

Effects of diets for three growing stages by rumen inocula donors on *in vitro* rumen fermentation and microbiome

Ryukseok Kang^{1#}, Huseong Lee^{2,3#}, Hyeonsu Seon², Cheolju Park², Jaeyong Song⁴, Joong Kook Park⁴, Yong Kwan Kim⁵, Minseok Kim^{2*} and Tansol Park^{1*}

¹Department of Animal Science and Technology, Chung-Ang University, Anseong 17546, Korea

²Division of Animal Science, Chonnam National University, Gwangju 61186, Korea

³Graduate School of Agricultural Science, Tohoku University, Sendai 980-0845, Japan

⁴Nonghyup Feed Co., LTD., Seoul 05398, Korea

⁵Seogwiposi Chuckhyup, Seogwipo 63585, Korea



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#These authors contributed equally to this work.

*Corresponding author

Minseok Kim

Division of Animal Science, Chonnam National University, Gwangju 61186, Korea.

Tel: +82-62-530-2128

E-mail: mkim2276@jnu.ac.kr

Tansol Park

Department of Animal Science and Technology, Chung-Ang University, Anseong 17546, Korea.

Tel: +82-31-670-3256

E-mail: tansol@cau.ac.kr

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ORCID

Ryukseok Kang

<https://orcid.org/0009-0001-6202-1283>

Abstract

Hanwoo and Jeju Black cattle (Jeju Black) are native breeds of Korean cattle. Jeju Black cattle are recognized as natural monuments and are known to exhibit slower growth rates compared to Hanwoo. While several studies have analyzed the genetic characteristics of these cattle, there has been limited research on the differences in their microbiome. In this study, rumen fluid was obtained from three Hanwoo steers and three Jeju Black steers, and three different diets (total mixed rations [TMRs] for growing, early fattening, and late fattening periods) were used as substrates for *in vitro* fermentation. The *in vitro* incubation was conducted for 3 h and 24 h following a 2 × 3 factorial arrangement. After both incubation periods, fermentation characteristics were analyzed, and ruminal microbiome analysis was performed using 16S rRNA gene sequencing, employing both QIIME2 and PICRUST2. The results revealed significant differences in the ruminal microbiota due to the inoculum effect. At the phylum level, Patescibacteria and Synergistota were found to be enriched in the Jeju Black inoculum-treated group. Additionally, using different inocula also affected the relative abundance of major taxa, including Ruminococcus, Pseudoramibacter, Ruminococcaceae CAG-352, and the [*Eubacterium*] *ruminantium* group. These microbial differences induced by the inoculum may have originated from varying levels of domestication between the two subspecies of donor animals, which mainly influenced the fermentation and microbiome features in the early incubation stages, although this was only partially offset afterward. Furthermore, predicted commission numbers of microbial enzymes, some of which are involved in the biosynthesis of secondary metabolites, fatty acids, and alpha amylase, differed based on the inoculum effect. However, these differences may account for only a small proportion of the overall metabolic pathway. Conversely, diets were found to affect protein biosynthesis and its related metabolism, which showed differential abundance in the growing diet and were potentially linked to the growth-promoting effects in beef cattle during the growing period. In

Huseong Lee
<https://orcid.org/0000-0003-1931-2007>
 Hyeonsu Seon
<https://orcid.org/0000-0003-1618-0897>
 Cheolju Park
<https://orcid.org/0000-0003-2013-8210>
 Jaeyong Song
<https://orcid.org/0000-0002-8613-5605>
 Joong Kook Park
<https://orcid.org/0000-0002-9959-0578>
 Yong Kwan Kim
<https://orcid.org/0009-0009-1316-6986>
 Minseok Kim
<https://orcid.org/0000-0002-8802-5661>
 Tansol Park
<https://orcid.org/0000-0002-4480-4524>

Competing interests

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

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Availability of data and material

Upon reasonable request, the datasets of this study can be available from the corresponding author.

Authors' contributions

Conceptualization: Song J, Kim M.
 Data curation: Kang R, Lee H.
 Formal analysis: Kang R, Lee H, Seon H.
 Methodology: Song J, Kim M, Park T.
 Software: Kang R, Lee H.
 Validation: Song J, Kim M, Park T.
 Investigation: Park C, Song J, Park JK, Kim YK, Kim M.
 Writing - original draft: Kang R, Lee H.
 Writing - review & editing: Kang R, Lee H, Seon H, Park C, Song J, Park JK, Kim YK, Kim M, Park T.

Ethics approval and consent to participate

All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Chonnam National University, Korea (CNU IACUC-YB-2020-7).

conclusion, this study demonstrated that using different inocula significantly affected *in vitro* fermentation characteristics and microbiome features, mainly in the early stages of incubation, with some effects persisting up to 24 h of incubation.

Keywords: *In vitro* fermentation, Korean native cattle, Growing stages, Rumen microbiome, Functional prediction

INTRODUCTION

Korean native cattle can be separated into four distinct breeds: Hanwoo (brown), Chikso (brindle), Heugu (black), and Jeju heugu (Jeju Black) [1–2]. Hanwoo constitutes a significant portion of the Korean native cattle population and is predominantly found on the Korean Peninsula, while Jeju Black cattle have been exclusively maintained on Jeju Island [3]. The Jeju Black cattle breed is believed to have been imported from the Mongol area in the 13th century and is currently classified as an endangered species due to a significant reduction in its population (Domestic Animal Diversity Information System, DAD-IS, FAO). In recognition of its cultural and ecological importance, Jeju Black cattle have been registered as a natural monument in Korea, and a specific breeding program has been implemented to conserve its population (Jeju Black Cattle, Cultural Heritage Administration, Korea, <http://english.cha.go.kr/>). Jeju Black beef is renowned for its high content of oleic acid, linoleic acid, and unsaturated fatty acids, making it a premium beef product in Korea [4]. However, its limited growth rate compared to that of Hanwoo and its habitat-specific characteristics have posed challenges for successful breeding programs [5]. While several studies have investigated the genetic differences between Jeju Black cattle and Hanwoo [2–4], research comparing their ruminal microbiome has been limited.

In Korea, beef feeding systems are structured into three distinct stages: the growing period, early fattening period, and late fattening period, each aimed at producing high-marbled beef. The growing period, which lasts up to 12 months of age, focuses on the development of bones, internal organs, and digestive organs, and is followed by the early fattening period, during which intramuscular fat deposition is induced through high concentrate feeding. The final stage, late fattening, concludes at 29–30 months, aiming to maximize intramuscular fat deposition by reducing the forage to concentrate ratio in the diet [6]. Increased concentrate feeding typically leads to a higher fermentation rate of ruminal microbiota, resulting in a rapid decrease in ruminal pH [7–9], elevated gas production, and propionate production [10–12].

In vitro digestibility can be influenced by various factors, such as the species of the rumen fluid donor animals and the type of feed used for their maintenance [13]. Differences in eating behavior, rumination, digestive tract physiology, ruminal retention time, and ruminal microbiome among donor animal species can impact the initial microbial and nutritional conditions of rumen inocula, ultimately influencing the overall fermentation characteristics in *in vitro* studies [13,14]. *In vitro* studies using rumen contents from different ruminant species as donors have demonstrated differences in nutrient degradability, microbial protein synthesis [15], and methane production [16,17]. Rumen samples from animals fed similar diets tend to exhibit comparable microbial compositions. For instance, consistent differences in gas production were observed in rumen samples collected from six different sheep fed the same diet, while *in vitro* degradation results were similar among nine different sheep receiving the same diet in the same environment [18,19].

In this study, we analyzed the differences in the ruminal microbiome and its relationship with ruminal fermentation under *in vitro* conditions using rumen inocula collected from both Hanwoo and Jeju Black cattle. Simultaneously, we investigated the effects of three different diets commonly fed during the growing, early fattening, and late fattening periods of beef cattle in Korea on the

rumen fermentation characteristics, microbiota, and its functions.

MATERIALS AND METHODS

Animals and rumen fluid sampling

This study used six growth stages of Hanwoo and Jeju Black steers (body weight [BW], 400 ± 24.0 kg) to obtain rumen fluid samples. The steers were divided into two groups according to breeds of brown ($n = 3$; aged 16 months) and black ($n = 3$; aged 20 months) and housed in a tie stall at the same barn at the Seogwipo Korea Federation of Livestock Cooperatives Farm. This barn was equipped with an individual tie stanchion, feed bin, automatic waterer, and mineral block, and was bedded with wood shavings. Hanwoo and Jeju Black steers (one of each) were randomly assigned to three treatment groups (growing period total mixed ration [TMR], early fattening period TMR, and late fattening period TMR). The diet was formulated to meet the nutritional requirements of the National Research Council (NRC) [20] for beef cattle. The animals were adapted to the treatment diets for 2 weeks before the trial. The diet was fed twice daily during the adaptation period (at 09:00 and 17:00 h). Rumen fluid (approximately 200 mL) was collected from each Hanwoo and Jeju Black steer via a stainless steel stomach tubing at 2 h post-feeding and transferred to the laboratory using preheated thermos bottles. Each of the six rumen fluids was used for DNA extraction. Additionally, the rumen fluids of Hanwoo and Jeju Black cattle fed the early fattening period TMR were separately strained through four layers of cheesecloth and were maintained in a 39°C incubator before mixing with McDougall's buffer solution [21]. All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Chonnam National University, Korea (CNU IACUC-YB-2020-7).

Donor animal feeds and *in vitro* substrates

Growing period, early fattening period, and late fattening period TMR diets were obtained from Seogwipo Korea Federation of Livestock Cooperatives Farm. All feed samples were dried and milled to pass through a 1-mm mesh screen in a Wiley mill (Model 4, Thomas Scientific, Swedesboro, NJ, USA). The dry matter (DM) was determined by drying the samples to a constant weight at 65°C. Samples were analyzed for crude protein (CP), crude fat (CF), and crude ash according to the methods described by the Association of Official Analytical Collaboration (AOAC) [22]. The moisture was calculated as $\times 100$ by the AOAC [22]. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were evaluated as described by Van Soest et al. [23,24] using a fiber analyzer (ANKOM2000, ANKOM Technology, Macedon, NY, USA). The chemical compositions of the formulated TMR diets are shown in Table 1.

In vitro incubation procedures and experimental design

McDougall's buffer solution was heated at 39°C, flushed with O₂-free CO₂ for 20 min, and then mixed with the rumen fluid in a 2 : 1 (vol : vol) ratio under anaerobic conditions. Next, 40 mL of the buffered rumen fluid was dispensed into a 120 mL gas-tight serum bottle filled with bubbled O₂-free CO₂ and was then flushed into the headspace of the serum bottles, which were then closed with a butyl rubber stopper containing a rubber septa with an aluminum seal, respectively, and incubated at 39°C. The DAISYII apparatus contained four 4-L digestion vessels, which slowly rotated in a digestion chamber that was maintained at 39.5°C. Samples to be analyzed were heat sealed into ash-free and N-free filter bags and inserted into the digestion vessels. The *in vitro* experiment was conducted for 3 h and 24 h under a 2 × 3 factorial arrangement using two breeds (Hanwoo and Jeju Black) and three substrates (growing period TMR, early fattening period TMR,

Table 1. Ingredients and chemical composition (% dry matter basis) of diets

Composition	Compositions (%)		
	Growing	Early fattening ¹⁾	Late fattening
Ingredients			
TMF base	22	23.5	23.5
Ground corn	14.2	17.2	20
Cotton seed	3	4	5
DDGS	4	4.8	5
Citrus juice pulp	15	15	15
Molasses	5	5	5
Timothy hay	2	-	-
Alfalfa	2	-	-
Tall fescue	4	4	3
Orchard grass	4	3	2
green barley	13	11	8
Total mixture ²⁾	0.2	0.2	0.2
Forumen	0.2	0.2	0.2
Water	11.3	12	13
Chemical composition			
DM	56.31	64.15	56.55
CP	14.14	14.62	14.82
EE	3.85	4.17	4.40
CF	14.62	15.60	12.91
Ash	7.89	8.05	7.48

¹⁾Diet of donor animals.

²⁾Minerals and vitamins mixture, vitamin A 28,000 IU; vitamin D3 4,000 IU; vitamin E 80 IU; Mn 80 ppm; Zn 100 ppm; Fe 70 ppm; Cu 50 ppm; Co 0.5 ppm; I 2.0 ppm; Se 1.0 ppm.

TMF, total mixed fermentation; DDGS, dried distillers grain with solubles (USA); DM, dry matter; CP, crude protein; EE, ether extract; CF, crude fiber.

and late fattening period TMR) as factors.

Fermentation parameters analyses

In vitro total gas measurements were calculated from the headspace gas pressure measured by a pressure transducer (Sun Bee Instrument, Seoul, Korea). The pH of the rumen fluid was measured with a pH meter (Orion Star A211 bench-top pH meter, Thermo Scientific, Bremen, Germany) and the residual rumen fluid samples were stored at -20°C immediately for DM digestibility and volatile fatty acids (VFA) and ammoniacal-N ($\text{NH}_3\text{-N}$) analysis. After thawing, 10 mL of the sample was mixed with 1 mL of HgCl_2 2% (wt/vol) solution and briefly centrifuged at $2,000\times g$ for 10 min at 4°C to remove feed particles. The supernatants were then used for DNA extraction and VFA and $\text{NH}_3\text{-N}$ analysis. The VFA concentration was determined using gas chromatography (Varian CP-3800, Varian, Walnut Creek, CA, USA). The $\text{NH}_3\text{-N}$ concentration was determined using a Multiplate spectrophotometer (Benchmark PlusTM, Bio-Rad, Tokyo, Japan) at 625 nm as described by Chaney and Marbach [25].

DNA extraction and 16S rRNA gene sequencing

Total community DNA was extracted from 42 rumen fluid samples (six from donor animals and 36 from the *in vitro* rumen fermentation experiment) using a Mini-Beadbeater-16 (BioSpec

Products, Bartlesville, OK, USA) and the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) [26]. To amplify 16S rRNA gene amplicons, a library was generated from each DNA sample using the 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTA CHVGGGTATCTAATCC-3') primers targeting the V3-V4 region, and was sequenced on the MiSeq platform (Illumina, San Diego, CA, USA). Pairs of reads were merged using the Fast Length Adjustment of SHort reads (FLASH) program [8].

Metagenomic analysis of rumen microbiota

The sequences were analyzed using QIIME2 (version 2022.11) [27]. Primers were trimmed using Cutadapt [28]. After trimming off the adapter and primer sequences, DADA2 was performed for filtering by quality score (≥ 25) and removal of chimeric sequences [29].

Bacteria and archaea were classified using scikit-learn [30] with the weighted SILVA reference database (v.138) [31]. Further taxonomic filtration was done to remove the amplicon sequence variants (ASVs) labeled as 'unassigned,' 'chloroplast,' or 'mitochondria.' The average rarefied abundance table was made by averaging the 1,000 times repeated rarefaction outputs at 1,170 ASVs computed by q2-repeat-rarefy [32].

α -diversity measurements including species richness (observed ASVs and Chao1 estimates), evenness, Shannon's index, and Simpson's index were calculated based on the aforementioned repeatedly rarefied ASV abundance table. Comparison of the overall microbiotas between the Hanwoo inoculum-treated group (HWG) and Jeju Black inoculum-treated group (JBG), and between the growing, early-fattening, and late-fattening diets were done using principal coordinates analysis (PCoA) based on the Bray-Curtis distance matrix [33].

Functional prediction of prokaryotic microbiota

Functional microbial features were predicted from the 16S ASVs using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) [34]. The normalized counts of the predicted enzyme commission (EC) numbers were used to define the overall functional dissimilarities between HWG and JBG, and between diets. Principal components analysis (PCA) based on the Bray-Curtis distance matrix was used to analyze overall functional dissimilarities. PCA outputs were visualized using the ggfortify package of R (4.2.2) [35].

Statistical analysis

Fermentation data and α -diversity metrics results were statistically analyzed separately for 3 h and 24 h of the *in vitro* incubation using the GLIMMIX procedure of SAS 9.4 (SAS Institute, Cary, NC, USA), where inocula (HWG and JBG), diets, interaction between inoculum, and diet effects were set as fixed effects. Permutational multivariate analysis of variance (PERMANOVA) was used to statistically analyze the overall microbiotas and their functional dissimilarities between time, inoculum, and diet effects with interactions using vegan and the pairwiseAdonis package of R (4.2.2) with 9,999 random permutations followed by multiple-test corrections with the Benjamini-Hochberg correction [36]. For statistical analysis of microbiome data, major microbial features including classified taxa at the phylum, family, and genus level and microbial functions represented by predicted ECs selected when the average relative abundance of each feature was over 0.1% across all samples were mainly discussed in this study. Differentially abundant microbial phyla, families, genera, and EC numbers were determined using Microbiome Multivariable Associations with Linear Models 2 (MaAsLin2) [37] with the Benjamini-Hochberg corrected p -value (q -value) 0.05 as the cutoff for significant differences. Relative abundances of microbial taxa and predicted EC numbers were normalized with a centered-log ratio and used for the MaAsLin2 analysis. Except

for the relative abundance data, statistical significance was declared at $p \leq 0.05$.

RESULTS

In vitro fermentation characteristics

In vitro fermentation characteristics according to inoculum and diet effects are shown in Table 2. The pH was high in the JBG at both incubation times ($p < 0.0500$). $\text{NH}_3\text{-N}$ was higher in the JBG after 3 h of incubation ($p = 0.0147$), while the opposite was found after 24 h ($p = 0.0273$). Total gas production and dry matter digestibility (DMD) was higher in HWG at both incubation times ($p < 0.0500$) but the total VFA concentration was enriched more in the HWG at only the earlier incubation period ($p < 0.0001$). Among the major VFAs, the molar proportion of acetate and valerate showed opposite trends at 3 h of incubation, while no significant differences were found in any VFA profile at 24 h.

At 3 h of incubation, the total gas production was higher in the growing diet compared to that

Table 2. Ruminal fermentation characteristics at 3 h and 24 h of incubation

Measurements	Inoculum		Diet			SEM	<i>p</i> -values		
	HWG	JBG	Growing	Early fattening	Late fattening		Inoculum	Diet	Inoculum × Diet
3 h of incubation									
pH	6.84 ^b	6.86 ^a	6.84	6.86	6.86	0.01	0.0238	0.2480	0.1561
Gas (mL)	16.44 ^a	12.44 ^b	15.50 ^a	14.00 ^{ab}	13.83 ^b	0.57	< 0.0001	0.0372	0.3080
DMD	35.13 ^a	34.19 ^b	34.83 ^a	33.37 ^b	35.78 ^a	0.34	0.0127	0.0002	0.0042
$\text{NH}_3\text{-N}$ (mg/dL)	0.54 ^b	0.82 ^a	0.50	0.71	0.82	0.07	0.0147	0.0636	0.1058
Total VFA (mM)	29.28 ^a	24.19 ^b	26.36 ^b	25.63 ^b	28.23 ^a	0.73	< 0.0001	0.0054	0.0712
Acetate (%)	47.69 ^a	44.82 ^b	46.64	45.32	46.80	0.45	0.0002	0.0785	0.5411
Propionate (%)	21.45	22.28	19.85	22.68	23.06	0.97	0.6771	0.3706	0.3288
A:P ratio	2.81	2.01	3.20	2.00	2.03	0.39	0.3325	0.3938	0.3844
Isobutyrate (%)	4.57	3.64	5.65	3.47	3.19	0.76	0.5590	0.3888	0.3711
Butyrate (%)	11.92	12.96	11.79	12.93	12.62	0.38	0.1905	0.4597	0.3718
Isovalerate (%)	5.46	5.83	6.31	5.53	5.10	0.33	0.5758	0.3371	0.3278
Valerate (%)	8.91 ^b	10.46 ^a	9.75 ^{ab}	10.07 ^a	9.23 ^b	0.23	< 0.0001	0.0124	0.1702
24 h of incubation									
pH	6.64 ^b	6.67 ^a	6.67	6.65	6.65	0.01	0.0257	0.2149	0.1184
Gas (mL)	74.83 ^a	66.56 ^b	69.75	71.25	71.08	1.16	< 0.0001	0.5770	0.4477
DMD	59.34 ^a	55.74 ^b	56.84	56.93	58.85	0.60	0.0010	0.1229	0.9205
$\text{NH}_3\text{-N}$ (mg/dL)	0.60 ^a	0.37 ^b	0.38 ^b	0.67 ^a	0.41 ^b	0.06	0.0273	0.0449	0.3565
Total VFA (mM)	48.15	50.29	46.83	49.50	51.33	0.98	0.2863	0.1995	0.6846
Acetate (%)	50.69	50.85	50.97	51.02	50.32	0.21	0.6994	0.3361	0.2701
Propionate (%)	26.53	26.84	25.87 ^b	26.32 ^b	27.88 ^a	0.25	0.3286	0.0004	0.3993
A:P ratio	1.91	1.90	1.97 ^a	1.94 ^{ab}	1.81 ^b	0.02	0.6432	0.0017	0.1851
Isobutyrate (%)	2.12	2.06	2.18	2.08	2.01	0.04	0.4049	0.1952	0.6494
Butyrate (%)	11.16	11.14	11.30 ^a	11.32 ^a	10.83 ^b	0.08	0.9120	0.0099	0.7247
Isovalerate (%)	3.44	3.37	3.52	3.40	3.29	0.05	0.5602	0.2572	0.6365
Valerate (%)	6.07	5.73	6.16	5.86	5.67	0.11	0.1446	0.2119	0.5916

^{a,b}Superscripts indicate statistically significant differences ($p < 0.05$).

HWG, Hanwoo inoculum-treated group; JBG, Jeju Black inoculum-treated group; DMD, dry matter digestibility; VFA, volatile fatty acids; A, acetate; P, propionate.

of the late fattening diet. Additionally, the DMD were significantly lower in the early fattening diet with a significant interaction between the inoculum and diet ($p = 0.0042$), while the total VFA concentration was only high in the late fattening diet. For each VFA profile, no significant diet effect was found except with valerate, and a significant difference was seen between the early and late fattening diets. At 24 h of incubation, the pH, gas production, DMD, and total VFA concentration did not differ by diet, and only $\text{NH}_3\text{-N}$ was significantly higher in the early fattening diet compared to that of the other diets. Additionally, opposite profiling was found between propionate and butyrate. Increasing the molar proportion of propionate at the end of the fattening period resulted in the lowest A:P ratio by the late fattening diet. For all measurements at 24 h of incubation, no significant interaction effect between the inoculum and diet was found ($p > 0.100$).

Difference of ruminal microbiota in inoculum and diet effects

Diversity measurements of the ruminal microbiota

No significant differences were found in any of the analyzed α -diversity measurements between both inoculums ($p > 0.1$) (Table 3). At 3 h of incubation, the observed features of the HWG were higher than those of the JBG ($p = 0.0268$), but after 24 h of incubation, the difference by inoculum treatment was offset. No significant diet effects were found in any α -diversity measurements. Overall, the microbial community structures significantly differed by incubation time and inoculum ($q < 0.01$) using the Bray-Curtis distance matrix with no significant diet and interaction effects seen among the fixed effects ($p > 0.05$) (Fig. 1).

Table 3. α -diversity measurements

Measurements ¹⁾	Inoculum		Diet			SEM	p -values		
	HWG	JBG	Growing	Early fattening	Late fattening		Inoculum	Diet	Inoculum \times Diet
0 h rumen sample									
Observed ASVs	722	738	-	-	-	24.057	0.7877	-	-
Chao1 estimates	1,523	1,429	-	-	-	89.729	0.6545	-	-
Evenness	0.91	0.93	-	-	-	0.006	0.1200	-	-
Shannon's index	8.63	8.84	-	-	-	0.088	0.2845	-	-
Simpson's index	0.99	1.00	-	-	-	0.001	0.2261	-	-
3 h of incubation									
Observed ASVs	181 ^A	156 ^B	178	163	164	5.804	0.0268	0.3801	0.0938
Chao1 estimates	263	241	246	233	277	10.736	0.3346	0.2677	0.6825
Evenness	0.90	0.90	0.90 ^a	0.90 ^a	0.89 ^b	0.002	0.8856	0.0802	0.6731
Shannon's index	6.72 ^a	6.51 ^b	6.75	6.58	6.52	0.061	0.0610	0.2040	0.1344
Simpson's index	0.99 ^a	0.98 ^b	0.99	0.99	0.98	0.001	0.0990	0.1480	0.2051
24 h of incubation									
Observed ASVs	248	233	247	235	240	6.890	0.2936	0.8524	0.7304
Chao1 estimates	340	343	368 ^a	316 ^c	341 ^b	10.003	0.8463	0.0863	0.1553
Evenness	0.89	0.88	0.89	0.88	0.89	0.002	0.4127	0.5128	0.4736
Shannon's index	7.05	6.94	7.04	6.94	7.01	0.043	0.2416	0.7052	0.9264
Simpson's index	0.99	0.98	0.99 ^a	0.98 ^b	0.99 ^a	0.001	0.7649	0.0881	0.8584

¹⁾Goods coverage of all 0 h rumen samples were $\geq 67.3\%$, 3 h incubation samples were $\geq 94.8\%$, and 24 h incubation samples were $\geq 90.9\%$.

^{A,B}Superscripts indicate statistically significant differences ($p < 0.05$).

^{a,b}Superscripts indicate statistical tendency of differences ($p < 0.1$).

HWG, Hanwoo inoculum-treated group; JBG, Jeju Black inoculum-treated group; ASVs, amplicon sequence variants.

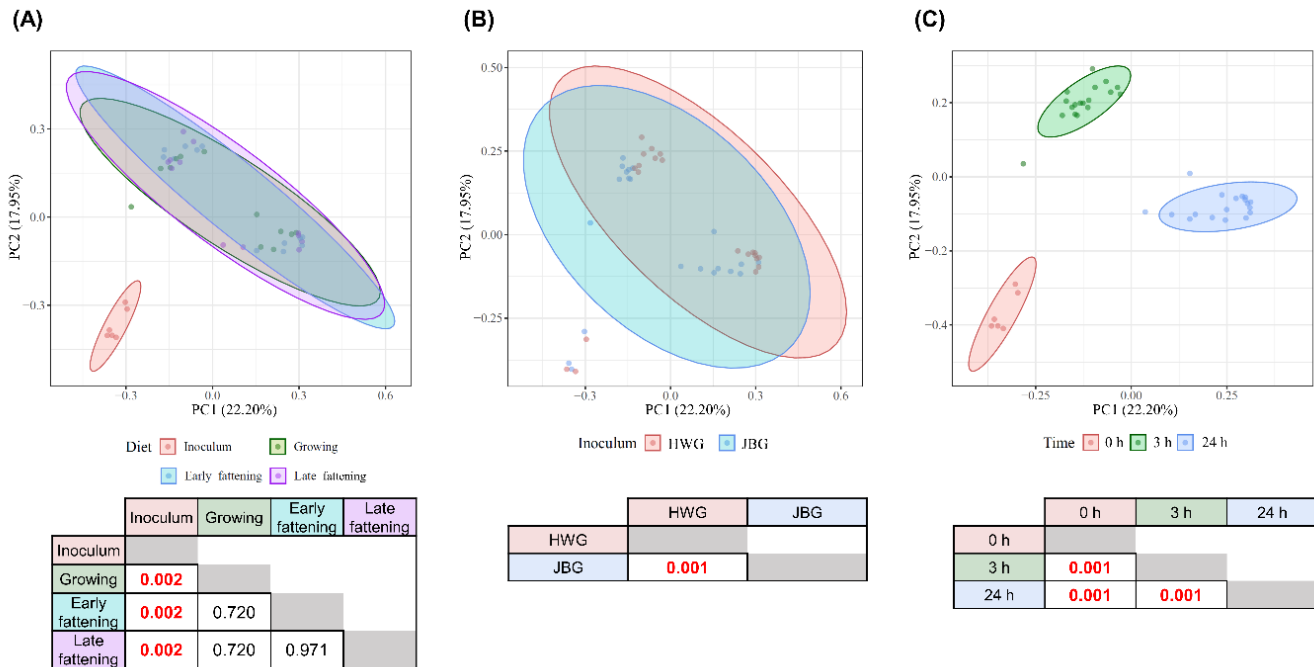


Fig. 1. Overall microbial community represented by the repeatedly rarefied amplicon sequence variant (ASV) table. Principal coordinates analysis (PCoA) based on Bray-Curtis distance matrix was used for visualization with statistical analysis outputs by permutational multivariate analysis of variance (PERMANOVA) for (A) diet, (B) inoculum, and (C) incubation time effects. HWG, Hanwoo inoculum-treated group; JBG, Jeju Black inoculum-treated group.

Overall taxonomic composition of major microbial taxa

Among the major taxa across all 42 samples, at the phylum level, Firmicutes (80.44%) was primarily dominant, followed by Proteobacteria (8.48%) and Bacteroidetes (2.55%). Lachnospiraceae (17.54%), Ruminococcaceae (14.31%), and Planococcaceae (10.56%) were the three most dominant families, while *Solibacillus* (9.77%), *Succinivibrio* (7.40%), and an unclassified genus within Lachnospiraceae (6.99%) were found as major genera across all samples (Supplementary Data Tables S1 and S2).

Taxonomic composition differences after 3 h of incubation

Among the major phyla selected in this study, only Patescibacteria was more enriched in the JBG than in the HWG (6.39% vs. 3.86%, respectively) after 3 h of incubation (Fig. 2A). Among the major families, Planococcaceae, Peptostreptococcaceae, and Saccharimonadaceae were differentially abundant in the HWG, while Clostridiaceae, Bifidobacteriaceae, Atopobiaceae, Eubacteriaceae, and Ruminococcaceae were differentially abundant in the JBG (Fig. 2B). At the genus level, *Ruminococcus*, *Pseudoramibacter*, and Ruminococcaceae CAG-352 were more enriched in the HWG, while *Blautia*, *Clostridium sensu-stricto-1*, *Paeniclostridium*, *Solibacillus*, Oscillospiraceae NK4A214 group, and an uncultured genus within Ruminococcaceae were more enriched in the JBG (Fig. 2C). At the family level, Ruminococcaceae was significantly enriched in the growing diet, and at the genus level, Ruminococcaceae CAG-352 was also enriched in the growing diet, while the [*Eubacterium*] *coprostanoligenes* group was enriched in the late fattening diet (Fig. 3).

Taxonomic composition differences after 24 h of incubation

Among the major phyla, Patescibacteria and Synergistota were differentially abundant in JBG

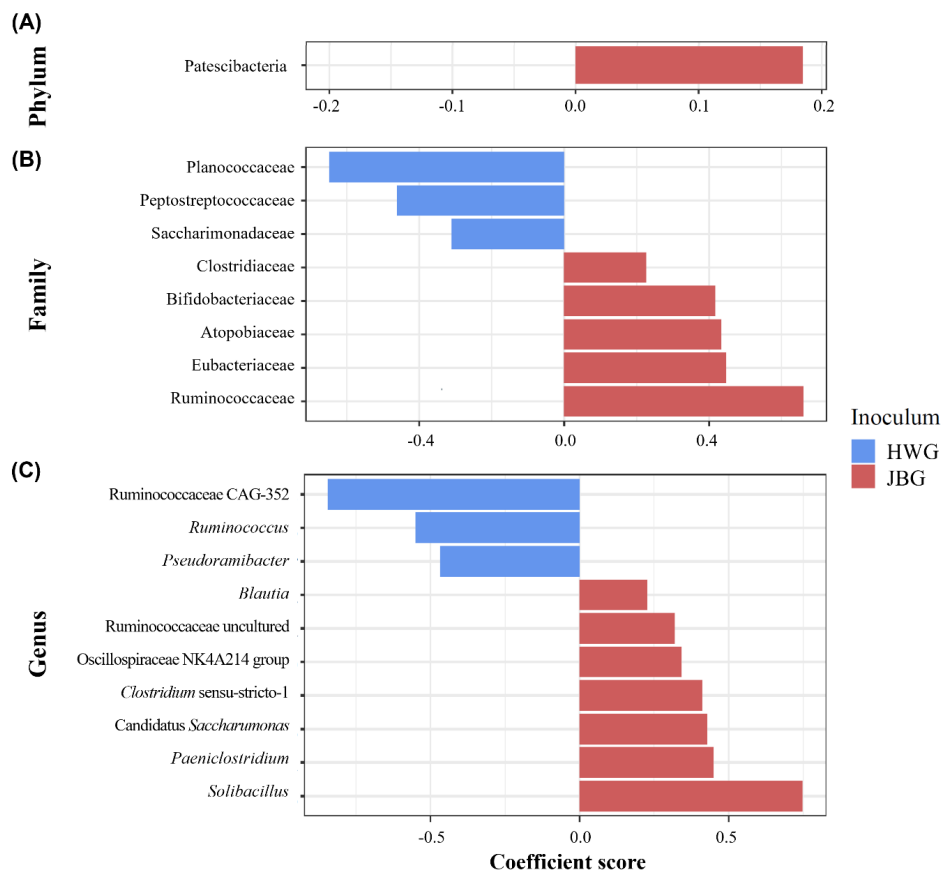


Fig. 2. Differentially abundant major microbial taxa (at least $\geq 0.1\%$ of the average relative abundance across all samples) at the phylum (A), family (B), and genus levels (C) by inoculum effect at 3 h of incubation. Statistical analysis was performed using MaAsLin2 with $q = 0.05$ as the cutoff. HWG, Hanwoo inoculum-treated group; JBG, Jeju Black inoculum-treated group.

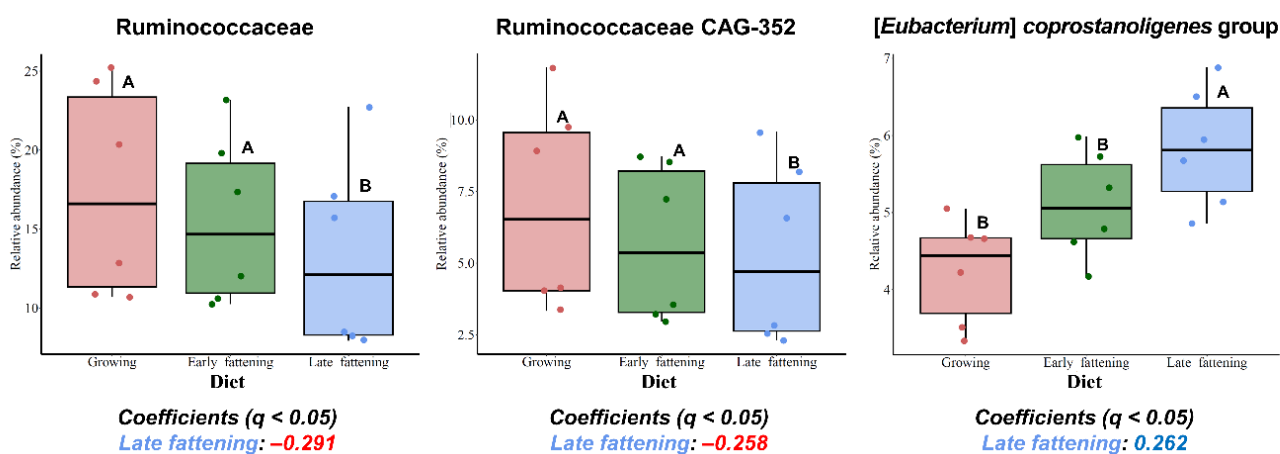


Fig. 3. Differentially abundant major microbial taxa (at least $\geq 0.1\%$ of the average relative abundance across all samples) by diet effect after 3 h of incubation. Statistical analysis was performed using MaAsLin2 with $q = 0.05$ as the cutoff.

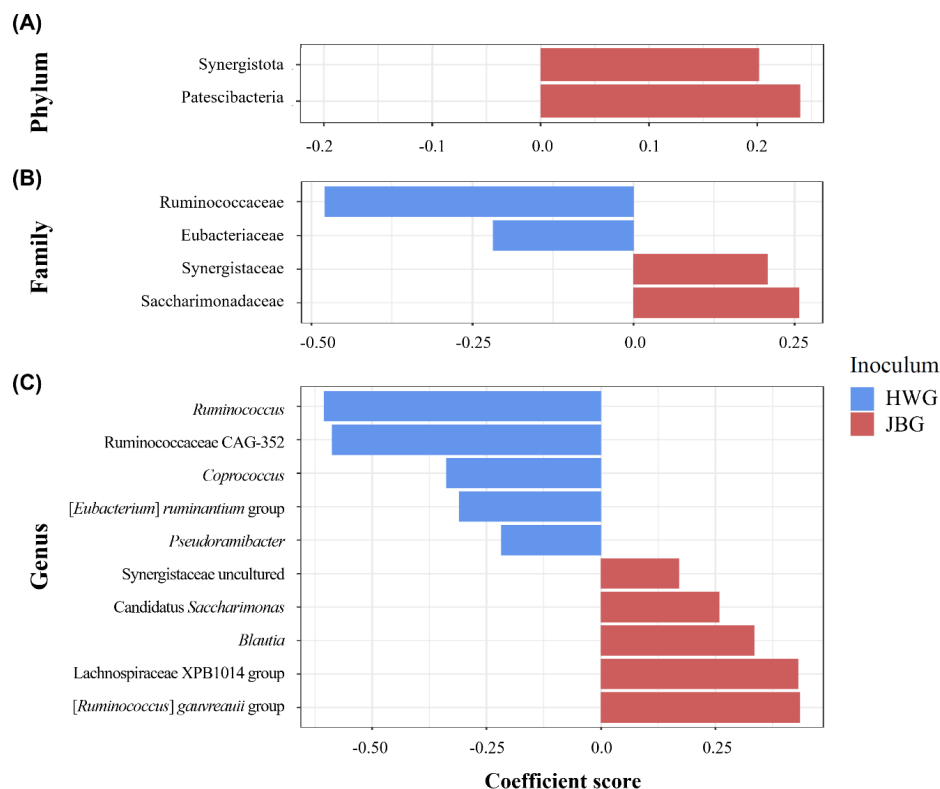


Fig. 4. Differentially abundant major microbial taxa (at least $\geq 0.1\%$ of the average relative abundance across all samples) at the phylum (A), family (B), and genus levels (C) by inoculum effect at 24 h of incubation. Statistical analysis was performed using MaAsLin2 with $q = 0.05$ as the cutoff. HWG, Hanwoo inoculum-treated group; JBG, Jeju Black inoculum-treated group.

(Fig. 4A). At the family level, Ruminococcaceae and Eubacteriaceae were significantly enriched in the HWG, while Synergistaceae and Saccharimonadaceae were significantly enriched in the JBG (Fig. 4B). At the genus level, the predominance of *Ruminococcus*, Ruminococcaceae CAG-352, *Coprococcus*, [*Eubacterium*] *ruminantium* group, and *Pseudoramibacter* were confirmed in the HWG, while *Candidatus Saccharimonas*, *Blautia*, Lachnospiraceae XPB1014 group, [*Ruminococcus*] *gauvreauii* group, and uncultured genera within Synergistaceae were predominant in the JBG (Fig. 4C). At the genus level, the relative abundance of the uncultured genus within Ruminococcaceae was significantly higher in the growing diet compared to that of the fattening period diets (Fig. 5).

Functional results of ruminal microbiota in inoculum and diet effects

Overall microbial functional community analysis

Based on the predicted microbial functions represented by the EC numbers analyzed using PCA visualization and PERMANOVA, it was shown that only the *in vitro* incubation time significantly affected the overall functional microbial community and all pairwise comparisons between the three time points differed significantly ($q = 0.001$), while the inoculum and diet effects did not show a significant difference ($q > 0.05$) (Fig. 6). No significant interaction effect was found between the time, inoculum, and diet effects ($q > 0.05$).

Comparison of functional features by inoculum effect

Comparative analysis of functional features to analyze inoculum and diet effects during *in vitro*

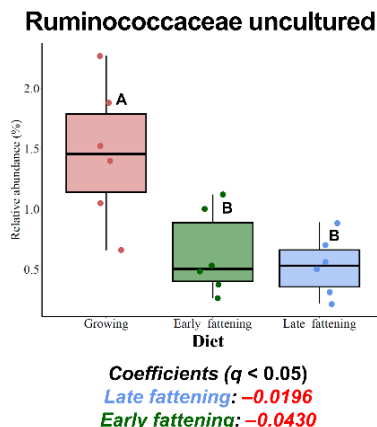


Fig. 5. Differentially abundant major microbial taxa (at least $\geq 0.1\%$ of the average relative abundance across all samples) by diet effect after 24 h of incubation. Statistical analysis was performed using MaAsLin2 with $q = 0.05$ as the cutoff.

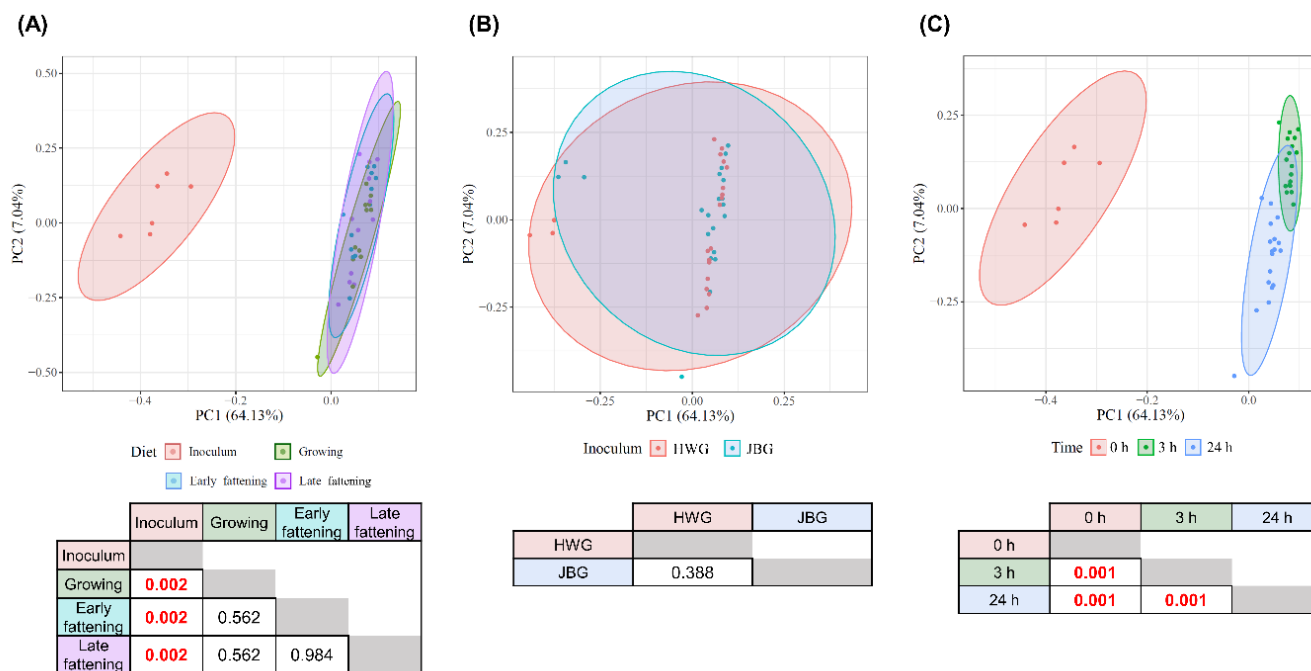


Fig. 6. Overall microbial functional community represented by enzyme commission profiles. Principal components analysis (PCA) based on Bray-Curtis distance matrix was used for visualization with statistical analysis outputs by permutational multivariate analysis of variance (PERMANOVA) for (A) diet, (B) inoculum, and (C) incubation time effects. HWG, Hanwoo inoculum-treated group; JBG, Jeju Black inoculum-treated group.

incubation was done using MaAsLin2 based on the relative abundance of the predicted microbial EC numbers. Among the major EC numbers, 190 features differed by inoculum effect at 3 h of incubation (Supplementary Data Table S3). After 24 h of incubation (Table 4), seven specific EC numbers including four transferases (EC 2), two hydrolases (EC 3), and one lyase (EC 4) differed significantly by inoculum effect. The discrepancy pattern of those seven specific EC numbers was also similar at 3 h of incubation.

Table 4. Differentially abundant predicted microbial enzyme commission (EC) numbers (at least $\geq 0.1\%$ average relative abundance across all samples) by inoculum effect at 24 h of incubation¹⁾

Enzymes	EC number	Relative abundance (%)		SEM	Coefficient	p-value	q-value
		HWG	JBG				
Site-specific DNA-methyltransferase (adenine-specific)	EC:2.1.1.72	0.576	0.496	0.0138	-0.0238	0.0003	0.0464
Beta-ketoacyl-[acyl-carrier-protein] synthase II	EC:2.3.1.179	0.222	0.192	0.0035	-0.0138	< 0.0001	0.0189
Uridine kinase	EC:2.7.1.48	0.164	0.182	0.0033	0.0088	0.0003	0.0497
2-C-methyl-D-erythritol 4-phosphate cytidyltransferase	EC:2.7.7.60	0.215	0.191	0.0042	-0.0101	0.0002	0.0416
Alpha-amylase	EC:3.2.1.1	0.149	0.087	0.0082	-0.0202	< 0.0001	0.0228
Phosphoribosyl-ATP diphosphatase	EC:3.6.1.31	0.174	0.157	0.0026	-0.0072	0.0001	0.0324
Tryptophan synthase	EC:4.2.1.20	0.346	0.318	0.0043	-0.0157	< 0.0001	0.0176

¹⁾Statistical analysis was performed using MaAsLin2 with $q = 0.05$ as the cutoff.

HWG, Hanwoo inoculum-treated group; JBG, Jeju Black inoculum-treated group; ATP, adenosine triphosphate.

Table 5. Differentially abundant predicted microbial enzyme commission (EC) numbers (at least $\geq 0.1\%$ average relative abundance across all samples) by diet effect at 3 h of incubation¹⁾

Enzymes	EC number	Relative abundance (%)			SEM	Coefficient	p-value	q-value
		Growing	Early fattening	Late fattening				
2-oxoacid oxidoreductase (ferredoxin)	EC:1.2.7.11	0.204 ^b	0.204 ^b	0.215 ^a	0.0031	0.0178	0.0024	0.0294
Purine-nucleoside phosphorylase	EC:2.4.2.1	0.146 ^b	0.148 ^b	0.150 ^a	0.0024	0.0110	0.0004	0.0072
Adenosylcobinamide-GDP ribazoletransferase	EC:2.7.8.26	0.107 ^b	0.107 ^b	0.108 ^a	0.0013	0.0052	0.0025	0.0299
S-ribosylhomocysteine lyase	EC:4.4.1.21	0.123 ^a	0.119 ^{ab}	0.114 ^b	0.0025	-0.0119	0.0020	0.0259
Asparagine synthase (glutamine-hydrolyzing)	EC:6.3.5.4	0.189 ^a	0.184 ^b	0.184 ^b	0.0018	-0.0070	0.0019	0.0245

¹⁾Statistical analysis was performed using MaAsLin2 with $q = 0.05$ as cutoff.

^{a,b}Superscripts indicate statistically significant differences ($p < 0.05$).

GDP, guanosine diphosphate.

Comparison of functional features by diet effects

Five EC numbers differed by diets (Table 5). Among these, one oxidoreductase (EC 1) and two transferases (EC 2) were significantly enriched in the late fattening diet, while lyase (EC 4) and ligase (EC 6) were enriched in the growing diet. These results were obtained only at 3 h of incubation, and there were no significantly different functional features at 24 h of incubation.

DISCUSSION

Inoculum effect on ruminal microbiota

The present study investigated the impact of different inocula on the ruminal microbiota *in vitro*. Our results demonstrated significant differences in the microbial community composition between two inoculum-treated groups, as evident from the distance matrix analyzing the compositional dissimilarity. These findings are consistent with previous studies, which highlighted how the overall microbial community structures in rumen fluid can be influenced by various factors, including breed and cattle subspecies [38,39]. In this experiment, the donor animals were provided with an early fattening period diet and raised on Jeju Island. Rumen fluid samples were collected through stomach sampling techniques. As noted by Hagey et al. [40], the microbial composition can exhibit variations depending on the sampling method employed. At the phylum level, it was observed that Firmicutes increased, and Bacteroidota (Bacteroidetes) decreased when compared to rumen fistula fluid filter through cheesecloth. In a separate study, certain members of the Firmicutes

phylum, such as Christensenellaceae and Lachnospiraceae, were reported to have potentially higher abundance when sampling liquid-based fluid via stomach sampling [41]. Furthermore, numerous factors, including geographical location and diet, have been identified as influential contributors to the microbial diversity and quantity within the digestive tract [42]. Under high-concentrate feeding conditions, Firmicutes accounted for over 70% of the detected microbes. At the genus level, *Prevotella* experienced a significant decrease under high-concentrate feeding conditions when compared to low-concentrate diet [43].

During the early incubation time, there were only transient differences in richness, while more pronounced compositional differences emerged later, resulting in a significant disparity in the overall microbial community between the two inocula-treated groups. This effect can be attributed to the distinct physical and physiological traits of the highly domesticated Hanwoo cattle and the relatively native Jeju Black cattle. Gut size and ruminal passage rate are known to influence the rumen microbial composition [44,45], possibly leading to the acquisition of distinct rumen microbiota by the two groups.

At the family and genus levels, certain members of the ruminal microbiota were significantly enriched in the HWG. Notably, carbohydrate fermenting bacteria such as *Ruminococcus*, *Pseudoramibacter*, and Ruminococcaceae CAG-352 from the Firmicutes phylum were identified. *Ruminococcus* species are well-known cellulolytic bacteria, including *R. flavefaciens* and *R. albus* [46–48]. *Pseudoramibacter* consumes glucose or pyruvate and produces acetate [49], while uncultured genus CAG-352 is primarily fibrolytic [50]. These genera likely contributed to fibrolysis more rapidly in the HWG, resulting in a significantly higher total VFA concentration and acetate proportion during the early incubation time. The [*Eubacterium*] *ruminantium* group, which was mainly found in rumen fluid, is hemicellulolytic (mainly xylan) [51,52] and produces lactate, butyrate, and formate [53]. *Coproccoccus* ferments aromatic compounds into organic acids such as acetate and benzoate [54]. *C. catus* [55], *C. eutactus*, and *C. comes* [56] are reported to produce succinate, which is a precursor of propionate. These two genera, which appeared to be enriched only at the later incubation time in the HWG, may have increased production of other VFAs rather than acetate, thus offsetting the difference in VFA profiles at 24 h of incubation.

Similarly, certain microbial taxa were enriched in the JBG, including Patescibacteria (also known as Candidate Phyla Radiation [CPR]), which possesses surface proteins facilitating its attachment to other microorganisms like bacteria and methanogens [57]. Additionally, Synergistota can oxidize acetate into H₂ and CO₂ [58] and may provide short-chain fatty acids and sulfate to methanogens or sulfate-reducing bacteria [59]. Given the differences in the grazing environment and degree of domestication for Jeju Black cattle, methane production might be higher compared to that of Hanwoo [60]. However, methane production was not measured in this study, and further research is required to directly correlate the abundances of these enriched microbial taxa with methane production.

The analysis of relative abundance in 3 h and 24 h samples did not reveal significant differences in the *in vitro* fermentation characteristics between the two groups. However, it was observed that certain bacterial taxa associated with the JBG showed fermentative traits that might explain the distinct VFA profiles observed, especially during the early stages of incubation. Notably, the *Blautia* species including *B. hydrogenotrophica*, *B. schinkii*, and *B. producta* were found to produce acetate, lactate, or succinate [61]. Similarly, the [*Ruminococcus*] *gaurvreauii* group was identified as a major acetate producer [62,63]. *Candidatus Saccharimonas* was positively correlated with propionate production in dairy cows [64], while the Oscillospiraceae NK4A214 group was negatively correlated with acetate and propionate proportions in young goats [65]. Additionally, the abundance of *Solibacillus* was found to be positively correlated with valerate under acidosis conditions in dairy

cows [65].

Taken together, by using different inocula, the differentially abundant bacterial genera have some features that can make differences in *in vitro* fermentation and that might be responsible for the VFA profiles at the beginning of the incubation period and the overall *in vitro* digestibility.

Diet effect on ruminal microbiota

The present study investigated the impact of different diets on the ruminal microbiota. Surprisingly, no significant differences were observed in the overall ruminal microbiota in response to the three different diets. This lack of variation was reflected in all analyzed α -diversity measurements, indicating that the number of microbes, evenness, and overall diversity of the ruminal microbiota remained consistent under *in vitro* conditions. While the diets were formulated to meet the nutritional requirements for each stage of growth and fattening, the relatively small variation in the chemical composition of the experimental diets may have resulted in only mild differences during *in vitro* incubation. This assumption is further supported by the lack of differences in digestibility measurements and minor variations in fermentation characteristics. It is important to consider these subtle differences when interpreting the microbiome data in this study.

Although limited studies have explored the relationship between the [*Eubacterium*] *coprostanoligenes* group and the forage-to-concentrate ratio, a previous study involving feed additives reported a positive correlation between this genus and ruminal pH [66]. The prevalence of the [*Eubacterium*] *coprostanoligenes* group in the late fattening diet could have influenced the ruminal pH, thereby potentially explaining the lack of significant differences in ruminal fermentation characteristics during later incubation times. The role of this bacterial group in pH regulation highlights the complexity of ruminal microbial interactions and their potential impact on the fermentation process.

Ruminococcaceae is a prominent taxon within the Firmicutes phylum in the rumen [67,68] known for its cellulolytic and fibrolytic capabilities [69,70]. In the present study, Ruminococcaceae exhibited differential abundance in the growing diet, which contained more fiber-rich ingredients such as timothy hay and alfalfa. Interestingly, uncultured and tentative genera within the Ruminococcaceae family, namely Ruminococcaceae CAG-352 and an uncultured Ruminococcaceae genus, were enriched at 3 h and 24 h of incubations, respectively. Previous research has shown that some uncultured/unclassified genera within Ruminococcaceae, including Ruminococcaceae UCG-011 and UCG-010, were enriched in the intensive forage feeding condition [70,71]. The high relative abundance of Ruminococcaceae CAG-352 at 3 h of incubation, which ranged from 29.0% to 48.6%, may exert a notable impact at the family level. The presence and activity of these specific taxa could be linked to fiber digestion.

Microbial functional differences by inoculum effect

In contrast to the overall microbial community, the functional microbial community did not differ significantly due to the inoculum effect. These findings are consistent with previous studies in the rumen, highlighting that different microbial communities may share similar functional potential [72].

Notably, six EC numbers were enriched in the HWG, including 2-C-methyl-D-erythritol 4-phosphate cytidyltransferase (EC:2.7.7.60), phosphoribosyl-adenosine triphosphate (ATP) diphosphatase (EC:3.6.1.31), and tryptophan synthase (EC:4.2.1.20), which contain nucleic acid, phosphate, or nucleoside triphosphate (NTP), and were involved in the biosynthesis of secondary metabolites, and beta-ketoacyl-[acyl-carrier-protein] synthase II (EC:2.3.1.179), which was involved in fatty acid biosynthesis. However, the predicted microbial function difference was not related to fermentation characteristics or the ruminal microbiota diversity. Additionally,

the enrichment of alpha-amylase in the HWG did not lead to significant changes in glucose fermentation or ruminal pH, suggesting that other factors may play a more significant role in determining these outcomes.

Microbial functional differences by diet effect

S-ribosylhomocysteine lyase (EC:4.4.1.21) and asparagine synthase (glutamine-hydrolyzing) (EC:6.3.5.4) were found to be enriched in the growing diet at 3 h of incubation. These enzymes are involved in protein biosynthesis, specifically the synthesis of cysteine, methionine, aspartate, and glutamate. The enrichment of these microbial enzymes in the growing diet at an earlier incubation time related to protein biosynthesis resulted in the numerically and significantly enhanced nitrogen utilization efficiency at both 3 h and 24 h of incubation, respectively. Previous studies have reported a negative relationship between $\text{NH}_3\text{-N}$ concentration in the rumen and the number of microbial proteins derived from non-protein nitrogen [73,74]. Microbial proteins synthesized from multiple nitrogen sources serve as a crucial source of amino acids for ruminants [75,76]. The increased nitrogen utilization efficiency observed in this study suggests that the diet for the growing period potentially promoted beef cattle growth by delivering a greater amount of nitrogen sources to the small intestine [77].

In conclusion, this study used Jeju Black and Hanwoo cattle, both of which belong to the *Bos primigenius* species, as rumen fluid donors for an *in vitro* fermentation experiment with three different diets corresponding to the fattening stages of beef cattle. Our results suggest that ruminal microbiota differences present in the two different inocula may influence the early stages of fermentation and microbial composition. However, these differences were only partially offset after 24 hours of incubation. While the inoculum significantly affected the overall microbial community, it did not seem to exert a substantial impact on the functional microbial community. Among the differentially abundant taxa affected by the inoculum effect, *Patescibacteria*, *Ruminococcus*, *Pseudoramibacter*, *Blautia*, and *Solibacillus* could potentially contribute to the observed differences in rumen fermentation characteristics and overall microbial community structure. Dietary effects were not significant based on the alpha- and beta-diversity measurements, which showed minor differences in the bacterial taxa. However, it is essential to consider that the rumen fluid used in the experiment came from donor animals that were adapted to the diets for only 2 weeks. Thus, the original differences present in the inoculum, as indicated by specific microbial taxa, overall diversity, and functional features, should be carefully considered while interpreting the potential diet effects. In conclusion, this study provides valuable insights into the rumen microbiome and functional characteristics of Hanwoo and Jeju Black cattle.

SUPPLEMENTARY MATERIALS

Supplementary materials are only available online from: <https://doi.org/10.5187/jast.2023.e109>.

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