of aflatoxin B_1 (AFB_1) and lactic acid bacteria (LAB) on glutathione turnover in the tissue of lambs (4 animals per group, 16 in total)

Item	Treatment				SEM	*P*-value		
	Control	LAB	AFB_1	AFB_1 + LAB		LAB	AFB_1	interaction
Liver								
GSH (mg/g)	25.93	33.74^{A}	12.19	20.24^{B}	1.702	< 0.001	< 0.001	0.899
GSTs (U/g)	317.40	326.20^{a}	209.90	236.80^{B}	10.660	< 0.001	< 0.001	0.046
GR (U/g)	25.83	38.57^{a}	13.95	20.60^{B}	2.183	0.001	< 0.001	0.214
Duodenal mucosa								
GSH (mg/g)	34.29	46.73^{A}	20.19	25.19	2.217	< 0.001	< 0.001	0.022
GSTs (U/g)	372.40	433.80^{A}	224.30	294.60^{B}	17.150	< 0.001	< 0.001	0.666
GR (U/g)	39.07	41.55^{a}	24.04	28.37^{B}	1.551	< 0.001	< 0.001	0.204

GSH = glutathione, GSTs = glutathione s-transferases, GR = glutathione reductase, SEM = standard error of the means
^{a,A,B}statistically different from: ^{a}Control, $P < 0.05$; ^{A}Control, $^{B}AFB_1$, $P < 0.01$

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Table 4. Effect of aflatoxin B_1 (AFB_1) and lactic acid bacteria (LAB) on the AFB_1 residues in the tissues of lambs (4 animals per group, 16 in total)

Item	AFB_1 residue (μg/kg)				AFB_1-DNA adduct (μg/kg)			
	AFB_1	AFB_1 + LAB	SEM	*P*-value	AFB_1	AFB_1 + LAB	SEM	*P*-value
Liver	43.35	28.61	1.212	< 0.001	15.99	14.62	1.238	0.294
Kidney	36.57	20.94	1.585	< 0.001	21.07	16.76	0.967	0.001
Plasma	7.57	4.68	0.263	< 0.001	6.62	1.75	0.243	< 0.001
Feces	60.96	39.23	2.609	< 0.001	16.30	1.10	0.797	< 0.001

SEM = standard error of the means

were focused on non-ruminant animals, there is very limited literature dealing with LAB and GSH pathways in ruminants. Probiotics in fermented milk were found to show a significant hepatoprotective effect by enhancing glutathione peroxidase, superoxide dismutase, catalase and GSTs in AFB_1-induced hepatic damage of rats (Kumar et al. 2012). Similarly, probiotics counteracted the negative effects of AFB_1 on serum AST, ALT, malondialdehyde, total protein, albumin, globulin and glutathione pathway in chickens (Liu et al. 2018a; Salem et al. 2018). Meanwhile, the circular GSH in the body can be synthesized by the host, gut co-residents and supplemented LAB, so the role of GSH from the supplemented probiotics in the gut lumen against the toxicity is worthy of further exploration.

Lactic acid bacteria reduced AFB_1 residue and AFB_1-DNA adduct. Results of the effect of LAB on AFB_1 residue in tissues are presented in Table 4. The LAB diet reduced ($P < 0.001$) the tissue residues of AFB_1 in the liver, kidney, plasma and faeces by 34, 43, 38 and 36%, respectively (Table 4). These data imply that supplementation of LAB to the AFB_1 diet reduces AFB_1 absorption into the circulatory system and increases AFB_1 decomposition and metabolism in the gastrointestinal tract of lambs. These findings were consistent with the studies where probiotics reduced AFB_1 levels in the liver, kidney and serum of chickens (Liu et al. 2018a; Salem et al. 2018), and probiotic yoghurt reduced AFB_1 biomarkers in the urine of children (Nduti et al. 2016). However, with the adsorption by yeast cells, AFB_1 increased the diameter of yeast cells, suggesting there is an advantage since a larger cell would be able to adsorb mycotoxins more efficiently (Dogi et al. 2017). Whether the cell sizes of probiotics in the present study are influenced by AFB_1 will be warranted by further study.

In the present study, the AFB_1 diet with LAB supplementation lowered ($P \leq 0.001$) the contents of AFB_1-DNA adduct in the liver, kidney, plasma and faeces by 71, 20, 74 and 93%, respectively, further indicating that LAB can interfere with AFB_1 absorption and consequently decrease its genotoxicity. Kumar et al. (2011) found that probiotic-fermented milk reduced DNA damage, tumour incidence and the mRNA levels of *C-myc*, *Bcl-2*, *Cyclin D-1* and *Rasp-21* in the hepatic cells of rats. Slizewska et al. (2010) reported that supplementation with a probiotic preparation decreased the extent of DNA damage in the faecal water of chickens. Jebali et al. (2018) observed that *Lactobacillus plantarum* decreased AFB_1-induced DNA damage, upregulated *caspase-3*, *caspase-9*, *CYP3A-13*, *Bax* and *p53*, and downregulated the expression of *TNFα* and *Bcl-2* and their target proteins. Additionally, AFB_1 can bind plasma protein or amino acids and interfere with their functions (Guengerich et al. 1996), but this information is unavailable in ruminants, which needs further study.

CONCLUSION

The present study concluded that the toxicity of AFB_1 was reflected in decreased growth performance and GSH activity, and increased AFB_1 residue and AFB_1-DNA adduct in tissues. The mixture of LAB strains, *Lactobacillus acidophilus*, *Lactobacillus plantarum* and *Enterococcus faecium* was shown to effectively enhance the growth performance of lambs and remove AFB_1 from tissues, as well as to increase DNA protection by increasing GSH turnover and reducing AFB_1 residue and DNA adduct. There were also interactions on BWG and liver GSTs that resulted in the more pronounced effect of LAB in the AFB_1 basal diet. Therefore, LAB play a crucial role in AFB_1 removal and can be used as a bioactive additive for AFB_1 reduction in ruminant feed.

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