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RECEIVED 24 April 2024

ACCEPTED 17 September 2024

PUBLISHED 09 October 2024

## CITATION

Jeong J, Yu C, Kang R, Kim M and  
Park T (2024) Application of  
propionate-producing bacterial consortium  
in ruminal methanogenesis inhibited  
environment with bromoethanesulfonate as a  
methanogen direct inhibitor.  
*Front. Vet. Sci.* 11:1422474.  
doi: 10.3389/fvets.2024.1422474

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# Application of propionate-producing bacterial consortium in ruminal methanogenesis inhibited environment with bromoethanesulfonate as a methanogen direct inhibitor

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Methane production in ruminants is primarily due to the conversion of metabolic hydrogen (H<sub>2</sub>), produced during anaerobic microbial fermentation, into methane by ruminal methanogens. While this process plays a crucial role in efficiently disposing of H<sub>2</sub>, it also contributes to environmental pollution and eliminating methane production in the rumen has proven to be challenging. This study investigates the use of probiotics, specifically propionate-producing bacteria, to redirect accumulated H<sub>2</sub> in a methane-mitigated environment. For this objective, we supplemented experimental groups with *Lactiplantibacillus plantarum* and *Megasphaera elsdenii* for the reinforced acrylate pathway (RA) and *Selenomonas ruminantium* and *Acidipropionibacterium thoenii* for the reinforced succinate pathway (RS), as well as a consortium of all four strains (CB), with the total microbial concentration at 1.0 × 10<sup>10</sup> cells/mL. To create a methane-mitigated environment, 2-bromoethanesulfonate (BES) was added to all experimental groups at a dose of 15 mg/0.5 g of feed. BES reduced methane production by 85% *in vitro*, and the addition of propionate-producing bacteria with BES further decreased methane emission by up to 94% compared with the control (CON) group. Although BES did not affect the alpha diversity of the ruminal bacteriome, it reduced total volatile fatty acid production and altered beta diversity of ruminal bacteria, indicating microbial metabolic adaptations to H<sub>2</sub> accumulation. Despite using different bacterial strains targeting divergent metabolic pathways (RA and RS), a decrease in the dominance of the [*Eubacterium*] *ruminantium* group suggesting that both approaches may have a similar modulatory effect. An increase in the relative abundance of *Succiniclasticum* in the CB group suggests that propionate metabolism is enhanced by the addition of a propionate-producing bacterial consortium. These findings recommend using a consortium of propionate-producing bacteria to manage H<sub>2</sub> accumulation by altering the rumen bacteriome, thus mitigating the negative effects of methane reduction strategies.

## KEYWORDS

ruminant, methane emission, methanogen direct inhibition, probiotics, alternative hydrogen sink, propionate production

## Introduction

The generation of methane gas (CH<sub>4</sub>) in ruminants is an excretory process that has evolved over a long time as a result of microbial fermentation in the rumen, making it impossible to completely inhibit CH<sub>4</sub> production (1). Methanogenesis in the rumen causes a significant loss of gross energy (2–12%) and far greater global warming than does carbon dioxide (CO<sub>2</sub>), causing a negative impact on the environment (2). These factors underscore the critical need for strategies to reduce CH<sub>4</sub> emissions from ruminant livestock. Several methods to mitigate CH<sub>4</sub> emissions, such as defaunation (3), the use of probiotics (4, 5), feed additives, including plant extracts (6–8), bacteriocins (9), nitrates and sulfates (10), fumarates (11, 12), synthetic compounds like 3-nitrooxypropanol (13, 14), bromo-organic compounds from seaweed (15, 16), and ionophores like monensin (17), and adjustments in feed composition, including altering the forage-to-concentrate ratio (18) and supplementing dried distillers grains with solubles (19), have been employed. These strategies can be broadly classified into the following categories based on their mechanisms: indirect and direct approaches (20, 21). Indirect mechanisms aim to reduce the substrates available for methanogens by inhibiting fibrolytic bacteria, protozoa, or fungi (22). While effective in reducing methane, these microorganisms are essential for the digestion of fibrous feed, and their inhibition can adversely affect animal nutrition and productivity. Conversely, direct strategies aim to suppress the population of methanogenic archaea directly, thus significantly lowering methane production. Although this method seems optimal, it presents challenges in maintaining animal productivity. Suppressing methanogens disrupts normal microbial fermentation (23, 24), leading to the accumulation of hydrogen (H<sub>2</sub>), which in turn hinders the re-oxidation processes of NADH, NADPH, and FADH<sub>2</sub>. This reduction may potentially decrease the production of volatile fatty acids (VFAs) (25, 26), resulting in microbial adaptation that could eventually counteract methane reduction efforts. Therefore, managing H<sub>2</sub> concentration when using direct methanogen inhibitors is a crucial factor that must be carefully considered and addressed.

Hydrogenotrophic bacteria that induce H<sub>2</sub> uptake through alternative H<sub>2</sub> sinks, such as dissimilatory sulfate reduction, nitrate reduction, propionate production, and reductive acetogenesis, compete with ruminal methanogens (27). However, the reduced efficiency of H<sub>2</sub>-capture by propionate producers and reductive acetogens, combined with the limited availability of electron acceptors for nitrate and sulfate reducers, makes it difficult for these hydrogenotrophic bacteria to dominate in the rumen (10, 24, 27). Thus, in an environment where ruminal methanogens are reduced by specific inhibitors, hydrogenotrophic bacteria with lower H<sub>2</sub> affinity

may benefit from the absence of their dominant competitors. Moreover, the increased H<sub>2</sub> concentration resulting from methanogen-specific inhibitors can enhance the availability of H<sub>2</sub> and provide an opportunity to boost the dominance of hydrogenotrophic bacteria (24).

VFAs, including acetate, propionate, and butyrate, are key end-products of microbial fermentation in the rumen, serving as significant energy sources for ruminants and substrates for methanogenesis (28). Unlike the acetate and butyrate production pathways, the propionate production pathway consumes a greater amount of hydrogen than it generates. As a result, it functions as a net hydrogen sink, which may diminish the availability of hydrogen for methanogenesis through competitive interactions (1). Efforts to enhance propionate production, aiming to limit methanogenesis by leveraging this competitive mechanism, have been explored (27, 29, 30). Despite these efforts, the affinity of propionate producers for H<sub>2</sub> is lower than that of methanogens, thus maintaining the dominance of methanogens and limited methane reduction, which was typically restricted to approximately 10–15% in normal rumen conditions (1).

A comparative study of gene expression patterns associated with H<sub>2</sub> metabolism between high- and low-methane-emitting animals (31) revealed similar expression levels of enzymes involved in H<sub>2</sub> production. However, enzymes mediating various alternative H<sub>2</sub> sinks that induce H<sub>2</sub> uptake were activated in low-methane-emitting animals. Additional research has shown increased gene and transcript abundance related to lactate and propionate production as well as butyrate conversion from acetate, which all contribute to H<sub>2</sub> uptake in low-methane-emitting animals (32, 33). Based on these findings, increasing the dominance of microbes that contribute to alternative H<sub>2</sub> sinks, such as propionate production in the rumen, to induce H<sub>2</sub> uptake appears to be a feasible approach for methane mitigation.

Considering these factors comprehensively, we hypothesized that using a bacterial consortium focused on the propionate production pathway (24, 34), combined with the application of a direct methanogen inhibitor (2-bromoethanesulfonate, BES) (35–37) will not only aid in addressing the issue of accumulated H<sub>2</sub> but also help in further reducing methane production. Furthermore, we suggest that utilizing a consortium of bacteria involved in the propionate pathway (34) rather than individual strains could more effectively modulate the rumen microbiome. This strategy promotes pathway-based enhancement and represents a synergistic approach that could be practically applied on farms to achieve a more substantial reduction in methane emissions.

## Materials and methods

### Selection and preparation of microbes

The selection of bacterial strains for the *in vitro* fermentation experiment was based on their involvement in the metabolic pathways responsible for propionate production in the rumen (acrylate and succinate pathways) (Table 1). *Lactiplantibacillus plantarum* (KCTC 3103), *Megasphaera elsdenii* (KCTC 5187), and *Acidipropionibacterium thoenii* (KCTC 5343) were obtained from the Korean Collection for Type Cultures (KCTC, Jeongseup, Republic of Korea). *Selenomonas ruminantium* (DSM 2150) was obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ; Braunschweig, Germany). *L. plantarum* is a transient, facultative anaerobic bacterium,

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Abbreviations: CH<sub>4</sub>, methane gas; CO<sub>2</sub>, carbon dioxide; H<sub>2</sub>, hydrogen; VFA, volatile fatty acid; BES, 2-bromoethanesulfonate; RCM, reinforced clostridial medium; OD, optical density; CON, control group; RA, reinforced acrylate pathway group; RS, reinforced succinate pathway group; CB, consortium group; NH<sub>3</sub>-N, ammonia nitrogen; DMD, dry matter digestibility; NDFD, neutral detergent fiber digestibility; DW, distilled water; q-PCR, quantitative real-time polymerase chain reaction; RBB + C, repeated bead-beating plus column; HiFi, high-fidelity; ASV, amplicon sequence variant; OTU, operational taxonomic unit; NMDS, non-metric multidimensional scaling; HSD, honestly significant difference; dDM, degraded dry matter; A:P ratio, acetate-to-propionate ratio; PERMANOVA, permutation analysis of variance; BCVFA, branched-chain volatile fatty acid.

TABLE 1 Bacterial strains used in the *in vitro* fermentation experiment for enhancing the propionate production pathway.

Bacterial strain	Associated pathway	Group	
<i>Lactiplantibacillus plantarum</i>	Acrylate–lactate production	RA	CB
<i>Megasphaera elsdenii</i>	Acrylate–propionate production		
<i>Acidipropionibacterium thoenii</i>	Succinate–propionate production	RS	
<i>Selenomonas ruminantium</i>	Succinate–propionate production		

Standardization of total cell number:  $1.0 \times 10^{10}$  cells/mL for the reinforced acrylate (RA) pathway group,  $5.0 \times 10^9$  cells/mL of *L. plantarum* (KCTC 3103) and *M. elsdenii* (KCTC 5187); for the reinforced succinate (RS) pathway group,  $5.0 \times 10^9$  cells/mL of *S. ruminantium* (DSM 2150) and *A. thoenii* (KCTC 5343); for the consortium (CB) group,  $2.5 \times 10^9$  cells/mL of *L. plantarum*, *M. elsdenii*, *S. ruminantium*, and *A. thoenii*.

which is generally introduced through the food in the rumen (38). *L. plantarum* has the potential for lactate production to facilitate the acrylate pathway for propionate production (34) and reduction in methanogenesis by inoculation of *L. plantarum* BX62 strain (39). *M. elsdenii*, *A. thoenii*, and *S. ruminantium* are obligate anaerobic bacteria residing in the rumen. *M. elsdenii* is involved in the acrylate pathway and has been reported to produce not only butyrate by fermenting glucose but also propionate through lactate fermentation (1, 40, 41). Among the various propionic acid bacterial strains, *A. thoenii* has a potential for methane mitigation (30). *S. ruminantium* is reported as a propionate producer and succinate utilizer (27). Additionally, *A. thoenii* and *S. ruminantium* can use lactate as a substrate for propionate production (27). These bacterial strains were cultured in reinforced clostridial medium (RCM) supplemented with 10% clarified rumen fluid under strict anaerobic conditions. The cultures were preserved at  $-80^\circ\text{C}$  in 20% glycerol. For the *in vitro* fermentation experiment, 1% volume aliquots (100  $\mu\text{L}$ ) of each strain's frozen stock were inoculated into RCM with 10% clarified rumen fluid and incubated at  $37^\circ\text{C}$  under strict anaerobic conditions. Incubation times were 48 h for all strains except for *L. plantarum*, which was incubated for 24 h. Microbial growth was quantified and standardized to an optical density of 1.0 at 600 nm ( $\text{OD}_{600}$ ). Cultures achieving this growth level were temporarily stored at  $4^\circ\text{C}$  for up to 12 h before cell harvesting. Considering the inverse relationship between cell concentration and cell volume at an OD of 1.0 (42), the cell volume was evaluated by following instructions in Bergey's manual (43, 44) and a previous study (45). Subsequently, the desired cell quantities for each strain were established using the calculated cell volume (46) to precisely ascertain the required cell counts for every strain. While the reinforced acrylate pathway (RA) and reinforced succinate pathway (RS) group each requires a cell count of  $5.0 \times 10^9$  cells/mL per species, the consortium group (CB) group requires one of  $2.5 \times 10^9$  cells/mL. For cell harvesting, cultures were transferred to 2-mL screw cap tubes equipped with O-rings in an anaerobic workstation (Whitley DG250; Don Whitley Scientific, England) and centrifuged at 10,000 g for 10 min at  $4^\circ\text{C}$ . After centrifugation, the supernatant was removed, and the cells were washed twice with an anaerobic salt solution (comprising  $\text{K}_2\text{HPO}_4$  1.0 g/L,  $\text{KH}_2\text{PO}_4$  1.0 g/L,  $\text{NaHCO}_3$  10.0 g/L,  $\text{NaCl}$  2.0 g/L,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.5 g/L in anoxic solution adjusted to pH 6.5–6.7) to remove the residual medium components. The final cell

suspension volume was adjusted to 1 mL per tube by combining each bacterial strain within their respective groups using the same anaerobic salt solution, in preparation for inoculation. Prepared bacterial inoculants were stored at  $4^\circ\text{C}$  until use, removed 30 min before inoculation to allow temperature equilibration, and then introduced into fermentation bottles as part of the inoculation procedure.

## Experimental design

The *in vitro* fermentation experiment was structured based on a completely randomized design, with each of the treatment groups replicated five times. The groups were organized as follows: (1) CON, which served as the control group; (2) BES, 15 mg of 2-bromoethanesulfonate per 0.5 g of basal diet was added (137502-25G, Sigma-Aldrich, St. Louis, MO, USA) (35); (3) RA, the reinforced acrylate pathway group, which included the same BES concentration along with *L. plantarum* and *M. elsdenii*; (4) RS, the reinforced succinate pathway group, which included the same BES concentration along with *S. ruminantium* and *A. thoenii*; and (5) CB, the consortium group for propionate production pathway, incorporating the same concentration of BES and all four microbial strains. BES was included in all the treatment groups except the CON group to assess its effectiveness in creating a methane-reducing environment. The overall microbial dose for the groups receiving microbial supplementations (RA, RS, and CB) was based on the estimated cell counts and then set at  $1.0 \times 10^{10}$  cells/mL to ensure a consistent level of microbial influence across these treatments.

## *In vitro* fermentation experiment

For the *in vitro* fermentation experiment, the stomach tubing method was used to collect rumen fluid from three Hanwoo cows located at the Nonghyup Co., Ltd. research farm. The time required to reach our laboratory from this farm is approximately 30 min. The procedures were authorized by the Institutional Animal Care and Use Committee (IACUC) at Chung-Ang University (202401030036) and conducted in compliance with the ethical standards outlined in the IACUC guidelines for animal welfare. Collection was performed before the morning feeding at 7:30 a.m. To ensure the preservation of sample integrity, the rumen fluid was transported to the laboratory within 30 min in an airtight, insulated container pre-flushed with 99.999%  $\text{CO}_2$  gas, which was passed through a pre-heated copper column to maintain a stable temperature and anaerobiosis. Upon arrival at the laboratory, the rumen fluid underwent filtration through two cheesecloth layers to eliminate particulate impurities. Subsequently, the filtered rumen fluid was combined with a buffer solution at twice its volume, as specified previously (47), and the mixed inoculum's pH was adjusted to 6.7. The inoculum was continuously flushed with oxygen-free  $\text{CO}_2$  to maintain anaerobic conditions. The experimental substrate was the same as the feed used at the research farm [comprising pellet concentrate and oat hay (Table 2)]. It was ground through a 1.0-mm sieve and oven-dried at  $55^\circ\text{C}$  for 48 h before being placed in the fermentation bottles. Each 125-mL fermentation bottle was loaded with 0.5 g of this feed with a forage-to-concentrate ratio of 1:1 and 50 mL of the prepared inoculum.

TABLE 2 Composition of diet used in the *in vitro* fermentation experiment.

Item (% of DM)	Oat hay	Pellet concentrate
Dry matter	91.56	89.19
Crude protein	3.55	16.20
Crude fat	2.15	4.08
Crude fiber	27.40	8.66
Crude ash	5.02	6.95
Calcium	0.09	1.22
Phosphorus	0.09	0.65
ADF	30.87	13.23
NDF	54.17	31.10

DM, dry matter; ADF, acid detergent fiber; NDF, neutral detergent fiber.

All procedures were performed under anaerobic conditions. After inoculating 1 mL of the pre-prepared microbial tube, the inoculated bottles were immediately sealed with butyl rubber stoppers and aluminum caps and placed in a shaking incubator. The incubator conditions were set to 39°C with a shaking speed of 60 rpm, and the samples were incubated for 48 h.

## Sample collection and fermentation measurements

After incubation, total gas and CH<sub>4</sub> production, pH, ammonia nitrogen (NH<sub>3</sub>-N), VFA concentration, dry matter digestibility (DMD), and neutral detergent fiber digestibility (NDFD) were analyzed. The headspace gas pressure of the fermentation bottle was recorded using a pressure transducer (L20000DCV3, Laurel Electronics, Inc., Costa Mesa, CA, USA) and simultaneously collected in a gas bag using a rubber stopper, needle, and 3-way cock setup (48). Gas pressure was recorded and collected at intervals of 3, 6, 12, 24, 36, and 48 h during incubation (49), with continuous monitoring to ensure that the pressure did not exceed 480 mbar and prevent microbial growth inhibition (50). Gas volume was estimated by converting mbar readings into mL by injecting air into a similarly sized fermentation bottle filled with distilled water (DW) and an equal volume of inoculum for calibration under standard atmospheric pressure (48). CH<sub>4</sub> concentration from the collected gas (2 mL) was determined using gas chromatography (YL6500 GC system, Youngin Chromass, Anyang, Republic of Korea) equipped with a thermal conductivity detector and a packed GC column (G3591-80055, Agilent Technologies Inc., Santa Clara, CA, USA), employing a gas-tight syringe (1010 TLL, Hamilton company, Reno, NV, USA) for injection. CH<sub>4</sub> measurements were taken in duplicates within 2 days of collection. After 48 h of incubation using a cut tip, 1.8 mL of inoculum was pipetted into a 2-mL microcentrifuge tube. Subsequently, the residual inoculum was transferred to a 50-mL conical tube for pH analysis using a pH meter (MW150, Milwaukee Instruments, Inc., Rocky Mount, NC, USA). The inoculum was then placed in a pre-weighed R510 nylon bag (Ankom Technology, Macedon, NY, USA), carefully transferring the remaining particles with DW. The bag was gently squeezed 2–3 times before drying in an oven at 65°C for 72 h to measure DMD, ensuring all sampling

procedures were carefully conducted using ice to prevent additional microbial fermentation and to avoid unnecessary DM loss. For VFA and NH<sub>3</sub>-N analyses, samples were prepared by centrifuging 1.8 mL of the inoculum at 16,000 g for 15 min at 4°C. For VFA analysis, 1.0 mL was mixed with 0.2 mL of 25% (w/v) metaphosphoric acid; for NH<sub>3</sub>-N analysis, 0.5 mL was mixed with 0.1 mL of 0.2 M sulfuric acid (H<sub>2</sub>SO<sub>4</sub>). VFA samples were stored at –80°C and NH<sub>3</sub>-N samples, at 4°C. The remaining pellet was stored at –80°C to use later for extraction during quantitative real-time polymerase chain reaction (qPCR) and microbiome analyses. VFA concentration was quantified using gas chromatography (7890B, Agilent Technologies Inc., Santa Clara, CA, USA) with an autosampler (7693A, Agilent Technologies Inc., Santa Clara, CA, USA), flame ionization detector, and a capillary column (Nukol Fused silica capillary column, Supelco, Bellefonte, PA, USA). The carrier gas was nitrogen (N<sub>2</sub>), with a makeup flow set at 30 mL/min and a column flow set at 1 mL/min. The detector and inlet temperatures were set to 220°C. The initial oven temperature was set to 90°C, which was then increased to 200°C at a rate of 15°C/min, followed by a hold time of 2 min. Subsequently, the temperature was raised to 220°C at a rate of 20°C/min, with a final hold time of 3 min for measurement. The split ratio was set at 10:1, and the injection volume was 0.8 µL. NH<sub>3</sub>-N concentration was determined using a micro plate spectrophotometer (INNO, LITEK, Seongnam, Republic of Korea) by following the colorimetric method of Chaney and Marbach (51) with modifications by Hamid et al. (49) using ammonium chloride as a standard. After the DMD analysis, the maximum amount of DM in the R510 nylon bag was transferred to a F57 filter bag (Ankom Technology, Macedon, NY, USA) for NDFD measurement using a fiber analyzer (A200, Ankom Technology, Macedon, NY, USA), in accordance with the Ankom NDF procedure and incorporating the use of heat-stable  $\alpha$ -amylase.

## Metagenomic DNA extraction

Total metagenomic DNA was extracted from the microbial pellets using the repeated bead-beating plus column purification (RBB + C) method by following a previously described protocol (52). For the preparation of qPCR standards, genomic DNA from the pure cultures of the four bacterial strains was extracted using the AccuPrep® Genomic DNA Extraction Kit (Bioneer, Daejeon, Republic of Korea). This process also incorporated mechanical lysis with bead-beating to ensure a thorough breakdown of cell walls for optimal DNA yield. Additional qPCR standards were prepared from aliquots of the extracted metagenomic DNA samples, ensuring a broad representation of the microbial DNA present in the samples for accurate quantification.

## High-fidelity long-read sequencing

For the analysis of the bacteriota, samples were amplified and sequenced at Macrogen Inc. (Seoul, Republic of Korea) using the HiFi long-read sequencing platform PacBio Sequel IIE system (Pacific Biosciences, CA, USA). The 16S rRNA gene bacterial universal primer pairs used were 27F (5'-AGRGTTGATYMTGGCTCAG-3') and 1492R (5'-GYTACCTTGTTACGACTT-3'). The resultant raw fastq

files were then imported into QIIME2 environment<sup>1</sup> (53) for comprehensive microbiome analysis. After demultiplexing, the sequences were quality-filtered, and the denoising process, which involves removing primer and adaptor sequences as well as eliminating chimeric sequences, was performed using the DADA2 plugin (54). Amplicon sequence variants (ASVs), with a mean length of 1,457 nucleotides, were taxonomically classified using the Silva 138 99% operational taxonomic units (OTUs) reference database (55) with the confidence threshold set at 0.8. The minimal sequence count across all samples was 19,752, and repeated rarefaction was conducted 1,000 times at this minimum sequence count to normalize data across the samples (56). The differences in rumen bacterial diversity within individual samples (alpha diversity) and comparative diversity between samples (beta diversity) were analyzed based on the repeatedly rarefied ASV table. Beta diversity was visualized using non-metric multidimensional scaling (NMDS) plots based on the Bray–Curtis dissimilarity distance matrices.

## Quantitative real-time PCR

Primer sets for the quantification of specific microbial populations were chosen based on previous studies (see [Supplementary Table 1](#)). Unique gene regions were targeted for *L. plantarum* and *A. thoenii*, while the primer sets for other microbes primarily focused on 16S rRNA gene sequences, except the 18S rRNA gene sequence used for protozoa. Initial conventional PCR amplifications were performed to generate qPCR standards using 1  $\mu$ L genomic DNA per reaction in a PCR thermal cycler (TP 600, TaKaRa, Kusatsu, Gunma, Japan). The amplification conditions, including cycling parameters and temperature settings, were adapted from established protocols corresponding to each primer set. The specificity of each primer set was verified and confirmed using TestPrime<sup>2</sup> (57) and confirmed by gel electrophoresis on a 1.5% agarose gel. After verification, the PCR products were purified using the AccuPrep PCR/Gel Purification Kit (Bioneer, Daejeon, Republic of Korea). The concentration of nucleic acids in the purified amplicons was measured using a NanoDrop One microvolume UV–Vis spectrophotometer (Thermo Fisher Scientific, Wilmington, NC, USA), facilitating the calculation of copy numbers per mL for standard curve preparation. These standards were subsequently stored at  $-20^{\circ}\text{C}$  until needed. Microbial quantification was performed on the QuantStudio 1 system (Thermo Fisher Scientific, Wilmington, NC, USA). Each qPCR reaction comprised 1  $\mu$ L template DNA (genomic or metagenomic) added to 15  $\mu$ L reaction mixture. This mixture included 0.075  $\mu$ L of each primer (forward and reverse, both at 100  $\mu\text{M}$  concentration), 7.5  $\mu$ L PowerUp SYBR Master Mix (2X), and 6.35  $\mu$ L ultra-pure water. For total protozoa quantification, 2  $\mu$ L template DNA was used. The qPCR amplification protocol was executed in accordance with the specific guidelines for each primer set, ensuring accurate and reliable microbial quantification.

## Statistical analyses

The Shapiro–Wilk test was conducted to examine whether microbial fermentation parameters followed a normal distribution. Levene's test was utilized to assess the equality of variances among the groups. Such parameters were then analyzed using the Proc Glimmix Procedure in SAS, version 9.4 (SAS Institute, Cary, NC, USA), incorporating Tukey's honestly significant difference (HSD) test to account for supplementation effects as a fixed effect. Conversely, for data sets, including total gas production, pH, methane yield (measured in mL/g of degraded dry matter [dDM]), and the acetate-to-propionate ratio (A:P ratio), which diverged from normal distribution, the non-parametric Kruskal–Wallis H test followed by Dunn's post-hoc test, was applied. Significance of the supplementation effects was declared at  $p \leq 0.05$ , while tendency was noted when  $0.05 < p \leq 0.10$ . Microbial diversity analysis encompassed alpha diversity metrics, such as Chao1, Simpson, Shannon, and Faith's Phylogenetic Diversity, processed in QIIME2 (53). The diversity indices were analyzed using the Proc Glimmix Procedure (SAS, version 9.4, SAS Institute, Cary, NC, USA) with Tukey's HSD test. Beta diversity was analyzed using permutation analysis of variance (PERMANOVA) tests, executed 9,999 times using the `adonis2` function within the `vegan` package in R (58), with results corrected for multiple comparisons using the Benjamini–Hochberg method based on the Bray–Curtis dissimilarity distance matrix. To investigate the multivariate association in the ruminal bacteriome, microbial compositional data were analyzed using MaAsLin 2 (59), focusing on major taxa present in all groups at 100% occurrence. Significant differences were determined based on a false discovery rate-adjusted  $p$ -value ( $Q$ -value) of  $\leq 0.05$  by applying the Benjamini–Hochberg procedure for multiple test corrections. Pearson correlation analysis was conducted using PROC CORR in SAS, version 9.4 (SAS Institute, Cary, NC, USA) with the results visualized in a heatmap generated in R, facilitating the interpretation of correlations between microbial fermentation parameters and differentially abundant microbial relative abundances, while for the supplemented bacterial strains, copy numbers were used instead of relative abundances.

## Results

### Fermentation characteristics

The results from the 48-h *in vitro* fermentation experiment, detailing the fermentation characteristics, are summarized in [Table 3](#) and [Figure 1](#). The incorporation of BES markedly decreased total gas production ( $p < 0.0001$ ); however, it had no significant impact on DMD, NDFD, and pH. The  $\text{NH}_3\text{-N}$  levels showed a tendency to decrease in the RA group. The supplementation of propionate-producing bacteria in conjunction with BES (in the RA, RS, and CB groups) led to a greater reduction in methane production ( $p < 0.0001$ ) than that in the BES-only group. Although BES addition initially lowered total VFA production significantly ( $p < 0.0001$ ), the incorporation of some propionate-producing bacteria (particularly in the RA and CB groups) was found to restore VFA levels that were comparable with those in the CON group. Specifically, the acetate concentration significantly increased in the RA and CB groups with microbial supplementation, although these levels did not fully match

1 <https://qiime2.org/>

2 <https://www.arb-silva.de/search/testprime/>

those of the CON group. However, the levels of other VFAs, such as propionate and butyrate, were restored to those observed in the CON group with RA and CB supplementation. The valerate concentration was significantly higher in the RA and CB groups than that in the CON group. Overall, the addition of propionate-producing bacteria along with BES not only increased the methane mitigation potential but also restored the VFA patterns disrupted by BES treatment.

## Diversity analyses

Analysis of alpha and beta diversity was conducted using 16S rRNA gene long-read sequencing data from 25 rumen fluid samples, achieving 100% Good's coverage across all samples. Alpha diversity indices, including Chao1, Shannon, and Simpson, showed no significant differences between the groups (Table 4). Beta diversity analysis based on Bray–Curtis dissimilarity demonstrated a significant distinction ( $Q < 0.001$ ) between the CON and BES-supplemented groups. Nonetheless, the addition of microbial supplements (RA, RS, and CB) did not significantly alter the beta diversity when compared with that of the BES-only group (Figure 2), indicating that the propionate-producing bacterial consortium did not significantly shift the overall beta diversity of the rumen microbiome under conditions aimed at mitigating methane production.

## Investigation of multivariate association in ruminal bacteriome

MaAsLin2 analysis at the phylum level revealed significant changes ( $Q \leq 0.05$ ) in the relative abundances of the major taxa present in all groups (see Supplementary Figure 1). The abundance of Verrucomicrobiota significantly dropped in the BES group (coefficient =  $-0.43$ ), whereas that of Fibrobacterota and Desulfobacterota both increased (coefficients =  $0.26$  and  $0.24$ , respectively). No significant differences were found among the groups

receiving BES supplementation. At the genus level, 28 genera exhibited significant differences between the CON and BES groups (Figure 3). Comparing the BES group with the groups receiving additional probiotics (Figure 4) showed that one genus, [*Eubacterium*] *ruminantium* group, in the RA group; six genera in the RS group, including [*Eubacterium*] *ruminantium* group, Erysipelotrichaceae UCG-006, Bacilli RF39, *Solobacterium*, Erysipelotrichaceae UCG-002, and Erysipelatoclostridiaceae UCG-004; and six genera in the CB group, including *Succiniclasticum*, *Acidaminococcus*, Butyricocccaceae UCG-009, Bacilli RF39, *Solobacterium*, and Erysipelotrichaceae UCG-002, showed significant changes. The abundances of Bacilli RF39, *Solobacterium*, Erysipelotrichaceae UCG-002, and Erysipelatoclostridiaceae UCG-004 were significantly lower in both the CB and RS groups than the abundances of those in the BES group. Additionally, the abundance of [*Eubacterium*] *ruminantium* was significantly lower in the RA and RS groups than the abundance of that in the BES group.

## Quantitative analysis using qPCR

The effects of the experimental treatments on the abundance of specific bacterial strains, total bacteria, protozoa, and methanogens in the rumen microbiome were evaluated using qPCR, and the results are summarized in Table 5. The inclusion of BES did not significantly alter the absolute abundance of total bacteria and protozoa. Similarly, the supplementation of propionate-producing bacterial strains did not significantly affect the abundance of these microbial groups. However, the inclusion of BES significantly decreased the absolute abundance of total methanogens. Excluding *M. elsdenii*, the addition of other specific bacterial strains (RA:  $5.0 \times 10^9$  cells/mL *L. plantarum* and *M. elsdenii*, RS:  $5.0 \times 10^9$  cells/mL *S. ruminantium* and *A. thoenii*, CB:  $2.5 \times 10^9$  cells/mL *L. plantarum*, *M. elsdenii*, *S. ruminantium*, and *A. thoenii*) caused a significant increase in the abundance of these added strains when compared with the CON and BES-only groups. The significant rise in the abundance of *A. thoenii* was directly

TABLE 3 Fermentation parameters measured over 48 h in the *in vitro* fermentation experiment.

Parameter	Group					SEM	<i>p</i> -value
	CON	BES	BES (+)				
			RA	RS	CB		
DMD (%)	67.54	67.45	67.54	66.95	68.48	4.47	0.6725
NDFD (%)	54.34	54.36	55.20	54.90	52.30	5.94	0.4296
pH	6.50	6.50	6.50	6.51	6.50	3.10	0.4399
NH <sub>3</sub> -N (mg/dL)	22.73 <sup>a</sup>	20.57 <sup>ab</sup>	20.32 <sup>b</sup>	22.00 <sup>ab</sup>	21.18 <sup>ab</sup>	4.11	0.0573
Total gas (mL)	115.97 <sup>A</sup>	103.54 <sup>B</sup>	102.16 <sup>B</sup>	101.56 <sup>B</sup>	102.21 <sup>B</sup>	4.72	<0.0001
CH <sub>4</sub> (mL)	4.58 <sup>A</sup>	0.66 <sup>B</sup>	0.27 <sup>C</sup>	0.28 <sup>C</sup>	0.33 <sup>C</sup>	3.11	<0.0001
CH <sub>4</sub> (mL/g dDM)	13.56 <sup>A</sup>	1.97 <sup>B</sup>	0.80 <sup>C</sup>	0.85 <sup>C</sup>	0.97 <sup>C</sup>	3.23	<0.0001

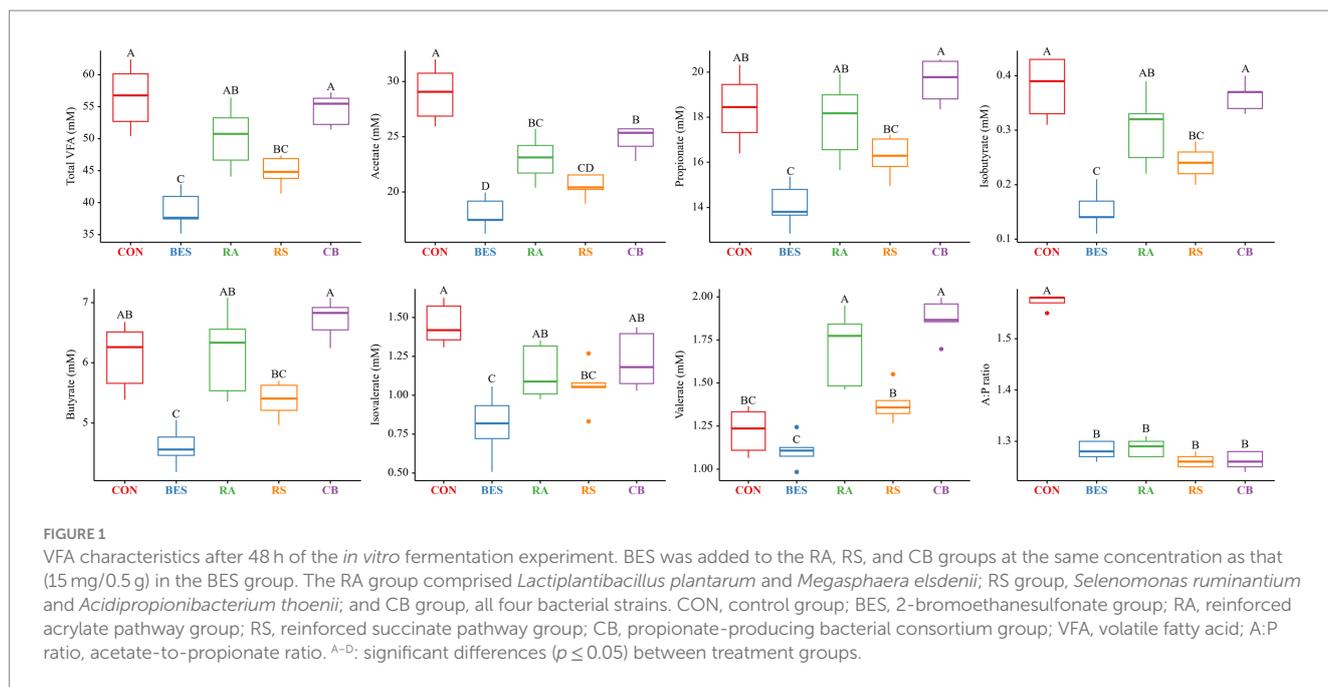
BES was added to the RA, RS, and CB groups at the same concentration as that (15 mg/0.5 g) in the BES group.

The RA group comprised *Lactiplantibacillus plantarum* and *Megasphaera elsdenii*; RS group, *Selenomonas ruminantium* and *Acidipropionibacterium thoenii*; and CB group, all four bacterial strains.

CON, control group; BES, 2-bromoethanesulfonate group; RA, reinforced acrylate pathway group; RS, reinforced succinate pathway group; CB, propionate-producing bacterial consortium group; CH<sub>4</sub> (mL/g dDM), quantity of methane production per degraded gram of dry matter; NH<sub>3</sub>-N, ammonia nitrogen; DMD, dry matter digestibility; NDFD, neutral detergent fiber digestibility; SEM, pooled standard error of the mean.

<sup>A-C</sup>: significant differences ( $p \leq 0.05$ ) between treatment groups.

<sup>a-b</sup>: tendency ( $0.05 < p \leq 0.10$ ) between treatment groups.



**TABLE 4** Alpha diversity analysis after 48 h in the *in vitro* fermentation experiment.

Alpha diversity index	Group					SEM	p-value
	CON	BES	BES (+)				
			RA	RS	CB		
Chao1	454	464	471	443	432	42.6	0.6015
Evenness	0.931	0.929	0.931	0.931	0.929	0.0030	0.6442
Faith's phylogenetic diversity	27.769	26.921	27.620	26.146	25.422	1.41	0.0751
Shannon	8.212	8.218	8.267	8.180	8.129	0.12	0.4739
Simpson	0.995	0.995	0.995	0.995	0.995	0.0003	0.2661

BES was added to the RA, RS, and CB groups at the same concentration as that (15 mg/0.5 g) in the BES group.

The RA group comprised *Lactiplantibacillus plantarum* and *Megasphaera elsdenii*; RS group, *Selenomonas ruminantium* and *Acidipropionibacterium thoenii*; and CB group, all four bacterial strains.

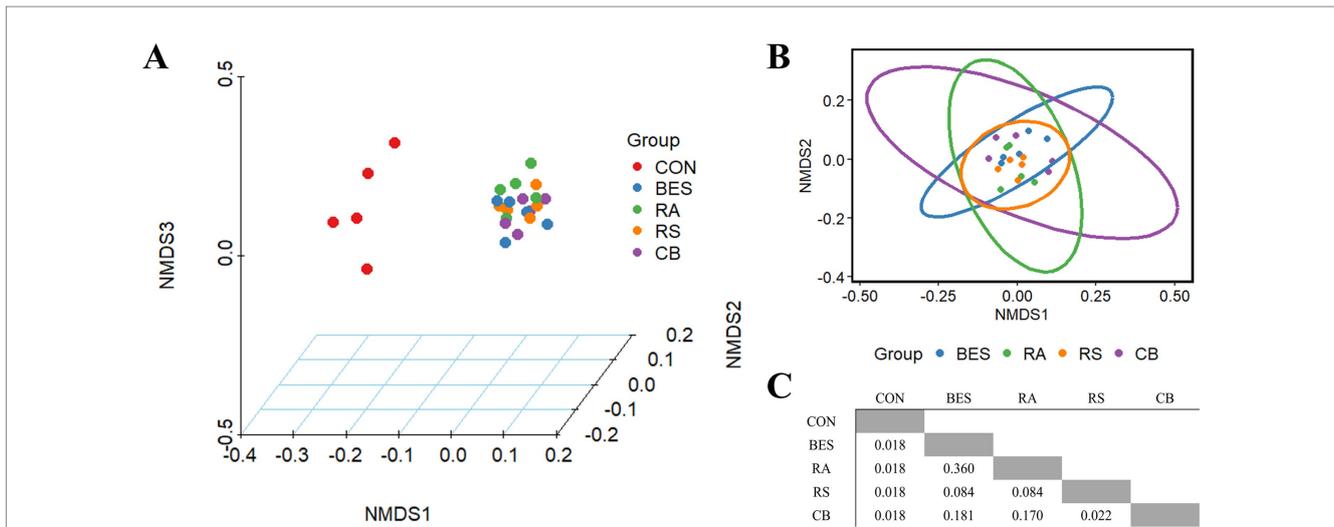
CON, control group; BES, 2-bromoethanesulfonate group; RA, reinforced acrylate pathway group; RS, reinforced succinate pathway group; CB, propionate-producing bacterial consortium group; SM, pooled standard error of the mean.

proportional to the quantity added, underscoring the successful colonization and growth of this specific strain under the experimental conditions.

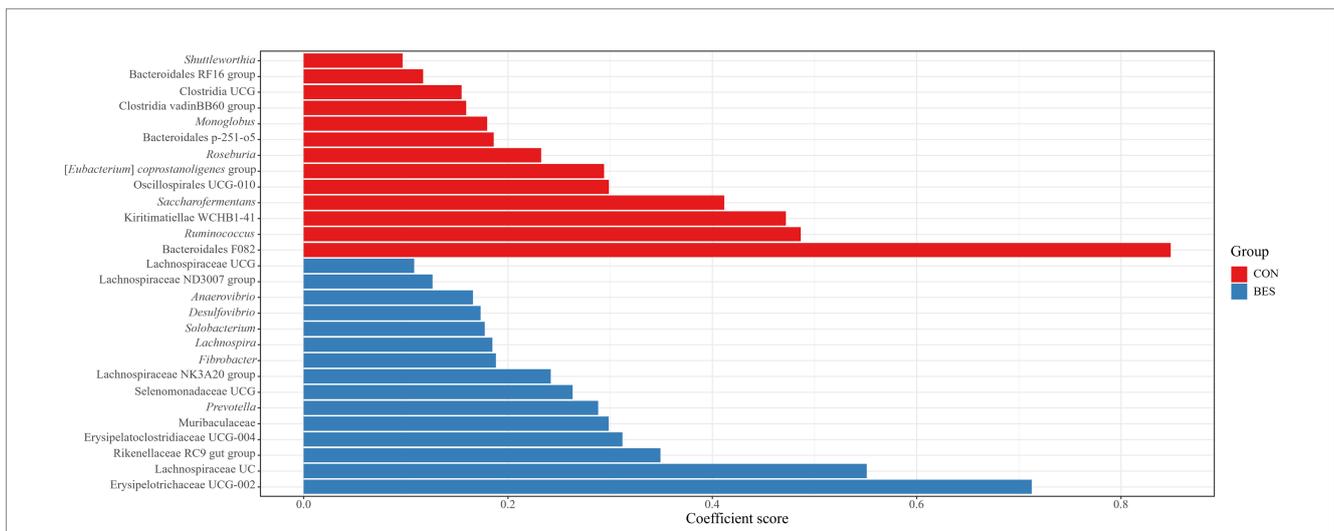
## Discussion

Consistent with previous studies, our findings confirmed that the addition of BES significantly reduces methanogenesis (35, 37). Moreover, the introduction of propionate-producing bacteria into a methane-mitigated environment demonstrated not only the potential for further reducing methane emissions but even the possibility of restoring total VFA in some groups (RA and CB). The acetate concentration, which contributes to H<sub>2</sub> production in metabolic pathways (1), increased in the RA and CB groups beyond the levels observed in the BES-only group. The increase in acetate concentration in the RA and CB groups compared with that in the BES group

indicates that microbial fermentation patterns are enhanced in a methanogen-suppressed environment. While acetate concentration was found to not have returned to the baseline levels observed in the CON group, in light of previous research finding (31) suggesting that ruminal bacteria can sense H<sub>2</sub> concentrations and adjust metabolic pathways in the rumen where H<sub>2</sub> has accumulated, the increase in acetate concentration observed in the RA and CB groups compared to the BES group suggests that the addition of propionate-producing bacteria may help partially resolve the issue of H<sub>2</sub> accumulation. Furthermore, compared with acetate concentration, the increased butyrate concentration observed in the RA and CB groups may reflect the conversion of acetate to butyrate. This conversion also contributes to the H<sub>2</sub> sink (1). The rise in propionate concentrations underscores the effective role of added bacterial strains in enhancing propionate metabolism, either directly or indirectly. The propionate production pathway does not efficiently compete with methanogenesis for metabolic H<sub>2</sub> (24); hence, previous attempts to



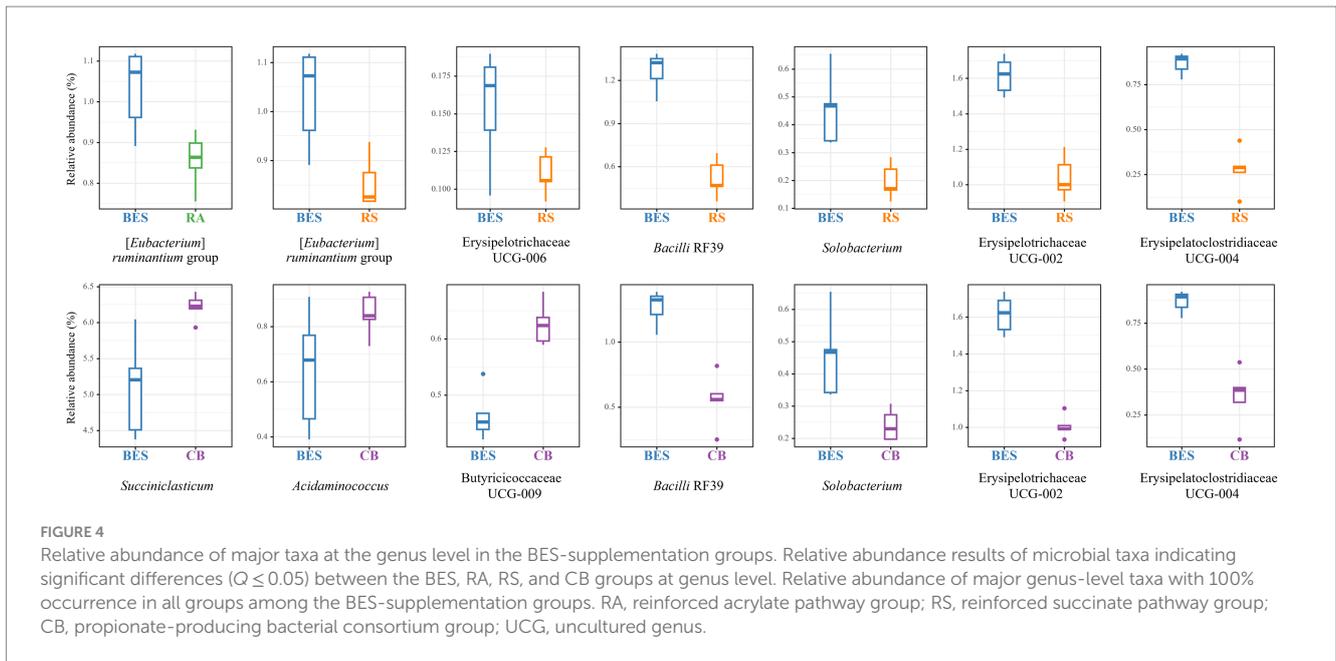
**FIGURE 2** NMDS plot based on Bray–Curtis distance matrix. BES (15 mg/0.5 g of feed) was added to all the groups except the control group. Three-dimensional NMDS plot including the control group (A), two-dimensional NMDS plot comparing the BES-supplemented groups (B), Q-values resulting from pairwise multiple tests adjusted using the Benjamini–Hochberg method (C). NMDS, non-metric multidimensional scaling; BES, 2-bromoethanesulfonate group; RA, reinforced acrylate pathway group; RS, reinforced succinate pathway group; CB, propionate-producing bacterial consortium group; VFA, volatile fatty acid; A:P ratio, acetate-to-propionate ratio.



**FIGURE 3** Analysis of multivariate associations in the ruminal bacteriome at the genus level between the CON and BES groups. Coefficient scores of ruminal bacteriome data showing significant differences ( $Q \leq 0.05$ ) between the CON and BES groups at genus level. BES, 2-bromoethanesulfonate group; UC, unclassified; UCG, uncultured genus.

utilize propionate-producing bacteria as probiotics in standard rumen conditions *in vitro* have not yielded significant success (30). This observation suggests that methane-mitigating strategies aimed at altering the metabolic pathways of ruminal fermentation could be more effective when used in conjunction with direct inhibitors of methanogens (24). The elevation of branched-chain volatile fatty acids (BCVFAs, including isobutyrate and isovalerate) and valerate to levels comparable with those in the control group likely serves as a stimulant for cellulolytic bacteria (60), although this was not observed in our study. The addition of bacterial strains along with BES did not cause significant differences in the alpha diversity

indices; however, significant shifts in beta diversity were observed. This may be attributed to the methanogenesis-inhibiting environment created by BES. The distinction in beta diversity without an alpha diversity alteration suggests that while the composition of the bacterial community changes, the overall richness and evenness of bacteriota remain stable, which indicates a balanced ecosystem adjusting to methane metabolism shifts. Ruminal bacteria are expected to fill their respective niches, thus forming a balanced ecosystem. Nevertheless, these alterations resulted in deficient VFA production in the BES-only group, which was effectively resolved by the addition of propionate-producing bacteria, as demonstrated by



**TABLE 5** Quantitative real-time polymerase chain reaction analysis for absolute quantification of microbial groups and supplemented bacterial strains.

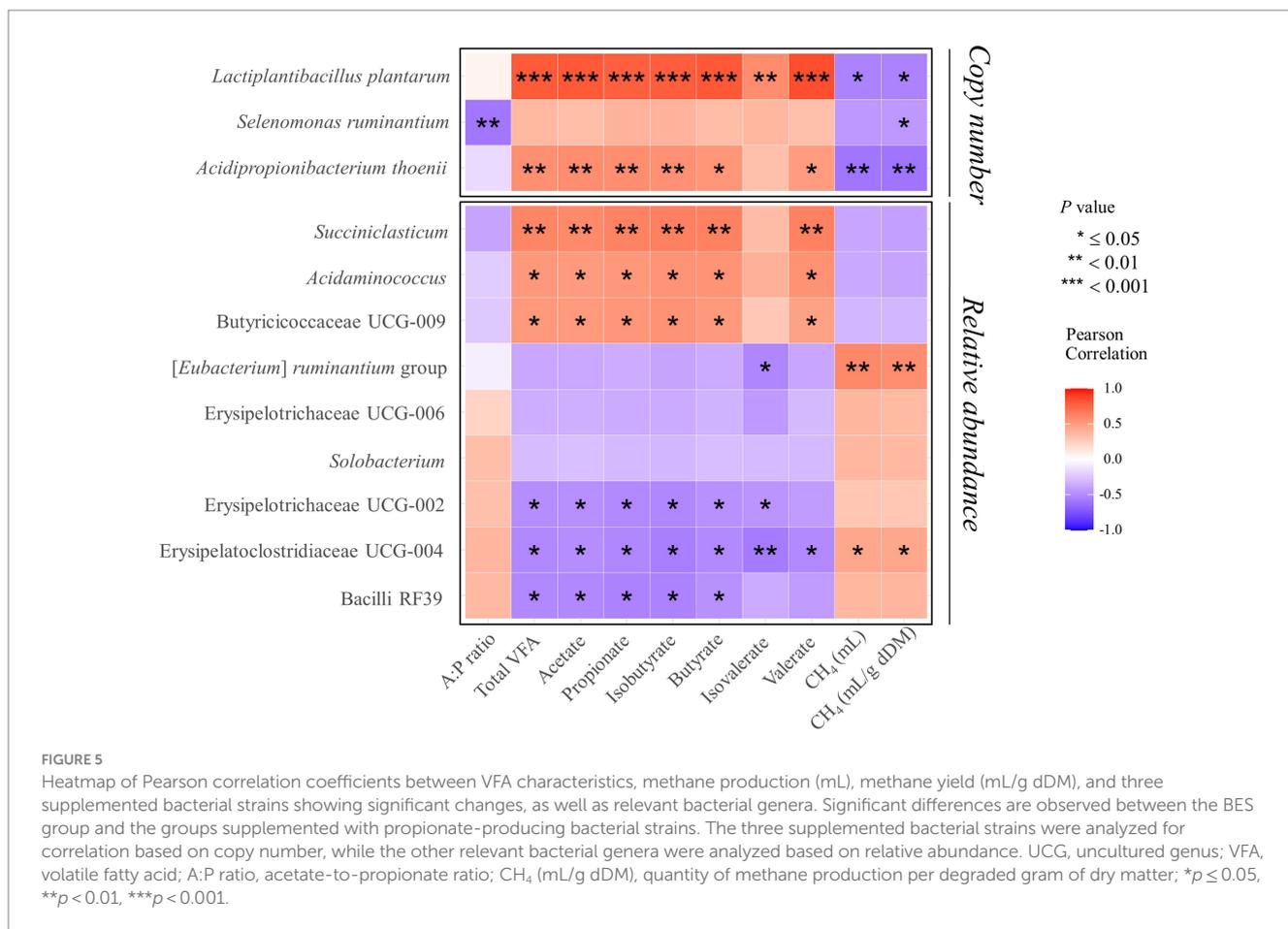
Item	Group					SEM	p-value
	CON	BES	BES (+)				
			RA	RS	CB		
<b>Absolute abundance, log copies/mL</b>							
Total bacteria	10.30	10.26	10.33	10.27	10.34	0.06	0.1141
Total protozoa	8.97	8.96	8.97	8.94	8.97	0.01	0.9574
Total methanogen	8.17 <sup>A</sup>	7.17 <sup>B</sup>	7.19 <sup>B</sup>	7.06 <sup>B</sup>	6.89 <sup>B</sup>	0.18	<0.0001
<i>Lactiplantibacillus plantarum</i>	1.40 <sup>C</sup>	1.74 <sup>BC</sup>	5.97 <sup>A</sup>	2.41 <sup>B</sup>	5.72 <sup>A</sup>	0.45	<0.0001
<i>Megasphaera elsdenii</i>	7.83	7.85	8.03	7.84	7.89	0.17	0.3892
<i>Selenomonas ruminantium</i>	8.73 <sup>C</sup>	8.76 <sup>BC</sup>	8.91 <sup>AB</sup>	8.93 <sup>A</sup>	8.94 <sup>A</sup>	0.01	0.0008
<i>Acidipropionibacterium thoenii</i>	5.00 <sup>CD</sup>	4.87 <sup>D</sup>	5.05 <sup>C</sup>	6.68 <sup>A</sup>	6.39 <sup>B</sup>	0.07	<0.0001

CON, control group; BES, 2-bromoethanesulfonate group; RA, reinforced acrylate pathway group; RS, reinforced succinate pathway group; CB, propionate-producing bacterial consortium group; SEM, pooled standard error of the mean.

<sup>A-D</sup>: significant differences ( $p \leq 0.05$ ) between treatment groups.

the effects observed in the RA and CB groups. To ascertain these pattern changes, qPCR analysis of the added bacterial strains and major microbial groups were conducted to accurately monitor select taxa in conjunction with MaAsLin2 analysis. Alongside the absence of significant differences in DMD and NDFD, no significant changes were observed in the absolute abundance of total bacteria and total protozoa. Excluding *M. elsdenii*, the addition of the other three bacterial strains significantly enhanced their abundance, suggesting their functional relevance in the BES-supplemented environment. The three bacterial strains were found to have a negative correlation with methane yield (mL/g dDM) (Figure 5). Additionally, two of the bacterial strains, *L. plantarum* and *A. thoenii*, exhibited a negative correlation with methane production (mL). *L. plantarum* and *A. thoenii* were also positively correlated with propionate concentration, which may suggest their role in enhancing propionate levels. Conversely, *S. ruminantium* displayed a negative correlation

with the A:P ratio, which may support its involvement in propionate metabolism. While the bacterial strains were assisted by the direct inhibitor BES, these findings are consistent with results from previous studies (27, 30, 39), reinforcing the understanding of the effects of supplementation with these bacterial strains on methane and propionate dynamics. Moreover, BES significantly reduced the absolute abundance of methanogens, which is known to induce an imbalance in H<sub>2</sub> metabolism and the accumulation of large amounts of H<sub>2</sub> in the rumen (24). This prompts the growth of H<sub>2</sub>-utilizing bacteria, such as *Anaerovibrio* (61), *Desulfovibrio* (62), and *Fibrobacter* (27), whereas the growth of H<sub>2</sub>-producing cellulolytic bacteria, such as *Ruminococcus* (63) and *Saccharofermentans* (64), is lowered. The addition of BES has been shown to negatively impact the relative abundance of *Roseburia*, a bacterial genus associated with an alternative propionate-producing pathway (1). This underscores the critical role of direct inhibitors and highlights the importance of



selecting suitable microbial additives to bypass their effects. The high-concentration BES treatment caused a dramatic shift in the microbial distribution between the CON and BES groups. However, there are limitations, including that the experiment was conducted only once, and the BES effect was so strong that it overshadowed the impact of both the co-treated bacterial strains and pathway-specific approaches. Despite these differences, the abundance of the [*Eubacterium*] *ruminantium* group, one of the representative ruminal fibrolytic bacteria (65), was reduced in both the RA and RS groups, suggesting that even with the use of varying probiotic strains and targeting divergent metabolic pathways, a consortium-based approach might have similar modulatory effects on the rumen microbiota. The reduction in the relative abundance of certain bacterial taxa in the RS and CB groups, including *Solobacterium*, which is known to be associated with high residual methane emissions (66), suggest that adding *S. ruminantium* and *A. thoenii*, or a propionate-producing bacterial consortium in conjunction with the methanogen-specific inhibitor, could further enhance methane reduction. When all the four probiotic strains were added in the CB group, the relative abundances of *Succiniclasticum*, *Acidaminococcus*, and *Butyricococcaceae* UCG-009 significantly increased; this correlated with restoration of the total VFA profile (Figure 5). Alongside the results showing that some strains of *Succiniclasticum* and *Acidaminococcus* have a negative correlation with methane yield in

dairy cows (67), the qPCR-validated successful colonization and expected fermentation outputs of supplemented bacterial strains coupled with the increased relative abundances of a major bacterial taxon *Succiniclasticum* (68), which is closely linked to propionate metabolism, demonstrate the efficacy of employing probiotic application of propionate-producing bacterial consortium for not only directing propionate production and achieving additional methane mitigation but also significantly restoring the VFA production profile in the methane-mitigated rumen environment.

In conclusion, our research findings indicate that incorporating propionate-producing bacteria is an effective biochemical approach both for augmenting methane reduction efforts and replenishing the total VFA production, which is often diminished because of abnormal H<sub>2</sub> metabolism caused by the action of direct methanogenesis inhibitors *in vitro*. Bromo-compounds, such as BES, are classified as class I ozone-depleting substances (69); hence, they cannot be used for methane reduction. Instead, by integrating this strategy with the addition of available methanogen-specific inhibitors, such as 3-nitrooxypropanol (14, 70), the careful selection of probiotic candidates can further improve the availability of energy resources for the host animals. This is achieved by dual modulation of the rumen microbiome and fermentation profiles, which offers a comprehensive solution to improve both environmental and nutritional outcomes of ruminal fermentation.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: <https://www.ncbi.nlm.nih.gov/>, PRJNA1098854.

## Ethics statement

The animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at Chung-Ang University. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

## Author contributions

JJ: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft. CY: Data curation, Methodology, Validation, Writing – review & editing. RK: Conceptualization, Investigation, Software, Visualization, Writing – review & editing. MK: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. TP: Conceptualization, Data curation, Funding acquisition, Investigation, Project administration, Supervision, Validation, Visualization, Writing – review & editing.

## Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This research was supported by the Chung-Ang University Graduate Research Scholarship, 2023. Additionally, this work was carried out with the support of the Cooperative Research Program for Agriculture Science and Technology Development (Project No. 1395077484), Rural Development Administration, Republic of Korea.

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## Acknowledgments

The biological resources used in this research were distributed from KCTC and DSMZ. We thank Prof. Jakyem Seo and Dr. Jaeyong Song, and their laboratory members for providing guidance in establishing the *in vitro* experimental procedures, procedural assistance, and rumen fluid.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fvets.2024.1422474/full#supplementary-material>

### SUPPLEMENTARY FIGURE S1

Relative abundance of major bacterial taxa at the phylum level showing significant differences between the groups Relative abundance results of major phylum-level microbial taxa with 100% occurrence in all groups, indicating significant differences ( $Q \leq 0.05$ ) between the groups. CON, control group; BES, 2-bromoethanesulfonate group; RA, reinforced acrylate pathway group; RS, reinforced succinate pathway group; CB, propionate-producing bacterial consortium group; SEM, pooled standard error of the mean.

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