Polish Journal of Microbiology
2021, Vol. 70, No 2, 175–187
https://doi.org/10.33073/pjm-2021-015

ORIGINAL PAPER

Comparative Analysis of the Microbiota Between Rumen and Duodenum of Twin Lambs Based on Diets of Ceratoides or Alfalfa

ZACCHEAUS PAZAMILALA AKONYANI#, FENG SONG#, YING LI, SUDE QIQIGE and JIANGHONG WU*

College of Animal Science and Technology, Inner Mongolia University for the Nationalities, Tongliao, China

Submitted 21 November 2020, revised 6 February 2021, accepted 6 March 2021

Abstract

In our previous study, diet directly impacted the microbiota of the rumen in twin lambs. The duodenum is the first part of the small intestine, so we seek to determine whether there is a difference in the digesta between the two feed groups HFLP (high fiber, low protein) and LFHP (low fiber, high protein), and its impact on the biodiversity and metabolism of the duodenum. Results showed that the number of Operational Taxonomic Units (OTUs) in the duodenum (2,373 OTUs) was more than those in the rumen (1,230 OTUs), and 143 OTUs were significantly different in the duodenum between the two groups. The two most predominant phyla were *Bacteriodetes* and *Firmicutes*, but this ratio was reversed between the rumen and duodenum of lambs fed different feedstuffs. The difference in the digesta that greatly changed the biodiversity of the rumen and duodenum could affect the microbial community in the gastrointestinal tract (GIT). Sixteen metabolites were significantly different in the duodenum between the two groups based on the metabolome analysis. The relationships were built between the microbiome and the metabolome based on the correlation analysis. Some metabolites have a potential role in influencing meat quality, which indicated that the diet could affect the microbiota community and finally change meat quality. This study could explain how the diet affects the rumen and duodenum's microbiota, lay a theoretical basis for controlling feed intake, and determine the relationship between the duodenum's microbiota and metabolism.

K e y w o r d s: correlation analysis, digesta, metabolome, 16S rRNA sequencing, sheep

Introduction

Many environmental factors such as nutrition, habitat, and host genetics impact the components and functions within the gut microbiome (Ussar et al. 2016). The rumen of sheep envelops a complicated microbiota and acts as the primary location for fermentation of consumed feed (McCann et al. 2017), which directly impacts sheep's health and physiological functions.

The rumen is the largest compartment of ruminants where plant cell walls and other herbage materials are degraded by intricate microbial communities predominated by bacteria (Sirohi et al. 2012). The bacteria systematically decompose plant cell materials (Flint and Bayer 2008) and break down plant biomass, serving as a link between the sheep and the nutrients absorbed by the sheep. The succeeding rumen fermentation manufactures ammonia and short-chain fatty acids (SCFAs), including acetate, butyrate, and propionate. In a previous study, we found that diet directly impacts the microbiota structure of the rumen and affects the metabolic process in sheep muscle (Wu et al. 2020). However, the nutritional components that entered the small intestines were not analyzed.

The duodenum, the first part of the small intestines, is where absorption of nutrients begins due to its ability to receive partially degraded food from the stomach. The duodenum is the most proximal phase of degradation, and it represents the most oversized diameter, the densest villi, and the deepest part within the small intestines. The duodenum takes fluid and bile produced by the pancreas and the liver, thereby assisting the intestines in breaking fat, protein, and starch (Faichney 1969; DeGregorio et al. 1982; Lewis and Dehority 1985). Few studies have been done regarding the microbiota of the duodenum, and there is a need to analyze the metabolome of the duodenum, compare it with the microbiota of the rumen, to determine the structure of the bacterial community in the lambs based on two different feeds.

Zaccheaus P. Akonyani and Feng Song contribute equally to this work and are co-first authors.
* Corresponding author: J. Wu, College of Animal Science and Technology, Inner Mongolia University for the Nationalities, Tongliao, China; **e-mail: wujianghonglong@126.com**

Experimental

Materials and Methods

Preparation of feed pellets. In our previous study (Wu et al. 2020), two categories of feed pellets were prepared. One category was alfalfa (Medicago sativa) which belongs to the LFHP group. The other category was ceratoides (*Ceratoides arborescens*) belonging to the HFLP group.

Experimental animals. To avoid the influence of genetic background, four pairs of 3 months old twins Sunit lambs with an average weight of 24 ± 2.3 kg were used in the experiment. The details of how the twin lambs were grouped, monitored, fed, slaughtered, and their genomic DNA extracted can be seen in Wu et al. (2020). For microbiome analysis, the liquid phases of duodenum content were separated by squeezing them through four gauze layers (1 mm mesh). The fluid was divided into two parts, centrifuged at 500 g for 30 min at 4°C to isolate residual particles and preserved at –80°C.

16S rRNA sequencing of duodenum and rumen. To determine the structure of the bacterial community in the lambs fed based on two different dietary requirements, the 16S rRNA microbiome in the duodenum was sequenced. Microbiome DNA was extracted using the E.Z.N.A.® Stool DNA Kit according to the manufacturer's instructions. Bacterial 16S genes were enlarged from microbiome DNA using V3-V4 region primers and arranged in sequence using the Illumina MiSeq PE300. A total of 181,562 tags were obtained from 8 specimens, with an average of 22,695.25 tags per specimen after being filtered and merged. The UCLUST (Edgar 2010) algorithm of QIIME (version 1.8.0) (Caporaso et al. 2010) was used to group the various tags with 97% of similarity and to obtain the Operational Taxonomic Units (OTUs). The OTUs were carefully annotated and carried out by the Silva database (Quast et al. 2013). The Mothur software version 1.30 (Schloss et al. 2009) and UniFrac techniques (Lozupone and Knight 2005) were used in calculating the alpha and beta diversity. The 16S rRNA microbiome in the rumen was obtained from the NCBI SRA data under BioProject PRJNA659928. The same analysis pipeline was used for rumen microbiome.

Duodenum metabolome analysis. In this research, metabolites were separated from the sheep's duodenum and analyzed with liquid chromatography combined with mass spectrometry (LC/MS). An untargeted metabolic analysis was done on the duodenum of the four pairs of the twin lambs. The variation between the two groups (HFLP and LFHP) was identified by using principal components analysis (PCA) and orthogonal partial least squares discriminant analysis (PLS-DA) (Bylesjö et al. 2006): p-value ≤ 0.05 + VIP ≥ 1. The p-value was tested by Mann-Whitney-Wilcoxon Test/ Student's t-test, and the VIP (Variable Important in the Projection) is the PLS-DA first principal component. To increase the sample size for metabolic analysis, two new samples in each group were created and averagely mixed to produce more sample size. Sample A and B were mixed to produce E (1:1, v/v), and samples C and D were mixed to form F (1:1, v/v). Ions were assigned to metabolites based on online databases, including Human Metabolome Database (HMDB; https://www.hmdb.ca), Biofluid Metabolites Database (http://metlin.scripps.edu), mzCloud (https://www.mzcloud.org), Lipid Maps (https://www.lipidmaps.org), and MassBank (https://www.massbank.jp).

Joint analysis of microbiota and metabolome. Spearman's correlation analysis between the microbiota and metabolites was carried out, coefficients were produced (Looney and Hagan 2007), and was significant ($p \leq 0.05$). The screening condition was $|rho| > 0.8$. Based on the corresponding relationship between the final metabolite and OTUs, the information was inputted into Cytoscape software (https://cytoscape.org) to draw a network diagram.

Results

The microbial diversity of the rumen and duodenum. Based on Simpsons and Shannon indices, there was a significant difference in the rumen's microbial diversity between the HFLP and the LFHP groups ($p < 0.05$). This could be the effect of the high fiber content in the HFLP diet (Table II). However, there was no significant difference in the duodenum's microbial diversity between the two groups (Table III). The diversity of the microbiota in the HFLP group was higher

Table I
Nutritional components of two feed stuffs.

	DM (%)	GE (MJ/kg DM)	CP (% DM)	CF (% DM)	ADF (% DM)	NDF (% DM)
LFHP	89.4	16.3	16.1	2.4	25.2	46.2
HFLP	90.5	15.8	11.8	2.2	29.6	57.5

LFHP – low fiber high protein level, HFLP – high fiber low protein level, ADF – acid detergent fiber, CF – crude fat, CP – crude protein, DM – dry matter, GE – gross energy, NDF – neutral detergent fiber

Table II
The diversity analysis of the rumen microbiota of twin lambs.

Items	HFLP	LFHP	*p*-value
Shannon	5.055 ± 0.1589	4.235 ± 0.181	0.014
Simpsons	0.019 ± 0.004	0.066 ± 0.041	0.116
Observed species (Obs)	992.000 ± 68.474	930.000 ± 18.037	0.154
Ace	1051.658 ± 64.182	992.341 ± 26.875	0.102
Chao 1	1059.470 ± 67.920	1017.360 ± 29.399	0.165

The HFLP group was fed with ceratioids and the LFHP group of twin lambs was fed with alfalfa in the phylum of the rumen. Presented parameters are sample estimators of total species with sample identifications (Sample ID). While Obs, Ace, and richness (Chao 1) are used to describe the species number, Simpson and Shannon's indices are used to indicate how diverse the rumen microbiota is.

Table III
The diversity analysis of duodenum microbiota of twin lambs.

Items	HFLP	LFHP	*p*-value
Shannon	7.855 ± 0.880	7.828 ± 0.838	0.964
Simpsons	0.985 ± 0.006	0.986 ± 0.005	0.391
Observed species (Obs)	966.750 ± 293.842	905.000 ± 247.818	0.781
Chao 1	1278.063 ± 241.678	1223.815 ± 327.198	0.843

The HFLP group was fed with ceratoides, the LFHP group of twin lambs was fed with alfalfa in the phylum of the duodenum. Presented parameters are sample estimators of total species with sample identifications (Sample ID). While Obs and richness (Chao 1) are used to describe the species number, Simpsons and Shannon's indices are used to indicate how diverse the duodenum microbiota is.

than the LFHP group in the rumen. However, that pattern could not be entirely detected in the duodenum. The diversity of the microbiota in the duodenum was higher than those in the two groups' rumen.

Phylum community distribution of the rumen and duodenum. On the phylum level, the abundance of 11 phyla of the rumen and duodenum was calculated and used to draw histograms for comparisons. A greater number of sequences obtained from the phylum class pertained to the phyla *Bacteroidetes*, and *Firmicutes* but at the duodenum's phylum level, a larger proportion of the sequences obtained related to *Firmicutes,* and *Bacteroidetes*. The major bacteria in the rumen's phylum level were *Bacteriodetes*, but were replaced with *Firmicutes* in the duodenum. The major bacteria were higher in the LFHP group than the HFLP group for the rumen and duodenum (Fig. 1 and 2).

Genus community distribution of the rumen and duodenum. A detailed examination of the relative abundance of bacterial OTUs showed that the two different feeds have a varied influence on both the rumen and duodenum's microbiota. According to the 16s rRNA gene of the rumen and duodenum bacteria sequences, 1,230 OTUs and 2,373 OTUs among eight rumen and duodenum samples were identified. In the duodenum, 143 OTUs were significantly different between the two groups.

Ninety-two non-identical genera were designated from the sequences at the genus level. The genera were among almost all specimens. *Prevotella* was abundant in the rumen of the LFHP group compared to the HFLP group (Fig. 3a). Unclassified *Lachnospiraceae* were prevalent in the LFHP group's duodenum compared to the HFLP group (Fig. 3b). The high protein in the LFHP feed increases the genera *Prevotella* and unclassified *Lachnospiraceae* of the rumen and duodenum.

Comparison between the microbiota of ceratoides (HFLP) and alfalfa (LFHP) feeds, and their *Firmicutes*/*Bacteriodetes* (F:B) ratio in the rumen and duodenum. The F:B ratio in the rumen microbiota of the twin lambs fed on the LFHP pellets was 0.104, and that of the HFLP pellets was 0.275 (Fig. 4a and 4b). The F:B ratio in the duodenum microbiota of the twin lambs fed on the LFHP pellets was 6.419, and that of the HFLP pellets was 5.356 (Fig. 4c and 4d). There was a change in the F:B ratios in the rumen and duodenum even under the different feeds.

Differential expression metabolites between two groups. After the data was filtered, 3,696 stable metabolic features were detected. A partial least squares discriminant analysis (PLS-DA) was performed between the two groups to identify metabolic differences in duodenum of twin sheep fed different diets (Fig. 5). The results showed that 407 significantly different metabolites

Duodenum Phylum	A1	B1	C1	D1	A2	B2	C2	D2
Actinobacteria	1.71	4.93	3.45	6.91	1.15	0.80	1.49	10.21
Bacteroidetes	12.46	0.37	7.95	1.88	1.62	21.04	11.33	0.14
Cyanobacteria	0.04	0.05	0.03	0.22	0.21	0.11	0.24	0.41
Elusimicrobia	0.06	0.00	0.01	0.00	0.00	0.02	0.00	0.00
Firmicutes	71.23	74.94	65.81	43.86	88.03	66.20	68.63	74.56
Lentisphaerae	0.04	0.00	0.08	0.03	0.00	0.07	0.01	0.00
Proteobacteria	2.47	0.08	2.46	1.33	0.33	1.73	1.00	0.15
Spirochaetes	0.40	0.01	0.47	1.23	0.09	0.78	0.56	0.00
Synergistetes	0.00	0.01	0.12	0.10	0.00	0.05	0.06	0.00
Tenericutes	0.42	0.02	0.29	0.12	0.17	0.38	0.24	0.01
Others	11.168	19.579	19.325	44.301	8.3986	8.83	16.45	14.52
Rumen Phylum								
Actinobacteria	0.12	0.66	1.71	0.29	0.03	0.13	0.06	0.01
Bacteroidetes	73.88	68.19	68.22	70.14	86.20	85.20	89.14	85.65
Cyanobacteria	0.18	0.15	0.12	0.02	0.06	0.01	0.03	0.03
Elusimicrobia	0.04	0.02	0.02	0.00	0.04	0.01	0.01	0.04
Firmicutes	12.58	15.84	23.98	24.91	8.75	11.16	7.81	8.33
Lentisphaerae	1.47	2.75	0.73	1.28	0.60	0.38	0.52	0.61
Proteobacteria	9.09	10.20	2.37	1.18	1.48	0.55	0.36	1.05
Spirochaetes	0.19	0.15	0.19	0.64	0.40	0.23	0.20	0.41
Synergistetes	0.08	0.40	0.10	0.05	0.02	0.02	0.02	0.01
Tenericutes	1.60	0.69	0.91	0.79	1.93	1.13	1.17	2.81
Others	0.77	0.95	1.65	0.69	0.49	1.19	0.67	1.07

Fig. 1. This figure shows a comparison between the phylum composition of the bacteria in the rumen and duodenum of twin lambs. The letters A1-D1 represent HFLP group, and A2-D2 represent LFHP group.

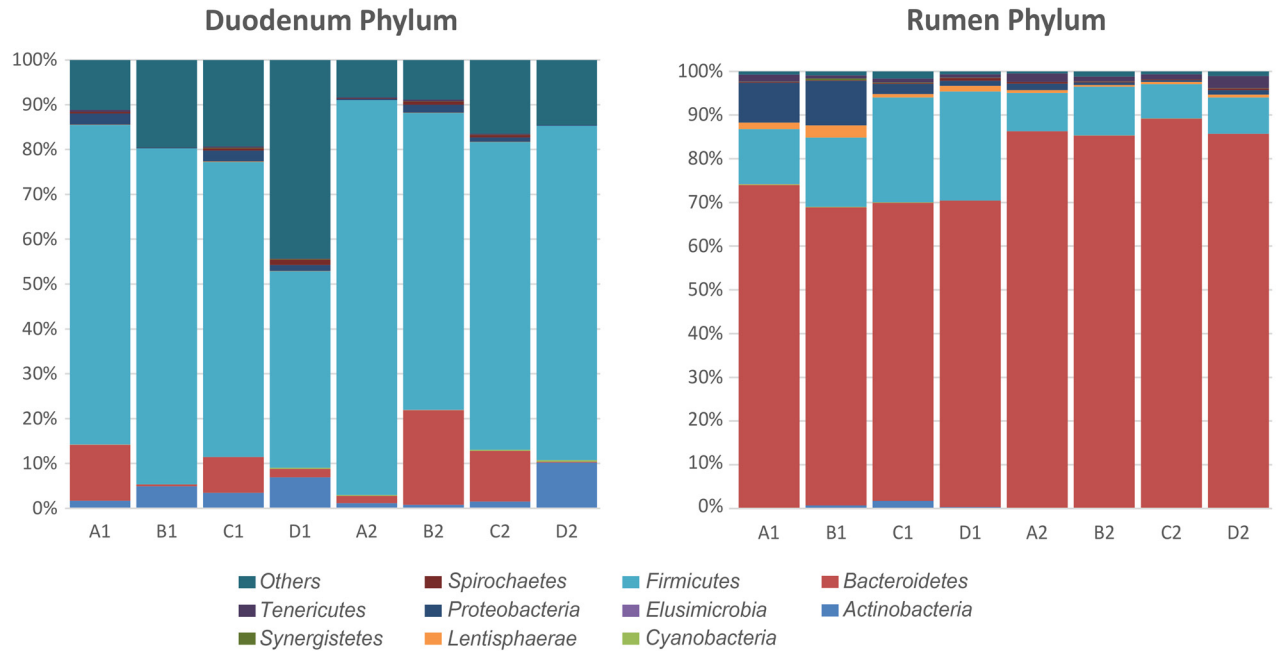

Fig. 2. The phylum composition of rumen and duodenum of twin lambs. The letters A1-D1 represent the HFLP group, and the A2-D2 represent the LFHP group of phylum and duodenum, respectively.

(p-value ≤ 0.05) were screened between the two groups. In the HFLP group, 273 of the metabolites showed a lower expression, and 134 of the metabolites showed a higher expression. Tandem mass spectrometry (MS/MS) was used to detect 16 significantly different metabolites indicated in the heat map (Fig. 6). The HFLP group increased 7 of the metabolites while that of the LFHP group influenced 9 of the metabolites. Some of the metabolites discussed are adenosine, taurine, L-alanine, and nicotinic acid (Fig. 7). It was detected that there was a significant difference between the HFLP diet and the LFHP diet (p-value ≤ 0.05).

The correlation analysis indicated that the abundance of 5,6-dihydrouracil correlated positively with

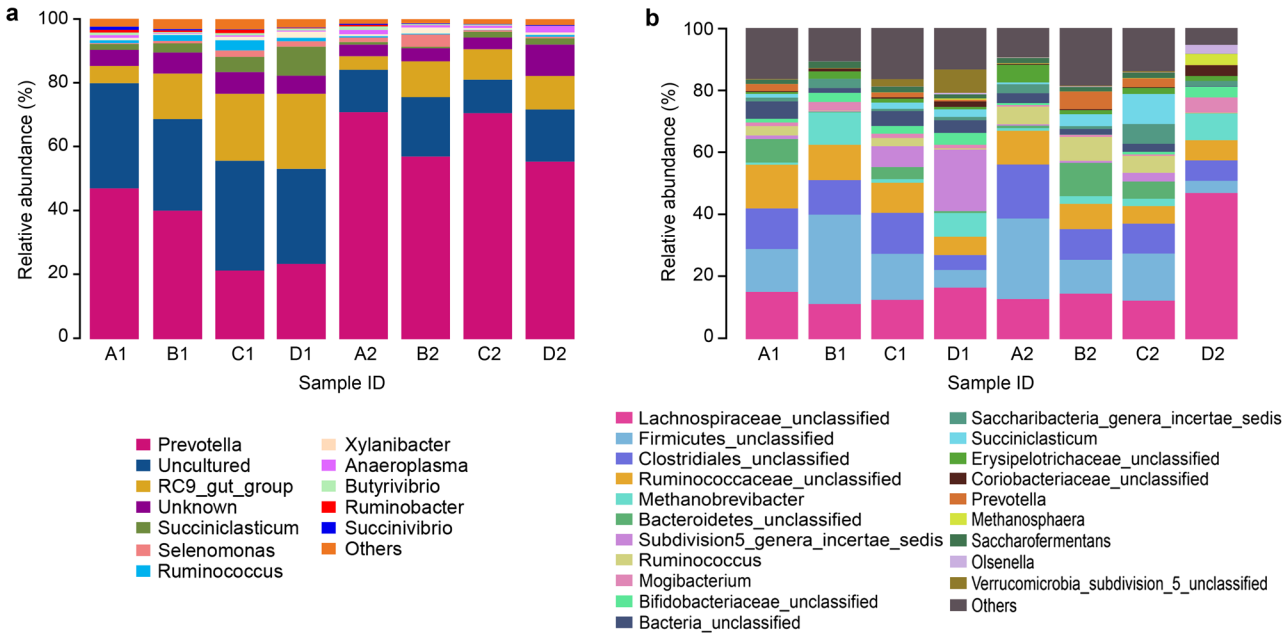

Fig. 3. The genus community distribution of rumen and duodenum.

a) The genus community distribution of rumen. The letters A1-D1 represent the first group fed with HFLP and A2-D2 represents the second group of twin lambs fed with LFHP in the genus. b) The genus community distribution of duodenum. The letters A1-D1 represent the first group fed with HFLP and A2-D2 represents the second group of twin lambs fed with LFHP in the genus.

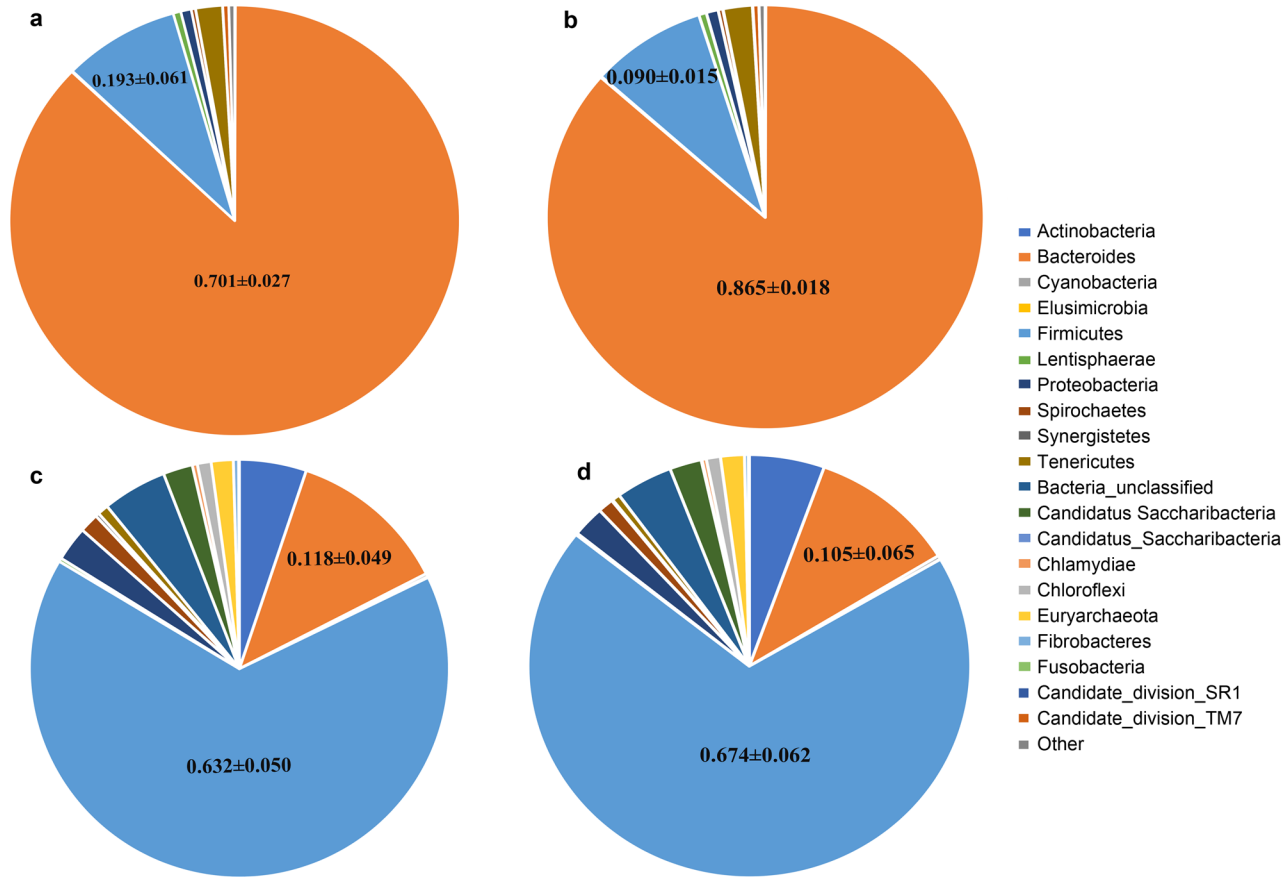

Fig. 4. Comparison between HFLP and LFHP's microbiota and their F:B ratio in the rumen and duodenum.

The letters a) and b) represent HFLP pellets and LFHP pellets, respectively, of the rumen. Enterotypes were strongly associated with feeds a) and b) which show LFHP pellets (*Bacteroidetes*) against HFLP pellets (*Firmicutes*). LFHP pellets displayed a substantial increase in *Bacteroidetes* and a reduction in *Firmicutes*. The letters c) and d) represent HFLP and LFHP pellets, respectively, of the duodenum. Enterotypes were strongly associated with feeds c) and d) which show LFHP pellets (*Bacteroidetes*) against HFLP pellets (*Firmicutes*). HFLP pellets displayed an increase in *Firmicutes* and a reduction in *Bacteroidetes*.

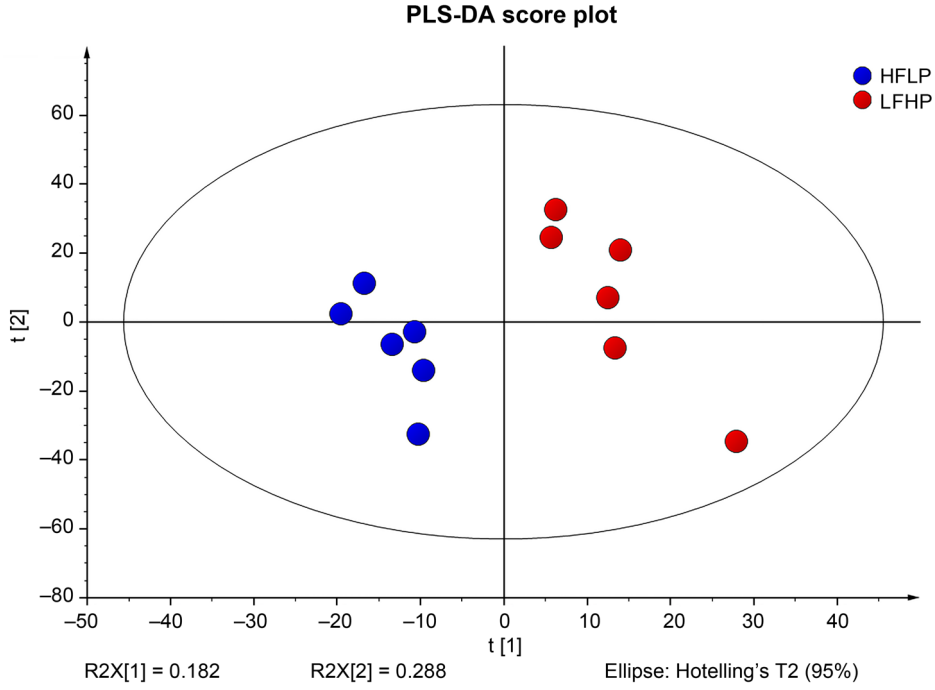

Fig. 5. PLS-DA score plot came from liquid chromatography combined with mass spectrometry (LC/MS) spectra for the HFLP group (blue dots) and the LFHP group (red dots).

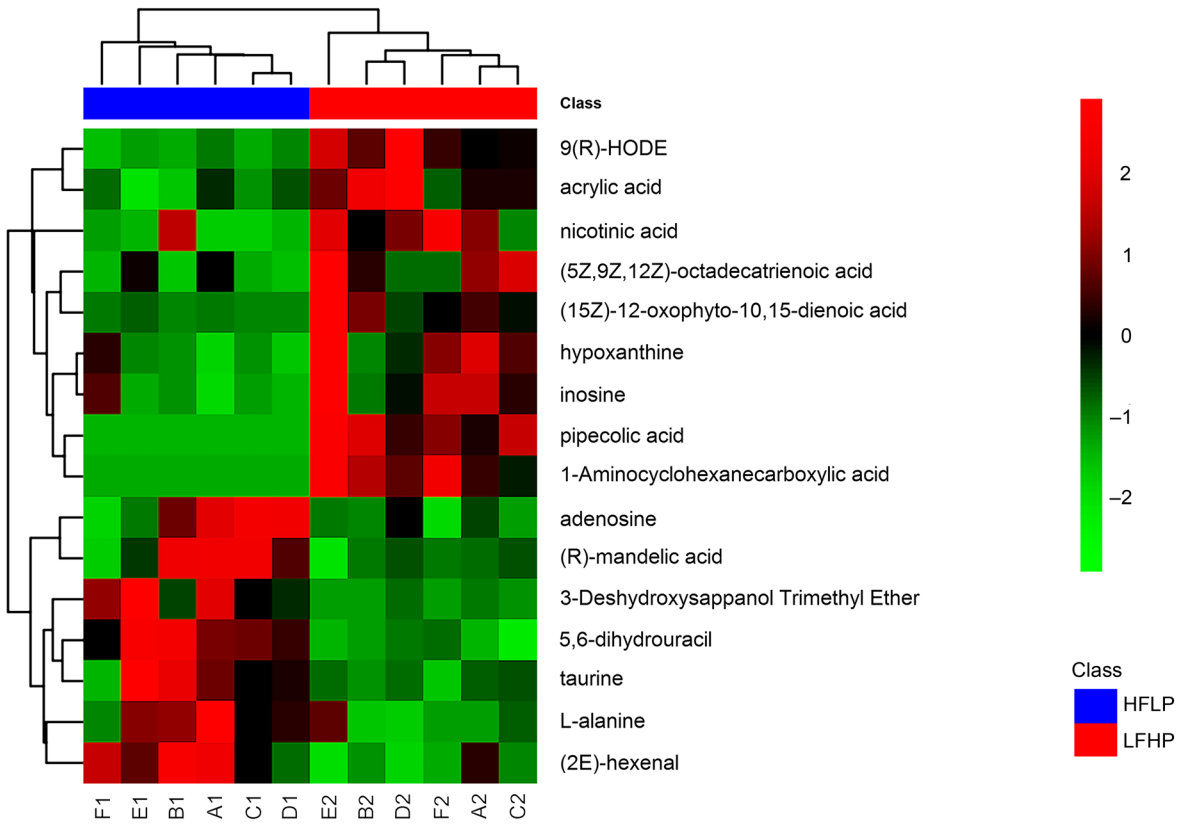

Fig. 6. Heat map built by the 407 significantly different metabolites (p-value ≤ 0.05) in the HFLP and LFHP groups. The red color represents the LFHP group, and the blue represents the HFLP group.

adenosine, and negatively correlated with 9(R)-HODE and acrylic acid. Adenosine was observed to be positively correlated with 5,6-dihydrouracil, and correlated negatively with 9(R)-HODE and acrylic acid. 9(R)-HODE had a positive correlation with acrylic acid, and correlated negatively with 5,6-dihydrouracil and

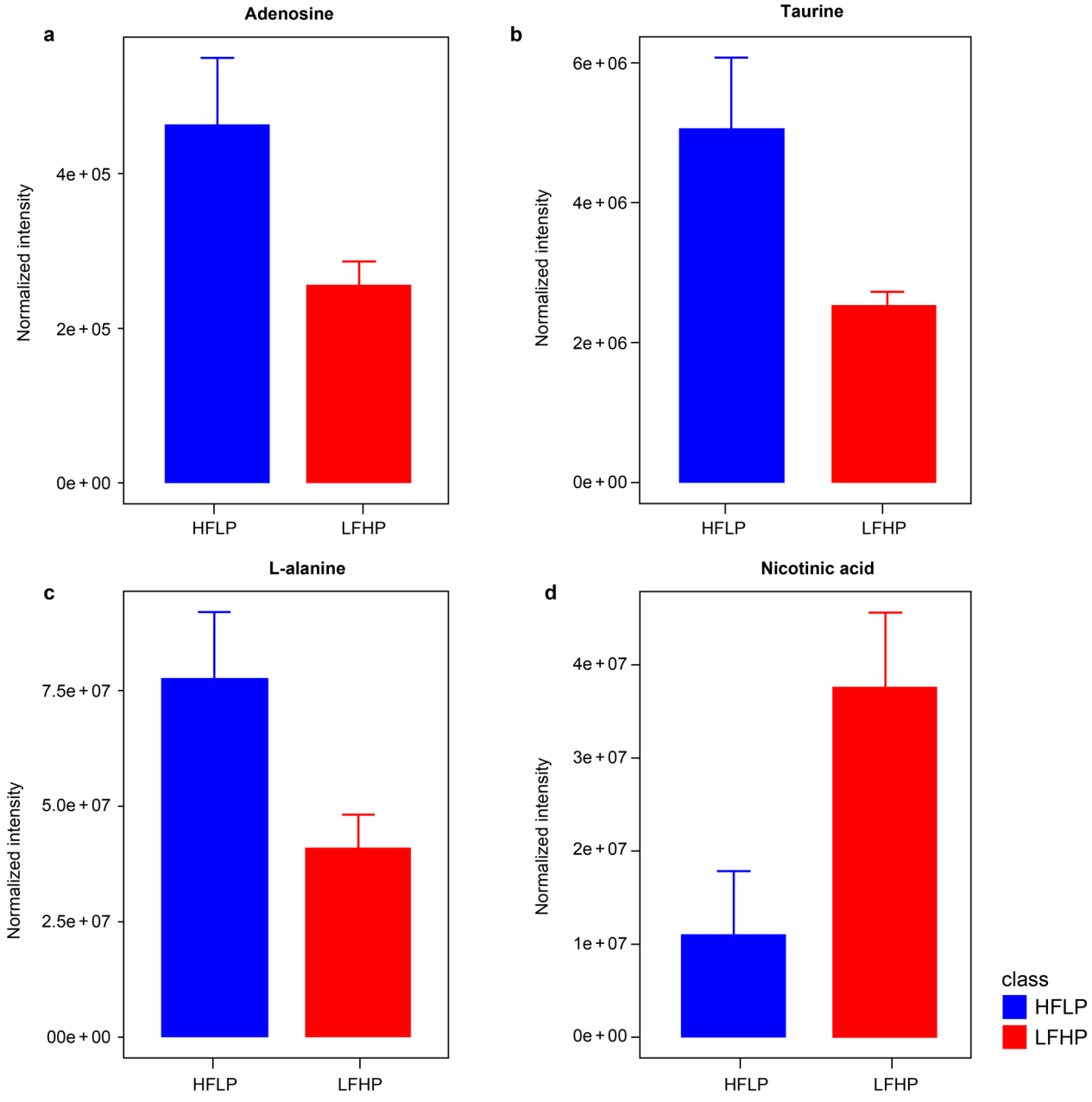

Fig. 7. The relationship between the two feed (LFHP and HFLP) groups and the metabolites.

adenosine. Also, acrylic acid was seen to be correlated positively with 9(R)-HODE but negatively correlated with 5,6-dihydrouracil, and adenosine (Fig. 8).

Relationship between duodenal microbiota and metabolites. Correlation analysis between the microbiota and metabolites was carried out to find the possible coexistence. 79 correlations were related positively ($|rho| > 0.8$, p-value ≤ 0.05) while 194 correlations were related negatively ($|rho| > 0.8$, p-value ≤ 0.05) between the OTUs and the metabolites. *Prevotella* and 1-aminocyclohexanecarboxylic acid had the greatest positive correlations ($r = 0.84$, p-value ≤ 0.05). *Methanobrevibacter* and 3-deshydroxysappanol trimethyl ether were detected to have the greatest negative correlations ($r = -0.79$, p-value ≤ 0.05) between the bacteria and metabolites.

The bacterium *Candidatus saccharibacteria* (OTU31, OTU970) is positively correlated with the metabolites 9(R)-HODE and acrylic acid and correlated negatively with 5,6-dihydrouracil, adenosine, and L-alanine. The bacterium *Cyanobacteria sreptophyta* (OUT217) was found to be correlated positively with 9(R)-HODE and acrylic acid but correlated negatively with 5,6-dihydrouracil, adenosine, and L-alanine. The bacteria *Firmicutes blautia*, *Firmicutes Firmicutes* unclassified, and *Firmicutes Eubacterium* sp. C2 (OTU224, OTU769, OTU798) which all belong to phylum *Firmicutes* were positively correlated with 9(R)-HODE and acrylic acid but negatively correlated with 5,6-dihydrouracil, adenosine, and L-alanine. The bacterium *Planctomycees planctomycetaceae* (OTU1882) correlated

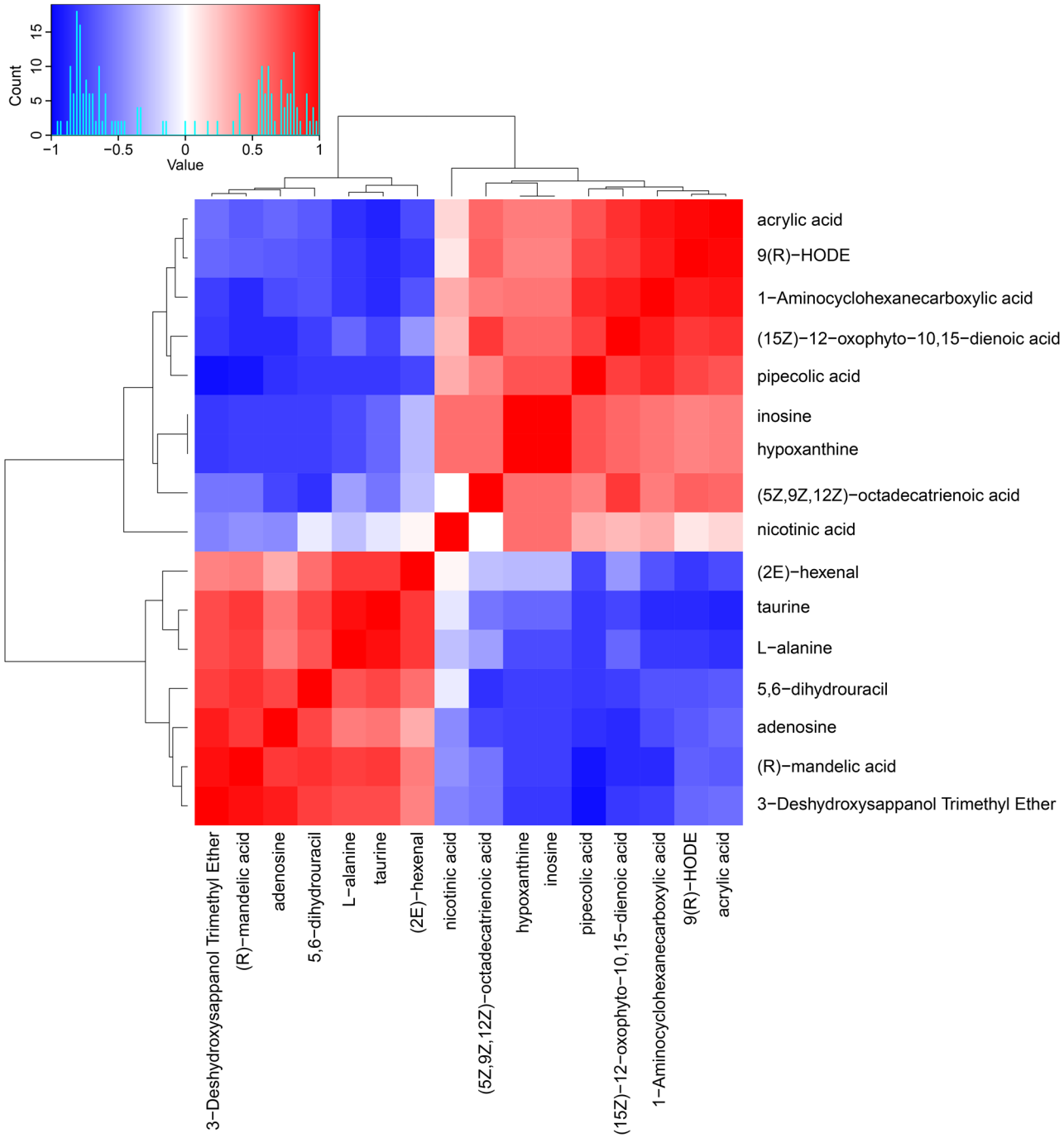

Fig. 8. Metabolites correlation heat map. The rows and columns in the figure represent different metabolites, and different colors represent different correlations. The red color represents a positive correlation, and the blue represents a negative correlation among the metabolites. The deeper the color in the heat map, the better the metabolism.

positively with 5,6-dihydrouracil, adenosine, and L-alanine but correlated negatively with 9(R)-HODE and acrylic acid (Fig. 9).

Discussion

Many former researchers on ruminants only concentrate on the rumen microbiomes. However, we decided to compare the rumen and duodenum microbiomes using two feed types and the duodenal metabolism to investigate the relationship between microbiota and metabolome because metabolites can influence the meat quality and health of the host (Xu et al. 2013; Muroya et al. 2019).

The biodiversity of the rumen and duodenum microbiota. The biodiversity of the rumen was higher when fed with ceratoides (HFLP) than when fed with alfalfa pellets (LFHP) (Table II). The outcome of this work is compatible with our previous research, which

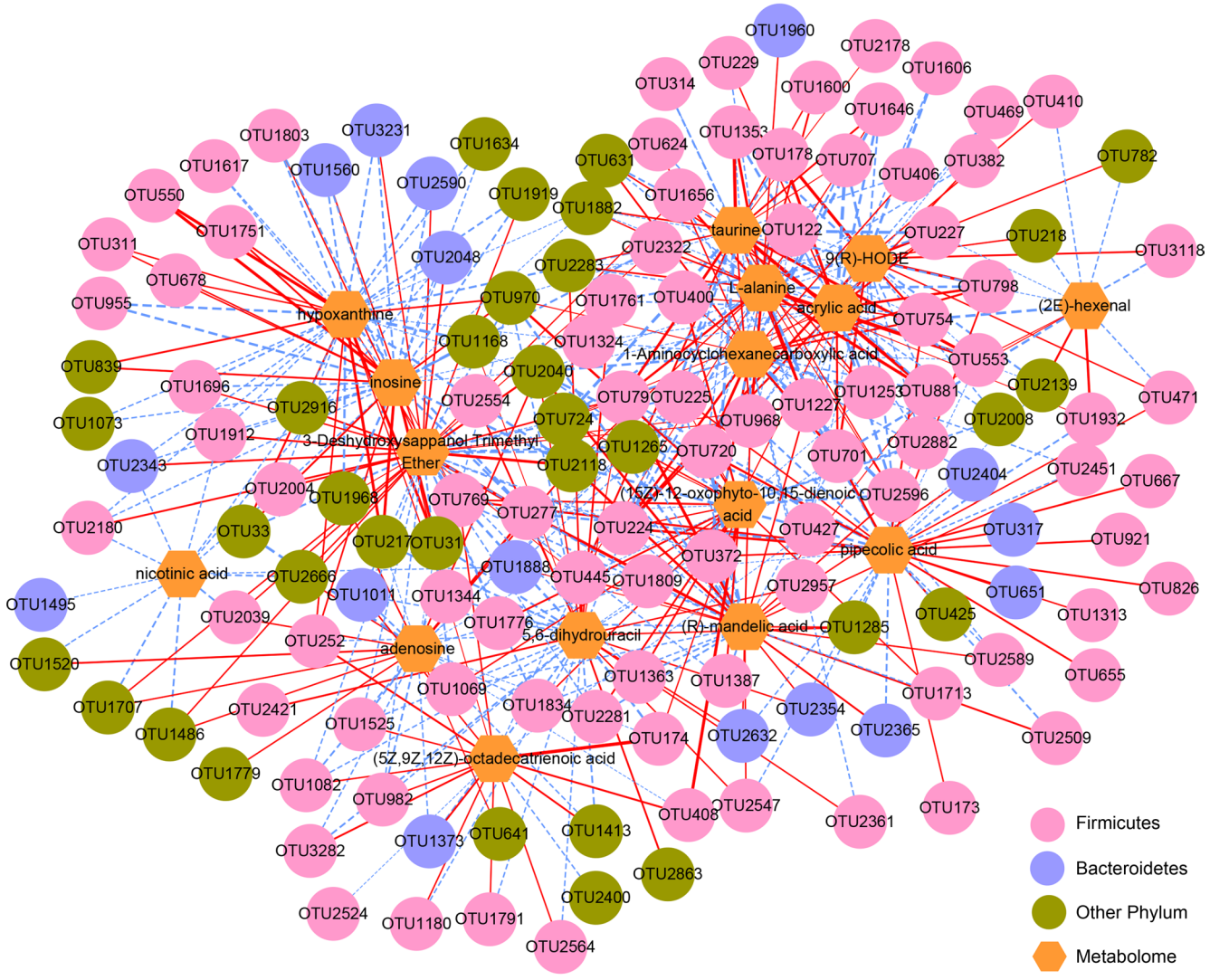

Fig. 9 This figure shows the relationship between OTUs, metabolites, and the microbiota. The nodes represent different OTUs related to bacteria, which belong to *Firmicutes*, *Bacteriodetes*, and other phyla. The orange diamonds indicate 16 metabolites detected. The lines indicate the correlations between the OTUs, bacteria, and metabolites. The solid red connecting lines show a positive correlation, and the blue connecting dot line indicates a negative correlation between the OTUs, bacteria, and metabolites. The line thickness shows how strong the correlation was.

reveals that the microbiota in the lambs' rumen fed a high fiber diet (HFLP) were more diverse than those fed a low fiber diet (LFHP) (Wu et al. 2020). A study shows that *Firmicutes* and *Bacteroidetes* are the major predominant phyla of microorganisms in the gut community of terrestrial animals (Qin et al. 2010). In this research, the most predominant phyla in the rumen's microbiota of the twin lambs were *Bacteroidetes* and *Firmicutes* (Fig. 1 and 2), which are more associated with the breakdown of carbohydrates and proteins. Results of this kind have been revealed in earlier reports (Hook et al. 2011). *Bacteroidetes* aids in the degradation of starch in the rumen (Stevenson and Weimer 2007).

The LFHP diet influenced the genus *Prevotella* and the phylum *Bacteroides* of the rumen (Fig. 3). Results of this kind have been detected in earlier research (Zhang et al. 2014). The genus *Prevotella* plays a significant role in breaking down dietary protein to ammonia, which can reduce the utilization of dietary amino acids. The degradation of peptides in the rumen is associated with the breakdown approach in which dietary protein is disintegrated into ammonia, thereby leading to an inexpedient utilization of dietary amino acids (Walker et al. 2005).

There was no significant difference between the two diets in the duodenum in this study (Table III). Regardless of diet, amino acid profiles of the duodenal digesta were similar due to the presence of forage (Merchen et al. 1986). In this study, *Firmicutes* was relatively abundant in the duodenum's phylum level, which confirms a research study where *Firmicutes* were the predominant phylum among all the bacterial groups across the GIT besides those within the omasum and abomasum, in which *Bacteroidetes* were more prevalent (Wang

et al. 2017). The *Lachnospiraceae* was found to be the most abundant genus in the duodenum (Fig. 3). This finding agrees with a research study that revealed that other *Firmicutes* members showing a great abundance in lamb's gut were *Lachnospiraceae* (Palomba et al. 2017).

The Firmicutes/Bacteroidetes ratio (F:B) in the rumen and duodenum's microbiota. The microbiota's F:B ratio was reversed between the rumen and the duodenum due to differences in the organs and functions. The most potent organ, which degrades and converts plant materials to SCFAs in the ruminants, is the rumen (Wang et al. 2020). It possesses the complex microbiota that plays a vital role in the fermentation of feed and energy metabolism, and the SCFAs provide more than 70% of the energy to guarantee host growth and reproduction performance (Flint et al. 2007). Several researchers suggested that bacteria detected in intestinal contents as they go through the abomasum could come from lysed cells (Waghorn et al. 1990; Koenig et al. 1997; Hristov 2007), and the role of the duodenal microbiota in terms of its function in feed degradation is likely to be different from that of the ruminal bacterial community.

A study revealed that including dried distillers' grains with solubles (DDGS) at the detriment of corn bran decreased the flow of bacteria from the rumen (Castillo-Lopez et al. 2014). The DDGS consists of about 31% crude protein, 34% neutral detergent fiber, 12% fat, and 5% starch (Paz et al. 2013). Also, the rumen's microbiota clustered differently compared to that of the duodenum showing different bacterial diversity between ruminal bacteria and the duodenal digesta. Therefore, including the DDGS in the feeds would increase the flow of saturated fatty acids to the duodenum and cause a shift in the rumen and duodenum's bacterial diversity (Castillo-Lopez et al. 2014).

The small intestine, which comprises the duodenum, is a long and coiled tube where the remaining degradable activities occur. Villi that line the small intestine are the main site where nutrients are absorbed and are distributed to the whole body. Amino acids, fatty acids, and sugars which are the end products of digestion, are absorbed from the small intestines, enters the lymph, and distributed (National Research Council 2007). Another study revealed that when fat was injected into the duodenum of lambs, it was absorbed quickly, but when introduced to the rumen, absorption was slow and took several days (Heath and Morris 1962).

The feed delivered plays a crucial function in the F:B ratio (Ramirez Ramirez et al. 2012). The F:B ratio of the microbiota appreciably changed in the rumen when the twin lambs consumed either LFHP or HFLP feeds (5.356 and 6.419), respectively (Fig. 4a and 4b). There was also a change in the F:B ratio of microbiota in the duodenum when the twin lambs consumed either LFHP or HFLP feeds (0.275 and 0.104), respectively (Fig. 4c and 4d).

The more significant F:B ratio in fecal specimens is related to an increase in human weight (Ley et al. 2006), and a study found that the frequency of particular microbial phylotypes could be affected within the offspring of farm animals such as cattle due to the sire breed when utilizing disparate feeds (Hernandez-Sanabria et al. 2013). Also, the changes of the F:B ratios in this current research agrees with a previous study that analyzed the F:B ratio in mice and humans, where changes in the GIT were demonstrated to affect obesity and the capability of the host to harvest energy (Krajmalnik-Brown et al. 2012). It implies that the shift in the F:B ratio in this study could result from the different kinds of feed.

The relationship between the two feed (LFHP and HFLP) groups and the metabolites. Adenosine can influence meat quality. Apart from influencing the components of the gut microbiota, the type of diet can also regulate metabolic homeostasis in twin lambs. In the presence of low protein, meat quality improves in the HFLP group (Fig. 7a). A study summarized that Tibetan sheep meat was preferred to Small-tailed Han sheep meat even though variations between the breeds were not much; however, meat quality was enhanced in the two breeds with the growth of the nutritional energy level when a low-protein feed was given (Jiao et al. 2020). The presence of adenosine in this study could influence the health and regulate the sheep's immune system. It reduces the production of tumor necrosis factor (TNF), induces the manufacture of Nitric Oxide (NO), and plays a vital role in maintaining tissue perfusion (Adanin et al. 2002).

Taurine was influenced by the HFLP feed (Fig. 7b). Taurine present in this study is vital when inspecting the relationship between taurine and palatability. A study mentioned that ribose 5-phosphate, and pyrrolidone carboxylic acid or taurine were natural antecedents of 4-hydroxy-5-methyl-3(2H)-furanone, which is a taste part removed from beef broth and has a caramel-like and burnt chicory smell (Tonsbeek et al. 1968; Weenen et al. 2005). The abundance of taurine in lambs and much more in beef could also enhance the beef's nutritional value apart from contributing to flavor (Purchas et al. 2004). Taurine is important for meat quality, and increasing the concentration of taurine in mutton could be a future breeding objective for Sunit sheep.

The metabolite L-alanine, which was influenced by HFLP diet (Fig. 7c) could benefit the host by reducing tuberculosis. Tuberculosis is caused by *Mycobacterium tuberculosis*, and it is a major health issue globally. A study discovered that exogenous L-alanine can lead to the manufacturing of reactive oxygen species in *Mycobacterium smegmatis* by accelerating the tricarboxylic

cid cycle and/or primary metabolism synergizing with fluoroquinolones, which, in the long run, results in the destruction of *M. smegmatis* (Zhen et al. 2020).

The LFHP diet could increase nicotinic acid in the twin lambs (Fig. 7d). A study showed that replacing dietary protein with non-protein nitrogen depresses nicotinic acid (Buziassy and Tribe 1960). Another study revealed that both nicotinamide and insulin-induced hypoglycemia reductions in free fatty acid enhanced growth hormones released in dairy cows, and each of these cases provided a possible function for glucose and free fatty acid in modulating the growth hormone-releasing factor, which stimulates the release of growth hormones in ruminants (Reynaert et al. 1975; Sartin et al. 1988).

The joint analysis of metabolites and microbiota in the duodenum. The bacteria, which all belong to phylum *Firmicutes* (OTU224, OTU769, and OTU798) correlated negatively with adenosine (Fig. 9). The bacteria *Planctomycetes planctomycetaceae* (OTU1882) correlated positively with adenosine and L-alanine. Early research states that *Planctomycetes* contain a strong proline- and cysteine-rich proteins envelope and not a peptidoglycan cell wall (Liesack et al. 1986). Also, *Candidatus saccharibacteria* (OTU31, OTU970), and *Cyanobacteria sreptophyta* (OUT217) correlated negatively with adenosine and L-alanine. Recently, whole genomes of *Saccharibacteria*, acquired via metagenomics, reported that a few members ferment metabolites, glucose, and various sugars and produce lactate (Albertsen et al. 2013). *Cyanobacteria* has various unique roles that include the ability to restore nitrogen, synthesize vitamin B and K21, syntrophically manufacture hydrogen, and obligate anaerobic fermentation (Di Rienzi et al 2013).

Based on the relationship between the bacteria and metabolites, *Methanobrevibacter* can produce methane in the gut. Methanogenic archaea represented by *Methanobrevibacter ruminantium* produce ruminant's methane and is found in ruminants fed on varied kinds of feeds worldwide (Leahy et al. 2010).

Conclusions

This study shows how 16S rRNA sequencing combined with metabolome analysis may be used in discovering new and significant influences on the functions of a microbe inside the host. The results showed that the diet could directly affect the diversity of rumens' microbiota but not the microbiota in duodenum. There was a shift in the F:B ratio in the rumen and duodenum of the twin lambs even under the different feeds. We found that some bacteria had a relationship with the metabolites. In summary, these findings could provide knowledge of how the diet affects the microbiota of the rumen and duodenum, lay a theoretical basis for controlling feed intake, and determine the relationship between the duodenum's microbiota and metabolism.

Acknowledgments

This project was funded by the National Natural Science Foundation of China (Grant Number 31560623), the Innovation Foundation of IMAAAHS (2017CXJJM03-2). We are grateful to Emily Minter for helping to edit and revise this manuscript.

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

Literature

Adanin S, Yalovetskiy IV, Nardulli BA, Sam AD 2nd, Jonjev ZS, Law WR. Inhibiting adenosine deaminase modulates the systemic inflammatory response syndrome in endotoxemia and sepsis. Am J Physiol Regul Integr Comp Physiol. 2002 May;282(5):R1324–R1332. https://doi.org/10.1152/ajpregu.00373.2001

Albertsen M, Hugenholtz P, Skarshewski A, Nielsen KL, Tyson GW, Nielsen PH. Genome sequences of rare, uncultured bacteria obtained by differential coverage binning of multiple metagenomes. Nat Biotechnol. 2013 Jun;31(6):533–538. https://doi.org/10.1038/nbt.2579

Buziassy C, Tribe DE. The synthesis of vitamins in the rumen of sheep. I. The effect of diet on the synthesis of thiamine, riboflavin, and nicotinic acid. Aust J Agr Res. 1960;11(6):989–1001. https://doi.org/10.1071/AR9600989

Bylesjö M, Rantalainen M, Cloarec O, Nicholson JK, Holmes E, Trygg J. OPLS discriminant analysis: combining the strengths of PLS-DA and SIMCA classification. J Chemometrics. 2006;20:341–351. https://doi.org/10.1002/cem.1006

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010 May;7(5):335–336. https://doi.org/10.1038/nmeth.f.303

Castillo-Lopez E, Ramirez Ramirez HA, Klopfenstein TJ, Anderson CL, Aluthge ND, Fernando SC, Jenkins T, Kononoff PJ. Effect of feeding dried distillers grains with solubles on ruminal biohydrogenation, intestinal fatty acid profile, and gut microbial diversity evaluated through DNA pyro-sequencing. J Anim Sci. 2014 Feb;92(2):733–743. https://doi.org/10.2527/jas.2013-7223

DeGregorio RM, Tucker RE, Mitchell GE Jr, Gill WW. Carbohydrate fermentation in the large intestine of lambs. J Anim Sci. 1982 Apr;54(4):855–862. https://doi.org/10.2527/jas1982.544855x

Di Rienzi SC, Sharon I, Wrighton KC, Koren O, Hug LA, Thomas BC, Goodrich JK, Bell JT, Spector TD, Banfield JF, et al. The human gut and groundwater harbor non-photosynthetic bacteria belonging to a new candidate phylum sibling to Cyanobacteria. Elife. 2013 Oct 1;2:e01102. https://doi.org/10.7554/eLife.01102

Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics. 2010 Oct 1;26(19):2460–2461. https://doi.org/10.1093/bioinformatics/btq461

Faichney GJ. Production of volatile fatty acids in the sheep caecum. Aust J Agr Res. 1969;20(3):491–498. https://doi.org/10.1071/AR9690491

Flint HJ, Bayer EA. Plant cell wall breakdown by anaerobic microorganisms from the Mammalian digestive tract. Ann N Y Acad Sci. 2008 Mar;1125:280–288. https://doi.org/10.1196/annals.1419.022

Flint HJ, Duncan SH, Scott KP, Louis P. Interactions and competition within the microbial community of the human colon: links between diet and health. Environ Microbiol. 2007 May;9(5):1101–1111. https://doi.org/10.1111/j.1462-2920.2007.01281.x

Heath TJ, Morris B. The absorption of fat in sheep and lambs. Q J Exp Physiol Cogn Med Sci. 1962 Apr;47:157–169. https://doi.org/10.1113/expphysiol.1962.sp001587

Hernandez-Sanabria E, Goonewardene LA, Wang Z, Zhou M, Moore SS, Guan LL. Influence of sire breed on the interplay among rumen microbial populations inhabiting the rumen liquid of the progeny in beef cattle. PLoS One. 2013;8(3):e58461. https://doi.org/10.1371/journal.pone.0058461

Hook SE, Steele MA, Northwood KS, Dijkstra J, France J, Wright AD, McBride BW. Impact of subacute ruminal acidosis (SARA) adaptation and recovery on the density and diversity of bacteria in the rumen of dairy cows. FEMS Microbiol Ecol. 2011 Nov;78(2):275–284. https://doi.org/10.1111/j.1574-6941.2011.01154.x

Hristov AN. Comparative characterization of reticular and duodenal digesta and possibilities of estimating microbial outflow from the rumen based on reticular sampling in dairy cows. J Anim Sci. 2007 Oct;85(10):2606–2613. https://doi.org/10.2527/jas.2006-852

Jiao J, Wang T, Zhou J, Degen AA, Gou N, Li S, Bai Y, Jing X, Wang W, Shang Z. Carcass parameters and meat quality of Tibetan sheep and Small-tailed Han sheep consuming diets of low-protein content and different energy yields. J Anim Physiol Anim Nutr (Berl). 2020 Jul;104(4):1010–1023. https://doi.org/10.1111/jpn.13298

Koenig KM, Rode LM, Cohen RD, Buckley WT. Effects of diet and chemical form of selenium on selenium metabolism in sheep. J Anim Sci. 1997 Mar;75(3):817–827. https://doi.org/10.2527/1997.753817x

Krajmalnik-Brown R, Ilhan ZE, Kang DW, DiBaise JK. Effects of gut microbes on nutrient absorption and energy regulation. Nutr Clin Pract. 2012 Apr;27(2):201–214. https://doi.org/10.1177/0884533611436116

Leahy SC, Kelly WJ, Altermann E, Ronimus RS, Yeoman CJ, Pacheco DM, Li D, Kong Z, McTavish S, Sang C, et al. The genome sequence of the rumen methanogen *Methanobrevibacter ruminantium* reveals new possibilities for controlling ruminant methane emissions. PLoS One. 2010 Jan 28;5(1):e8926. https://doi.org/10.1371/journal.pone.0008926

Lewis SM, Dehority BA. Microbiology and ration digestibility in the hindgut of the ovine. Appl Environ Microbiol. 1985 Aug;50(2):356–363. https://doi.org/10.1128/AEM.50.2.356-363

Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity. Nature. 2006 Dec 21;444(7122):1022–1023. https://doi.org/10.1038/4441022a

Liesack W, König H, Schlesner H, Hirsch P. Chemical composition of the peptidoglycan-free cell envelopes of budding bacteria of the *Pirella/Planctomyces* group. Arch Microbiol. 1986;145:361–366. https://doi.org/10.1007/BF00470872

Looney SW, Hagan JL. 4 statistical methods for assessing biomarkers and analyzing biomarker data. In: Rao CR, Miller JP, Rao DC, editors. Handbook of statistics. Amsterdam (The Netherlands): Elsevier; 2007;27. p.109–147. https://doi.org/10.1016/S0169-7161(07)27004-X

Lozupone C, Knight R. UniFrac: a new phylogenetic method for comparing microbial communities. Appl Environ Microbiol. 2005 Dec;71(12):8228–8235. https://doi.org/10.1128/AEM.71.12.8228-8235.2005

McCann JC, Elolimy AA, Loor JJ. Rumen microbiome, probiotics, and fermentation additives. Vet Clin North Am Food Anim Pract. 2017 Nov;33(3):539–553. https://doi.org/10.1016/j.cvfa.2017.06.009

Merchen NR, Firkins JL, Berger LL. Effect of intake and forage level on ruminal turnover rates, bacterial protein synthesis and duodenal amino acid flows in sheep. J Anim Sci. 1986 Jan;62(1):216–225. https://doi.org/10.2527/jas1986.621216x

Muroya S, Oe M, Ojima K, Watanabe A. Metabolomic approach to key metabolites characterizing postmortem aged loin muscle of Japanese Black (Wagyu) cattle. Asian-Australas J Anim Sci. 2019 Aug;32(8):1172–1185. https://doi.org/10.5713/ajas.18.0648

National Research Council. Nutrient requirements of small ruminants: sheep, goats, cervids, and New World camelids. Washington (USA): The National Academies Press; 2007. https://doi.org/10.17226/11654

Palomba A, Tanca A, Fraumene C, Abbondio M, Fancello F, Atzori AS, Uzzau S. Multi-omic biogeography of the gastrointestinal microbiota of a pre-weaned lamb. Proteomes. 2017 Dec 18; 5(4):36. https://doi.org/10.3390/proteomes5040036

Paz HA, Castillo-Lopez E, Ramirez-Ramirez HA, Christensen DA, Kononoff PJ. Invited review: Ethanol co-products for dairy cows: there goes our starch … now what? Can J Anim Sci. 2013;93(4):407–425. https://doi.org/10.4141/cjas2013-048

Purchas RW, Rutherfurd SM, Pearce PD, Vather R, Wilkinson BH. Concentrations in beef and lamb of taurine, carnosine, coenzyme Q(10), and creatine. Meat Sci. 2004 Mar;66(3):629–637. https://doi.org/10.1016/S0309-1740(03)00181-5

Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, Nielsen T, Pons N, Levenez F, Yamada T, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 2010 Mar 4;464(7285):59–65. https://doi.org/10.1038/nature08821

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 2013 Jan;41(Database issue):D590–D596. https://doi.org/10.1093/nar/gks1219

Ramirez Ramirez HAR, Nestor K, Tedeschi LO, Callaway TR, Dowd SE, Fernando SC, Kononoff PJ. The effect of brown midrib corn silage and dried distillers' grains with solubles on milk production, nitrogen utilization and microbial community structure in dairy cows. Can J Anim Sci. 2012; 92(3):365–380. https://doi.org/10.4141/cjas2011-133

Reynaert R, De Paepe M, Marcus S, Peeters G. Influence of serum free fatty acid levels on growth hormone secretion in lactating cows. J Endocrinol. 1975 Aug;66(2):213–224. https://doi.org/10.1677/joe.0.0660213

Sartin JL, Bartol FF, Kemppainen RJ, Dieberg G, Buxton D, Soyoola E. Modulation of growth hormone-releasing factor stimulated growth hormone secretion by plasma glucose and free fatty acid concentrations in sheep. Neuroendocrinology. 1988 Dec; 48(6):627–633. https://doi.org/10.1159/000125073

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, et al. Introducing Mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol. 2009 Dec;75(23):7537–7541. https://doi.org/10.1128/AEM.01541-09

Sirohi SK, Singh N, Dagar SS, Puniya AK. Molecular tools for deciphering the microbial community structure and diversity in rumen ecosystem. Appl Microbiol Biotechnol. 2012 Sep;95(5):1135–1154. https://doi.org/10.1007/s00253-012-4262-2

Stevenson DM, Weimer PJ. Dominance of *Prevotella* and low abundance of classical ruminal bacterial species in the bovine rumen

revealed by relative quantification real-time PCR. Appl Microbiol Biotechnol. 2007 May;75(1):165–174. https://doi.org/10.1007/s00253-006-0802-y

Tonsbeek CHT, Plancken AJ, Weerdhof TVD. Components contributing to beef flavor. Isolation of 4-hydroxy-5-methyl-3(2H)-furanone and its 2,5-dimethyl homolog from beef broth. J Agr Food Chem. 1968;16(6):1016–1021 https://doi.org/10.1021/jf60160a008

Ussar S, Fujisaka S, Kahn CR. Interactions between host genetics and gut microbiome in diabetes and metabolic syndrome. Mol Metab. 2016 Jul 18;5(9):795–803. https://doi.org/10.1016/j.molmet.2016.07.004

Waghorn GC, Shelton ID, Sinclair BR. Distribution of elements between solid and supernatant fractions of digesta in sheep given six diets. New Zeal J Agr Res. 1990;33(2):259–269. https://doi.org/10.1080/00288233.1990.10428418

Walker ND, McEwan NR, Wallace RJ. A pepD-like peptidase from the ruminal bacterium, *Prevotella albensis*. FEMS Microbiol Lett. 2005 Feb 15;243(2):399–404. https://doi.org/10.1016/j.femsle.2004.12.032

Wang J, Fan H, Han Y, Zhao J, Zhou Z. Characterization of the microbial communities along the gastrointestinal tract of sheep by 454 pyrosequencing analysis. Asian-Australas J Anim Sci. 2017 Jan; 30(1):100–110. https://doi.org/10.5713/ajas.16.0166

Wang Q, Wang Y, Wang X, Dai C, Tang W, Li J, Huang P, Li Y, Ding X, Huang J, et al. Effects of dietary energy levels on rumen fermentation, microbiota, and gastrointestinal morphology in growing ewes. Food Sci Nutr. 2020 Nov 10;8(12):6621–6632. https://doi.org/10.1002/fsn3.1955

Weenen H, Kerler J, van der Ven JGM. The Maillard reaction in flavour formation. In: Swift KAD, editor. Flavours and fragrances. Woodhead Publishing Series in Food Science, Technology and Nutrition. Cambridge (England): Woodhead Publishing; 2005. p. 153–170. https://doi.org/10.1533/9781845698249.3.153

Wu J, Yang D, Gong H, Qi Y, Sun H, Liu Y, Liu Y, Qiu X. Multiple omics analysis reveals that high fiber diets promote gluconeogenesis and inhibit glycolysis in muscle. BMC Genomics. 2020 Sep 24;21(1):660. https://doi.org/10.1186/s12864-020-07048-1

Xu X, Xu P, Ma C, Tang J, Zhang X. Gut microbiota, host health, and polysaccharides. Biotechnol Adv. 2013 Mar-Apr;31(2):318–37. https://doi.org/10.1016/j.biotechadv.2012.12.009

Zhang R, Zhu W, Zhu W, Liu J, Mao S. Effect of dietary forage sources on rumen microbiota, rumen fermentation and biogenic amines in dairy cows. J Sci Food Agric. 2014 Jul;94(9):1886–1895. https://doi.org/10.1002/jsfa.6508

Zhen J, Yan S, Li Y, Ruan C, Li Y, Li X, Zhao X, Lv X, Ge Y, Moure UAE, et al. L-Alanine specifically potentiates fluoroquinolone efficacy against *Mycobacterium* persisters via increased intracellular reactive oxygen species. Appl Microbiol Biotechnol. 2020 Mar;104(5):2137–2147. https://doi.org/10.1007/s00253-020-10358-9