The response of ruminal fermentation, epithelium-associated microbiota, and epithelial barrier function to severe feed restriction in pregnant ewes

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ABSTRACT: The objective of this study was to evaluate the changes in the ruminal fermentation, epithelium-associated microbiota, and ruminal epithelial barrier function in response to severe feed restriction (SFR) in pregnant ewes. Sixteen pregnant ewes (108 d of gestation) were randomly blocked and assigned to 1 of 2 treatments: control (CON, $n = 8$) and SFR ($n = 8$). Ewes were fed a common diet with a 60:40 forage to concentrate ratio for 7-d baseline period followed by a SFR challenge period. Ewes on the SFR treatment were restricted to 30% of the base for 15 d. At the end of the experimental period, all animals were slaughtered and then ruminal contents and ruminal epithelial tissue were collected. Results showed that ruminal pH was greater in SFR group ($P = 0.040$) compared with CON group, while SFR decreased ($P < 0.05$) the concentrations of ruminal acetate, propionate, butyrate, and total volatile fatty acid. A plot of principal coordinate analysis and analysis of molecular variance revealed that the composition of ruminal epithelial bacterial communities in the CON group was distinct from that of the ruminal epithelial microbiome in the SFR animals. At the genus level, SFR increased the abundance of unclassified Neisseriaceae,

Comamonas, and *Papillibacter*, and decreased the proportion of *Howardella*, *Desulfobulbus*, and *Suttonella* ($P < 0.05$) compared with CON group. The metagenome of ruminal epithelium-associated microbiota predicted by PICRUSt revealed that the SFR significantly affected 14 metabolic pathways, and 9 were significantly enriched in the SFR group. In particular, SFR markedly increased relative abundances of dominant gene families involved in amino acid metabolism ($P = 0.003$), cellular processes and signaling ($P = 0.021$), and lipid metabolism ($P = 0.001$). The real-time PCR results showed SFR decreased the mRNA expression of *IL-10* ($P = 0.003$) and upregulated the mRNA expression of *IL-6* ($P = 0.003$) and *TLR4* ($P = 0.021$). The mRNA expression of *Claudin-1* ($P = 0.001$) and *ZO-1* ($P = 0.009$) were lower in the SFR group compared with the CON group. Generally, our data suggest that SFR decreased most ruminal fermentation parameters, altered the composition of rumen epithelium-associated microbiota, and compromised the barrier function of rumen epithelium. These findings are of great importance for understanding the alteration in the rumen function following SFR in pregnant ewes.

Key words: ewe, microbiota, rumen epithelium, ruminal fermentation, severe feed restriction

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INTRODUCTION

There are several factors (pregnancy, lactation, and severe stress) that result in the decrease of feed intake and negative effects on production performance in sheep. In pregnant ewes,

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substantial requirements of nutrition are necessary for the fetal growth and mammary gland development. However, during pregnancy, the voluntary feed intake declines because rumen volume decreases due to the fetuses growth and fat deposits in the abdominal cavity (Forbes, 1977; Forbes, 1980). During late pregnancy, a severe decrease in feed intake in ewes carrying multiple fetuses will lead to negative energy balance and result in metabolic disorder such as pregnancy toxemia. Feed restriction (FR) studies mainly focused on the fetus and lamb growth (Heasman et al., 1999; Ford et al., 2007; Tygesen et al., 2007), maternal homeorhetic adaptation (Tygesen et al., 2008), and metabolic profile (Cal-Pereyra et al., 2015). However, little information is available on the changes of the rumen physiology of pregnant ewes during FR, in spite of the fact that rumen is one of the most responsive tissues to dietary restriction (Britton and Krehbiel, 1993). More than 75% of the metabolizable energy supply is provided through VFAs which generated from the microbial fermentation. Although they only comprise 1% to 2% of the total rumen microorganisms, the epithelium-associated microbiota represents a direct link with the host, relates to urea cycle, maintains the structure and integrity of epithelium, and secures rumen environment (Cheng et al., 1979; Cheng and Costerton, 1980). It has been reported that FR decreases the gut barrier function and stimulates bacterial translocation in human (Genton et al., 2015). But less is known about the effect of FR on the epithelium-associated microbiota and epithelial barrier function.

The aim of this study was to examine the effect of severe feed restriction (SFR) on ruminal fermentation, the composition of the epithelium-associated microbiota and epithelium barrier function in pregnant ewes.

MATERIALS AND METHODS

The experimental design and procedures for this study were approved by the Animal Care and Use Committee of Nanjing Agricultural University, following the requirements of the Regulations for the Administration of Affairs Concerning Experimental Animals (The State Science and Technology Commission of P. R. China, 1988).

Animal, Diets, and Experimental Design

Sixteen ewes aged 2 to 3 yr (body weight: 61.5 ± 1.2 kg, 108 d of gestation) were fed ad libitum for 7 d to determine the feed intake baseline.

During the subsequent 15-d treatment period, sheep were randomly allocated to either ad libitum access to feed in the control group (CON; $n = 8$) or feed restriction to 30% of the feed intake baseline (SFR; $n = 8$) in individual pens.

The diet consisting of 0.4 kg concentrate, 0.6 kg oat hay, and 2.2 kg rye silage was mixed and offered twice a day at 0900 and 1500 h daily. Animals had free access to water. The nutrient compositions and nutrition level of the diets are shown in Table 1. The body weight of pregnant ewes was measured before the morning feeding at the beginning and the end of the experiment. The fetuses were removed from the ewes and fetal body weight was recorded.

Rumen pH and Fermentation Parameters

At the end of the experiment, sheep were slaughtered after 4-h interval of morning feeding. Immediately after slaughter, a representative sample of rumen digesta (at least 200 mL) was used to determine the pH value and then strained through 4 layers of cheesecloth. A sample of the ruminal fluid was preserved in 25% (wt/vol) metaphosphoric acid (5 mL ruminal fluid:1 mL metaphosphoric acid) and stored at -20°C until later analysis for VFA concentration. The rumen epithelial tissues were collected as described by Liu et al. (2013). Briefly, within 5 min after slaughter, a segment of the rumen wall from the ventral sac was collected, and the ruminal epithelium was separated from the muscular and serosal layers by blunt dissection,

Table 1. Ingredient and nutrition composition of the experimental diets (dry matter basis)

Items	Diet
Ingredient composition, % DM	
Rye silage	42.31
Oat hay	34.62
Corn meal	12.00
Soybean	5.77
Barley	4.15
Mineral and vitamin supplement ¹	1.15
Nutrition composition	
ME, MJ/kg DM	11.64
CP, % of DM	14.71
Crude fat, % of DM	2.95
NDF, % of DM	48.32
ADF, % of DM	29.09
Ash, % of DM	7.35

¹Contained 9% to 13% Ca, 14% to 18% sodium chloride, 6.6% P, 537 mg/kg Mn, 7.2 mg/kg Co, 540 mg/kg Cu, 2,422 mg/kg Zn, 32 mg/kg I, 353 mg/kg nicotinamide, 89 g/kg sodium bicarbonate, 216 kIU/kg vitamin A, 54 kIU/kg vitamin D₃, 1,080 IU/kg vitamin E.

and immediately washed 3 times in ice-cold PBS. The samples were divided into 2 portions. The first portion of the tissue sample was cut into pieces of 0.5×0.5 cm and immediately frozen in liquid nitrogen for RNA extraction. The second tissue sample portion was cut to approximately 2×2 cm pieces, scraped for microbiota which attached to the rumen epithelium using a germ-free glass slide, immediately transferred into liquid nitrogen, and then stored at -80°C until microbial DNA extraction.

Physiological Parameter Measurements

Rumen pH was measured by a portable pH meter (PB-10; Sartorius, Gottingen, Germany). Volatile fatty acids were measured by gas chromatography (GC-14B; Shimadzu, Japan; capillary column film thickness: $30\text{ m} \times 0.32\text{ mm} \times 0.25\text{ }\mu\text{m}$; column temperature = 130°C ; injector temperature = 180°C ; detector temperature = 180°C) (Qin, 1982). Crotonic acid was used as the internal standard.

Microbial DNA Isolation

A total of 250 mg underlying rumen epithelial tissue in each sample was used for DNA extraction. The DNA was isolated and purified by using a QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany). The DNA samples were quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Madison, WI). The absorption ratio (260/280 nm) of all samples was greater than 1.80, indicating high DNA purity. The extracted DNA samples were stored at -80°C until further processing.

PCR Amplification, DNA Illumina MiSeq Sequencing, and Data Analysis

The V3–V4 regions of bacterial 16S rRNA genes were amplified using primers 338F (5'-barcode-ACTCCTRCGGGAGGCAGCAG-3') and 806R (5'-GGACTACCVGGGTAT CTAAT-3') as described by Mao et al. (2015). The cycling parameters included an original denaturation at 95°C (2 min), performed with 25 cycles at 95°C (30 s), and then annealed at 55°C (30 s), 72°C (30 s), and finally extended at 72°C (5 min). Amplicons which separated from 2% agarose gels were purified by the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA) following the instruction. Meanwhile, the quality of PCR product was tested by QuantiFluor-ST (Promega, Durham,

NC). PCR production was applied to construct a sequencing library with an Illumina TruSeq DNA Sample Preparation Kit (Illumina, San Diego, CA). Illumina TruSeq PE Cluster and Sequencing by Synthesis (SBS) Kits were used to perform cluster generation, template hybridization, isothermal amplification, linearization, blocking and denaturation, and hybridization of the sequencing primers. Paired-end sequencing 2×250 bp was performed to sequence all libraries on an Illumina MiSeq platform according to standard protocols.

For data analysis, raw Illumina FASTQ (Illumina, San Diego, CA) files were demultiplexed, quality filtered, and analyzed by the Quantitative Insights into Microbial Ecology (QIIME). With the criteria, the sequences, more than 200 bp in length, lack of ambiguous base “N,” more than 25 in average base quality score, were labeled as high quality ones, then were continued to analyze. Operational taxonomic units (OTUs) were clustered with a 97% similarity cutoff using UPARSE, and chimeric sequences were identified using UCHIME (Edgar, 2010). The most abundant sequences within each OTU aligned with the SILVA database (version 100; 2009) using PyNAST (Caporaso et al., 2010), and were assigned to representative sequences using the QIIME's Ribosomal Database Project (RDP) classifier. Rarefaction curves were applied to measure the depth of the sequencing. The α -diversity, including ACE, Chao, Shannon and Simpson index, was used to determine the richness and diversity of bacterial community. The β -diversity calculations were performed using QIIME. Principal coordinate analysis (PCoA) was used to compare groups of samples on the basis of unweighted UniFrac distance metrics, and to evaluate significant differences. An unweighted distance-based analysis of molecular variance (AMOVA) was conducted to assess the significant differences among the samples using the MOTHUR program (version 1.29) (Schloss et al., 2009). The raw sequence files are available from National Center for Biotechnology Information, with Sequence Read Archive under the accession number SRP 127672.

Functional Gene Prediction

In the present study, phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) was used to infer the metagenomic contribution of the ruminal epithelium-associated communities observed based on the Kyoto Encyclopedia of Genes and Genomes (KEGG). PICRUSt is a bioinformatics tool which uses

marker 16S rRNA genes to predict the metagenome gene's functional content (Langille et al., 2013). In the current study, these predictions are precalculated for genes in databases including the KEGG. The difference in predicted molecular functions of bacterial communities between the CON and SFR groups was performed by a principal component analysis (PCA) using the SIMCA-P software (SIMC 11.5) package (Umetrics, Umea, Sweden).

RNA Extraction and Real-Time PCR

Total RNA was extracted from rumen epithelium using Trizol (Takara Bio, Otsu, Japan). Briefly, 100 mg of rumen epithelium was placed inside liquid nitrogen to grind with 1 mL Trizol, followed by chloroform extraction, RNA precipitation, and ethanol rinse (Chomczynski and Sacchi, 1987). The RNA concentration was then quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Madison, WI). The absorption ratio (260/280 nm) of all samples was between 1.80 and 2.10, indicating high RNA purity. The concentration of RNA was adjusted to 500 ng/ μ L by using DNase/RNase-Free water and stored at -80°C . Total RNA (1 μ g) was carefully reverse-transcribed into cDNA using a PrimeScript RT reagent kit with gDNA Eraser (Takara Bio, Otsu, Japan) according to the manufacturer's instructions. cDNA was synthesized mixing 5* gDNA Eraser Buffer, gDNA Eraser Buffer, RNA, and RNase-Free dH_2O . The reaction was performed at 42°C for 2 min and kept at 4°C . Then, 5* Prime Script RT Enzyme Buffer, Prime Script RT Enzyme Mix I, and RNase-Free dH_2O were added using temperature program: 37°C for 15 min and 85°C for 5 s.

Real-Time Quantitative PCR

Genes related to tight junction were evaluated using real-time quantitative PCR. Real-time PCR of candidate genes and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) were carried out using the QuantStudio 5 Real-Time PCR Instrument (Applied Biosystems, Foster City, CA), with fluorescence detection of SYBR green dye. The amplification conditions were as follows: 95°C for 30 s, 40 cycles of 95°C for 5 s and 60°C for 30 s, followed by a dissociation curve step (95°C for 15 s, 60°C for 1 min, and 95°C for 15 s). The primers and amplicon sizes of cytokine, *TLR*, tight junction genes, and *GAPDH* were listed in Supplementary Table S1. All measurements were performed in triplicate. Of the candidate housekeeping genes

(*GAPDH* and β -actin), *GAPDH* was finally selected as a housekeeping gene for the accurate normalization in PCR, and all the results of the target genes expressions were calculated by the $2^{-\Delta\Delta\text{CT}}$ method.

Statistical Analysis

The ruminal fermentation parameters, the body weight gain, the fetuses weight, and the mRNA expression of cytokine, *TLR* genes, and tight junction proteins were analyzed using the independent-samples *t*-test in SPSS software packages (SPSS version 19 for Windows, SPSS Inc., Chicago, IL). The nonparametric Kruskal–Wallis test was applied to determine the relative abundance of microbial phyla, genera, OTUs, and KEGG pathways. Significance was declared at $P < 0.05$ and trends at $P < 0.10$.

RESULTS

Ewes' Weight Gain and Fetal Weight

At the start of the experimental period, no significant difference ($P = 0.240$) was observed in the average live weight between the 2 groups. At the end of the experiment, the live weight in the SFR group was lower ($P = 0.022$) compared with the CON group. The body weight gain was significantly greater ($P = 0.017$) in the CON group than that in SFR group. The body weight of fetuses from the CON group is greater ($P = 0.016$) compared with the SFR group (Fig. 1).

Figure 1. Effects of severe feed restriction (SFR) on the ewe's live weight. Ewes received the normal feeding in the CON group or SFR for 15-d treatment period. The value is depicted as means $\pm$ SEM ($n = 8$). Initial weight: the live weight of ewes at the start of the experiment of the adaptation period; final weight: the live weight throughout experimental period in the late gestation period; weight gain = final weight – initial weight; average fetal weight: the average weight of fetus after move from the amnion.

Rumen Fermentation

As shown in Table 2, SFR decreased the concentrations of acetate ($P < 0.001$), propionate ($P < 0.001$), butyrate ($P = 0.025$), valerate ($P = 0.039$), and total VFA ($P < 0.001$) compared with the CON group, whereas SFR increased ruminal pH ($P = 0.040$). There was no significant difference in the concentrations of isobutyrate ($P = 0.093$) and isovalerate ($P = 0.112$) between the 2 groups. The molar proportion of propionate was greater ($P = 0.022$) in the CON group than that in the SFR group, SFR did not affect the molar proportion of acetate ($P = 0.972$), butyrate ($P = 0.414$), and the ratio of acetate to propionate tended to increase ($P = 0.059$).

Sequencing and Diversity of Ruminal Epithelial Bacterial Communities

In the present study, a total of 569,320 valid reads were obtained, with an average of 35,582 sequences for each sample. Rarefaction curves were constructed to quantify the OTU coverage of sampling shown in Fig. 2. All the curves approached a plateau, indicating that the sampling depth was sufficient.

As shown in Table 3, alpha diversity results showed that SFR did not affect OTU numbers ($P = 0.473$), ACE ($P = 0.780$), Chao ($P = 0.959$), Shannon ($P = 0.798$), and Simpson index ($P = 0.969$). The PCoA profile based on the unweighted UniFrac metric was used to characterize β -diversity of the bacterial community across all samples (Fig. 3). The PCoA profile showed that the composition of the rumen epithelium-associated microbial community of the SFR and the CON groups were distinct (axis 1 + axis 2 = 66.7%).

AMOVA also showed that there was a significant difference in the composition of rumen epithelium-associated microbiota between the 2 groups ($P < 0.001$). The Venn diagram showed that with a share of 1,577 OTUs in both group, the CON group had 117 particular OTUs accounting for 6.9% of the CON group, while the SFR group had 106 OTUs, equal to 6.3% of the total OTUs (Fig. 4).

Taxonomic Composition of Ruminal Epithelial Bacterial Populations

At the phylum level, 20 phyla were found across all samples (Fig. 5A), and Firmicutes, Bacteroidetes, and Proteobacteria were the most dominant phyla, representing an average percentage of 41.86%, 32.01%, and 15.54%, respectively. The abundances of 3 phyla including Spirochaetae (CON vs. SFR, 3.98% vs. 2.70%), Fibrobacteres (CON vs. SFR, 2.52% vs. 2.80%), and Actinobacteria (CON vs. SFR, 1.19% vs. 1.35%) were greater than 1%. The abundances of other

Figure 2. Rarefaction curves based on operational taxonomic units (OTUs) at 3% divergence for each rumen epithelium. The CON group: CON-1, CON-2, CON-3, CON-4, CON-5, CON-6, CON-7, CON-8. The SFR group: SFR-1, SFR-2, SFR-3, SFR-4, SFR-5, SFR-6, SFR-7, SFR-8.

Table 2. Effect of severe feed restriction (SFR) on rumen pH and ruminal fermentation in pregnant ewes

Item	CON	SFR	SEM ¹	P-value
pH	5.85	6.10	0.062	0.040
Acetate, mM	70.83	47.35	3.384	<0.001
Propionate, mM	18.84	11.14	1.206	<0.001
Isobutyrate, mM	0.53	0.74	0.062	0.093
Butyrate, mM	11.16	8.20	0.685	0.025
Isovalerate, mM	0.79	1.09	0.091	0.112
Valerate, mM	1.11	0.79	0.079	0.039
Total volatile fatty acid, mM	103.27	69.30	5.094	<0.001
Acetate, mol %	68.68	68.63	0.631	0.972
Propionate, mol %	18.17	16.03	0.47	0.022
Butyrate, mol %	10.8	11.64	0.48	0.414
Acetate:propionate	3.83	4.30	0.125	0.059

¹SEM = standard error of the mean.

Table 3. Effects of severe feed restriction (SFR) on the alpha diversity of rumen epithelium-associated microbiota at the 3% dissimilarity level

Item	CON	SFR	SEM ¹	P-value
OTU number ²	1,138	1,090	32	0.473
ACE ³	1,310	1,295	25	0.780
Chao	1,314	1,311	29	0.959
Shannon	5.18	5.12	0.111	0.798
Simpson index	0.02	0.02	0.003	0.969

¹SEM = standard error of the mean.

²OTU = operational taxonomic unit.

³ACE = abundance-based coverage estimator.

Figure 3. Principal coordinate analysis (PCoA) of rumen epithelium-associated bacterial communities. The percentage of variation explained by PC1 (first principal component) and PC2 (second principal component) was indicated in the axis. CON = the control samples ($n = 8$); SFR = the severe feed restriction samples ($n = 8$).

14 phyla, including Candidate_division_TM7, BD1-5, Candidate_division_SR1, Tenericutes, Cyanobacteria, Synergistetes, Elusimicrobia, Lentisphaerae, unclassified Bacteria, Chloroflexi, SHA-109, Planctomycetes, Fusobacteria, and Verrucomicrobia were less than 1% of the sample (Supplementary Table S2). The abundances of Elusimicrobia ($P = 0.036$) and Lentisphaerae ($P = 0.012$) were greater in the SFR group than the CON group.

At the genus level, 172 genera were detected across all samples, 24 of which exhibited a great abundance ($>1\%$ of total sequences) shown in Fig. 5B. Results showed that SFR caused a significant increase in the relative abundance of unclassified Neisseriaceae ($P = 0.046$), *Comamonas* ($P = 0.002$), and *Papillibacter* ($P = 0.005$), whereas the abundance of *Howardella* ($P = 0.027$), *Desulfobulbus*

Figure 4. Venn diagrams displaying the distribution of operational taxonomic units (OTUs) between the control samples (CON, $n = 8$) and severe feed restriction group (SFR, $n = 8$) in epithelium-associated microbiota.

($P = 0.036$), and *Suttonella* ($P = 0.036$) showed a corresponding decrease.

At the OTU level, there were 25 dominant OTUs with a relative abundance $>1\%$ in at least 1 group, as listed in Table 4. And 3 most abundant OTUs in SFR group were OTU6 (5.41% of the total sequence, belonging to taxon unclassified Neisseriaceae and phylum Proteobacteria), OTU1 (4.97%, belonging to genus *Campylobacter* and phylum Proteobacteria), and OTU7 (3.32%, belonging to genus *Comamonas* and phylum Proteobacteria). Compared with the CON group, a significant increase in abundance of OTU37 (taxon *RC9_gut_group* and phylum Bacteroidetes, $P = 0.018$), OTU36 (genus *Incertae_Sedis* and phylum Firmicutes, $P = 0.009$), OTU938 (genus *Butyrivibrio* and phylum Firmicutes, $P = 0.002$), OTU6 (taxon unclassified Neisseriaceae and phylum Proteobacteria, $P = 0.046$), and OTU7 (genus *Comamonas* and phylum Proteobacteria, $P = 0.002$) was observed in SFR group. Conversely, the ruminal epithelium microbiota of the SFR group presented a significantly lower abundance of several OTUs than the CON group, including OTU30 (genus *Incertae_Sedis* and phylum Firmicutes, $P = 0.046$), OTU10 (genus *Howardella* and phylum Firmicutes, $P = 0.027$), OTU11 (genus *Butyrivibrio* and phylum Firmicutes, $P = 0.003$), OTU23 (genus *Suttonella* and phylum Proteobacteria, $P < 0.001$), and OTU46 (genus *Desulfobulbus* and phylum Proteobacteria, $P = 0.046$).

Predicted Metabolic Functions of Rumen Epithelial Bacteria

To provide an in-depth analysis of the molecular functions of epithelium microbiota, PICRUSt

Figure 5. Distributions of microbiota at phyla and genus level. (A). Relative abundances of phyla levels are depicted as mean values for the CON and severe feed restriction (SFR) group. (B) Distributions of genera in rumen epithelium of pregnant sheep with SFR. Only 24 genera which exhibited a great abundance ($>1\%$) were presented). $*P < 0.05$.

was used to predict the metagenomic contribution of the communities observed based on KEGG. A total of 39 gene families were identified across the samples (Supplementary Table S3). The PCA based on the relative abundance values of the KEGG

pathways showed a clear distinction between the clustering of the CON group and that receiving SFR (Fig. 6A). Among the 39 gene families, the majority of the genes belonged to membrane transport (11.89% in the epithelium-associated

Table 4. Effects of severe feed restriction (SFR) on relative abundance of 25 dominant operational taxonomic units (OTUs; % of total sequences in at least 1 group) in rumen epithelium

Phylum	Genus	OTU ID ²	Abundance ¹ , %		SEM	P-value
			CON	SFR		
Bacteroidetes	Unclassified Prevotellaceae	OTU2	6.69	2.05	1.373	0.093
		OTU4	2.59	2.01	0.506	0.916
	<i>RC9_gut_group</i>	OTU31	1.13	0.33	0.217	0.115
		OTU37	0.80	2.10	0.306	0.018
	<i>Prevotella</i>	OTU5	2.84	1.83	0.535	0.248
Fibrobacteres	<i>Blvii28_wastewater-sludge_group</i>	OTU18	0.33	1.03	0.220	0.599
	<i>Fibrobacter</i>	OTU71	0.85	1.10	0.165	0.600
Firmicutes	Unclassified Ruminococcaceae	OTU13	2.49	2.70	0.229	0.401
	Unclassified Lachnospiraceae	OTU12	1.31	1.12	0.195	0.248
	Unclassified Christensenellaceae	OTU3	2.11	1.38	0.600	1.000
		OTU74	0.94	1.45	0.184	0.172
	<i>Incertae_Sedis</i>	OTU30	1.07	0.72	0.097	0.046
		OTU36	0.28	1.10	0.236	0.009
	<i>Howardella</i>	OTU10	2.96	0.96	0.464	0.027
	<i>Butyrivibrio</i>	OTU14	1.86	1.57	0.284	0.529
		OTU9	1.77	2.30	0.340	0.834
		OTU11	1.08	0.12	0.153	0.003
Proteobacteria	Unclassified Neisseriaceae	OTU938	0.12	1.00	0.282	0.002
		OTU6	1.79	5.41	0.953	0.046
	<i>Suttonella</i>	OTU23	1.13	<0.01	0.253	<0.001
	<i>Halomonas</i>	OTU8	0.89	1.43	0.167	0.207
	<i>Desulfobulbus</i>	OTU46	1.08	0.46	0.143	0.046
	<i>Comamonas</i>	OTU7	0.82	3.32	0.581	0.002
	<i>Campylobacter</i>	OTU1	3.68	4.97	0.906	0.753
Spirochaetae	<i>Treponema</i>	OTU33	1.17	0.43	0.292	0.093

¹Values are represented by means; *n* = 8.²ID = OTU identifier.

microbiota of the CON group and 11.56% in the SFR group, respectively), amino acid metabolism (9.75% in the CON group, 10.08% in the SFR group), carbohydrate metabolism (9.85% in the CON group, 9.61% in the SFR group), replication and repair (8.95% in the CON group, 8.83% in the SFR group), and energy metabolism (5.91% in the CON group, 5.87% in the SFR group). Of the 39 gene families, 14 were significantly affected by SFR (Fig. 6B). The SFR group had a greater relative abundance of genes related to amino acid metabolism ($P = 0.003$), cellular processes and signaling ($P = 0.021$), lipid metabolism ($P = 0.001$), xenobiotics biodegradation and metabolism ($P = 0.009$), metabolism of terpenoids and polyketides ($P = 0.002$), metabolism of other amino acids ($P = 0.002$), endocrine system ($P = 0.001$), transport and catabolism ($P = 0.016$), excretory system ($P = 0.006$), and a lower percentage of gene families associated with genetic information processing ($P = 0.046$), transcription ($P = 0.021$), metabolism ($P = 0.046$), enzyme families ($P = 0.046$), and environmental adaptation ($P = 0.003$).

The mRNA Expression of Cytokine and TLR Genes in Rumen Epithelium

As shown in Fig. 7, SFR decreased the mRNA expression of *IL-10* ($P = 0.003$), and upregulated the mRNA expression of *IL-6* ($P = 0.003$) and *TLR4* ($P = 0.021$), whereas there were no differences in *IL-2* ($P = 0.505$) and *TLR3* ($P = 0.867$).

The mRNA Expression of Tight Junction Proteins in Rumen Epithelium

As compared with the CON group (Fig. 8), SFR decreased the mRNA expression of *Claudin-1* ($P = 0.001$) and *ZO-1* ($P = 0.009$), while did not affect the expression of *Claudin-4* ($P = 0.595$) and *Occludin* ($P = 0.066$).

DISCUSSION

In the late gestation of pregnant ewes, rapid fetal growth may produce a conflict in the ewes which increased nutrition demand and decreased feed intake, resulting in a lower live weight (Jyothi

Figure 6. Functional diversity of the ruminal epithelial bacteria. (A) Principal component analysis (PCA) of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of ruminal epithelial bacteria in the CON and severe feed restriction (SFR) group. (B) Effect of SFR on the relative abundances of gene families of rumen epithelium-associated bacteria in pregnant ewes ($n = 8$). Only the significantly different genes ($P < 0.05$) are presented.

Figure 7. Changes in relative mRNA expression of cytokine and TLR genes of ruminal epithelium in sheep with normal feeding (CON) and severe feed restriction (SFR). The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; a housekeeping gene) mRNA level was used to normalize the relative amount of each studied mRNA, and the $2^{-\Delta\Delta CT}$ method was used to analyze the data. In the figure, the value of control group (CON, $n = 8$) and severe feed restriction group (SFR, $n = 8$) represent by mean $\pm$ SEM.

Figure 8. Changes in relative mRNA expression of genes related to the tight junction of ruminal epithelium in sheep with normal feeding (CON) and severe feed restriction (SFR). The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; a housekeeping gene) mRNA level was used to normalize the relative amount of each studied mRNA, and the $2^{-\Delta\Delta CT}$ method was used to analyze the data. In the figure, the value of control group (CON, $n = 8$) and severe feed restriction group (SFR, $n = 8$) represent by mean $\pm$ SEM. ZO-1 = zonula occludin-1.

et al., 2014; Abba et al., 2015). The undernourished sheep which were limited in maternal intake during pregnancy gradually consumed maternal body reserves (crude protein and fat), impaired nutrient supply, and also slowed fetal growth (Luther et al., 2007). In the current study, in alignment with findings in previous studies of rats (Passos et al., 2000), our data showed the SFR resulted in less weight compared with the CON group, indicating that the insufficient nutrient supply affected the weight gain of pregnant ewes and the fetus growth when the ewes encountered feed restriction.

Results from the current study confirmed that a decrease in ruminal VFA concentration was responsive to the SFR, consistent with the previous study (Zhang et al., 2013), which reported that imposing feed restriction to 75%, 50%, or 25% of ad libitum intake resulted in VFA concentrations that were, respectively, 71%, 55%, and 38% of that measured at the baseline value. In accordance with findings in limit-fed steers (Soto-Navarro et al., 2000), the current study also showed that SFR decreased the molar proportion of propionate, indicating that FR may alter the pattern of ruminal fermentation. In addition, our findings also showed that SFR increased the ruminal pH, which may be partly explained by the reduction in ruminal total volatile fatty acid concentration in the SFR group.

Changes in the Composition and Structure of the Rumen Epithelium-Associated Microbiota

As a fractional representative of rumen microbiota, rumen epithelium-associated microbiota has been reported to be distinctly diverse at the taxonomic identification and the function from rumen content-adherent microbiota (Cho et al., 2006). However, the study of ruminal epithelium-associated microbiota has not received much attention and research, in particular the effect of undernutrition on the composition and function of ruminal epithelium-associated microbiota. The present study provides the first characterization of the rumen epithelium-associated bacterial community in pregnant sheep under SFR. In the present study, there were no differences in indices including ACE, Chao, Shannon, and Simpson, indicating that SFR did not alter the α -diversity of ruminal epithelium-associated bacterial community. These results indicated that the bacterial community attached to the rumen wall of individual sheep was relatively stable in spite of a transition from a normal feeding condition to an SFR feeding condition. This stability may be due to the tissue-specific metabolic activities of

adherent populations and may reflect the fact that this population relies less on digesta for function. However, the present study detected a discrepancy in the taxonomic composition of rumen epithelial bacteria between the CON and SFR group by PCoA and AMOVA. In the current study, the alteration in the composition of epithelial microbiome may contribute to the reduction of fermentable substrate favoring the growth of bacteria, and the increased ruminal pH. Indeed, many previous studies revealed that the rumen microbial ecosystem was directly linked to the nutrition level and rumen pH (Belanche et al., 2012; Wang et al., 2016).

The experimental data describing the composition of rumen epithelial populations under conditions of SFR in detail are lacking. In the current study, at the phylum level, our data revealed that the composition of the ruminal epithelial bacterial community in sheep was similar to that found in the rumen epithelium from dairy cattle (Schären et al., 2017), heifers (Petri et al., 2013), and goats (Liu et al., 2015). The phyla Firmicutes, Bacteroidetes, and Proteobacteria were the most prevalent in rumen epithelium-associated microbiota of sheep, indicating that the members of these phyla play a key role in maintaining the rumen epithelial microbial community. At the genus level, the current study revealed that SFR highlighted the presence of a great proportion of unclassified Neisseriaceae, *Comamonas*, and *Papillibacter*, with decreased the abundance of *Howardella*, *Desulfobulbus*, and *Suttonella*. Of these changed genera, the great abundance of *Comamonas* in rumen epithelium was also found in the tissue-associated community of heifers (Petri et al., 2013). In this experiment, the reason for the increase in the abundance of genus *Comamonas* in rumen epithelium-associated community of SFR animals is unclear and still requires further investigation. *Desulfobulbus* was reported to be related to propionate production and sulfur reduction (Parkes and Calder, 1985). In the current study, a lower abundance of *Desulfobulbus* was in accordance with the decreased propionate proportion in rumen. A previous study has reported that the growth of *Howardella* spp. was stimulated by acetate (Cook et al., 2007); thus, a lower abundance of *Howardella* in the SFR group may be explained by the lower concentration of acetate. In the present study, SFR significantly decreased the abundance of *Howardella*, *Desulfobulbus*, and *Suttonella* and increased the abundance of unclassified Neisseriaceae, *Comamonas*, and *Papillibacter*, indicating that SFR may break homeostasis of rumen epithelium-associated microbial community.

The current study used PICRUSt to infer putative metagenomes from 16S rRNA gene profiles at KEGG level 2. The distinct difference of predicted functional pathways in PCA between the 2 groups indicated that alterations of bacterial communities were also likely to alter the functional capability of the rumen epithelium-associated bacterial community. Among the pathways, and in alignment with the previous report (Wang et al., 2017), the current study showed that the most abundant functional categories were those corresponding to the functions of membrane transport, amino acid metabolism, carbohydrate metabolism, replication and repair, and energy metabolism, which are all essential for bacteria survival (Langille et al., 2013). In particular, the relative abundances of the gene families related to amino acid and lipid metabolism, and cellular processes and signaling increased in the SFR group. In contrast, microbial genes related to genetic information processing, transcription, metabolism, and enzyme families were less abundant in the SFR group than the CON group. Of these altered gene families, a greater abundance of genes related to amino acid and lipid metabolism in the SFR group may be related to the decrease in the feed intake, as SFR provided a decreased supply of carbohydrates and that epithelial bacteria may derive energy from amino acid and lipid fermentation. In addition, the decrease in the abundance of genes related to genetic information processing, transcription, metabolism, and enzyme families in the SFR group may relate to the adaptation of ruminal epithelial microbiota to the decreased nutrient state caused by SFR.

The Effect of SFR on Inflammatory Response and Barrier Function of Rumen Epithelium

Interestingly, our data revealed that SFR decreased the mRNA expression of *IL-10* and upregulated the mRNA expression of *IL-6* and *TLR4*. Previous studies revealed that *IL-6* is a pro-inflammatory cytokine and *IL-10* is an anti-inflammatory cytokine (Ohzato et al., 1992; Moore et al., 2001), and *TLR4* is a signaling receptor in innate immunity that is a specific immunologic response to systemic bacterial infection and epithelial injury (Kawai and Akira, 2006). In this study, the changes in the expression of *IL-6*, *IL-10*, and *TLR4* suggest that SFR induced inflammation in ruminal epithelium. One possible explanation for this result is that SFR results in the increase in the abundance of some pathogenic and opportunistic pathogenic bacteria which could induce inflammation. Actually,

the present study revealed that SFR resulted in the enrichment of Neisseriaceae populations which are known to have deleterious effects leading to mucosal injury (Wong et al., 2015). Hence, this result revealed that the inflammatory response that occurs in the ruminal epithelium in SFR group may partly arise from these deleterious effects of pathogenic and opportunistic pathogenic bacteria attached to the rumen epithelium.

It was reported that FR drastically increased the passive movement of 3-O-methyl- α -D-glucose, a small hydrophilic molecule, across the ruminal epithelium, indicating a reduction in barrier function (Gäbel and Aschenbach, 2002). Zhang et al. (2013) reported that severe short-term FR restricted to 25% of voluntary intake compromised the barrier function. In the present study, our data showed that FR decreased the mRNA expression of *Claudin-1* and *ZO-1*, implying that SFR could potentially impair the tight junctions between cells in the rumen epithelium. These results also support the negative effects of SFR on barrier function of ruminal epithelium. There are 2 possible explanations for the impairment of tight junction observed in the SFR group. First, a possible explanation for the impairment of tight junction is that FR may result in lower rates of ruminal epithelial cell proliferation which negatively impacts ruminal epithelial integrity and structure (Steele et al., 2015). Second, previous studies have revealed that intestinal mucosal barrier damage in colitis may mostly result from the production of inflammatory cytokines in response to commensal bacteria (Lupp et al., 2007; Strober et al., 2007). As mentioned earlier, in the present study, our data revealed that SFR increased the mRNA expression of pro-inflammatory cytokine *IL-6* and decreased the mRNA expression of anti-inflammatory cytokine *IL-10*. In addition, our data revealed that the abundance of pathogenic and opportunistic pathogenic bacteria such as Neisseriaceae populations was higher in SFR group compared with the CON. Thus, these findings indicate that the impairment of tight junction in SFR group might be caused by the production of inflammatory cytokines in response to the increase in the percentage of these pathogenic bacteria attached to ruminal epithelium.

CONCLUSION

This is the first study to examine the effects of SFR on ruminal fermentation, composition and potential function of epithelium-associated bacterial community, and transcription abundance

of genes associated with the barrier functionality of ruminal epithelial tissue in pregnant ewes. The results of this study suggest that SFR resulted in a lower VFA concentration, especially the content of acetate, propionate, and butyrate. This study also demonstrated that SFR had a considerable effect on the composition and function of ruminal epithelium-associated microbial communities. Additionally, the regulation of genes involved in inflammatory response and ruminal barrier function indicates that SFR may compromise the barrier function of rumen epithelium. These findings provide previously unknown novel information about the bacterial community following a period of SFR, and enhance our understanding of the alteration in rumen fermentation and ruminal epithelial barrier function following SFR in pregnant ewes.

SUPPLEMENTARY DATA

Supplementary data are available at *Journal of Animal Science* online.

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Conflict of interest statement. None declared.

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