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Running Title (within 10 words)	GSM-Driven Synbiotics for Methane Mitigation in Fermented TMR
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(Unstructured) Abstract (up to 350 words)

Methane is a major greenhouse gas with a global warming potential 28 times greater than that of carbon dioxide, making reduction of livestock emissions a critical challenge. Current mitigation strategies, such as feed management, chemical additives, and algae supplementation, face limitations related to safety, supply instability, and economic feasibility. To address these issues, we investigated the development of methane-reducing feeds utilizing synbiotics. In a preliminary study, we evaluated the methane reduction potential of *Komagataeibacter intermedius* and *Zygosaccharomyces parabailii* (KZ), two SCOBY-derived strains, and identified prebiotics that promote their colonization. Genome-scale metabolic (GSM) models were constructed for three hydrogen-producing bacteria, five methanogens, and the KZ strain, and their metabolic networks were compared. Five prebiotics (glycerol, oleic acid, menaquinone-7, Gly-Leu, and Gly-Asn-L) were identified as substrates utilized by KZ but not by hydrogen-producing bacteria or methanogens. Coculture experiments confirmed that combined prebiotic treatment significantly enhanced KZ growth. *In vitro* rumen fermentation assays demonstrated reduced methane emissions in synbiotic-treated groups, with the combined prebiotic treatment resulting in the strongest inhibition. Based on these findings, a synbiotic-based fermented total mixed ration (FTMR) was developed. Synbiotic supplementation alleviated acidification, increased acetic acid production, and enriched bacterial and fungal populations. Microbial community analysis revealed higher abundance of lactic acid bacteria and non-*Saccharomyces* yeasts, indicating improved fermentation and enhanced feed quality. In conclusion, the synbiotic-based FTMR stabilized pH, altered organic acid profiles, and reshaped microbial communities, with synergistic interactions between lactic acid bacteria and yeast supporting a more stable fermentation environment. These results demonstrate that GSM modeling is effective for designing synbiotic-based feeds and that the newly developed synbiotic FTMR improves fermentation quality. Notably, this study focused on prebiotic identification, synbiotic formulation, are fermentation characterization; direct evaluation of the methane reduction efficacy of FTMR under *in vitro* and *in vivo* conditions remains a subject for future investigation.

Keywords (3 to 6): Synbiotics, Genome-scale metabolic model, Fermented total mixed ration, Enteric methane mitigation

Introduction

Climate change due to global warming is one of the most pressing challenges facing humanity. Methane is a potent greenhouse gas with a global warming potential (GWP) about 28 times greater than that of carbon dioxide, despite being present at lower atmospheric concentrations [1]. Enteric fermentation in ruminants is a major source of methane emissions, accounting for approximately 40% of global agricultural methane emissions [2] and representing the largest share of greenhouse gas emissions from livestock farming [3]. Reducing methane emissions from ruminant production has therefore become a critical goal in the fight against climate change.

In the rumen, microorganisms ferment carbohydrates to produce volatile fatty acids (VFAs) such as acetate, propionate, and butyrate. The hydrogen generated during this process is primarily consumed by methanogenic archaea (methanogens) and converted into methane. Various methane reduction strategies have been reported, including changes in feed composition and the use of chemical or natural additives [4–6].

However, these methods face significant limitations for commercialization, including supply constraints, high costs, and concerns about long-term safety and efficacy. As a result, biological methane reduction strategies using microorganisms have recently attracted considerable attention. Studies have shown that hydrogen metabolic pathways compete with propionate-producing and acetate-accumulating bacteria, and that activation of alternative hydrogen utilization pathways can significantly reduce methane production [7–9].

Lactic acid bacteria (LAB) supplementation, for example, can lower methane production indirectly by shifting fermentation pathways and altering organic acid profiles, rather than directly inhibiting methanogenesis [10]. These findings imply that microbiological strategies in the rumen offer feasible approaches for methane mitigation.

Total mixed ration (TMR) and fermented TMR (FTMR) are widely used feed types for ruminants due to their nutritional and management benefits. Conventional TMRs require complex control of moisture, microbial activity, and fermentation balance among feed ingredients, whereas FTMR employs LAB and fiber-degrading enzymes during fermentation to accumulate beneficial metabolites, thereby extending shelf life and improving overall feed quality [11]. FTMRs have therefore attracted attention as a strategy to enhance both storability and nutritional value. Nonetheless, broader application of FTMRs is limited by insufficient understanding of the functional microorganisms that can be leveraged for efficient fermentation and practical use. The fermentation process of TMR generates metabolites such as lactic acid, acetic acid, propionic acid, and butyric acid [12, 13]. It is therefore essential to identify which metabolites most effectively promote fermentation and microbial colonization, and to elucidate the microbial activities they induce. We recently demonstrated that microbial consortia derived from Symbiotic Culture of Bacteria and Yeast (SCOBY) exhibit significant methane-mitigating effects in fermentation systems, implying their potential as functional inoculants for FTMR development [14].

Synbiotics have attracted attention as a strategy to improve the storability and nutritional value of feed through fermentation, because they enhance fermentation quality by promoting microbial colonization and function [15]. To optimize microbial fermentation further, targeted prebiotic development is needed beyond simple fiber- and oligosaccharide-focused research [16]. Meanwhile, genome-scale metabolic (GSM) models have emerged as useful tools for predicting microbial metabolic pathways and substrate utilization, as well as for identifying which precursors and substrates stimulate the growth and metabolic activity of specific strains [17]. Such models can be applied to predict effective prebiotic candidates for targeted probiotics.

In this study, we developed a synbiotic FTMR by combining methane-reducing microorganisms with targeted prebiotics that enhance their growth and evaluated its effects on nutritional and microbiological functional properties during fermentation. The scope of this study was limited to prebiotics identification, synbiotic formulation, and fermentation characterization of the FTMR. The direct assessment of methane reduction efficacy of FTMR under *in vitro* and *in vivo* conditions, as well as the elucidation of the underlying mechanisms, are designated as future research directions.

Methods and Materials

Isolation and identification of microorganisms derived from kombucha

Kombucha was used as the source of microbial inoculum in this study and was cultured under controlled laboratory conditions [18]. To prepare the fermentation medium, 0.5% (w/v) green tea leaves were infused in 400 mL of distilled water at 70 ℃ for 5 min. After removing the tea leaves, 10% (w/v) sucrose was added and dissolved completely. The medium was then sterilized by autoclaving and cooled to room temperature. Subsequently, 20% (v/v) of the previously prepared kombucha stock was inoculated into the sterile medium. The culture vessel was covered with sterile gauze to maintain aerobic conditions and incubated statically at 25 ℃ for 21 d.

To isolate the microorganisms, present in the kombucha, lactic acid bacteria were cultured on *Lactobacilli* MRS agar (Difco, West Chester, PA, USA) and incubated at 37 ℃ for 3 d. Yeasts were isolated using Yeast Peptone Dextrose agar (YPD; Difco) and Potato Dextrose Agar (PDA; Difco), followed by incubation at 25 ℃ for 3 d. The dominant bacterial and yeast strains were identified by Macrogen (Seoul, Korea). The 16S rRNA gene and the internal transcribed spacer (ITS) region were sequenced for bacterial and yeast identification, respectively. Species-level identification was performed by analyzing the obtained sequences using the NCBI BLAST tool.

GSM Model Construction and Analysis

Genome-scale metabolic modeling (GSM) was applied to two experimental strains, *Komagataeibacter intermedius* and *Zygosaccharomyces parabailii*. In addition to SCOBY-derived microbes, ruminal microorganisms essential for methane production were also included in the GSM analysis. Five representative methanogens found in the rumen (*Methanobrevibacter ruminantium*, *M. gottschalkii*, *M. smithii*, *M. olleyae*, and *M. millerae*) and three hydrogen-producing microorganisms (*Ruminococcus albus*, *R. flavefaciens*, and *Butyrivibrio fibrisolvens*) were selected. The genome sequences of each microorganism were obtained in GBFF format from the NCBI GenBank database, and the GSMs were constructed using the KBase platform (https://www.kbase.us/).

Model construction was automated using the “Build Metabolic Model” tool, and metabolic pathway analysis was conducted using Flux Balance Analysis (FBA). The modeling conditions were set to anaerobic conditions, complete media, and D-glucose as the carbon source to mimic the rumen environment, with biomass production defined as the objective function for calculating metabolic flux. To identify prebiotic candidates based on GSM analysis, the utilization status of each compound by the selected microorganisms was analyzed. Compounds that were not taken up by the five methanogenic and three hydrogen-producing microorganisms but were utilized by the two experimental strains were shortlisted as candidate prebiotics. From these candidates, selections were further refined based not only on their predicted high metabolic flux contributions to the target strains but also on their economic feasibility and practical usability for industrial applications.

Measurement of SCOBY Growth Under Different Prebiotic Supplementation Conditions

Three different prebiotic treatments were designed to promote the growth of the defined SCOBY: (1) amino acids and vitamin supplementation (AA + Vit), (2) carbohydrates and vitamin supplementation (Car + Vit), and (3) a mixed treatment containing all prebiotics (Car + AA + Vit). For amino acid supplementation, glycyl-L-leucine (0.04g/L; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) and Nα-glycyl-L-asparagine (0.011g/L; Tokyo Chemical Industry Co., Ltd.) were used. The carbohydrate sources included glycerol (0.555g/L; Biosesang Inc., Seongnam, Korea) and oleate (12 mL/L; Daejung Chemicals & Metals Co., Ltd., Siheung, Korea). Menaquinone-7 (0.1 mg/L; Sigma-Aldrich, St. Louis, MO, USA) was used as the vitamin source.

The culture media were prepared by described in the previous section. Subsequently, 20% (v/v) of previously prepared kombucha stock was inoculated into the sterile medium. The culture vessel was covered with sterile gauze to maintain aerobic conditions and incubated statically at 25 ℃ for 21 d. The experimental groups were treated with AA + Vit, Car + Vit, and Car + AA + Vit, and a control group was included for comparison. To measure optical density, 10 mL of culture medium was dispensed into a glass tube, placed in an optical reader, and the OD value was measured. Microbial growth was monitored by measuring the optical density at 600 nm (OD_{600}) at 7-d intervals throughout the incubation period.

Evaluation of methane reduction efficacy under *in vitro* fermentation of prebiotics

Rumen fluid samples were collected from fistulated Holstein steers with rumen fistulas at the experimental farm of Seoul National University (Pyeonchang, Korea). The collected rumen fluid was filtered through a 1 mm mesh sterile gauze (Daihan Medical, Seoul, Korea) and degassed using a vacuum pump. The flask was sealed with a rubber stopper and maintained under anaerobic conditions by purging with nitrogen gas using a vacuum lock.

The rumen buffer was prepared following the method of Goering & Van Soest (1970), containing Na_2HPO_4 2.85 g/L, KH_2PO_4 3.1 g/L, $MgSO_4 \cdot 7H_2O$, 0.3 g/L, $NaHCO_3$ 17.5 g/L, NH_4HCO_3 2.0 g/L, tryptone 2.5 g/L, L-cysteine-HCl 0.625g/L, and resazurin sodium salt 10 mg/L. A mineral solution consisting of $CaCl_2 \cdot 2H_2O$ 1.32 g/L, $MnCl_2 \cdot 4H_2O$ 1.0 g/L, $CoCl_2 \cdot 6H_2O$ 0.1 g/L, and $FeCl_3 \cdot 6H_2O$ 0.8 g/L was added. The final buffer was adjusted to pH 7.0 using HCl.

The basal diet consisted of a commercial feed mix (Purinafeeds, Seoul, South Korea) containing corn and soybean meal, supplemented with ground rice straw. Both the feed mix and straw were ground for 20 min using a grinder (HR3757, Philips, Amsterdam, Netherlands) and sterilized.

The *in vitro* fermentation was conducted in 240 mL serum bottles, each containing 0.5 g of ground feed mix, 0.5 g ground rice straw, 20 mL of rumen buffer, 20 mL of rumen fluid, and 1 mL of the experimental treatment sample. The bottles were sealed with rubber stoppers. The control group received a 1 mL of 0.85% saline solution instead of the treatment sample. Fermentation was carried out in an anaerobic chamber (COY Laboratory Products, Grass Lake, MI, USA) at 39 ℃ and 180 rpm. Samples were collected at 10, 20, and 30 h of incubation. All treatments were prepared in triplicate.

Methane and carbon dioxide production were analyzed using a gas chromatograph (ChroZen GC system, Youngin Chromass, Anyang, Korea). The analytical column system included a molecular sieve 13× (Supleco, Bellefonte, PA, USA) and a Porapak-N (Supleco). To ensure methanogenic activity, initial gas conditions were set to 3% hydrogen and 3% carbon dioxide, with nitrogen as the background gas. A 5-mL of headspace gas was sampled from each flask and injected into the GC via a gas-tight syringe. An automatic injector system transferred 250 μL of the sample to the GC valve. The carrier gas (helium) flow rate was maintained at 3.0 mL/min.

Values are presented as the mean ± standard deviation (SD) and were derived from triplicate experiments. GraphPad Prism 9 software was used to statistically analyze the experimental data. Multiple comparisons were performed using the false discovery rate (FDR) approach, specifically the two-stage step-up method. An unpaired t-test assuming individual variance for each row was used to compare the groups. The results were considered statistically significant at $p < 0.05$. Significance levels are indicated by asterisks as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$; ns indicates no statistically significant difference.

Chemical analysis of fermented total mixed ration (FTMR)

The total mixed ration (TMR) was supplied by Hampyeong Livestock Cooperative (Korea) and consisted of fish meal pellets, timothy hay, alfalfa hay bales, Cheonji base (a commercial mineral and vitamin premix), corn distillers' grains, molasses, probiotics, and Daumin. The TMR was supplemented with KZ consortium at concentrations of 5% and 10% (v/w), and the moisture content was adjusted to 40% using sterile distilled water. Prepared TMR samples (200 g each) were vacuum-sealed and subjected to anaerobic fermentation at 25 ℃ for up to 14 d (n = 3 per treatment). The proximate composition of the control (CON) and treatment groups (5% Mix and 10% Mix) is presented in Table 1.

The moisture content was determined by pre-drying samples in an oven at 65 °C for 48 h, equilibrating at room temperature for 24 h, and then drying approximately 2 g of the pulverized sample in an aluminum weighing dish at 135°C for 2 h using the atmospheric pressure drying method. The samples were cooled in a desiccator for 30 min before weighing, and the weight loss indicated moisture content. Crude protein was measured by the Kjeldahl method (AOAC 976.05): 0.5 g of sample was digested with 12 mL of sulfuric acid and a catalyst mixture at 420°C for 1 h, with nitrogen quantified using a Kjeltec Auto 8400 Analyzer (FOSS, Sweden) and converted to crude protein by multiplying the nitrogen amount by 6.25. Crude fat was analyzed using 0.5 g sample sealed in an Ankom nylon filter bag, dried at 102 ± 2°C for 23 h, cooled, and extracted with diethyl ether for 60 min. Fat content was determined by the difference in weight after extraction. Crude fiber was determined by sequentially treating 0.5 g of sample in an Ankom nylon filter bag with 1.25% H_2SO_4 and 1.25% NaOH and measuring the weight loss, analyzed using a DELTA Fiber Analyzer (Ankom Technology). Ash content was established by combusting approximately 2 g of sample in a muffle furnace at 600°C for 3 h after preheating, followed by cooling and weighing. Organic matter content was calculated by subtracting ash content from total dry weight. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were quantified by sequential extraction of 0.5 g of sample in a nylon filter bag with neutral and acid detergent solutions using an Ankom DELTA Fiber Analyzer; residues after extraction were used to calculate NDF and ADF, respectively.

FTMR samples were collected on days 0, 7, and 14. For each sampling point, 10 g of sample was mixed with 40 mL of 0.85% saline solution and homogenized using a stomacher (BagMixer 400, Interscience, Saint-Nom, France) for 30 min. The homogenate was filtered through a sterile filter bag and used for pH measurement, microbial enumeration, and high-performance liquid chromatography (HPLC) analysis.

The pH of the FTMR extracts was measured using a pH meter (Sarter 300, OHAUS, Parsippany, USA). After pH measurement, the samples were centrifuged at 4,500 × g for 10 min, and the supernatant was filtered through a 0.25 μm membrane filter.

Metabolite concentrations, including sucrose, glucose, fructose, lactate, acetate, and ethanol, were determined using HPLC (1260 Infinity system, Agilent Technologies, Santa Clara, USA) according to the method described by Jeong et al. (2024). A Rezex-ROA Organic Acid H+(8%) column (Phenomenex Inc., Torrance, USA) was used with 0.005 N sulfuric acid as the mobile phase. The flow rate was set at 0.6 mL/min, and the column temperature was maintained at 50 ℃.

Microbial population and diversity analysis

FTMR extracts collected on days 0, 7, and 14 were subjected to 10-fold serial dilutions in 0.85% saline solution. Diluted samples were plated using the spread plate method. Bacterial populations were cultured on plate count agar (PCA), consisting of tryptone (5 g/L), yeast extract (2.5 g/L), dextrose (1 g/L), and agar (16 g/L). Yeast populations were cultured on yeast extract glucose chloramphenicol (YGC) agar, composed of yeast extract (5 g/L), dextrose (20 g/L), chloramphenicol (0.1 g/L), and agar (16 g/L). All plates were incubated aerobically at 25 ℃ for 72 h. After incubation, colony-forming units (CFUs) were enumerated to quantify bacterial and yeast populations.

For DNA extraction, the pellet obtained from centrifuged FTMR extract was transferred to a bead-beating tube and vortexed for 30 min. Genomic DNA was extracted using a DNeasy PowerSoil Pro kits (QIAGEN, Germany) according to the manufacturer’s protocol. The concentration and purity of the extracted DNA were assessed using a spectrophotometer (NanoDrop, IMPLEN, Germany). Next-generation sequencing (NGS) analysis was outsourced to Macrogen (Seoul,Korea).

For bacterial community analysis, the V3-V4 region of the 16S rRNA gene was amplified using the following primer set: forward (5’–CCTACGGGNGGCWGCAG–3’) and reverse (5’-GACTACHVGGGTATCTAATCC-3’). For yeast community profiling, the ITS3-4 region was targeted using the primer set: forward (5’-GCATCGATGAAGAACGCAGC-3’) and reverse (5’-TCCTCCGCTTATTGATATGC-3’) in the first-round PCR. PCR conditions included an initial denaturation at 95 ℃ 3 min, followed by 25 cycles of denaturation at 95 ℃ for 30 s, annealing at 55 ℃ for 30 s, and extension at 72 ℃ for 30 s, with a final extension at 72 ℃ for 5 min. Following amplification, sequencing libraries were prepared using the Herculase II Fusion DNA Polymerase Nextera XT Index V2 Kit (Illumina, San Diego, USA). Sequencing was performed on the Illumina MiSeq platform.

Statistical analysis

Data for methane and carbon dioxide concentrations, metabolite profiles, and microbial populations were expressed as the mean ± standard deviation (SD). Statistical analyses were conducted using two-way ANOVA, followed by Tukey’s multiple comparison test, implemented in GraphPad Prism version 8.0.2. Groups not sharing the same letter were considered statistically significantly different at $p < 0.05$.

Results

Isolation and identification of microorganisms

Seven microbial species, including bacterial and yeast strains, were isolated and identified from kombucha (Table 2). The bacterial isolates were *Komagataeibacter intermedius* (Accession No. PP851368), *Komagataeibacter swingsii* (PX059175), and *Gluconoacetobacter hansenii* (PX059176). The yeast strains included *Zygosaccharomyces bisporus* (PX058846), *Zygosaccharomyces parabailii* (PX058844), *Brettanomyces bruxellensis* (PX058845), and *Dekkera bruxellensis* (PX058843). Colony morphology and relative abundance assessments revealed *K. intermedius* and *Z. parabailii* as the dominant species. These two strains, collectively referred to as KZ, were co-cultured under controlled laboratory conditions, and the resulting mixed culture was employed in synbiotic-based FTMR production.

Screening of Prebiotic Candidates for KZ

Genome-scale metabolic (GSM) analysis was used to screen prebiotic candidates for KZ. Metabolic models were constructed for three representative hydrogen-producing rumen bacteria (*Ruminococcus albus, R. flavefaciens,* and *Butyrivibrio fibrisolvens*), five methanogenic archaea (*Methanobrevibacter ruminantium, M. gottschalkii, M. smithii, M. olleyae,* and *M. millerae*), and the KZ strains. The uptake states of potential substrates were compared across these models (Figure 1).

A total of 15 compounds were identified that were utilized exclusively by KZ but not by the hydrogen-producing bacteria or methanogens (Figure 1; Table 3). These included small carbohydrates (e.g., glycerol and oleate), cofactors (e.g., menaquinone-7, CoA, and Fe^{3+}), and several dipeptides (e.g., Gly-Leu, Gly-Asn-L, and Gly-Met). To avoid redundancy, five representative compounds were selected based on predicted utilization rates, feasibility in cultivation, and commercial availability (Table 4): glycerol, oleate, menaquinone-7, Gly-Leu, and Gly-Asn-L. These were categorized into carbohydrates, vitamins/minerals, and amino acids, and subsequently used in *in vitro* microbial studies.

Effect of Prebiotic Supplementation on KZ Growth

To assess the growth-promoting effects of the selected prebiotics, KZ cultures were grown under four supplementation conditions: amino acids + carbohydrates (AA + Car), amino acids + vitamin (AA + Vit), carbohydrates + vitamin (Car + Vit), and a mixed group containing all three (Car + AA + Vit). Concentrations were standardized (e.g., glycerol 0.555 g/L, oleate 12 mL/L, Gly-Leu 0.04 g/L, Gly-Asn-L 0.011 g/L, and menaquinone-7 0.1 mg/L).

The Car + AA + Vit group showed a significant increase in absorbance compared to the control group (0.217 ± 0.020 vs. 0.176 ± 0.022; $p < 0.05$; Figure 2). The AA + Vit and Car + Vit groups exhibited non-significant upward trends. Overall, supplementation with the mixed prebiotics effectively promoted the growth of KZ.

Inhibitory Effects of Synbiotic Culture on Methane and Carbon Dioxide Production

To evaluate methane-reduction efficacy prior to FTMR application, an *in vitro* rumen fluid fermentation assay was conducted. Experimental groups included a control without additives, KZ alone, and KZ combined with different prebiotics (AA + Vit, Car + Vit, Car + AA + Vit). Methane concentrations (% v/v) increased over time in all groups; however, synbiotic-treated groups consistently produced less methane than the control group (Figure 3). The Car + AA + Vit group showed the greatest suppression (70.5% and 49.2% reduction at 10 and 20 h, respectively; $p < 0.05$ vs. controls), with borderline significance compared to the SCOBY group ($p = 0.0869$). Other groups (AA + Vit, Car + Vit, and SCOBY) showed reductions ranging from 59 to 66%. Carbon dioxide concentrations also increased across all groups, but synbiotic supplementation consistently lowered CO_2 production relative to the controls ($p < 0.01$).

pH Changes in TMR During Fermentation with Synbiotics

Based on the *in vitro* rumen fluid fermentation assay, the combined prebiotic treatment (Mix) demonstrated the strongest growth-promoting and methane-reducing effects. Accordingly, the synbiotic mixture composed of KZ, amino acids, vitamins, and carbohydrates (KZ + AA + Vit + Car) was incorporated into the FTMR to provide methane-mitigating functions. TMR was supplemented with synbiotics at three concentrations (CON, 5% Mix, and 10% Mix) and fermented anaerobically for 0, 7, and 14 d.

Following supplementation, pH values were monitored to assess acidification patterns (Table 5). The initial pH of the control group was 5.20, whereas the 5% Mix and 10% Mix groups showed significantly lower values of 5.11 and 5.02, respectively ($p < 0.05$; Table 5). As fermentation progressed, pH declined across all treatments, but the rate and extent of acidification varied. On day 7, the 10% Mix group exhibited a higher pH (4.92) than the controls (4.86), and by day 14, the 5% Mix group (4.73) remained significantly higher than the controls (4.66) ($p < 0.05$). These results indicate that synbiotic supplementation moderates the rate of acidification during fermentation.

Metabolite Changes in FTMR with Synbiotics and Fermentation Time

Metabolite profiling was conducted to evaluate the effects of synbiotic supplementation on fermentation dynamics (Figure 4). At day 0, sucrose, glucose, and fructose concentrations were high across all groups but declined rapidly. By day 7, sucrose and fructose were below the detection limit in all treatments. At day 14, the 10% Mix group retained the highest glucose concentration (3.23 g/L), while the controls showed the lowest (1.78 g/L); however, no statistically significant difference was observed ($p > 0.05$).

Organic acids and ethanol were also measured. Acetate concentrations remained low during the first 7 d but increased significantly by day 14. The levels in the 5% Mix group (2.33 g/L) tended to be higher than those in the controls (1.84 g/L) and 10% Mix (1.73 g/L), although the differences were not statistically significant. Lactate concentrations steadily increased across all groups, with no significant treatment differences. Ethanol levels significantly increased over time, peaking in the 10% Mix group (7.25 g/L) at day 14. These findings suggest that synbiotic supplementation modulated carbon metabolism and microbial activity during anaerobic storage.

Dynamics of Culturable Bacteria and Fungi in FTMR

Viable counts of bacteria and fungi were monitored over 14 d (Figure 5). At day 0, bacterial and fungal counts did not differ among controls, 5% Mix, and 10% Mix ($p > 0.05$).

By day 7, bacterial populations increased significantly in all groups (log CFU/g, $p < 0.05$), with the 10% Mix group showing the greatest increase throughout fermentation. The 5% Mix group displayed moderately higher counts at day 7, but by day 14, levels were lower than those of the 10% Mix group.

Fungal populations followed a similar pattern: all groups showed significant increases from day 0 to day 7 ($p < 0.05$). Beyond day 7, fungal counts plateaued, with no further differences among treatments at day 14. All groups maintained elevated fungal levels during later fermentation stages. Overall, synbiotic supplementation, particularly at the highest concentration, significantly stimulated bacterial growth throughout fermentation, while fungal populations increased early and stabilized regardless of treatment.

Dynamics of Microbial Community in FTMR Influenced by Synbiotic Supplementation and Fermentation Time

The α-diversity of bacterial and fungal communities was assessed using the Shannon and Gini-Simpson indices, which revealed temporal variation associated with fermentation time and synbiotic supplementation (Table 6). Across the fermentation period, the diversity of both bacteria and fungi decreased, indicating ecological succession from an initially heterogeneous community to a more specialized consortium adapted to prolonged anaerobic conditions. The control group generally exhibited lower diversity, whereas the 10% Mix group tended to maintain higher diversity throughout fermentation. Although the differences were not statistically significant, the trends implied that synbiotic supplementation may support a more diverse and stable microbial network through enhanced trophic and metabolic interactions.

β-diversity, assessed using principal coordinate analysis (PCoA) (Figure 6), revealed distinct trajectories for bacterial and fungal communities. For bacteria, Principal Component 1 (PC1) explained 95.91% of the variance, separating samples primarily by fermentation stage rather than treatment. The 10% Mix group had the highest PC1 score at day 0, reflecting a more heterogeneous and metabolically active bacterial composition early in fermentation. Over time, bacterial communities shifted toward negative PC1 values. Fungal communities (PC1 = 76.32%) displayed the opposite trend, clustering at negative PC1 initially but progressing toward positive PC1 by day 14. This pattern implies replacement of early facultative yeasts by stress-tolerant taxa. The 10% Mix group showed the largest displacement along PC2 at day 7, indicating pronounced fungal restructuring, likely driven by synergistic bacterial–fungal interactions that enhanced carbon metabolism and fermentation stability.

At the phylum level (Figure 7A), bacterial communities initially included Bacillota (formerly Firmicutes), Actinomycetota, Cyanobacteriota, and Pseudomonadota. The 10% Mix group exhibited high baseline abundances of Cyanobacteriota (29.9%) and Pseudomonadota (32.9%). As fermentation progressed, Bacillota rapidly increased, becoming dominant (> 90%) in all groups by day 14. In fungal communities (Figure 7B), Ascomycota was dominant at baseline, but unclassified fungal taxa increased significantly in synbiotic-treated groups. By day 7, the 10% Mix group reached 40.9% unclassified taxa, while the 5% Mix group peaked at 43.0% by day 14.

At the genus level (Figure 7C), bacterial communities before fermentation included *Weissella*, *Staphylococcus*, *Pantoea*, and *Pseudomonas*. During fermentation, the levels of LAB such as *Companilactobacillus*, *Fructilactobacillus*, *Ligilactobacillus*, and *Levilactobacillus* increased markedly. By day 14, *Fructilactobacillus* dominated the 10% Mix group (33.9%, Figure 6E). At the species level, early communities were dominated by *Weissella jogaejeotgali* and unclassified *Cyanophyceae* species, particularly in the 10% Mix group. These taxa were gradually replaced by LAB species such as *Companilactobacillus nuruki* and *Fructilactobacillus fructivorans*. Spoilage-associated species, including *Staphylococcus saprophyticus* and *Pantoea agglomerans*, decreased markedly over time.

Among fungal communities (Figure 7D), *Saccharomyces* initially dominated all groups (75 – 79%). While *Saccharomyces* remained dominant in the control group, the 5% and 10% Mix groups developed more diverse fungal profiles. Non-*Saccharomyces* genera such as *Starmerella*, *Trichosporonoides*, and *Brettanomyces* were consistently detected in synbiotic-treated groups, indicating broader and more diverse fungal compositions compared to the controls.

Discussion

Methane is one of the most problematic greenhouse gases emitted by the livestock industry. Efforts to reduce methane emissions have been ongoing for decades and are essential for achieving sustainable livestock production. To this end, various compounds and probiotics have been incorporated into feed formulations to mitigate emissions. In this study, we propose synbiotics-based FTMR as a potential strategy for enteric methane reduction. GSM-based prebiotic prediction was used to compare the metabolic networks of methanogens, hydrogen-producing bacteria, and SCOBY (*Komagataeibacter intermedius* and *Zygosaccharomyces parabailii*). We identified substrates that the SCOBY community (KZ strains) could selectively utilize, but which were not accessible to methanogens or hydrogen-producing bacteria, making them suitable as prebiotics. These candidates promoted the growth of target microorganisms with methane-mitigating potential, and their combination as synbiotics effectively reduced methane production *in vitro*. The compounds identified through GSM screening (glycerol, oleate, menaquinone-7, Gly-Leu, and Gly-Asn-L) enhanced the growth and metabolic activity of KZ, with combined treatments showing stronger growth-promoting effects than single substrates. This finding is consistent with recent studies demonstrating that GSM models can predict substrate utilization by specific microbial strains and selectively enhance their growth[19]. For example, one study predicted and experimentally validated four compounds (L-serine, L-threonine, D-mannitol, and γ-aminobutyric acid) specifically utilized by target *Pseudomonas* strains based on GSM [20]. This approach, however, has limitations. GSM predicts fluxes using static genome-based networks, and its accuracy depends on genome annotation quality, gap-filling, and environmental parameter settings [21]. In this study, the predicted uptake and metabolic profiles of candidate compounds were not experimentally validated, underscoring the need for recalibration and further testing, such as isotope tracing [22, 23]. In addition, the experiment was conducted under static *in vitro* conditions (green tea-based medium, 25 °C, 14 d of stationary incubation), which cannot fully replicate the dynamic rumen environment. Although the *in vitro* results demonstrated methane reduction, the stability and efficacy of this mitigation strategy must be confirmed *in vivo*.

The pH is a critical indicator of feed quality, as inappropriate values can affect stability, storability, and digestibility in ruminants. After 14 d of fermentation, the synbiotic-treated feed had a higher pH than that of the untreated control feed. A recent study similarly reported that the pH of silage containing yeast varied significantly with the yeast content and storage period. The addition of yeast and prebiotics to the FTMR can modulate fermentation dynamics, often stabilizing or elevating pH without compromising quality or aerobic stability. Importantly, excessively low pH (< 4.2) may indicate over-acidification and potential palatability issues [24], whereas moderately higher pH values (4.5 – 4.8) are generally associated with optimal lactic acid fermentation, improved aerobic stability, and reduced spoilage by undesirable microorganisms [25].

Furthermore, to investigate metabolic changes during fermentation, we analyzed metabolites in FTMR. We observed increased levels of acetic acid and ethanol accompanied by a decrease in lactic acid. The *Z. parabailii* strain used in this study is resistant to lactic acid and versatile in carbohydrate metabolism, enabling ethanol production under variable conditions. This observation is consistent with reports that *K. intermedius* strains efficiently oxidize ethanol to acetate [26]. It should be noted that methane production was not directly measured in the FTMR experiment; therefore, a direct causal link between FTMR fermentation and methane reduction cannot be established. To date, direct evidence linking SCOBY or kombucha-derived microbial consortia to enteric methane mitigation remains limited. Nevertheless, the functional characteristics of SCOBY- particularly the synergistic interaction between yeasts and acetic acid bacteria – suggest potential relevance to methane reduction mechanisms. Our findings further support this notion, as the synbiotic intervention reshaped the fermentation profile by increasing ethanol and acetate concentrations. From a thermodynamic perspective, ethanol functions as an electron carrier serving as an alternative metabolic hydrogen (H_2) sink, diverting reducing equivalents away from the CO_2 reduction pathway utilized by hydrogenotrophic methanogens [27-28]. Furthermore, yeast supplementation has been shown to stimulate acetate-producing bacteria that competitively consume H_2, thereby suppressing methanogenesis [29]. Within this context, competition among functional microbial groups for shared substrates such as H_2 can lead to competitive exclusion between methanogens and alternative hydrogenotrophs [30], suggesting that the yeast-LAB consortium introduced via FTMR may limit substrate availability for hydrogenotrophic methanogens, thereby indirectly restricting methanogenic proliferation through multi-layered microbial interactions [31-32].

The impact of synbiotic treatment on microbial community structure in fermented feed was also evident. Integrated multi-omics analyses have shown that the emergence and persistence of specific beneficial bacteria are critical for maintaining fermentation quality and stability, underscoring the functional importance of core microbiomes [33]. Bacterial and fungal populations play complementary roles in fiber degradation, substrate transformation, and the modulation of rumen fermentation dynamics. Dominant bacterial taxa such as *Fructilactobacillus*, *Companilactobacillus*, and *Weissella* primarily drive lactic acid production and contribute to pH stabilization. Fungal members, particularly yeasts, including *Zygosaccharomyces*, *Starmerella*, *Brettanomyces*, and filamentous forms, are pivotal in depolymerizing complex polysaccharides and generating growth factors or cross-feeding metabolites such as ethanol, CO_2, and vitamins that sustain bacterial growth through syntrophic interactions [34]. This consortium mirrors the native rumen ecosystem, in which anaerobic fungi (e.g., *Neocallimastrix* spp.) and yeasts act synergistically with prokaryotes to optimize fiber degradation and harvest energy [35].

In this study, the early stage of fermentation was characterized by diverse genera, including *Weissella*, *Pantoea*, and *Staphylococcus*. As fermentation progressed, communities became increasingly dominated by LAB, particularly *Companilactobacillus* and *Fructilactobacillus*. This succession reflects established trends in silage and fermentation research: heterogeneous early populations transition into LAB-centered communities under selective fermentation conditions [36]. The accelerated proliferation of LAB in synbiotic treatments may stem from their competitive advantage under the prevailing substrate composition. Notably, *Fructilactobacillus* exhibits high fermentation efficiency on fructose- and sucrose-rich substrates, supporting its late-stage dominance in the 10% Mix group [37]. Non-Saccharomyces yeasts such as *Starmerella* and *Brettanomyces* also contributed to ethanol and acetic acid production, influencing overall metabolic flux through cross-feeding interactions with

LAB. Previous studies on kombucha fermentation have shown that yeast–acetic acid bacteria interactions underpin the pH and organic acid balances that drive microbial succession and metabolic outcomes [38].

Our findings imply that synbiotic intervention not only promotes the predominance of beneficial LAB strains but also reorganizes complex interdomain microbial networks, ultimately improving fermentation quality under the present *in vitro* conditions.

While the *in vitro* results provide mechanistic insight into the synbiotic's potential, several limitations must be acknowledged. Batch culture systems cannot fully replicate the dynamic rumen environment, including continuous digesta passage, salivary buffering, and complex host-microbe interactions. Consequently, the observed reductions in methane production and shifts in fermentation profiles should not be directly extrapolated to *in vivo* conditions without further validation. Future research should therefore prioritize longitudinal *in vivo* trials to confirm the sustained efficacy of synbiotic FTMR on methane yield and animal performance, ensuring the practical applicability of this approach in commercial livestock production. The present study was intentionally scoped to fermentation quality characterization, and the direct evaluation of FTMR-associated methane mitigation *in vitro* and *in vivo* is reserved for future investigation.

In summary, synbiotic supplementation enhanced fermentation quality, as evidenced by stabilized pH, distinctive organic acid profiles, and marked shifts in microbial community structure. Synergistic interactions between LAB and yeasts contributed to a more stable fermentation environment and facilitated the predominance of beneficial microorganisms. These findings suggest that strategic reconstitution of the microbial community through synbiotics holds promise as an approach to mitigate enteric methane emissions (Figure 8), though further *in vivo* validation is needed to confirm these promising findings.

Acknowledgments

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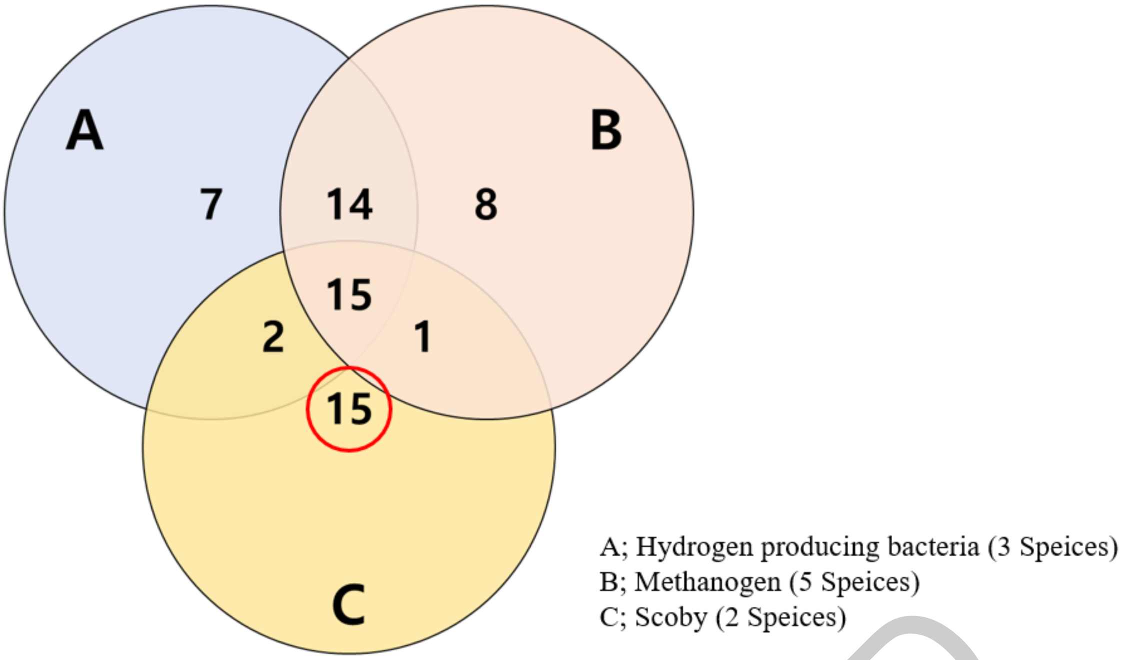

Figure 1. Identification of common and differential metabolites, that are utilized by indicated microbes by using GSM analysis. Three microbial groups were analyzed using genome-scale metabolic models (GSMs): hydrogen-producing bacteria (Group A; *Ruminococcus albus*, *Ruminococcus flavefaciens*, *Butyrivibrio fibrisolvens*), methanogen(Group B; *Methanobrevibacter ruminantium*, *Methanobrevibacter gottschalkii*, *Methanobrevibacter smithii*, *Methanobrevibacter olleyae*, *Methanobrevibacter millerae*), and SCOBY(Group C; *Komagataeibacter intermedius*, *Zygosaccharomyces parabailii*). The diagram illustrates the overlap in metabolite uptake profiles among the groups. A total of 15 compounds (highlighted in red; Glycerol, Oleate, Menaquinone 7, CoA, Fe3, Mg, Ala-L-Thr-L, Gly-Leu, meso-2,6-Diaminopimelate, Gly-Asn-L, Gly-Met, Gly-Tyr, Gly-Asp-L, Ala-His, Cys-Gly) were identified as uniquely utilized by the KZ group but not by hydrogen-producing bacteria or methanogens, and were considered potential prebiotic candidates for further evaluation.

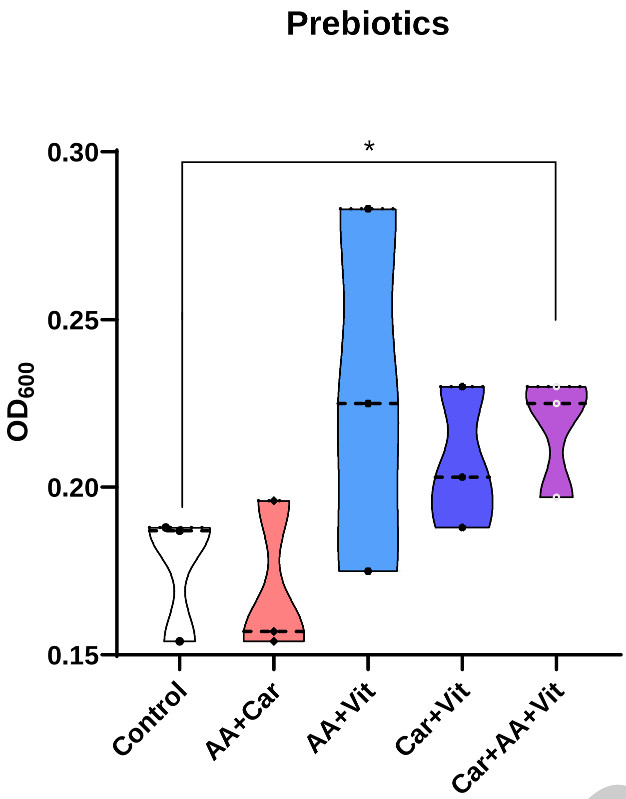

Figure 2. Effects of supplementation of potential mixtures of selected prebiotics for KZ growth. Violin plots represent the optical density (OD_{600}) values of KZ cultures grown under different prebiotic treatment. Treatments included combinations of amino acids (AA; Gly-Leu, Gly-Asn-L), carbohydrates (Car; Glycerol, Oleate), and vitamins (Vit; Menaquinone-7), as well as a mixed treatment group (Mix) and a control group without added prebiotics. KZ cultures were incubated statically at 25 °C for 14 d using green tea and sucrose-based medium supplemented with the respective prebiotics. Microbial growth evaluation was conducted by measuring OD_{600} values at 7-day. Each dot represents an individual replicate (n =3). One-tailed unpaired T-test was conducted for statistic analysis($^{*}p \leq 0.05$).

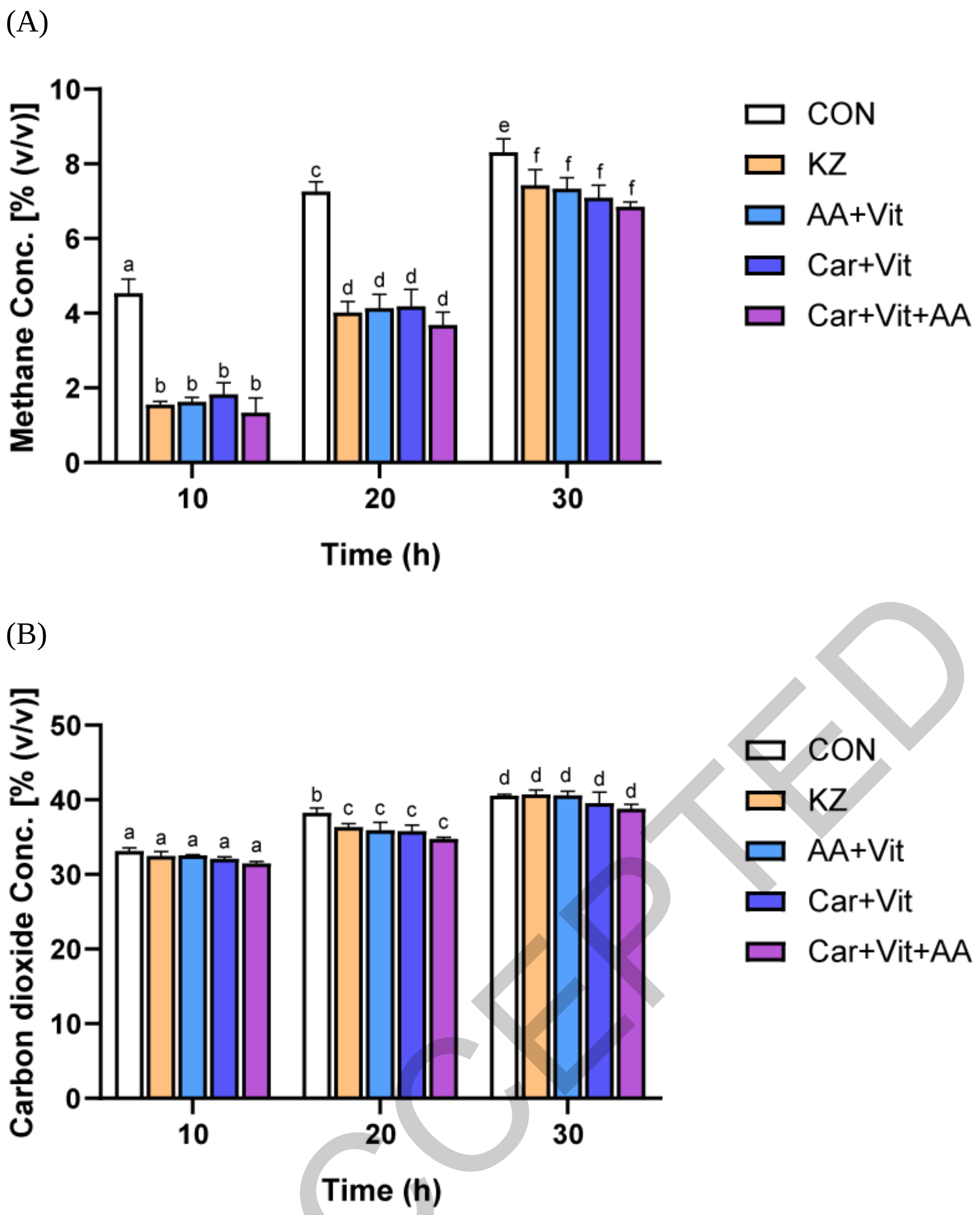

Figure 3. Effects of KZ and/or prebiotics supplementation on ruminal methane and carbon dioxide emissions during in vitro fermentation. Bar plots represent methane (A) and carbon dioxide (B) concentrations under different prebiotic treatments. Treatments included combinations of amino acids (AA; Gly-Leu, Gly-Asn-L), carbohydrates (Car; Glycerol, Oleate), and vitamins (Vit; Menaquinone-7), as well as a mixed group (Mix) and a control group without prebiotics. KZ (*Komagataeibacter intermedius* and *Zygosaccharomyces parabailii*) were incubated statically at 25°C for 21 days in a green tea and sucrose-based medium supplemented with the respective prebiotics. Methane and carbon dioxide concentrations were analyzed by gas chromatography at 10-hour intervals. Data are presented

as mean ± SD (n = 3). Statistical analysis was performed using two-way ANOVA followed by Tukey’s multiple comparisons tested in Prism (ver 8.0.2). Groups sharing the same letter are not significantly different ($p > 0.05$).

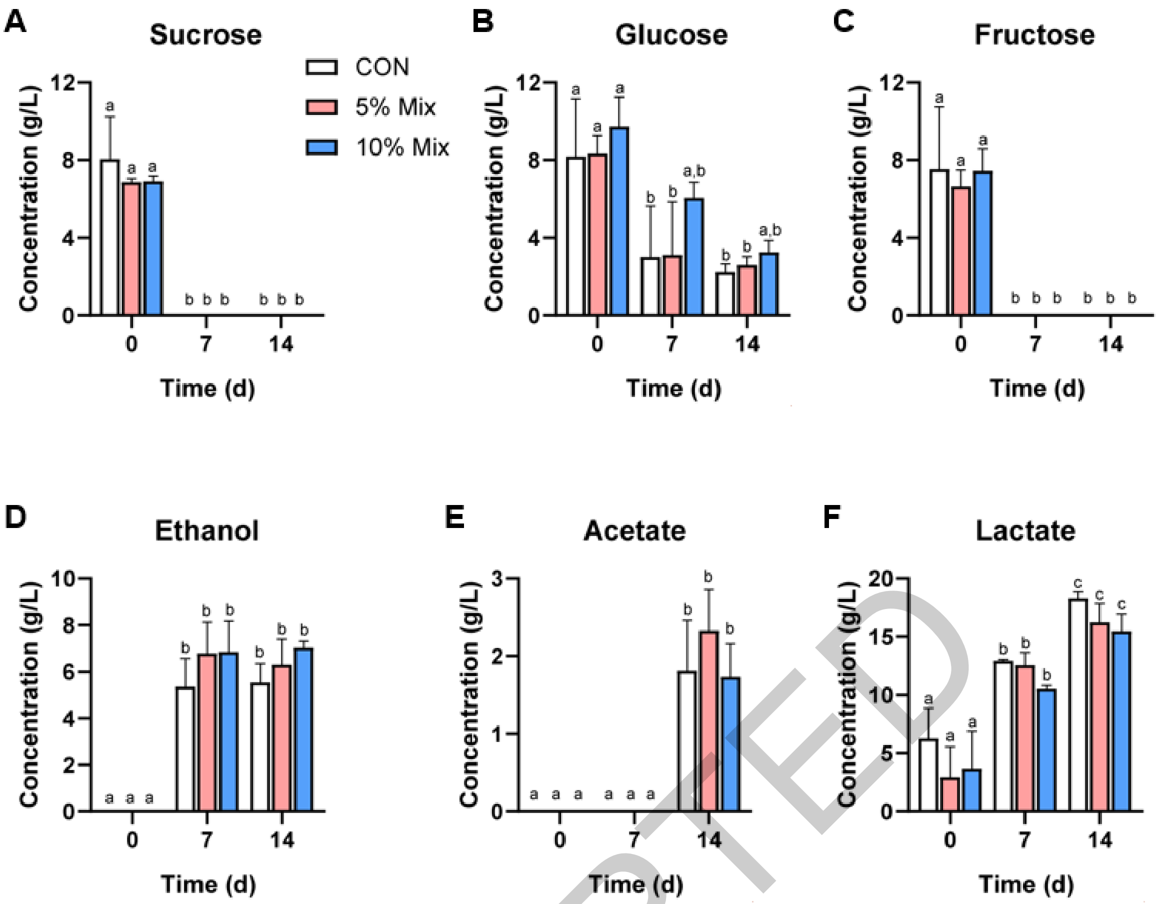

Figure 4. Effects of Synbiotic supplementation alters metabolite profiles in TMR during fermentation process. Bar plots represent the metabolite contents in FTMR under different concentrations of synbiotics and fermentation times. Synbiotics consisted of combinations of KZ, amino acids (Gly-Leu, Gly-Asn-L), carbohydrates (Glycerol, Oleate), and vitamins (Menaquinone-7). FTMR samples were fermented at 25 °C for 14 d under anaerobic conditions, supplemented with the varing concentrations of synbiotics. Metabolite contents were analyzed by HPLC at 7-day intervals. Each dot represents an individual replicate (n = 3). Two-way ANOVA followed by Tukey's multiple comparisons test was conducted for statistical analysis in Prism (ver 8.0.2). Groups sharing the same letter are not significantly different($p < 0.05$).

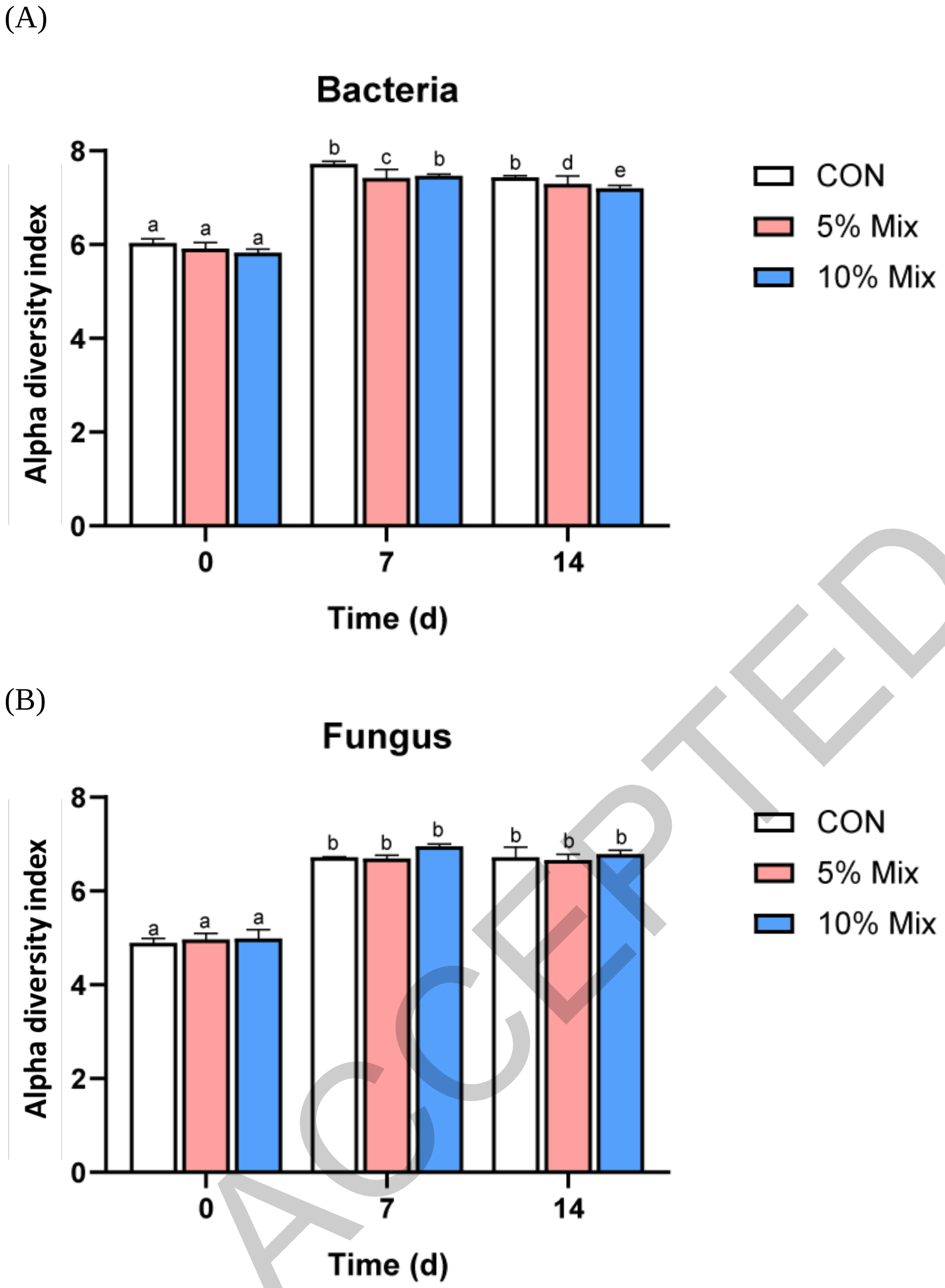

Figure 5. α-diversity indices for bacteria and fungus in synbiotics-treated FTMR after 14 days fermentation. Microbial diversity and evenness were respectively presented as Shannon index in bacterial (A) and fungal (B) community. FTMR samples were supplemented with synbiotics as follows: CON (untreated control); 5% Mix (5% combination of Gly-Leu, Gly-Asn-L, glycerol, oleate, menaquinone-7, and KZ); 10% Mix (10% combination of Gly-Leu, Gly-Asn-L, glycerol, oleate, menaquinone-7, and KZ). All samples were fermented anaerobically at 25 °C for 14 d. QIIME (v1.9.0) was utilized for alpha diversity metrics to represent species complexity within individual samples.

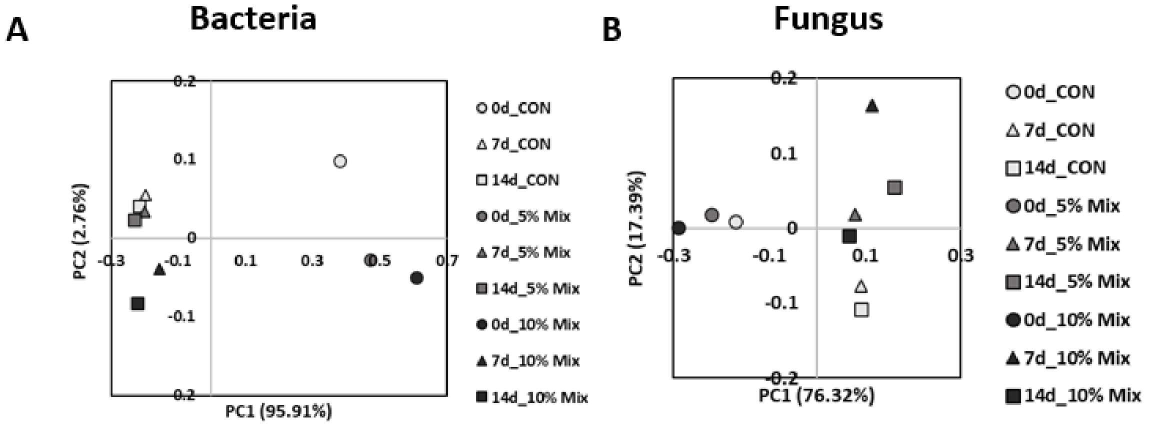

Figure 6. PCoA analysis for bacteria and fungus in synbiotics-treated TMR during fermentation process. Synbiotics supplementation altered bacterial (A) and fungal (B) community structured during FTMR fermentation, as shown by PCoA, with distinct temporal shifts and group-specific clustering patterns. FTMR samples were supplemented with synbiotics as follows: CON (untreated control); 5% Mix (5% combination of Gly-Leu, Gly-Asn-L, glycerol, oleate, menaquinone-7, and KZ); 10% Mix (10% combination of Gly-Leu, Gly-Asn-L, glycerol, oleate, menaquinone-7, and KZ). All samples were fermented anaerobically at 25 °C for 14 d. The mafft (v7.475) and FastTreeMP (v2.1.10) were used for multiple sequence alignment and phylogenetic tree construction. QIIME (v1.9.0) was utilized for calculating distance matrix.

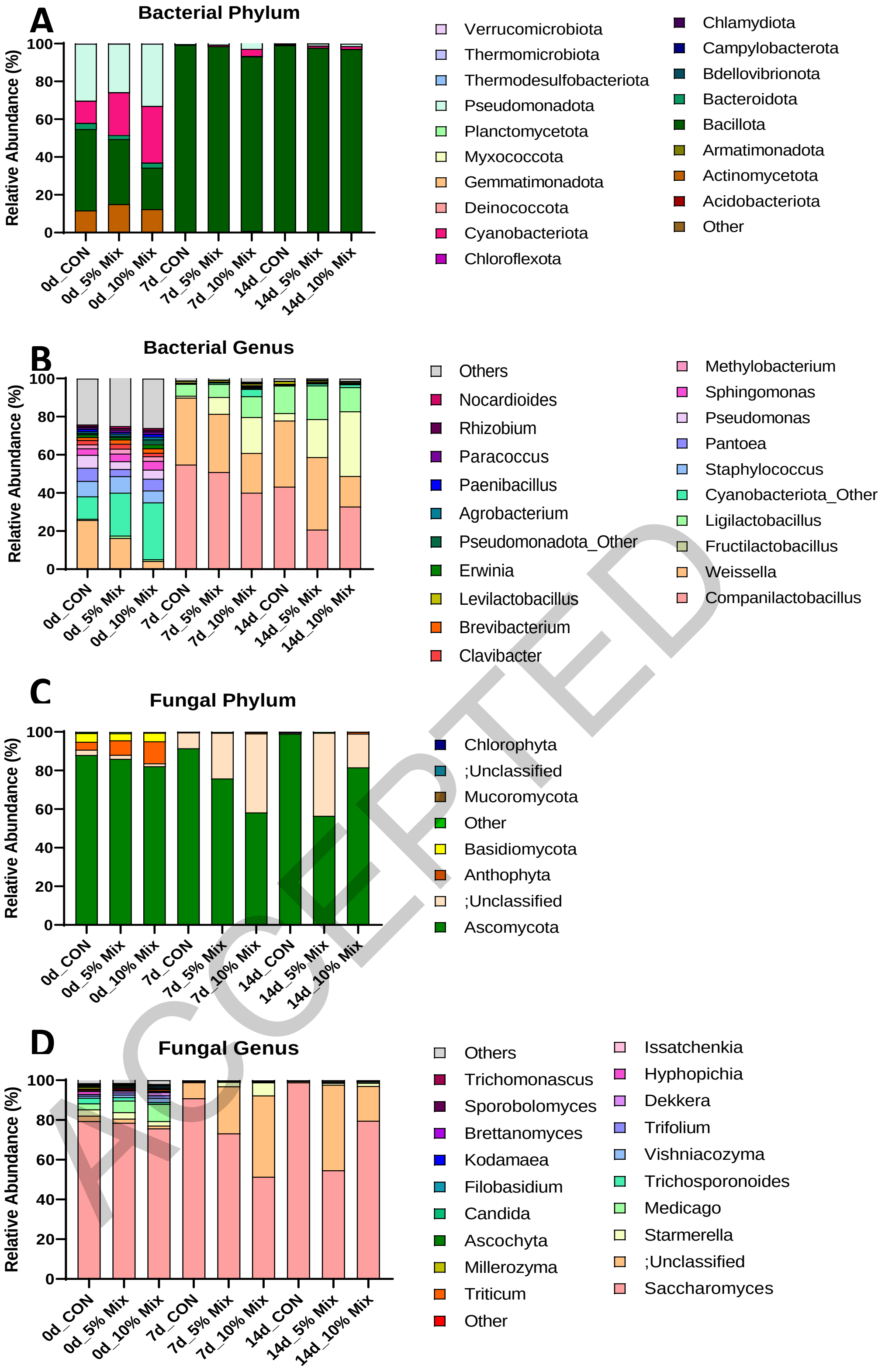

Figure 7. Taxonomic shifts in bacterial and fungal communities of FTMR during synbiotic-supplemented fermentation. (A) Bacterial phyla, (B) fungal phyla, (C) bacterial genera, (D) fungal genera. The relative abundance of 16S rRNA and ITS 3-4 reads assigned to each taxon is shown. FTMR samples were supplemented with synbiotics as follows: CON (untreated control); 5% Mix (5% combination of Gly-Leu, Gly-Asn-L, glycerol, oleate, menaquinone-7, and KZ); 10% Mix (10% combination of Gly-Leu, Gly-Asn-L, glycerol, oleate, menaquinone-7, and KZ). All samples were fermented anaerobically at 25 °C for 14 d.

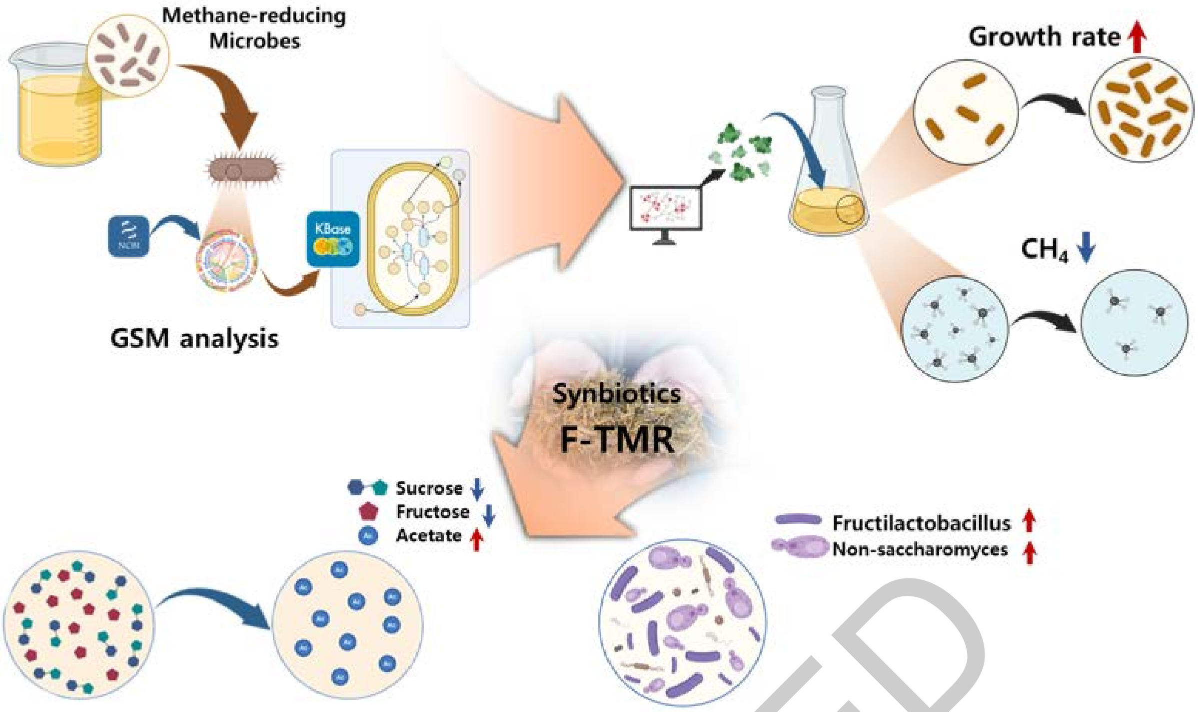

Figure 8. Workflow for producing potential methane-reducing candidates. Rumen-derived microbial samples were initially isolated and subjected to genomic characterization. Genome sequences were retrieved from public databases (NCBI) and reconstructed into genome-scale metabolic models using KBase. Metabolic pathway analysis was performed to predict potential prebiotic candidates. Selected prebiotics were co-cultured with probiotics and validated using in vitro fermentation assays. Subsequently, synbiotics were formulated, and changes in metabolites and microbial composition were analyzed.

Table 1. Chemical composition of experimental diet (FTMR)

Items (%) [2]	Treatments [1]		
	CON	5% Mix	10% Mix
Crude protein	9.79	9.71	10.16
Crude fat	1.63	1.55	1.75
Crude fiber	10.33	10.71	9.87
Crude ash	5.23	5.14	5.17
NDF	22.19	24.2	24.46
ADF	11.36	11.67	10.93

1)CON (untreated control); 5% Mix (5% combination of Gly-Leu, Gly-Asn-L, glycerol, oleate, menaquinone-7, and KZ); 10% Mix (10% combination of Gly-Leu, Gly-Asn-L, glycerol, oleate, menaquinone-7, and KZ)

2)NDF (Neutral detergent fiber); ADF (Acid detergent fiber)

Table 2. List of isolated bacterial and yeast strains from kombucha

	The most matched organisms	Identities (%)	Accession No.
Bacteria	*Komagataeibacter intermedius*	99.86	NR_026435.1
	Komagataeibacter swingsii	99.36	NZ_NKUB01000060.1
	Gluconoacetobacter hansenii	99.72	NR_026133.1
Yeast	*Zygosaccharomyces bisporus*	92.99	KC881074.1
	Zygosaccharomyces parabailii	99.82	CP019500.1
	Brettanomyces bruxellensis	99.81	CP063131.1
	Dekkera bruxellensis	99.08	JX094777.1

Table 3. Detailed information of metabolites, that are utilized by indicated microbes by using GSM analysis

	A	B	C
O_2	+	+	+
Mn_2^+	+	+	+
Zn_2^+	+	+	+
Sulfate	+	+	+
L-Arginine	+	+	+
Cu_2^+	+	+	+
Ca_2+	+	+	+
Cl^-	+	+	+
Co_2^+	+	+	+
K^+	+	+	+
Mg	+	+	+
Spermidine	+	+	+
Thiamin	+	+	+
Folate	+	+	+
Myristic acid	+	+	+
Heme	+	+	-
L-Lysine	+	+	-
L-Aspartate	+	+	-
BIOT	+	+	-
L-Leucine	+	+	-
L-Histidine	+	+	-
L-Asparagine	+	+	-
L-Valine	+	+	-
L-Threonine	+	+	-
D-Arabinose	+	+	-
Riboflavin	+	+	-
L-Isoleucine	+	+	-
1,2-Diacyl-sn-glycerol dioctadecanoyl	+	+	-
L-Serine	+	+	-
D-Glucose	+	-	+
L-Tryptophan	+	-	+
4-Hydroxybenzoate	-	+	+
S-Adenosyl-L-methionine	+	-	-
GTP	+	-	-
L-Glutamine	+	-	-
Pyridoxal	+	-	-
Nicotinamide ribonucleotide	+	-	-
TTP	+	-	-
PAN	+	-	-
Glycine	-	+	-
L-Alanine	-	+	-
H^+	-	+	-
L-Tyrosine	-	+	-
L-Cysteine	-	+	-
Putrescine	-	+	-
Ala-Ala	-	+	-
ddca	-	+	-
fe3	-	-	+
gly-asn-L	-	-	+
ala-L-Thr-L	-	-	+
Ala-His	-	-	+
Gly-Met	-	-	+
Menaquinone 7	-	-	+
Gly-Leu	-	-	+
Gly-Phe	-	-	+
Gly-Tyr	-	-	+
Glycerol	-	-	+
meso-2,6-Diaminopimelate	-	-	+
Cys-Gly	-	-	+
ocdca	-	-	+

A; Hydrogen producing bacteria(3 Speices)
B; Methanogen(5 Speices)
C; Scoby(2 Speices)

Table 4. Detailed information for selectively utilizable metabolites for KZ

Exchange	Formula	Uptake states(g/mol)	Excretion states
Carbohydrate			
Glycerol	$C_3H_8O_3$	100.000	None
Oleate	$C_{18}H_{35}O_2$	2.160	None
Mineral			
Menaquinone 7	$C_{46}H_{64}O_2$	0.186	None
Fe_3	Fe	0.093	None
Mg	Mg	0.065	None
Amino acid			
ala-L-Thr-L	$C_7H_{14}N_2O4$	10.804	None
Gly-Leu	$C_8H_{16}N_2O_3$	7.096	None
meso-2,6-Diaminopimelate	$C_7H_{14}N_2O_4$	5.665	None
gly-asn-L	$C_6H_{11}N_3O_4$	4.258	None
Gly-Phe	$C_{11}H_{14}N_2O_3$	3.244	None
Gly-Met	$C_7H_{14}N_2O_3S$	2.785	None
Gly-Tyr	$C_{11}H_{14}N_2O_4$	2.533	None
gly-asp-L	$C_6H_9N_2O_5$	2.060	None
Ala-His	$C_9H_{14}N_4O_3$	1.720	None
Cys-Gly	$C_5H_{10}N_2O_3S$	0.255	None

Table summarizes metabolites predicted to be selectively utilized by KZ(*Komagataeibacter intermedius, Zygosaccharomyces parabailii*) but not by hydrogen-producing bacteria or methanogens, as determined via GSM modeling on the KBase platform. The listed compounds include carbohydrates, minerals, and amino acids, with their molecular formulas, uptake states (g/µmol), and excretion statuses

Table 5. Effects of synbiotics supplementation on pH changes in total mixed ration (TMR) during fermentation period.

Days of Ensiling	Treatment		
	CON	5% Mix	10% Mix
0	5.20 ± 0.03^{a}	5.11 ± 0.01^{b}	5.02 ± 0.0^{c}
7	4.86 ± 0.01^{a}	4.75 ± 0.11^{ab}	4.92 ± 0.03^{b}
14	4.66 ± 0.03^{a}	4.76 ± 0.04^{b}	4.72 ± 0.08^{ab}

1) CON (untreated control); 5% Mix (5% combination of Gly-Leu, Gly-Asn-L, glycerol, oleate, menaquinone-7, and KZ); 10% Mix (10% combination of Gly-Leu, Gly-Asn-L, glycerol, oleate, menaquinone-7, and KZ)

^{a,b,c}Means with different superscripts in the same row differ significantly ($p < 0.05$). Means sharing at least one superscript letter are not significantly different.

Table 6. Effects of synbiotics supplementation on alpha diversity indices in total mixed ration (TMR) during fermentation period.

Index	Days of Ensiling	Treatment		
		CON	5% Mix	10% Mix
Shannon	0	6.13	6.27	6.49
	7	2.04	2.38	3.24
	14	2.68	3.08	3.15
Gini-Simpson	0	0.93	0.95	0.96
	7	0.61	0.67	0.79
	14	0.71	0.80	0.82

[1)] CON (untreated control); 5% Mix (5% combination of Gly-Leu, Gly-Asn-L, glycerol, oleate, menaquinone-7, and KZ); 10% Mix (10% combination of Gly-Leu, Gly-Asn-L, glycerol, oleate, menaquinone-7, and KZ)