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Characterization of the microbial communities along the gastrointestinal tract of sheep by 454 pyrosequencing analysis

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Objective: The gastrointestinal tract of sheep contain complex microbial communities that influence numerous aspects of the sheep's health and development. The objective of this study was to analyze the composition and diversity of the microbiota in the gastrointestinal tract sections (rumen, reticulum, omasum, abomasum, duodenum, jejunum, ileum, cecum, colon, and rectum) of sheep.

Methods: This analysis was performed by 454 pyrosequencing using the V3-V6 region of the 16S rRNA genes. Samples were collected from five healthy, small tailed Han sheep aged 10 months, obtained at market. The bacterial composition of sheep gastrointestinal microbiota was investigated at the phylum, class, order, family, genus, and species levels.

Results: The dominant bacterial phyla in the entire gastrointestinal sections were *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*. In the stomach, the three most dominant genera in the sheep were *Prevotella*, unclassified *Lachnospiraceae*, and *Butyrivibrio*. In the small intestine, the three most dominant genera in the sheep were *Escherichia*, unclassified *Lachnospiraceae*, and *Ruminococcus*. In the large intestine, the three most dominant genera in the sheep were *Ruminococcus*, unclassified *Ruminococcaceae*, and *Prevotella*. *R. flavefaciens*, *B. fibrisolvens*, and *S. ruminantium* were three most dominant species in the sheep gastrointestinal tract. Principal Coordinates Analysis showed that the microbial communities from each gastrointestinal section could be separated into three groups according to similarity of community composition: stomach (rumen, reticulum, omasum, and abomasum), small intestine (duodenum, jejunum, and ileum), and large intestine (cecum, colon, and rectum).

Conclusion: This is the first study to characterize the entire gastrointestinal microbiota in sheep by use of 16S rRNA gene amplicon pyrosequencing, expanding our knowledge of the gastrointestinal bacterial community of sheep.

Keywords: Pyrosequencing, Microbiota, Gastrointestinal Tract Sections, Sheep

INTRODUCTION

A huge number of various microorganisms live in the gastrointestinal tract of ruminants, about ten times their body's own cells in number. Sheep is a ruminant animal where the gut is a fermentative chamber for a complex and dynamic microbial population. Symbiotic bacterial community is crucial for the host health in many aspects, such as in balancing the immune response, digesting the nutrients, and mediating the host physiology. In addition, symbioses between microbiota and host can facilitate the development of the latter's gastrointestinal tract. Especially, bacteria in the ruminant gut play a major role in the biological degradation of dietary fibers. Ruminant digestion relies on the bulk of cellulose hydrolysis bacteria. However, the microbiota composition and diversity are affected by many factors, such as diet composition, host genetics, and environment, and so on. Therefore, it is now recognized that a better and sufficient understanding of the composition and diversity of

the gastrointestinal microbial community is required to further enhance the growth and gut health of ruminants.

In the past, the conventional culture-based technique was used to isolate and characterize the gastrointestinal microbiota of ruminants. Indeed, even if the culture-based technique has successfully isolated key representative bacteria, it is not sufficient to characterize the entire microbial populations, because a large majority of gastrointestinal microbiota is not culturable. A recent article indicated that some unculturable microbiota were abundant in the rumen, which play an important role in the ruminal fermentation [1]. Over the last 10 years the development of high-throughput sequencing techniques has allowed for a considerable increase in knowledge of the microbial diversity of the ruminant gut. Roche 454 pyrosequencing platform provides new approaches for researchers to investigate complex microbial communities. This platform does not required a clone library, and has high throughput efficiency and sensitivity. The application of this approach enabled us to successfully analyze several samples at a time, which significantly reduced experimental cost and improved efficiency.

Although the small tail Han sheep is an important sheep breed with many favorable features, including fast growth, strong reproduction, stable genetic performance, and good adaption, data on its gut microbiota are limited. Rumen contents and feces in ruminants are often used to assess gastrointestinal microbiota [2,3], however, these sections do not represent the composition and diversity of microbiota in the entire gastrointestinal tract. Especially, the forestomach of ruminants, namely, rumen, reticulum and omasum contribute to the digestion of the cellulose substances in the diet. The small intestine is responsible for absorption of water, nutrients, and electrolytes. However, knowledge on the microbiota in the gastrointestinal tract of ruminants is limited. Recent studies explored the composition of the gastrointestinal microbiota in dairy cattle and Brazilian Nelore breed of cattle [4,5]. However, no studies to date have evaluated the entire gastrointestinal bacterial community in adult sheep using pyrosequencing, especially small tail Han sheep, which is a main sheep breed in Tianjin. The objective of this study was to characterize the gastrointestinal bacterial community of small tail Han sheep using pyrosequencing of 16S rRNA gene amplicons. Understanding the composition and structure of the microbiota in the gastrointestinal tract of sheep may be useful for developing ruminant production and management.

MATERIALS AND METHODS

Animals and sampling

The use and care of the animals used in this study were approved by the Animal Care Advisory Committee of the Tianjin Academy of Agricultural Sciences. The health of the sheep was monitored continuously before and during the experimental period. Five,

healthy, small tailed Han sheep aged 10 months and weighing between 55 kg were obtained from a commercial feedlot, and fed the same feed. The diets were consisting of 57.78% green hay, 12.22% alfalfa, 16.67% corn, 8.89% soybean meal, wheat bran 3.33%, 0.11% $CaHPO_4$, 0.89% NaCl, and 0.11% premix. One kilogram of premix contains: $FeSO_4 \cdot 7H_2O$ 170 g; $CuSO_4 \cdot 5H_2O$ 70 g; $MnSO_4 \cdot 5H_2O$ 290 g; $ZnSO_4 \cdot 7H_2O$ 240 g; $CoCl_2 \cdot 6H_2O$ 510 mg; KI 200 mg; $NaSeO_3$ 130 mg; vitamin A (VA) 1,620,000 IU; VD_3 324,000 IU; VE 540 IU; VK_3 150 mg; VB_{12} 0.9 mg; VB_5 450 mg; Calcium pantothenate 750 mg; Folic acid 15 mg. Following the standard livestock management practices, all sheep were placed in disinfected individual pens with *ad libitum* water and feed access. No animals showed signs of disease or ill health. Physiological and biochemical indexes of small tail Han sheep were shown in Table 1. Fresh samples (20 g) were collected from three regions of the gastrointestinal tract, namely, the stomach (rumen, reticulum, omasum, and abomasum), small intestine (duodenum, jejunum, and ileum), and large intestine (cecum, colon, and rectum). Each sample was preserved at –20°C and shipped on dry ice to the Tianjin University, Tianjin, China, for analysis of the microbiota.

DNA extraction

The digesta of every gastrointestinal part which contains solid

Table 1. Physiological and biochemical indexes of small tail Han sheep

Physiological indexes	Measured values
Rectal temperature (°C)	39.01 ± 0.77
Heart rate (times/min)	75.20 ± 5.38
Respiration rate (times/min)	19.07 ± 4.92
Blood physiological indexes	
WBC (white blood cell count) ($\times 10^9$/L)	8.49 ± 3.84
RBC (red blood cell count) ($\times 10^{12}$/L)	11.00 ± 0.18
MCH (mean corpuscular hemoglobin) (pg)	11.23 ± 2.73
MCV (mean corpuscular volume) (fL)	39.30 ± 0.31
HGB (hemoglobin) (g/L)	120.79 ± 8.04
HCT (hematocrit) (%)	34.49 ± 0.88
PLT (platelet count) ($\times 10^9$/L)	550.25 ± 25.55
Lymphocyte cell (%)	66.53 ± 7.16
Basophil cell (%)	0.44 ± 0.03
Acidophilic cell (%)	3.29 ± 1.08
Monocytes (%)	5.72 ± 0.09
Blood biochemical indexes	
LDH (lactate dehydrogenase) (U/L)	433.54 ± 45.86
CA (total calcium) (mmol/L)	2.38 ± 0.12
CHOL (total cholesterol) (mmol/L)	3.78 ± 0.32
GLU (glucose) (mmol/L)	2.56 ± 0.37
BUN (Blood urea nitrogen) (mmol/L)	5.25 ± 0.45
CHE (cholinesterase) (U/L)	640.01 ± 2.22
AKP (alkaline phosphatase) (U/L)	325.05 ± 69.13
ALB (albumin) (g/L)	30.18 ± 3.00
GLO (globulin) (g/L)	23.75 ± 3.09
TP (total protein) (g/L)	55.03 ± 6.24
GPT (glutamic-pyruvic transaminase) (U/L)	30.15 ± 5.06
GOT (glutamic-oxalacetic transaminase) (U/L)	81.01 ± 15.32

and liquid was homogenized by mixing. Total DNA was extracted from the digesta samples containing solids and liquids (100 mg each) according to the instructions in E.Z.N.A stool DNA kit (Omega Bio-Tek, Doraville, CA, USA). For better analysis of gram-positive bacteria DNA, a second incubation at 95°C for 10 min was performed following the initial incubation at 70°C for 13 min in the protocol. All procedures were performed on ice. The final elution volume was 200 µL, and DNA concentration was determined by a Nano Drop spectrophotometer (Nano Drop Technologies, Wilmington, DE, USA).

PCR amplification of the V3-V6 region of bacterial 16S rRNA genes

The DNA extracted from each sample was amplified with a set of primers targeting the hypervariable V3-V6 region of the 16S rRNA gene. The forward primer was 338F: 5'-ACTCCT-ACGGGAGGCAGCAG-3' and the reverse primer was 1046R: 5'-CGACAGCCATGCANCACCT-3' [6]. The forward primer contained the sequence of the Titanium A adapter and a 9-bp barcode sequence. The reverse primer contained the sequence of the Titanium B adapter. The amplification mix contained 5 U of FastStart High Fidelity polymerase, 1×FastStart High Fidelity Reaction Buffer, 0.2 mM deoxynucleoside triphosphates, 0.4 µM of each primer, and 50 ng of template DNA in a reaction volume of 50 µL. Polymerase chain reaction (PCR) was performed on MyCyclerTM Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) set as follows: 94°C for 4 min; 25 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 50 s; and 72°C for 10 min. The PCR amplicon products were visualized using 1% agarose gels, extracted from the gels, and then purified using a SanPrep PCR Purification Kit (Sangon Biotech, Shanghai, China) according to the manufacturer's instructions.

454 Pyrosequencing and data analysis

The PCR products of the V3-V6 region of the 16S rRNA genes were sequenced using a Roche 454 FLX Titanium sequencer. The 16S rRNA raw sequence data were processed using the quantitative insights into microbial ecology (QIIME, University of Colorado, Boulder, CO, USA) version 1.7.0 software pipeline. The sequences were quality-filtered using default QIIME parameters, and poor-quality sequences were eliminated from the data sets (i.e. sequences where lengths were less than 200 bp or more than 1,000 bp, number of ambiguous bases exceeded a limit of six, there was a missing quality score, the mean quality score was below 25, the maximum homopolymer run exceeded a limit of six, and the number of mismatches in the primer exceeded a limit of two). Chimeras were detected and excluded using the software Black Box Chimera Check (B2C2) (Research of Testing Laboratory of the South Plains, Lubbock, TX, USA). Qualified sequences were clustered into operational taxonomic units (OTUs) with a 97% similarity threshold using the UCLUST algorithm (Tiburon, CA, USA).

A representative sequence was chosen from each OTU by selecting the first sequence. The representative sequences were taxonomically classified using the ribosomal database project (RDP) classifier, and the confidence threshold was set at 0.8. The relative abundances of the phylum and class levels were plotted as bar graph. The relative abundances of the order, family and genus levels were created as heatmap. The relative abundance of the species was shown as a table. Representative sequences were aligned against the Greengenes core set using Python Nearest Alignment Space Termination (PyNAST) software (University of Colorado, Boulder, CO, USA). The minimum aligned sequence length was set at 150 bp and the minimum percent identity was set at 75%. A subsequent phylogenetic tree was built using FastTree (Lawrence Berkeley National Laboratory, Berkeley, CA, USA). To account for unequal sequencing depth across samples, subsequent analyses were performed on a randomly selected subset of 1,038 sequences per sample. For alpha diversity measurements, the estimated number of OTUs in each sample using the Chao1 index, the diversity of the populations using the Shannon index, and the amount of phylogenetic branch length observed in each sample (phylogenetic distance [PD]) were determined. For beta diversity, differences in the microbial communities between the gastrointestinal sections were investigated using the phylogeny-based unweighted UniFrac distance metric.

RESULTS

Richness and diversity analysis

Fifty samples from the 10 different gastrointestinal tract sections (rumen, reticulum, omasum, abomasum, duodenum, jejunum, ileum, cecum, colon, and rectum) of 5 sheep were used in our experiments. The pyrosequencing pipeline yielded 363,298 16S rRNA gene sequence reads from all gastrointestinal tract samples. After quality-filtering with QIIME default settings, a total of 252,030 16S rRNA sequence reads were retained from all gastrointestinal microbiota samples, including 30,350 reads from the rumen, 24,965 reads from the reticulum, 63,825 reads from the omasum, 34,825 reads from the abomasum, 11,960 reads from the duodenum, 6,035 reads from the jejunum, 31,795 reads from the ileum, 26,550 reads from the cecum, 8,840 reads from the colon, and 12,885 reads from the rectum (Table 2). The average length of the quality-checked and filtered sequences was 605 bp. The maximum length was 818 bp, and the minimum length was 200 bp.

To compare the bacterial species richness and diversity in the entire gastrointestinal tract sections, the bacterial richness and diversity levels were analyzed using the observed species, Chao1 estimator, Shannon index, and PD index. OTUs were grouped at the 97% similarity level. To account for unequal sequencing depth across samples, subsequent analyses were performed on a randomly selected subset of 1,038 sequences

Table 2. Alpha diversity estimates of microbiota associated with the gastrointestinal tract sections of sheep[1]

Sample	Total reads	Total OTUs	Reads subsampled	Observed species	Chao1	Shannon	PD
Rumen	6,070±766	651±222	1,038	457.200±133.200	1,071.704±303.257	8.113±1.421	26.055±4.415
Reticulum	4,993±419	633±195	1,038	454.000±119.100	1,057.464±299.901	8.065±1.077	24.866±3.778
Omasum	12,765±1,222	789±353	1,038	572.100±203.300	1,819.647±401.110	8.525±1.609	32.331±4.994
Abomasum	6,965±891	703±347	1,038	521.500±194.600	1,315.026±307.458	8.334±1.522	30.340±4.636
Duodenum	2,392±432	362±109	1,038	326.000±79.500	669.954±178.903	7.004±0.778	20.650±2.330
Jejunum	1,207±367	322±99	1,038	316.100±77.900	640.548±162.316	6.957±0.765	20.924±2.421
Ileum	6,359±808	648±272	1,038	476.200±123.000	1,260.505±338.792	7.993±0.993	26.626±3.909
Cecum	5,310±776	644±268	1,038	447.600±108.800	916.780±183.394	8.102±1.399	24.127±3.111
Colon	1,768±564	526±190	1,038	372.000±99.100	721.966±137.115	7.585±0.820	20.432±2.027
Rectum	2,577±367	615±213	1,038	445.700±105.200	975.539±159.533	7.947±0.986	26.944±4.383

[1] Data are expressed as average±standard deviation (n = 5 sheep). The number of observed species, Chao 1 estimator, Shannon index and phylogenetic distance (PD) index were analyzed after reads subsampled to 1,038 reads. The operational taxonomic units (OTUs) were defined with 3% dissimilarity.

per sample. This number was chosen to avoid exclusion of samples with a lower number of sequence reads from further analysis. As shown in Table 2, the highest microbial richness of samples were found in the stomach (rumen, reticulum, omasum, and abomasum), and the average of the observed species were between 454.000 and 572.100; the average of Chao1 index varied from 1,057.464 to 1,819.647. Ileum and large intestine (cecum, colon, and rectum) samples had moderate microbial richness, and the average of the observed species were between 372.000 and 476.200; the average of Chao1 index varied from 721.966 to 1,260.505. While duodenum and jejunum samples had lowest microbial community richness (observed species 326.000±79.500 and 316.100±77.900, Chao1 index 669.954±178.903 and 640.548±162.316). Similarly, the stomach samples had the highest microbial diversity (the average of Shannon index were between 8.065 and 8.525, the average of PD index varied from 24.866 to 32.331). Ileum and large intestine samples had moderate microbial diversity, and the average of Shannon

index were between 7.585 and 8.102; the average of PD index varied from 20.432 to 26.944. While duodenum and jejunum samples had lowest microbial community diversity (Shannon index 7.004±0.778 and 6.957±0.765, PD index 20.650±2.330 and 20.924±2.421).

Sheep gastrointestinal microbiota composition at the phylum level

To describe the composition of the gastrointestinal microbiota, a taxon-dependent analysis was carried out using the RDP classifier. The results, shown in Figure 1, describe the average distribution of DNA sequences into phyla (n = 5 sheep). A total of 15 bacterial phyla were identified across the entire gastrointestinal tract sections. *Firmicutes* and *Bacteroidetes* were the most abundant phyla in all gastrointestinal tract sections, and comprised 52.3%±15.7% and 32.4%±4.4% of the total sequences, followed by *Proteobacteria* (7.5%±2.1%). The remaining sequences were *Verrucomicrobia* (2.0%±0.7%), *Actinobacteria*
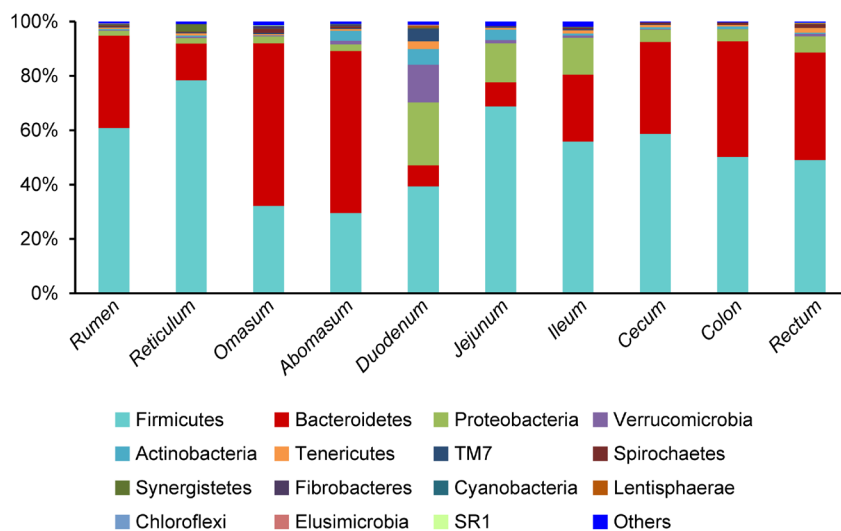

Figure 1. Ribosomal database project (RDP) classification of the sequence reads from different gastrointestinal tracts of sheep at the phylum level. The abscissa (X-axis) represents the different gastrointestinal tract sections, and the ordinate (Y-axis) represents the average relative abundance of the bacterial phyla (n = 5 sheep).

(1.7%±0.5%), *Tenericutes* (1.0%±0.1%), and a number of low-abundance phyla. The low-abundant phyla were TM7, *Spirochaetes, Synergistetes, Fibrobacteres, Cyanobacteria, Lentisphaerae, Chloroflexi, Elusimicrobia*, and Sulfur River 1 (SR1). The proportion of sequences that could not be assigned to a phylum using the RDP classifier ranged from 0.3% to 1.9%. When bacterial composition was compared regionally, the bacterial composition of the each gastrointestinal tract section showed no significant differences between them. The phylum *Firmicutes* dominated all bacterial communities along the gastrointestinal tract except for in the omasum and abomasums, where *Bacteroidetes* was predominant. The phylum *Bacteroidetes* (7.7±1.9% and 8.9±1.3%) was less abundant in the duodenum and jejunum than other gastrointestinal tract sections. In the contrast, the phyla *Proteobacteria* (13.5% to 23.2%) and *Actinobacteria* (3.7% to 5.8%) were more abundant in the small intestine than the stomach and large intestine. Although the phyla *Verrucomicrobia* had the low abundance in the gastrointestinal tract sections, the *Verrucomicrobia* in the duodenum accounted for 13.8%-±2.9% of the total sequences.

Sheep gastrointestinal microbiota composition at the class level

The results, shown in Figure 2, describe the average distribution of DNA sequences into classes (n = 5 sheep). A total of 25 bacterial classes were identified across the entire gastrointestinal tract sections. *Clostridia* and *Bacteroidia* were the most abundant classes in all gastrointestinal tract sections, and comprised 50.3%±13.2% and 32.3%±4.1% of the total sequences, followed by *Gammaproteobacteria* (6.6%±1.7%). The remaining sequences were *Verruco-5* (1.9%±0.7%), *Actinobacteria* (1.7%±0.5%),

Bacilli (1.3%±0.2%), *Mollicutes* (1.0%±0.1%), and a number of low-abundance classes. The results showed that several classes contributed to the differences in community composition between different gastrointestinal tract sections. In the stomach, the three most dominant classes in the sheep were *Clostridia* (49.2%±7.7%), *Bacteroidia* (41.6%±5.6%), and *Gammaproteobacteria* (1.7%±0.3%). It is worth mentioning that *Actinobacteria* (1.2%±0.4%) was also abundant in the stomach. In the small intestine, the three most dominant classes in the sheep were *Clostridia* (50.0%±9.9%), *Gammaproteobacteria* (15.8%±3.3%), and *Bacteroidia* (13.6%±4.1%). Classes *Verruco-5* (5.2%±1.1%), *Bacilli* (4.0%±0.9%), and TM7-3 (1.8%±0.2%) were significantly more abundant in the small intestine than other gastrointestinal tract sections. In the large intestine, the three most dominant classes in the sheep were *Clostridia* (52.0%±9.7%), *Bacteroidia* (38.6%±6.3%), and *Gammaproteobacteria* (4.1%±1.6%).

Sheep gastrointestinal microbiota composition at the order level

Differences in bacterial average abundance at the order level for the sheep gastrointestinal tract sections are shown as a heatmap in Figure 3 (n = 5 sheep). Regardless of which gastrointestinal sections they occurred in, the 8 orders , including *Clostridiales* (24.8% to 70.2%), *Bacteroidales* (7.6% to 59.7%), *Aeromonadales* (0.2% to 3.4%), contaminated aquifer clone WCHB1-41 (0.1% to 13.8%), *Coriobacteriales* (0.2% to 4.6%), *Bifidobacteriales* (0.1% to 4.9%), RF39 (0.2% to 1.6%), and *Erysipelotrichales* (0.1% to 1.1%) were defined as the core orders, because they were found in all gastrointestinal tract sections of sheep. The results showed that several orders contributed to the differences in community composition between different
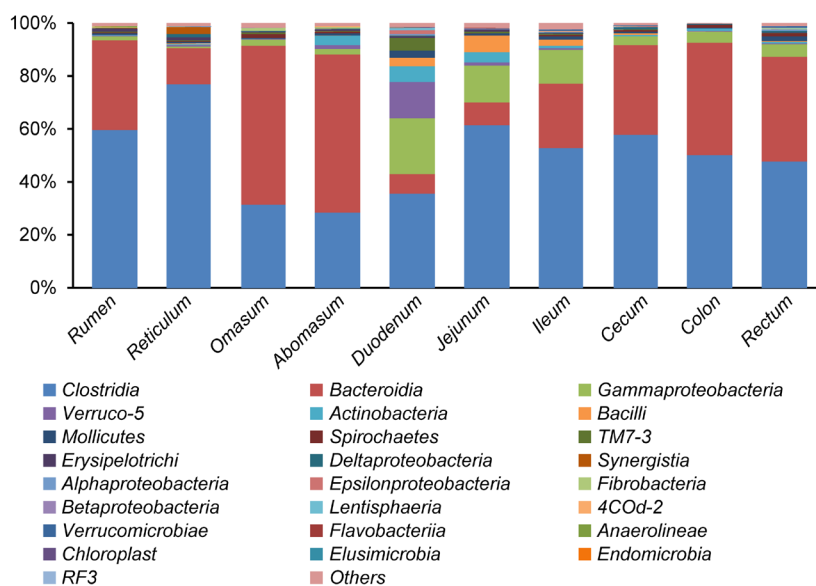

Figure 2. Ribosomal database project (RDP) classification of the sequence reads from different gastrointestinal tracts of sheep at the class level. The abscissa (X-axis) represents the different gastrointestinal tract sections, and the ordinate (Y-axis) represents the average relative abundance of the bacterial classes (n = 5 sheep).
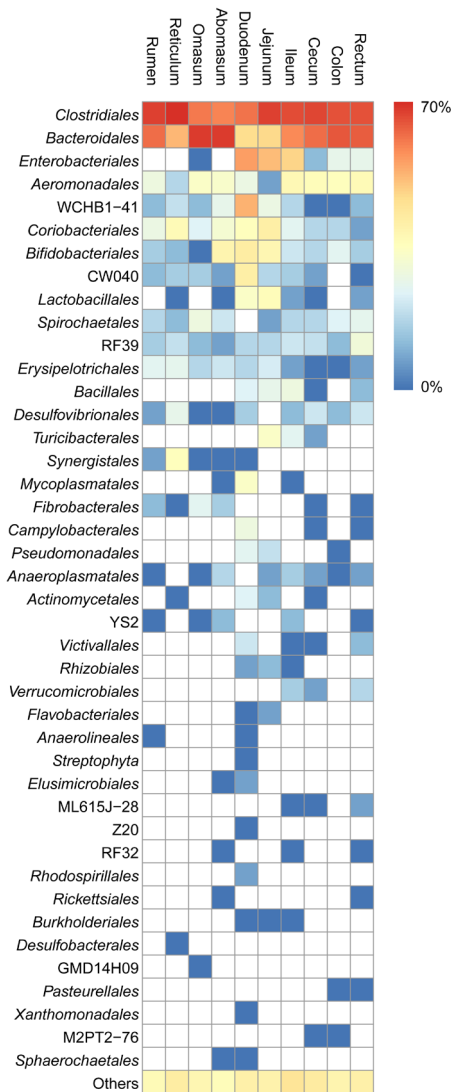
Figure 3. Heatmap at the order level among the sheep gastrointestinal tract sections. Heatmap columns show normalized average relative abundance (n = 5 sheep) and provide a comparison of the differences in abundance between different orders in the same section. Heatmap rows show normalized average relative abundance and provide a comparison of the differences in abundance between different sections in the same order.

gastrointestinal tract sections. In the stomach, the three most dominant orders in the sheep were *Clostridiales* (45.1%±-6.6%), *Bacteroidales* (41.6%±5.6%), and *Coriobacteriales* (1.8%±0.2%). In the small intestine, the three most dominant orders in the sheep were *Clostridiales* (44.3%±6.9%), *Bacteroidales* (13.6%±4.1%), and *Enterobacteriales* (13.4%±3.1%). Orders contaminated aquifer clone WCHB1-41 (5.2%±1.7%), oral clone CW040 (1.8%±0.8%), and *Lactobacillales* (4.6%±1.8%) were significantly more abundant in the small intestine than other gastrointestinal tract sections. In the large intestine, the three most dominant orders in the sheep were *Clostridiales* (48.2%±6.3%), *Bacteroidiales* (38.6%±6.3%), and *Aeromonadales* (2.9%±0.4%).

Sheep gastrointestinal microbiota composition at the family level

Differences in bacterial average abundance at the family level for the sheep gastrointestinal tract sections are shown as a heatmap in Figure 4 (n = 5 sheep). Regardless of which gastro-
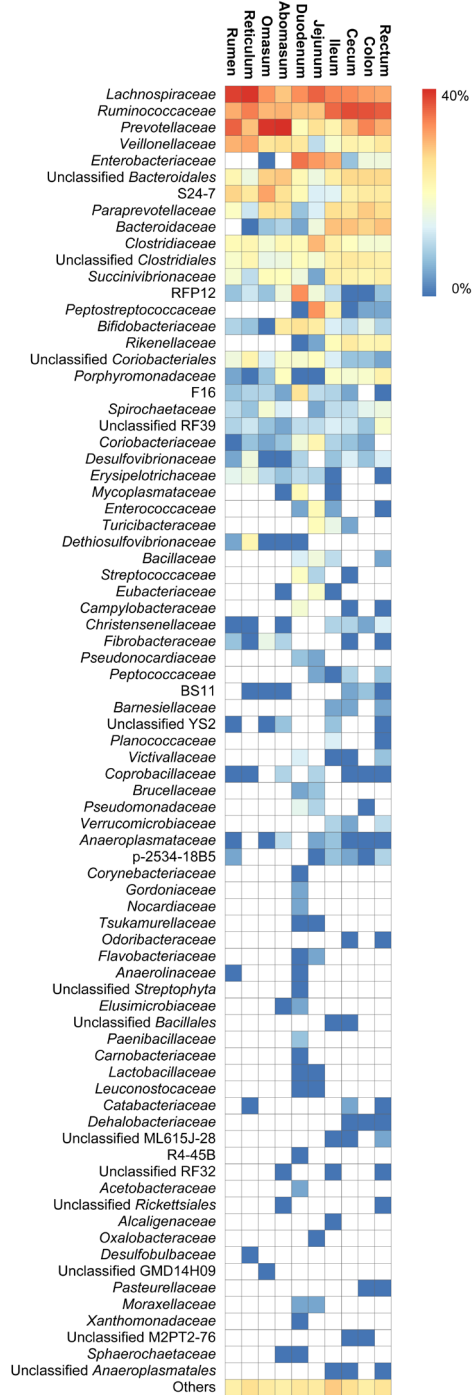

Figure 4. Heatmap at the family level among the sheep gastrointestinal tract sections. Heatmap columns show normalized average relative abundance (n = 5 sheep) and provide a comparison of the differences in abundance between different families in the same section. Heatmap rows show normalized average relative abundance and provide a comparison of the differences in abundance between different sections in the same family.

intestinal sections they occurred in, the 11 families, including *Lachnospiraceae* (7.1% to 37.5%), *Ruminococcaceae* (7.2% to 28.4%), *Prevotellaceae* (2.2% to 40.1%), *Veillonellaceae* (0.5% to 11.0%), S24-7 (a member of *Bacteroidales*) (0.7% to 11.0%), *Paraprevotellaceae* (0.3% to 6.8%), *Clostridiaceae* (1.4% to 8.5%), *Succinivibrionaceae* (0.2% to 3.4%), RFP12 (a member of WCHB1-41) (0.1% to 13.8%), *Bifidobacteriaceae* (0.1% to 4.9%), and *Porphyromonadaceae* (0.1% to 3.0%) were defined as the core families, because they were found in all gastrointestinal tract sections of sheep. The results showed that several families contributed to the differences in community composition between different gastrointestinal tract sections. In the stomach, the three most dominant families in the sheep were *Prevotellaceae* (26.7%±3.1%), *Lachnospiraceae* (22.8%±2.7%), and *Ruminococcaceae* (11.1%±3.3%). It is worth mentioning that *Veillonellaceae* (7.4%±1.6%) and S24-7 (6.5%±2.0%) were also abundant in the stomach. In the small intestine, the three most dominant families in the sheep were *Lachnospiraceae* (17.5%-±2.9%), *Enterobacteriaceae* (13.4%±3.4%), and *Ruminococcaceae* (11.9%±2.1%). Families *Peptostreptococcaeae* (5.4%±0.9%), RFP12 (5.2%±0.4%), and *Clostridiaceae* (4.6%±1.1%) were significantly more abundant in the small intestine than other gastrointestinal tract sections. In the large intestine, the three most dominant families in the sheep were *Ruminococcaceae* (26.7%±3.6%), *Lachnospiraceae* (12.1%±2.2%), and *Prevotellaceae* (11.0%±-2.3%). Families *Bacteroidaceae* (7.1%±1.5%) and *Paraprevotellaceae* (5.6%±0.9%) were significantly more abundant in the large intestine than other gastrointestinal tract sections.

Sheep gastrointestinal microbiota composition at the genus level

A total of 120 genera were identified in the entire gastrointestinal tract sections of sheep (n = 5 sheep). Of these, 31 genera were found to be abundant present at ≥0.5% of gastrointestinal tract bacterial sequences (Figure 5). The 31 genera accounted for 79.0%±22.7% of the total sequences for the gastrointestinal tract sections. Regardless of which gastrointestinal sections they occurred in, the 15 genera, including *Prevotella* (2.2% to 40.2%), unclassified *Lachnospiraceae* (3.5% to 15.3%), *Ruminococcus* (2.0% to 14.2%), unclassified *Ruminococcaceae* (3.0% to 12.5%), unclassified S24-7 (0.7% to 11.0%), CF231 (a member of *Paraprevotellaceae*) (0.2% to 4.9%), unclassified RFP12 (0.1% to 13.8%), unclassified *Clostridiaceae* (0.4% to 6.1%), unclassified *Bifidobacteriaceae* (0.1% to 3.6%), *Clostridium* (0.1% to 2.3%), *Oscillospira* (0.1% to 3.2%), unclassified *Veillonellaceae* (0.1% to 4.1%), *Succinivibrio* (0.2% to 2.5%), *Anaerovibrio* (0.1% to 2.2%), and *Coprococcus* (0.2% to 1.9%) were defined as the core genera, because they were found in all gastrointestinal tract sections of sheep. The results showed that several genera contributed to the differences in community composition between different gastrointestinal tract sections. In the stomach, the three most dominant genera in the sheep were *Prevotella*
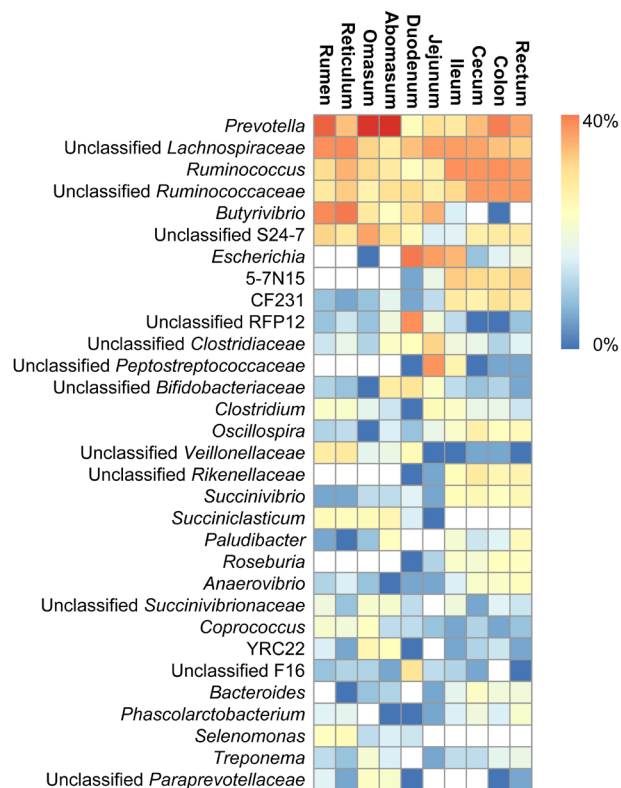

Figure 5. Heatmap at the genus level among the sheep gastrointestinal tract sections (more than 0.5% of the total DNA sequences). Heatmap columns show normalized average relative abundance (n = 5 sheep) and provide a comparison of the differences in abundance between different genera in the same section. Heatmap rows show normalized average relative abundance and provide a comparison of the differences in abundance between different sections in the same genus.

(26.7%±3.1%), unclassified *Lachnospiraceae* (9.8%±2.0%), and *Butyrivibrio* (9.5%±2.1%). Genus unclassified S24-7 (6.5%±-2.0%) was significantly more abundant in the stomach than other gastrointestinal tract sections. In the small intestine, the three most dominant genera in the sheep were Escherichia (12.6%±3.0%), unclassified *Lachnospiraceae* (10.4%±2.7%), and *Ruminococcus* (6.3%±1.9%). Genera unclassified *Peptostreptococcaceae* (5.3%±0.8%) and unclassified RFP12 (5.2%±0.4%) were significantly more abundant in the small intestine than other gastrointestinal tract sections. In the large intestine, the three most dominant genera in the sheep were *Ruminococcus* (13.0%±2.9%), unclassified *Ruminococcaceae* (12.3%±2.6%), and *Prevotella* (12.1%±2.2%). Genus 5-7N15 (5.6%±0.9%) was significantly more abundant in the large intestine than other gastrointestinal tract sections.

Sheep gastrointestinal microbiota composition at the species level

Due to the V3-V6 region of 16S rRNA gene (about 600 bp) being amplied, limited species were identified in present study. A total of 22 species are shown in Table 3, *R. flavefaciens* (average relative abundance was 2.5% of total sequences), *B.*

Table 3. RDP classification of the sequence reads from different gastrointestinal tracts of sheep at the abundant species level (%)[1]

Species	Rumen	Reticulum	Omasum	Abomasum	Duodenum	Jejunum	Ileum	Cecum	Colon	Rectum
Ruminococcus flavefaciens	3.8±0.7	7.9±1.0	4.0±1.3	2.9±1.0	0.9±0.4	0.9±0.5	1.1±0.6	1.2±0.5	1.1±0.4	1.0±0.4
Butyrivibrio fibrisolvens	4.3±0.8	6.4±1.1	2.2±1.1	1.5±1.0	2.8±1.1	5.4±1.7	0.7±0.5	NA	0.1±0.1	NA
Selenomonas ruminantium	1.9±0.5	2.4±0.6	0.5±0.1	0.7±0.3	0.6±0.3	NA	NA	NA	NA	NA
Ruminococcus bromii	1.0±0.2	0.8±1.4	1.6±0.7	0.6±0.2	0.1±0.1	0.1±0.1	0.1±0.1	0.4±0.2	0.3±0.3	0.3±0.1
Bifidobacterium pseudolongum	NA	NA	NA	0.1±0.1	NA	1.3±0.6	0.2±0.2	0.3±0.1	0.6±0.3	0.3±0.2
Clostridium butyricum	NA	NA	NA	0.1±0.1	0.1±0.1	0.1±0.1	1.1±0.3	0.8±0.4	0.2±0.1	0.1±0.1
Fibrobacter succinogenes	0.3±0.1	0.1±0.1	1.0±0.6	0.4±0.2	NA	NA	NA	0.1±0.1	NA	0.1±0.1
Pseudobutyrivibrio ruminis	0.3±0.1	1.0±0.3	0.6±0.2	0.1±0.1	NA	NA	NA	NA	NA	NA
Prevotella ruminicola	0.2±0.1	NA	0.6±0.3	1.0±0.7	NA	0.1±0.1	NA	NA	NA	NA
Prevotella copri	0.2±0.1	NA	0.1±0.1	0.1±0.1	0.4±0.2	NA	0.4±0.2	NA	0.1±0.1	0.3±0.2
Ruminococcus calidus	0.6±0.2	0.3±0.2	0.2±0.1	0.2±0.1	0.1±0.1	NA	NA	NA	NA	NA
Pseudomonas fragi	0.1±0.1	0.1±0.1	NA	0.1±0.1	0.4±0.2	0.4±0.2	0.1±0.1	0.2±0.1	NA	NA
Desulfovibrio D168	0.1±0.1	0.7±0.4	NA	0.1±0.1	0.3±0.2	NA	NA	NA	NA	NA
Megasphaera elsdenii	NA	NA	0.5±0.3	0.3±0.2	NA	NA	NA	NA	NA	NA
Campylobacter fetus	NA	NA	NA	0.3±0.2	0.3±0.1	NA	NA	NA	0.1±0.1	0.1±0.1
Dialister invisus	0.2±0.1	0.5±0.3	NA	NA	NA	NA	NA	NA	NA	NA
Bacillus foraminis	NA	NA	NA	NA	NA	NA	0.5±0.3	NA	NA	0.2±0.1
Prevotella stercorea	NA	NA	NA	NA	0.1±0.1	NA	NA	0.5±0.3	NA	NA
Pseudomonas veronii	NA	NA	NA	0.1±0.1	0.5±0.2	NA	NA	NA	NA	NA
Clostridium perfringens	NA	NA	NA	NA	NA	0.2±0.1	0.3±0.2	NA	NA	NA
Sharpea azabuensis	0.1±0.1	NA	NA	0.2±0.1	NA	0.2±0.1	NA	NA	NA	NA
Sharpea p-3329-23G2	0.1±0.1	NA	NA	0.2±0.2	NA	0.2±0.2	NA	NA	NA	NA

[1] Data are expressed as average ± standard deviation (n = 5 sheep).
NA, not found in this gastrointestinal tract section.

fibrisolvens (average relative abundance was 2.3% of total sequences), and *S. ruminantium* (average relative abundance was 0.6% of total sequences) were three most dominant species in the sheep gastrointestinal tract. The species *R. flavefaciens* (2.9% to 7.9%), *S. ruminantium* (0.5% to 2.4%), *R. bromii* (0.6% to 1.6%), *F. succinogenes* (0.1% to 1.0%), *P. ruminis* (0.1% to 1.0%), *P. ruminicola* (0.0% to 1.0%), *R. calidus* (0.2% to 0.6%), *Desulfovibrio* D168 (0.0% to 0.7%), *M. elsdenii* (0.0% to 0.5%), and *D. invisus* (0.0% to 0.5%) were more abundant in the stomach than the other gastrointestinal sections. Meanwhile, the species *B. pseudolongum* (0.0% to 1.3%), *C. butyricum* (0.1% to 1.1%), *P. copri* (0.0% to 0.4%), *P. fragi* (0.0% to 0.4%), *B. foraminis* (0.0% to 0.5%), and *P. stercorea* (0.0% to 0.5%) were more abundant in the small intestine and large intestine than the stomach. It is worthy that *P. veronii* (0.5%±0.2%) has high abundance in the duodenum of sheep, and *C. perfringens* (0.2%±0.1% and 0.3%±0.2%) has high abundance in the jejunum and ileum of sheep.

Principal coordinates analysis

The diversities between samples from different gastrointestinal tract sections (i.e. beta diversity) were compared, and principal coordinates analysis (PCoA) of unweighted UniFrac distances (an evaluation of community membership that does not consider abundances) was carried out. The PCoA plot, shown in Figure 6, indicated that the microbial communities of gastrointestinal tract in sheep were separated into three groups according to similarity of community composition which include the stomach (rumen, reticulum, omasum, and abomasum), small intestine (duodenum, jejunum, and ileum), and large intestine (cecum, colon, and rectum).

DISCUSSION

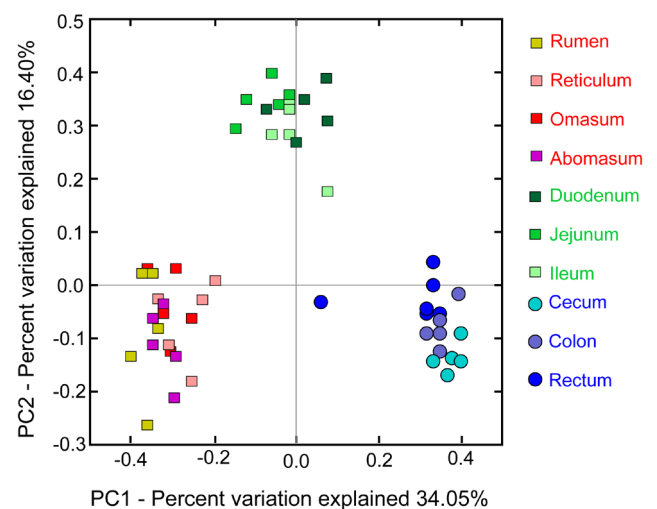In our study, higher bacterial richness and diversity were ob-

Figure 6. Beta diversity estimates for the gastrointestinal microbiota of sheep. Using the unweighted UniFrac distance metric to measure phylogenetic distances between samples from different gastrointestinal sections.

served in the stomach and large intestine than in the small intestine, which was also previously observed in the dairy cattle and the Brazilian Nelore breed of cattle [4,5]. The distal gut environment of the ruminant is more complex than the proximal gut. Most of the dietary constituents are digested in the stomach. The small intestine is much longer than the other sections, and small intestine has high concentration of bile salt and digestive enzymes so that the bacteria are difficult to grow. The partial microbial digestion that also takes place in the large intestine of ruminants could also explains the detection of high richness and diversity in the large intestine. Different oxygen tension and physiological roles in different gastrointestinal tract sections may lead to this result. The PCoA plot in the present study is in agreement with on the Nelore cattle [4] and shows that the samples from adjacent gastrointestinal tract segment (stomach, small intestine, and large intestine) harbor microbial communities more similar than from other segments.

The phylum level, the structure of bacterial community in the gastrointestinal tract was similar to that found in Chinese Mongolian sheep [7] and Holstein dairy cattle [5]. However, with regard to the abundance of these predominant phyla, there were some differences found among these studies. These differences might be due to variations in species, diets, living environment, and analysis methods. In general, the microbiota of the stomach, ileum, and large intestine exhibited greater abundances of *Firmicutes* and *Bacteroidetes*, while the duodenum and jejunum showed higher relative abundances of *Firmicutes* and *Proteobacteria*. A recent study on the dairy cattle Illumina-based method found that among the gastrointestinal tract, the high abundance of phylum *Proteobacteria* was found in the small intestine [5]. *Firmicutes* in the ruminants is known to degrade the fibre and cellulose [8]. *Bacteroidetes* is known to aid the digestion of complex carbohydrates, and also ferment organic matter [9]. The cause of the high abundance of *Proteobacteria* in the small intestine is not entirely clear, and future studies are needed to clarify this issue. To our knowledge, TM7 (0.0% to 4.6%) and SR1 (0.0% to 0.1%) phyla were found in the sheep gastrointestinal tract for the first time. TM7, which has not yet been cultivated in a laboratory, and is also reported in the feces of grass hay-fed horses [10]. SR1 has been reported in the rumen of cow using pyrosequencing method [11], however the role of this bacteria in the sheep gastrointestinal tract remains unknown.

Fifteen genera were considered as the core genera because these were existed in the all gastrointestinal tract sections. Of these 15 genera, several of unclassified genera, including S24-7, CF231, and RFP12, were detected. Therefore, this study provides detailed information regarding to both known bacteria and unclassified bacteria. A study on the rumen bacterial diversity of 80 to 110-day-old goats using 16S rRNA sequencing indicated that as the age of the goat increases, S24-7 showed

an increasing trend [12]. Therefore, that the 10-months sheep in the present study had a high abundance of S24-7 in the stomach is reasonable. CF231, family *Paraprevotellaceae*, was the third top genus of the phylum *Bacteroidetes* in the rumen of steers [13]. However, in our present study, CF231 was high abundance in the ileum and large intestine, and the stomach was of low abundance. Different species could produce this discrepancy. The function of CF231 will need to be studied in the future. Family in the RFP12, order of *Verrucomicrobia* was the second most abundant genus among all horse feces [14]. However, in our present study, RFP12 was of high abundance in the jejunum, which was never reported before for ruminants. Therefore, the function of RFP12 will need to be characterized in the future. Of these 15 genera, a total of 11 genera, including *Prevotella*, unclassified *Lachnospiraceae*, *Ruminococcus*, unclassified *Ruminococcaceae*, CF231, unclassified RFP 12, unclassified *Clostridiaceae*, *Clostridium*, *Oscillospira*, unclassified *Veillonellaceae*, and *Coprococcus*, have been reported to dominate the yaks rumen using Illumina MiSeq sequencing method (Majorbio Bio-Pharm Technology Co., Ltd, Shanghai, China) [15]. *Succinivibrio* was previously detected in the rumen of cow using pyroseuquencing method [11]. *Anaerovibrio* was seen throughout the gastrointestinal tract of 3-week-old preweaned calves using pyrosequencing method [16]. Unclassified *Bifidobacteriaceae* was previously found in the ileum of goats using pyrosequencing method [17]. Therefore, some core genera could be shared in the most of the ruminants.

A primary finding of our study is that there are differences in microbiota composition between the segments of the gastrointestinal tract. This is in agreement with previous studies that have reported significant changes in the microbiota within the gastrointestinal tract as digesta passes from one segment to another [4]. *Prevotella* in the stomach or the large intestine was identified the most abundant and important genus, which was in agreement with the sequencing of rumen or cecum samples of ruminants [18,19]. Prevotella, which has a unique mucin glycoprotein degradation capability, might exploit this, resulting in the host's increased growth and survival, and also can degrade the hemicelluloses and xylans, which promotes the digestion of the feed [20]. *Butyrivibrio* (average relative abundance is 9.5% of total sequences) dominated in the stomach of sheep in the present study. The abundance of *Butyrivibrio* was in line with the previous report on the goat rumen microbiota, using cloned 16S rRNA gene analysis, where it accounted about 10.0% [21] and dairy cattle four stomach microbiota, using Illumina MiSeq (Department of Computer Science, North Arizona University, Flagstaff, AZ, USA) platform analysis, where *Butyrivibrio* accounted for about 5.1% [5]. Therefore, even if the different methods and different ruminant breeds were used, *Butyrivibrio* dominated the stomach of ruminants. Dunne et al. demonstrated that *Butyrivibrio* could modulate the secretion of hemicelluloses-degrading enzymes [22], which

supported the notion that this organism made an important contribution to polysaccharide degradation in the rumen. In addition, *Butyrivibrio* is an important butyrate producer, and promotes the stomach epithelium proliferation. *Ruminococcus* dominated in the small intestine and large intestine, and it account for 6.3%±1.9% and 13.0%±2.9% of total sequences, respectively. *Ruminocuccus* is also found in the 3-week old preweaned calves, and it dominated in the jejunum, however it is less abundant genus in the large intestine [16]. This disagreement may be due to the variance of the host age, diet and breed. *Ruminococcus* was found to produce carbohydrate active enzymes, and degraded the carbohydrate from the diet [23]. It is noteworthy that *Escherichia* (12.6%±3.0%) accounted a large amount in the small intestine, which is unexpected before. A review study [24] indicated *Proteobacteria* represented between 5% and 40% of the bacteria detected in the ileum of pigs, and it is well known that *Escherichia* was the largest genus in the *Proteobacteria*. Therefore, whether pigs or ruminants, Escherichia may have the same physiological roles in the small intestine. A high abundance of unclassified *Lachnospiraceae* was observed in the stomach and small intestine in the present study, which agrees with a review on the status of the phylogenetic diversity census of ruminal microbiomes that summarized *Lachnospiraceae* was one of the largest taxons [1]. A study on the ileum of goats using pyrosequencing also indicated that unclassified *Lachnospiraceae* dominated in the ileum [17]. *Lachnospiraceae* is known to be a beneficial bacterium in the human intestine, because of the role it plays in the fermentation of carbohydrates to short chain fatty acids [25]. Maybe *Lachnospiraceae* has the similar role in the gastrointestinal tracts of sheep. A high abundance of unclassified *Ruminococcaceae* (12.3%±2.6%) was observed in the large intestine from our study, which is in accordance with the report on adult goat cecal luminal content microbiota [26]. Most of the *Ruminococcaceae* also act as major degraders of resistant polysaccharides, such as starch and cellulose, and contributes a range of degradative enzyme systems that allow the host to break up plant cell walls [27]. In our study, the abundant genera in the large intestine does not in agree with the cattle fecal microbiota identified using pyrosequencing [28]. They suggested that *Clostridium* (19.7%) and *Bacteroides* (10.4%) predominated in the feces of cattle. However, in the present study, *Clostridium* and *Bacteroides* only accounted 0.6% and 1.3% of total sequences in the rectum, respectively. The large variation in the abundant genera could be attributed to the differences in the breed, and differences between feces and rectal samples. Therefore, the feces should not be used instead of rectal samples.

Perhaps, the most salient finding in the present study was the characterization of the microbial composition in the sheep gastrointestinal tract at the species level. As Illumina sequencing, the most popular method nowadays, would not be able to identify to the species owing to short sequencing length. However, pyrosequening has a long sequencing length, so it overcomes this problem. *R. flavefaciens*, *R. bromii*, and *F. succinogenes* are the most dominant cellulolytic bacteria in the rumen and feces of ruminant [1,7]. Our results demonstrated that *R. flavefaciens* was the most abundant species in the gastrointestinal tract. This result supports the previous finding that *R. flavefaciens* dominated in the rumen of goats [21]. However, the abundances of *R. bromii* and *F. succinogenes* were different in different ruminant species. The three dominant cellulolytic bacteria are consistently higher in the stomach than in the large intestine and small intestine. The stomach provides low pH and cellulosic materials, therefore is convenient for the cellulolytic bacteria growth. Another species, *B. fibrisolvens*, dominated in the stomach and small intestine of sheep, which is a hemicellulolytic bacterium commonly isolated from the rumen of cattle, sheep, and deer [29]. *B. pseudolongum* dominated in the small intestine and large intestine, which has the activity of degrading the pectin and glucose in the rabbit cecum [30]. *P. ruminicola* is also reported in the rumen of cattle, and it is associated with ruminal carbohydrate and protein fermentation [2].

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

ACKNOWLEDGMENTS

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