JAST (Journal of Animal Science and Technology) TITLE PAGE

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ARTICLE INFORMATION	Fill in information in each box below
Article Type	Research article
Article Title (within 20 words without abbreviations)	Crude protein levels adjusted by Distiller's dried grains with solubles reshape rumen microbiota in Hanwoo steers during early fattening period
Running Title (within 10 words)	DDGS-adjusted CP alters rumen microbiota structure
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Competing interests	No potential conflict of interest relevant to this article was reported.
Funding sources State funding sources (grants, funding sources, equipment, and supplies). Include name and number of grant if available.	This study was supported by the Cooperative Research Program for Agriculture Science and Technology Development (Project No. RS-2020-RD009319), Rural Development Administration, Republic of Korea. The funders played no role in this study or in the preparation of the manuscript.
Acknowledgements	Not applicable.
Availability of data and material	Full-length 16S rRNA sequences were deposited in the NCBI SRA under BioProject PRJNA1244067. All datasets analyzed in the present study are available from the corresponding author upon request.
Authors' contributions Please specify the authors' role using this form.	Conceptualization: Seo JK, Seo SW. Data curation: Kim HB. Formal analysis: Kim HB, Cho HJ. Methodology: Kim HB, Cho HJ. Software: Kim HB. Validation: Moon JB, Park TS. Investigation: Moon JB. Writing - original draft: Kim HB. Writing - review & editing: Seo JK, Seo SW, Cho HJ, Park TS, Moon JB, Kim HB.
Ethics approval and consent to participate	The use of animals and protocols for this experiment was reviewed and approved by the Chungnam National University Animal Research Ethics Committee (202103A-CNU-027).

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Abstract

Distiller's dried grains with solubles (DDGS), a by-product typically used in cattle diets as a protein source, is a promising feed ingredient for CH_4 mitigation because of its high ether extract level. This study investigated how increasing levels of crude protein (CP) adjusted by DDGS affect rumen microbiota in relation to CH_4 mitigation and animal productivity. A total of 24 Hanwoo steers in the early fattening period (16 months old, 504 ± 33.0 kg) were studied over 16 weeks, including a 2-week adaptation period. Tall fescue hay and four concentrate mixes were used as experimental diets: 15% CP in dry matter (DM) (low CP [LCP]), 18% CP (lower middle CP [LMCP]), 19% CP (higher middle CP [HMCP]), and 21% CP (high CP [HCP]). The rumen microbiota was analyzed using full-length 16S rRNA sequencing. Dietary CP levels did not significantly alter alpha diversity, whereas beta diversity, based on the weighted UniFrac distance, showed clear separation between the LCP and HCP groups. Among the major taxa, 18 bacterial taxa were differentially abundant among the treatments, and 22 showed linear associations with dietary CP levels. Notably, *Selenomonas dextrinosolvens*, the unclassified species Gammaproteobacteria, *Ruminobacter amylophilus*, and *Lentimicrobium saccharophilum* exhibited a negative linear association with dietary CP levels, whereas the unclassified species Bacteroidales and *Eubacterium ventriosum* showed a positive linear association. The predicted KEGG modules related to amino acid biosynthesis were enriched in LCP compared to HCP and were negatively associated with increasing CP levels. The unclassified species Gammaproteobacteria and *L. saccharophilum* were negatively correlated with one or more CH_4 measurements. Dietary CP levels adjusted by DDGS supplementation influenced shifts in rumen bacterial composition and had functional implications in Hanwoo steers during the early fattening period. Specifically, certain bacterial taxa related to lipid metabolism and predicted metabolic functions related to amino acid biosynthesis were linearly associated with dietary CP levels as adjusted by DDGS. This study provides a valuable insight into how dietary CP levels adjusted by DDGS shape rumen microbial communities and their predicted functional profiles, with potential implications for optimizing CP utilization in Hanwoo steers.

Keywords: Crude protein; DDGS; Rumen microbiota; Methane mitigation; Animal productivity, Hanwoo steer

Introduction

Hanwoo is a native breed of Korean cattle renowned for its superior meat quality, particularly marbling (1). To produce high-quality meat, the Hanwoo beef industry generally utilizes long-term fattening periods, which can be divided into two periods: an early fattening period (at 13–21 months of age) and a late fattening period (at 22–29 months of age) (2). The emerging focus on shortening the fattening period is driven by several factors, including reduction in GHG emissions, lower production costs, and shifts in consumption trends (3). The early fattening period is a crucial stage marked by significant muscle development and a notable increase in the average daily gain (ADG) (2). Several studies have reported improvements in growth performance with a high-crude protein (CP) diet. Xia, Rahman (4) reported a significant positive effect on ADG and gain-to-feed ratio in Holstein bulls. In addition, Jeong, Seong (5) reported improved ADG and carcass quality grade in late-fattening Hanwoo steers fed high levels of CP.

Enteric methane (CH_4) emissions from ruminants represent both an environmental concern and a loss of feed energy, thereby reducing production efficiency in cattle (6-8). Consequently, dietary strategies have been explored to mitigate CH_4 emissions while improving animal performance. Among these, distiller's dried grains with solubles (DDGS), a by-product of the bioethanol industry, have gained attention due to their high level of CP content (an average of 30%) and ether extract (EE) content (7–17%), which may influence rumen fermentation and microbial composition (9-12). Several previous studies have evaluated the potential CH_4 mitigation potential of including DDGS at up to 40% DDGS in ruminant rations and consistently reported a reduction in CH_4 production (13-16). Taken together, these findings suggest that supplementation of ruminant rations with DDGS is a promising strategy for CH_4 mitigation in animal husbandry.

The rumen is an extremely anaerobic environment in which digestion and fermentation of ingested feed occur and are orchestrated by diverse rumen microbiota. Dietary changes can alter rumen microbiota and fermentation patterns (17, 18). Therefore, the investigation of shifts in rumen microbiota using high-throughput sequencing techniques plays a pivotal role in linking our understanding of the relationship between dietary alterations and growth performance. Although there is substantial evidence supporting the reduction of CH_4 through the inclusion of DDGS in ruminant diets, there remains a scarcity of data regarding the influence of DDGS and elevated dietary protein levels on CH_4 production and the composition of rumen microbiota.

A previous feeding trial showed that increasing dietary CP levels through DDGS supplementation improved ADG and reduced CH_4 yield (ppm/kg of dry matter intake [DMI]) and CH_4 intensity (ppm/kg of ADG) during the early fattening period in Hanwoo steers (19). Based on these findings, we hypothesized that increasing CP levels adjusted by DDGS would alter rumen microbial composition and associated metabolic functions, potentially contributing to the observed changes in CH_4 and ADG. Therefore, the objective of this study was to evaluate the effects of increasing dietary CP levels in the concentrate mix on rumen microbiota and their predicted functional profiles using full-length 16S rRNA sequencing.

Materials and Methods

Animals, diets, and procedures

The use of animals and protocols for this experiment were reviewed and approved by the Chungnam National University Animal Research Ethics Committee (202103A-CNU-027). The feeding trial was conducted at the Center for Animal Science Research, Chungnam National University, Korea, and detailed information regarding animal management, chemical analysis of feed ingredients, and details of sample collection have been reported previously (19). Briefly, 24 Hanwoo steers in the early fattening period (16 months old, 504 ± 33.0 kg of BW) were used in this experiment. Steers were grouped according to their initial BW and breeding values for carcass weight and arranged in a completely randomized block design (20). The feeding experiment lasted for 16 weeks, including a 2-week adaptation period. Tall fescue hay and four concentrate mixes were used as the experimental diets. The experimental concentrate mixes comprised four different levels of CP: 15% CP of dry matter (DM) (low CP [LCP]), 18% CP of DM (lower middle CP [LMCP]), 19% CP of DM (higher middle CP [HMCP]), and 21% CP of DM (high CP [HCP]). Diets were formulated to meet nutrient requirements according to Korean feeding standards for Hanwoo steers during the early fattening period (2). The CP content was adjusted primarily through DDGS supplementation, which inherently alters both protein and lipid content. To minimize differences in dietary lipid levels, hydrogenated fat was added to the formulations, resulting in comparable EE content across experimental concentrate mixes. The diet formulation and estimated chemical composition of the experimental diets are presented in Table 1 and Supplemental Table 1, respectively. All Hanwoo steers were fed concentrate diets twice daily at 08:00 and 18:00, while forage and drinking water were provided ad libitum throughout the experiment.

Collection of rumen fluid and DNA extraction

Rumen fluid was collected three times on three consecutive days (day 1, 14:00; day 2, 11:00; and day 3, 07:00) using an oral stomach tube (21). Briefly, the initially obtained rumen fluid (approximately 200 mL) was discarded, and 500 mL of rumen fluid was collected. The collected rumen fluid was transferred to the laboratory for further analysis. Following this, the rumen fluid (2 mL) was centrifuged at 20,000 × g for 20 min at 4°C, and the remaining pellet was stored at -80°C until DNA extraction. Microbial genomic DNA was extracted according to the manufacturer's protocol (QIAamp Fast DNA Stool Kit, Hilden, Germany), with a bead-beating step (22). Briefly, the ruminal pellet was mixed with 1 mL of InhibitEX buffer and sterile zirconia beads (0.3 g of 0.1 mm and 0.1 g of 0.5 mm). The mixture was then homogenized for 20 min using a Vortex-Genie 2 homogenizer (Scientific Industries Inc., Hohemia, NY, USA). The samples were heated at 70 ℃ for 5 minutes and subsequently at 95 ℃ for 5 min. The remaining steps were performed according to the manufacturer's recommendations. After DNA extraction, quantity and quality were measured using a NanoDrop (ND-200, Allsheng, Hangzhou, China). The purified DNA was stored at -20 °C until 16S rRNA gene sequencing.

Full-length 16S rRNA gene sequencing

Sequencing libraries were prepared following the PacBio amplicon template preparation and sequencing protocols to amplify the region between 27F and 1492R within the 16S rRNA gene, using a primer pair with symmetric barcoded adapters (27F: 5′-AGRGTTYGATYMTGGCTCAG-3′ and 1492R: 5′-RGYTACCTTGTTACGACTT-3′) (23, 24). The first amplification was carried out with an initial denaturation at 94 °C for 5 min, followed by 35 cycles consisting of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 90 s, concluding with a final elongation at 72 °C for 5 min. The resulting amplicons were purified using AMPure beads (Agencourt Bioscience, Beverly, MA, USA). DNA concentration was measured using Quant-IT PicoGreen (Invitrogen), and its quality was evaluated with a TapeStation D5000 Screen Tape (Agilent Technologies, Waldbronn, Germany). For PacBio Sequel library preparation, pooled amplicon DNA (500 ng) was used. Library preparation was performed using a PacBio SMRTbell Expert Template Prep Kit 2.0 (Pacific Biosciences, Menlo Park, CA, USA). SMRTbell templates were annealed using Sequel II Bind Kit 2.1 (Pacific Biosciences, Menlo Park, CA, USA). The Sequel II Sequencing Kit 2.0 (Pacific Biosciences, Menlo Park, CA, USA) and SMRT cells 8M Tray (Pacific Biosciences, Menlo Park, CA, USA) were used for sequencing. The data per sample were captured for each SMRT cell with a 10 h movie time on the PacBio Sequel sequencing platform by Macrogen (Seoul, Korea).

The following procedures adhered to the PacBio Sample Net-Shared Protocol, which can be accessed at http://pacificbiosciences.com/.

Metataxonomic analysis of rumen microbiota

Barcode sequences were trimmed using Cutadapt (Version 4.1) (25). A general metataxonomic analysis was performed based on Quantitative Insights into Microbial Ecology 2 (QIIME2, Version 2024.2) (26). Briefly, the divisive amplicon denoising algorithm 2 (DADA2) plugin was used to remove primer sequences, denoise low-quality reads (Q score < 25), and eliminate chimeric sequences (Supplemental Table 2). Amplicon sequence variants (ASVs) were taxonomically classified based on the National Center for Biotechnology Information (NCBI) 16S rRNA gene database, which was constructed following the RESCRIPt protocol (27). Alpha diversity, including the Chao1 estimate, observed ASVs, Faith's phylogenetic diversity, evenness, and Shannon and Simpson indices, was assessed based on the rarefied ASV tables using randomly selected 1,263 ASVs per sample. The rarefaction depth was selected based on sequencing depth distribution across samples to retain sufficient reads while minimizing sample loss, and was confirmed to be adequate for capturing rumen microbial diversity as indicated by the saturation of rarefaction curves (Supplemental Figure 1). The overall ruminal microbiota shaped by different levels of dietary CP was compared using principal coordinate analysis (PCoA) based on unweighted and weighted UniFrac distance matrices and visualized using the Plotly package in R (version 4.2.1). The number of shared and unique bacterial taxa at the phylum and species levels were visualized using the VennDiagram package in R (version 4.2.1). For taxonomic analysis, only taxa present in ≥ 30% of all rumen samples were defined as core bacterial taxa and were primarily evaluated in this study.

Prediction of functional profiles

Functional profiles of the rumen microbiota were inferred from full-length 16S rRNA gene sequences using phylogenetic investigation of communities by reconstruction of unobserved states 2 (PICRUSt2, version 2.5.0) (28). Predictions were based on ASV-level data and the corresponding feature (biological observation) table. Subsequently, the predicted metabolic functions were mapped to the Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs and modules using the manually curated KEGG BRITE database. Principal component analysis (PCA) was conducted to assess differences in predicted KEGG orthologs based on Bray–Curtis dissimilarities across varying dietary CP levels and was visualized using the ggfortify package in R (29).

Statistical analysis

The examination of variable normality was conducted using the Shapiro–Wilk test using the UNIVARIATE procedure in SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Data from the metataxonomic analysis, which did not exhibit a normal distribution after several transformations, including log, square root, and arcsine, were analyzed using the non-parametric Kruskal–Wallis rank sum test, utilizing the Kruskal–Wallis test function in the stats package in R (version 4.2). If a statistical trend was observed (Kruskal–Wallis $P < 0.10$), Dunn's post hoc test with the Benjamini–Hochberg adjustment (false discovery rate [FDR]-*P*) using the DunnTest function in the FSA package was conducted. Microbiome multivariate association with linear models (MaAsLin2) was performed using compound Poisson linear models with default parameters to assess the linear association of microbiota and KEGG modules with increasing CP levels (30). Permutational multivariate analysis of variance (PERMANOVA) was used to analyze the statistical differences in the PCoA and PCA results among treatments with 9,999 random permutations in QIIME2 and R, respectively. Spearman correlation coefficients between rumen microbiota and animal performance including growth, rumen fermentation, blood metabolite, and CH_4 parameters reported in the previous study (19) were computed using the microbiome package in R and visualized using the ggplot2 package in R. Statistical significance was set at $P < 0.05$, and a statistical tendency was considered at $0.05 \leq P \leq 0.10$.

Results

Changes in animal performance

Growth performance and feed intake were obtained from the associated feeding trial (19). Briefly, DMI, including both concentrate and forage, did not differ significantly among treatments ($P > 0.10$), whereas crude protein intake increased linearly with increasing dietary CP levels ($P < 0.001$). In addition, increasing dietary CP levels through DDGS supplementation tended to increase ADG ($P = 0.068$) and significantly decreased CH_4 yield (ppm/kg DMI; $P = 0.014$) and CH_4 intensity (ppm/kg ADG; $P = 0.008$).

Changes in alpha and beta diversity by dietary crude protein level

One steer in the LMCP group was excluded from the experiment because of health-related issues. In total, 225,648 reads were obtained from 23 Hanwoo steers, with an average of 9,811 ± 3,215 reads per sample (Supplemental Table 2). After denoising and quality filtering based on the DADA2 plugin, an average of 4,097 ± 1865 reads were retained, with a total of 94,222 reads (Supplemental Table 2), which resulted in greater than 95.3%

of Good's coverage for all samples (data not shown). The rarefaction curves of alpha diversity measurements, including Chao1, observed ASVs, Faith's phylogenetic diversity, evenness, Shannon index, and Simpson index, almost approached the saturation plateau, indicating that the sequence depth for rarefaction was adequate (Supplemental Figure 1). Various levels of dietary CP did not lead to notable alterations in any of the alpha diversity measurements of rumen microbiota (Figure 1 and Table 2). PCoA based on the weighted UniFrac distance demonstrated a significant impact of the dietary CP level on rumen microbiota (Figure 2B, *P* = 0.0293), especially between the LCP and HCP groups (pairwise comparison, *Q*-value = 0.0132), whereas there were no significant differences based on the unweighted UniFrac distance (Figure 2A, *P* = 0.1976).

Bacterial community composition

The shared and unique microbiota at the phylum and species levels are shown in Figure 3 (A and B). At the phylum level, eight phyla were common to all treatments, whereas unique phyla were observed exclusively in the LCP and HCP groups (Figure 3A). At the species level, 50 of the 112 species (44.6%) were shared among all treatments. The number of unique species observed exclusively in the LCP, LMCP, HMCP, and HCP groups was six, eight, six, and seven, respectively. However, none of these unique species were considered part of the core microbiota, as their occurrence was below 30% across the samples (Figure 3B).

Figure 3 (C and D) shows the distribution of major core phyla and core species present in ≥30% of samples, with an average relative abundance greater than 0.5%. Regardless of the dietary treatment, the most dominant phylum was Bacteroidota, comprising an average of 76% of each community, while Bacillota was the second most abundant phylum, representing an average of 16.4% (Figure 3C). At the species level, 47 of the 112 species were identified as core species, representing an average of 96.7% of the community (Figure 3D). *Prevotella ruminicola* and *Prevotella brevis* were the predominant species, constituting approximately 50% of the total population (Figure 3D).

Changes in rumen microbiota by dietary crude protein level

Eighteen bacterial taxa were identified as differentially abundant between the dietary CP levels using the Kruskal–Wallis test with Dunn's post hoc analysis, demonstrating at least a statistical tendency (Figure 4A and Supplemental Table 3). At the phylum level, Bacteroidota showed a significantly higher relative abundance in the HCP group than in the LCP group (Figure 5B, *P* = 0.0433), whereas Pseudomonadota exhibited a significantly higher relative abundance in the LCP group than in the HCP group (Figure 4B, *P* = 0.0129). The relative abundance of the Bacteroidales lineage from the class to the species level was significantly enriched in the HCP group, whereas the *Succinivibrio dextrionosolvens* lineage from the phylum to the species level was significantly enriched in the LCP group (Figure 4 and Supplemental Table 3).

A linear association between rumen microbiota and dietary CP levels was identified using MaAsLin2 (Figure 5). The analysis detected 23 linear associations from the phylum to the species level, demonstrating a statistical tendency (Figure 5A and Supplemental Table 4). At the phylum level, the relative abundance of Bacteroidota increased significantly with increasing dietary CP levels (Figure 5B; *Coefficient* = 0.070, *FDR-P* = 0.0491). Conversely, the Pseudomonadota and Bacillota to Bacteroidota ratios exhibited opposite associations (Figure 5B; Pseudomonadota, *Coefficient* = -0.731 and *FDR-P* = 0.0361; Bacillota to Bacteroidota ratio, *Coefficient* = -0.320, and *FDR-P* = 0.0275).

At the species level, three species within the class Gammaproteobacteria (*S. dextrinosolvens*, unclassified species [US_] Gammaproteobacteria, and *Ruminobacter amylophilus*) and *Lentimicrobium saccharophilum* exhibited a negative linear association with dietary CP levels (Figure 5B; *Coefficient* = -0.730, -0.801, -1.276, and -0.821, and FDR-*P* = 0.0361, 0.0927, 0.0698, and 0.0768, respectively). In contrast, US_Bacteroidales, *Eubacterium ventriosum*, and *Anaeroplasma abactoclasticum* showed positive linear associations (Figure 5B; *Coefficient* = 0.284, 0.947, and 1.269; *FDR-P* = 0.0361, 0.0698, and 0.0927, respectively).

Differences in predicted functional profiles

To compare the differences in overall bacterial function across different dietary CP levels, functional profiles were predicted based on full-length 16S rRNA gene sequencing data using PICRUSt2. PCA based on the KEGG ortholog levels showed a statistical tendency among the dietary treatments (Figure 6A, PERMANOVA, *P* = 0.0712), revealing distinct clusters between the LCP and HCP treatments (Figure 6A, pairwise comparison, Pseudo-*F* = 4.0401 and *Q* = 0.0236). At the KEGG module level, 15 KEGG modules were differentially abundant among the dietary treatments (Figure 6B, *P* < 0.05). Of the 15 differentially abundant KEGG modules, seven were related to amino acid metabolism, three to carbohydrate metabolism, and five to energy metabolism. In the amino acid metabolism, seven KEGG modules (M00015, Proline biosynthesis, glutamate to proline; M00025, Tyrosine biosynthesis, chorismite to HPP to tyrosine; M00040, Tyrosine biosynthesis, chorismite to arogenate to tyrosine; M00029, Urea cycle; M00034, Methionine salvage pathway; M00134, Polyamine biosynthesis, arginine to ornithine to putrescine and M00948, Hydroxyproline degradation, trans-4-hydroxy-L-proline to 2-oxoglutarate) were significantly enriched in the LCP group compared to the HCP group (Figure 6B). Among the three differentially abundant KEGG modules related to carbohydrate metabolism, M00307 (Pyruvate oxidation, pyruvate to acetyl-CoA) was significantly lower in the LCP group than in the other groups, whereas two modules (M00373, Ethylmalonyl pathway, and M00741, Propanoyl-CoA to succinyl-CoA) exhibited significant differences between the HMCP and HCP treatments (Figure 6B). In energy metabolism, two KEGG modules (M00149, Succinate dehydrogenase and M00144, NADH:quinone oxidoreductase) were significantly enriched in the HCP group, whereas M00374 (dicarboxylate-hydroxybutyrate cycle) was significantly lower in the LCP group than in the other groups (Figure 6B). Additionally, two KEGG modules (M00150, Fumarate reductase, and M00595, Thiosulfate oxidation by SOX complex, thiosulfate to sulfate) were enriched in the LCP group (Figure 6B).

MaAsLin2 analysis showed that 13 significant linear associations were identified between the KEGG modules and increased dietary CP levels (Table 3). Among these, seven were associated with amino acid metabolism, two with carbohydrate metabolism, and four with energy metabolism. Seven KEGG modules related to amino acid metabolism (M00040, Tyrosine biosynthesis, chorismate to arogenate to tyrosine; M00136: GABA biosynthesis, prokaryotes, putrescine to GABA; M00134, Polyamine biosynthesis, arginine to ornithine to putrescine; M00025, Tyrosine biosynthesis, chorismate to HPP to tyrosine; M00034, Methionine salvage pathway; M00029, Urea cycle; and M00021, Cysteine biosynthesis, serine to cysteine) showed a negative association with increasing dietary CP levels. In contrast, two KEGG modules related to carbohydrate metabolism (M00307, Pyruvate oxidation, pyruvate to acetyl-CoA; M00532, Photorespiration) were positively associated with increasing dietary CP levels. Among the identified KEGG modules related to energy metabolism, M00150 (Fumarate reductase) was negatively associated with increasing levels of dietary CP, whereas three KEGG modules (M00149, Succinate dehydrogenase; M00144, NADH:quinone oxidoreductase, prokaryotes; and M00153, Cytochrome bd ubiquinol oxidase) were positively associated.

Correlation between the rumen microbiota and animal performance

Correlation analysis using Spearman’s correlation was conducted between animal performance metrics and 11 bacterial species that showed differential abundance or a linear relationship. Various strong relationships (Coefficient $|\rho| > 0.4$ and $P < 0.10$) were identified (Figure 7). In terms of growth performance, US_Bacteroidales were positively correlated with total and concentrated CP intake, whereas *S. dextrinosolvens* and *R. amylophilus* were negatively correlated. Additionally, US_Bacteroidales and *Anaeroplasma abactoclasticum* were positively correlated with ADG. Regarding rumen fermentation parameters, ammonia nitrogen (NH_3-N) was positively correlated with US_Bacteroidales and *A. abactoclasticum*, but negatively correlated with *S. dextrinosolvens*, *L. saccharophilum*, and *R. amylophilus*. In the volatile fatty acid (VFA) profiles, the propionate proportion was negatively correlated with US_Bacteroidales and *Treponema bryantii* and positively correlated with *S. dextrinosolvens*, whereas the acetate-to-propionate ratio showed the opposite correlation. The butyrate proportion was negatively correlated with *S. dextrinosolvens*, US_Gammaproteobacteria, *L. saccharophilum*, and *R. amylophilus*, and positively correlated with US_Bacteroidales. Among the blood metabolites, blood urea nitrogen (BUN) was negatively correlated with *S. dextrinosolvens*, *L. saccharophilum*, and *R. amylophilus* and positively correlated with US_Bacteroidales. Non-esterified fatty acids were negatively correlated with US_Bacteroidales and *T. bryantii*, and positively correlated with *S. dextrinosolvens*. US_Bacteroidales, US_Christensenellaceae, and *A. abactoclasticum* negatively correlated with one or more CH_4 concentrations, whereas *S. dextrinosolvens*, US_Gammaproteobacteria, *L. saccharophilum*, and *R. amylophilus* were positively correlated.

Discussion

The growing emphasis on sustainable livestock production has prompted the exploration of various feed ingredients capable of mitigating CH_4 emissions, which are a major GHG. DDGS, a byproduct of the bioethanol industry, has garnered attention in this context because of its high CP and EE contents, leading to a potential dual benefit: optimizing CP content while simultaneously reducing CH_4 emissions (11, 12). In a previous feeding trial conducted by our colleagues, it was noted that increasing CP levels through DDGS supplementation resulted in increased ADG levels and a concurrent decrease in CH_4 yield (ppm/kg of DMI) and CH_4 intensity (ppm/kg of ADG) from eructation (19). Importantly, DMI was similar across treatments in the feeding trial, indicating that the observed differences were primarily driven by dietary CP levels rather than feed intake variation. In the present study, we identified bacterial taxa potentially associated with both ADG enhancement and CH_4 reduction, resulting from increased CP levels modulated by DDGS supplementation.

In the present study, we observed that increasing the levels of CP through supplementation with up to 29.4% DDGS in a concentrate mix did not result in alteration of the alpha diversity in the rumen microbiota of Hanwoo steers during the early fattening period. This finding is consistent with several previous studies involving DDGS, which also reported no significant differences in rumen microbiota richness and diversity across various ruminant species, including beef cattle (31, 32), dairy cattle (33), and lambs (34, 35). In terms of beta diversity, our analysis revealed a significant separation between the LCP and HCP groups when evaluated using a weighted UniFrac distance matrix, whereas no significant differences were observed using the unweighted UniFrac distance matrix. In contrast to our findings, previous studies reported no notable separation or differences when DDGS was included in up to 50% of diets (33-35). A possible explanation for this discrepancy may be related to differences in dietary CP levels. While previous studies increased DDGS inclusion while maintaining similar CP levels among treatments, the present study increased CP levels by adjusting the proportion of DDGS in the diet. Therefore, the observed differences in microbial community structure may be attributed to the combined effects of both CP level and DDGS inclusion, and their individual contributions cannot be fully distinguished in this experimental design. The divergence in findings between weighted and unweighted UniFrac distances is also noteworthy. Generally, weighted UniFrac distance accounts for both the presence or absence and relative abundance of microbiota, whereas unweighted UniFrac distance considers only the presence or absence of these microbial structures (36). This observation suggests that the differences in the overall structure of rumen microbiota between the LCP and HCP groups were predominantly driven by variations in the relative abundance of certain bacterial taxa. These findings imply that bacterial populations with higher abundances play a significant role in differentiating rumen bacterial communities. Biologically, this suggests that the varying CP levels primarily influence the relative abundance of established taxa rather than altering the overall phylogenetic composition, reflecting changes in the activity of core microbiota under different CP levels adjusted by DDGS supplementation.

Dietary shifts can affect rumen bacterial communities, which are influenced by their preference for various nutrient sources, including different types of carbohydrates (structural carbohydrates vs. non-fiber carbohydrates [NFC]), lipids (saturated fatty acids vs. unsaturated fatty acids), and CP (37-39). Despite dietary variations, Bacteroidota and Bacillota remained the core phyla in the rumen and are dominant regardless of the diet type (40-42). Consistent with previous findings, these phyla constituted at least 86% of the rumen samples in this study. An increase in CP levels adjusted by DDGS significantly increased Bacteroidota levels and reduced the Bacillota to Bacteroidota ratio. This aligns with previous studies reporting similar trends with varying levels of DDGS inclusion (43, 44). However, there are inconsistencies in the responses of these phyla to different DDGS levels; some studies observed no differences up to 25% DDGS inclusion (31, 32), whereas others reported opposite trends to our findings at 20% inclusion (33). Castillo-Lopez et al. (35) suggested that varying DDGS levels might induce changes in microbial composition by affecting diet composition, particularly crude fat and starch content. Interestingly, the phylum Pseudomonadota and its members, including *S. dextrinosolvens*, *R. amylophilus*, and US_Gammaproteobacteria, decreased linearly with increasing CP levels. Pseudomonadota is known for its role in carbohydrate degradation in high-grain diets (45, 46), and its members, such as *S. dextrinosolvens* and *R. amylophilus* are amylolytic and produce succinate via glucose metabolism (47, 48). Typically, succinate is rapidly converted to propionate in the rumen (49, 50), as evidenced by a linear decrease in propionate levels in a previous feeding trial (19). Despite the linear decreases in Succinivibrionaceae species and propionate, the CH_4 yield (ppm/kg of DMI) and CH_4 intensity (ppm/kg of ADG) from eructation decreased linearly with higher CP levels adjusted by DDGS (19). This suggests that CH_4 reduction is influenced by factors other than propionate production, which is an alternative H_2 sink in the rumen. In the present study, US_Bacteroidales and *E. ventriosum* were enriched with increasing CP levels, as adjusted by DDGS. Previous research has noted that various unclassified species belonging to Bacteroidales may play a role in biohydrogenation (51). A positive correlation between conjugated linoleic acid in serum and *E. ventriosum* suggests its involvement in this process (52). DDGS, which is rich in unsaturated fatty acids, primarily linoleic acid (C18:2) and oleic acid (C18:1) (53), can mitigate CH_4 production through direct toxicity against microbiota and biohydrogenation in the rumen environment (54-57). Biohydrogenation in the rumen offers dual advantages; it acts as a detoxification mechanism and as an alternative H_2 sink for methanogenesis (54, 58). In this study, the EE content among treatments was maintained by supplementation with hydrogenated fat, focusing on the differences in fatty acid composition, particularly between saturated and unsaturated fatty acids. The high inclusion level of DDGS, leading to elevated unsaturated fatty acid levels, may have enriched US_Bacteroidales and *E. ventriosum* and potentially inhibited methanogens, thereby reducing CH_4 yield. However, as this study did not explore changes in methanogen populations, further research is needed to clarify the interactions between methanogens and biohydrogenation-related rumen microbiota, specifically US_Bacteroidales and *E. ventriosum* in CH_4 production.

The distinct bacterial community structures observed with increasing CP levels adjusted by DDGS led to significant differences in the overall predicted functional features between the LCP and HCP groups. Typically, ingested dietary proteins undergo digestion and fermentation into amino acids, peptides, and ammonia in the rumen, primarily through proteolytic bacteria (59). Consequently, higher CP levels could directly influence the functional attributes of rumen microbiota, affecting metabolic outcomes such as NH_3-N concentration in the rumen and BUN concentration. A previous feeding trial confirmed this, showing a linear increase in NH_3-N concentration in the rumen and BUN concentration with higher dietary CP levels adjusted by DDGS (19). The present bacterial functional analysis predicted using PICRUSt2 revealed significant shifts in KEGG modules related to amino acid biosynthesis, including those for methionine (M00034), tyrosine (M00025 and M00040), proline (M00015), and putrescine (M00134). Bacterial amino acid synthesis is an important microbial function that may contribute to maintaining amino acid availability under conditions of limited dietary protein. Previous study in human gut has suggested that gut microbiota can partially compensate for dietary protein deficiency through enhanced microbial amino acid synthesis (60). In this context, the increased representation of amino acid biosynthesis pathways observed in the LCP group may reflect a potential microbial adaptive response to lower CP availability. However, this interpretation should be approached cautiously, as the functional predictions are based on PICRUSt2 and the mechanisms may differ in the rumen ecosystem. Additionally, M00150 (Fumarate reductase, prokaryotes) was more prevalent in the LCP group than in the higher CP group, suggesting a decrease in fumarate-to-succinate metabolism with increasing CP levels. This reduction might be related to the decreased proportion of propionate observed in an earlier feeding trial. Moreover, the linear enrichment of M00307 (Pyruvate oxidation) and M00144 (NADH:quinone oxidoreductase) was observed. The conversion of pyruvate to acetyl-CoA in the rumen involves enzymatic reactions involving pyruvate formate lyase and pyruvate-ferredoxin oxidoreductase (61). In the pyruvate-ferredoxin oxidoreductase pathway, included in M00307, pyruvate is converted into acetyl-CoA, CO_2, and reduced ferredoxin (61). The reduced ferredoxin is reoxidized by ferredoxin—NAD^+ oxidoreductase, which generates NADH (62). NADH accumulation can inhibit microbial growth and fermentation under anaerobic conditions (63, 64). A previous study linked the enzymatic reaction of NADH dehydrogenase and the reduction of unsaturated fatty acids to NADH re-oxidation in butyrate production (65). Hence, the observed enrichment of biohydrogenation-related bacteria, especially US_Bacteroidales and *E. ventriosum*, may be associated with the linear increase in ruminal butyrate proportion in the preceding feeding trial.

Among the nine bacterial species that showed a significant correlation with at least one aspect of animal performance, US_Bacteroidales exhibited notable positive correlations with CP intake, CP-related metabolites (such as ruminal butyrate proportion and BUN), and ADG, all of which increased linearly with increasing CP levels adjusted by DDGS in the preceding feeding trial. Members of the order Bacteroidales are commonly recognized as carbohydrate degraders in anaerobic environments (66, 67). However, a recent metagenomic analysis has shown that uncultured Bacteroidales populations possess the genetic capabilities for protein degradation, suggesting that they are significant contributors as protein degraders (68, 69). Although there is no definitive evidence linking US_Bacteroidales to increased ADG in ruminants, one possible explanation is its role in enhancing the utilization of

CP and lipids. Interestingly, both US_Gammaproteobacteria and *L. saccharophilum*, which decreased linearly with increasing CP levels adjusted by DDGS, showed negative correlations with at least one CH_4 indicator. Gammaproteobacteria encompass approximately 250 genera and are associated with pathogenicity and methanotrophy (70, 71). Although Gammaproteobacteria methylotrophs have not been conclusively identified in the rumen, they have been detected in other anaerobic environments (72, 73). Additionally, *L. saccharophilum*, initially isolated from methanogenic granular sludge, can ferment glucose to acetate, formate, and hydrogen, suggesting its potential involvement in hydrogenotrophic methanogenesis (74).

The findings of this study highlight the potential of DDGS-based dietary strategies to modulate rumen microbial communities and associated metabolic functions. However, several limitations should be considered. The relatively small sample size per group (n = 5-6) may limit the statistical power to detect subtle changes, particularly among low-abundance taxa. In addition, this study relied on predicted functional profiles using PICRUSt2, which infers gene content based on 16S rRNA data and may not fully capture the actual metabolic activity of the rumen microbiome. Therefore, the functional interpretations should be considered with caution. Future studies integrating metagenomic or metatranscriptomic approaches are needed to better elucidate the mechanisms underlying CH_4 mitigation and nutrient utilization associated with DDGS supplementation.

Conclusion

In summary, varying CP levels adjusted by DDGS influenced shifts in the rumen microbiota and their functional implications in Hanwoo steers during the early fattening period. This study revealed that increasing dietary CP levels by adjusting the DDGS induced a linear increase in US-Bacteroidales and *E. ventriosum*, which are related to lipid metabolism in the rumen. These changes in bacterial community structure were accompanied by alterations in predicted metabolic functions, including key KEGG modules related to amino acid, carbohydrate, and energy metabolism. Importantly, our results demonstrate that dietary CP levels can modulate rumen microbial composition and functional potential. The positive correlation between certain bacterial taxa, such as US_Bacteroidales, and animal performance indicators, such as ADG and ruminal butyrate proportion, underscores the intricate relationship between diet, microbiota, and animal productivity. Overall, this study provides insights into how dietary CP levels adjusted by DDGS shape rumen microbial communities and their predicted functional profiles, contributing to a better understanding of diet-microbiome interactions in ruminants.

Competing Interests

No potential conflict of interest relevant to this article was reported.

Acknowledgments

Not applicable.

Author's Contributions

No potential conflict of interest relevant to this article was reported.

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Tables

Table 1. Analyzed chemical composition (g/kg DM or as stated) of experimental diets

Item[1]	Treatment[2]				Tall fescue
	LCP	LMCP	HMCP	HCP	
Chemical composition					
DM, g/kg as fed	886	886	886	886	888
OM	911	916	918	920	941
CP	147	178	193	208	69
NDICP	26	27	27	27	13
ADICP	12	13	14	15	9
aNDF	271	286	293	301	640
ADF	134	131	129	128	404
ADL	38	37	36	36	62
EE	69	62	59	56	13
Ash	89	84	82	80	59
Ca	15	13	12	10	3
P	6	6	7	7	1
NFC	450	417	400	383	232
TDN	747	740	736	733	557
NE_m, MJ/kg DM	8.1	8	7.9	7.8	5
NE_g, MJ/kg DM	5.4	5.3	5.2	5.2	2.7

[1] DM, dry matter; OM, organic matter; CP, crude protein; NDICP, neutral detergent fiber insoluble CP; ADICP, acid detergent insoluble CP; aNDF, neutral detergent fiber analyzed using a heat-stable amylase and expressed inclusive of residual ash; ADF, acid detergent fiber; ADL, acid detergent lignin; EE, ether extract; NFC, non-fiber carbohydrate; TDN, total digestible nutrients; NE_m, net energy for maintenance; NE_g, net energy for growth.

[2] LCP, concentrate mix consisting of low crude protein (CP); LMCP, concentrate mix consisting of lower-middle CP; HMCP, concentrate mix consisting of higher-middle CP; HCP, concentrate mix consisting of high CP.

Table 2. Linear association between alpha diversity measurements and dietary crude protein levels[1]

Items	Coefficient	Standard error	FDR-*P*[2]
Chao1	-0.038	0.022	
Observed ASVs	-0.015	0.016	0.336
Faith’s phylogenetic diversity	0.093	0.045	0.444
Evenness	0.132	0.108	0.336
Shannon index	0.103	0.085	0.398
Simpson index	0.124	0.113	0.398

ASV, amplicon sequence variant.

[1] Linear associations were evaluated using Microbiome multivariable association with linear models (MaAsLin2).

[2] Statistical *P* values from MaAsLin2 were adjusted using Benjamini–Hochberg adjustment (FDR-*P*).

Table 3. Significant linear associations between KEGG modules and dietary crude protein levels[1]

KEGG modules	KEGG BRITE[2] level 2	Coefficient	Standard error	FDR-*P*[2]	Description
M00025	AA metabolism	-1.596	0.5020	0.0433	Tyrosine biosynthesis, chorismate to arogenate to tyrosine
M00040	AA metabolism	-1.176	0.2899	0.0196	GABA biosynthesis, prokaryotes, putrescine to GABA
M00021	AA metabolism	-0.527	0.1185	0.0122	Polyamine biosynthesis, arginine to ornithine to putrescine
M00034	AA metabolism	-0.065	0.0192	0.0310	Tyrosine biosynthesis, chorismate to HPP to tyrosine
M00136	AA metabolism	-0.051	0.0108	0.0094	Methionine salvage pathway
M00134	AA metabolism	-0.034	0.0094	0.0236	Urea cycle
M00029	AA metabolism	-0.024	0.0072	0.0310	Cysteine biosynthesis, serine to cysteine
M00307	CA metabolism	0.039	0.0101	0.0218	Pyruvate oxidation, pyruvate to acetyl-CoA
M00532	CA metabolism	0.031	0.0082	0.0218	Photorespiration
M00150	EN metabolism	-0.609	0.1450	0.0179	Fumarate reductase, prokaryotes
M00149	EN metabolism	0.067	0.0203	0.0350	Succinate dehydrogenase, prokaryotes
M00144	EN metabolism	0.066	0.0179	0.0236	NADH:quinone oxidoreductase, prokaryotes
M00153	EN metabolism	0.052	0.0156	0.0343	Cytochrome bd ubiquinol oxidase

[1] Linear associations were evaluated using Microbiome multivariable association with linear models (MaAsLin2).

[2] Only Kyoto encyclopedia of Genes and Genomes (KEGG) modules for amino acid metabolism (AA), carbohydrate metabolism (CA), and energy metabolism (EN) are presented.

[2] Statistical *P* values from MaAsLin2 were adjusted using Benjamini–Hochberg adjustment (FDR-*P*).

Figure legends

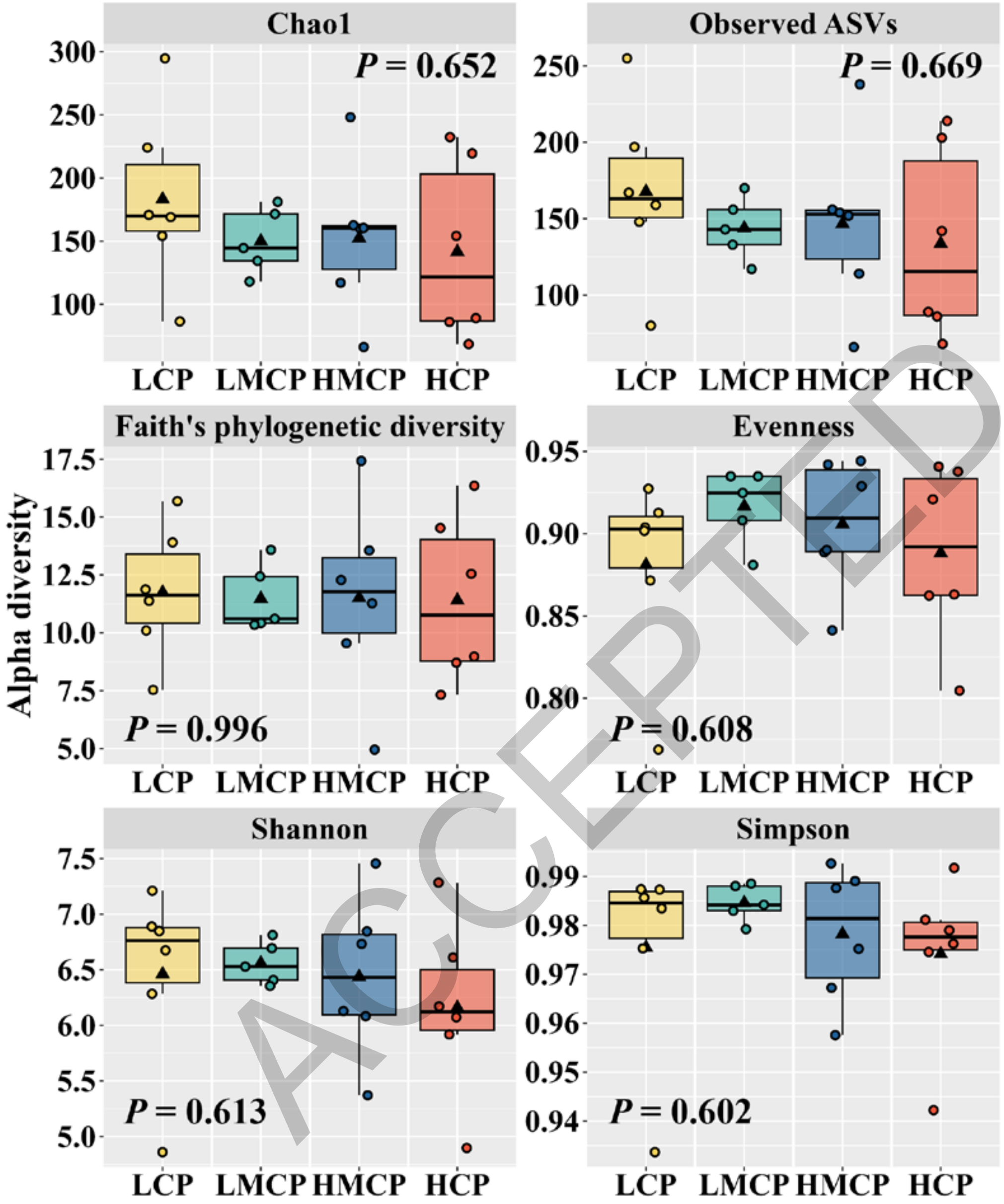
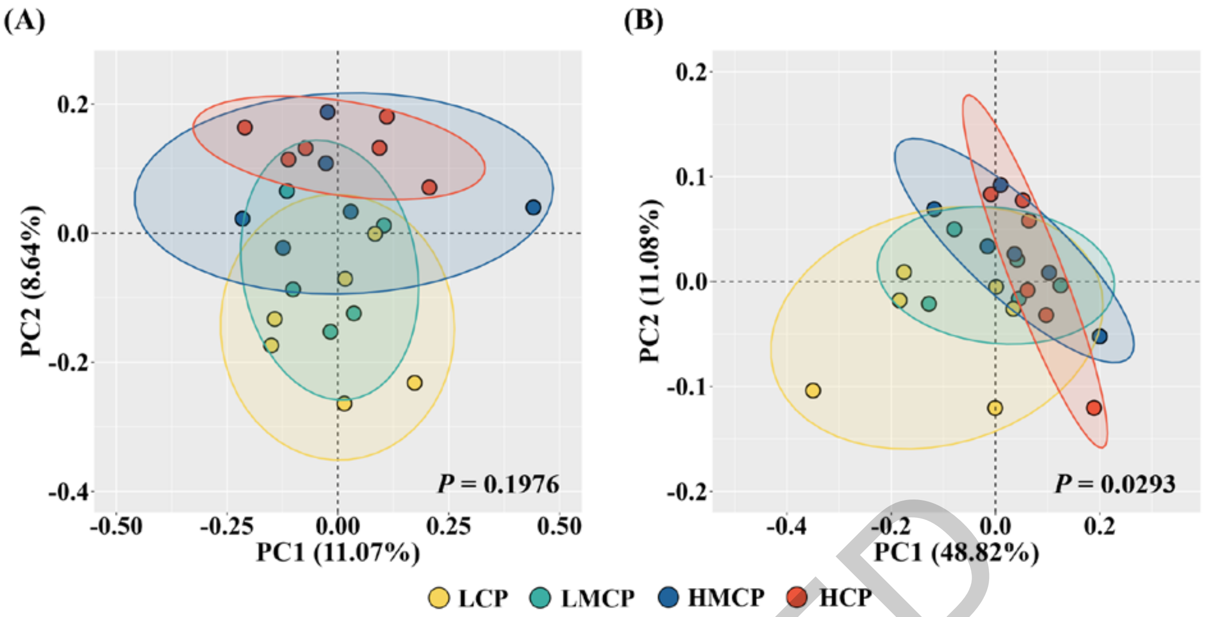


Figure 1. Differences in alpha diversity measurements of the rumen microbiota.



Pairwise comparison		Pseudo-*F*	*Q*-value
LCP	LMCP	1.6870	0.3138
LCP	HMCP	2.7979	0.1302
LCP	HCP	4.1376	0.0276
LMCP	HMCP	0.6832	0.8267
LMCP	HCP	1.2721	0.3138
HMCP	HCP	0.5277	0.8662

Pairwise comparison		Pseudo-*F*	*Q*-value
LCP	LMCP	1.0093	0.6699
LCP	HMCP	1.2146	0.2904
LCP	HCP	1.4974	0.0132
LMCP	HMCP	0.8823	0.9594
LMCP	HCP	1.1449	0.2904
HMCP	HCP	0.7379	0.9594

Figure 2. Principal coordinate analysis based on (A) unweighted and (B) weighted UniFrac distance matrices of rumen microbiota. Permutational multivariate analysis of variance was used to compare differences in the rumen microbiota.

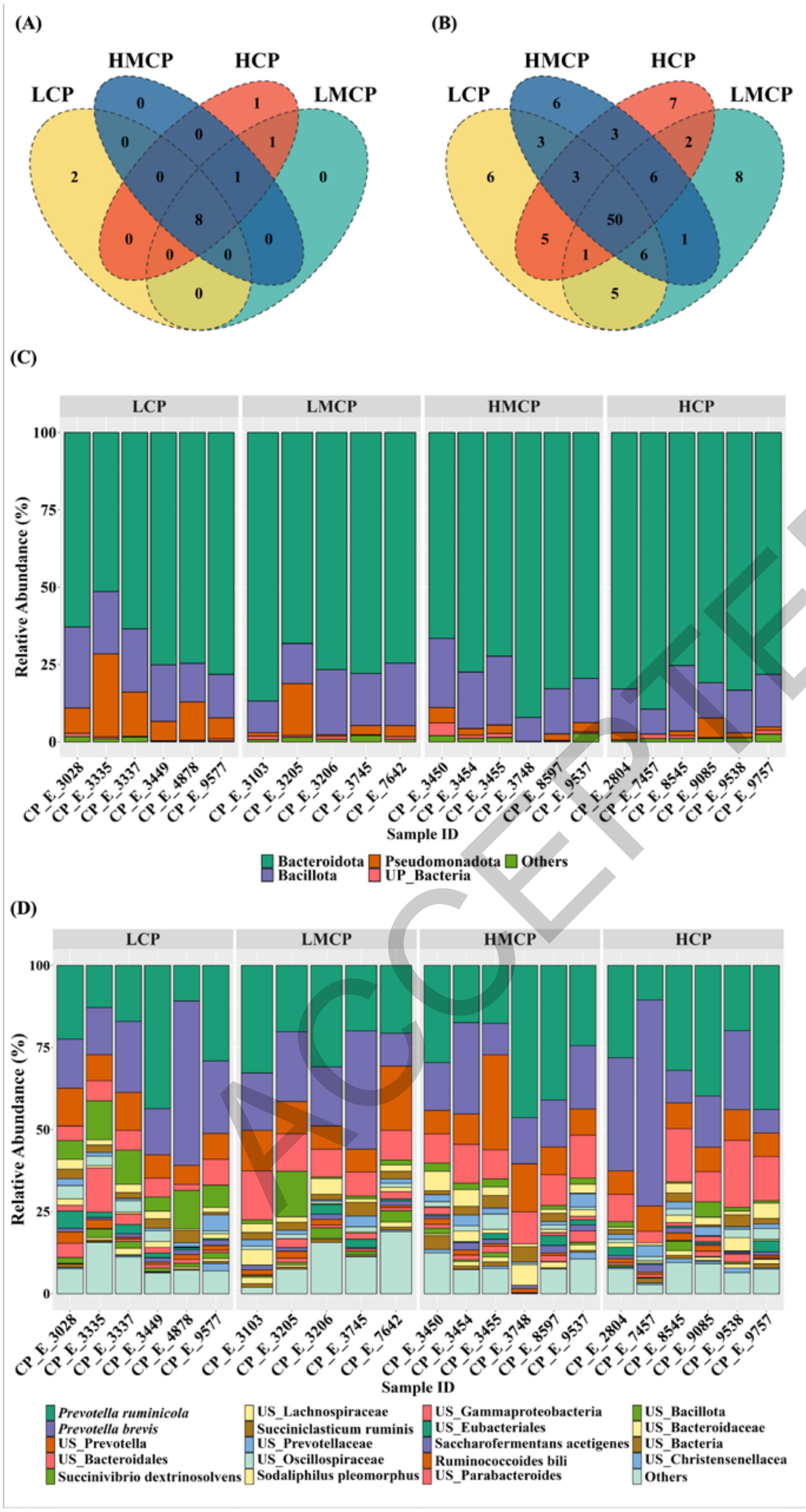


Figure 3. Distribution of rumen microbiota according to dietary CP adjusted using DDGS. (A-B) Venn diagram depicting the shared and unique microbiota at the (A) phylum and (B) species levels in the rumen microbiome. (C-

D) Taxa bar plot of the predominant rumen microbiota at the (C) phylum and (D) species levels. Only taxa having ≥ 30% of occurrence and 0.5% of relative abundance are visualized.

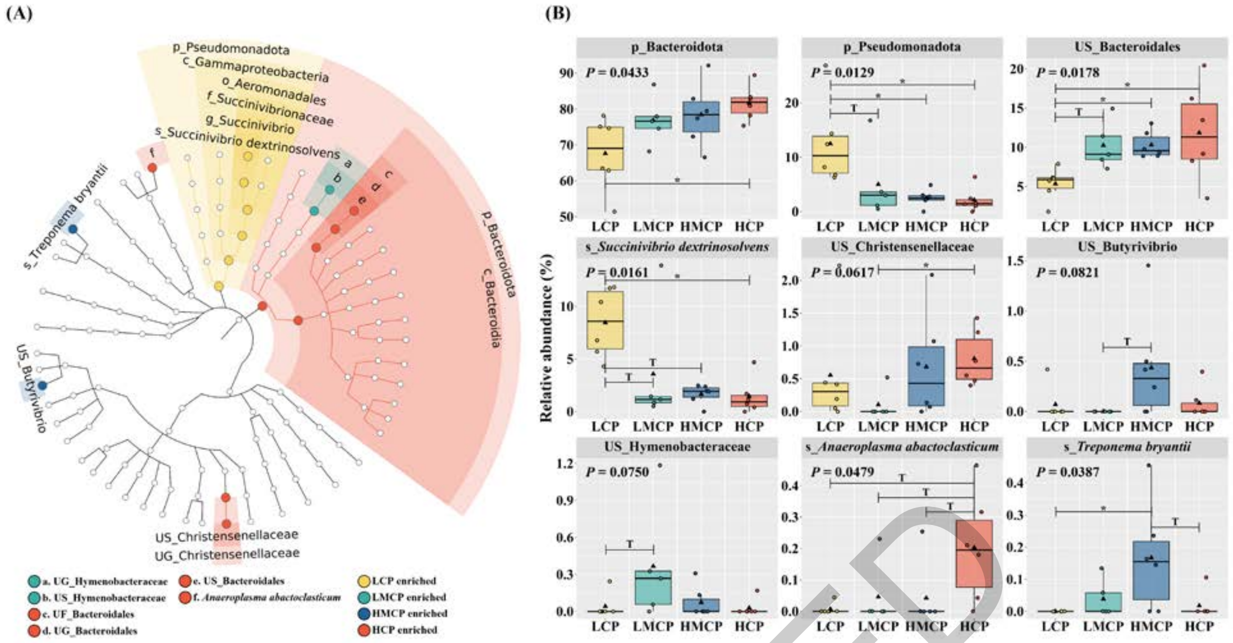


Figure 4. Differentially abundant rumen microbiota depending on dietary crude protein levels. (A) Cladogram describing the overall differences in rumen microbiota. (B) Differentially abundant phyla and species. Differentially abundant microbiota were identified based on the Kruskal–Wallis test, followed by Dunn's post hoc test with Benjamini–Hochberg adjustment; only microbial taxa detected in at least 30% of occurrences among the treatments were evaluated. p_, phylum; c_, class; o_, order; f_, family; g_, genus; s_, species; US_, unclassified species; T, 0.05 ≤ *P* ≤ 0.10; *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

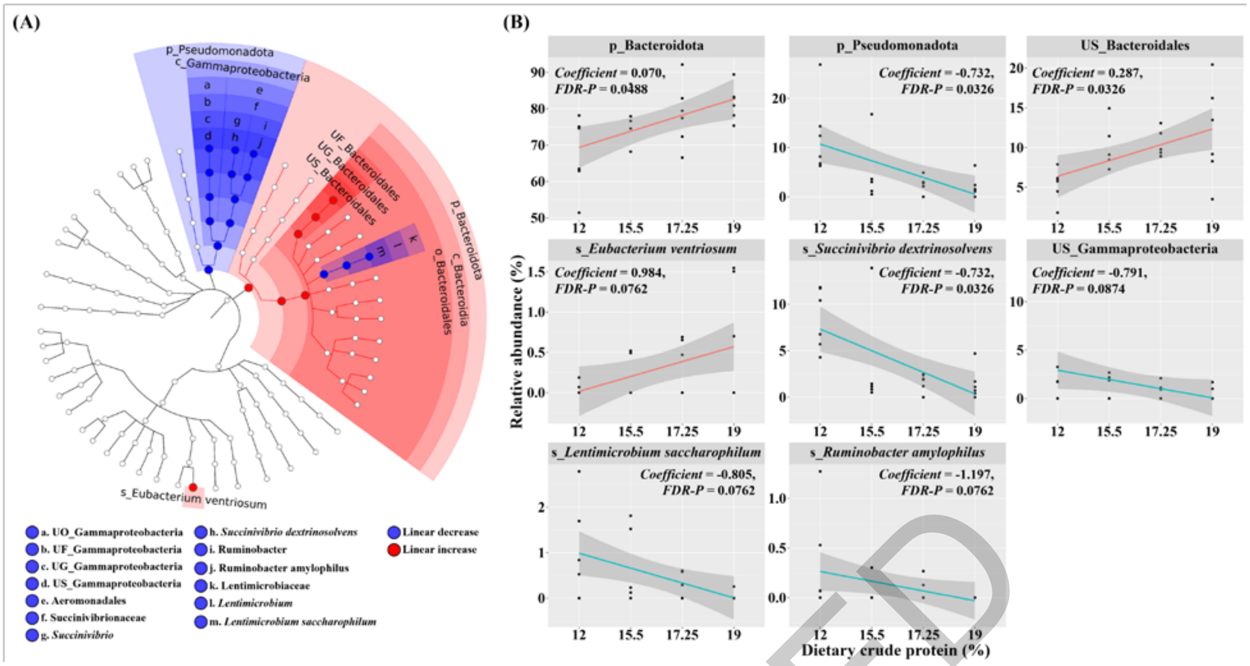
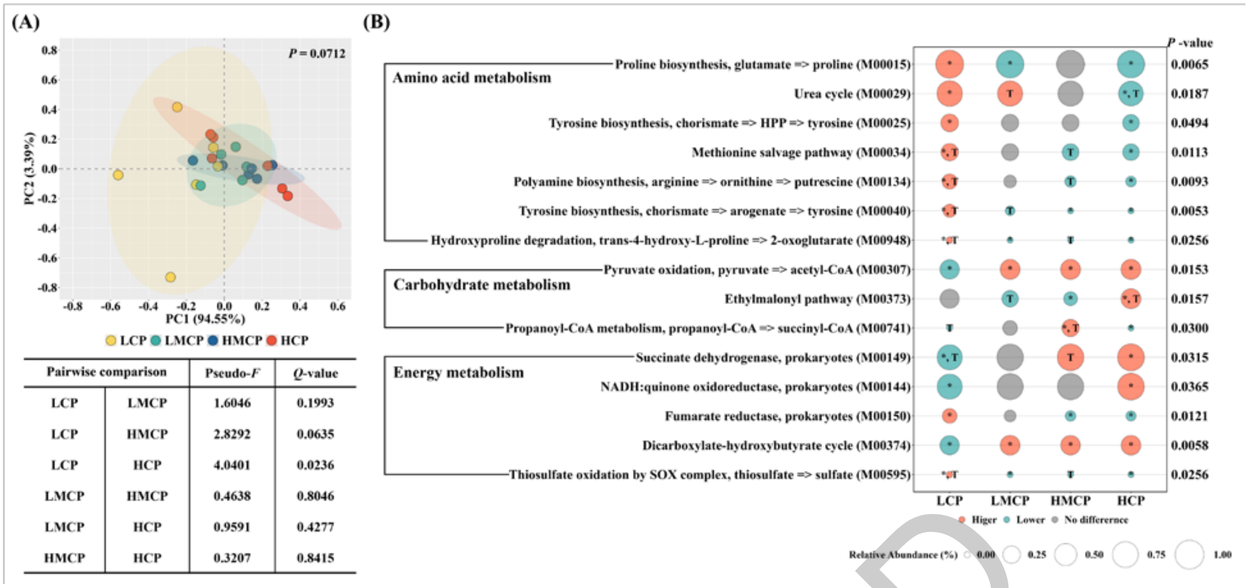


Figure 5. Linear association between the ruminal microbiota and dietary crude protein levels. (A) Cladogram showing a linear association between microbial taxa and crude protein levels. (B) Linear associations at phylum and species levels. Only microbial taxa detected in ≥ 30% of the occurrences among the treatments were evaluated. Linear associations were evaluated using Microbiome multivariable association with linear models (MaAsLin2). Statistical *P* values from MaAsLin2 were adjusted using Benjamini–Hochberg adjustment (*FDR-P*).



Pairwise comparison		Pseudo-*F*	*Q*-value
LCP	LMCP	1.6046	0.1993
LCP	HMCP	2.8292	0.0635
LCP	HCP	4.0401	0.0236
LMCP	HMCP	0.4638	0.8046
LMCP	HCP	0.9591	0.4277
HMCP	HCP	0.3207	0.8415

Figure 6. Overall changes in the predicted functional profiles according to dietary crude protein levels. Predicted functional profiles were determined using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (version 2.5.0) and matched based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. (A) Principal component analysis plot based on KEGG orthologs. (B) Differentially enriched KEGG modules. Only significant KEGG modules belonging to amino acid, carbohydrate, and energy metabolism at KEGG Brite level 2 were visualized.

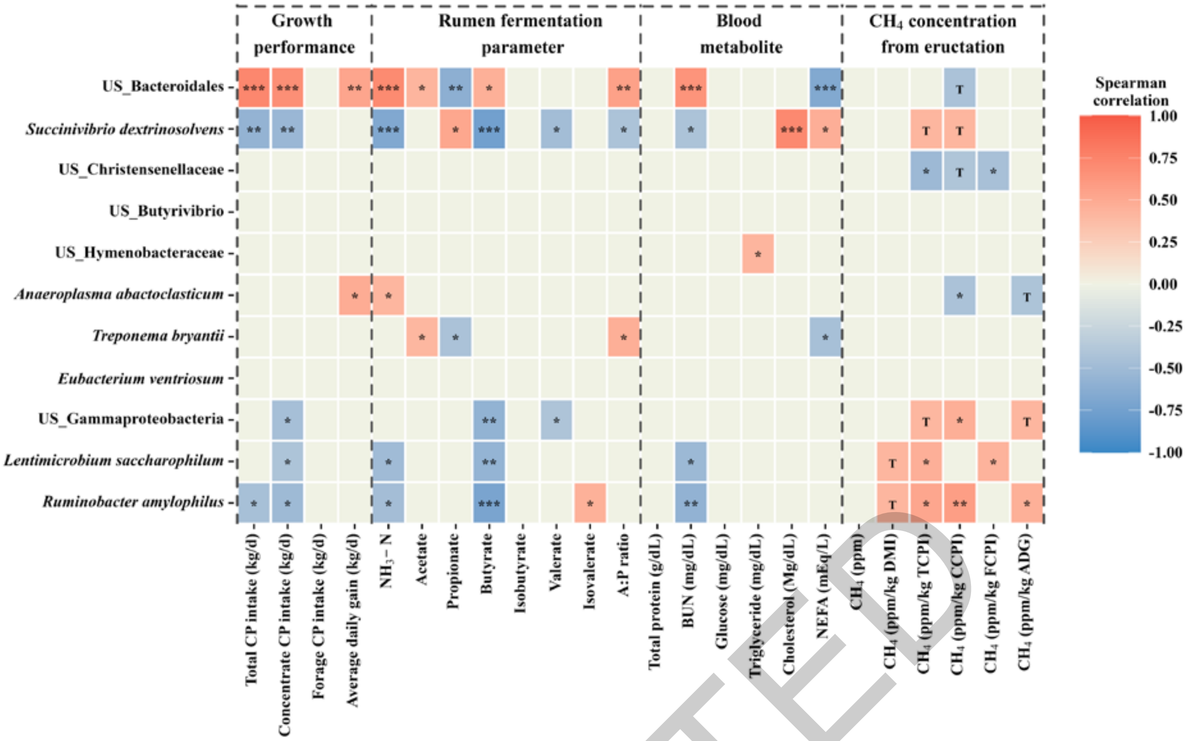


Figure 7. Correlation analysis of rumen microbiota and animal characteristics. Spearman's correlation coefficient between rumen microbiota and animal characteristics was calculated, and correlations above $|r| = 0.4$ and $P < 0.10$ were visualized. Only rumen microbiota detected in at least one statistical tendency on the Kruskal–Wallis test or microbiome multivariable association with linear models analysis (MaAsLin2) were evaluated. The degree of each correlation is indicated by the intensity of the color based on the color key on the right-hand side. US_, unclassified species; T, $0.05 \leq P \leq 0.10$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.