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Impact of feeding regimens on the composition of gut microbiota and metabolite profiles of plasma and feces from Mongolian sheep

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Mongolian sheep are an indigenous ruminant raised for wool and meat production in China. The gut microbial community plays an important role in animal performance and metabolism. The objective of this study was to investigate the effects of two feeding regimens on the diversity and composition of gut microbiota and metabolite profiles of feces and plasma from Mongolian sheep. A total of 20 Mongolian sheep were assigned to one of two feeding regimens: free grazing (FG) and barn confinement (BC). When samples were collected, the average live weights of the sheep were 31.28 ± 1.56 kg and 34.18 ± 1.87 kg for the FG and BC groups, respectively. At the genus level, the FG group showed higher levels of *Bacteroides*, *RC9_gut_group*, *Alistipes*, *Phocaeicola*, *Barnesiella*, and *Oscillibacter*, and lower levels of *Succinivibrio*, *Treponema*, and *Prevotella*, compared to the BC group. The butyric acid content in feces was lower in the FG group ($P < 0.05$). Higher levels of palmitic acid, oleic acid, alpha-linolenic acid, L-carnitine, L-citrulline, and L-histidine, and lower levels of L-tyrosine, L-phenylalanine, and L-kynurenine were found in the plasma of the FG sheep. Moreover, there were substantial associations between several gut microbiota genera and alterations in feces and plasma metabolites especially those involved in the metabolism of butyric acid, linolenic acid, and L-tyrosine. Feeding regimens can not only influence the composition of gut microbiota, but also alter metabolic homeostasis in sheep.

Keywords: feeding regimens, gut microbiota, metabolite, mongolian sheep

Introduction

In many countries, including China, grassland protection and conservation is becoming ever more important. The implementation of new government policies has placed pressure on the traditional practice of farming grazing domesticated animals. Consequently, grazing is gradually being phased out and replaced with concentrated animal feeding operations similar to feedlot rearing. It has been demonstrated that, at least for sheep, feeding regimens can affect their overall health (Celi *et al*., 2017), growth characteristics (Resconi *et al*., 2009), and even meat quality (Joy *et al*., 2008; Wang *et al*., 2018a). Moreover, several studies have indicated that the diversity and composition of gut microbes in animals, including cattle, pigs, and geese, appear to be related to their nutrition, which depends largely on their feeding regimens (Lazarevic *et al*., 2009; Xia *et al*., 2018; Zhao *et al*., 2018).

Recent advances in high-throughput sequencing have increased our understanding of the composition and interactions of complex bacterial populations in the animal gastrointestinal tract (Kim *et al*., 2019). The gut microbiota is now known to play important roles in enhancing host nutritional acquisition, stimulating immune modulation, and maintaining homeostasis in response to lifestyle changes (Kau *et al*., 2011; Clemente *et al*., 2012). However, recent research has shown the relationship both the microbial composition and high omega-3 concentration in animals diets, feeding paradigms, and probiotics (Costantini *et al*., 2017; Wang *et al*., 2017; Xue *et al*., 2017). Research has explored, by means of metabolomics, the effects of the gut microbiota on metabolic function.

Metabolomics, as a part of systems biology, is an innovative, high-throughput bioanalytical method that has been used in recent years for detecting plasma, fecal, rumen, and tissue metabolite biomarkers from humans and animals (Karisa *et al*., 2014; Warner *et al*., 2015; Xia *et al*., 2018; Zhao *et al*., 2018). Gas chromatography-mass spectrometry (GC-MS), nuclear magnetic resonance (NMR), and liquid chromatography-mass spectrometry (LC-MS) are the main analytical tools in metabolomic studies. Microbial metabolites such as short-chain fatty acids (SCFAs) are used primarily as an energy source in the host. Guida and Venema (2015) showed that SCFAs are ligands for G protein-coupled receptors, GPCR41 and GPCR43, which are closely related to fat metabolism genes (FAS, ACC, and PPARγ). Meanwhile, knowledge of metabolite concentrations is valuable for understanding the health and productivity of individual sheep (Malau-Aduli and Holman, 2015). Quantifying key hematological metabolite concentrations has been used to estimate the response of sheep to diet changes (Hegarty *et al*., 2006). In addition, Laeger *et al*. (2012) observed that a form of plasma metabolites improved to increase body tissue reserves and efflux toward higher milk production. However, there is limited

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information available that compares the effects of pasture grazing and feeding in the barn on fecal microbes and plasma metabolites in sheep.

The objective of this study was to use 16S rRNA gene sequencing and LC-MS-based metabolomics to investigate the effects of two feeding regimens, free grazing and barn confinement, on the composition of gut microbiota and metabolites from feces and plasma in Mongolian sheep.

Materials and Methods

Animals and diets

In total, 20 Mongolian sheep were kept indoors for 3 months together with their mothers. At an average live weight of 10 ± 0.5 kg, the lambs were allocated randomly to one of two feeding groups, free-grazed (FG) or barn-confined (BC), with 10 lambs (five male and five female) per group. For 9 months, the FG sheep were allowed to graze freely on grassland that consisted mainly of *Stipa gobica*, *Stipa breviflora*, and *Cleistogenes squarrosa*, whereas the BC sheep were fed a diet consisting of corn (60%), hay (38%), and minerals (2%) for 9 months. When samples were collected, the average live weights of the sheep were 31.28 ± 1.56 kg and 34.18 ± 1.87 kg for the FG and BC groups, respectively. The animal experiments were carried out according to the guidelines outlined by the National Institute of Animal Health, China (GB 14925-2001) and the Inner Mongolia Agriculture University Animal Care (Permit number: IMAU-2017323).

Sample collection

After 9 months of feeding, 20 blood and fecal samples were collected in the morning before feeding. Blood samples were collected from the jugular vein into EDTA tubes. The samples were immediately placed on ice and centrifuged at 3,000 × *g* for 10 min at 4°C. The resulting plasma was stored at -80°C until analysis. Fecal samples were aseptically collected in sterile plastic tubes, immediately frozen in liquid nitrogen, and stored at -80°C until analysis.

16S rRNA microbial community analysis

Total bacterial metagenomic DNA was extracted from 20 fecal samples using a QIAamp DNA Stool Mini Kit (No. 51504; Qiagen) according to the manufacturer's instructions. The V4-V5 hypervariable regions of the bacterial 16S rRNA gene were amplified with primers 515F (5′-GTGCCAGCM GCCGCGGTAA-3′) and 907R (5′-CCGTCAATTCMTTT RAGTTT-3′) using a thermocycler PCR system (GeneAmp 9700, ABI). The PCR reactions were conducted using the following program: 3 min of denaturation at 95°C, 27 cycles of 30 sec at 95°C, 30 sec of annealing at 55°C, and 45 sec of elongation at 72°C, and a final extension at 72°C for 10 min. The PCR reactions were performed in triplicate in 20-μl mixtures containing 4 μl of 5× FastPfu Buffer, 2 μl of 2.5 mM dNTPs, 0.8 μl of each primer (5 μM), 0.4 μl of FastPfu Polymerase, and 10 ng of template DNA. The resulting PCR products were extracted from a 2% agarose gel and further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences) and quantified using QuantiFluor™-ST (Promega) according to the manufacturer's instructions.

Purified amplicons were pooled in equimolar amounts and paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Illumina) according to the standard protocols by Majorbio Bio-Pharm Technology Co., Ltd.. The raw reads were submitted to the NCBI Sequence Read Archive database under ID number PRJNA515122.

The raw sequencing data were quality-filtered by Trimmomatic and merged by FLASH (Magoč and Salzberg, 2011; Bolger *et al*., 2014). Filtered sequences were clustered into operational taxonomic units (OTUs) according to representative sequences using UPARSE (version 7.0; http://drive5.com/uparse/). The taxonomy of each 16S rRNA gene sequence was analyzed using an RDP Classifier algorithm (http://rdp.cme.msu.edu/) against the Silva 128 database (https://www.arb-silva.de) using a confidence threshold of 80%. Alpha and beta diversities were calculated based on the *de novo* taxonomic tree constructed by the representative chimera-checked OTU set using FastTree (Price *et al*., 2010). To assess the microbiota structure of different samples, principal coordinate analysis (PCoA) based on the weighted UniFrac distances derived from the phylogenetic tree was performed (Lozupone and Knight, 2005). The differences between groups were compared using STAMP software (http://kiwi.cs.dal.ca/Software/STAMP) (Parks and Beiko, 2010).

Metabolite profiling analysis of plasma and fecal samples

Plasma metabolite analysis : Plasma metabolite analyses were performed using an ultra-high performance liquid chromatography (UHPLC) system (1290 Infinity LC; Agilent Technologies) coupled to a quadrupole time-of-flight (Q-TOF) instrument (AB Sciex Triple TOF 6600). For hydrophilic interaction liquid chromatography (HILIC) separation, samples were analyzed using a 2.1 mm × 100 mm ACQUITY UPLC BEH 1.7-μm column (Waters). The autosampler and column compartment were maintained at 4 and 25°C, respectively. The mobile phase consisted of solvents A (25 mM ammonium acetate and 25 mM ammonium hydroxide in water) and B (acetonitrile). The elution gradient program for the plasma samples was as follows: 85% B for 1 min; linear reduction to 65% in 11 min; reduction to 40% in 0.1 min and maintenance for 4 min; and then increase to 85% in 0.1 min, with a 5-min re-equilibration period. The flow rate was 0.3 ml/min.

The electrospray ionization source conditions were set as follows: Ion Source Gas 1 (Gas1) as 60, Ion Source Gas 2 (Gas2) as 60, curtain gas as 30, source temperature at 600°C, and IonSpray Voltage Floating at ± 5,500 V. In MS-only acquisition, the instrument was set to acquire over the *m/z* range of 60 to 1,000 Da, and the accumulation time for the TOF MS scan was set at 0.20 sec/spectra. In auto MS/MS acquisition, the instrument was set to acquire over the *m/z* range of 25 to 1,000 Da, and the accumulation time for product ion scan was set at 0.05 sec/spectra. The product ion scan was acquired using information-dependent acquisition with high-sensitivity mode selected. The parameters were set as follows: collision energy fixed at 35 V with ± 15 eV; declustering potential at 60 V (+) and -60 V (−); exclusion of isotopes within 4 Da; and candidate ions to monitor per cycle set to six.

The raw MS data from the plasma samples were first processed using ProteoWizard (version 3.0.8789). After nor-

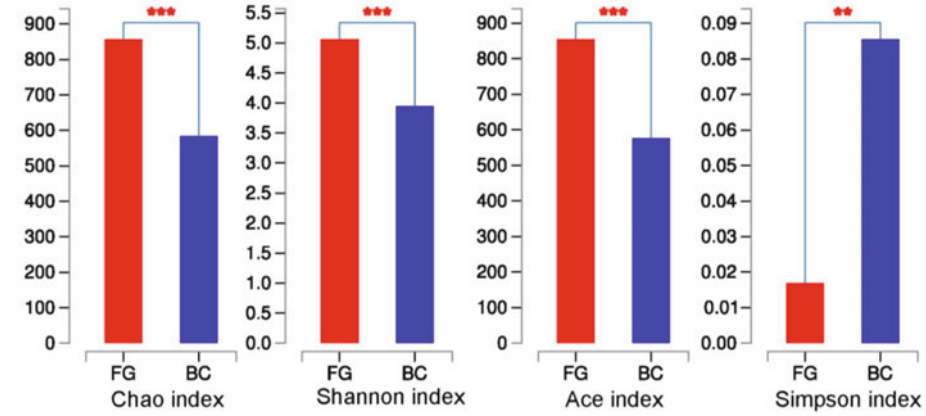

Fig. 1. Differences in alpha-diversity of gut microbiota between groups. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

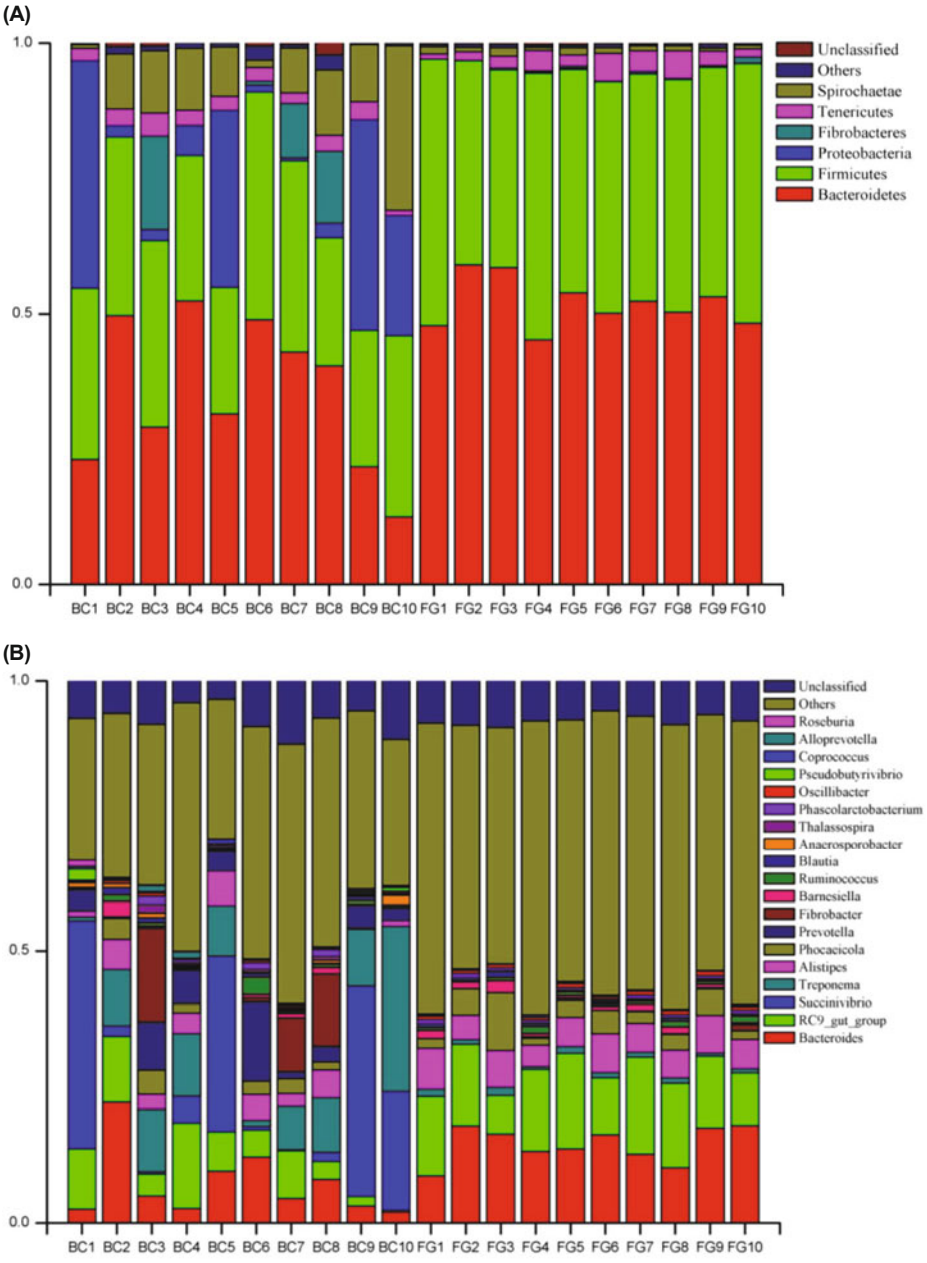

Fig. 2. Distribution and composition of gut microbiota. Color-coded bar plot shows the average bacterial phylum/genus distributions in all samples. Data in this figure were scaled up to 100%. (A) phylum, (B) genus.

malization to total peak intensity, the processed data were uploaded into MetaboAnalyst software (www.metaboanalyst.ca) for further analysis. The assigned ions of the metabolite were identified through database searches including the Human Metabolome Database (http://www.hmdb.ca/), the Biofluid Metabolites Database (http:// metlin.scripps.edu), and MassBank (http://www.massbank.jp/).

Fecal metabolite analysis : Twenty fecal samples, each weighing approximately 1.0 g, were diluted with 2.0 ml of 0.9% NaCl solution, followed by the addition of 1 ml of 0.5 M sulfuric acid. The diluted solution was kept on ice for 30 min before centrifugation at 10,000 rpm for 10 min at 4°C. Supernatants (1.0 ml) were extracted using 2 ml of diethyl ether by vortex mixing for 2 min, centrifugation (10,000 rpm/min) for 10 min, followed by incubation at 4°C for 30 min. The extracts were stored at -20°C prior to analysis. Extracts (1 µl) were separated by injection into a TR-Wax MS fused silica capillary column (Thermo, 30 m × 0.25 mm i.d. × 0.25-µm film thickness) in a Thermo 1300 GC system and detected using a Thermo ISQ ion trap MS system operating in full scan mode. The operating conditions of the column were as follows: the initial temperature of 100°C was maintained for 0.5 min, then increased to 180°C at 8°C/min and maintained for 1 min, increased again to 200°C at 20°C/min, and finally maintained at 200°C for 5 min. The injector temperature was maintained at 250°C, the volume of the injected sample was 1 µl, helium was used as the carrier gas at a constant flow rate of 1.2 ml/min, the split ratio was 10:1, the ion source temperature was 250°C, and the scan mass range was m/z 40 to 450. The SCFAs present were identified by their specific retention times under these GC conditions.

Statistical analysis

Statistical comparisons were analyzed by SPSS version 19.0 (SPSS Inc.). Values of P less than 0.05 were considered statistically significant.

Results

Alpha diversity of gut microbiota

The high quality dataset contained a total of 489,435 sequences from all of the fecal samples. A total of 1359 OTUs were obtained in all samples, and for each OTU, the relative abundance was plotted for each sample in which the OTU was present.

Alpha diversity was estimated by four indices, namely, Chao, Ace, Simpson, and Shannon. The results indicated that the Chao ($P < 0.001$) and Ace ($P < 0.001$) indices were significantly higher in the FG group than in the BC group (Fig. 1). The Simpson ($P < 0.01$) and Shannon ($P < 0.001$) diversity

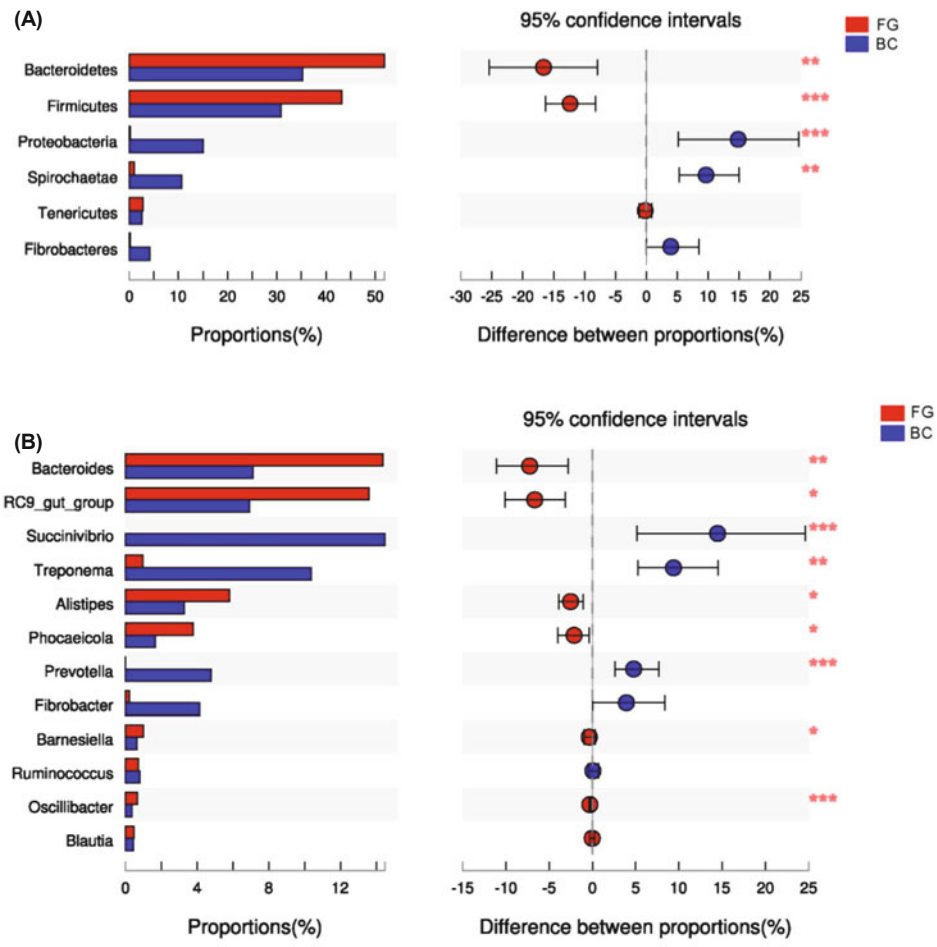

Fig. 3. Differences in gut microbiota between groups. (A) phylum, (B) genus. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

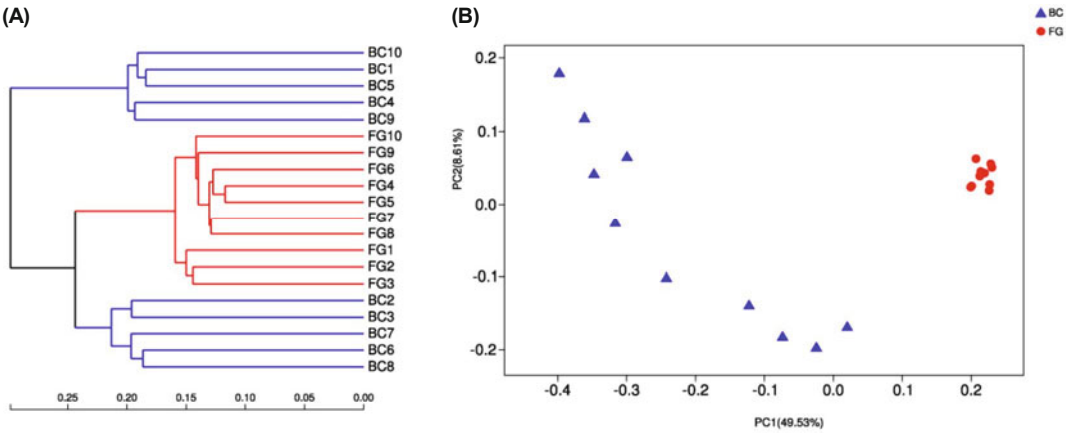

Fig. 4. Principal coordinates analysis (PCoA) of the dissimilarity between the microbial samples. Figures were constructed using weighted UniFrac distances. (A) Hierarchical dendrograms of each sample at the OTU level. Red and blue represent FG group and BC group, respectively. Each line represents one sample. (B) The principal coordinate analyses of 20 Mongolian sheep fecal samples from two feeding groups was shown. The first two axes of the principal coordinate analysis are represented with principal coordinate axis 1 on the x-axis (49.53% variability) and principal coordinate axis 2 on the y-axis (8.61% variability).

indices were significantly different between the two groups (Fig. 1). These results suggest that the alpha diversity of gut microbes was higher in the FG sheep than in the BC sheep.

Composition of gut microbiota

The microbiota composition of fecal samples from Mongolian sheep was profiled at different taxonomic levels. A total of six main phyla were identified in the feces using 16S rRNA gene sequencing (Fig. 2A). In the FG group, all sequences were classified into three main phyla (relative abundance over 1%): *Bacteroidetes* predominated (51.87%), followed by *Firmicutes* (43.24%) and *Tenericutes* (2.83%) (Fig. 2A). In the BC group, *Bacteroidetes* was also the most abundant (35.24%), followed by *Firmicutes* (30.89%), *Proteobacteria* (15.01%), *Spirochaetae* (10.63%), *Fibrobacteres* (4.15%), and *Tenericutes* (2.63%) (Fig. 2A). Obvious differences in both groups were also found in the relative abundances of four phyla (*Bacteroidetes*, *Firmicutes*, *Spirochaetae*, and *Proteobacteria*). The proportions of *Bacteroidetes* ($P < 0.01$) and *Firmicutes* ($P < 0.001$) were significantly higher in the FG group than in the BC group, and the proportions of *Spirochaetae* ($P < 0.01$) and *Proteobacteria* ($P < 0.001$) were increased in the BC group compared with the FG group (Fig. 3A).

Sequences from the samples revealed 62 genera. The top 19 genera (relative abundance over 0.2%) are listed in Fig. 2B. The sequences that could not be classified into known genera were designated as "unclassified." At the genus level, *Bacteroides* (14.37%), *RC9_gut_group* (13.60%), *Alistipes* (5.80%), and *Phocaeicola* (3.79%) were the predominant genera (relative abundance over 1%) in the FG sheep fecal samples using 16S rRNA gene sequencing. In the BC group, there were eight prevalent genera that each accounted for over 1% of the total microbiota population, namely, *Succinivibrio* (14.48%), *Treponema* (10.36%), *Bacteroides* (7.12%), *RC9_gut_group* (6.93%), *Prevotella* (4.78%), *Fibrobacter* (4.15%), *Alistipes* (3.28%), and *Phocaeicola* (1.68%). At the genus level, the variation in the composition of fecal microbiota was significantly different ($P < 0.01$) between the two groups (Fig. 3B). The abundances of *Bacteroides*, *RC9_gut_group*, and *Alistipes* increased, and those of *Prevotella* and *Treponema* decreased, in the FG group compared with the BC group. In addition, the genus *Succinivibrio* was not detected in the FG group.

Beta diversity of gut microbiota

PCoA was used to identify differences in fecal microbiota between the two groups. The PCoA plots with weighted UniFrac distance matrices showed that samples from the same feeding pattern group were closer, whereas samples from different feeding patterns had distinct differences (Fig. 4B). These results were confirmed by the construction of hierarchical dendrograms (Fig. 4A).

Fecal metabolic profiling

Fecal metabolites (SCFAs) were examined in the samples by measuring the concentrations of acetic, propionic, butyric, isobutyric, valeric, and isovaleric acids (Table 1). The concentrations of total SCFA and butyric acid were significantly higher ($P < 0.05$) in the BC group than in the FG group. However, the feeding regimens did not affect the concentrations of acetic, propionic, valeric, isobutyric, and isovaleric acid ($P > 0.05$).

Plasma metabolic profiling

The plasma metabolic profiles of Mongolian sheep were obtained using LC-MS. Using the orthogonal partial least squ-

Table 1. Effect of feeding regimens on fecal metabolites

	FG	BC
Total SCFA (μg/g)	213.86 ± 30.26[b]	324.44 ± 75.45[a]
Acetic acid	21.54 ± 1.88	19.74 ± 5.02
Propionic acid	73.47 ± 24.13	73.56 ± 14.99
Isobutyric acid	18.01 ± 1.30	18.94 ± 2.44
Butyric acid	54.13 ± 8.87[b]	163.51 ± 20.51[a]
Isovaleric acid	44.08 ± 3.27	46.19 ± 5.78
Valeric acid	2.61 ± 0.36	2.50 ± 0.36

[a,b] Significant differences between feeding regimen ($P < 0.05$).

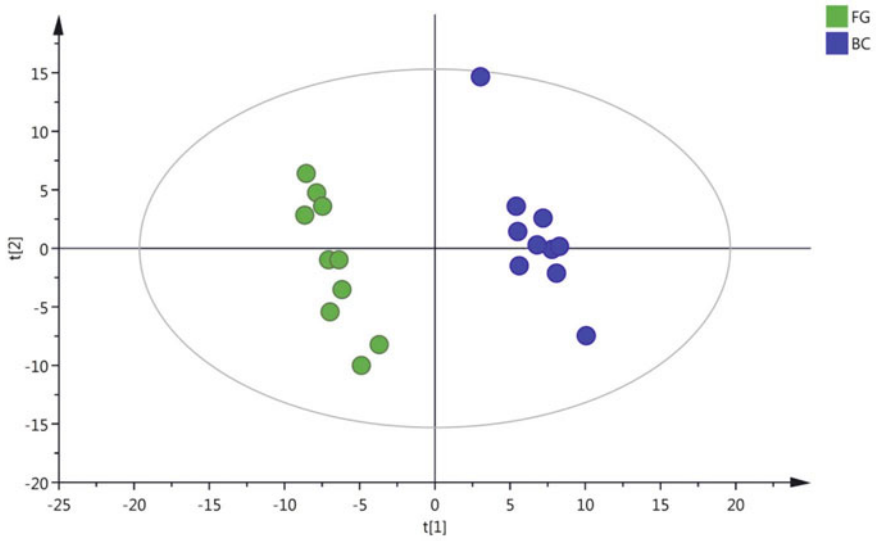

Fig. 5. **OPLS-DA score plots of plasma samples from the FG and BC groups, R2[X]=0.146, R2X[2]=0.105, Ellipse: Hotelling's T2 (95%).**

ares discriminant analysis (OPLS-DA) model, different metabolic compositions were found between the FG and BC groups, indicating that the feeding regimens resulted in distinct biochemical changes (Fig. 5).

According to multivariate statistical analysis in combination with three criteria (Table 2), the compounds identified

Table 2. **Different plasma metabolic profile between FG group and BC group**

NO.	RT (min)	Metabolites	VIP	m/z	Adduct Ion	Formula	BC/FG	Pathways
B1	1.068	Arachidic acid	9.95466	311.2953	(M-H)-	C20H40O2	↓ **	Fatty acid metabolism
B2	1.169	Oleic acid	21.6824	281.2488	(M-H)-	C18H34O2	↓ *	Fatty acid metabolism
B3	1.205	gama-Linolenic acid	12.7572	277.2173	(M-H)-	C18H30O2	↓ **	Fatty acid metabolism
B4	1.207	alpha-Linolenic acid	2.19371	555.441	(2M-H)-	C18H30O2	↓ **	Fatty acid metabolism
B5	1.216	cis-9-Palmitoleic acid	7.42893	253.2174	(M-H)-	C16H30O2	↓ *	Fatty acid metabolism
B6	1.219	Palmitic acid	11.9161	255.2334	(M-H)-	C16H32O2	↓ *	Fatty acid metabolism
B7	1.236	Pentadecanoic Acid	5.00982	241.2174	(M-H)-	C15H30O2	↓ **	Fatty acid metabolism
B8	1.255	Myristic acid	6.770	227.202	(M-H)-	C14H28O2	↓ *	Fatty acid metabolism
B9	3.006	Valeric acid	1.88184	101.0604	(M-H)-	C5H10O2	↑ *	Fatty acid metabolism
B10	8.505	Betaine	17.0752	118.0853	(M+H)+	C5H11NO2	↑ **	Fatty acid metabolism
B11	11.608	L-Carnitine	3.38068	162.1119	(M+H)+	C7H15NO3	↓ *	Fatty acid metabolism
B12	1.285	Taurolithocholic acid	1.34668	501.3322	(M+NH4)+	C26H45NO5S	↑ **	Bile acid biosynthesis
B13	4.592	Chenodeoxycholate	2.13438	410.3244	(M+NH4)+	C24H40O4	↑ **	Bile acid biosynthesis
B14	6.259	Taurocholate	1.29825	516.2963	(M+H)+	C26H45NO7S	↑ **	Bile acid biosynthesis
B15	6.492	Glycodeoxycholic acid	2.03615	450.319	(M+H)+	C26H43NO5.H2O	↓ **	Bile acid biosynthesis
B16	7.012	Cholic acid	3.26227	426.3193	(M+NH4)+	C24H40O5	↑ *	Bile acid biosynthesis
B17	7.899	Glycocholic acid	2.93053	466.3139	(M+H)+	C26H43NO6	↓ **	Bile acid biosynthesis
B18	9.262	Taurine	6.16771	126.021	(M+H)+	C2H7NO3S	↑ **	Amino acids and related
B19	7.929	L-Phenylalanine	2.8351	166.0854	(M+H)+	C9H11NO2	↑ **	Amino acids and related
B20	8.010	L-Kynurenine	1.13303	209.0909	(M+H)+	C10H12N2O3	↑ *	Amino acids and related
B21	8.552	L-Isoleucine	1.83746	132.1007	(M+H)+	C6H13NO2	↓ *	Amino acids and related
B22	9.347	L-Tyrosine	1.69955	182.0802	(M+H)+	C9H11NO3	↑ *	Amino acids and related
B23	10.923	L-Creatine	1.5844	132.0757	(M+H)+	C4H9N3O2	↓ **	Amino acids and related
B24	11.388	L-1-Methylhistidine	3.06554	170.0916	(M+H)+	C7H11N3O2	↑ **	Amino acids and related
B25	11.793	L-Glutamine	2.37386	147.0755	(M+H)+	C5H10N2O3	↑ **	Amino acids and related
B26	12.354	L-Citrulline	3.01884	176.102	(M+H)+	C6H13N3O3	↓ *	Amino acids and related
B27	13.24	L-Carnosine	2.04392	225.099	(M-H)-	C9H14N4O3	↓ *	Amino acids and related
B28	13.339	L-Histidine	1.26952	156.0756	(M+H)+	C6H9N3O2	↓ *	Amino acids and related
B29	17.086	N6-Methyl-L-lysine	2.3607	161.1274	(M+H)+	C7H16N2O2	↑ **	Amino acids and related

BC/FG, Barn confined group compared to Free grazed group
↑, up-regulated; ↓, down-regulated
*$P < 0.05$, **$P < 0.01$

in the two groups of plasma samples were mainly related to amino acids, fatty acids, and bile acid biosynthesis.

A total of 29 compounds were identified (Table 2), of which 12 were amino acids, namely, taurine (B18), L-phenylalanine (B19), L-kynurenine (B20), L-isoleucine (B21), L-tyrosine (B22), L-creatine (B23), L-glutamine (B24), L-citrulline (B25), L-histidine (B26), L-carnosine (B27), L-methyl histidine (28), and N6-methyl-l-lysine (29). Nine fatty acids were identified, namely, arachidic acid (B1), oleic acid (B2), gamma-linolenic acid (B3), alpha-linolenic acid (B4), cis-9-palmitoleic acid (B5), palmitic acid (B6), pentadecanoic acid (B7), myristic acid (B8), and valeric acid (B9). Metabolites of bile acid biosynthesis included taurolithocholic acid (B12), chenodeoxycholate (B13), taurocholate (B14), glycodeoxycholic acid (B15), cholic acid (B16), and glycocholic acid (B17). A few of the compounds identified are involved in the biosynthesis of secondary metabolites, including betaine (B10) and L-carnitine (B11).

The variation trend of the metabolites is described in a heat map (Fig. 6). Twenty metabolites (B1–B8, B11–B14, B16, B18–B20, B22, B24, B25, and B29) were significantly decreased and nine metabolites (B9, B10, B15, B17, B21, B23, and B26–B28) were observably increased in the BC group compared with the FG group.

Correlation between gut microbes, fecal metabolites, and plasma metabolites in Mongolian sheep

To explore the correlations between gut microbes and fecal metabolites, we built a correlation heat map based on Pearson's correlation coefficients. Clear correlations were present

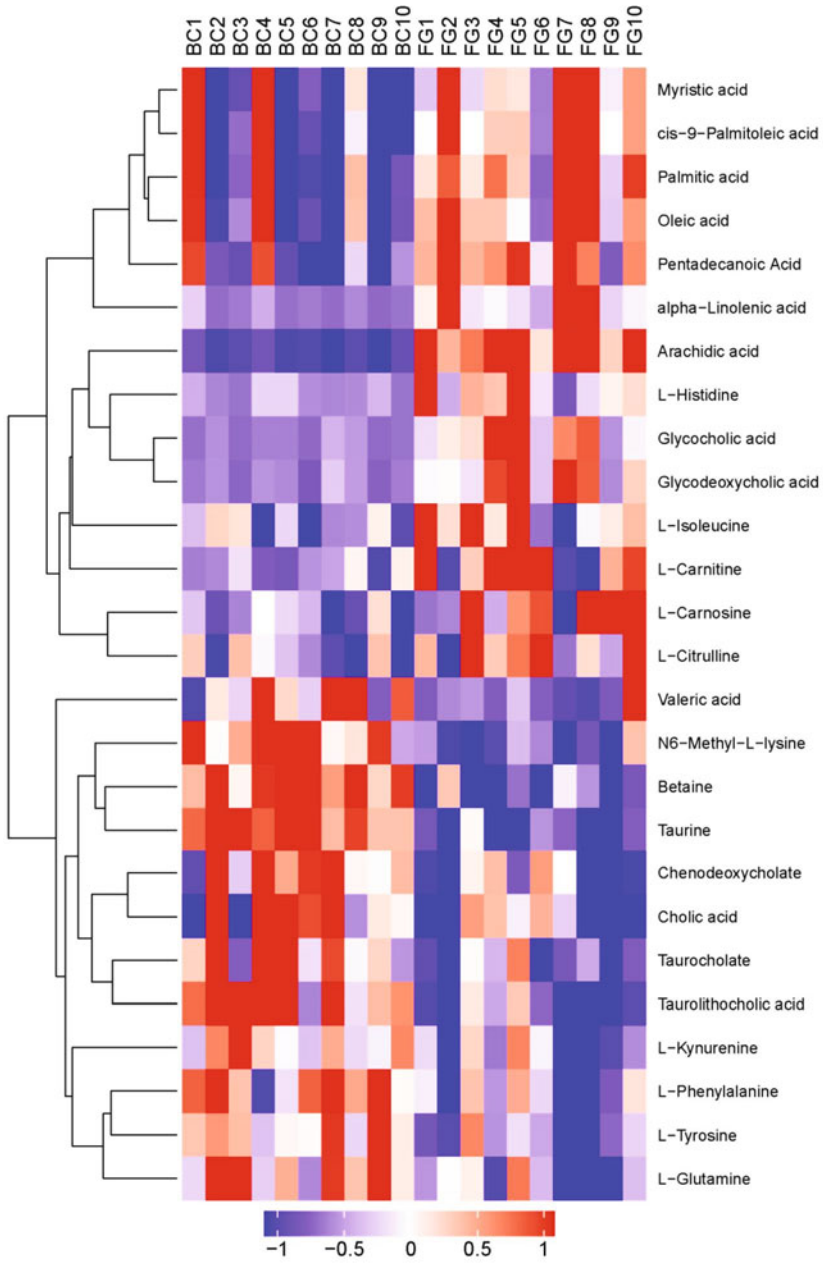

Fig. 6. Heat map summarizing fold changes of significantly altered metabolites in the LC/MS data of plasma samples. Red and blue represent higher and lower concentrations of metabolites in the BC group, respectively, compared with FG group.

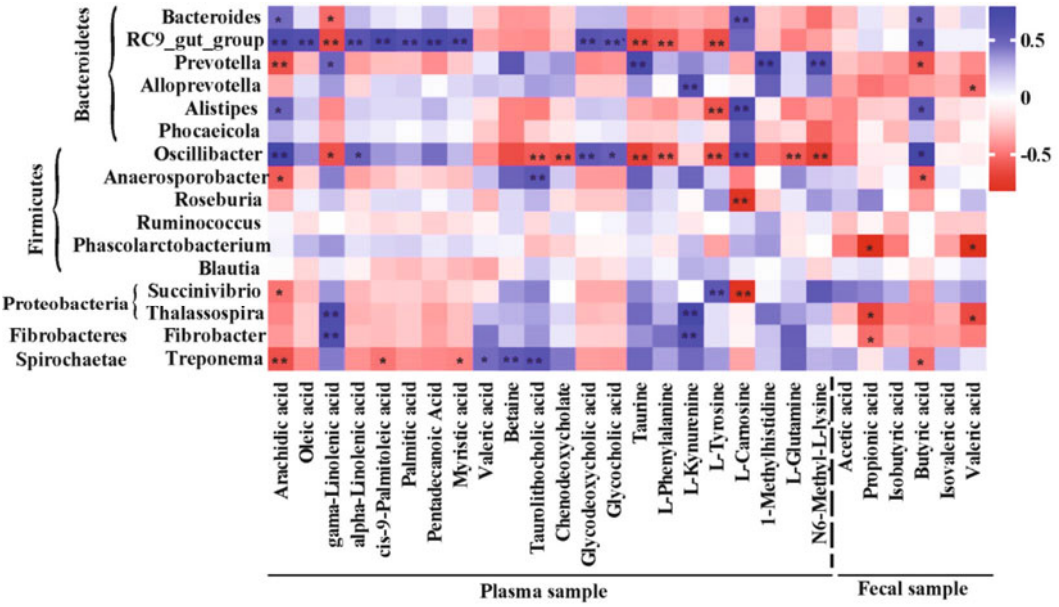

Fig. 7. Heat map summarizing the correlation of gut microbiota genera and feces and plasma metabolites between the FG and BC groups. Red represents the minimum correlation coefficient and blue the maximum.

between gut microbes and SCFAs ($r > 0.4$ or $r < -0.4$, $P < 0.05$) (Fig. 7). For example, *Thalassospira* and *Phascolarctobacterium* were negatively correlated with propionic acid and valeric acid. Interestingly, butyric acid was positively correlated with *Alistipes*, *Bacteroides*, *RC9_gut_group*, and *Oscillibacter*, and negatively correlated with *Prevotella*, *Treponema*, and *Anaerosporobacter*.

The correlation between gut microbes and fatty acid metabolism is presented in Fig. 7. For example, arachidic acid (B1) was positively corrected with *Bacteroides*, *RC9_gut_group*, and *Alistipes*, and negatively correlated with *Prevotella*, *Anaerosporobacter*, and *Succinivibrio*. Meanwhile, *Thalassospira*, *Fibrobacter*, and *Prevotella* were positively corrected with gamma-linolenic acid (B3), whereas *Bacteroides*, *RC9_gut_group*, and *Oscillibacter* were negatively corrected with gamma-linolenic acid (B3). In addition, *Treponema* was negatively corrected with arachidic acid (B1), palmitic acid (B6), and myristic acid (B8).

The correlations between gut microbes and bile acid biosynthesis and amino acids are also presented in Fig. 7. For example, *RC9_gut_group* and *Oscillibacter* were positively corrected with glycodeoxycholic acid (B15) and glycocholic acid (B17); however, *Oscillibacter* was negatively corrected with taurolithocholic acid (B12) and chenodeoxycholate (B13). The correlation between gut microbes and amino acids is shown in Fig. 7. *Bacteroides* was positively corrected with L-carnosine (B27). *RC9_gut_group* was negatively corrected with taurine (B18), L-phenylalanine (B19), and L-tyrosine (B22). *Oscillibacter* was negatively corrected with L-phenylalanine (B19), L-tyrosine (B22), L-carnosine (B27), L-glutamine (B25), and N6-methyl-l-lysine (B29), and positively corrected with L-carnosine (B27). *Succinivibrio* was positively correlated with L-kynurenine (B20) and N6-methyl-l-lysine (B29), and negatively corrected with L-tyrosine (B22).

Discussion

In this study, 16S rRNA gene sequencing and LC-MS-based metabolomics were employed to explore the effect of feeding regimens on the gut microbial community, fecal metabolites, and plasma metabolic phenotype of Mongolian sheep. The results demonstrated differences in the abundance of phyla and genera in the gut microbiota, and showed that diet affected the metabolic composition of fecal and plasma samples from sheep. Furthermore, the altered gut microbiota had significant correlations with fecal and plasma metabolites, suggesting that feeding regimens not only altered the gut microbial community, but also affected the metabolic phenotype of Mongolian sheep, resulting in an imbalance of host metabolites. These findings may show the effect of feeding regimens on the health and meat quality of Mongolian sheep, thus increasing our understanding of the significance of the gut microbiota. This is important because it suggests that gut microbiota can be modulated to improve growth, gut health, and fat accumulation.

Many researchers have recently shown that diet can lead to changes in the gut microbiota. In this study, we found alterations in the abundance of four major phyla, namely, *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Spirochaetae* between the FG and BC sheep. Moreover, changes at the genus level, including higher levels of *Bacteroides*, *RC9_gut_group*, *Alistipes*, *Phocaeicola*, and *Oscillibacter*, and lower levels of *Succinivibrio*, *Treponema*, *Prevotella*, and *Fibrobacter*, were observed in the grazing sheep. *Bacteroides* can break down complex polysaccharides, enhance nutrient utilization, and develop, and thus improve, the host's immune system (Backhed *et al.*, 2004). *Bacteroides* can also maintain intestinal microecological balance (Zhang *et al.*, 2018). Su *et al.* (2014) and Zhou *et al.* (2018) reported that *RC9_gut_group*, which belongs to *Rikenellaceae*, usually ferments carbohydrates or

proteins and might play a crucial role in improving lipid metabolism. As expected, the barn-feeding regimen increased the abundance of *Prevotella* (a type of starch degrader) in the gut of Mongolian sheep. Similarly, a previous study reported that feeding a high-grain diet increased the proportion of *Prevotella* in the colons of goats (Ye *et al*., 2016). Another study showed that *Succinivibrio*, which is a type of amylolytic bacteria, specializes in fermenting succinate and converting it to propionate, thereby providing energy for the body (Zhang *et al*., 2018). Interestingly, our study did not find *Succinivibrio* in the FG sheep. Therefore, further studies are required to explore the roles of these key bacteria in different feeding regimens.

The SCFAs in the fecal metabolites consisted mainly of acetic, propionic, and butyric acids. Strong evidence has indicated that SCFAs fulfill most of the energetic requirements of animals and play important roles in the maintenance of animal health and growth (Tan *et al*., 2014). Recently, metabolomic studies found that fecal metabolite changes were connected to diseases caused by changed gut microbiota, such as obesity and type II diabetes (Le Gall *et al*., 2011). In this study, we found that butyric acid levels differed significantly between the two feeding regimens. The signaling activities of butyric acid are mediated by GPCRs to promote energy storage by increasing adipogenesis and inhibiting adipocyte lipolysis (Ge *et al*., 2008). Importantly, these receptors are localized in adipose tissue, suggesting that adipose tissue is an important target of metabolites produced by gastrointestinal microbes (Guida and Venema, 2015).

With respect to the metabolome, studies on pasture feeding have revealed that the metabolic pathways and metabolites in plasma, rumen, and fecal samples from ruminant animals are altered (Frey *et al*., 2018; O'Callaghan *et al*., 2018). Several of the altered metabolites, such as tryptophan, cholic acid, and phenylalanine acid, are metabolic byproducts of gastrointestinal tract microbes. Recently, studies on plasma metabolites have revealed that changes in metabolic phenotypes are connected with diseases such as obesity and diabetes (Moon *et al*., 2018; Wang *et al*., 2018b). In this study, the plasma metabolic profile of grazing sheep was determined and found to be significantly different from that of confined sheep. Moreover, a total of 29 plasma metabolites were found to be biomarkers in grazing sheep. Among those metabolites, alpha-linolenic acid was described as an important muscle biomarker in grazing sheep in our previous study (Wang *et al*., 2018a). Furthermore, Sun *et al*. (2015) suggested that high-fat diets affect bile acid metabolism in animals. In this study, the bile acid metabolite products of cholic acid (B16) and taurolithocholic acid (B12) were identified and considered to be candidates for biomarkers associated with confined sheep.

Increasingly, studies have reported that changes in gut microbes associated with metabolites could be used to explore the mechanism of development of diseases such as obesity (Wolf and Lorenz, 2012; Ottosson *et al*., 2018). In our study, we observed that butyric acid was positively correlated with *Bacteroides*, *RC9_gut_group*, *Alistipes*, and *Oscillibacter*, and negatively correlated with *Prevotella*, *Anaerosporobacter*, and *Treponema*. SCFA concentrations are sensed by specific GPCRs (GPR43 and GPR41), which are involved in the regulation of lipid and glucose metabolism (den Besten *et al*., 2013). Acetate preferentially activates GPCR43, and butyrate preferentially activates GPCR41 (Brown *et al*., 2003). GPCR41 signaling is required for the gut microbiota to promote host adiposity. Interestingly, our study showed that *RC9_gut_group* and *Oscillibacter* were positively correlated with alpha-linolenic acid in plasma, which further indicates that a high abundance of *RC9_gut_group* and *Oscillibacter* in grazing sheep could promote the deposition of alpha-linolenic acid in mutton (Wang *et al*., 2018a). Moreover, gut bacteria altered the distribution of amino acids in plasma. This suggests that the gut microbiota affected the bioavailability of amino acids to the host. Importantly, amino acids could also serve as precursors for the synthesis of SCFAs by bacteria, suggesting interplay between microbial activity and host amino acid and SCFA homeostasis (Davila *et al*., 2013; Neis *et al*., 2015). In addition, our study found that glycodeoxycholic acid and glycocholic acid displayed significant positive correlations with *RC9_ gut_group*. The bile acids that escape the enterohepatic circulation pass into the colon where they undergo bacterial metabolism (Gérard, 2014). Therefore, they might modulate lipid, glucose, and energy metabolism in the host.

Conclusion

In this study, 16S rRNA gene sequencing and LC-MS-based metabolomics were combined to investigate the effects of feeding regimens on the gut microbiota and metabolites of feces and plasma from sheep. The results indicated that the feeding regimens not only influenced the composition and diversity of gut microbiota, but also altered plasma and fecal metabolites. In addition, correlation analysis indicated that several gut genera were strongly correlated with changes in both the production of butyric acid and plasma metabolites. Overall, regulated gut microbiota-related metabolites may be useful biomarkers to probe the functional effects of grazing or aid in the development of microbiota-based animal fodder.

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Conflict of Interest

No conflict of interest declared.

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