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Effects of Sampling Techniques and Sites on Rumen Microbiome and Fermentation Parameters in Hanwoo Steers

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We evaluated the influence of sampling technique (cannulation vs. stomach tube) and site (dorsal sac vs. ventral sac) on the rumen microbiome and fermentation parameters in Hanwoo steers. Rumen samples were collected from three cannulated Hanwoo steers via both a stomach tube and cannulation, and 16S rRNA gene amplicons were sequenced on the MiSeq platform to investigate the rumen microbiome composition among samples obtained via 1) the stomach tube, 2) dorsal sac via rumen cannulation, and 3) ventral sac via rumen cannulation. A total of 722,001 high-quality 16S rRNA gene sequences were obtained from the three groups and subjected to phylogenetic analysis. There was no significant difference in the composition of the major taxa or alpha diversity among the three groups ($p > 0.05$). Bacteroidetes and Firmicutes represented the first and second most dominant phyla, respectively, and their abundances did not differ among the three groups ($p > 0.05$). Beta diversity principal coordinate analysis also did not separate the rumen microbiome based on the three sample groups. Moreover, there was no effect of sampling site or method on fermentation parameters, including pH and volatile fatty acids ($p > 0.05$). Overall, this study demonstrates that the rumen microbiome and fermentation parameters are not affected by different sampling techniques and sampling sites. Therefore, a stomach tube can be a feasible alternative method to collect representative rumen samples rather than the standard and more invasive method of rumen cannulation in Hanwoo steers.

Keywords: 16S rRNA gene amplicon sequencing, fermentation parameters, rumen microbiome, stomach tube, cannulation

Introduction

Regular collection and analysis of rumen samples from cattle are important for investigating the composition of the rumen microbiome, which greatly contributes to the effective digestion of plant materials and rumen fermentation [1]. Rumen cannulation is the standard method for collecting rumen samples that are used to analyze the rumen microbiome and fermentation parameters [2]. However, the invasiveness of the surgical procedure of cannulation limits the number of cattle that can be sampled. Therefore, the stomach tube has been proposed as an alternative method of collecting a greater number of rumen samples to increase the statistical power of the analysis. However, there is still debate as to whether stomach tube collection is a useful alternative with equivalent results on the rumen microbiome by comparison to those obtained with cannulation.

Several studies have shown that the stomach tube is a feasible alternative to rumen cannulation in sheep, goats, calves, and cattle based on comparison of the composition of rumen microbiomes using the traditional polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) method [3–5]. In addition, Paz *et al*. [6] showed that the rumen microbiome was similar between stomach

tube and cannulization samples in Holstein and Jersey cattle using next-generation sequencing. Moreover, some studies showed similar rumen fermentation parameters such as pH, volatile fatty acids (VFA), and ammonia between the two sampling techniques [3–5, 7], whereas others demonstrated differences [8, 9]. Paz *et al*. [6] indicated that discarding the first 200 ml of the rumen fluid could help to minimize saliva contamination that can influence the fermentation parameters.

The Hanwoo cattle breed native to Korea is unique owing to its particularly smaller size [7]; accordingly, the stomach tube that is typically used for Holstein cattle is too thick for effective use in collecting rumen samples from Hanwoo cattle. Therefore, a new stomach tube with reduced thickness needs to be designed for rumen sampling in Hanwoo steers with an insertion depth corresponding to the suggestion of Shen *et al.* [7]. Furthermore, the objective of this study was to compare the composition of the rumen microbiome and the fermentation parameters of the rumen fluid in Hanwoo cattle between samples collected using the modified stomach tube and cannulation.

Materials and Methods

Animals and Rumen Sampling

All experimental procedures were approved and performed under the guidelines of the National Institute of Animal Science Institutional Animal Use and Care Committee in Korea.

Three ruminally cannulated Hanwoo steers (32 months old; 605 ± 18 kg of body weight) were individually housed and fed a diet composed of 80% concentrate and 20% mixed hay (45% tall fescue, 45% orchardgrass, and 10% Kentucky bluegrass).

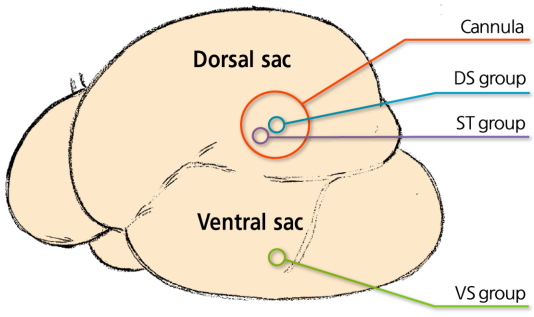

Fig. 1. Collection of rumen samples from Hanwoo steers.
Nine rumen samples were collected from three Hanwoo steers: 1) ST group = three samples collected via the stomach tube from three animals, 2) DS group = three samples collected from the dorsal sac via rumen cannulation from three animals, and 3) VS group = three samples collected from the ventral sac via rumen cannulation from three animals.

The following nine samples of rumen digesta were collected from three Hanwoo steers at 2 h post-feeding: 1) three samples collected via the stomach tube from three animals (ST group), 2) three samples collected from the dorsal sac via rumen cannulation from three animals (DS group), and 3) three samples collected from the ventral sac via rumen cannulation from three animals (VS group) (Fig. 1). All nine rumen digesta samples were strained through eight layers of cheesecloth and stored at −80°C until extraction of metagenomic DNA and analysis of fermentation parameters.

For sampling via the stomach tube, a new stomach tube (probe head length = 13 cm, probe head diameter = 3 cm, tube length = 210 cm, tube diameter = 1 cm) was manually developed for use in Hanwoo cattle. Rumen digesta samples (about 200 ml) were obtained using a vacuum pump (Suction-Pump, Welch & Thomas, USA).

DNA Extraction and Next-Generation Sequencing

Metagenomic DNA was extracted from the nine rumen samples using the RBB+C bead-beating method [10]. The V3-V4 region of 16S rRNA genes was amplified from each DNA sample with the universal primers 341F and 805R [11]. The resultant nine 16S rRNA gene amplicon libraries were sequenced on the MiSeq platform (Illumina, USA). Paired reads were assembled using the FLASH program [12], and the assembled sequences were processed using the programs in the QIIME software package 1.9.1 [13]. After the sequences were demultiplexed and quality-filtered, chimeric sequences were detected using the ChimeraSlayer program [14]. All non-chimeric sequences were classified into taxa using the Greengenes reference database [15], and operational taxonomic units (OTUs) were calculated at a 97% sequence similarity threshold using the uclust program [16]. The number of OTUs was normalized by subsampling 70,000 sequences from each rumen sample, and used to build a phylogenetic tree with the FastTree program [17]. The alpha diversity was determined through various metrics (number of OTUs, Chao1, PD_whole_tree distance, and Shannon diversity index), and the beta diversity was determined through evaluation of the principal coordinate analysis (PCoA) plot on the unweighted UniFrac distance matrix.

Fermentation Parameters

The pH was measured immediately from the strained rumen digesta samples using a pH meter (Mettler-Toledo AG, Switzerland). The VFA concentration in the rumen samples was analyzed by gas chromatography (Agilent 7890, Agilent Technologies Inc., USA) as described previously [18].

Statistical Analysis

The mean proportion of each taxon identified among the total sequences and the mean abundances of fermentation parameters of the rumen fluid were compared among the three sample groups using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons post-hoc test in XLSTAT statistical

software (Addinsoft, USA). A significant difference was determined at $p < 0.05$.

Nucleotide Sequence Accession Numbers

The 16S rRNA gene sequences obtained in this study are available from the EMBL European Nucleotide Archive (PRJEB25166).

Results

Rumen Microbiome Composition

A total of 722,001 clean sequences were obtained from the nine rumen samples. Each sample was represented by >70,000 sequences. Taxa that accounted for ≥0.2% of the total sequences on average were regarded as "major taxa" and used for statistical comparison among the three sample groups. The 722,001 sequences were assigned to 20 phyla, with Bacteroidetes (45.2–68.4%) representing the most dominant phylum, followed by Firmicutes (27.4–45.4%) in each sample; these two phyla accounted for approximately 90% of the total sequences in all nine samples together. Verrucomicrobia was the third most dominant phylum, accounting for 0.8–5.0% of the total sequences in each sample. Moreover, the proportions of these three phyla did not differ ($p > 0.05$) among the three sample groups (Table S1). The proportions of TM7, Proteobacteria, Actinobacteria, Spirochaetes, Tenericutes, Chloroflexi, Planctomycetes, Synergistetes, and WPS-2 accounted for 0.2–0.8% of the total sequences on average across the nine samples with no difference ($p > 0.05$) among the three sample groups detected, except for Chloroflexi, which was greater ($p < 0.05$) in the DS group than in the ST group. The remaining eight minor phyla identified, Lentisphaerae, SR1, Armatimonadetes, Fibrobacteres, LD1, Elusimicrobia, Fusobacteria, and GN02, accounted for <0.2% of the total sequences across the nine samples on average.

The Bacteroidetes sequences were assigned to six major families, including Prevotellaceae (18.3–53.5%), Paraprevotellaceae (1.8–4.6%), S24-7 (1.2–4.8%), RF16 (0.3–2.0%), BS11, and Bacteroidaceae (0.2–0.5%), that accounted for ≥0.2% of the total sequences across the nine samples on average (Table S1). Proportions of these six major families did not differ ($p > 0.05$) among the three sample groups, except for putative family S24-7, which was greater ($p < 0.05$) in the DS group than in the ST group. Four major genera of Bacteroidetes were identified, including *Prevotella* (18.3–53.4%), YRC22 (0.7–2.4%), CF231 (0.8–1.4%), and BF311 (0.1–0.5%), with no difference in proportion ($p > 0.05$) among the three sample groups (Fig. 2).

The Firmicutes sequences were assigned to seven major families, including Ruminococcaceae (4.0–11.3%), Lachnospiraceae (4.9–17.1%), Veillonellaceae (6.3–12.5%), Erysipelotrichaceae (1.0–1.3%), Clostridiaceae (0.3–1.5%),

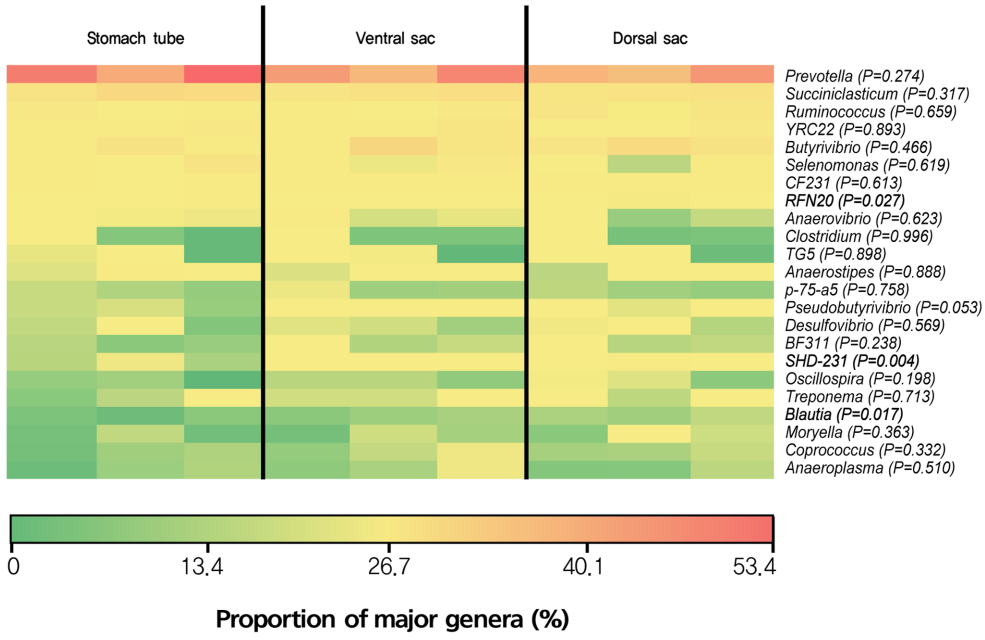

Fig. 2. Heatmap showing the proportion of major genera among the three sample groups.
Genera that accounted for ≥0.2% of the total sequences on average were regarded as "major" genera. Bold *p*-values indicate groups that significantly differ ($p < 0.05$).

Table 1. Rumen microbial diversity statistics among the three sample groups.

Sample group	Sampling type	No. of sequences	No. of OTUs	Chao1	Shannon diversity index	PD_whole_tree
Stomach tube (n = 3)	Subsampled reads[1]	70,000[2]	17,384[a]	76,039[a]	10.507[a]	774[a]
Dorsal sac (n = 3)	Subsampled reads[1]	70,000[2]	19,484[a]	83,296[a]	11.162[a]	900[a]
Ventral sac (n = 3)	Subsampled reads[1]	70,000[2]	18,662[a]	81,515[a]	10.927[a]	852[a]

[1]Means among the three sample groups were compared using ANOVA followed by Tukey's test.

[2]The number of OTUs was normalized by randomly subsampling 70,000 sequences from each rumen sample.

[a,b,c]Means with different superscript letters within a column indicate individual subsamples showing a significant difference ($p < 0.05$).

Christensenellaceae (0.1–2.3%), and Mogibacteriaceae (0.3–1.6%), that accounted for ≥0.2% of the total sequences on average across the nine samples, with no difference ($p > 0.05$) among the three sample groups. There were 14 major genera of Firmicutes identified, including *Succiniclasticum* (2.7–7.8%), *Butyrivibrio* (1.1–9.1%), *Ruminococcus* (0.4–3.0%), and *Selenomonas* (0.3–3.3%), along with RFN20, *Anaerostipes*, *Anaerovibrio*, *Pseudobutyrivibrio*, *Clostridium*, *Moryella*, p-75-a5, *Coprococcus*, *Oscillospira*, and *Blautia*, that accounted for ≥0.2% of the total sequences across the nine samples on average, with no difference ($p > 0.05$) among the three sample groups, except for the genera RFN20 and *Blautia* (Fig. 2). The proportion of RFN20 was greater ($p < 0.05$) in the ST group than in the DS group, while that of *Blautia* was greater ($p < 0.05$) in the DS group than in the ST group.

The remaining 10 major phyla identified included the 10 major families RFP12 (0.5–1.1%), F16 (0.6–0.9%), Desulfovibrionaceae (0.1–0.5%), Succinivibrionaceae (0.0–0.3%), Coriobacteriaceae (0.2–1.2%), Spirochaetaceae (0.2–0.7%), Anaeroplasmataceae (0.1–0.4%), Anaerolinaceae (0.2–1.0%), Pirellulaceae (0.2–0.9%), and Desulfovibrionaceae (0.1–0.6%) (Table S1), with no difference ($p > 0.05$) among the three sample groups, except for Anaerolinaceae, which showed a greater proportion ($p < 0.05$) in the DS group than in the ST group. The proportion of the putative genus SHD-231 placed within Anaerolinaceae was also greater ($p < 0.05$) in the DS group than in the ST group (Fig. 2). The proportions of the remaining four major genera, *Desulfovibrio*, *Treponema*, *Anaeroplasma*, and TG5, among the 10 phyla also did not differ ($p > 0.05$) among the three sample groups (Fig. 2).

Rumen Bacterial Diversity

The alpha diversity indices (number of OTUs, Chao1, PD_whole_tree distance, Shannon diversity index, and Simpson diversity index) did not differ ($p > 0.05$) among the three sample groups (Table 1). Moreover, the beta diversity PCoA plot indicated that the rumen microbiomes of the three sample groups were not separated, whereas the individual microbiomes of the three animals were distinct (Fig. 3). These results indicate that rumen microbial diversity in Hanwoo steers is not affected by different sampling techniques and sampling sites.

Fermentation Parameters

The pH values and VFA concentrations did not differ ($p > 0.05$) among the three sample groups (Table 2), indicating that fermentation parameters are not affected by different sampling techniques and sites.

Discussion

In the present study, we designed a new stomach tube for collecting the rumen digesta from Hanwoo cattle because

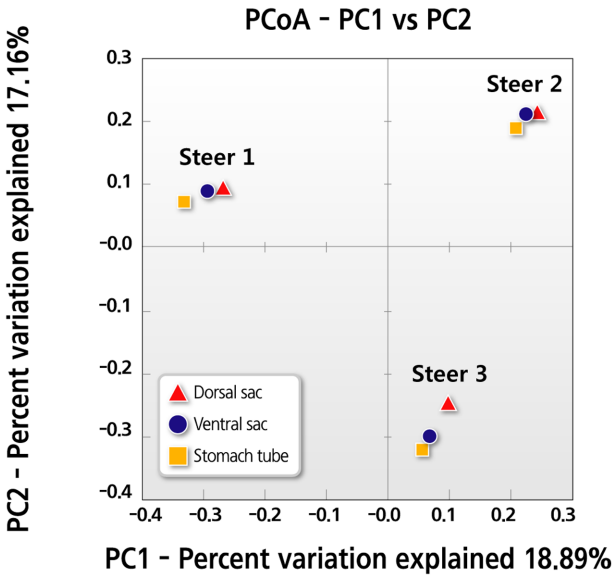

Fig. 3. Unweighted principal coordinate analysis (PCoA) displaying correlations among the three sample groups.
Unweighted PCoA was conducted using nine individual subsamples with 70,000 sequences, indicating no separation of the rumen microbiome based on different sampling techniques and sampling sites.

Table 2. Fermentation parameters among the three sample groups[1].

Item	Stomach tube (n = 3)	Dorsal sac (n = 3)	Ventral sac (n = 3)	p-value	SEM
pH	6.27	6.07	6.15	0.735	0.176
Total VFA, mM	123.57	122.60	110.55	0.852	17.887
Acetate, %	54.68	55.06	54.69	0.943	0.877
Propionate, %	19.69	19.49	19.70	0.986	0.999
Isobutyrate, %	1.61	1.59	1.61	0.990	0.118
Butyrate, %	18.02	17.88	18.10	0.986	0.945
Others, %	6.00	5.99	5.90	0.973	0.339
A:P	2.79	2.85	2.79	0.963	0.172

[1]Means among the three sample groups were compared using ANOVA followed by Tukey's test.
VFA, volatile fatty acids; A:P, acetate:propionate

of the lower body size compared to other cattle breeds. We identified that a probe with a head diameter of 3 cm could be successfully used to collect rumen digesta samples from Hanwoo steers with a body weight greater than 250 kg. The stomach tube was inserted via the esophagus until the probe head first made contact with and slightly sank into the rumen digesta. The head of the stomach tube was then repositioned by pulling it out and reintroducing it into the stomach several times. After repositioning, we identified that the probe head of the introduced stomach tube was at a similar position (insertion depth of stomach tube = 190 cm) in the dorsal rumen sac at which the rumen cannula was installed (Fig. 1), indicating that the sampling site via stomach tube collection is not biased compared to that used via cannulation in Hanwoo steers. By contrast, in Chinese Holstein cattle, Shen *et al*. [7] demonstrated that the probe head of the stomach tube was located at a position below the rumen cannula. This discrepancy may result from the different body types of Holstein and Hanwoo cattle.

Overall, we found that there was no impact of different sampling techniques and sampling sites on the composition of the rumen microbiome in the collected samples based on next-generation sequencing. This observation is similar to previous studies on rumen microbiomes in calves, sheep, goats, and beef cattle using the traditional PCR-DGGE method [3–5] and a recent study on rumen microbiomes in Holstein and Jersey cattle using next-generation sequencing [6]. Therefore, the stomach tube can be a feasible alternative method to collect representative samples of the rumen digesta from Hanwoo steers, which will contribute to investigating the composition of the rumen microbiome by increasing the number of animals that can be sampled.

Although most of the major taxa identified were not affected by the different sampling techniques, the proportions of three putative taxa, S24-7, RFN20, and SHD-231, significantly differed among the three sampling approaches. Because these taxa were only putatively classified based on sequences recovered from uncultured bacteria, they may not be well-defined and should be newly reclassified to novel taxa with the update of reference taxonomy databases in the future. Therefore, the observed differences in these three taxa may not actually have resulted from the different sampling techniques but rather reflect inaccurate taxonomy definition. Although the known genus *Blautia* also showed a significant difference in proportion between the two sampling techniques, this difference may be attributed to low repeatability due to the relatively small number of sequence reads obtained, as described previously [19].

The fermentation parameters of the rumen fluid collected from Hanwoo steers were also not affected by the different sampling techniques. As suggested previously [6], we discarded the first 200 ml of the rumen fluid to reduce potential contamination by saliva. This step is considered to minimize any potential alteration of fermentation parameters due to factors other than sampling technique. Therefore, the present study demonstrates that rumen samples collected via the stomach tube can accurately reflect fermentation parameters such as pH and VFA, as described in previous studies [4, 5, 7]. The fermentation parameters of the rumen fluid were also similar between the samples collected from the ventral sac and the dorsal sac in Hanwoo steers. This finding is in contrast to that of a previous study showing that the sampling site significantly affected the values of fermentation parameters of the rumen fluid in Chinese Holstein cattle [7]. This discrepancy may be attributed to the probing step applied to reduce saliva contamination and the different body types of the cattle sampled.

In conclusion, this study demonstrates that use of a

stomach tube and sampling from different sites (ventral sac vs. dorsal sac) do not affect the composition of the rumen microbiome or the fermentation parameters of rumen fluid collected from Hanwoo steers. Moreover, we have confirmed that discarding the first 200 mL of rumen fluid may help to minimize saliva contamination that could potentially alter fermentation parameters. Therefore, the stomach tube method is a feasible alternative to rumen cannulation for collecting representative rumen samples to analyze the rumen microbiome and fermentation parameters in Hanwoo steers.

Acknowledgments

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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