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Effects of Dietary Natural Mycotoxins Exposure on Performance, Biochemical Parameters and Milk Small Molecule Metabolic Pathways of Lactating Cows

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Abstract: The presence of mycotoxins in feed has the potential to cause significant detriment to animal and human health, and even severe economic implications. Previous studies on the effects of mycotoxins mainly focused on the addition of commercially available mycotoxins into feeds in animals. In the present study, corn meal and cottonseed were kept in warm and humid conditions to allow for mycotoxins produced and then used to substitute 50% and 100% of normal corn meal and cottonseed in diets for lactating cows for 14 days. The results showed that aflatoxin M1, deoxynivalenol, aflatoxin B1, and zearalenone were primary mycotoxins in milk from cows fed the diets. Compared with the control group, feeding the diets containing mildewy corn meal and cottonseed reduced feed intake, milk yield, and milk fat, protein and lactose productions ($p > 0.05$). No significant difference was observed in the acetate and valerate concentrations, acetate to propionate ratio, and the calculated CH_4 production in rumen fluid ($p > 0.05$), whereas, the propionate, butyrate, isovalerate concentrations were affected ($p < 0.05$) depending on the content and type of natural mycotoxins. Serum creatinine and total glyceride concentrations were influenced with corn meal and cottonseed fully replaced with the mildewy feeds. Metabolic pathways for small molecule metabolites in milk were altered by dietary mycotoxin exposures, and the changes were mainly associated with amino acid metabolism, glucose metabolism, and energy metabolism. However, cows exposed to natural mycotoxins in the diets were still in healthy conditions and had low somatic cell count in milk.

Keywords: mycotoxin; metabolomics; dairy cow; milk

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1. Introduction

Milk containing mycotoxins is a critical safety issue for milk quality. The occurrence of mycotoxins in milk is likely from mycotoxin-contaminated feeds that are fed to lactating cows. The maximum permissible limit of mycotoxins in feeds has been extensively established worldwide [1–4]. For instance, the allowable limit of aflatoxin B1 in feed for lactating cows is 20 μg/kg in America [2], and 10 μg/kg and 5 μg/kg in China and the European Union respectively [3,4]. The vomitoxin limit in the European Union is 1270–1750 μg/kg and 5000–10,000 μg/kg in the United States of America (USA). The *fumonisin* limit is 5–100 μg/kg in the USA, and 2000 μg/kg in the European Union [2,3]. Ma et al. [5] investigated contamination of mycotoxins in feedstuffs in China and found that aflatoxin B1, zearalenone, and deoxynivalenol were widely present in all the feedstuffs studied, and

these toxins, as well as α-zearalenol, were detected in 50 raw milk samples [6]. Hence, aflatoxins, zearalenones, and deoxynivalenol are three main mycotoxins present in milk. In particular, aflatoxin M1 is a potent toxic mycotoxin which is classified as a Group 1 human carcinogen by the International Agency for Research on Cancer (IARC) [7]. Furthermore, aflatoxin M1 is the only mycotoxin that has been established for the maximum residual limit in milk around the world [8,9].

Mycotoxins found in milk are from mycotoxins-contaminated feeds fed to lactating dairy cows. Dairy cattle feeds are extremely susceptible to mycotoxins infection and contamination during harvest, production, processing, and storage [10,11]. Environmental temperature and humidity, feed surface moisture, and substrate are the major influencing factors for mycotoxins growth. A 25–30 °C temperature is most suitable for fungus growth and mycotoxins reproduction, and 28–32 °C is the optimum toxigenic temperature for *Aspergillus flavus*. Feed moisture exceeding 13% may cause mildew growth and mycotoxin production. Furthermore, the mycotoxins produced by different raw material substrates are also different. Aflatoxins are derived from a group of secondary metabolites that are synthesized by *Aspergillus flavus* and *Aspergillus parasiticus*, which can be found in maize, cottonseed, walnut, peanut, etc., [12,13]. Zearalenone is a secondary metabolite produced by various *Fusarium* species that are frequently present in cereal grains, such as maize, barley, oats, and sorghum [14]. Deoxynivalenol, also known as vomitoxin, is one of the most commonly occurring mycotoxins of the group B trichothecenes produced by *Fusarium* molds [15,16]. Deoxynivalenol pollution commonly occurs in temperate regions, and is frequently found in foods or feeds, such as wheat, barley, oats, and maize [17]. Because of the presence of mycotoxins in various feedstuffs, mycotoxin contamination of feeds for dairy cows is almost unavoidable. Therefore, a suitable storage and transportation condition is of particular importance. A significant amount of work has also been focused on the adverse effect of mycotoxins on the performance and health of dairy cows [18–20], especially aflatoxins B1. Studies have found that aflatoxin B1 contamination of feeds for dairy cows significantly reduced feed intake, feed efficiency, and milk yield, affected the serum biochemical parameters and detoxification metabolism, and caused immune system change and organ damage [19,21]. Additionally, mycotoxins contamination also influenced the animal byproducts that cause harmful effects on food safety and human health resulting in significant economic losses. Similarly detrimental effects have also been reported for deoxynivalenol and zearalenone contaminations in dairy cows [22–24]. In these studies, pure mycotoxins were used. However, alone they are unable to accurately mimic the actual situations of factor and degree in mildew, because one species of fungi may produce more than one mycotoxin concomitantly, and different fungal species may coexist in the same feed [11], which means its guiding significance for the production process of dairy cows is limited. Fewer research has been carried out using natural mycotoxins in feeds and the effects of combined mycotoxins.

In the present study, we examined the effects of mycotoxins, namely aflatoxin (B1, B2), zearalenone, and deoxynivalenol naturally present in maize and cottonseeds in dairy cows to reflect the normal farming practice. The effects of these toxins on feed intake, milk production and composition, rumen fermentation, and some small molecular metabolites in milk were determined.

2. Materials and Methods

2.1. Animals, Management, and Sample Preparation

The use of the animal and the experimental procedures were approved by the Animal Care and Use Committee of the Institute of Animal Science, Chinese Academy of Agricultural Sciences (No. IAS15020; Date: 16 July 2015). The experiment was performed at Ningxia Sinofarm Dairy Farm, Helan, Ningxia, China. The temperature and humidity recorded at the Farm were respectively 25–36 °C and 20–30%, from August to September in 2018. Forty multiparous Holstein cows in late lactation (lactation length: 263 ± 22 days, milk yield: 21.1 ± 2.6 kg/d, parity: 2.5–3.5 times) were used in this study. Cows were

randomly divided into five groups, eight animals per group. The basal diet (Table 1), containing cornmeal and cottonseed, was formulated to meet the nutrient requirement of lactating cows for production of 25 kg/d of milk (Ministry of Agriculture of China, 2004 [25]).

Table 1. Ingredients and chemical composition of the experimental diet.

Item	Amount (%)
Ingredients, % of DM	
Soybean extract	1.07
Corn DDGS	10.60
Wheat bran	4.00
Soybean meal	8.88
Whole cottonseed	4.14
Cornmeal	22.51
Spray grain corn	8.65
Premix [1]	2.09
Yeast	0.05
Carb-fine	0.47
Sodium bicarbonate	1.12
Salt	0.14
Corn silage	21.77
Oat grass	10.19
Alfalfa hay	4.33
Nutrient, % of DM	
Crude protein	17.65
Fat	4.15
Neutral detergent fiber	29.49
Nonfiber carbohydrate	39.50
Calcium	0.80
Phosphorus	0.48
Ash	9.27
Energy (Mcal/Kg)	
Metabolizable energy	2.88
Net energy	1.67

[1] Premix consisted of vitamin A, vitamin D3, vitamin E, copper sulfate, ferrous sulfate, zinc sulfate, manganese sulfate, sodium selenite, and was formulated to provide (per kg of DM): 35 mg of Cu, 70 mg of Fe, 55 mg of Mn, 110 mg of Zn, 80 mg of Co, 110 mg of Se, 140,000 IU of vitamin A, 18,000 IU of vitamin D3, and 6000 IU of vitamin E.

To mimic natural mildewy conditions, the mildew feed was prepared under the local temperature and humidity conditions in Ningxia. Two tons of corn meal and 1.5 tons of cottonseed were selected, and 35% and 28.5% water was added to corn meal and cottonseed respectively and mixed for 30–40 min by a self-propelled TMR vehicle. Then they were placed in separate feed bunks mildew for 15 days. Mildew corn meal and cottonseed were subsequently spread out and dried for 5–7 days until the water content was under 10% to prevent secondary mildew. Mildew cottonseed and corn meal were stored in separate feed bunks, but the mildew corn meal needed to be smashed again by a pulverizer. Mycotoxin concentrations of dry matter in the mildew feed raw material and experimental diets were measured, and the results are shown in Tables 2 and 3. Five groups of the animals were then randomly allocated to five diets as following [20]:

- Control (Cont) group: fed the basal diet;
- 50Cot group: 50% cottonseed in the basal diet was substituted with the moldy cottonseed;
- 100Cot group: all cottonseed in the basal diet were substituted with the moldy cottonseed;
- 50CotCorn group: 50% cottonseed and 50% corn meal in the basal diet were substituted with the moldy cottonseed and moldy corn meal;

- 100CotCorn: all cottonseed and corn meal in the basal diet substituted with the moldy cottonseed and moldy cornmeal.

Table 2. Comparison of major mycotoxins' levels (μg/kg dry matter) [1] in corn meal and cottonseed naturally mildewed and non-mildewed.

Item (μg/kg)	Non-Mildewed		Mildewed	
	Corn Meal	Cottonseed	Corn Meal	Cottonseed
Aflatoxins	<3	3	3	>150 [2]
Zearalenones	<272	40	490	84
Deoxynivalenol	<350	0	1250	ND [3]

[1] The mycotoxins in corn meal and cottonseed were determined by immunochromatography [26]. [2] >150, exceed the maximum limit of detection of 150 μg/kg. [3] ND, not detected.

Table 3. The concentrations of major mycotoxins (μg/kg dry matter) [1] in the experimental diets for dairy cows.

Item (μg/kg)	Control	50Cot	100Cot	50CotCorn	100CotCorn
Aflatoxin B1	0.02	28.67	61.34	30.10	59.91
Zearalenones	160.33	161.25	165.14	216.85	248.34
Deoxynivalenol	1654.31	1672.03	1697.74	1736.91	1791.16
Aflatoxin B2	0.83	1.58	2.82	1.44	2.98
Aflatoxin G1	12.94	13.07	12.09	12.66	14.38
Aflatoxin G2	ND [2]	ND	ND	ND	ND
Lysergol	ND	ND	ND	ND	ND
Sterigmatocysin	ND	ND	ND	ND	ND
T-2 Toxin	ND	ND	ND	ND	ND
HT-2 Toxin	ND	ND	ND	ND	ND
Zearalanoe	ND	ND	ND	ND	ND
α-Zearalenol	ND	ND	ND	ND	ND

[1] The mycotoxins residue in diets were determined by liquid chromatography-mass spectrometry/mass spectrometry method [27]. [2] ND, not detected.

Cows were kept in individual stable. The diets were prepared as total mixed ration and fed three times daily at 08:30, 16:30, and 00:30, and milked three times per day at 08:00, 16:00, and 24:00. Fresh drinking water was available all the time. The experiment lasted for 28 days, including 14 days on the basal diet (adaptation period), 14 days on the experimental diets (experimental period).

2.2. Sample Collection and Preparations

The feed offered and refusal were weighed daily to calculate feed intake of individual cows during the experimental period. Feed and refusal were sampled daily, pooled at the end of the experiment, and dried at 65 °C for 48 h. Dried samples were ground and passed through a 40-mesh sieve and stored at −2 °C until analysis.

Milk samples were collected on day 1, 2, 3, 5, 7, 13, and 14 of the experimental period. At each milking, the milk yield was recorded by a digital milk meter (Bou-matic Ltd., Madison, WI, USA), and a sample was collected. Three milk samples for each day were pooled, and four aliquots (each 50 mL) were stored at −20 °C. One aliquot was added with bronopol to prevent spoilage of milk and stored at −4 °C before the determination of milk composition. One aliquot milk sample on day 14 was stored at −20 °C for analysis of the metabolites. The rest milk samples were stored at −20 °C for determination of the mycotoxin concentrations.

Blood samples, each 15–20 mL, were drawn from the jugular vein before the morning feeding on day 14 of the experimental period. Plasma was harvested by centrifugation at 3200× *g* for 15 min, and eight aliquots were stored at −20 °C until analyses of blood parameters.

The ruminal fluid from each cow was collected with an oral stomach tube (A1141K, Anscitech, Wuhan, China) 2 h after the morning feeding on day 14 of the experimental period. The oral stomach tube was rinsed with water and the first 80–100 mL of liquid was discarded to avoid cross contamination between cows. A total of 50 mL of rumen fluid was filtered through four layers of gauze. The pH value of the fluid was measured immediately, and the sample was stored at −20 °C for analyses of fermentation parameters.

2.3. Sample Analyses

The chemical composition of feeds and refusal samples were analyzed for dry matter (DM) (Association of Official Analytical Chemists [AOAC] 930.5), ash content (AOAC 942.05), crude protein (CP) (AOAC 984.13), neutral detergent fiber (NDF) (AOAC 2002.04), and acid detergent fiber (ADF) (AOAC 973.18) [28]. Milk samples were analyzed for milk protein, fat, lactose, total solids, milk urea nitrogen (MUN), and somatic cell count (SCC) using an automatic milk composition analyzer (Combi Milkoscan FT + & Fossomatic FC, Foss Electric, Hillerod, Denmark). Analysis of mycotoxin residues in milk using a liquid chromatograph-mass spectrum was conducted following the method reported by Huang et al. [6].

The plasma samples were analyzed for biochemical parameters using an Auto-Analyzer 7020 (Hitachi High-Technologies Corporation, Tokyo, Japan) with commercial kits (DiaSys Diagnostics Systems GmbH, Frankfurt, Germany). The biochemical parameters included albumin (ALB), globulin (GLOB), creatinine (CR), alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin to globulin ratio (A/G), alkaline phosphatase (ALP) activity, total bilirubin (TBiL), direct bilirubin (DBiL), indirect bilirubin (IBiL), γ-glutamyl transpeptidase (GGT), total cholesterol (TC), total triglyceride (TG), uric acid (UA), and urea.

The rumen fluid samples were analyzed for rumen fermentation parameters using a gas chromatography equipped with a flame ionization detector (GC-7890, Agilent Technologies Corporation, Santa Clara, CA, USA) [29]. The rumen liquid (1 mL) was deproteinized with 200 μL of 25% metaphosphoric acid, and centrifuged at 13,000× *g* for 10 min at 4 °C. The supernatant was filtered through a 0.22 μm filter before the GC analysis. The GC parameters were set as follows: injector temperature 200 °C, column temperature 180 °C, detector temperature 200 °C; carrier gas was high purity (99.99%) nitrogen (Beijing Beiwen Gas Co., Ltd., Beijing, China), total pressure 85 kPa, circulation flow rate 7 mL/min, oxygen flow rate (39.9%, Beijing Beiwen Gas Co., Ltd., Beijing, China) 30 mL/min, and air flow rate 350 mL/min.

Small molecular metabolites in milk samples were determined by ultra-performance liquid chromatography (UPLC) (ACQUITY UPLC I-Class, Waters, Manchester, UK), equipped with high-resolution mass spectrometers (MS/MS, ESI-QTOF/MS, Xeyo G2-5, Waters, Manchester, UK). Milk (0.1 mL) and was transferred into 2 mL Axygen centrifuge tube (Corning, Tewksbury, NY, USA) and mixed with 1.4 mL methanol (with 0.1% formic acid), and vortexed for 30 s. Samples were kept at −20 °C for a few min to accelerate protein precipitation, then centrifuged at 12,000× *g* for 10 min at 4 °C. The supernatant (1 mL) was collected into an injection vial (with filter membrane), and stored at −20 °C for later UPLC-MS/MS analysis. Milk small molecular metabolites were determined according to methods in the reference [30].

2.4. Data Calculation and Statistical Analysis

Data on dry matter intake, milk yield, milk composition, serum biochemical parameters, and rumen fluid volatile fatty acids (VFAs) were preliminary processed using Excel 2010 and then subjected to one-way ANOVA. The general linear model procedure was used for all of them and then Student Newman Keuls post hoc tests on the differences between the means once the ANOVA indicated a significant effect of the treatment (Version 10.0, SAS Institute Inc., Cary, NC, USA). Significance was declared when p value was ≤ 0.05. Graphs were produced using Origin 2021 (OriginLab Corporation, Northampton, MA, USA).

The LC-MS/MS data were analyzed with multivariate analysis using the Progenesis QI software (Waters, Wilmslow, UK), subjected to searching HMDB database for compound matching. MetaboAnalyst 3.0 (https://www.metaboanalyst.ca (accessed on 14 February 2022)) was used to analyze the pathways mostly relevant to mycotoxin contamination in the milk metabolic pathways through data input, compound identification matching, pathway analysis (including pathway enrichment analysis and pathway topology analysis).

3. Results

3.1. Mycotoxin Residues in Milk

Feeding the cows with the diets containing mildewy corn meal and cottonseed resulted in a significant increase in the major mycotoxin residues in milk compared with the control group (Figure 1). Aflatoxin M1 and deoxynivalenol concentrations were significantly greater in groups 100Cot, 50CotCorn, and 100CotCorn ($p < 0.05$), and aflatoxin B1 and zearalenone concentrations were significantly greater in group 100Cot ($p < 0.05$) compared with the control group.

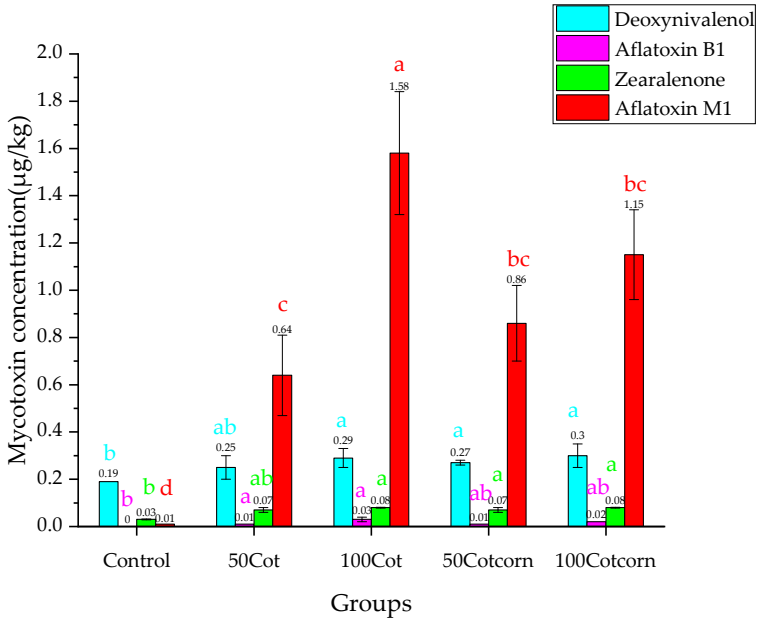

Figure 1. Concentrations of major mycotoxins in milk of dairy cows fed diets containing mildewy corn meal and cottonseed. Different letters (a, b, c, and d) denote significant differences ($p < 0.05$).

3.2. Feed Intake, Milk Yield, and Milk Composition

As shown in Table 4, feeding the cows with the diets containing mildewy corn meal and cottonseed significantly affected the production performances including feed intake, milk composition, and yield. When compared with the control group, the dry matter intake was significantly lower in groups 100Cot, 50CotCorn, and 100CotCorn ($p < 0.05$), but not in group 50Cot ($p > 0.05$). The milk yield was greater in group 50Cot ($p < 0.05$), but not different in groups 100Cot, 50CotCorn, and 100CotCorn ($p > 0.05$). The content of 4% fat-corrected milk (4% FCM) and energy-corrected milk (ECM) was similar between the control and group 50Cot ($p > 0.05$), and significantly lower in groups 100Cot, 50CotCorn, and 100CotCorn ($p < 0.05$). The milk fat concentration was lowered in groups 50Cot and 100Cotcorn ($p < 0.05$), but did not differ in groups 100Cot and 50CotCorn ($p > 0.05$). The milk protein concentration was low only in group 100CotCorn ($p < 0.05$). The milk lactose concentration was greater in group 50Cot ($p < 0.05$). The total solids concentration was low only in group 100CotCorn ($p < 0.05$). SCC was lower in groups 50Cot, 50CotCorn, and 100Cotcorn ($p < 0.05$) than the control. There was no significant change in the feed efficiency ratio (FER) and MUN concentration among the five groups ($p > 0.05$).

Table 4. Productive performance and milk composition of dairy cows fed diets containing mildewy corn meal and cottonseed.

Item	Control	50Cot	100Cot	50CotCorn	100CotCorn	SEM	*p*-Value
Productive performance [1]							
DMI (kg/d)	20.71 [a]	20.99 [a]	19.99 [b]	19.58 [bc]	19.36 [c]	0.08	<0.01
MY (kg/d)	21.30 [b]	23.91 [a]	20.93 [b]	19.74 [b]	19.77 [b]	0.24	<0.01
4% FCM (kg/d)	23.18 [ab]	24.64 [a]	22.14 [b]	20.82 [b]	20.83 [b]	0.43	<0.01
ECM (kg/d)	25.63 [ab]	27.25 [a]	24.54 [b]	23.29 [b]	23.27 [b]	0.02	<0.01
FER	1.25	1.32	1.22	1.21	1.20	0.35	0.08
Milk composition							
Fat (%)	4.52 [a]	4.20 [b]	4.37 [ab]	4.37 [ab]	4.18 [b]	0.03	0.01
Protein (%)	3.84 [a]	3.77 [a]	3.83 [a]	3.99 [a]	3.41 [b]	0.03	<0.01
Lactose (%)	4.98 [b]	5.10 [a]	4.94 [b]	4.94 [b]	5.03 [ab]	0.01	<0.01
Total solids (%)	14.01 [a]	13.63 [a]	13.85 [a]	13.96 [a]	13.27 [b]	0.06	<0.01
SCC (10^4/mL) [2]	14.96 [a]	7.39 [b]	14.44 [a]	7.52 [b]	9.09 [b]	1.08	<0.01
MUN (μg/mL) [2]	12.94	13.07	12.09	12.66	14.38	0.29	0.16

[abc] Means with different superscript letters in the same row indicate significant difference ($p < 0.05$). [1] DMI, dry matter intake; MY, milk yield; 4%FCM, 4% fat-corrected milk = 0.4 × milk (kg) + 15 × fat (kg) [31]; ECM, energy-corrected milk = 0.327 × milk (kg) + 12.95 × fat (kg) + 7.20 × protein (kg) [31]; FER, feed efficiency ratio = ECM/DMI [32]. [2] SCC, somatic cell count; MUN, milk urea nitrogen.

3.3. Serum Biochemical Parameters

Biochemical parameters in serum are shown in Table 5. Compared with the control group, the creatinine (CR) concentration was significantly lower only in group 100CotCorn ($p < 0.05$), and total glyceride concentration (TG) was greater in groups 100Cot and 100CotCorn ($p < 0.05$). No significant difference was observed in the other parameters, such as total protein, albumin, globulin, alanine aminotransferase, aspartate aminotransferase, the albumin/globulin ratio, alkaline phosphatase, total bilirubin, direct bilirubin, indirect bilirubin, γ-glutamyl transpeptidase, total cholesterols, and uric acid ($p > 0.05$).

Table 5. Blood metabolites of dairy cows fed diets containing mildewy corn meal and cottonseed.

Item [1]	Control	50Cot	100Cot	50CotCorn	100CotCorn	SEM	*p*-Value
TP (g/L)	73.06	72.79	72.71	73.86	72.76	0.82	0.99
ALB (g/L)	36.10	34.39	35.50	37.55	36.40	0.38	0.09
GLOB (g/ L)	36.96	38.40	37.21	36.31	36.88	0.88	0.96
CR (μmol/L)	75.88 [a]	66.56 [ab]	66.75 [ab]	72.43 [a]	64.00 [b]	1.50	0.01
ALT (U/L)	27.75	26.63	28.43	29.25	27.75	0.69	0.81
AST (U/L)	67.29	68.00	71.86	67.57	72.86	1.88	0.81
A/G	1.01	0.91	0.98	1.08	0.99	0.03	0.47
ALP (U/L)	53.76	58.11	78.35	81.95	70.00	4.00	0.09
TBiL (μmol/L)	8.88	10.97	9.82	12.25	10.60	0.42	0.11
DBiL (μmol/L)	1.92	2.42	2.40	2.54	2.46	0.08	0.09
IBiL (μmol/L)	6.97	8.55	7.42	9.71	8.14	0.36	0.11
GGT (U/L)	35.16	33.23	33.98	33.98	37.20	1.19	0.85
TC (mmol/L)	5.88	6.35	5.72	6.67	6.08	0.20	0.61
TG (mmol/L)	0.04 [b]	0.04 [b]	0.06 [a]	0.06 [ab]	0.07 [a]	0.003	<0.01
UA (μmol/L)	27.28	24.95	27.23	16.74	25.23	1.40	0.08
Urea (mmol/L)	3.89	3.57	3.89	3.67	3.86	0.07	0.55

[abc] Means with different superscript letters in the same row indicate significant difference ($p < 0.05$). [1] TP, total protein; ALB, albumin; GLOB, globulin; CR, creatinine; ALT, alanine aminotransferase; AST, aspartate aminotransferase; A/G, albumin/globulin; ALP, alkaline phosphatase; TBiL, total bilirubin; DBiL, direct bilirubin; IBiL, indirect bilirubin; GGT, γ-glutamyl transpeptidase; TC, total cholesterols; TG, total triglyceride; UA, uric acid.

3.4. Rumen Function

Rumen fermentation parameters are shown in Table 6. Compared with the control, no significant difference was observed in the acetate concentration, the acetate to propionate ratio, valerate concentration, the total VFA concentration, and the calculated CH_4 production for the other four groups ($p > 0.05$). The propionate concentration was significantly greater in group 50Cot; the isobutyrate concentration was significantly greater in groups 50CotCorn and 100CotCorn; the butyrate concentration was lower in group 100Cot; and the isovalerate concentration was significantly greater in group 50Cot than those for the control ($p < 0.05$).

Table 6. Rumen fermentation parameters in rumen fluid of dairy cows fed diets containing mildewy corn meal and cottonseed.

Item	Control	50Cot	100Cot	50CotCorn	100CotCorn	SEM	*p*-Value
Acetate (mmol/L)	65.15	73.74	58.96	75.35	65.27	2.07	0.07
Propionate (mmol/L)	21.79 ^{b}	26.71 ^{a}	21.56 ^{b}	23.12 ^{ab}	22.44 ^{ab}	0.61	0.05
Acetate/Propionate	3.01	2.84	2.99	3.14	3.04	0.04	0.16
Isobutyrate (mmol/L)	0.74 ^{a}	0.87 ^{a}	0.80 ^{a}	1.02 ^{b}	1.09 ^{b}	0.03	<0.01
Butyrate (mmol/L)	12.68 ^{ab}	14.04 ^{a}	9.78 ^{b}	12.76 ^{ab}	13.48 ^{ab}	0.49	0.05
Isovalerate (mmol/L)	1.43 ^{bc}	1.64 ^{abc}	1.24 ^{c}	1.85 ^{ab}	2.08 ^{a}	0.08	<0.01
Valerate (mmol/L)	1.48 ^{ab}	1.90 ^{a}	1.32 ^{b}	1.67 ^{ab}	1.70 ^{ab}	0.06	0.02
TVFA (mmol/L) [1]	101.27	116.89	99.63	124.29	114.07	2.32	0.12
CH_4 (mmol/L) [2]	28.27	31.64	27.26	32.62	28.55	0.76	0.11

^{abc} Means with different superscript letters in the same row indicate significant difference ($p < 0.05$). [1] TVFA, total volatile fatty acids. [2] CH_4 production (mmol/ L) = 0.45 × acetate (mmol/ L) − 0.275 × propionate (mmol/ L) + 0.4 × butyrate (mmol/L) [33].

3.5. Mycotoxin-Induced Metabolomic Changes in Milk

The enrichment analysis and path impact values of different pathways were performed by using the metabolomics view map originated from pathway topology analysis (MetaboAnalyst3.0). The pathways ranked in the top four were glycine, serine, and threonine metabolism, pyruvate metabolism, taurine and hypotaurine metabolism, and citrate cycle (TCA cycle), and the corresponding pathway impact values were 0.48, 0.42, 0.35, and 0.33, respectively (Figure 2).

Principal component analysis (PCA) was performed to obtain an overview of the metabolites in milk among the five groups. The values of abscissa and ordinate were used as pathway impact and pathway enrichment of differential metabolites in metabolic pathways, in other words, the greater the number of differential metabolites means that the corresponding pathway was more important. As shown in Figure 3, the difference between groups 50CotCorn and 100CotCorn was greater than that between the other three groups.

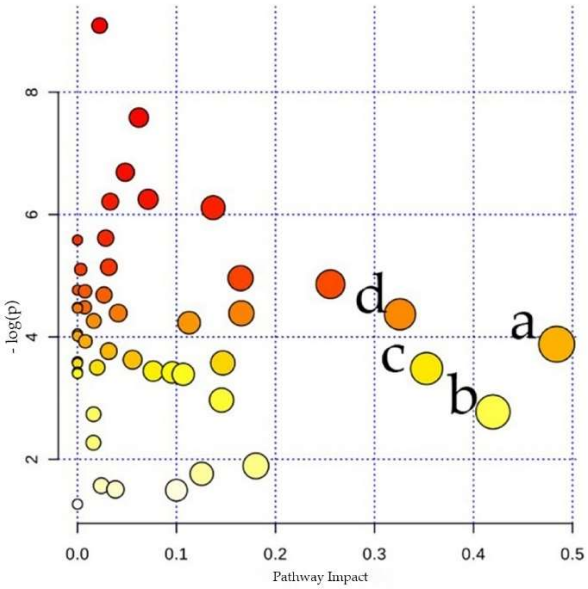

Figure 2. Metabolome view map showing the matched pathways according to the *p* values obtained from pathway enrichment analysis and the pathway impact values produced by pathway topology analysis. (**a**) Glycine, serine and threonine metabolism; (**b**) pyruvate metabolism; (**c**) taurine and hypotaurine metabolism; (**d**) citrate cycle (TCA cycle). In the map, dots with larger sizes and darker colors represent greater pathway enrichment and greater pathway impact values, respectively.

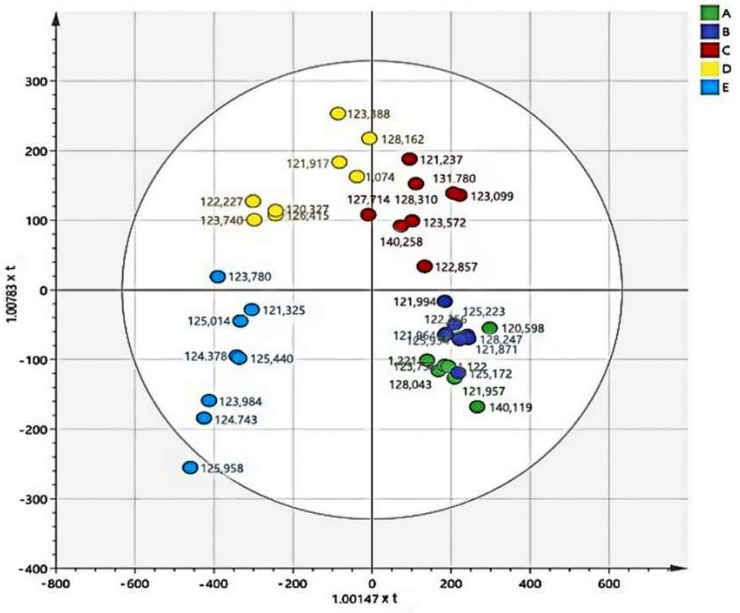

Figure 3. Principal component analysis plot for metabolites in milk detected by using LC-MS/MS. Green (A) Control group; mineral blue (B) 50Cot group; red (C) 100Cot group; yellow (D) 50CotCorn group; azure (E) 100CotCorn group.

4. Discussion

Mycotoxin contamination is an important topic with a significant impact on feed and food safety. So far, although numerous studies on mycotoxin addition have been reported, relatively little has been focused on the natural mildew. In comparison to the major literature [18,21,33,34] that added single pure mycotoxin, this study has a higher mycotoxin level in feed by natural mildew.

4.1. Natural Mycotoxins Residues in Milk

In the present study during the 14 days of experiments, feeding dairy cows with diets containing mildewy corn meal and cottonseed for 14 days resulted in substantial increases of natural mycotoxin residues in milk, particularly aflatoxin M1 from the diets containing mildewy cottonseed. Aflatoxin M1 was derived from oxidation of four hydroxy derivatives of aflatoxin B1 in the hepatic microsomal mixed function oxidase system in cows [18]. A number of countries have set up strict legal regulations on aflatoxin M1 residue in milk, and the maximum allowance is 0.05 μg/kg milk by the European Union and 0.5 μg/kg milk in China, the USA, and Japan [8,9]. Previous studies have shown that aflatoxin M1 excreted in milk can reach 0.08–0.39 μg/kg when 20 μg/kg and 40 μg/kg aflatoxin B1 is added to Holstein cows' feed [18,21]. In the present study after the 14 days of experiments, the natural mycotoxin M1 residue was 0.64–1.58 μg/kg in milk of the cows fed the moldy feeds, exceeding the limits and the literature. Additionally, the *aflatoxin M1* conversion in milk was 1.91–2.86%, which was slightly higher than some reports [18,21]. This may be attributed to the effect of aflatoxin B1 content, individual differences between cows, and synergistic effects of mycotoxins. The contents of aflatoxin M1 in 100Cot group is significantly higher than that obtained with 100CotCorn (Figure 1) even if the initial contents of these groups in aflatoxin B1 is almost the same. Therefore, interaction (antagonism) between molecules of Cot and Corn could lead to the reduction of aflatoxin M1 in 100CotCorn.

4.2. Effects of Natural Mycotoxin in Feed Intake, Milk Yield and Milk Composition

Feed intake, milk yield and milk composition are important reference indexes for the performance of lactating cows, and to a certain extent, also reflect the health state of cows. Feeding a diet containing mildewy components to ruminant can cause many adverse effects, such as a loss of appetite, decreases in feed intake and milk production [19]. In the present study during the 14 days of experiments, there was a decreasing trend for the dry matter intake, milk yield, 4% FCM, and ECM with the increasing amounts of mildewy corn meal and cottonseed in the diet. Our results are similar to the finding by Jones et al. where feeding 140 dairy cows with a diet containing 20 μg/kg Aflatoxin B1 decreased their feed intake and milk production [34]. However, Battacone et al. [35] found feeding lactating sheep with diets containing 32 and 64 μg/kg AFB1 had no influence on sheep's performance, which is in agreement with no obvious influence of dietary aflatoxin B1 on milk production in lactating cows by Kutz [36]. The reason for the no detrimental effects of the dietary mycotoxin levels on the productive performance of sheep and cows might be attributed to the dietary mycotoxin level not exceeding the threshold for toxic effects.

In the present experiment during the 14 days of experiments, we found feeding diets containing mildewy corn meal and cottonseed changed milk compositions, such as fat, protein, and total milk solids, as well as somatic cell counts. The finding agrees with previous studies that observed a high correlation between dietary aflatoxin B1 level and milk composition in lactating cows [21,37]. Numerous studies have also demonstrated the effect of dietary mycotoxin contamination on milk composition. Keese et al. [37] fed 27 German Holstein lactating dairy cows with a diet containing 4.4 mg/kg vomitoxin and found that milk fat concentration was increased, however, when the dietary vomitoxin exceeded 4.6 mg/kg, the milk yield increased but milk fat and milk protein concentrations decreased. Queiroz et al. [38] found similar results. MUN is a crucial indicator to the evaluation of dietary energy level, protein metabolism in the body, and reproductive performance in dairy cows [36]. In the present study during the 14 days of experiments, no significant difference in MUN between the five groups might imply no significant change in the metabolism by natural mycotoxins in the diets. SCC is an indicator to the health status of lactating cows [39]. In cows that are healthy or have no intramammary infection and mastitis, SCC should be lesser than 200,000/mL [40]. In the present study during the 14 days of experiments, SCC was below 200,000/mL for all cows, and showed no difference between the groups, indicating good healthy conditions of the cows under the farming management and conditions.

4.3. Effects of Natural Mycotoxin in Serum Biochemical Parameters

Biochemical parameters in serum could reflect the healthy and metabolic status of the organs (liver and kidneys in particular) and the whole body [18,19]. The effect of dietary mycotoxins on serum biochemical parameters is not conclusive. For example, Battacone et al. [35] found that dairy goats fed diets contaminated with aflatoxin B1 (2.30 and 5.03 μg/kg) showed no change in plasma ALP, AST, and ALT levels. This finding agrees with a report by Wang et al. [21]. However, Queiroz et al. [38] found that when aflatoxin B1 in diets for lactating cows reached 75 μg/kg, the plasma concentration of haptoglobin was reduced, indicating a non-specific immune stress occurring in the body. In the present study during the 14 days of experiments, there was no significant difference in most of the serum biochemical parameters between the control and the other four treatments, likely due to the low level of the mycotoxin in the diets. Our results are supported by the findings from a previous study [21].

4.4. Effects of Natural Mycotoxins in Rumen Fermentation

VFAs and CH_4 are byproducts of ruminal fermentation [41], which are often used to evaluate the efficiency of ruminal microbial fermentation [18]. Several reports have shown that mycotoxin contamination of feeds or diet affects rumen fermentation [20,21,41,42]. Data from the present study during the 14 days of experiments are broadly consistent with the results by Jiang et al. [42] and Santos et al. [43]. Individual VFAs, namely propionate, isobutyrate, butyrate, isovalerate, and valerate were affected by the dietary levels of natural mycotoxins in the present study. In addition, there was no significant change in the acetate to propionate ratio, indicating that the primary pattern of rumen fermentation was not affected by natural mycotoxins present in mildewy corn meal and cottonseed during the 14 days of experiments. The results agree with the report by Wang et al., where aflatoxin B1 and mildewy cottonseed were studied [20,21].

4.5. Milk Metabolomic Pathway Changes Induced by Natural Mycotoxins

In the current study, the changes in small molecule metabolites in milk was used an LC-MS/MS metabolic method. The results indicate that dietary mycotoxin exposure significantly affected the amino acid metabolism, carbohydrate metabolism, and energy metabolism in the mammary gland (Figures 2 and 3). Similar studies have shown that dietary aflatoxin B1 exposure and feeding diet containing mycotoxins-contaminated cottonseed significantly affected rumen fermentation and metabolites in both plasma and milk in lactating cows by using NMR-based metabolomic method [20,21].

Previous research showed that amino acids metabolism was affected by mycotoxins [20]. Serine is derived from 3-phospho-D-glycerate, and glycine is derived from serine [44]. Threonine is an essential amino acid that is derived from aspartate in bacteria and plants, and metabolized to generate glycine and serine [45]. In the present study, glycine, serine, and threonine metabolism had the greatest impact score in the metabolic pathway analysis, indicating that dietary mycotoxin exposure may result in a disorder of amino acids metabolism. Glycine, serine, and threonine are all glucose-producing amino acids [46]. Studies have shown that the change in glucose metabolism may be an important metabolic alteration caused by mycotoxin exposure [47]. Therefore, dietary mycotoxin exposure may affect amino acid metabolism and glucose metabolism in the mammary gland.

Pyruvate is the intermediate product of glycolytic metabolism [48], which plays an important role in glucose metabolism, amino acid metabolism, citrate cycle, and fatty acid metabolism [49]. Pyruvate realizes the mutual conversion of sugars, fats, and amino acids in the body through the acetyl CoA and tricarboxylic acid cycles. More importantly, pyruvate is the end product of glycolysis and a raw material for gluconeogenesis. In this study, the detection and pathway analysis of small molecular metabolites in milk showed that pyruvate metabolism was significantly enriched after the dietary mycotoxin exposure. The tricarboxylic acid (TCA) cycle is a central hub of carbohydrate, lipid, and amino acid metabolism [50] and also the primary pathway that the body procures energy from and for

the biosynthesis of raw materials. Cheng et al. [46] reported that dietary AFB1 exposure may disrupt the tricarboxylic acid cycle in goats, further affecting energy metabolism. Similar results were found in the present study that the tricarboxylic acid cycle may be disrupted by residual mycotoxins in milk. A similar phenomenon has also been observed in other animal studies. Yarru [51] and Wan [52] found that feeding chickens with aflatoxin-contaminated feeds may affect the ATP synthesis pathway and reduce energy production and gene expression. In summary, dietary mycotoxin exposure may cause adverse effects on glucose metabolism in milk and in animal health.

Taurine and hypotaurine metabolic pathways are the main energy metabolic pathways in which hypotaurine is oxidized to taurine by hypotaurine dehydrogenase [53]. Studies have found that the stabilization of taurine level has a positive effect on lowering blood lipids, blood sugar, and serum triglyceride levels, maintaining reproductive function, improving obesity, and reducing liver fat accumulation [44]. The present study discovered that the taurine and hypotaurine metabolism pathways had a greater impact score in the metabolic pathway enrichment analysis, indicating that dietary mycotoxins exposure may affect the body's energy metabolism. Meantime, pyruvate metabolism is an important energy metabolic pathway responsible for glycolysis to produce energy in organisms [48]. The study also found that dietary mycotoxin exposure affected pyruvate metabolism in milk.

5. Conclusions

In summary, feeding lactating cows with diets containing natural mildewy corn meal and cottonseed reduced feed intake, milk yield, and milk fat, protein and lactose productions, but no significant effect was found in the majority of biochemical parameters. Additionally, metabolic pathways of small molecule metabolites in milk also changed. In conclusions, this study may provide a guide to the production practices.

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Institutional Review Board Statement: The study (Protocol Number: IAS15020; Date of approval: 16 July 2015) was approved by the Animal Care and Use Committee of the Institute of Animal Science, Chinese Academy of Agricultural Sciences.

Informed Consent Statement: Not applicable.

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