Lactobacillus rhamnosus GG modulates gastrointestinal absorption, excretion patterns, and toxicity in Holstein calves fed a single dose of aflatoxin B₁

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ABSTRACT

The aim of the present study was to evaluate the effects of Lactobacillus rhamnosus GG (LGG; ATCC 53013) on growth performance and hepatotoxicity in calves fed a single dose of aflatoxin B₁ (AFB₁) and to investigate the absorption, distribution, and elimination of AFB₁ and the hydroxylated metabolite aflatoxin M₁ (AFM₁) in rumen fluid, blood, and excretions. Twenty-four male Holstein calves were blocked for body weight and age and were randomly assigned to 1 of 3 treatment groups: (1) untreated control, (2) treated with 4.80 mg of AFB₁ (AFB₁ only), or (3) treated with 1 × 10¹⁰ cfu of LGG suspension and 4.80 mg of AFB₁ (AFB₁ plus LGG). The calves received LGG suspension in 50 mL of phosphate-buffered saline daily via oral administration for 14 d before and on the day they received a single oral dose of AFB₁. Body weight was recorded at the beginning of the study (before LGG administration), at the day of AFB₁ administration, and at the end of the trial. Rumen fluid, blood, urine, and feces samples were collected continuously for 96 h after AFB₁ administration. Average daily gain (ADG) and plasma biochemical parameters were analyzed, and concentrations of AFB₁ and AFM₁ in the samples were determined for monitoring excretion pattern and toxicokinetics. The results showed that ADG was lower in AFB₁-treated animals; LGG administration partially mitigated the decrease in ADG (0.85 ± 0.08 vs. 0.76 ± 0.18 kg of gain/d). The AFB₁ treatment increased plasma aspartate aminotransferase, alkaline phosphatase, and lactate dehydrogenase levels. Administration of LGG alleviated the AFB₁-induced increase in plasma enzymes activity. The excretion patterns of AFB₁ and AFM₁ were surprisingly regular; toxins were rapidly detected in all samples after a single oral dose of AFB₁, and the peak of toxins concentrations was sequentially reached in rumen fluid, plasma, urine, and feces (except AFM₁ in rumen fluid), followed by an exponential decrease. The excretion curves showed that AFB₁ and AFM₁ concentrations were the highest in feces and urine, respectively. Administration of LGG decreased the concentrations of free AFB₁ and AFM₁ in rumen fluid and reduced the release of toxins into plasma and urine. Toxicokinetic parameters (except for the time of maximum concentration and the terminal half-life) were reduced by LGG administration. In conclusion, the absorption, distribution, and excretion of AFB₁ and AFM₁ were rapid in calves fed a single dose of AFB₁. Urine was the main route for the excretion of AFM₁, and the clearance pattern from the peak of concentration was well fitted by exponential decreasing function. Administration of LGG reduced the absorption of AFB₁ in the gastrointestinal tract by increasing the excretion via the feces, thus alleviating the hepatotoxic effect of AFB₁.

Key words: Lactobacillus rhamnosus GG, aflatoxin B₁, excretion, toxicokinetics

INTRODUCTION

The occurrence of mycotoxins in foods and animal feeds has been frequently reported in many countries, and aflatoxins are among the most toxic types (Kim et al., 2017). Aspergillus flavus, Aspergillus parasiticus, and Aspergillus nomius, which are known to contaminate corn, sorghum, rice, peanuts, tree nuts, figs, ginger, nutmeg, and milk, produce aflatoxins before and during crop harvesting, in storage, and after processing and manufacturing (Ellis et al., 1991; FDA, 2012). Many reports have shown high levels of contamination of raw agricultural products with aflatoxin B₁ (AFB₁), which is the most prevalent and toxic of all aflatoxins, exceeding the maximum permissible limit (Chen et al., 2013; Guchi, 2015; Waliyar et al., 2015). Those limits vary extensively between countries. In European Union countries, the maximum level of AFB₁ in complete feed...
for calves is 0.01 mg/kg (European Commission, 2003), whereas in the United States, the action level of total aflatoxins in young animal feed and ingredients is 0.02 mg/kg (FDA, 2000). In China, the maximum level of AFB1 in ingredients is 0.05 mg/kg (China General Administration of Quality Supervision, Inspection and Quarantine, 2001).

When AFB1-contaminated food or feed is consumed, the toxin is metabolized to aflatoxin M1 (AFM1) and secreted into the tissues, biological fluids, and milk of lactating animals (Zarba et al., 1992). Both AFB1 and AFM1 are hepatotoxic, carcinogenic, teratogenic, and immunosuppressive, and they inhibit several metabolic systems, thus causing liver, kidney, and heart damage (Diaz, 2005a). The 2 toxins are classified in group 1 as human carcinogens by the International Agency for Research on Cancer of the World Health Organization (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2002).

Many physicochemical methods have been reported to remove AFB1 from food or feed. Among these methods, the use of adsorbents made from bentonite and esterified glucomannan is regarded as a promising and practical way to reduce aflatoxin absorption in the gastrointestinal tract (Diaz et al., 2004; Kabak et al., 2006). However, most of these methods usually cause unwanted alteration of food properties (Diaz, 2005b). Therefore, recent studies have focused on biological detoxification, especially via microbe-mediated mechanisms—either adsorption or degradation. Of the lactic acid bacteria, Lactobacillus rhamnosus GG (LGG) efficiently binds AFB1 with peptidoglycan in the bacterial cell wall, thus playing an important role (Kim et al., 2017).

Lactobacillus rhamnosus GG (ATCC 53103), a gram-positive bacterium that was isolated by Barry R. Goldin and Sherwood L. Gorbach (Silva et al., 1987), has been used as a probiotic bacterium due to its resistance to gastric acid and bile and strong avidity for human intestinal mucosal cells (Walter, 2008). Lactobacillus rhamnosus GG has powerful adhesive properties and can exclude or reduce pathogenic adherence as well as produce substances antagonistic to foodborne pathogens (Gorbach, 2000). In addition, LGG shows a strong ability to sequester aflatoxins. The study by El-Nezami et al. (1998) was the first to report that the elimination of AFB1 was a rapid process involving the removal of approximately 80% of AFB1 in the presence of LGG. Peltoenen et al. (2001) evaluated the adsorption capacity of 20 different strains to AFB1 and showed that 2 Lactobacillus amylovorus strains and 1 Lactobacillus rhamnosus strain rapidly bound and removed more than 50% of AFB1. Bovo et al. (2014) found no differences in the AFB1 binding capacity between live and lyophilized LGG (Howaru LYO 40 DCU; Danisco Brasil Ltda, Pirapaisinho, Brazil) at pH 3.0 and 6.0. Gratz et al. (2007) clearly showed that AFB1 transport can be reduced by LGG binding in the Caco-2 model. These results suggested that the bioavailability and toxicity of AFB1 were reduced rapidly by the binding of LGG and the toxin. Deabes et al. (2012) evaluated whether LGG (ATCC 53013) could remove aflatoxins in vivo and found that oral administration of 1 × 1010 cfu of LGG for 7 d to male albino mice significantly counteracted oxidative stress and protected against aflatoxin-induced genotoxicity. Gratz et al. (2006) suggested that LGG reduced the absorption of AFB1 in the intestines, preventing weight loss and reducing the hepatotoxicity caused by a high dose of AFB1 (4.8 μmol/kg of BW) in rats. These studies indicated that LGG is effective in removing AFB1 in vitro and in vivo in certain animal species. These authors hypothesized that AFB1 is not removed by metabolism but rather is physically bound to molecular components of the bacterium.

This in vitro evaluation method, however, has its limitations in simulating gastrointestinal tract conditions. There is also little information available on the effect of LGG on aflatoxin toxico-kinetics and excretion pattern in ruminants. Practically, the use of highly contaminated feed by dairy farmers is unlikely; however, a single accidental feeding of contaminated feed may occur (Battacone et al., 2003, 2012). In this study, we hypothesized that LGG would sequester AFB1 in the gastrointestinal tract of calves and reduced the amount of AFB1 absorbed into the circulatory system. Therefore, a single-dose in vivo experiment in Holstein male calves was conducted to investigate the effects of LGG on AFB1 excretion and toxicity.

**MATERIALS AND METHODS**

**Preparation of AFB1**

The AFB1 used in the study was produced by culture of a toxigenic A. flavus strain (no. 3.4409), which was purchased from the China General Microbiological Culture Collection Center (Beijing, China). The carrier for AFB1 consisted of 50% corn grit, 25% rice, and 25% barley. After mixing thoroughly, the water content of the carrier was adjusted to 35%, and it was placed into 2-L gas-permeable cap polyethylene plastic buckets and sterilized by autoclaving (121°C, 20 min). The carrier was inoculated with A. flavus and incubated at 25°C for 4 wk. To prevent fungal growth, the inoculated carrier was autoclaved (121°C, 20 min) and dried at 65°C for 48 h (Xiong et al., 2015). The concentrations of aflatoxins in culture materials were analyzed by the HPLC method described by the China Feed Industry Stan-
respectively; the AFG1 and AFG2 concentrations were 24.01 and 0.25 mg/kg, respectively; the AFG1 and AFG2 concentrations were undetectable.

**Bacterial Strain and Growth Conditions**

*Lactobacillus rhamnosus* GG (ATCC 53013) was purchased from the China Center of Industrial Culture Collection (Beijing, China) and was originally sourced from the American Type Culture Collection. The lyophilized strain was reactivated in De Man, Rogosa and Sharpe broth (Aoboxing Bio-Tech Co. Ltd., Beijing, China) at 37°C and 200 rpm for 24 h and grown under these conditions until a high concentration of cells (>10^10 cells/mL) was obtained. Bacterial pellets (~10^10 cells) were collected by centrifugation (3,200 × g, 4°C, 10 min) and washed twice with PBS (0.01 M phosphate, 0.15 M sodium chloride, pH 7.3). Bacterial cell concentration was estimated by measuring the optical density of samples at 600 nm and comparing the results with a standard curve (obtained by combining the results from flow cytometry and spectroscopy over a range of LGG concentrations; Lahtinen et al., 2004).

**Animals and Experimental Design**

The experiment was conducted at the Experimental Practice and Demonstration Center of Northeast Agricultural University (Harbin, China). The experimental protocol was conducted in accordance with the practices outlined in the *Guide for the Care and Use of Agriculture Animals in Research and Teaching* (FASS, 2010).

Twenty-four male Holstein calves (mean ± SD; age = 120.04 ± 4.20 d, BW = 126.39 ± 6.62 kg) were blocked for BW and age before being randomly assigned to 1 of 3 treatment groups (8 calves per group): (1) untreated control (CON), (2) treated with 4.80 mg of AFB1 (AFB1 only), or (3) treated with 1 × 10^10 cfu of LGG suspension and 4.80 mg of AFB1 (AFB1 plus LGG). The calves received a suspension of LGG in 50 mL of PBS daily via oral administration for 14 d before and on the day they received a single oral dose of AFB1. The AFB1 dose was obtained by mixing 0.2 kg of AFB1 grain carrier with standard pellet concentrate feed (Gushi A&H Group Co. Ltd., Harbin, China). The amount of AFB1 administered in this experiment corresponded to approximately 0.038 mg/kg of BW. The calves in the CON and AFB1 only groups received no AFB1 grain carrier or PBS to eliminate the influence of diet.

The calves were raised in individual pens (1.5 × 2.5 m²) on straw bedding with a front metal gate allowing access to feed. All calves were fed the same diet twice daily at 0600 and 1800 h. The calves were allowed free access to feed and water ad libitum throughout the 18-d feeding trial.

**BW, Sample Collection, and Analysis**

Body weight was recorded at the beginning of the study (before LGG administration), at the day of AFB1 dosing, and at the end of the study. Samples of rumen fluid, blood, urine, and feces were collected at 0, 2, 4, 8, 12, 16, 24, 36, 48, 60, 72, and 96 h after AFB1 administration. Rumen fluids were collected using an oral stomach tube. The first 100 mL of rumen fluid was discarded before sample collection. The tube was cleaned with ethanol and then washed with water to prevent the contamination of rumen fluid between calves. Rumen fluids were filtered with double-layer gauze and centrifuged (3,000 × g, 15 min, 4°C), and the supernatants were collected into clean tubes. Blood samples of all calves were collected from the jugular vein into heparinized tubes (Vacutainer, Huawei Medical Appliances Co. Ltd., Yangzhou, China) and centrifuged (3,000 × g, 15 min, 4°C). Plasma fractions were transferred into clean tubes. Urine samples of almost 30 mL were taken by manually stimulating the genitals of calves. Then, the samples were acidified with 0.1 N HCl to pH 4.5 and centrifuged (3,000 × g, 15 min, 4°C). The supernatants were then transferred into clean tubes. Feces samples weighing almost 100 g were taken directly from the calves' rectum. The samples were homogenized and transferred into clean tubes. All the samples were stored at −20°C until later analysis.

Plasma samples were sent to Heilongjiang Electric Power Hospital (Harbin, China) for analysis of the concentrations of total protein, albumin, globulin, albumin/globulin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), AST/ALT, alkaline phosphatase, triglyceride, and lactate dehydrogenase (LDH). Samples were analyzed using an AU680 Beckman Coulter analyzer (Beckman Coulter Commercial Enterprise China Co. Ltd., Shanghai, China).

The AFB1 and AFM1 concentrations in all samples were analyzed using an immunoaffinity column purification technique and determined by HPLC using the method described by the China Feed Industry Standardization Technical Committee (2014) and the China Ministry of Health (2016). Briefly, feces extract solutions were obtained by thoroughly homogenizing feces pellets with 80% methanol (in 10% NaCl, vol/vol). The homogenates were filtered through glass microfiber filters (110 mm in diameter, 1.5 μm in pore size; Whatman, Buckinghamshire, UK). One aliquot of plasma, urine, feces extract, and rumen fluid was di-
luted in double-distilled water and then passed through a Vicam AflaTest WB immunoaffinity column (Vicam, Watertown, MA). Following elution, the samples were evaporated to dryness under nitrogen and redissolved with the mobile phase for injection into the HPLC apparatus. Concentration of AFB$_1$–albumin adduct in plasma samples was determined using the HPLC method. Briefly, aliquots of each plasma sample (150 μL) were digested with pronase (pronase:total protein, 1:4 wt:wt) at 37°C for 3 h. The digests were purified by a solid phase cartridge (MAX cartridges, Waters Technology China Co. Ltd., Shanghai, China), which was preprimed with methanol and equilibrated with water. The loaded cartridge was sequentially washed with water, 70% methanol, and 1% ammonium hydroxide in methanol at a flow rate of 1 mL/min. The purified AFB$_1$ adducts were eluted with 2% formic acid in methanol, dried in a CentriVap concentrator (Labconco Corp., Kansas City, MO), and reconstituted for HPLC fluorescence detection. Adduct concentrations were adjusted to total plasma albumin content to account for variations in volume (Sabbioni et al., 1990; Qian et al., 2013; Mitchell et al., 2014).

The HPLC system consisted of a Waters binary pump (model 600) and a Waters 2475 fluorescence detector connected to a postcolumn photochemical derivatization unit (PriboFastKRC, PriboLab Pte. Ltd, Qingdao, China). A reversed-phase C18 column (Zorbax SB, 250 × 4.6 mm, 5 μm i.d.; Agilent Technologies Inc., Palo Alto, CA) was used to separate the sample constituents. Detection of AFB$_1$ was performed using a mobile phase of methanol and water (45:55 vol:vol) and 1% phosphoric acid; excitation and emission wavelengths in the detector were set at 360 and 440 nm, respectively. Detection of AFM$_1$ was performed using a mobile phase of acetonitrile and water (25:75 vol:vol) and 1% phosphoric acid; excitation and emission wavelengths in the detector were set at 360 and 430 nm, respectively. Detection of AFM$_1$–albumin adduct was performed using a mobile phase of methanol and 0.2 M sodium phosphate buffer (pH 7.4, 50:50 vol:vol); excitation and emission wavelengths in the detector were set at 405 and 470 nm, respectively. Separation was performed at ambient temperature at a flow rate of 1.0 mL/min. For both the AFB$_1$ and AFM$_1$ standard solutions and sample extracts, the injection volume was 20 μL; for AFB$_1$–albumin adducts, the injection volume was 100 μL. Quantification was based on the peak area and retention time compared with external standards run between every 10 samples. The limits of detection for AFB$_1$, AFM$_1$, and AFB$_1$–albumin adduct using this method were 300 ng/mL, 10 pg/mL, and 0.4 pg/mL, respectively.

**Statistical Analysis**

The data were analyzed using SAS/STAT software (version 9.2; SAS Institute Inc., Cary, NC). All data were tested for normality before analysis using Levene’s test. Data on ADG were analyzed using the GLM procedure that included the treatment effect and error term. Plasma biochemical parameters were analyzed as a randomized complete block design with repeated measures when applicable by a mixed model that included the fixed effects of treatment, time (h) after AFB$_1$ administration, and treatment × time interactions and the random effects of the block and calf within the block. The data for the plasma biochemical parameters obtained from the 0-h time point were added to the model as covariates in the statistical analysis concerned.

Data of the excretion variables of aflatoxin in rumen fluid, plasma, urine, and feces were compared by nonparametric 1-way ANOVA (Mann-Whitney U test; Gratz et al., 2006; Aoudia et al., 2008; Boudra et al., 2013). The clearance pattern of AFM$_1$ by excretion into the urine after the peak of concentration was reached (AFM$_1$ in μg/L) and was studied by adapting the following exponential function to the data:

\[
\text{AFM}_1 = a \exp (b \times t),
\]

where \(a\) is the intercept, \(b\) is the exponential parameter, and \(t\) is the time from the administration of AFB$_1$ in hours (Battacane et al., 2003, 2012).

Noncompartmental analysis was used to estimate the toxicokinetic parameters of AFB$_1$ and AFM$_1$ in the urine of calves using Kinetica version 4.0 (Thermo/Innaphase, Philadelphia, PA; Grün et al., 2009; Boudra et al., 2013): \(\text{Cmax}\) and \(\text{Tmax}\) were peak values defined as the maximum concentration and the time required to reach it, respectively; 
\(\text{AUC}_{0-\infty}\) was the area under the time concentration curve, which was calculated using the trapezoidal method between the first and last measurable concentrations; 
\(\text{AUC}_{0,\text{last}}\) was extrapolated to infinity by the ratio of the last measurable concentration to the terminal slope \(k_e\), 
\(\text{AUC}_{0,\infty} = \text{AUC}_{0,\text{last}} + \text{AUC}_{\text{last},\infty}\); and \(T_{1/2}\) was the terminal half-life determined as the ratio of the natural logarithm of 2 to the terminal slope \(k_e\). The ratio of \(\text{Cmax}\) and \(\text{AUC}\) to the administered dose was calculated to investigate the toxicokinetic linearity. These toxicokinetic parameters were statistically analyzed using the GLM procedure that included the treatment effect and error term.

Statistically significant differences among treatments were assessed using Tukey’s adjustment test. Significance was declared when \(P \leq 0.05\). Data are reported as the least squares means ± standard error of the mean.
RESULTS AND DISCUSSION

No calves showed any evident clinical or health disturbances related to AFB$_1$ administration throughout the entire experimental period. The dose of AFB$_1$ selected in this study was comparable with levels that animals fed naturally contaminated feeds might receive. Although the feed commonly used in the diet of calves does not seem to pose a very high risk for high aflatoxin contamination, sporadic ingestion of relatively high doses of AFB$_1$ can occur. This occurrence could be relevant in light of the consequent levels of AFM$_1$ in contaminated milk from dairy animals (Battacone et al., 2012; Britzi et al., 2013; Xiong et al., 2015) or health problems in calves and growing cattle (Baines et al., 2013).

Growth Performance

The BW of calves at the beginning of our study were similar across the 3 groups (126.21 ± 6.54, 126.80 ± 7.40, and 126.16 ± 6.80 kg for the CON, AFB$_1$ only, and AFB$_1$ plus LGG groups, respectively). No difference was found in ADG among the calves in 3 groups before AFB$_1$ dosing (Figure 1). However, after AFB$_1$ administration, a lower ADG was observed for the AFB$_1$ only calves, although the difference was not significant compared with the CON calves (0.76 ± 0.18 vs. 0.96 ± 0.20 kg/d; $P = 0.08$). Furthermore, the AFB$_1$ plus LGG calves showed an alleviation of this growth faltering effect compared with the calves in the AFB$_1$ only group (0.85 ± 0.08 vs. 0.76 ± 0.18 kg/d; $P = 0.09$; Figure 1), although the ADG of AFB$_1$ plus LGG calves was lower than that before AFB$_1$ administration. Our results were in good agreement with the report published by Lynch et al. (1971), who found significant reductions in a dose-dependent manner in feed intake and BW gain in the AFB$_1$-fed calves. Additionally, our data showed that the administration of LGG alone could not have a big effect on BW gain and that the critical function of LGG might be associated with reducing the availability of free AFB$_1$ within the intestinal tract, thus reducing its toxicity.

Plasma Parameters

To assess the hepatotoxicity of AFB$_1$ administration, plasma biochemical parameters were measured. The results of the ANOVA are shown in Table 1. The AFB$_1$ treatment significantly increased plasma AST, alkaline phosphatase, and LDH levels ($P < 0.01$, $P = 0.02$, and $P < 0.01$, respectively). The results of Qian et al. (2013) showed that ALT was released into the blood quickly after a single dose of AFB$_1$, with levels decreasing back to normal after a stable period. However, no difference in ALT activity was observed after AFB$_1$ treatment compared with the CON calves in our experiment ($P > 0.05$). The AST and ALT activities were characteristic of early and sensitive indicators of hepatocyte injury, before bile duct proliferation and periportal necrosis (Qian et al., 2013). The results showed that AST was a more sensitive indicator in evaluating AFB$_1$-induced liver injury. Administration of LGG reduced the fluctuation in plasma enzyme activity induced by AFB$_1$, with a significant effect on AST and LDH (both $P < 0.05$) compared with the AFB$_1$ only calves. These findings indicated that the probiotic treatment increased AFB$_1$ retention within the gastrointestinal tract and may consequently reduce the toxic effects of a single dose of AFB$_1$ in calves. No significant differences were observed for total protein, albumin, globulin, ALT, or triglyceride levels ($P > 0.05$; Table 1), suggesting that the concentration of AFB$_1$ administered in this trial had only low and transient negative effects on hepatocytes.

Aflatoxin Solubilization, Absorption, and Excretion in the Gastrointestinal Tract

We did not detect AFB$_1$ or AFM$_1$ in any of the samples before AFB$_1$ administration or in the CON group samples after a single dose of AFB$_1$.

In Rumen Fluid. The results of AFB$_1$ solubilization and AFM$_1$ secretion into rumen fluid are shown in
Figure 2A and B. Following a single dose of AFB1, the toxin rapidly distributed in rumen fluid and digesta with rumination and rumen motility. Concentration of AFB1 in rumen fluid collected at 2 h was the highest in all samples, which indicated that the peak was reached after approximately 2 h. The AFB1 clearance in rumen fluid was rapid: it decreased to a relatively low concentration by 48 h and was undetected by 96 h after administration. Large differences in AFB1 concentrations in the rumen fluid among individuals were observed, potentially due to different degrees of rumen filling and water intake between calves. Furthermore, relatively low concentrations of AFM1 were detected and reached a peak at 12 h in rumen fluid. The AFM1 in the rumen fluid may be a consequence of metabolite formation by cytochrome P450 enzyme activity and release by the rumen wall or of rumen microbial activity and salivary circulation. Sulzberger et al. (2017) found that oral supplementation of clay to dairy cattle reduced the amount of free AFB1 in rumen fluid. Similarly, LGG administration reduced the concentration of AFB1 and AFM1 in rumen fluid before AFB1 was completely solubilized. This finding indicated that the binding of toxin to LGG was very rapid.

In Plasma. Furthermore, we evaluated the excretion of AFB1 and AFB1–albumin adduct in plasma (Figure 2C and D). The peak of AFB1 concentration was reached approximately 4 h after the administration of AFB1; then, the concentration decreased quickly in the plasma. Previous studies have indicated that AFB1 was absorbed through the duodenum and then was mainly metabolized in the liver; unabsorbed AFB1 was excreted into the feces (Gratz et al., 2006). The presence of AFB1 in plasma indicated that it was being absorbed and transported in the gastrointestinal tract and other tissues. Gallo et al. (2010) showed that both AFB1 and its hydroxyl derivative AFM1 were detectable in the plasma only 5 min after administration of an oral bolus containing 4.9 mg of AFB1. In the current experiment, the peak of AFB1–albumin adduct concentration appeared at 8 h, with another gentle peak observed at 36 h. The oscillatory pattern in plasma may be due to variations in rumen activity in calves and may depend on the gradual release of the AFB1–albumin adduct from tissue to the blood, on the liver conversion rate of AFB1 to AFM1, and on the excretion of toxins in urine and feces (Battacone et al., 2003). The second absorption phase may be a consequence of enterohepatic cycling of toxin. By comparison, the peak of AFB1–albumin adduct concentration was reached later, indicating that AFB1 was metabolized mainly between 4 and 8 h after a single dose of AFB1.

In Feces. Figure 2E and F shows the concentrations of AFB1 and AFM1 excreted in feces. The concentration peaks of both toxins occurred at 16 h, which were later than those in rumen fluid, blood, or urine; however, the peak values of toxins were higher than those in the other 3 samples. The presence of AFB1 reflected unabsorbed toxin rather than systemic uptake and subsequent biliary excretion because bile mainly contains conjugated rather than free aflatoxin metabolites. The presence of AFM1 in feces suggested that this metabolite was formed by enterocytes, which are known to possess cytochrome P450 enzyme activity necessary for the conversion of AFB1 to AFM1 (Gratz et al., 2006). The peak values of toxins concentration indicated that feces was the main route for AFB1 excretion. Other studies have also reported that feces contains both AFB1 and AFM1, with higher AFB1 concentration, regardless of whether exposure to AFB1 was short term or long term (Firmin et al., 2011). Helferich et al. (1986) showed that oral administration of [14C]-AFB1 to lactating dairy goats

**Table 1. Least squares means of plasma parameters for calves after a single oral dose of aflatoxin B1 (AFB1; 4.80 mg/animal) with or without Lactobacillus rhamnosus GG (LGG) administration (n = 8 calves/group)**

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment</th>
<th>SEM</th>
<th>Effect (<em>P</em>-value)</th>
<th>Treatment</th>
<th>Time</th>
<th>Treatment × time</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP (g/L)</td>
<td>CON</td>
<td>63.73</td>
<td>0.47</td>
<td>0.33</td>
<td>0.43</td>
<td>0.92</td>
</tr>
<tr>
<td>ALB (g/L)</td>
<td>AFB1 only</td>
<td>34.05</td>
<td>0.70</td>
<td>0.24</td>
<td>0.17</td>
<td>0.47</td>
</tr>
<tr>
<td>GLB (g/L)</td>
<td>AFB1 plus LGG</td>
<td>31.35</td>
<td>0.16</td>
<td>0.63</td>
<td>0.29</td>
<td>0.45</td>
</tr>
<tr>
<td>ALB/GLB</td>
<td></td>
<td>1.09</td>
<td>0.02</td>
<td>0.10</td>
<td>0.13</td>
<td>0.38</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td></td>
<td>75.48</td>
<td>1.87</td>
<td>0.01</td>
<td>0.02</td>
<td>0.42</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td></td>
<td>24.84</td>
<td>1.02</td>
<td>0.28</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>AST/ALT</td>
<td></td>
<td>2.97</td>
<td>0.16</td>
<td>0.44</td>
<td>&lt;0.01</td>
<td>0.39</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td></td>
<td>169.72</td>
<td>6.74</td>
<td>0.07</td>
<td>0.04</td>
<td>0.38</td>
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<tr>
<td>TG (mmol/L)</td>
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<td>0.01</td>
<td>0.52</td>
<td>0.04</td>
<td>0.84</td>
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<tr>
<td>LDH (U/L)</td>
<td></td>
<td>829.23</td>
<td>19.22</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.24</td>
</tr>
</tbody>
</table>

*Means within a row with no common superscripts differ (*P* ≤ 0.05) after adjustment by Tukey’s test.

*a*TP = total protein; ALB = albumin; GLB = globulin; AST = aspartate aminotransferase; ALT = alanine aminotransferase; ALP = alkaline phosphatase; TG = triglyceride; LDH = lactate dehydrogenase.
resulted in an excretion rate of feces AFB1 of 52%, indicating that feces was the main route of AFB1 excretion. Furthermore, our data showed that the concentrations of AFB1 and AFM1 in feces between the AFB1 only and AFB1 plus LGG groups were similar but were slightly greater than the concentrations in the AFB1 plus LGG groups.

Figure 2. Effects of *Lactobacillus rhamnosus* GG (LGG) on aflatoxins concentration in rumen fluid, blood, and excretions of calves fed a single dose of aflatoxin B1 (AFB1; 4.80 mg/animal). Each data point represents the mean (±SD) of the 8 animals. (A) AFB1 in rumen fluid; (B) aflatoxin M1 (AFM1) in rumen fluid; (C) AFB1 in plasma; (D) AFB1–albumin adduct in plasma; (E) AFB1 in feces; (F) AFM1 in feces; (G) AFB1 in urine; (H) AFM1 in urine.
group. This effect was mainly due to the complexing of LGG with AFB$_1$ and AFM$_1$ in the gastrointestinal tract, thus increasing the excretion via the feces.

**In Urine.** Aflatoxin M$_1$ has been identified as the main metabolite of AFB$_1$ in urine for humans and laboratory animals, indicating that urine is one of the most important pathways to excrete AFB$_1$ from the body (Hsieh et al., 1994; Thieu and Pettersson, 2009). Last, we evaluated AFB$_1$ and AFM$_1$ excretion in the urine (Figure 2G and H). The AFM$_1$ was excreted in urine by blood circulation. After oral administration, AFB$_1$ and AFM$_1$ were rapidly detected in urine, confirming the intense and rapid absorption of AFB$_1$ in the gastrointestinal tract and its active metabolism in the liver. Peak concentrations of both toxins were reached at 8 h, and the peak value of AFM$_1$ in urine was higher than the peak value in feces. Following the peak, the concentrations of 2 toxins decreased rapidly, reaching relatively low levels in urine collected 36 h after administration in calves. Urine is one of the organic fluids in which aflatoxin metabolites persist the longest after removal of toxin feed (Fernández et al., 1997). The results of the present study indicated that urine was an important vehicle for the elimination of AFM$_1$. Helferich et al. (1986) found that oral administration of [14C]-AFB$_1$ to lactating dairy goats resulted in 30.3% of the total amount of radioactive material being found in urine, indicating that urine was not the main route of AFB$_1$ excretion. Fernández et al. (1997) showed that AFM$_1$ concentration was higher than AFB$_1$ in the urine of dairy goats fed a diet with 5 mg/kg per day of aflatoxin, indicating that AFM$_1$, but not AFB$_1$, was mainly excreted in urine.

The results of the solubilization or excretion of toxins in rumen fluid, blood, feces, and urine showed some common characteristics. The excretion pattern (Figure 2A–H) of AFB$_1$ and its metabolite was surprisingly regular, with toxins detected rapidly in samples after a single oral dose of AFB$_1$. Peak concentrations were sequentially reached in rumen fluid, plasma, urine, and feces (except AFM$_1$ in rumen fluid), followed by an exponential decrease. Moreover, the excretion of both AFB$_1$ and AFM$_1$ was highly variable in all samples. In these excretion curves, AFB$_1$ and AFM$_1$ concentrations were the largest in feces and urine, respectively. Administration of LGG decreased the concentrations of AFB$_1$ and AFM$_1$ in rumen fluid, plasma, and urine but increased the concentrations in feces. The results indicated that the absorption of AFB$_1$ in the gastrointestinal tract of calves was rapid. However, the amount of absorption was competitively reduced by LGG administration. The high variability in toxin concentrations could result from high individual variability among animals, probably related to either differences in plasma volumes or differences in passage of the toxin through the gastrointestinal mucosae (Gallo et al., 2010). Differences in AFB$_1$ carryover found among species in the literature may also be explained by extensive species variability in the expression and catalytic activity of hepatic enzyme families (e.g., cytochrome P450 and glutathione transferase) involved in the biotransformation and detoxification of AFB$_1$ (Pier, 1992; Sun et al., 2016). In our experiment, these data also suggested that LGG was able to retain additional AFB$_1$ and AFM$_1$ inside the gastrointestinal tract of calves, most likely by binding both AFB$_1$ and AFM$_1$ to the bacterial surface. This binding process occurred immediately after AFB$_1$ administration or AFM$_1$ formation.

**Aflatoxin Clearance Pattern and Toxicokinetic Parameters in Urine**

The presence of AFM$_1$ in the urine indicated AFB$_1$ contamination in the diet and absorption into the tissues after ingestion. Aflatoxin M$_1$ is the most sensitive indicator of aflatoxin exposure (Fernández et al., 1997). Therefore, the clearance pattern of AFM$_1$ (Figure 3) and the toxicokinetics of AFB$_1$ and AFM$_1$ (Table 2) in the urine of calves were further analyzed.

The equations fitting the data of urine AFM$_1$ clearance from the peak were represented by the following exponential decreasing functions:

\[
\text{AFB}_1 \text{ only treatment: } \text{AFM}_1 (\mu g/L) = 162.66 \exp (-0.057 \text{h}), \quad R^2 = 0.9734, \quad \text{and}
\]

**Figure 3.** Clearance pattern of aflatoxin M$_1$ (AFM$_1$) in the urine of calves fed a single dose of aflatoxin B$_1$ (AFB$_1$; 4.80 mg/animal) with or without *Lactobacillus rhamnosus* GG (LGG) administration (n = 8 calves/group).
AFB1 plus LGG treatment: AFM1 (μg/L) = 92.315 exp (−0.057 h), R² = 0.9680.

Understanding the absorption and clearance pattern of AFM1 will be beneficial for diagnosing poisoning in farming practices. Our results showed that the clearance of AFM1 in urine occurred mainly in the first 2 d after the administration of AFB1, with a 93.10% decrease in AFM1 concentration compared with the peak value of toxin concentration in the AFB1 only group (Figure 3). Additionally, as shown in Figure 3, the initial part of the clearance curve did not fit perfectly with the data, but the fitting degree increased with time. This case might be due to the fact that the exponential model simulated only the clearance of toxin, but aflatoxins were still absorbed into the body circulation from the gastrointestinal tract at the initial part of the clearance curve. Table 2 showed the results of the toxicokinetics of AFB1 and AFM1 in urine; Cmax, Tmax, AUC0-last, AUC0-∞, and the ratios of Cmax and AUC to the administered dose were significantly decreased by LGG administration (P < 0.01 for both AFB1 and AFM1). In addition, Cmax and AUC0-last could be taken to indirectly represent the amount of toxin. Administration of LGG reduced Cmax by 44.50 and 40.14% for AFB1 and AFM1 compared with the AFB1 only treatment calves, respectively; for AUC0-last, the reduction was 44.34 and 43.31%, respectively. However, there was no significant effect on Tmax in urine between AFB1 only and AFB1 plus LGG calves (P = 0.33 for AFB1; P = 0.15 for AFM1).

Administration of LGG increased the passage rate of AFB1 and its metabolite AFM1 in the gastrointestinal tract; therefore, the concentrations of 2 toxins in feces were greater in the AFB1 plus LGG calves. This effect of sequestering the toxin mainly occurred in rumen fluid. It is worth noting that the complexing of AFB1 by LGG had more rapid kinetics than absorption of AFB1 by the gastrointestinal tract. Some in vitro studies suggested that AFB1 is not removed by degradation but rather becomes physically bound to peptidoglycans of bacteria (Haskard et al., 2001; Vosough et al., 2014). However, further research is needed to establish which components of the peptidoglycan layer of LGG are responsible for the binding of AFB1, if LGG is applied commercially in ruminant products and feeds.

CONCLUSIONS

The absorption, distribution, and excretion of AFB1 and AFM1 were rapid in calves fed a single dose of AFB1 and emerged by the appearance of toxins in rumen fluid, blood, and excretion. Urine was the main route for the excretion of AFM1, and the clearance pattern from the peak of concentration was well fitted by an exponential decreasing function. Administration of LGG reduced the absorption of AFB1 in the gastrointestinal tract by increasing excretion via the feces, thus

Table 2. Toxicokinetics of aflatoxin B1 (AFB1) and its main metabolite aflatoxin M1 (AFM1) in the urine of calves after a single oral dose of AFB1 (4.80 mg/animal)

<table>
<thead>
<tr>
<th>Toxicokinetic parameter</th>
<th>AFB1 only</th>
<th>AFB1 plus LGG</th>
<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cmax (μg/L)</td>
<td>34.43</td>
<td>19.11</td>
<td>2.55</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Cmax/dose</td>
<td>0.91</td>
<td>0.50</td>
<td>0.07</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>8.50</td>
<td>8.00</td>
<td>0.25</td>
<td>0.33</td>
</tr>
<tr>
<td>AUC0-last [h × (μg/L)]</td>
<td>562.59</td>
<td>313.14</td>
<td>36.69</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>AUC0-last/dose</td>
<td>14.80</td>
<td>8.24</td>
<td>0.94</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>AUC0-∞ [h × (μg/L)]</td>
<td>595.94</td>
<td>321.94</td>
<td>38.80</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>AUC0-∞/dose</td>
<td>15.68</td>
<td>8.47</td>
<td>1.02</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>T1/2 (h)</td>
<td>15.76</td>
<td>13.17</td>
<td>0.40</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>AFM1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cmax (μg/L)</td>
<td>148.12</td>
<td>88.67</td>
<td>9.13</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Cmax/dose</td>
<td>3.90</td>
<td>2.34</td>
<td>0.24</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>9.00</td>
<td>8.00</td>
<td>0.46</td>
<td>0.15</td>
</tr>
<tr>
<td>AUC0-last [h × (μg/L)]</td>
<td>2,545.88</td>
<td>1,443.25</td>
<td>153.45</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>AUC0-last/dose</td>
<td>67.00</td>
<td>37.98</td>
<td>4.04</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>AUC0-∞ [h × (μg/L)]</td>
<td>2,594.75</td>
<td>1,473.13</td>
<td>156.06</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>AUC0-∞/dose</td>
<td>68.28</td>
<td>38.77</td>
<td>4.11</td>
<td>&lt;0.01</td>
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<tr>
<td>T1/2 (h)</td>
<td>12.26</td>
<td>12.27</td>
<td>0.13</td>
<td>0.98</td>
</tr>
</tbody>
</table>

1Cmax = maximum concentration; Tmax = time to reach Cmax; AUC0-last = area under the time-concentration curve; AUC0-∞ = AUC0-last extrapolated to infinity; T1/2 = terminal half-life.

2Lactobacillus rhamnosus GG.
alleviating the hepatotoxic effect. In addition, LGG administration significantly affected the toxicokinetics of AFB1 and AFM1 in the urine of calves.

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