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ABSTRACT

This study compared the rumen microbiota and fermentation characteristics between Hanwoo steers with low and high methane emissions. Eighteen steers (seven months old) were fed an identical diet for 60 days, and their methane emissions were measured using the GreenFeed system at nine months of age. Based on their methane emission data, steers in the top 25% (high-methane group, HM; n = 4) and bottom 25% (low-methane group, LM; n = 4) were selected for analysis of rumen microbiota and fermentation characteristics. Rumen samples were collected, and the microbial composition was analyzed using metataxonomics based on full-length bacterial 16S rRNA gene sequencing on the PacBio Sequel II platform and archaeal 16S rRNA gene sequencing on the Illumina MiSeq platform. The data were processed using the QIIME2 pipeline and the MicrobiomeAnalyst platform. Rumen fermentation characteristics were analyzed using gas chromatography. Compared with the HM group, steers in the LM group exhibited significantly lower methane production (g/day), yield (g/kg DMI), and intensity (g/kg metabolic body weight (MBW)), but similar body weight and dry-matter intake, while showing greater average daily gain. Moreover, higher propionate concentrations and greater abundances of *Prevotella* and *Succinivibrio* were found in the LM group. Methane yield was negatively correlated with the relative abundances of *Prevotella* and *Succinivibrio*, as well as with propionate concentration. However, no significant differences in alpha diversity metrics or overall microbiota composition were observed between the two groups. Similarly, no clear separation between the HM and LM groups was observed in beta-diversity analyses for either bacterial or archaeal communities. In the archaeal community, *Methanobrevibacter* was dominant, and no significant differences in archaeal taxonomic composition were observed between the two groups. The ciliate protozoal population was lower in the LM group than in the HM group, whereas the abundances of total bacteria, total

archaea, and total fungi did not differ significantly. These findings suggest that the specific rumen microbial taxa and fermentation profiles, rather than overall microbial community diversity, were associated with the differences in methane emissions.

Keywords: Hanwoo steers; metataxonomic analysis; methane emissions; rumen microbiota

Introduction

Rapid industrialization has steadily increased greenhouse gas (GHG) emissions, thereby accelerating global warming by trapping more heat in Earth's atmosphere [1]. Methane (CH_4) is a major GHG emitted from agricultural production and has a greater global warming potential than carbon dioxide (CO_2) [1]. In the livestock industry, CH_4 is produced through enteric fermentation and manure management, with most CH_4 from enteric fermentation in ruminant animals [2]. These CH_4 emissions have raised environmental concerns and led to energy losses of up to 12% in ruminants, thereby reducing feed efficiency and overall performance [3]. Therefore, mitigating enteric CH_4 emissions benefits both environmental sustainability and ruminant productivity.

Ruminants have a unique digestive system in which feed is fermented by a complex rumen microbiota [4, 5]. This fermentation produces volatile fatty acids (VFAs), ammonia (NH_3), and gases such as CH_4, CO_2, and hydrogen (H_2) [6]. H_2, in particular, is generated primarily during acetate formation [7], and it is a key substrate for methanogenesis driven by methanogenic archaea, resulting in CH_4 as the final product [8].

To date, various strategies, including animal and feed management, diet formulation, and rumen manipulation, have been evaluated to mitigate enteric CH_4 emissions from ruminants [6, 8, 9]. However, these mitigation approaches have generally shown limited long-term effectiveness and increased costs, making large-scale on-farm application challenging [9-11]. This highlights the need for alternative strategies that can reduce CH_4 emissions without compromising rumen fermentation or animal productivity.

Recently, genetic selection of ruminants with lower CH_4 emissions has attracted increasing attention [11]. This strategy is prompted by findings that CH_4 emissions and feed efficiency traits

are influenced by the coevolutionary and symbiotic relationships between a host and its rumen microbiota [12]. Moreover, studies have reported that the composition of the rumen microbiota is moderately heritable, suggesting that selective breeding of animals with lower CH_4-producing microbiota may provide a sustainable long-term strategy for reducing CH_4 emissions [13, 14].

Although studies on dairy and beef cattle have investigated the associations between rumen microbiota and CH_4 emissions, the key features of rumen microbiota associated with methane emissions remain poorly defined [15, 16]. Moreover, because rumen microbiota composition and host genetics differ among breeds, breed-specific investigations are necessary to elucidate the microbial and physiological mechanisms associated with CH_4 emissions [17]. Hanwoo cattle are the predominant native beef breed in Korea, representing most of the national beef cattle population [18-20]. Therefore, targeting CH_4 mitigation in Hanwoo can substantially reduce GHG emissions in the Korean beef industry. However, no study has examined the rumen microbiota in Hanwoo cattle with divergent CH_4 emissions. The present study investigated the rumen fermentation characteristics and microbiota associated with divergent CH_4 production in Hanwoo steers raised under the same dietary and management conditions.

Materials and Methods

Animal ethics

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Chonnam National University (Approval number: CNU IACUC-YB-2024-118), and all methods were performed in accordance with the relevant guidelines and regulations.

Animals, CH_4 measurements, and experimental design

Eighteen Hanwoo steers aged seven months (average body weight (BW): 233.4 ± 27.0 kg) were recruited from multiple farms and maintained under identical dietary and environmental conditions for 60 days. During the experimental period, the steers were fed *ad libitum* a commercial diet, and the dry-matter intake (DMI) was recorded manually for each steer. Each steer was weighed at the beginning and end of the experimental period. Table 1 presents the ingredients and chemical composition of the experimental diet.

After the 60-day feeding period, the CH_4 and CO_2 emissions from all 18 steers (average BW: 273.0 ± 23.8 kg; nine months of age) were measured using a GreenFeed system (C-Lock Inc., Rapid City, SD, USA) as described previously [21]. Before gas measurement, the steers were trained for one month to adapt to the GreenFeed system. The gas emissions from each steer were then recorded at eight different time points (00:00, 03:00, 06:00, 09:00, 12:00, 15:00, 18:00, and 21:00) over three consecutive days. A small quantity of concentrate pellets was provided to attract the steers to the feeding hood of the GreenFeed system and to maintain a proper head position during CH_4 measurements. Each steer was allowed 6 min of access to the GreenFeed system. Breath sample data from individual steers were sent to C-Lock Inc., where gas emissions were calculated and reported as CH_4 production (g/d; d), CH_4 yield (g/kg DMI), and CH_4 intensity (g/kg MBW).

Among the 18 steers, high-methane-yield (top 25%, HM; n = 4) and low-methane-yield (bottom 25%, LM; n = 4) steers were selected for comparative analysis of ruminal fermentation characteristics, microbiota, and growth performance between the two groups. The coefficient of

variation (CV, %) of methane emissions was calculated for each animal from measurements obtained over three consecutive days.

Ruminal sampling and fermentation characteristics

After the gas measurements, the stomach tubing technique was used to collect ruminal samples before morning feeding, as described previously [22]. The first 200 mL of the ruminal samples were discarded to avoid contamination from saliva. The pH of the ruminal samples was immediately measured using a pH meter (ST300, OHAUS, New Jersey, USA), while the remaining ruminal samples were stored at -80℃ for the analysis of VFAs and ammonia (NH_3-N), as well as for DNA extraction.

VFAs were analyzed using a previously described method [23]. Briefly, the preserved ruminal fluid samples were centrifuged at 6,000 ×g and 4℃ for 15 min, and 1 mL of the supernatant was mixed with 200 μL of 25% metaphosphoric acid and incubated at 4℃ for 30 min. Following incubation, the mixture was centrifuged at 6,000 ×g and 4℃ for 15 min, and the supernatant was filtered through a 0.45-μm filter and then placed in a 2-mL screw vial for VFA analysis, which was performed using a gas chromatograph (Trace 1310, Thermo Fisher Scientific, USA) equipped with a flame ionization detector and a capillary column (60 m × 0.25 mm × 0.25 um; Agilent Technologies Inc, California, USA). The oven regimen was as follows: the temperature was set to 80℃ for 1 min, then increased at 15℃ per min to 230℃ and maintained at 230℃ for 3 min. Nitrogen was used as the carrier gas at a flow rate of 2.2 mL/min.

Ammonia nitrogen (NH_3-N) was analyzed using the method described by Chaney and Marbach [24]. The centrifuged rumen fluid (20 μl each) was mixed with 1 mL of phenol color reagent and

1 mL of alkali–hypochlorite reagent. The mixture was then incubated at 37℃ for 15 min. Subsequently, 1 mL of the sample mixture was transferred to a VIS cuvette, and the optical density was measured at 630 nm using a spectrophotometer (Eppendorf Biospectrometer® basic, Hamburg, Germany).

DNA extraction and metataxonomic analysis

The repeated bead-beating plus column method was used to extract total microbiota DNA from the collected ruminal samples [25]. The DNA quality and quantity were assessed using a Biospectrometer (Eppendorf Biospectrometer® basic, Hamburg, Germany). For bacterial community analysis, the full length of 16S rRNA genes was amplified using universal primers 27F (5′-AGRGTTYGATYMTGGCTCAG-3′) and 1492R (5′-RGYTACCTTGTTACGACTT-3′), and the resultant amplicons were sequenced on the PacBio Sequel II platform by Macrogen (Seoul, Korea). For archaeal community analysis, the 16S rRNA gene was amplified using archaeal-specific primers Ar915aF (5′-AGGAATTGGCGGGGGAGCAC-3′) and Ar1386R (5′-GCGGTGTGTGCAAGGAGC-3′), and the amplicons were sequenced on the Illumina MiSeq platform (2 × 300 bp, Macrogen, Seoul, Korea).

The raw 16S rRNA gene sequence data were processed and analyzed using QIIME2 (version 2023.05) [26]. For bacterial community analysis, PacBio Sequel II full-length 16S rRNA sequences were processed from demultiplexed single-end reads, which were quality-filtered (Q > 25). Denoising, chimera removal, and clustering of sequences into amplicon sequence variants (ASVs) were performed using the DADA2 denoise-ccs plugin [27]. Primer sequences were trimmed within the denoise-ccs command with a maximum mismatch allowance of 2. Sequences

were subsequently filtered using a length range of 1,000 -1,600 bp, and a maximum expected error rate of 2.0.

For archaeal MiSeq data, paired-end reads were quality-filtered and merged prior to denoising using the DADA2 pipeline, after which chimera removal and ASV clustering were performed using similar filtering criteria as applied to the PacBio data. Denoising statistics for bacterial sequences and archaeal sequences are presented in Supplementary Tables 1 and 2, respectively.

Bacterial ASVs were taxonomically classified against the Silva 138 reference database [28], while archaeal ASVs were classified based on the NCBI database [29]. Species-level classification was not conducted for archaeal taxa because partial 16S rRNA gene sequencing did not provide sufficient taxonomic resolution for reliable species assignment. Microbial community data were analyzed using the web-based MicrobiomeAnalyst platform [30, 31]. Alpha diversity metrics, including Chao1, Shannon's, and Simpson's indices, were computed, while beta diversity analysis was performed using principal coordinates analysis (PCoA) based on the Bray–Curtis dissimilarity. Functional prediction and pathway annotations were performed using PICRUSt2 [32] with reference to the Kyoto Encyclopedia of Genes and Genomes database [33]. Nearest Sequenced Taxon Index (NSTI) scores were calculated to assess the accuracy of PICRUSt2 predictions. All ASVs were below the max NSTI cut-off of 2.0 and were retained for downstream analyses.

Quantitative real-time PCR (qPCR) assays

The absolute abundances of total bacteria, total archaea, total fungi, and ciliate protozoa were quantified using respective universal primers (Table 2) on a qTOWER real-time PCR system (Analytic Jena AG, Jena, Germany). The target of each microbial group was cloned into the

pGEM-T Easy Vector (Promega, USA), and the resulting plasmid DNA was used as the standard for group-specific qPCR. The copy number concentration of each standard was calculated from the PCR product length and DNA mass concentration. Each standard was serially diluted, and 10^2 to 10^{10} copies of each standard were used per reaction to generate standard curves.

Each qPCR reaction was performed in a total volume of 20 μL consisting of 10 μL of Prime Q-Mastermix (Genet Bio, Korea), 1 μL of each forward and reverse primer (10 μM), 1 μL of template DNA, and 7 μL of DEPC-treated water (Thermo Fisher Scientific, USA). The amplification conditions involved initial denaturation at 94°C for 10 min, followed by 40 cycles at 94°C for 30 s, 60°C for 1 min, and 72°C for 30 s. Each qPCR assay was performed in triplicate for both the standards and samples, along with no-template controls. Absolute abundance of each microbial group was expressed as copy numbers of the target per mL of ruminal samples, as described previously [34, 35].

Statistical analysis

The Shapiro–Wilk test in the R software package was used to assess the normality of all numeric data. The absolute abundance data for microbial groups (total bacteria, total archaea, ciliate protozoa, and total fungi) were log-transformed prior to analysis. Differences in fermentation characteristics (pH, VFAs, and NH_3-N), production performance variables (BW and DMI), and the log-transformed absolute abundances of microbial groups between the HM and LM groups were compared using Student's *t*-test in the R software package (version 4.2.1).

Differentially abundant taxa between these two groups were analyzed using the ANCOM-BC in QIIME2 [36]. The same method was applied to identify differentially abundant predicted

functional pathways derived from PICRUSt2 analysis. For both taxonomic and predicted functional pathway analyses, *p*-values were adjusted for multiple comparisons using the Benjamini–Hochberg method. Furthermore, alpha diversity metrics were compared using the nonparametric Mann–Whitney test, while beta diversity differences were assessed using PERMANOVA with 999 permutations [37].

Spearman rank correlation analysis was performed to evaluate associations among differentially abundant genera, rumen fermentation characteristics, and methane yield using R software package (version 4.2.1). Only variables showing significant differences between the HM and LM groups were included in the correlation analysis and visualization. Statistical significance was declared at $p < 0.05$.

Results

Enteric gas emissions and growth performance

Table 3 summarizes the growth performances and enteric gas emissions of the HM and LM groups. No significant differences were observed in initial BW, final BW, or DMI between the two groups. However, average daily gain (ADG) was significantly greater in the LM group than in the HM group ($p < 0.05$). Among the gas emission variables, CH_4 production (g/d), CO_2 production (g/d), CH_4 yield (g/d/kg DMI), and CH_4 intensity (g/d/kg MBW) were significantly greater in the HM group than in the LM group ($p < 0.05$). Methane measurements showed coefficients of variation ranging from 13.8% to 26.4% (mean = 19.9%).

Rumen fermentation characteristics

Data on the rumen fermentation characteristics of the HM and LM groups are shown in Table 4. The rumen concentration of propionate was significantly higher in the LM group than in the HM group ($p < 0.05$). However, the remaining fermentation characteristics did not differ significantly between the two groups.

Rumen bacterial composition

Across all eight samples of both groups, Bacteroidota and Firmicutes were the two predominant phyla, accounting for 45.4% and 38.4% of the total sequences, respectively (Fig. 1A). Verrucomicrobiota was the third most abundant phylum (6.9%), followed by Proteobacteria (4.4%), Fibrobacterota (2.2%), Cyanobacteria (1.5%), Spirochaetota (0.3%), Planctomycetota (0.3%), Patescibacteria (0.2%), and Actinobacteria (0.2%) (Fig. 1A). The remaining phyla collectively represented 0.1% of the total sequences. They were grouped as “Others” (Fig. 1A).

At the genus level, the top-10 known genera with the a relative abundances greater than 0.5% across all eight samples were *Prevotella* (16.6%), *Ruminococcus* (6.2%), *Succiniclasticum* (3.3%), *Fibrobacter* (2.2%), *Saccharofermentans* (1.8%), *Succinivibrio* (1.4%), *Butyrivibrio* (1.1%), *Ruminobacter* (0.8%), *Monoglobus* (0.6%), and *Pseudobutyrivibrio* (0.5%) (Fig. 1B). The remaining genera, including low-abundance known genera and unclassified groups, collectively accounted for 65.4% of the total sequences and were grouped as “Others” (Fig. 1B).

At the species level, only 10 taxa were assigned to known species across all eight samples (Fig. 1C). Of these known species, *Fibrobacter succinogenes* was the most dominant, representing 2.1% of the total sequences across all eight samples, followed by *Ruminococcus flavefaciens* (1.2%),

Prevotella ruminicola (1.0%), *Butyrivibrio fibrisolvens* (0.5%), *Streptococcus lutetiensis* (0.4%), *Ruminococcus albus* (0.1%), and *Lachnobacterium bovis* (0.1%) (Fig. 1C). *Kandleria vitulina*, *Butyrivibrio proteoclasticus*, and *Lactobacillus ruminis* each represented less than 0.1% of the total sequences. The remaining 94.5% of the total sequences could not be assigned to any known species and were grouped as "Others" (Fig. 1C).

Rumen archaeal composition

Methanobacteriota was the dominant phylum, representing 99.9% of the total sequences across all eight samples, and the remaining 0.1% was assigned to the phylum Thermoplasmatota (Fig. 2A). At the genus level, *Methanobrevibacter* was the most abundant genus, accounting for 98.3% of the total sequences across all eight samples, followed by *Methanosphaera* (1.1%), *Methanobacterium* (0.5%), and *Methanomassiliicoccus* (0.1%) (Fig. 2B).

Differentially abundant taxa

In the bacterial community, ANCOM-BC analysis showed no significant phylum-level differences. However, at the genus level, *Prevotella* and *Succinivibrio* were more abundant in the LM group than in the HM group ($p < 0.05$) (Fig. 3). At the species level, no significant differences were found between the two groups. No archaeal taxa differed significantly in relative abundance between the LM and HM groups.

Spearman rank correlation analysis revealed that methane yield was negatively correlated with the relative abundances of *Prevotella* ($r = -0.838$, $p < 0.01$) and *Succinivibrio* ($r = -0.735$, $p < 0.05$), as well as with propionate concentration ($r = -0.862$, $p < 0.01$) (Fig. 4). In addition,

Prevotella abundance was positively correlated with *Succinivibrio* (r = 0.778, $p < 0.05$), whereas no significant correlations were observed between propionate concentration and either genus (Fig. 4). No significant correlations were observed for other microbial genera or fermentation parameters.

Alpha and beta diversity

For the bacterial community, no alpha diversity metrics, including observed ASVs, Chao1 richness estimate, Shannon, and Simpson indices, differed significantly between the HM and LM groups, with effective sizes being small or medium (Table 5). Similarly, the PCoA plot based on Bray–Curtis dissimilarity showed no significant differences in the overall bacterial community structure between the two groups (Fig. 5A).

In the archaeal community, no significant differences were observed in the number of observed ASVs, Chao1 richness estimate, or the Simpson index between the two groups, with effective sizes being small or medium (Table 5). However, the Shannon index was significantly greater in the HM group than in the LM group ($p < 0.05$) (Table 5). Consistent with this, no clear separation between the two groups was observed in the PCoA plot based on Bray–Curtis dissimilarity (Fig. 5B).

Predicted functional features

In the bacterial community, PICRUSt2-based functional prediction indicated that genes associated with the degradation of glycosaminoglycan and other glycan were significantly more abundant in the LM group than in the MM group ($q < 0.01$; Fig. 6). However, in the archaeal

community, no significant differences were observed in the predicted functional profiles between the two groups.

Real-time PCR assays

Real-time PCR showed that ciliate protozoal populations were lower in the LM group than in the HM group ($p < 0.05$) (Fig. 7). The absolute abundance of total bacterial, total archaeal, and fungal populations did not differ significantly between the two groups (Fig. 7).

Discussion

The significantly lower CH_4 production, yield, and intensity observed in the LM group, despite the identical dietary and environmental conditions, indicated that the variations in CH_4 emissions were likely influenced by host phenotypic factors. CH_4 emission traits have been reported to be moderately heritable [13], supporting that the rumen microbiota features are important factors influencing CH_4 emissions and may serve as markers for selective breeding to provide environmental benefits in the cattle industry. In the present study, although no differences in BW or DMI were observed between the HM and LM groups, ADG was greater in the LM group. Collectively, these findings suggest that selective breeding of Hanwoo cattle with inherently low CH_4 emissions may help mitigate environmental impacts while maintaining growth performance.

The characteristics of rumen fermentation differed only with respect to propionate concentration, which was significantly higher in the LM group than in the HM group. Propionate is an important H_2 sink, as its production competes with methanogenesis for available H_2 in the rumen. When CH_4 production is inhibited, excess H_2 can be diverted to propionate production [38]. This corroborates

298 the negative correlation between CH_4 production and propionate concentration observed in the
299 present study and previous studies [7, 39]. Although the propionate concentration differed
300 significantly between the two groups, the total VFA concentrations did not, suggesting potential
301 differences in specific rumen fermentation pathways rather than overall fermentation activity.
302 Unlike previous studies that used anti-methanogenic feed additives to manipulate ruminal
303 fermentation, no dietary interventions were applied in the present study. Therefore, the increased
304 propionate levels observed in the LM group may be attributed to more active propionate-producing
305 microbial pathways or alternative H_2-utilizing mechanisms [7, 39].

306 *Prevotella* was significantly more abundant in the LM group than in the HM group, which is
307 consistent with previous reports of a negative correlation between *Prevotella* relative abundance
308 and CH_4 production [40, 41]. As a genus known to be predominantly involved in the degradation
309 of proteins and carbohydrates in the rumen, *Prevotella* mainly produces succinate as a
310 fermentation byproduct [4]. Succinate is a key intermediate in the succinate–propionate pathway
311 for propionate production by other rumen bacteria [42]. As this pathway competes with
312 methanogenesis for H_2, increased abundance of *Prevotella* may indirectly reduce CH_4 emissions
313 [7]. Moreover, because *Prevotella* is a predominant genus in the rumen across dietary and
314 environmental conditions [5], modulating its abundance could be an effective strategy for
315 mitigating CH_4 emissions in ruminants.

316 Similarly, *Succinivibrio* was significantly more abundant in the LM group than in the HM group,
317 consistent with previous studies showing that it was negatively correlated with CH_4 production
318 [43, 44]. *Succinivibrio* ferments carbohydrates mainly to succinate, which is subsequently
319 converted to propionate, thereby serving as an alternative H_2 sink [45, 46]. The enrichment of

320 *Succinivibrio* in the LM group could shift rumen H_2 flow toward propionate production rather than
321 methanogenesis, thereby mitigating CH_4 emissions in the Hanwoo steers.

322 Correlation analysis also showed that methane yield was negatively correlated with the relative
323 abundances of *Prevotella* and *Succinivibrio*, whereas no significant correlations were observed
324 between propionate concentration and either genus. This suggests that the negative association of
325 these genera with methane production may be linked to succinate production and H_2-utilization
326 pathways rather than directly to propionate concentration. Although succinate-utilizing bacteria
327 such as *Selenomonas ruminantium* and *Succiniclasticum ruminis* are known to produce propionate
328 [47, 48], no significant differences in these taxa were observed between the two groups in the
329 present study. Therefore, the increased propionate production may be associated with altered
330 metabolic activity without significant taxonomic shifts, or with the involvement of other unknown
331 succinate-utilizing microbes. However, as succinate concentrations were not directly measured in
332 the present study, this interpretation should be viewed with caution until it is validated by further
333 research.

334 Except for the Shannon index for the archaeal community, no significant differences were found
335 in alpha-diversity metrics or in overall community composition of bacteria or archaea between the
336 HM and LM groups. These findings suggest that phenotypic variations in CH_4 emissions may not
337 be substantially associated with overall microbiota apha-diversity or community composition of
338 bacteria or archaea. Previous studies have shown that rumen microbial diversity is influenced by
339 multiple factors, including diet, breed, and environment [5, 49]. Because these factors were
340 controlled in the present study, the CH_4-associated phenotype might not have been significantly
341 affected by the overall microbial diversity or composition. Furthermore, the absence of differences

could reflect the inherent stability and host-specific regulation of the rumen microbiota, as described previously [50, 51]. The observed difference in Shannon diversity of archaeal community may reflect potential variation within dominant taxa, such as *Methanobrevibacter*, at lower taxonomic resolution (e.g., species or strain level), which could not be resolved in the present study.

Functional prediction using PICRUSt2 analysis revealed that the LM group was enriched for pathways associated with glycan degradation, including degradation of glycosaminoglycan and other glycans. These pathways may be associated with enhanced breakdown of complex carbohydrates derived from dietary plant polysaccharides in the rumen. The enhanced breakdown of these glycans may increase the availability of fermentable substrates, which could, in turn, be indirectly associated with increased propionate formation. Because propionate formation serves as a hydrogen sink that competes with methanogenesis, this change in predicted functional potential may be associated with the lower methane emissions observed in the LM group. In contrast, no significant differences were observed in archaeal predicted functional profiles between the two groups, suggesting that archaeal functional potential was largely similar between HM and LM steers. However, these functional inferences are derived from 16S rRNA gene-based predictions and therefore should be interpreted with caution.

Although CH_4 emissions were significantly lower in the LM group than in the HM group, no significant differences in the total archaeal abundance or taxonomic composition were observed between the two groups. In contrast, the LM group exhibited a lower abundance of ciliate protozoa. These findings suggest that the reduced CH_4 emissions in the LM group may be associated with reduced interspecies H_2 transfer from protozoa to methanogens, rather than decreased methanogen

abundance. A lower protozoal abundance may lead to a reduced H_2 supply and weaker symbiosis between protozoa and methanogens, thereby potentially decreasing methanogenic activity in the rumen. Moreover, a previous study demonstrated that methanogen abundance remained similar among sheep with divergent CH_4 yields, whereas expression of key methanogenesis-related genes differed significantly with CH_4 production level [52]. Similarly, Kamke et al. [53] reported that differences in methane yield were associated with altered microbial gene expression and fermentation pathways affecting hydrogen metabolism. Collectively, these results indicate that variations in CH_4 emissions among animals are likely associated with microbial metabolic activity and syntrophic interactions between protozoa and methanogens than major differences with archaeal abundance or taxonomic composition. This interpretation is consistent with the present findings, which show that the differences in CH_4 emissions did not accompany significant variations in total archaeal abundance or taxonomic composition. However, because interspecies hydrogen transfer was not directly measured in the present study, this interpretation should be viewed with caution. Further studies directly quantifying interspecies hydrogen transfer are warranted.

A relatively low abundance of ciliate protozoa was observed in the present study, irrespective of CH_4 emission phenotype. This may be explained by the sampling method used (stomach tubing). Stomach tubing primarily retrieves the liquid fraction of rumen [22], while 63 to 90% of the rumen protozoa are within the solid fraction [54]. Therefore, the absolute abundance of protozoa should be interpreted with caution. However, because all rumen samples were collected using the same method, the comparison of relative differences in rumen protozoa between the two methane emission groups remains valid. In addition, protozoal diversity was not evaluated in the present

study, and future studies using sequencing-based approaches are needed to better characterize protozoal community structure and its potential role in methane emissions.

The relatively small sample size in the present study may limit statistical power to detect minor differences and restrict the generalizability of the findings. Therefore, the results should be interpreted with caution, and further studies with larger cohorts are warranted to validate the observed associations.

In the present study, full-length 16S rRNA gene sequencing was used to achieve more accurate taxonomic identification and finer resolution of bacteria at the species level. However, approximately 95% of the total sequences obtained from the rumen samples could not be assigned to any known species. This is likely due to the limited coverage of current reference databases [55, 56] and to the presence of numerous rumen microbial taxa that have not been cultured or taxonomically characterized [5, 57]. Studies employing advanced culturomics and genome-resolved metagenomics are required to isolate and characterize these uncultured taxa. This will expand the reference databases and enhance species-level resolution.

Conclusion

Some Hanwoo steers naturally produced significantly less CH_4 without compromised growth performance, while exhibiting greater ADG. These animals exhibited higher propionate concentrations and greater abundances of *Prevotella* and *Succinivibrio* within their rumen, indicating a shift toward more efficient H_2-utilizing fermentation pathways. The lower protozoal abundance in the LM group further supported the premise that limited interspecies H_2 transfer may contribute to reduced CH_4 emissions from LM steers. However, the overall microbiota

composition did not differ between the LM and HM groups. Collectively, these findings indicate that specific rumen microbial taxa and fermentation profiles, rather than overall rumen microbiota diversity or composition, may be associated with differences in CH_4 emissions. These microbial features may serve as potential biomarkers for breeding of low-CH_4 animals. However, their application in selective breeding strategies should be interpreted with caution until further validated in larger populations.

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561 **Tables**

562 **Table 1. Ingredients and chemical compositions of the diet fed to Hanwoo steers**

563

Item[1]	Feed composition
Feed ingredient, % of DM	
Rice straw	42.54
Corn grain	13.52
Wheat grain	6.49
Barley grain	2.36
Cane molasses	0.72
Corn steep liquor	0.86
Wheat bran	2.73
Corn gluten feed	10.34
Soybean meal	1.53
Rapeseed meal	1.72
Coconut meal	2.87
Palm kernel meal	6.03
DDGS	4.60
Lupin flake	1.72
Minerals and additives[2]	1.97
Chemical composition, % of DM	
CP	18.65
EE	3.18
CF	10.83
Ash	8.64
NDF	41.12
ADF	16.67
NFC[3]	28.42
NFE[4]	58.72

564 [1]DM = dry matter; CP = crude protein; EE = ether extract; CF = crude fiber; NDF = neutral detergent fiber; ADF = acid detergent
565 fiber; NFC = non-fiber carbohydrate; NFE = nitrogen-free extract.
566 [2]Minerals and additives included salt, limestone, a vitamin–mineral premix, a probiotic–enzyme complex additive, and a rumen
567 buffer.
568 [3]Calculated value from 100 – (% of CP + % of EE + % of Ash + % of NDF).
569 [4]Calculated value from 100 – (% of CP + % of EE + % of Ash + % of CF).
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573 **Table 2. PCR primers for quantitative real-time PCR assays**

Target taxon	Forward primer (5' → 3')	Reverse primer (5' → 3')	Size (bp)	References
Total bacteria	CGGCAACGAGCGCAACCC	CCATTGTAGCACGTGTGTAGCC	130	[58]
Total archaea	GAGGAAGGAGTGGACGACGGTA	ACGGGCGGTGTGTGCAAG	233	[59]
Ciliate protozoa	GCTTTCGWTGGTAGTGTATT	CTTGCCCTCYAATCGTWCT	233	[60]
Total fungi	GAGGAAGTAAAAGTCGTAACAAGGTTTC	CAAATTCACAAAGGGTAGGATGATT	120	[58]

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Table 3. Enteric gas emissions and growth performances of Hanwoo steers with high and low methane yields

Item	Phenotype		SEM	*P*-value
	HM	LM		
Initial BW, kg	249.250	217.475	9.557	0.120
Final BW, kg	285.250	264.500	7.900	0.241
ADG (kg/day)	0.400[b]	0.523[a]	0.030	0.026
DMI, kg/day	6.856	6.728	0.054	0.261
CH_4 production, g/day	170.525[a]	133.617[b]	7.228	< 0.001
CO_2 production, g/day	6,817.566[a]	5,644.327[b]	287.692	0.044
CH_4 yield, g/kg DMI[3]	24.871[a]	19.859[b]	0.971	< 0.001
CH_4 intensity, g/kg MBW[4]	2.458[a]	2.049[b]	0.092	0.016

HM, high-methane group; LM, low-methane group; SEM, standard error of the mean; BW, body weight; ADG, average daily gain; DMI, dry matter intake; MBW, metabolic body weight which was calculated using $BW^{0.75}$.

[a,b] Means with different superscripts within a row differ significantly ($p < 0.05$).

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Table 4. Rumen fermentation variables in Hanwoo steers with high and low methane yields

Item	Phenotype[1]		SEM[2]	*P*-value
	HM	LM		
pH	7.143	6.858	0.075	0.069
NH_3-N[3], mg/dL	6.190	7.439	0.601	0.341
Acetate, mM	43.092	51.300	2.750	0.174
Propionate, mM	10.005[b]	11.938[a]	0.435	0.012
Butyrate, mM	5.976	6.688	0.503	0.538
Isobutyrate, mM	0.486	0.579	0.034	0.210
Valerate, mM	0.066	0.146	0.030	0.185
Isovalerate, mM	0.570	0.737	0.057	0.201
A:P ratio[4]	4.303	4.284	0.149	0.957
Total VFAs[5]	60.179	71.386	3.584	0.153

584 [a,b] Means with different superscripts within a row differ significantly ($p < 0.05$).
585 [1]Phenotype = HM (high-methane-emitting group), LM (low-methane-emitting group)
586 [2]SEM = Standard error of the mean
587 [3]NH_3-N = Ammonia nitrogen
588 [4]A:P ratio = Acetate/propionate ratio
589 [5]Total VFAs = Total volatile fatty acid
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Table 5. Alpha diversity metrics of the rumen microbiota in Hanwoo steers with high and low methane yields.

Domain	Item	Phenotype		SEM	*p*-value	Cohen's d[1]	95% CI
		HM	LM				
Bacteria	Observed ASVs	393.500	428.000	22.828	0.686	-0.516	[-2.275, 1.243]
	Chao1	394.534	432.900	23.252	0.686	-0.568	[-2.333, 1.196]
	Shannon	7.903	8.052	0.097	0.343	-0.522	[-2.282, 1.237]
	Simpson	0.993	0.994	0.001	0.686	-0.408	[-2.156, 1.340]
Archaea	Observed ASVs	34.750	32.250	1.592	0.559	0.538	[-1.223, 2.299]
	Chao1	34.750	33.000	1.807	0.663	0.322	[-1.419, 2.064]
	Shannon	3.725[a]	3.004[b]	0.196	0.029	1.674	[-0.337, 3.685]
	Simpson	0.881	0.773	0.037	0.114	1.162	[-0.708, 3.033]

ASV, amplicon sequence variant; HM, high-methane group; LM, low-methane group; SEM, standard error of the mean; CI, confidence interval.

[1] Cohen's d represents standardized effective size between the HM and LM groups.

[a,b] Means with different superscripts within a row differ significantly ($p < 0.05$).

All samples from both HM and LM groups showed ≥ 99.8% Good's coverage.

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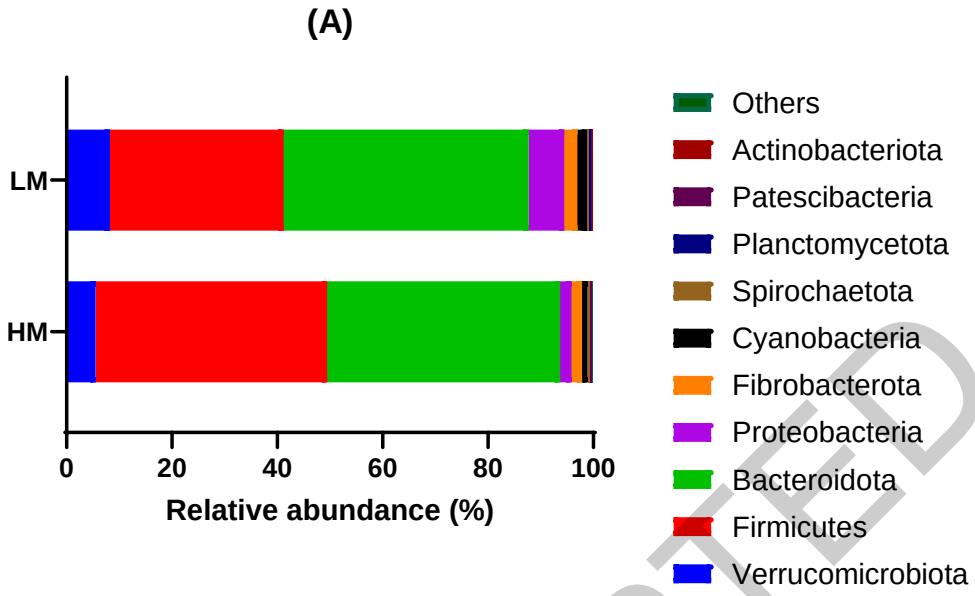
600 **Figures**



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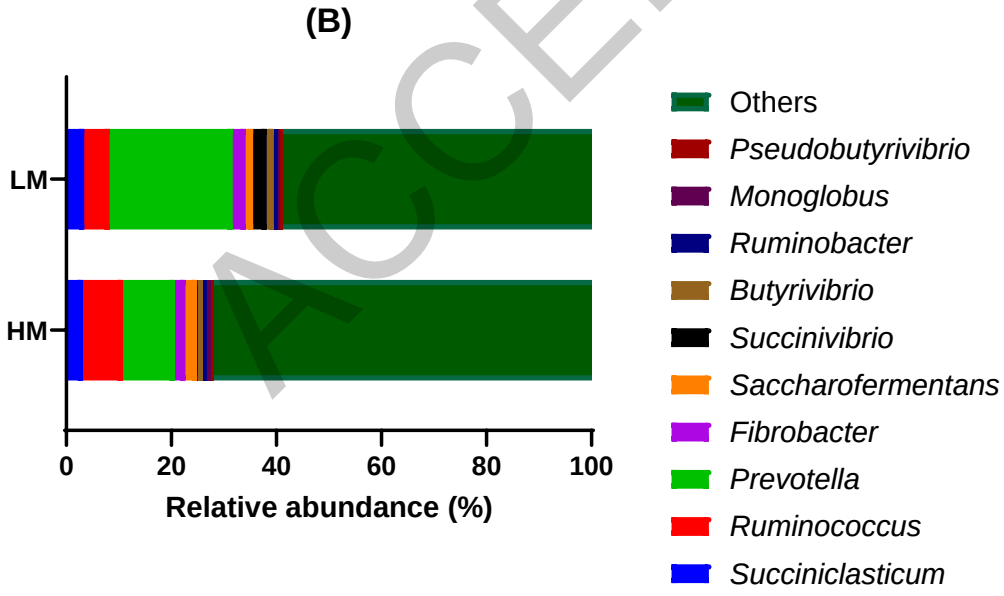

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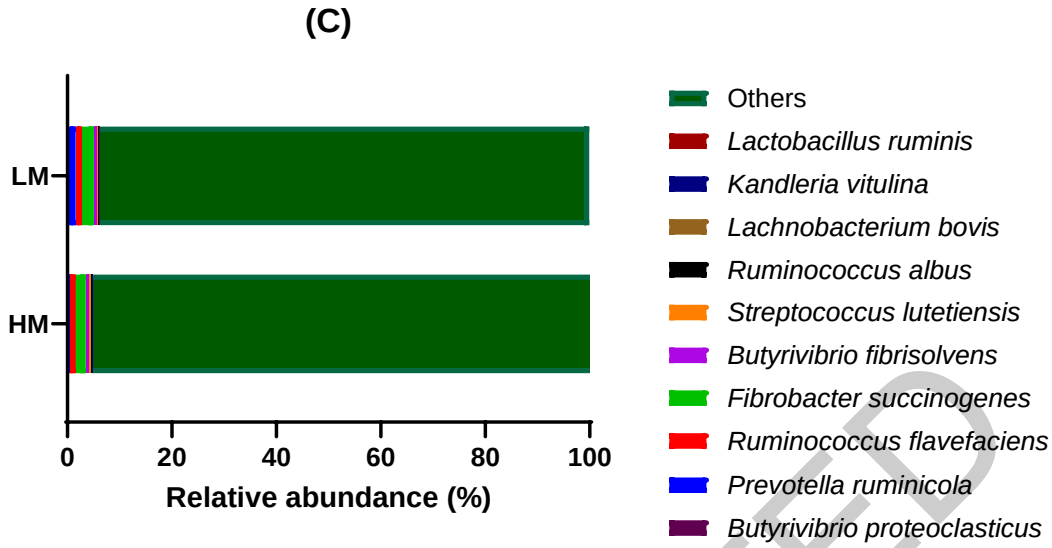


Fig. 1. Relative abundance of bacterial phyla (A), genera (B), and species (C) in Hanwoo steers with high and low methane yields. For each taxonomic level, only the 10 most abundant are shown, and all remaining taxa were collectively grouped as "Others". HM, high-methane group; LM, low-methane group.

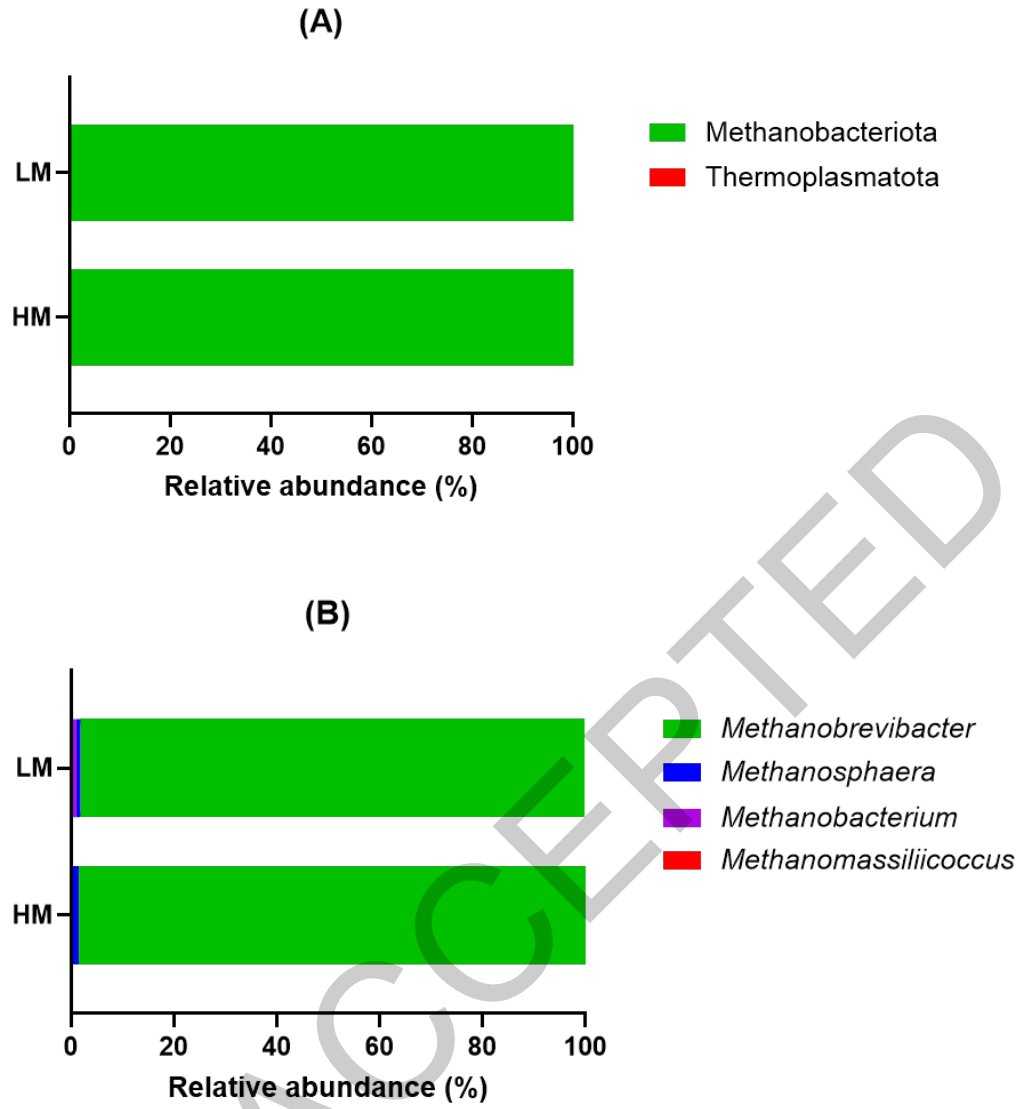
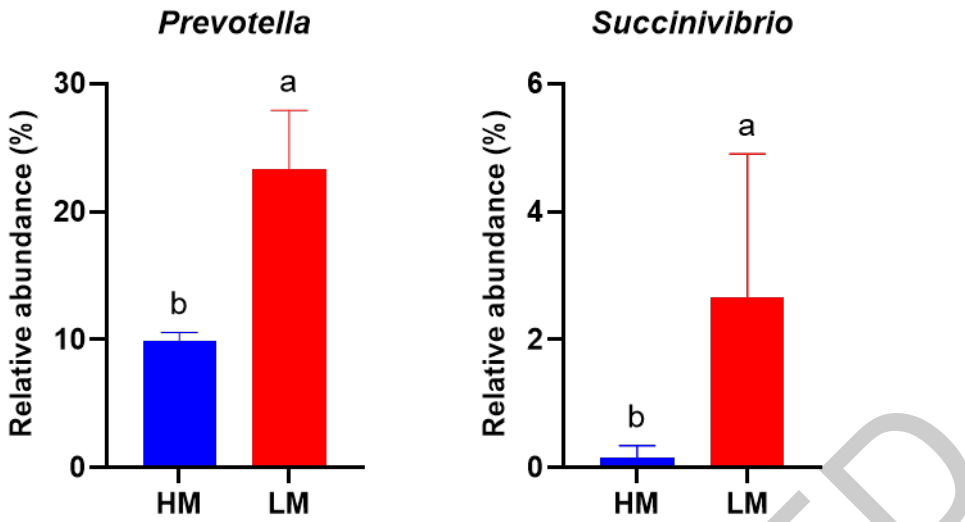


Fig. 2. Relative abundance of archaeal phyla (A), and genera (B) in Hanwoo steers with high and low methane yields. HM, high-methane group; LM, low-methane group.



625
626 **Fig. 3. Differentially abundant taxa in Hanwoo steers with high (HM) and low (LM) methane**
627 **yields.** Significant differences in the relative abundances of *Prevotella* and *Succinivibrio* were
628 identified using ANCOM-BC analysis. Means with different lowercase letters (a, b) indicate
629 significant differences between the HM and LM groups ($q < 0.05$).
630

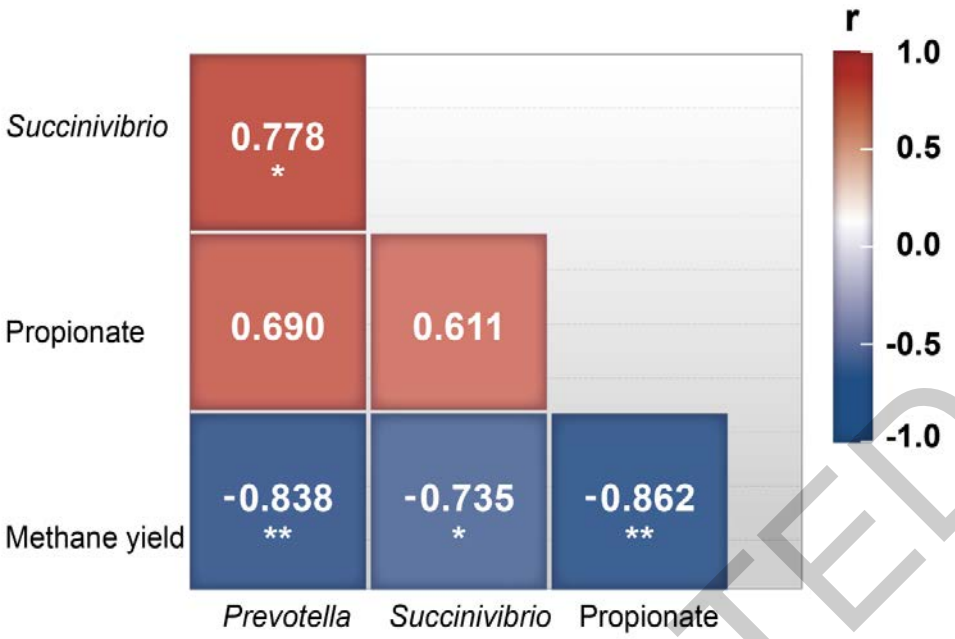
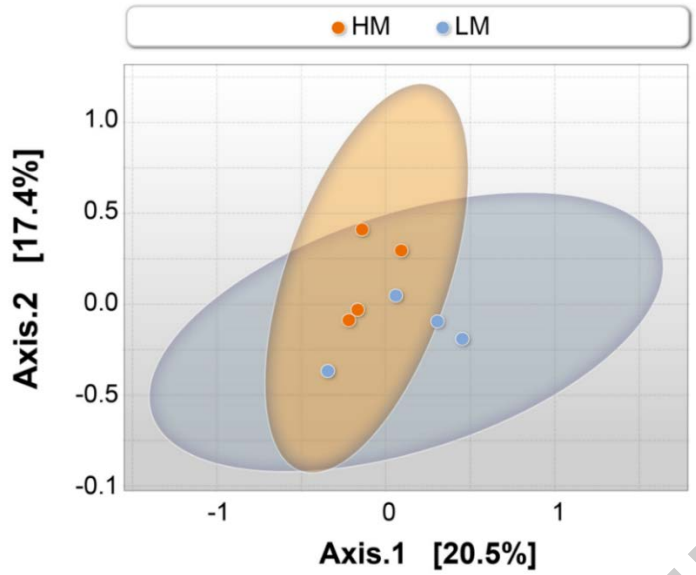


Fig. 4. Spearman correlation heatmap showing only significant correlations ($p < 0.05$) among selected bacterial genera, fermentation parameters, and methane yield. Non-significant correlations are not shown. Significance levels are indicated as $p < 0.05$ (*), and $p < 0.01$ (**).

(A)



(B)

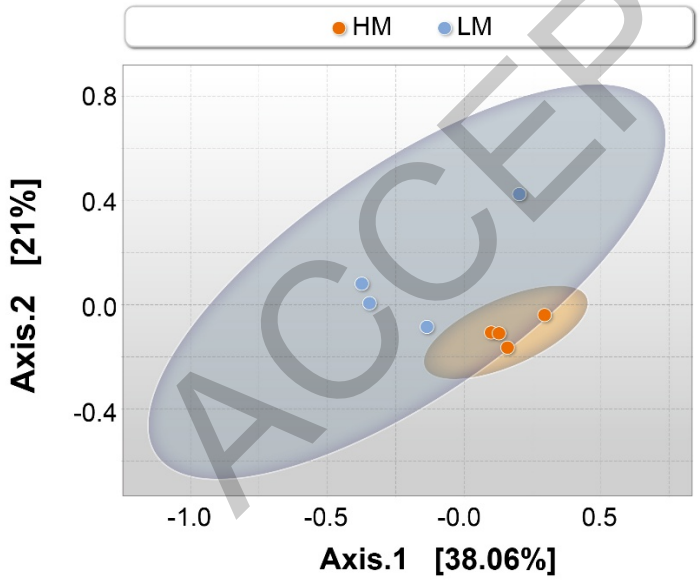


Fig. 5. Principal coordinates analysis (PCoA) plot based on Bray–Curtis dissimilarity matrix of bacterial (A) and archaeal (B) communities. No clear separation between the high-methane-yield (HM) and low-methane-yield (LM) groups was observed in the bacterial or archaeal communities ($p > 0.05$). Each point represents an individual sample, with red indicating the HM group and blue indicating the LM group.

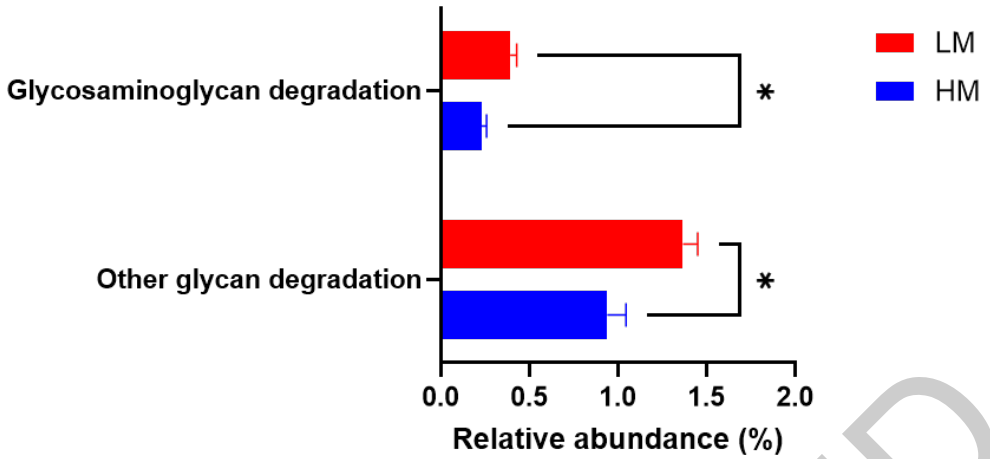


Fig. 6. Predicted functional features in Hanwoo steers with low (LM) and high (HM) methane yields. The relative abundance of glycan degradation pathways was significantly greater in the LM group. Asterisks (*) indicate significant differences between groups (ANCOM-BC, $q < 0.01$).

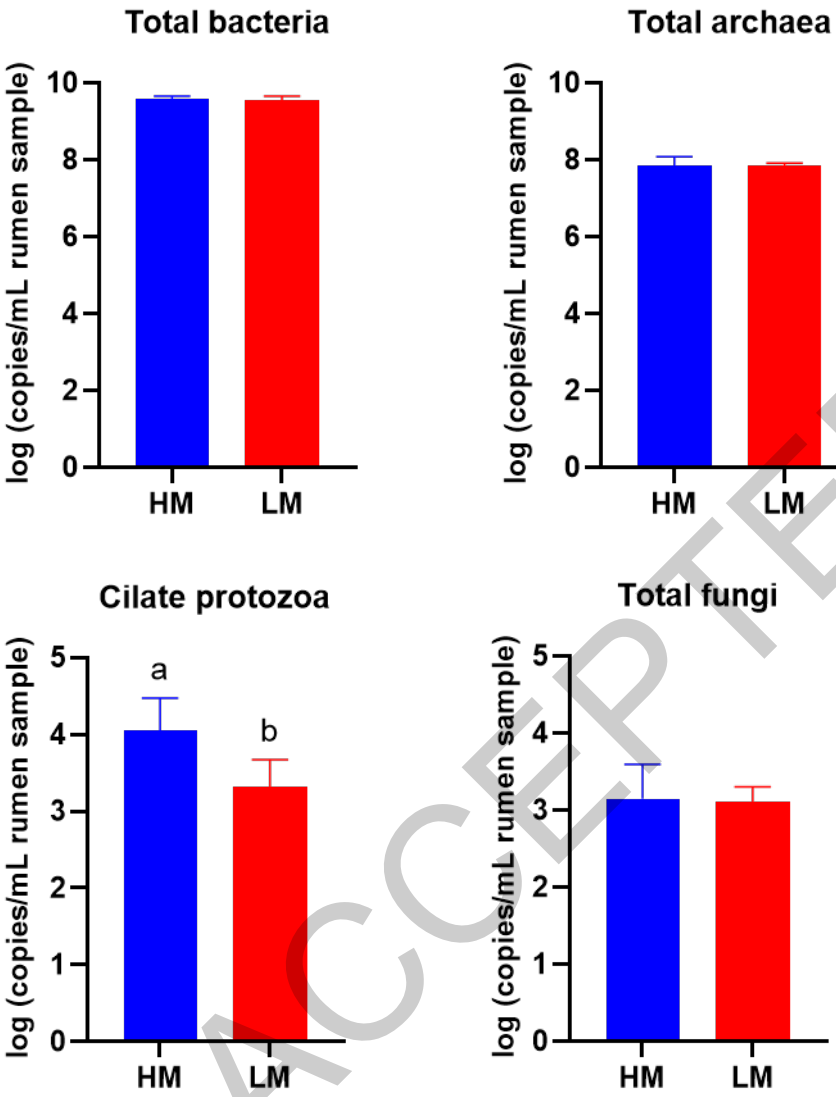


Fig. 7. Absolute abundance of rumen microbial populations quantified using real-time PCR in Hanwoo steers with high (HM) and low (LM) methane yields. Means with different lowercase letters (a, b) indicate significant differences between the HM and LM groups ($p < 0.05$).