The biochemical and metabolic profiles of dairy cows with mycotoxins-contaminated diets

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26 ABSTRACT

27 **Background:** Previous studies on the effects of mycotoxins have solely focused on their biochemical
28 profiles or products in dairy ruminants. Changes in metabolism that occur after exposure to mycotoxins, as
29 well as biochemical changes, have not been explored.
30 **Methods:** We measured the biochemical and metabolic changes in dairy cows after exposure to mycotoxins
31 using biochemical analyses and nuclear magnetic resonance. Twenty-four dairy cows were randomly
32 assigned to three different treatment groups. Control cows received diets with 2 kg uncontaminated
33 cottonseed. Cows in the 50% replacement group received the same diet as the control group, but with 1 kg
34 of uncontaminated cottonseed and 1 kg of cottonseed contaminated with mycotoxins. Cows in the 100%
35 replacement group received the same diet as the control, but with 2 kg contaminated cottonseed.
36 **Results:** The results showed that serum γ-glutamyl transpeptidase and total antioxidant capacities were
37 significantly affected by cottonseed contaminated with mycotoxins. There were also significant differences
38 in isovalerate and NH_3-N levels, and significant differences in the eight plasma metabolites among the three
39 groups. These metabolites are mainly involved in amino acid metabolism pathways. Therefore, the results
40 suggest that amino acid metabolism pathways may be affected by mycotoxins exposure.

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INTRODUCTION

Mycotoxins are toxic secondary metabolites produced mainly by pathogenic molds that infect plants or crops, and by fungi in the genera *Aspergillus*, *Fusarium*, and *Penicillium* (*Fink-Gremmels, 2008*). Several hundred mycotoxins from animal feed have been identified and different studies have shown that most feed samples were infected with at least one mycotoxin (*Rodrigues & Naehrer, 2012*). The main types of mycotoxins that are extremely harmful to animals and their products are aflatoxins, deoxynivalenol (DON), zearalenone (ZEA), T-2 toxin, fumonisin, and ochratoxin A (*Fink-Gremmels, 2008*; *Rodrigues & Naehrer, 2012*).

Because of the potential hazards of mycotoxins, the effect of mycotoxins on dairy animal health is a major concern (*Fink-Gremmels, 2008*). Dairy cows may be regularly exposed to a mixture of mycotoxins derived from different ingredients in their diets. However, previous studies focused on investigating basic blood biochemical parameters and animal performance suggest that dairy cows are more unresponsive to mycotoxins than monogastric livestock because mycotoxins are readily degraded by rumen microbes (*Dänicke et al., 2010*; *Pier, 1992*; *Santos & Fink-Gremmels, 2014*). This was supported by the result that milk production tended not to decrease in lactating ewes fed diets contaminated with aflatoxin B1 (AFB1) (*Battacone et al., 2009*). Additional data has found that even calves are able to tolerate moderate feeding with different amounts of grains that are naturally contaminated with *Fusarium* toxins (*Martin et al., 2010*). Nevertheless, it is possible that dairy ruminants are more vulnerable to mycotoxins under high pressure of milk production *(Jouany et al., 2005; Santos & Fink-Gremmels, 2014*). Previous studies have demonstrated that exposure to AFB1 or mixed mycotoxins can change the antioxidant states of dairy cows and goats (*Huang et al., 2018*; *Xiong et al., 2015*), and it is possible that these changes may be reflected at minor physical levels. Thus, the effects of mycotoxins on animal performance still require further investigation.

Some studies have examined the effects of mycotoxins from the perspectives of molecular biology and metabolomics. In particular, it has been shown that exposure to AFB1 can influence the Nrf2 signaling pathway via mitochondrial reactive oxygen species (ROS)-dependent signaling pathways, specifically by impairing mitochondria functions and activating the generation of ROS (*Liu & Wang, 2016*). Mycotoxins can thus impair metabolic status, so it is necessary to investigate the dangers of mycotoxin contamination. Nuclear magnetic resonance (NMR) spectroscopy is a relatively simple method that can provide rich information about biochemical processes that are sensitive to the dynamic metabolic status of the organism (*Maher & Rochfort, 2014*; *Nicholson & Wilson, 2003*). Many metabolomics studies in different organisms have been conducted using NMR after exposure to mycotoxins (*Cheng et al., 2017*; *Liu et al., 2013*; *Zhang et al., 2011*). AFB1 exposure can significantly increase glucose and fatty acid levels, but according to NMR analyses, it can reduce the concentrations of lactate, ketone bodies, and amino acids in the serum (*Cheng et al., 2017*). Specific blood metabolites of dairy goats were induced by AFB1 exposure, indicating that endogenous metabolic alterations occur after exposure to mycotoxins. Therefore, changes in blood metabolic substrates of dairy ruminants can reflect physical conditions when animals are affected by internal or external factors (*Cheng et al., 2017*; *Fink-*

83 *Gremmels, 2008; Huang et al., 2018*).

84   Despite several related studies, little research has been conducted on the potential metabolic signatures of

85 dairy cows when exposed to mycotoxins. In the present study, we assessed biochemical parameters that reflect

86 basic physiological conditions and changes in biomarkers based on plasma metabolomics using NMR

87 investigation.

88 MATERIALS AND METHODS

89 Preparation of diets contaminated with mycotoxins

90 Mycotoxins were cultivated and enriched on cottonseed contaminated with toxigenic fungi. We added water at

91 a ratio of 30% relative to the weight of the uncontaminated cottonseed, and uniformly mixed the water and

92 cottonseed. The wet cottonseed was then covered and placed in a relatively closed environment in order to

93 facilitate the growth of fungi and accumulation of mycotoxins in the mixture. We measured the concentration of

94 mycotoxins every 3 days (the cultivation period was set at 15 days, based on pilot experiments). When the AFB1

95 concentration in the cottonseed reached a suitable level, we dried the cottonseed out of the sunlight to stop the

96 accumulation of mycotoxins.

97 Determination of mycotoxins in contaminated cottonseed

98 An AFB1 concentration of 20 μg/kg in the diet (dry matter) was the target concentration based on the limit

99 specified by the European Union (EU) (*European Commission, 2002*). Additionally, the concentrations of two

100 other mycotoxins, ZEA and DON, were also tested.

101   The concentrations of AFB1, ZEA, and DON were determined using the ROSA FAST Quantitative Test

102 (Charm Sciences Inc, Lawrence, MA, USA). Five g of contaminated ground cottonseed was mixed with 5 mL

103 of methanol or water, and then shaken for 3 min. Next, 100 μL of the supernatant was removed and mixed with

104 1 mL of dilution buffer. Finally, 100 μL of the mixture was removed with a micropipette and spotted onto the

105 test strip placed in an incubator for 5 min. The results were visually interpreted by two trained technicians using

106 the ROSA Reader (ROSA Pearl Reader, Charm Sciences Inc., Lawrence, MA, USA). The results showed that 1

107 kg of contaminated cottonseed contained 20.08 μg AFB1 and 80.13 μg ZEA, but DON was not detected.

108 Animals, diets, and experimental design

109 The experiment was conducted at Ningxia Helan Sinofarm Dairy Farm (Ningxia, China) from December 17,

110 2015 to December 31, 2015 with an experimental period of 14 days. In total, 24 multiparous Holstein cows in

111 late lactation (lactation length = 283 ± 22 days, milk yield = 21.1 ± 2.6 kg/day, parity = 2.5-3.5 times) were

112 used in this study. The cows were raised in an open-sided, free stall barn with a sand bed, and were equipped

113 with neck clips to allow a measured feed intake. Each of the three treatments had eight individuals randomly

114 assigned to them. Cows in the control group received the uncontaminated diet (which included 2 kg of

115 uncontaminated cottonseed). Cows in the 50% replacement group received the same diet as the control group,

116 but the cottonseed component had 1 kg of uncontaminated cottonseed and 1 kg of cottonseed contaminated with

117 mycotoxins. Cows in the 100% replacement group received the same diet as the control group, but the cottonseed

118   component had 2 kg of cottonseed contaminated with mycotoxins (**Table 1**). Feedings occurred at 8:30, 16:30,

119   and 00:30.

120   All of the experimental procedures were approved by the Animal Care and Use Committee of the Institute

121   of Animal Science, Chinese Academy of Agricultural Sciences (Protocol: IAS15020). The diets were formulated

122   to meet and exceed the nutrient requirements for lactating Holstein cows (*NRC, 2001*). The ingredients, nutrients,

123   and energy contents of the basal diets are listed in **Table 1**.

124   **Sample collection and test methods**

125   **Diet samples**

126   Diet samples were weighed, dried at 65°C for 72 h, and stored at -20°C for subsequent analyses. The nutrient,

127   energy, and mineral ion indices were analyzed, including crude protein, fat, Ca, P, ash, non-fiber carbohydrate,

128   and neutral detergent fiber (**Table 1**).

129   **Blood parameters**

130   Blood samples were collected from the jugular vein using vacutainer tubes (with and without anticoagulant) after

131   milking on day 7. The samples were allowed to settle for about 1 h before centrifugation at $3000 \times g$ and 4°C

132   for 20 min to obtain serum, which was then frozen at -70°C for subsequent analyses. The serum samples were

133   analyzed to determine routine biochemical parameters using an Auto-Analyzer 7020 (Hitachi High-

134   Technologies Corp., Tokyo, Japan) with colorimetric commercial kits (DiaSys Diagnostics Systems GmbH,

135   Holzheim, Germany). Tested parameters included: alanine aminotransferase (ALT), aspartate aminotransferase

136   (AST), γ-glutamyl transpeptidase (GGT), alkaline phosphatase (ALP), total protein, albumin, globulin,

137   albumin/globulin ratio, urea, creatinine, uric acid, total bilirubin, direct bilirubin, indirect bilirubin, triglycerides,

138   and total cholesterol. Another set of serum samples for determining immune and antioxidant indices was sent to

139   Beijing CIC Clinical Laboratory (Beijing, China), where the concentrations of immunoglobulin M (IgM), IgA,

140   and IgG were determined using bovine immunoglobulin ELISA kits (Shanghai Meilian BioTech Company,

141   Shanghai, China). The methods used to determine the total antioxidant capacity (T-AOC), superoxide dismutase

142   (SOD), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) levels were described in previous

143   studies (*Cheng et al., 2017*; *Xiong et al., 2015*).

144   **Concentrations of volatile fatty acids (VFAs) and NH$_3$-N in rumen fluid**

145   Rumen fluid was collected using an oral stomach tube about 1 hr after the morning feeding on day 7, as described

146   in a previous study (*Shen et al., 2012*). The first 50-100 ml of flowing liquid was discarded to avoid

147   contamination with saliva, and the oral stomach tube was washed twice using fresh water before the next

148   sampling. The concentrations of rumen VFAs were determined using a gas chromatography system equipped

149   with flame ionization (GC 6890N, Agilent, Wilmington, DE). The rumen liquid was deproteinized with 2.0 mL

150   of 25% metaphosphoric acid and frozen before each VFA analysis. Helium was used as the carrier gas, and a

151   mixture of six species of VFAs (Supelco Inc., Bellefonte, PA, USA) was used as the identification standard. The

152   initial and final temperatures in the oven were 55°C and 195°C, respectively, and the detector and injector were

153   set to 250°C. The samples of deproteinized ruminal fluid were neutralized with potassium hydroxide solution

154 and then centrifuged at $1200 \times g$ for 10 min. Ruminal NH_3-N was analyzed with a UV-2000 ultraviolet visible

155 spectrophotometer (Unico Instrument Co. Ltd, Shanghai, China).

Metabolomic analysis of plasma by 1H NMR

157 Deuterium oxide (D_2O) and deuterated chloroform were purchased from Cambridge Isotope Laboratories, Inc.

158 (Tewksbury, MA, USA). In addition, 3-(trimethylsilyl) propionic-2,2,3,3,d4 propionic acid sodium salt was

159 purchased from Merck Inc. (Kirkland, QC, Canada). HPLC-grade methanol, methyl tert-butyl ether, water,

160 formic acid, and ammonium formate were purchased from Merck (Darmstadt, Germany).

161 Twenty-four frozen plasma samples were thawed at room temperature and 200 μl of each sample was mixed

162 with 400 μl of buffer (45 mM NaH_2PO_4/K_2HPO_4; 0.9% NaCl; pH: 7.4; 50% D_2O). After shaking and mixing,

163 the sample was centrifuged at 4°C and $16,099 \times g$ for 10 min. The supernatant was placed in a 5-mm NMR tube

164 for examination. A Bruker AVIII 600 MHz NMR (proton resonance frequency = 600.13 MHz, ultra-low

165 temperature probe; Bruker BioSpin GmbH, Rheinstetten, Germany) system was used for 1H NMR analysis of

166 the blood samples.

167 In order to attenuate the NMR signals to avoid the influence of macro-molecules, a water-pre-saturated

168 Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence was employed (recycle delay- 90° -(τ-180°-τ)$_n$-

169 acquisition). A one-dimensional CPMG pulse sequence with pre-saturated pressurized water was used to detect

170 the small molecule metabolites in each sample. The experimental parameters were as follows: spectral width,

171 12,000 Hz; waiting time, 2 s; mixing time, 100 ms; and sampling number, 32K. NMR spectra were manually

172 phased, baseline corrected, and referenced to TSP ($CH3$, $\delta 0.00$) using Bruker Topspin 3.0 software (Bruker

173 GmbH, Karlsruhe, Germany). NMR spectra were visually inspected using Amix 3.9.13 (Bruker, Biospin, Italy).

174 Finally, the NMR spectra were integrated over the 9.0-0.5 ppm range using an interval of 0.002 ppm and the

175 water peak (δ 5.20-4.20) was removed.

Data analysis

177 Blood biochemical parameters, antioxidant and immune indices, rumen fluid VFA levels, and NH_3-N data were

178 analyzed using analysis of variance in SPSS Statistics, and post-hoc tests were conducted (IBM SPSS Statistics

179 v19.0, SPSS Inc., Chicago, IL, USA). The statistical models included treatments as the fixed effects and cows

180 within treatment as the random effect. The data for food intake and milk yield before the first day of the treatment

181 period were used as a covariate in the statistical analysis. Tukey's multiple comparisons adjustment was used to

182 determine significant differences between least squares means. Statistically significant differences were accepted

183 at $P <$ 0.05.

184 The 1H NMR spectra were subjected to Fourier transformation, phase adjustment, baseline correction, and

185 calibration using MestReNova V7.0 software (Mestrelab Research SL, Santiago de Compostela, Spain). To

186 improve the signal-to-noise ratio, all of the spectra were multiplied by an exponential function of 1 Hz before

187 Fourier transformation. The 1H NMR spectra were referenced to an internal lactic acid CH_3 resonance at 1.33

188 μg/kg. The 1H NMR spectra were segmented into consecutive non-overlapping regions comprising 0.002 μg/kg

189 chemical shift "bins" between 0.5 and 9.0 μg/kg. The residual water peak at 4.18-6.70 μg/kg was removed from

190   the data. The normalized data were analyzed by multivariate analysis using SIMCA-P+ software (V11.0

191   Umetrics AB, Umea, Sweden). First, the 1H NMR spectra were analyzed using principal component analysis

192   (PCA) based on mean center scaling to reflect the overall differences. Next, the spectra were analyzed by

193   supervised methods of partial least-squares discriminant analysis (PLS-DA) and orthogonal partial least-squares

194   discriminant analysis (OPLS-DA) (*Lundstedt et al., 2007*). The quality of each model was determined based on

195   the goodness of fit parameter (R2) and a goodness of prediction parameter (Q2) (*Eriksson et al., 2008*). The

196   statistical significances of differences in the metabolite concentrations and appropriate correlation coefficients

197   were determined by OPLS-DA.

RESULTS

Blood biochemical, antioxidant, and immune parameters

200   There were no significant differences in most of the serum parameters (**Table 2**). However, there was a

201   significant difference in GGT between the control group and the 50% replacement group ($P < 0.05$), although

202   there was no significant difference in GGT between the control group and the 100% replacement group. In

203   addition, the difference in T-AOC between the control group and the 100% replacement group reached a very

204   significant level, but there was no significant difference between T-AOC in the control group and the 50%

205   replacement group.

Rumen function

207   The ruminal concentrations of VFAs and NH_3-N were used as indicators of rumen fermentation and the effects

208   of dietary treatments. We found that mycotoxins significantly increased ($P < 0.05$) the rumen NH_3-N

209   concentration in the 100% replacement group but not in the 50% replacement group (**Figure 1a**). In addition,

210   the contaminated cottonseed did not affect the total amount of rumen VFAs (**Figure 1b**). Interestingly, the

211   different levels of added contaminated cottonseed did not affect the concentrations of acetate, propionate,

212   butyrate, and valerate, but the contaminated cottonseed had significant effects on isovalerate ($P < 0.05$) (**Table**

213   **3**). The isovalerate concentrations in the control group differed from those in the 50% replacement group ($P <$

214   0.05), but there were no significant differences between the control group and the 100% replacement group.

Metabolomic profiling of plasma based on NMR analysis

216   Representative 1H NMR spectra (δ 9.0-0.5) of the plasma samples obtained from the control group, 50%

217   replacement group, and 100% replacement group are shown in **Figure 2**. These metabolites were identified and

218   compared based on data obtained from a previous study *(Nicholson & Wilson, 2003)* and the ChenomX spectral

219   database (Edmonton, AB, Canada). The data were subsequently analyzed using multivariate statistics, i.e., PCA,

220   PLS-DA, and OPLS-DA.

221   To obtain an overview of the data, PCA was performed to identify the two principal components (PC1 and

222   PC2), where the cumulative variance contribution rate of the 1H NMR spectra was 90.9% (PC1 = 81.5% and

223   PC2 = 9.4%) (**Figure 3A**). However, there was some overlap and one or two discrete values. **Figures 3B, 3C,**

224   **and 3D** show that the control and the 50% replacement group, the control and the 100% replacement group, and

225 the 50% replacement and 100% replacement group, respectively, were not well separated from each other, so it

226 was necessary to use supervised methods for further analysis. OPLS-DA increased the number of principal

227 components until the variance explained by the model (R2) or the predictive variance of the model (Q2) reached

228 2%. The validity of the model was established by conducting cross and permutation tests (200 times). After this

229 procedure, OPLS-DA score plots (left panel; **A**, **D**, and **G**) derived from 1H NMR spectra for plasma and the

230 corresponding coefficient loading plots (right panel; **B**, **C**, **E**, **F**, **H**, and **I**, in which, **B**, **E** and **H** are images

231 magnified 20 times) were shown in **Figure4**, the control and 50% replacement groups were discriminated with

232 R2X = 32.5% and Q2 = 0.4 (**Figure 4A**), the control and 100% replacement groups with R2X = 29.3% and Q2

233   = 0.374 (**Figure 4D**), and the 50% and 100% replacement groups with R2X = 26.5% and Q2 = 0.316 (**Figure**

234 **4G**).

235   The plasma spectra mainly contained signals from glycoproteins, glucose, amino acids, creatinine, and

236 citrate metabolites. The plasma concentrations of the eight metabolites differed significantly among the three

237 groups according to the results obtained by 1H NMR analysis (**Table 4**), particularly amino acids comprising

238 alanine, lysine, glutamine, and glycine, the carboxylic acids creatinine and citrate, glucose, and O-acetyl-

239 glycoprotein. Using the metabolomics view map derived from pathway topology analysis (MetaboAnalyst 3.0),

240 we found the enrichment analysis and path impact values of different pathways. The pathways ranked in the top

241 three were aminoacyl-tRNA biosynthesis, nitrogen metabolism and alanine, and aspartate and glutamate

242 metabolism (**Figure 5**).

243 ## DISCUSSION

244 **Biochemical parameters in blood**

245 Many components of blood can reflect physiological functions as well as immune and antioxidant activities in

246 the body. For example, GGT, AST, ALT, and ALP are used as liver function indicators (*Edrington et al., 1994*;

247 *Xiong et al., 2015*). We found no significant differences in most of the serum parameters in the control and two

248 treatments. However, two variables were affected by the intake of mycotoxins. The blood concentration of GGT

249 is considered an indicator of liver function in ruminants (*Osorio et al., 2014*; *Xiong et al., 2015*). Thus, in the

250 present study, the significant difference in GGT between the control group and 50% replacement group suggests

251 that liver function was affected by contaminated cottonseed, although it is unclear why the levels did not change

252 significantly in the 100% replacement group. Mycotoxins can influence liver function, but its effects fluctuate

253 according to toxin dose and duration. A daily intake of 3-128 µg/day of pure AFB1 for one week did not alter

254 the activities of several enzymes related to liver function in dairy sheep (*Battacone et al., 2005*), but the blood

255 activity level of ALT increased significantly in dairy ewes after the intake of a high dose of AFB1 (128 µg/day)

256 for two weeks (*Battacone, 2003*). In lambs fed 2.5 mg/kg AFB1 for 35–67 days, the serum GGT and AST levels

257 increased significantly (*Edrington et al., 1994*). However, in a different study, AFB1 did not affect the plasma

258 concentrations of AST, ALT, GGT, nor ALP in dairy cows (*Xiong et al., 2015*).

259   A previous study showed a slightly stronger reaction to 20 µg/kg AFB1 than 40 µg/kg AFB1, which was

260 reflected by the MDA levels (*Xiong et al., 2015*). Aspartate aminotransferase (AST), one of the most important

261 transaminases, is an indicator of liver function in clinical medicine used to judge whether the liver is damaged.

262 Data has shown that the group fed a meal without mycotoxins, the group fed AFB1, and the group fed AFB1

263 mixed with ZEA had similar serum AST levels, but the group fed AFB1 mixed with OTA showed significantly

264 higher levels than that of the three groups. Moreover, the group fed AFB1 mixed with OTA and ZEA had

265 significantly higher levels than that of the abovementioned four groups (*Huang et al., 2018*). These results may

266 hint that mycotoxin type and amount are key factors that can affect animal health performances.

267   The serum concentrations of IgM, IgA, and IgG in the present study did not change significantly, suggesting

268 that the contaminated cottonseed did not affect the immune functions of the dairy cows. Similar results were

269 found in other studies *(Korosteleva et al., 2007*; *Xiong et al., 2015*), but it is possible that the response to

270 mycotoxins may be reflected in other immune factors. Additional parameters were not tested in the current study,

271 and immune function measures are required for further comprehensive analysis.

272   Previous studies have shown that mycotoxins can influence animal oxidant stress levels. SOD and MDA

273 are used as indices of the degree of lipid peroxidation, and the SOD/MDA ratio can reflect free radical-induced

274 lipid peroxidation and the scavenging rate *(Surai, 2002*; *Xiong et al., 2015*). For example, mycotoxins increase

275 the MDA concentration, but decrease the SOD concentration in dairy goats (*Huang et al., 2018*). In the present

276 study, we found that the 100% replacement group had a lower concentration of serum T-AOC compared with

277 the other two groups. Since T-AOC is an indicator of overall biochemical antioxidant capacity, it reflects the

278 systemic ability of antioxidative enzymes and non-antioxidative enzymes to compensate for external stress and

279 the capacity to clear free radicals (*Surai, 2002*). Therefore, lower T-AOC levels may indicate that contaminated

280 cottonseed affected the overall antioxidant capacity of dairy cows. A previous study showed that the reaction to

281 20 µg/kg AFB1 was slightly stronger than the reaction to 40 µg/kg AFB1, which was reflected by the MDA level

282 and SOD/MDA ratio (*Xiong et al., 2015*). It should be noted that subtle changes may affect overall antioxidant

283 capacity in response to toxins (but not at a significant level), which may be sporadically detected in terms of

284 other oxidative indicators, such as SOD, MDA, and GSH-Px.

285 **Rumen function**

286 Feedstuffs (*e.g.*, plant constituents, fiber, and cellulose) are fermented in the rumen of dairy cows with the aid

287 of rumen microbes, bacteria, protozoa, and fungi. Evidence suggests that rumen microbiota can protect animals

288 by binding, deactivating, and degrading toxic molecules (*Fink-Gremmels, 2008*).

289   Rumen microorganisms convert carbohydrates into VFAs, and these organic acids are absorbed through

290 the gastrointestinal tract into the circulatory system to reach different tissues. VFAs are consequently an essential

291 energy resource for ruminants. The rumen concentration of VFAs has been used as an indicator of rumen

292 fermentation and the effects of dietary treatments *(Hall et al., 2015*; *Xiong et al., 2015*). In the present study, the

293 different levels of contaminated cottonseed affected isovalerate but did not affect the concentrations of total

294 VFAs, acetate, propionate, butyrate, isobutyrate, and valerate (**Figure 1 and Table 3**). A recent study suggested

295 that the concentration of total VFAs in rumen is not an appropriate indicator of ruminal fermentation or microbial

296   product formation because of the high daily variation in the amount of rumen fluid (*Hall et al., 2015*), and this

297   finding was supported by another study (*Xiong et al., 2015*). Thus, it may be more useful to investigate the levels

298   of specific types of VFAs. Isovalerate, isobutyric acid, and 2-methyl butyrate belong to the family of isoacids.

299   Previous studies have indicated that isoacids promote the growth of anaerobic bacteria in the rumen fluid and

300   degrade fiber in the rumen *(Liu et al., 2014)*. *In vitro* analyses of rumen fermentation have shown that isoacids

301   can promote the growth of micro-organisms and the degradation of fiber after adding a mixture of isobutyrate,

302   2-methyl butyrate, and isovalerate (*Gunter et al., 1990*). Thus, the significant differences in isovalerate levels in

303   the present study may indicate that rumen fermentation is influenced by contaminated cottonseed. However,

304   similar experiments were inconclusive in many previous animal studies. A similar study found that mixed

305   mycotoxins significantly increased NH_3-N and total VFA concentrations (*Kiyothong et al., 2012*). Another study

306   found that the acetate:propionate ratio in the rumen of dairy cows was influenced by supplementation with 20

307   μg/kg AFB1 (*Xiong et al., 2015*). Other studies found no evidence that feeding with isoacids improved

308   digestibility in the rumen (*Gunter et al., 1990*), with   one study in particular showing that supplementation with

309   isoacids does not change digestibility of dry material, fiber, and crude protein in steers (*McCollum et al., 1987*).

310   In the present study, the significant difference in rumen isovalerate between the control and 50%

311   replacement groups may suggest that rumen fermentation was affected by contaminated cottonseed. It is unclear

312   why the levels did not change significantly in the 100% replacement group. The experimental data of isovalerate

313   addition showed that the low, middle and high doses (100, 200, and 300 mg per DMI) were not consistent with

314   our expectations. The rumen isobutyrate content in the middle dose was the highest. Rumen isobutyrate levels

315   in the low dose group were the same as in the control group (*Liu et al., 2009*). Further research is needed to

316   clarify the relationships between mycotoxins, diet, and the concentrations of specific VFA in the rumen.

317   A suitable concentration of NH_3-N is an important mediator in rumen nitrogen metabolism. We found that

318   mycotoxins significantly increased the NH_3-N concentration in the rumen in the 100% replacement group

319   (**Figure 1**). The most suitable ruminal NH_3-N concentration for growth ranges between 5.0 and 28.0 mg/100 mL

320   (*Wanapat & Pimpa, 1999*). However, NH_3-N concentration in the 100% replacement group went beyond the

321   upper limit of this range, suggesting that the microbial protein decomposition capacity could be imbalanced

322   (*Wanapat & Pimpa, 1999*). The contaminated cottonseed may have affected the microbial degradation of

323   proteins and the synthesis of ammonia, for two possible reasons. First, isovalerate could have promoted the

324   utilization of microbial nitrogen to produce NH_3-N, thereby leading to increased microbial protein synthesis

325   (*Allison, 1969*). Second, the rumen NH_3-N concentration was reduced due to the decreased rate of protein

326   degradation. Our results showed that the decrease in isovalerate may have led to the reduced utilization of NH_3-N

327   by rumen microbes, or some other mechanism related to *in vivo* nitrogen retention may have been partially

328   blocked in the rumen (*Felix et al., 1980*).

329   **Analysis of plasma metabolites and metabolic pathways**

330   Feedstuffs can have important effects on the performances of dairy cows, and metabolomics can help us

331   accurately understand the causes of metabolic changes and their subsequent effects *(Cheng et al., 2017;*

332 *Sundekilde et al., 2013*). In the present study, eight plasma metabolites differed significantly among the three

333 groups. These metabolites were mainly involved in the metabolic pathways related to aminoacyl-*t*RNA

334 biosynthesis, nitrogen metabolism, and alanine, aspartate, and glutamate metabolism (**Table 4 and Figure 5**).

335 Interestingly, four amino acids included in the eight metabolites differed significantly in the present study,

336 indicating that amino acid metabolism was affected by the mycotoxins. Previous studies have shown that

337 mycotoxins can influence energy expenditure and protein metabolism (*Sun et al., 2014*; *Sundekilde et al., 2013*).

338 Generally, the balance of amino acids is regulated by the relative rates of synthesis and degradation. Studies

339 suggest that acylated amino acids to *t*RNA are the immediate precursors to protein synthesis and that each amino

340 acid is catalyzed by a specific synthase that combines with the corresponding *t*RNA (*Davis et al., 1999*; *Martin*

341 *et al., 1977*). However, the aminoacyl-*t*RNA concentration is very low and extremely unstable within cells and

342 tissues, and previous studies have shown that *t*RNA plays an important regulatory role in the gene expression

343 process within the dairy cow's mammary gland (*Bauman et al., 2006*; *Mackle et al., 2000*; *Wang et al., 2014*).

344 When an organism encounters external stress, the unloaded *t*RNA can act as an effector for the overall gene

345 expression levels in the cell, thereby allowing the organism to cope with an adverse environment. *t*RNA can be

346 used for this purpose in yeast and some mammalian cells. The nucleus monitoring system can continuously

347 monitor the integrity of *t*RNA, and in the absence of nutrition, the retrograde transport of *t*RNA into the nucleus

348 can effectively reduce the level of protein synthesis (*Davis et al., 1999*; *Martin et al., 1977*). Previous studies

349 have suggested that investigations of the effects of mycotoxins on animal metabolism should focus specifically

350 on amino acid metabolism.

351   Nitrogen metabolism (nitrogen balance) mediates the relationship between nitrogen intake and excretion.

352 Previous studies have indicated that feed constituents can influence nitrogen metabolism in dairy cows

353 (*Cantalapiedra-Hijar et al., 2014*; *Otto et al., 2003*). Amino acids are important nutrients in cow milk

354 (*Sundekilde et al., 2013*) and a major aspect of ruminant protein nutrition research involves studying the digestion

355 and absorption of feed proteins (*Cantalapiedra-Hijar et al., 2014*; *Castillo et al., 2001*). Nitrogen balance can

356 directly affect health status and pasture waste management (*Arriaga et al., 2009*). In the present study, the

357 nitrogen metabolism pathway was significantly affected, probably due to the effects of mycotoxins on the

358 synthesis and degradation of amino acids. The involved mechanism may influence glucogenic nutrients and the

359 utilization of amino acids. The amino acids that remain in the body after utilization by the liver and intestines

360 are transferred to the peripheral organs and tissues or excreted as waste. Thus, the visceral organs may play a

361 regulatory role in nitrogen metabolism in order to limit the systemic availability of absorbed amino acids to the

362 peripheral tissues (*Cantalapiedra-Hijar et al., 2014*; *Larsen et al., 2015*).

363   Our results also illustrate that mycotoxins affect glucose metabolites in the blood, thereby influencing

364 glucose metabolism. Previous studies have shown that mycotoxin-contaminated feed can affect glucose

365 metabolism, specifically by decreasing the activities of intestinal glucose transporters (*Bertrand & Applegate,*

366 *2013*; *Liu et al., 2013*; *Zhang et al., 2011*). Glucogenic amino acids are involved in the regulation of glucose

367 metabolism (*Xu et al., 2008*). Branched-chain amino acids may inhibit glycogenolysis in the liver and muscles,

368   and can enhance the alanine-glucose and lactic acid-glucose cycles (*Hayirli, 2006*). Alanine, aspartate, and
369   glutamate are glucogenic amino acids. Glutamate is a functional amino acid with important physiological
370   regulatory functions (*Duan et al., 2013*; *Zhang et al., 2013*). We found that feed contaminated with mycotoxins
371   had significantly affected alanine, aspartate, and glutamine metabolism pathways, indicating that body
372   gluconeogenesis is also influenced by the mycotoxins.

373   In the present study, we found significant differences in the O-acetyl-glycoprotein, citrate, and creatinine
374   concentrations among the three treatment groups. A previous study similarly showed that exposure to ZEA
375   significantly elevated the plasma levels of glucose and O-acetyl glycoprotein *(Liu et al., 2013)*. NMR has been
376   used to identify the milk metabolites obtained from two dairy cow breeds (Danish and Jersey Holstein), and they
377   found choline, creatinine, and citrate, which are also potential biomarkers (*O'Sullivan et al., 2013*; *Pinotti et al.,*
378   *2002*). Our findings suggest that O-acetyl-glycoprotein, citrate, and creatinine may be used as potential
379   biomarkers of altered metabolism.

CONCLUSION

381   Diets containing cottonseed contaminated with mycotoxins significantly influenced the blood GGT content and
382   T-AOC of dairy cows. Significant differences in isovalerate and NH_3-N concentrations were also found between
383   the control and treatment groups, indicating that the contaminated cottonseed may have influenced ruminal
384   function. Moreover, among the three treatment groups, there were significant differences in the eight plasma
385   metabolites mainly involved in the aminoacyl-*t*RNA biosynthesis, nitrogen metabolism, and alanine, aspartate,
386   and glutamate metabolism pathways. These differences suggest that amino acid metabolism pathways may be
387   important targets when investigating the effects of mycotoxin exposure in future research.

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Ingredients and mycotoxin composition of diets[1]

Notes: [1]Control group (0% of uncontaminated cottonseeds replaced in diet); 50% replacement group (50% of uncontaminated cottonseeds replaced with cottonseeds that were naturally contaminated with mycotoxins in diet) and 100% replacement group (100% of uncontaminated cottonseeds replaced with cottonseeds that were naturally contaminated with mycotoxins in diet). [2]Dried distillers grains with solubles. [3] ppb, μg/kg; AFB1, aflatoxin B1; ZEA, zearalenone; DON, deoxynivalenol. [4]ND, not detected.

1 **Table 1. Ingredients and mycotoxins composition of diets[1].**

Item	% DM
Ingredient	
Oats hay	2.24
Corn silage	45.57
Alfalfa hay	8.07
Soybean meal	4.86
DDGS[2]	5.58
Corn meal	11.88
Wheat bran	2.24
Extruded soy flour	0.56
Corn bran shotcrete	4.59
Cottonseed	4.48
Mineral meal	0.22
Diamond XP	0.13
NaHCO3	0.54
NaCl	0.07
Premix	1.12
Water	7.84
Nutrients, % of DM	
Crude Protein	17.65
Fat	4.15
Non fiber carbohydrate	39.5
Neutral detergent fiber	29.49
Ca	0.8
P	0.48
Ash	9.27
Energy (mcal/Kg)	
Metabolic energy	2.88
Net Energy	1.67
Mycotoxins (50% replacement) , ppb[3]	
AFB1	20.08
ZEA	85.13
DON	ND[4]

2 **Notes**: [1]Control group (0% of uncontaminated cottonseed replaced in diet); 50% replacement group (50% of the uncontaminated

3 cottonseed replaced with cottonseed contaminated with mycotoxins). [2]Dried distillers grains with solubles. [3]ppb, μg/kg; AFB1,

4 aflatoxin B1; ZEA, zearalenone; DON, deoxynivalenol. [4]ND, not detected.

Effects of cottonseed contaminated with mycotoxins[1] on the serum biochemical, antioxidant, and immune indices of dairy cows

Notes: [1] Control group (uncontaminated cottonseed), 50% replacement group (50% of the cottonseed replaced with cottonseed contaminated with mycotoxins), and 100% replacement group (100% of the cottonseed replaced with cottonseed contaminated with mycotoxins). [2] ALT, alanine aminotransferase ; AST, aspartate aminotransferase; GGT, γ-glutamyl transpeptidase; ALP, alkaline phosphatase; TP, total protein; ALB, albumin; GLOB, globulin; A/G, albumin/ globulin; UREA, urea; CR, creatinine; UA, uric acid; TBil, total bilirubin; DBil, directed bilirubin; IBiL, indirect bilirubin; TG, total triglyceride; TC, total cholesterol; GSH-PX, glutathione peroxidase ; MDA, malondialdehyde; T-AOC, total antioxidant capacity; SOD, superoxide Dismutase; IgG, immunoglobulin G; IgA, immunoglobulin A; IgM, immunoglobulin M. [3] Probability associated with the F-test based on differences between treatments [ab] Means in the same row with different superscripts are significantly different ($P < 0.05$) according to Tukey's test.

2   **Table 2. Effects of cottonseed contaminated with mycotoxins[1] on the serum biochemical, antioxidant, and immune indices**

3   **of dairy cows.**

Item[2]	Control	50% replacement	100% replacement	SEM	*P*-value[3]
ALT (U/L)	29.75	29.75	29.86	1.05	0.99
AST (U/L)	72.75	71.00	77.50	3.03	0.68
AST/ ALT	2.52	2.41	2.62	0.11	0.78
GGT (U/L)	43.09[a]	29.30[b]	34.45[a,b]	2.16	0.02
ALP (U/L)	90.31	67.61	71.19	12.36	0.74
TP (g/L)	73.96	73.53	73.53	0.97	0.98
ALB(g/L)	36.44	35.49	36.14	0.45	0.69
GLOB(g/L)	37.53	38.04	37.39	1.08	0.97
A/G	0.98	0.94	1.01	0.03	0.72
UREA(mmol/ml)	3.10	3.45	3.30	0.11	0.44
CR (μmol/L)	71.16	67.50	70.36	2.13	0.78
UA(μmol/L)	25.85	28.56	30.49	1.94	0.64
TBil(μmol/L)	11.05	9.82	9.82	0.53	0.46
DBil(μmol/L)	2.28	2.03	2.40	0.09	0.21
IBiL(μmol/L)	8.78	7.79	9.00	0.45	0.53
TG(mmol/ml)	0.05	0.05	0.06	0.004	0.74
TC(mmol/ml)	6.03	5.95	6.71	0.26	0.46
GSH-PX (U/ml)	669.0	661.5	687.8	12.48	0.70
MDA(nmol/ml)	12.42	12.77	8.02	1.18	0.19
SOD(U/ml)	106.9	108.9	107.4	0.81	0.57
SOD/ MDA	10.32	11.8	15.63	1.31	0.24
T-AOC(U/ml)	2.40[a]	2.39[a]	3.52[b]	0.26	0.009
IgG (μg/ml)	13.67	18.75	13.75	2.13	0.56
IgA (ng/ml)	59.43	50.82	53.57	4.46	0.43
IgM (ng/ml)	22.40	23.42	22.30	1.95	0.93

4   **Notes**: [1] Control group (uncontaminated cottonseed), 50% replacement group (50% of the uncontaminated cottonseed replaced

5   with cottonseed contaminated with mycotoxins), and 100% replacement group (100% of the uncontaminated cottonseed replaced

6   with cottonseed contaminated with mycotoxins).

7   [2] ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ-glutamyl transpeptidase; ALP, alkaline phosphatase;

8   TP, total protein; ALB, albumin; GLOB, globulin; A/G, albumin/ globulin; UREA, urea; CR, creatinine; UA, uric acid; TBil, total

9   bilirubin; DBil, directed bilirubin; IBiL, indirect bilirubin; TG, total triglyceride; TC, total cholesterol; GSH-PX, glutathione

10 peroxidase; MDA, malondialdehyde; T-AOC, total antioxidant capacity; SOD, superoxide Dismutase; IgG, immunoglobulin G;

11 IgA, immunoglobulin A; IgM, immunoglobulin M.

12 [3] Probability associated with the F-test based on differences between treatments.

13 [ab] Means in the same row with different superscripts are significantly different ($P < 0.05$) according to Tukey's test.

Effects of cottonseed contaminated with mycotoxins[1] on volatile fatty acid concentrations in the cow rumen

Notes: [1] Control group (uncontaminated cottonseed), 50% replacement group (50% of the cottonseed replaced with cottonseed contaminated with mycotoxins), and 100% replacement group (100% of the cottonseed replaced with cottonseed contaminated with mycotoxins). [2] Probability associated with the F-test based on differences between treatments. [abc] Means in the same row with different superscripts are significantly different ($P < 0.05$) according to Tukey's test.

2 **Table 3. Effects of cottonseed contaminated with mycotoxins[1] on volatile fatty acid concentrations in the cow rumen.**

Item (mmol/L)	Control	50% replacement	100% replacement	SEM	*P*-value[2]
Acetate	65.55	62.72	60.78	1.08	0.19
Propoinate	22.49	22.76	22.03	0.41	0.78
Acetate/Propoinate	2.93	2.77	2.77	0.06	0.43
Isobutyrate	0.66	0.72	0.75	0.02	0.27
Butyrate	12.73	12.31	12.21	0.36	0.84
Isovalerate	1.27 [a]	1.61 [b]	1.29 [ac]	0.05	0.008
Valerate	1.39	1.51	1.31	0.06	0.21

3 **Notes**: [1] Control group (uncontaminated cottonseed), 50% replacement group (50% of the uncontaminated cottonseed replaced

4 with cottonseed contaminated with mycotoxins), and 100% replacement group (100% of the uncontaminated cottonseed replaced

5 with cottonseed contaminated with mycotoxins).

6 [2] Probability associated with the F-test based on differences between treatments.

7 [abc] Means in the same row with different superscripts are significantly different ($P < 0.05$) according to Tukey's test.

OPLS-DA coefficients derived from the NMR spectra indicating the concentrations of metabolites present in plasma samples obtained from the three groups

(A) control, (B) 50% replacement group (50% of the uncontaminated cottonseed replaced with cottonseed contaminated with mycotoxins) and (C) 100% replacement group (100% of the uncontaminated cottonseed replaced with cottonseed contaminated with mycotoxins).

[a] Correlation coefficients where positive and negative signs indicate positive and negative correlations between the concentrations, respectively. Correlation coefficients of │ r │ > 0.666, 0.707, or 0.755 were used as the cutoff values for significant differences based on the discrimination significance at the $P = 0.05$ level, where df (degrees of freedom) = 7, 6, or 5; ''-'' indicates that the correlation coefficient │ r │ was less than 0.666, 0.707, or 0.755 .

[b] Multiplicity: s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublets; m, multiplet; br, broad resonance.

Table 4. OPLS-DA coefficients derived from the NMR data of plasma metabolites obtained from (A) control, (B) 50%
replacement group (50% of the uncontaminated cottonseed replaced with cottonseed contaminated with mycotoxins) and
(C) 100% replacement group (100% of the uncontaminated cottonseed replaced with cottonseed contaminated with
mycotoxins).

Metabolites[b]	r^a		
	A *vs.* B (*df*=7)	A *vs.* C (*df*=7)	B *vs.* C (*df*=7)
Ala(Alanine): 1.48(d)	-	-0.669	0.763
Lys(Lysine): 1.49(m), 1.74(m), 1.91(m)	0.743	-	0.697
Gln(Glutamine): 2.12(m), 2.42(m)	-	-	0.733
OAG(O-acetyl-glycoprotein): 2.13(s)	-	-	0.668
Cit(Citrate): 2.54(d), 2.68(d)	-	-0.748	0.695
Cr(Creatine): 3.03(s),3.93(s)	-	-	0.674
Gly(Glycine): 3.56(s)	-	-	0.731
Glucose: 3.42(t), 3.54(dd), 3.71(t), 3.73(m), 3.84(m), 3.25(dd), 3.41(t), 3.46(m), 3.49(t), 3.90(dd), 4.65(d), 5.23(d)	-	-	0.768

[a]Correlation coefficients where positive and negative signs indicate positive and negative correlations between the concentrations, respectively. Correlation coefficients of ｜r｜> 0.666, 0.707, or 0.755 were used as the cutoff values for significant differences based on the discrimination significance at the P = 0.05 level, where *df* (degrees of freedom) = 7, 6, or 5; ''-'' indicates that the correlation coefficient ｜r｜ was less than 0.666, 0.707, or 0.755.

[b]Multiplicity: s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublets; m, multiplet; br, broad resonance.

Figure 1

Effects of cottonseed contaminated with mycotoxins on the concentration of NH_3-N (a) and total VFA (Volatile Fatty Acid) (b) in the cows rumen.

Notes: Control group=uncontaminated cottonseed; 50% replacement group=50% of the uncontaminated cottonseed was replaced with cottonseed contaminated with mycotoxins; and 100% replacement group=100% of the uncontaminated cottonseed was replaced with cottonseed contaminated with mycotoxins
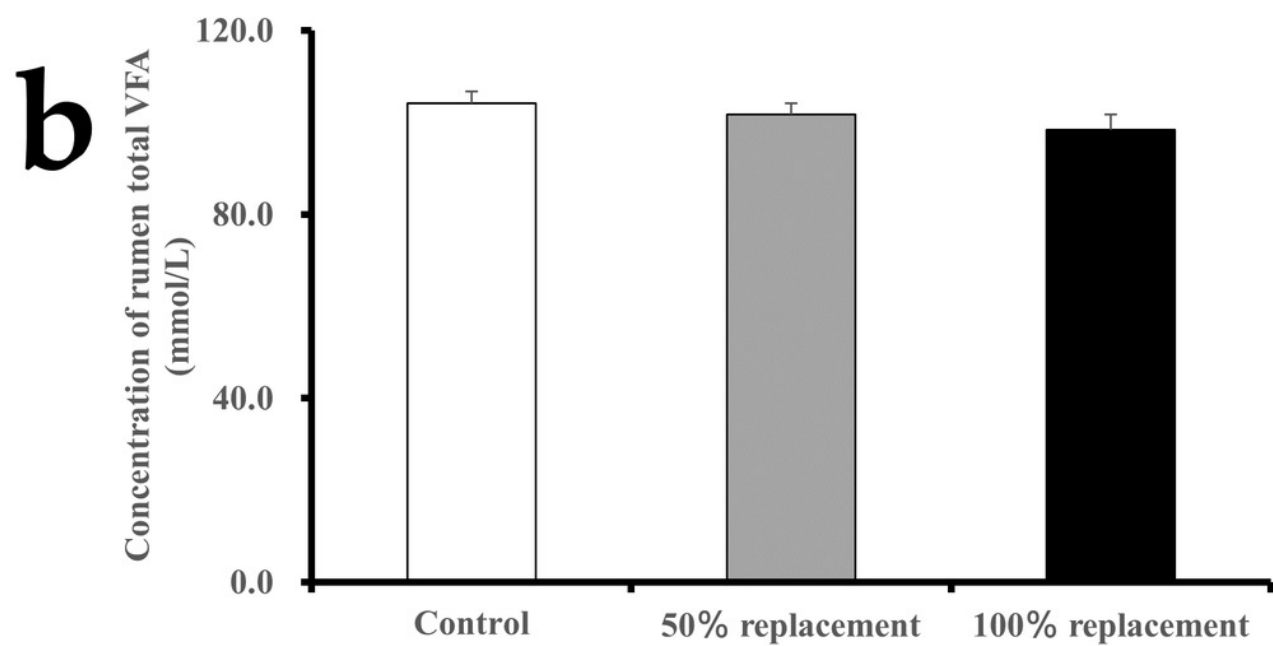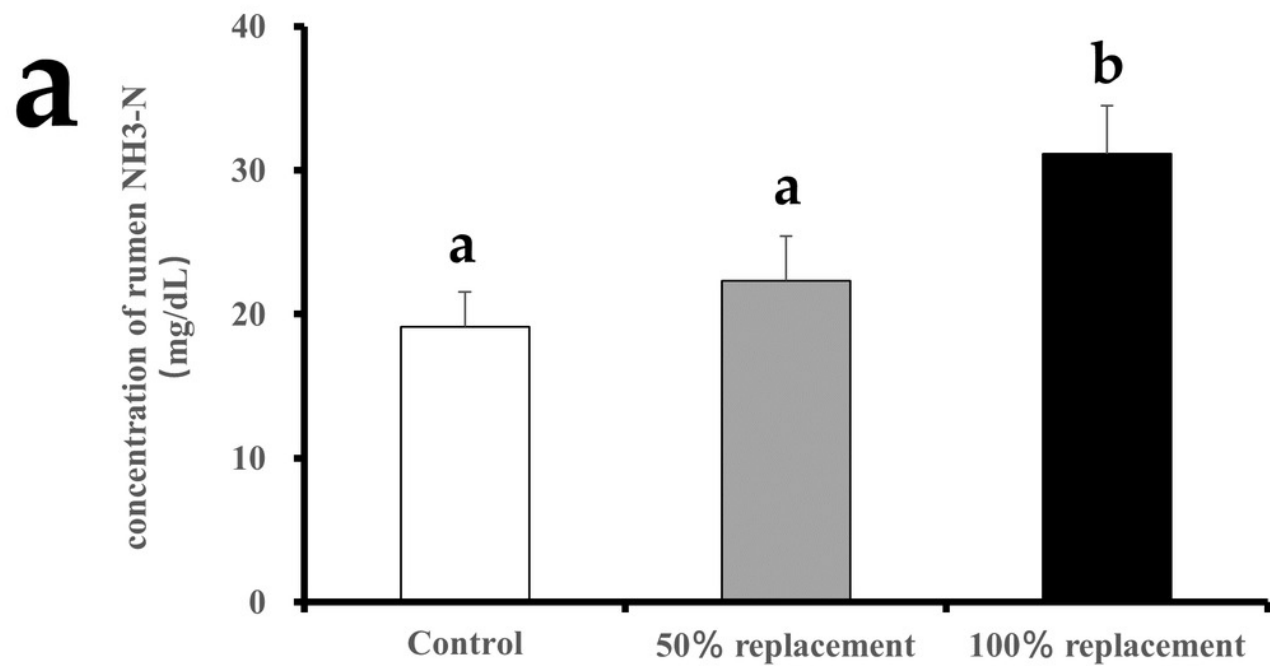
Figure 2

Representative 600 MHz 1D NOESY 1H-NMR spectra (δ0.5-5.5 and δ5.5-9.0) from cows plasma

(A) control group, (B) 50 % replacement group (50 % of the uncontaminated cottonseed was replaced with cottonseed contaminated with mycotoxins), and (C) 100 % replacement group (100 % of the uncontaminated cottonseed was replaced with cottonseed contaminated with mycotoxins). The δ5.5-9.0 region was magnified 16 times relative to the corresponding δ0.5-5.5 region for clarity. Abbreviations: Glu: Glutamate; NAG: *N*-acetyl glycoprotein signals; L1: LDL (Low Density Lipoprotein), CH_3-(CH_2)n-; L2: VLDL (Very Low Density Lipoprotein), CH_3-(CH_2)n-; L3: LDL, CH_3-(CH_2)n-; L4: VLDL, CH_3-(CH_2)n-; L5: VLDL, -CH_2; L6: lipid, -CH_2-CH=CH-; L7: lipid, -CH_2-C=O; L8: lipid, =CH-CH_2-CH=.
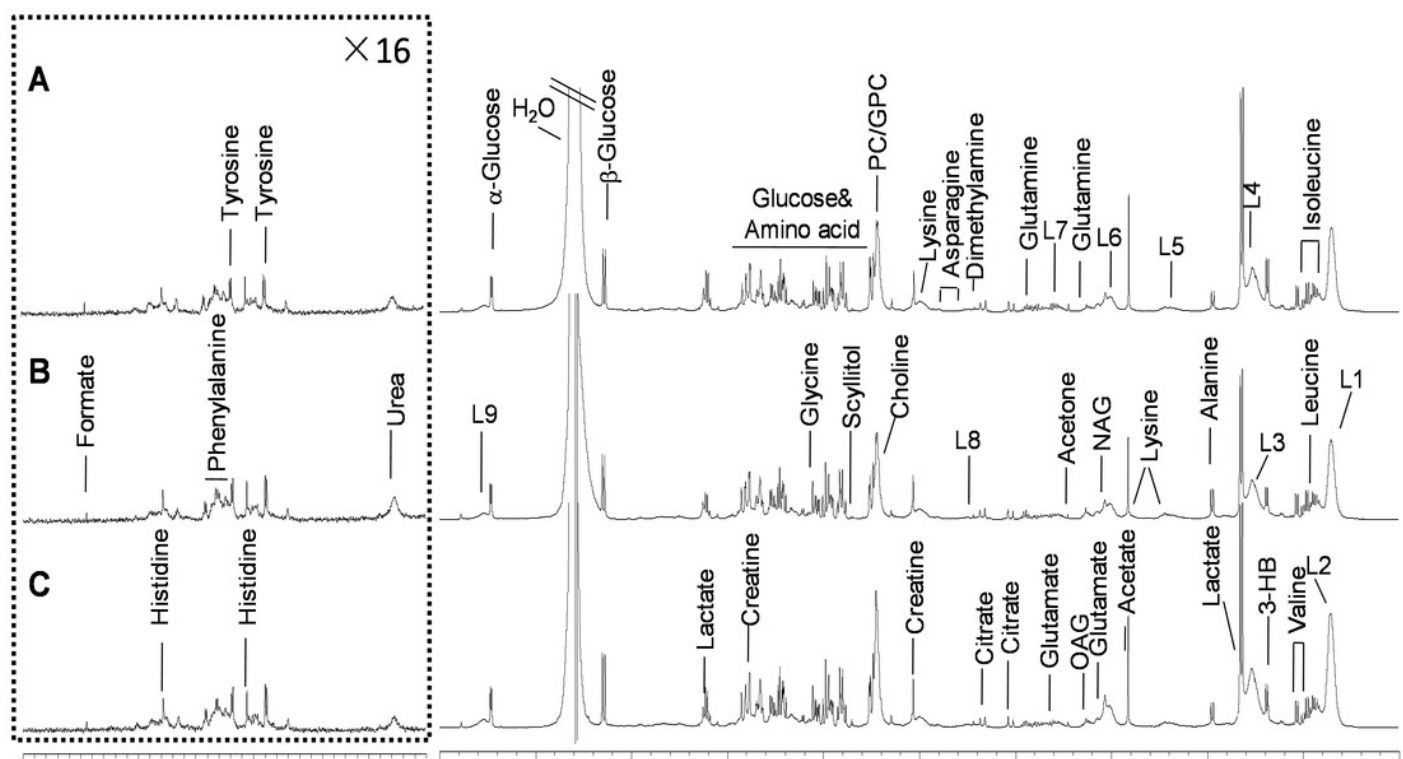
Figure 3

PCA plot based on the 1H NMR spectra for plasma obtained from different groups

control (I), black square; 50% replacement group (50% of the uncontaminated cottonseed was replaced with cottonseed contaminated with mycotoxins) (II), red spot; 100 replacement group (100% of the uncontaminated cottonseed was replaced with cottonseed contaminated with mycotoxins) (III), blue diamond.
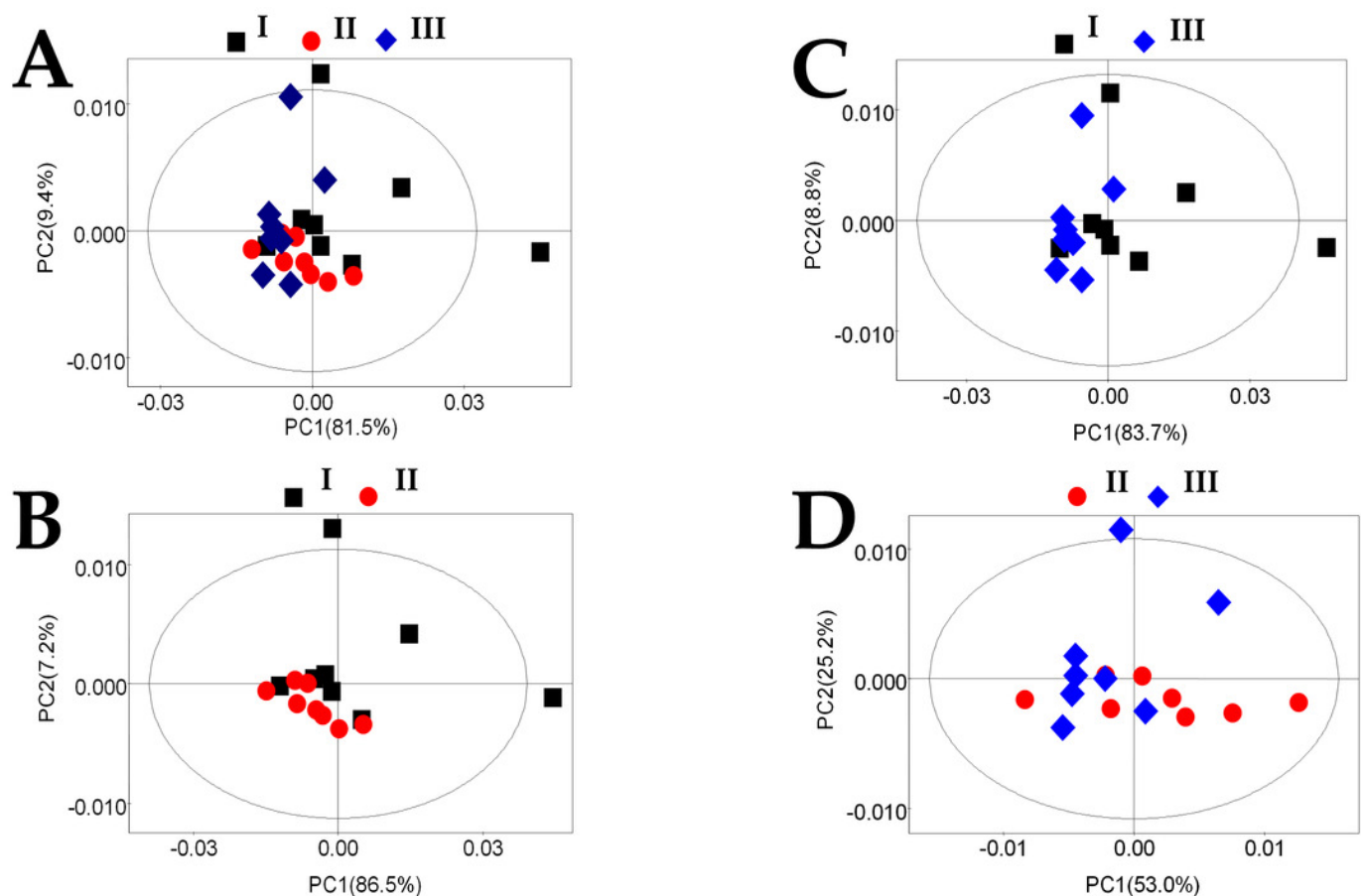
Figure 4

OPLS-DA score plots (left panel; A, D, and G) derived from 1H NMR spectra for plasma and the corresponding coefficient loading plots (right panel; B, C, E, F, H, and I, in which, B, E and H are images magnified 20 times)

control (I), black square; 50 % replacement group (50 % of the uncontaminated cottonseed was replaced with cottonseed contaminated with mycotoxins), (II), red spot; 100 % replacement group (100 % of the uncontaminated cottonseed was replaced with cottonseed contaminated with mycotoxins), (III), blue diamond. The color map shows the significant variations in metabolites between the two classes. Peaks in the positive direction indicate that metabolites were more abundant in the groups in the positive direction of the first principal component. Thus, metabolites that were more abundant in the three groups in the negative direction of the first primary component were shown as peaks in the negative direction.
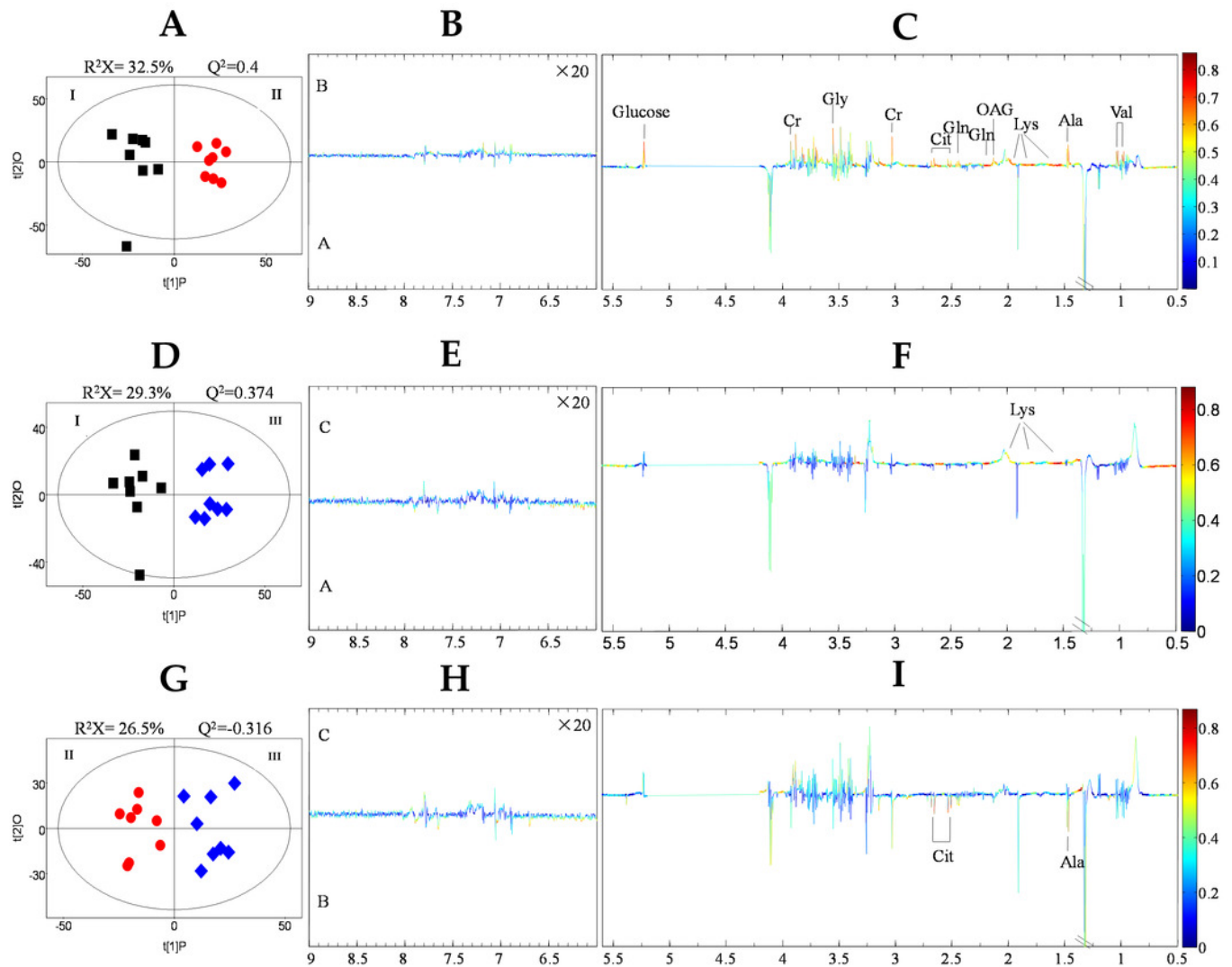
Figure 5

Metabolome view map showing the matched pathways according to the P-valuesobtained from pathway enrichment analysis and the pathway impact valuesproduced by pathway topology analysis

a, Aminoacyl-tRNA biosynthesis; b, nitrogen metabolism; c, alanine, aspartate, and glutamate metabolism. The x-axis represents the pathway impact and the y-axis represents pathway enrichment. In the map, dots with larger sizes and darker colors represent higher pathway enrichment and higher pathway impact values, respectively.