**ABSTRACT**

Aflatoxin contamination of feed poses a great risk to the global dairy industry. Analyzing the aflatoxin B1 (AFB1)-induced metabonomic changes in ruminants and screening potential biomarkers for early diagnosis of AFB1 exposure is urgently needed. Here, the effects of different doses (0, 50, and 500 μg/kg of the diet, dry matter basis) of AFB1 exposure on digestibility and performance of Saanen goats were studied, and a comprehensive untargeted metabolomic analysis was performed to reveal plasma metabonomic changes caused by the AFB1 exposure. In the current study, AFB1 exposure decreased total-tract nutrient digestibilities, nitrogen retention, total weight gain, and average daily gain of Saanen goats in a dose-dependent manner. Untargeted metabolomics revealed alterations in the plasma metabolome. A total of 3,310 and 1,462 ion peaks were obtained in positive and negative ion modes, respectively. Based on the screening criteria, 1,338 differential metabolites were detected between control and low-dose AFB1 (50 μg/kg) groups, 1,358 metabolites differed between control and high-dose AFB1 (500 μg/kg) groups, and 58 metabolites differed among all groups. Pathway analyses showed that choline metabolism in cancer and glycerophospholipid metabolism were significantly affected by the AFB1 treatments. Moreover, dysregulation of amino acid metabolism was also observed in AFB1 treated goats. The findings provided novel insights into the toxicity of AFB1 in ruminants. Exploring the underlying molecular causes of the changes may help the development of rapid diagnostic techniques and effective interventions for AFB1 intoxication.

**Key words:** aflatoxins, untargeted metabolomics, biomarker, ruminant aflatoxicosis

**INTRODUCTION**

Mycotoxins are toxic metabolites mainly produced by certain types of molds from *Fusarium, Claviceps, Penicillium, Alternaria, and Aspergillus* genera (Shi et al., 2018). Among all the identified mycotoxins, aflatoxins could be the most documented group due to their high toxicity and contamination rates. Aflatoxins are produced primarily by *Aspergillus* section *Flavi* (Blankson and Mill-Robertson, 2016). Aflatoxin B1 (AFB1) has been considered as the most carcinogenic and prevalent member, and it can be biotransformed into aflatoxin M1 via oxidative reactions, catalyzed by hepatic cytochrome P450 enzymes (Marin et al., 2013; Giovati et al., 2015). Aflatoxin M1 has a high transfer rate to animal products, especially milk (Giovati et al., 2015).

Aflatoxin B1 is very stable and, so far, the commonly used processing methods (e.g., cooking, baking, extrusion, and roasting) are unable to completely eliminate it (Marin et al., 2013; Adebo et al., 2017). Previous studies showed that ingestion of AFB1-contaminated feed could decrease performance, impair liver function, and increase disease susceptibility in cows and goats (Fink-Gremmels, 2008; Battaccone et al., 2012; Elgioushy et al., 2020). However, information on aflatoxins in ruminants is still limited (Elgioushy et al., 2020). Up to now, most studies on AFB1 toxicity were conducted on small laboratory animals such as rats or established cell lines, partly because purified and standard AFB1 is very expensive, the digestive systems of ruminants are more complicated, and they usually have greater feed intakes than laboratory animals (Zhang et al., 2011; Rotimi et al., 2017; Wang et al., 2021). Consequently, available studies on aflatoxicosis in production animals usually chose naturally contaminated feeds as AFB1 sources. However, these feeds may contain various mycotoxins because mycotoxin co-contamination is very common.
in feeds (Shi et al., 2018). The coexistence of multiple toxins in feeds could further complicate the treatment effects and make it difficult to identify the specific effects caused by AFB1.

At present, it is difficult to evaluate the global effects of aflatoxicosis in ruminants. The symptoms of aflatoxicosis are sometimes similar to many other better-known ailments (Ogunade et al., 2018). Thus, investigating the plasma metabolome alterations of AFB1-challenged ruminants may provide new insights into the toxicity of AFB1 and benefit the development of new mitigation strategies for aflatoxicosis.

Untargeted metabolomics aim to examine changes in metabolite profiles between control and experimental groups, which can detect metabolic alterations and screen potential markers for disease diagnosis (Luo et al., 2019). In this study, the untargeted metabolomics technique, based on ultra-HPLC combined with quadrupole time-of-flight MS, was applied to investigate the plasma metabolome alterations of AFB1-challenged Saanen goats. The effect of AFB1 exposure on performance, nutrient digestion, and nitrogen metabolism was also evaluated.

**MATERIALS AND METHODS**

**Animal and Experimental Design**

The procedure for animal care and handling was approved by the Institutional Animal Care and Use Committee, Southwest Minzu University (Chengdu, Sichuan, China), and the study complied with the *Guide for the Care and Use of Agricultural Animals in Research and Teaching* (FASS, 2010).

Eighteen healthy half-sib male Saanen goats with similar BW (31.78 ± 1.81 kg) and body condition, aged 6 mo, were selected as experimental animals. The experiment was conducted in a completely randomized design with 3 treatments and 6 repetitions. Before the experiment started, all goats were treated with ivermectin to prevent and control the parasites, and acclimated to the experimental facility and diet for 14 d. All goats were individually housed in 2-m² pens that were protected from wind and rain with free access to fresh water. Afterward, the goats were randomly assigned to 1 of the 3 treatments and subjected to a short-term AFB1 treatment protocol (Reed et al., 2018). The treatments were (1) control diet (CON; basal diet), (2) low-dose AFB1 diet (LD; oral dose of 50 μg/kg diet DM), and (3) high-dose AFB1 diet (HD; oral dose of 500 μg/kg diet DM). For the 2 AFB1 treatments, the AFB1 challenge was beginning on d 15 and continued for 14 d. The experimental diet (Table 1) was formulated and provided to meet the nutrition requirements of goats (NRC, 2007).

To minimize the interference of aflatoxins that already existed in the diet, all feedstuffs were free of mold contamination and screened for aflatoxins using the AgraQuant aflatoxins test Kit (Romer Labs), and the aflatoxins level in selected feedstuffs were below detection limits (<3 ppb). The amount of diet supplied was adjusted based on the real capability of consumption of the animals to control the orts.

Aflatoxin B1 standard was purchased from Pribolab Co., Ltd. and dissolved in methanol to prepare the concentration of 2 and 0.2 mg/mL as stock solutions that were stored in a refrigerator at 4°C (Cheng et al., 2017). The diet was provided twice daily (0630 and 1830 h). During the experimental period, the calculated amounts of AFB1 solutions were prepared, mixed with 200 g of TMR, and provided to the low-dose and high-dose groups before morning feeding. In the meantime, the CON group received similar quantities of methanol. To guarantee the correct ingestion of AFB1, the remaining quantity of the diet for each goat was provided only when it had consumed all the pre-weighed 200 g of TMR.

**Growth Performance**

All goats were weighted weekly at 0600 h before morning feeding and the ADG was calculated. The carcass study was conducted on d 29 and all goats were killed at the slaughterhouse using standard commercial procedures. Slaughter live weight was recorded immediately before slaughter. The tail, kidneys, pelvic fat, and

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>% of DM</th>
<th>Chemical composition</th>
<th>% of DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn grain</td>
<td>27.0</td>
<td>CP</td>
<td>15.8</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>6.00</td>
<td>Ether extract</td>
<td>2.7</td>
</tr>
<tr>
<td>Soybean hulls</td>
<td>9.30</td>
<td>OM</td>
<td>90.6</td>
</tr>
<tr>
<td>Cottonseed meal</td>
<td>3.00</td>
<td>NFC</td>
<td>29.5</td>
</tr>
<tr>
<td>DDGS</td>
<td>1.20</td>
<td>NDF</td>
<td>42.7</td>
</tr>
<tr>
<td>Molasses</td>
<td>1.20</td>
<td>ADF</td>
<td>23.2</td>
</tr>
<tr>
<td>Corn cob</td>
<td>8.40</td>
<td>Hemicellulose</td>
<td>19.5</td>
</tr>
<tr>
<td>Oat hay</td>
<td>16.0</td>
<td>Ca</td>
<td>1.33</td>
</tr>
<tr>
<td>Alfalfa hay</td>
<td>24.0</td>
<td>P</td>
<td>0.35</td>
</tr>
<tr>
<td>Premix</td>
<td>3.90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1Expressed as % of DM unless otherwise stated.
2NFC = 100 – CP – ash – NDF – ether extract.
3DDGS = distillers dried grains with solubles.
4Premix contained: Ca = 15 to 18%; P (minimum) = 8%; Mg (minimum) = 0.75%; NaCl = 27 to 33%; Cu = 2,500 to 2,700 mg/kg; Se (minimum) = 50 mg/kg; Zn (minimum), 4,000 mg/kg, K (minimum), 0.1%; vitamin A (minimum) = 600,000 IU/kg; vitamin D3 (minimum) = 60,000 IU/kg; vitamin E (minimum) = 4,000 IU/kg.
testes were retained in the carcass, and the HCW was recorded immediately after slaughter.

**Blood Sample Collection and Analyses**

Whole blood samples were collected aseptically from the left jugular vein of all goats after 3 h of morning feeding on d 14, 21, and 28 for the analyses of blood count analyses, and plasma samples were simultaneously processed using 10-mL vacutainer tubes containing EDTA as an anticoagulant. Complete blood count analyses were performed on a fully automated hematology analyzer (Mindray BC-1800). After centrifugation at 1,100 × g for 15 min at 4°C, the plasma was kept frozen at −20°C or −80°C until further analyses.

Untargeted plasma metabolomics analyses were performed on the plasma samples collected at d 28 by Biomarker Tech using Waters Xevo G2-XS Q-TOF mass spectrometer (Waters) coupled to an I-class Waters Acquity ultra-HPLC system. The mass spectrometer was equipped with an electrospray ionization source, and both positive and negative ion modes were performed.

In brief, the plasma sample was thawed at 4°C, and a 100-μL aliquot was taken from each sample and transferred into 500 μL of a pre-cooled methanol/acetonitrile solution (1:1, vol/vol). After vortex mixing and centrifuging (12,000 × g, 4°C) for 15 min, the supernatant was gathered and dried in a vacuum concentrator. Aqueous acetonitrile (acetonitrile: water = 1:1, vol/vol) was used to dissolve the dried supernatant, and vortex mixing and centrifuging (12,000 × g, 4°C) was performed to collect the resulted supernatant for further analyses. Chromatographic separation was conducted on an Acquity UPLC HSS T3 column (1.8 μm, 2.1 × 100 mm; Waters Corp.). The MassLynx 4.2 software (Waters) was used for controlling the acquisition of the mass data. In each data acquisition cycle, data were taken at both low and high collision energies. The quality control sample was prepared by mixing equal amounts of all samples and acetonitrile was used as a blank. The blank and quality control sample was first analyzed to monitor data quality and system stability. A detailed description of the metabolomics analyses could be found in a previously published study (Liu et al., 2020).

**Nutrient Digestibility and Nitrogen Retention**

From d 25 to 28, the total feces and urine of each animal were collected. Urine was collected into buckets containing HCl (10%, vol/vol) to maintain the pH below 3 and minimize nitrogen losses. The total feces and urine collected was weighed, mixed, and recorded daily (Shi et al., 2015). Aliquots urine (10%) and total fecal (20%) output were taken and stored at −20°C until chemical analyses to determine the digestibility and N retention. Nitrogen balances were calculated as the difference between the amount of N ingested and that lost in urine and feces.

**Chemical Analyses**

Feed and feces samples were ground using a Wiley mill with a 1-mm screen before analyses. AOAC International (2005) methods were used to analyze DM (method 934.01) and ash (method 942.05). Total nitrogen and ether extract were determined using the Dumas Dumas nitrogen analyzer and Soxhlet Extractor Sox 416 (Gerhardt), respectively. Neutral detergent fiber and ADF were analyzed by Fibertherm FT12 Fiber Analyzer (Gerhardt). The content of hemicellulose, cellulose, and NFC was evaluated based on the summative equation (NRC, 2001), where hemicellulose = NDF − ADF, cellulose = ADF − ADL, and NFC = 100 − CP − ash − NDF − ether extract.

**Data Processing and Statistical Analyses**

Plasma metabolomic raw data were collected by MassLynx 4.1 workstation and pretreated by the Progenesis QI software package for the extraction, alignment, deconvolution, and normalization of peaks. The software was set to automatically search the selected databases such as Human Metabolome Database (http://www.hmdb.ca/) and METLIN Metabolite Database (https://metlin.scripps.edu/) to identify the metabolites based on m/z, retention time, and fragment information. After being recognized and aligned, all data were normalized to the sum total ion intensity per chromatogram (Peng et al., 2022). Then, the data sets, including peak number (retention time-m/z pair), sample name, and normalized ion intensities, were imported into MetaboAnalyst 4.0 (https://www.metaboanalyst.ca/) to complete data preprocessing, including missing data estimation, median centering, and unit variance scaling. The detailed workflow for data preprocessing and metabolites identification was available in previous studies (Liu et al., 2020; Peng et al., 2022).

The multivariate statistical analyses were performed on the BMKCloud platform (www.biocloud.net). Principal components analysis and orthogonal partial least-squares discriminant analysis (OPLS-DA) were employed to visualize differences in metabolite profiles among samples. The adjusted P-value (false discovery rate) cutoff was set as 0.05 before the principal components analysis and OPLS-DA analysis. Differential metabolites were selected according to the following 3 conditions: minimum fold change (FC) > 1, P-value...
<0.05 in single-dimensional statistical analysis, and variable importance value (VIP) > 1 in the OPLS-DA model (Peng et al., 2022). Moreover, a permutation test was conducted to evaluate the quality of the OPLS-DA models. The goodness-of-fit was represented by $R^2_Y$, and the predictive ability was indicated by $Q^2$.

The differential metabolites were further annotated to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways by the KEGG PATHWAY database (http://www.kegg.jp/kegg/pathway.html). Furthermore, MetaboAnalyst 4.0 was also used to perform receiver operating characteristic (ROC) analysis based on the identified differential metabolites and the ROC curves were constructed by Random forest algorithms (Liu et al., 2020).

The effects of AFB$_1$ on apparent digestibility, nitrogen metabolism, growth performance, and blood cell counts were analyzed using one-way ANOVA by generalized linear model procedure in SAS 9.4 software (SAS Institute Inc.). The statistical model for analysis was $y_{ij} = \mu + T_i + e_{ij}$, where $y_{ij}$ is the observation, $\mu$ is the overall mean, $T_i$ is the effects of AFB$_1$ doses (low and high), and $e_{ij}$ is the random error. Duncan’s test was used for multiple comparisons if the major effect is significant at a 0.05 level. Orthogonal polynomial contrasts were performed to determine linear and quadratic effects of treatments on the responses analyzed. Significant differences were declared at $P \leq 0.05$, and trends were reported at $0.05 < P < 0.10$.

**RESULTS**

**Nutrient Digestibility and Nitrogen Balance**

Results on nutrient digestibility is shown in Table 2. Ingestion of AFB$_1$ quadratically decreased the digestibility of DM ($P = 0.033$) and OM ($P = 0.043$). The digestibility of ether extract ($P < 0.001$), CP ($P = 0.048$), NDF ($P = 0.013$), and ADF ($P = 0.004$) was decreased linearly with increasing AFB$_1$ doses.

Results on nitrogen metabolism (Table 3) showed that goats intoxicated with increasing AFB$_1$ levels ex-

---

### Table 2. Apparent digestibility (%) of nutrients in goats fed the experimental diets

<table>
<thead>
<tr>
<th>Item</th>
<th>AFB$_1$</th>
<th>SEM</th>
<th>$P$-value$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>50</td>
<td>500</td>
</tr>
<tr>
<td>DM</td>
<td>74.0</td>
<td>71.5</td>
<td>71.3</td>
</tr>
<tr>
<td>Ether extract</td>
<td>67.8</td>
<td>66.3</td>
<td>62.8</td>
</tr>
<tr>
<td>CP</td>
<td>75.2</td>
<td>72.7</td>
<td>70.9</td>
</tr>
<tr>
<td>OM</td>
<td>72.2</td>
<td>69.5</td>
<td>69.0</td>
</tr>
<tr>
<td>NDF</td>
<td>60.9</td>
<td>57.7</td>
<td>55.3</td>
</tr>
<tr>
<td>ADF</td>
<td>58.9</td>
<td>55.6</td>
<td>51.6</td>
</tr>
<tr>
<td>Hemicellulose</td>
<td>63.3</td>
<td>60.2</td>
<td>59.6</td>
</tr>
</tbody>
</table>

$^1$Zero = control diet without aflatoxin B$_1$ (AFB$_1$); 50 = 50 μg/kg AFB$_1$ per kg of dietary DM; 500 = 500 μg/kg AFB$_1$ per kg of dietary DM; n = 6 animals/treatment.

$^2$T = treatment effect; L = linear effect; Q = quadratic effect.

---

### Table 3. Nitrogen metabolism in goats fed the experimental diets

<table>
<thead>
<tr>
<th>Item$^1$</th>
<th>AFB$_1$</th>
<th>SEM</th>
<th>$P$-value$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>50</td>
<td>500</td>
</tr>
<tr>
<td>N intake (g/d)</td>
<td>28.8</td>
<td>28.8</td>
<td>28.8</td>
</tr>
<tr>
<td>Fecal N (g/d)</td>
<td>7.15</td>
<td>7.86</td>
<td>8.38</td>
</tr>
<tr>
<td>Urine N (g/d)</td>
<td>5.46</td>
<td>6.66</td>
<td>7.59</td>
</tr>
<tr>
<td>Digestible N (g/d)</td>
<td>21.6</td>
<td>20.9</td>
<td>20.4</td>
</tr>
<tr>
<td>Retained N (g/d)</td>
<td>16.2</td>
<td>14.3</td>
<td>12.8</td>
</tr>
<tr>
<td>N retention rate (%)</td>
<td>56.2</td>
<td>49.6</td>
<td>44.5</td>
</tr>
<tr>
<td>N bioavailability (%)</td>
<td>74.8</td>
<td>68.1</td>
<td>62.9</td>
</tr>
</tbody>
</table>

$^1$Digestible N = N intake − fecal N; retained N = N intake − fecal N − urine N; N retention rate = retained N/N intake; N bioavailability = retained N/digestible N.

$^2$Zero = control diet without aflatoxin B$_1$ (AFB$_1$); 50 = 50 μg/kg AFB$_1$ per kg of dietary DM; 500 = 500 μg/kg AFB$_1$ per kg of dietary DM; n = 6 animals/treatment.

$^3$T = treatment effect; L = linear effect; Q = quadratic effect.
creted more N through feces ($P = 0.048$) and urine ($P = 0.032$) in a linear manner. The digestible N ($P = 0.048$), retained N ($P = 0.002$), N retention rate ($P = 0.002$), and N bioavailability ($P = 0.012$) was decreased linearly with elevated AFB1 levels.

### Growth Performance

Table 4 showed the growth performance of the animals. All groups had similar initial BW at the beginning of the experiment. The final BW, carcass weight, and slaughter rate of the intoxicated goats were always lower than those in the CON group, but the difference was not statistically significant ($P > 0.05$). Exposure to increasing levels of AFB1 linearly decreased ($P = 0.029$) the total weight gain and ADG of the animals.

### Blood Cell Count Parameters

Complete blood cell count parameters are shown in Table 5. The levels of red blood cell count (RBC; $P = 0.028$), hemoglobin (HGB; $P = 0.013$), mean corpuscular volume (MCV; $P = 0.005$), and mean corpuscular hemoglobin ($P = 0.045$) were changed linearly with increasing AFB1 doses. No significant difference in the values of hematocrit, mean corpuscular hemoglobin concentration, and red cell distribution width index (RDW) were observed among groups.

### Metabolite Profiling of Plasma Samples

After checking the data integrity, a total number of 3,310 and 1,462 ion peaks were obtained in positive and negative ion modes, respectively. The supervised OPLS-DA analysis was constructed to visualize the alterations in metabolic profiles among the 3 groups (Supplemental Figure S1; https://doi.org/10.6084/m9.figshare.21194458.v2; Shi, 2022). The OPLS-DA models revealed good separations ($R^2$Y $> 0.9$, $Q^2$ $> 0.4$) among all groups except LD versus HD in negative ion mode. Apparent differences were observed in plasma metabolome among the treatments. The differences between the CON and HD were more obvious than that between CON and LD.

### Differential Metabolites and Their Identification

Based on the screening criteria, 1,307 (CON vs. LD), 1,319 (CON vs. HD), and 506 (LD vs. HD) differential metabolites were detected in positive ion mode, respectively. However, only 31 (CON vs. LD), 39 (CON vs. HD), and 6 (LD vs. HD) differential metabolites were detected in the negative ion mode. As shown in Figure 1, 98.2% of the differed metabolites in the positive mode were upregulated in LD and HD groups when compared with the CON group. Among the differed metabolites, 20 of them were only affected by the low-dose AFB1 (Supplemental Table S1; https://doi.org/10.6084/m9.figshare.21194458.v2; Shi, 2022), 17 metabolites were only affected by the high-dose AFB1 (Supplemental Table S2; https://doi.org/10.6084/m9.figshare.21194458.v2; Shi, 2022), and 58 metabolites were affected by both low- and high-dose AFB1 (Supplemental Table S3; https://doi.org/10.6084/m9.figshare.21194458.v2; Shi, 2022). Fold change of the metabolites was also calculated and included in Supplemental Tables S1–S3.

The 95 metabolites can be categorized into 20 classes; specifically, glycerophospholipids, carboxylic acids and derivatives, and organonitrogen compounds were the 3 most abundant classes. The relative average normalized levels of those metabolites were plotted in a heatmap created by the R package, pheatmap (Figure 2). The metabolites were clustered according to their Pearson correlation coefficients. Obvious alterations in the identified differential metabolites among groups can be observed in the heatmap.

The area under the curve (AUC) of the ROC curves was calculated using the trapezoidal rule and the AUC.

### Table 4. Growth performance of goats fed the experimental diets

<table>
<thead>
<tr>
<th>Item</th>
<th>AFB1</th>
<th>0</th>
<th>50</th>
<th>500</th>
<th>SEM</th>
<th>T</th>
<th>L</th>
<th>Q</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial BW (kg)</td>
<td>31.8</td>
<td>31.8</td>
<td>31.7</td>
<td></td>
<td>0.81</td>
<td>0.995</td>
<td>0.925</td>
<td>0.972</td>
</tr>
<tr>
<td>Final BW (kg)</td>
<td>33.7</td>
<td>33.3</td>
<td>32.3</td>
<td></td>
<td>0.94</td>
<td>0.542</td>
<td>0.284</td>
<td>0.843</td>
</tr>
<tr>
<td>Total weight gain (g)</td>
<td>1,883</td>
<td>1,533</td>
<td>550</td>
<td></td>
<td>401</td>
<td>0.081</td>
<td>0.029</td>
<td>0.694</td>
</tr>
<tr>
<td>ADG (g)</td>
<td>135</td>
<td>110</td>
<td>39.3</td>
<td></td>
<td>28.6</td>
<td>0.081</td>
<td>0.029</td>
<td>0.694</td>
</tr>
<tr>
<td>Carcass weight (kg)</td>
<td>16.6</td>
<td>15.8</td>
<td>15.6</td>
<td></td>
<td>0.58</td>
<td>0.475</td>
<td>0.372</td>
<td>0.409</td>
</tr>
<tr>
<td>Slaughter rate (%)</td>
<td>49.1</td>
<td>47.3</td>
<td>48.2</td>
<td></td>
<td>0.97</td>
<td>0.437</td>
<td>0.897</td>
<td>0.208</td>
</tr>
</tbody>
</table>

1Zero = control diet without aflatoxin B1 (AFB1); 50 = 50 μg/kg AFB1 per kg of dietary DM; 500 = 500 μg/kg AFB1 per kg of dietary DM; n = 6 animals/treatment.  
2T = treatment effect; L = linear effect; Q = quadratic effect.
values of the 95 metabolites (Supplemental Tables S1–S3) ranged from 0.81 to 1.

Metabolic Pathway Analyses

The KEGG enrichment analysis and topology analyses (MetaboAnalyst 5.0) were performed based on the 95 differential metabolites listed in Supplemental Tables S1–S3 (https://doi.org/10.6084/m9.figshare.21194458.v2; Shi, 2022) to investigate and map the metabolic differences among treatments. Pathways with an impact value greater than 0.1 or \( P < 0.05 \) were considered as significantly altered pathways and were labeled in Figure 3.

Pathway topology analysis revealed that the 58 metabolites differed among all groups enriched in 19 pathways, glycerophospholipid metabolism, tyrosine metabolism, caffeine metabolism, and cysteine and methionine metabolism were the most impacted pathways (pathway impact values >0.1). Based on KEGG enrichment analysis, the intestinal immune network for IgA production, small cell lung cancer, proteoglycans in cancer, glycosaminoglycan biosynthesis, and gastric cancer were significant metabolic pathways among treatments. Pathways with an impact value greater than 0.1 or \( P < 0.05 \) were considered as significantly altered pathways and were labeled in Figure 3.

Compared with the CON, the level of LysoPC \([18:3(6Z,9Z,12Z)]\), LysoPC \([22:5(4Z,7Z,10Z,13Z,16Z)]\), LysoPC \([20:5(5Z,8Z,11Z,14Z,17Z)]\), and triethanolamine in HD was significantly increased \((P < 0.05, FC > 2)\), and the level of sn-glycerol-3-phosphoethanolamine in LD was also increased \((P < 0.05, FC = 2.31)\). Notably, 12 of the 58 differential metabolites among all groups were enriched in the glycerophospholipid metabolism pathway, and their levels were significantly upregulated with increasing AFB1 dose \((P < 0.05)\). Moreover, 10 of them were also enriched in choline metabolism in cancer pathway together with diacylglycerols (DG) \([16:0/18:1(11Z)/0:0]\) and DG \([16:0/18:2(9Z,12Z)/0:0]\). Interestingly, DG \([16:0/18:1(11Z)/0:0]\) and DG \([16:0/18:2(9Z,12Z)/0:0]\) were also the 2 metabolites enriched in hepatocellular carcinoma and other 17 significant pathways.

### Table 5. Complete blood cell counts of goats fed the experimental diets

<table>
<thead>
<tr>
<th>Item</th>
<th>AFB(_1)</th>
<th>SEM</th>
<th>T</th>
<th>L</th>
<th>Q</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (10(^9)/L)</td>
<td>11.5</td>
<td>0.84</td>
<td>0.14</td>
<td>0.085</td>
<td>0.312</td>
</tr>
<tr>
<td>RBC (10(^12)/L)</td>
<td>19.8</td>
<td>0.65</td>
<td>0.040</td>
<td>0.028</td>
<td>0.165</td>
</tr>
<tr>
<td>HGB (g/L)</td>
<td>103</td>
<td>2.22</td>
<td>0.028</td>
<td>0.013</td>
<td>0.275</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>33.7</td>
<td>1.05</td>
<td>0.972</td>
<td>0.981</td>
<td>0.814</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>11.4</td>
<td>0.49</td>
<td>0.017</td>
<td>0.005</td>
<td>0.861</td>
</tr>
<tr>
<td>MCHC (g/L)</td>
<td>5.12</td>
<td>0.14</td>
<td>0.122</td>
<td>0.045</td>
<td>0.830</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>330</td>
<td>8.84</td>
<td>0.302</td>
<td>0.232</td>
<td>0.322</td>
</tr>
</tbody>
</table>

\(^1\)WBC = white blood cell count; RBC = red blood cell count; HGB = hemoglobin; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; RDW = red cell distribution width index.

\(^2\)Zero = control diet without aflatoxin B\(_1\) (AFB\(_1\)); 50 = 50 μg/kg AFB\(_1\) per kg of dietary DM; 500 = 500 μg/kg AFB\(_1\) per kg of dietary DM; n = 6 animals/treatment.

\(^3\)T = treatment effect; L = linear effect; Q = quadratic effect.
Figure 2. Heatmaps are generated by the R package pheatmap with standard settings. The colors from green to red indicate the normalized relative levels of metabolites. (A) Metabolites only affected by low-dose aflatoxin B1 (AFB1; 50 μg/kg AFB1 per kg of dietary DM); (B) metabolites only affected by high-dose AFB1 (500 μg/kg AFB1 per kg of dietary DM); (C) metabolites affected by both low- and high-dose AFB1; meta_288: sn-glycero-3-phosphoethanolamine; meta_1414: carbamazepine-O-quinone; meta_262: 4-(2-aminophenyl)-2,4-dioxobutanoic acid; meta_1411: 1-palmitoyl-sn-glycero-3-phosphocholine; meta_1310: 3,4-dihydroxyphenylacetaldehyde; meta_1588: α-[3-(nitrosoamino)propyl]-3-pyridinemethanol; meta_3274: 1-palmitoyl-sn-glycero-3-phosphocholine.
Figure 3. (A, C, and E) Kyoto Encyclopedia of Genes and Genomes pathway enrichment and (B, D, and F) topology analysis of metabolites differed among groups. (A and B) 20 metabolites only affected by low-dose aflatoxin B₁ (AFB₁; 50 μg/kg AFB₁ per kg of dietary DM); (C and D) 17 metabolites only affected by high-dose AFB₁ (500 μg/kg AFB₁ per kg of dietary DM); (E and F) 58 metabolites affected by both low- and high-dose AFB₁. The color of each circle changes according to the $P$-values (more significant changes of metabolites in the pathway are shown by darker colors), whereas the metabolite numbers and pathway impact score is depicted by the size of the circle. The most impacted pathways with the highest statistical significance scores are annotated for clarity.
Fifteen metabolites that differed among all groups (FC: 1.2–4.06, \( P < 0.05 \)) were associated with AA metabolism, including metabolic pathways associated with phenylalanine (ko00360; salicylic acid), arginine, and proline (ko00330; creatinine and pyrrole-2-carboxylic acid), lysine (ko00300 and ko00310; saccharopine, 2,4-diaminobutyric acid, and L-cystathionine), cysteine and methionine (ko00270; L-cystathionine and 5-methylthioribose), tyrosine metabolism (ko00350; 3,4-dihydroxyphenylacetaldehyde, homogentisic acid, leucodopachrome, 4-fumarylacetoacetic acid), and tryptophan metabolism (ko00380; 3-methyldioxyindole, indole, and tryptophanol, 6-hydroxymelatonin), and their content was significantly increased with elevated AFB1 doses.

**DISCUSSION**

To achieve more reliable results, animals used in this study were strictly selected before the experiment to minimize individual variations. Natural AFB1-contaminated feeds are usually co-contaminated with many other mycotoxins; therefore, we selected purified standard AFB1 to avoid the interference of other toxins derived from contaminated feeds. During the feeding experiment, all goats appeared to be clinically normal and no deaths resulted from the intoxication.

**Nutrient Digestibility and Nitrogen Balance**

Our results suggested that when the AFB1 level in the diet exceed 50 μg/kg, the total-tract digestibility of most nutrients in goats could be affected. This is in line with the previously published observations. For instance, Edrington et al. (1994) reported decreased intake, daily gain, and feed efficiency in growing lambs intoxicated with 2.5 mg/kg aflatoxin. Aflatoxin B1 exposure also decreased the apparent total-tract digestibility of DM and ether extract of pigs at different growing stages (Pu et al., 2021).

**Growth Performance**

The decreased growth performance agreed with previous observations. Fernández et al. (1996) reported that the reduction in BW is the most sensitive indicator of aflatoxin intoxication in growing lambs (Fernández et al., 1996). In another study, Fernández et al. (2000) also observed a nonsignificant decrease in BW and a significant decrease in ADG in lambs intoxicated with a diet contaminated with 2 ppm aflatoxin.

Sun et al. (2018) found that the AFB1-contaminated diet decreased BW and ADG of male rabbits. Feeding a diet supplemented with 280 μg/kg AFB1 tended to decrease the final BW and ADG of pigs (Pu et al., 2021). The decreased growth performance of intoxicated animals could have partly resulted from the suppressed nutrient digestibility and nitrogen retention.

**Blood Cell Count Parameters**

Hematological indices could reflect the pathological, physiological, and nutritional status of intoxicated animals. We found no significant difference in RBC, HGB, and MCV among groups before the AFB1 challenge (data not shown). Erythrocytes are the main medium for delivering oxygen to tissues and organs. The levels of RBC and HGB are associated with erythrocyte function, which delivers oxygen to organs and supports aerobic respiration and metabolism (Schaer et al., 2013). Increased RBC and HGB in lambs fed 2.5 ppm of aflatoxins have also been reported by Fernández et al. (1996). They inferred that the relative polycythemia was probably associated with the decrease in water intake because many studies observed an increase in hematological parameters in animal aflatoxicosis due to the hemoconcentration phenomenon. In another study, the MCV and HGB were elevated in mice treated with fumonisins B1 for 8 wk (Chen et al., 2021).

Zhang et al. (2020) found that orally administered with 0.75 mg/kg AFB1 decreased RBC and HGB, whereas MCV was not affected. The difference in results might be partly due to the discrepancy in animal species, dietary level, and sources (purified or naturally contaminated) of AFB1.

**Metabolite Profiling of Plasma Samples**

The parameters R²Y (model interpretation rate) and Q² (predictive ability of the model) were greater than 0.4 of most OPLS-DA models, indicating these models were stable and reliable. Cross-validation and permutation tests for these models showed that the Q² intercept is close to or underlies zero, indicating that there was no overfitting (Yong et al., 2021). Symbols representing different groups were distributed to different sides of the OPLS-DA score plots, suggesting that clear differences existed in the plasma metabolite profiles of different groups. Our results agreed well with previous studies that dense peaks and ions identified in plasma by positive modes are far more than that by negative modes, because most of the plasma metabolites (e.g., glycerophospholipid and AA) can be easier ionized at positive modes (Zhao et al., 2018).
**Differential Metabolites and Their Identification**

There is no uniform standard for differential metabolites and biomarker selection so far, and the differential metabolites selected may not be really useful as clinical biomarkers. Receiver operating characteristic curve analysis has been widely considered as an objective and statistically valid method for biomarker performance evaluation (Xia et al., 2013). The AUC values >0.8 means good utility, and >0.9 means excellent performance. Researchers suggested all metabolomic studies present ROC analysis results to facilitate further discovery and validation studies (Xia et al., 2013).

When taking the FC and AUC (FC >3, AUC >0.8) into consideration simultaneously, 5 metabolites between CON and LD, 6 metabolites between CON and HD, and 29 metabolites among the 3 groups could be considered as the most significantly altered metabolites, and might be used as biomarkers for discriminating CON, LD, and HD groups. Those metabolites might have the potential to be used as new biomarkers to diagnose aflatoxicosis in goats induced by different AFB1 doses. One limitation of our study was that the level of some reported blood biomarkers of aflatoxicosis in humans and animals, such as AFB1-albumin adduct and AFB-lysine adduct (Xue et al., 2016), was not determined. Further studies focused on comparing the identified metabolites with existing markers and validating their effectiveness in aflatoxicosis diagnosis are highly needed. Moreover, the AUC values could be affected by many factors such as small study sample sizes (Carter et al., 2016), more investigations with different animals, and larger sample sizes are encouraged.

To exert its carcinogenic potential, AFB1 must be metabolically activated by P450 enzymes in the liver, whereas the critical P450 enzymes in different animals have yet to be identified. Alpha-[3-(Nitrosoamino) propyl]-3-pyridinemethanol, 4-Oxo-1-(3-pyridyl)-1-butanone, and 3-hydroxyldocaine were related to cytochrome P450 metabolism (ko00980 and ko00982), and their levels were significantly upregulated (FC > 2.36, P < 0.01) with increasing doses of AFB1. In the human liver, P450s are critical for the formation of 4-Oxo-1-(3-pyridyl)-1-butanone (keto aldehyde), which could methylene DNA, and is believed to be important in the carcinogenic progress (Rendic and Guengerich, 2021). Moreover, car bamazepine-O-quinone, lidocaine, and S-(1,2-dichlorovinyl) glutathione were also related to cytochrome P450 metabolism (ko00980 and ko00982), and their content was significantly increased (FC > 2.38, P < 0.01) in LD when compared with CON. Further study is encouraged to evaluate the potential of these metabolites used as targets to block the activation of AFB1 in the liver.

Sphingosine has been reported as a pro-apoptotic factor in various cell types and may play important role in some apoptotic systems. It is also able to induce apoptosis in a mitochondrion-dependent manner (Cuvillier, 2002). In the present study, the content of plasma sphingosine was increased with AFB1 doses (Supplemental Table S3), and the relationship between sphingosine and cell apoptosis in ruminants deserves further research.

**Metabolic Pathway Analyses**

Based on our results, glycerophospholipid metabolism (ko00564) and choline metabolism in cancer (ko05231) pathways were the most affected pathways in AFB1 intoxicated goats, and many common metabolites were shared between them.

The elevation of plasma choline and phosphocholine might be related to the hepatotoxicity and carcinogenicity of AFB1. Zhang et al. (2011) observed a significant elevation of choline metabolites (choline, phosphocholine, glycerophosphocholine) in plasma, and increased lipids and AA (tyrosine, histidine, phenylalanine, leucine, isoleucine, and valine), choline, and nucleic acid metabolites in the liver of rats exposed to AFB1.

Choline metabolism could be affected in various types of cancers, and increased levels of phosphocholine and total choline-containing compounds have increasingly been considered as endogenous biomarkers of many cancers (Glunde et al., 2015). Detecting changes in choline metabolism in plasma could prove to be a useful way in early tumor identification (Glunde et al., 2015). Research confirmed that several enzymes in choline, ethanolamine, and phospholipid metabolism are involved in tumor progression and carcinogenesis (Cheng et al., 2016). Further studies on discovering the genes and signaling pathways leading to the changes in choline-containing metabolites are needed.

Glycerophospholipids are major lipid constituents of cell membranes and play vital roles in cell proliferation, differentiation, and apoptosis (van Meer et al., 2008). The content of phospholipids was increased during cell transformation and tumor progression (Cheng et al., 2016). Aberrant choline and ethanolamine phospholipid metabolism have been observed in all cancers so far (Cheng et al., 2016). Liver injury and disturbed expression of genes associated with lipid metabolism were also observed in rats exposed to 0.5 and 1 mg/kg of AFB1 for 7 d (Rotimi et al., 2017). Research on Hep3B cells reported similar findings. Wang et al. (2021) evaluated the effects of AFB1 exposure on metabolomic changes
of Hep3B cells, and a marked elevation in glycerophospholipids level was observed. The level of phosphocholine, 12 phosphatidylethanolamine, and 9 phosphatidylyserine metabolites was significantly increased in high-dose AFB1 cells. Moreover, elevated level of choline and phosphocholine in AFB1-exposed cells was also observed. Their results indicated disturbed structural integrity of cell membranes was induced by AFB1 because choline, phosphocholine, and phosphocholine are essential elements of them. Enzymes mediating the abnormal choline metabolism are being investigated as targets for cancer therapy. The role of the choline metabolism in ruminant AFB1 intoxication and the underlying molecular causes merits further investigation.

Dysregulation of AA metabolism was also observed in our study. The levels of the 15 differential metabolites among all groups related to AA metabolism were upregulated with increasing AFB1 levels. This is agreed with a previous study that AFB1 could induce significant changes in AA metabolism in Hep3B cells (Wang et al., 2021).

**CONCLUSIONS**

Exposure to AFB1 decreased total-tract nutrient digestibility, N retention, and ADG of Saanen goats. The ability of ruminants to inactivate AFB1 might be limited and the long-standing hypothesis that ruminants are less vulnerable to AFB1 may need to be reevaluated. Untargeted metabolomics successfully revealed the alterations in plasma metabolome. Metabolic changes in the high-dose group were more obvious than that in the low-dose group. Fifty-eight metabolites were significantly affected by both low and high doses of AFB1, and there is merit in the further investigation to explore the potential of using them as biomarkers for AFB1 intoxication diagnosis. There were remarkable changes in choline metabolism, glycerophospholipid metabolism, and AA metabolism pathways. Exploring the underlying molecular causes of the changes may help the development of rapid diagnostic techniques and preventive strategies for AFB1 intoxication.

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