



Article

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Effects of Administration of Prebiotics Alone or in Combination with Probiotics on In Vitro Fermentation Kinetics, Malodor Compound Emission and Microbial Community Structure in Swine

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Abstract: The objective of this study was to evaluate the effect of *Lactobacillus amylovorus*, *L. plantarum*, galacto-oligosaccharide (GOS) and their synbiotic formulations on pH, volatile fatty acids (VFA), malodor, and microbial ecological profiles through a 24-h in vitro fermentation model. Inclusion of GOS alone and in synbiotic combination with either probiotic resulted in consistently lower pH and higher total gas volumes at 12 and 24 h of incubation. Notably, concentrations of odorous compounds (hydrogen sulfide, H₂S and methyl mercaptan, CH₃SH) in the total gas produced were significantly lower in these GOS-containing treatments relative to the controls and probiotic-only-treated groups. However, although ammonia showed an initial relative reduction at 12 h, concentrations did not differ among treatments at 24 h. Further, the GOS-containing treatments had remarkably higher total and individual VFAs, including acetate, propionate, and butyrate, relative to controls and the probiotic-only treatments. Analysis of microbial composition and diversity showed clustering of GOS-containing treatments away from the controls and probiotic-only treatments at 12 and 24 h of incubation. Our study suggests that GOS supplementation (alone or in combination with *L. amylovorus* or *L. plantarum* probiotic strains) has the potential to increase VFA production in the swine gut while lowering emissions of malodorous compounds, except ammonia, in their manure.

Keywords: swine in vitro fermentation; galacto-oligosaccharides; *Lactobacillus amylovorus*; *Lactobacillus plantarum*



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1. Introduction

Microbial fermentation in the mammalian gut is an important contributor to a host's ability to utilize nutrients within consumed diets [1]. However, microbial fermentation in the intestinal tract as well as in the excreted manure yields malodorous compounds. And increased emissions of malodor result in air pollution, which can be a public nuisance to surrounding residential settlements [2]. The main molecules responsible for malodor in manure include sulfur-containing compounds (such as hydrogen sulfide, methyl mercaptan and dimethyl disulfide); ammonia and amines; indolic and phenolic compounds (such as methyl phenol, indole and 3-methyl indole); as well as some volatile fatty acids (VFAs, such as acetate and valerate) [2]. Most of these odorous molecules are a result of the fermentation of dietary proteins and peptides (or other nitrogenous macromolecules in the

diet), and more so when the peptides are rich in sulfur-containing amino acids [3]. It is for this reason that many methods proposed to reduce odor emissions in farms aim to reduce protein fermentation.

Manipulation of levels and sources of crude protein (CP) and dietary fiber (DF) in swine feed rations has been an effective strategy for reducing odor emission at the source [4]. Reducing CP content has the effect of lowering the amount of undigested proteins and peptides reaching the lower GIT, where the bulk of microbial fermentation takes place in monogastrics such as swine. To mitigate odor, it is also important that the protein source provide an amino acid pool that is balanced to the animal's physiological requirements; otherwise, many of the absorbed amino acids end up unutilized, resulting in their breakdown and excretion as urea in urine [5]. When urine urea mixes with manure, it is fermented by fecal microbiota into several odorous compounds, thus increasing the emission of mal-odor [6]. On the other hand, in manipulating dietary fiber content, the goal is to slow/delay its complete fermentation until the distal colon, where it is preferentially fermented by microbiota at the expense of proteins [4]. For this reason, slowly fermented insoluble DF sources are effective at mitigating protein fermentation since they reach the distal colon, where they present a preferred fermentation substrate. Prebiotics, which are a group of fibers that are not digested by mammalian endogenous enzymes, fit this requirement very well [7].

Prebiotics are fermented by select groups within the microbiota, which allows changes in microbial composition and/or activity in ways that benefit a host [7]. As in the dietary modulatory strategies above, prebiotic supplementation influences nutrient availability within the microbial ecosystem in the lower gut and subsequently in the manure. By altering fiber content in such environments, prebiotic supplementation influences the proliferation of saccharolytic microflora, thus shifting the balance further away from proteolytic microflora and/or activity. This, consequently, minimizes the accumulation of offensive, odorous gas products that are linked to protein fermentation. Galacto-oligosaccharides (GOS) are a common example of such prebiotics used in both human food and animal feed [8]. They are a group of oligosaccharides synthesized from lactose in a transgalactosylation reaction catalyzed mainly by β -galactosidases [9]. These GOS prebiotics are known to have a proliferative effect on members of the genera *Bifidobacterium* and *Lactobacillus* within the gut microbial environment [10]. This is important because several members of *Lactobacillus*, a predominantly saccharolytic genus, promote the health of pigs through their inhibitory effect on the largely proteolytic and pathogenic populations of *Enterobacteriaceae* [11]. Because of this interaction between prebiotics and the microflora, it is generally regarded that combining prebiotics such as GOS with probiotics would have an additive effect on the benefits conferred to a host. Generally, probiotics have been defined as "living microorganisms which when consumed in adequate amounts confer health benefits to a host" [12]. And for practical application in malodor mitigation, the probiotic strain to be used would need to have a recognized ability to suppress the production of odorous compounds in complex microbial environments.

Previous studies have demonstrated the odor-reducing potential of several probiotic strains [13–16], including some strains of *Lactobacillus plantarum* [17,18]. However, the effectiveness of microbial products in odor mitigation remains unsatisfactory [13,16]. Nonetheless, research into microbial methods of malodor mitigation is still nascent, and various combinations of probiotic strains should be evaluated, as well as their combinations with related approaches such as prebiotic application. In the current study, we, therefore, aimed to evaluate the effect of two *Lactobacillus* probiotic strains (*L. amylovorus* and *L. plantarum*), a prebiotic (galacto-oligosaccharide), and their synbiotic combinations on the fermentation kinetics, malodor emission and microbial community structure of finishing diets with swine fecal inoculum. We hypothesized that synbiotic combinations of a prebiotic galacto-oligosaccharide and probiotic *Lactobacillus* strains would have greater malodor mitigating effects compared to individual application of these supplements. And further, the individual applications would have a diminished malodor yield compared to controls.

2. Materials and Methods

2.1. Experimental Design

This study uses an in vitro fermentation model to evaluate the fermentation kinetics of fattening diets inoculated with rectally collected swine fecal samples as measured at three time points: 0, 12 and 24 h of incubation. Among the fermentation parameters evaluated were pH, total gas production, odor gas yield, and volatile fatty acids. Further, changes in the microbial ecosystems during the in vitro fermentation are evaluated using both quantitative real-time PCR and 16S rRNA gene sequencing techniques. The probiotics and the prebiotic were tested in five treatments together with a control, as described in Table 1 below. Each treatment group had 5 replicates for all time points, which were all tested in the subsequent assays unless otherwise stated.

Table 1. Fecal inoculum and the additives used in the experiment.

Category	Treatment *
Control	Fecal inoculum only (no additives)
T1	Fecal inoculum + <i>Lactobacillus amylovorus</i> 10 ⁷ (1 mL)
T2	Fecal inoculum + <i>Lactobacillus plantarum</i> 10 ⁷ (1 mL)
T3	Fecal inoculum + Oligo-galactans (0.5 g)
T4	Fecal inoculum + Oligo-galactans (0.5 g) + <i>L. amylovorus</i> 10 ⁷ (1 mL)
T5	Fecal inoculum + Oligo-galactans (0.5 g) + <i>L. plantarum</i> 10 ⁷ (1 mL)

* These treatments were added to 1 g of freeze-dried feed substrate.

2.2. Preparation of Freeze-Dried Fattening Diet (Fermentation Substrate)

The fermentation substrate, composed of a defined fattening swine diet (Table S1), was prepared based on a previously proposed method [19], which had two successive incubations with pepsin and pancreatin. Briefly, the weighed feed sample (1 g) was added into a 100-milliliter conical flask with a magnetic bar and 25 mL of 0.1 M phosphate buffer adjusted to pH 6.0 ± 0.05 and agitated for 2 min at room temperature. The pH of the mixture was adjusted to pH 2.0 ± 0.05 with 0.1 M hydrochloric acid, and 1 mL of pepsin (porcine, 703 U/mg; reference Sigma Aldrich P-7000) solution containing 250 mg of pepsin per mL of ultra-pure water was added. The capped flasks were incubated in a shaking incubator (150 rpm) at 37 °C for 4 h. After incubation, the flasks were taken out, 10 mL of 0.2 M phosphate buffer at pH 6.8 and a 5 mL solution of 0.6 M NaOH were added to the mixture, and the pH was adjusted to 6.8 ± 0.05 with 0.1 M NaOH. One milliliter of a pancreatin solution (porcine, grade IV, reference Sigma P-7545) containing 100 mg of pancreatin per mL of ultra-pure water was added to the mixture. The flasks were capped and re-incubated at 37 °C for 18 h in a shaking incubator. After the second incubation, the hydrolyzed samples were preheated at 80 °C for 15 min, followed by deep freezing at −80 °C for 6 h, and lyophilization at −80 °C in a high vacuum-free dryer (ilShinBioBase, Dongducheon, Republic of Korea) for 48 h to obtain the friable pellets for the in vitro fermentation.

2.3. Preparation of the Fecal Inoculant

Fresh fecal samples were rectally collected from 5 healthy crossbred Landrace and Yorkshire gestating sows. Prior to fecal collection, the selected animals had been monitored for 30 days to ensure their health and welfare were not compromised. The sows were monitored daily for signs of gastrointestinal conditions (feed intake/appetite, consistency and color of feces excreted, rectal prolapse); respiratory conditions (coughing and sneezing); urogenital conditions (discoloration of urine and vaginal discharge); as well as musculoskeletal conditions (skin discoloration, wounds and lameness). The animals did not display any abnormalities during this period of observation and were therefore judged healthy (and their fecal matter appropriate) for the purpose of this experiment. The fecal inoculum was collected, pooled and mixed, then immediately placed in a prewarmed thermoflask filled with CO₂ before transfer to the laboratory. The fresh feces (1/10 w/v)

were mixed with McDougall's buffer [20], stirred with a glass rod, and filtered through 4 layers of cheesecloth to remove insoluble and non-digested materials. The resulting suspension was homogenized and prewarmed in a shaking water bath incubator at 37 °C for 30 min, and CO₂ gas was injected to maintain anaerobic conditions.

2.4. Composition of the Additives

The amendments used in this study constitute two probiotic strains, *Lactobacillus amylovorus* and *Lactobacillus plantarum*, and galacto-oligosaccharides, and their combinations are shown in Table 1. These probiotic strains were isolated using a previously described procedure [21], and the cell-free supernatant of the broth cultures of *L. amylovorus* and *L. plantarum* was prepared by centrifugation at 10,000 rpm for 20 min at 4 °C. In treatments T1 and T2, 1 mL of broth cultures containing 1×10^7 log CFU/mL, respectively, were inoculated into the contents of the serum bottles. The synbiotic solutions in treatments T4 and T5 were composed of 1 mL of the probiotic strains (*L. amylovorus* and *L. plantarum*, respectively) and 0.5 g of the prebiotic (galacto-oligosaccharide).

2.5. In Vitro Fermentation

In vitro fermentation was performed using a modified method [22]. Firstly, 1 g of lyophilized feed (substrate) was added into the serum bottles (160 mL volume), followed by 100 mL of the fecal and McDougall buffer [20] homogenate, accompanied by a continuous CO₂ gas injection to maintain the anaerobic condition of the mixture. Secondly, determined quantities of the additives T1, T2, T3, T4 and T5 were immediately added into the fermentation bottles as described in Table 1, and a control (C) containing only the fecal inoculum was also used. Silicon stoppers were used to cover the bottles, and aluminum caps were used to seal the bottles. The fermentation bottles were incubated for 12 and 24 h at 37 °C in a shaking incubator set at 50 rpm. The initial pH of the fermentation bottle contents was measured before incubation. Records of data on changes in pH, total gas production, odor gas measurement, volatile fatty acids (VFA), and microbial population of the fermentation bottle content were determined at 0-, 12- and 24-h time points.

At the 12- and 24-h time points, fermentation bottles were removed from the incubator, and the accumulated gas was measured and collected immediately. Simultaneously, the pH of the reaction mixture was determined, and the fermentation bottles were placed in iced water to quench the fermentation reaction. Subsequently, the fermentation solution was filtered using 4 layers of cheesecloth, and the collected filtrate was sealed before being stored at −20 °C until VFA evaluation and DNA extraction.

2.6. Analysis of In Vitro Fermentation Parameters

Total gas production at each time point was determined by measuring the head-space gas pressure using a pressure transducer that interfaces with a computer to allow direct entry of pressure and volume data, as previously described [23]. We also manually measured the total gas produced using a calibrated plastic 50-milliliter syringe and recorded the volume from each fermentation bottle at every set time point. To measure the pH of the serum bottle contents, we used the Orion Star A211 Benchtop pH meter (Thermo Scientific, Waltham, MA, USA).

2.7. Odor Gas Measurement

The total gas from the serum bottles was collected into a 10 mL BD vacutainer. One milliliter (1 mL) of the gas produced during the fermentation was used to determine the hydrogen sulfide (H₂S), methyl mercaptan (CH₃SH), and dimethyl sulfide (C₂H₆S) concentrations. And another 1 mL of gas was used to determine concentrations of ammonia (NH₃). Concentrations of the odorous compounds within the gas produced were determined using a gas chromatography method on a sensor gas chromatography device (Nissha Fis Inc., Osaka, Japan) following the manufacturer's instructions. We used the ODSA-P2 model to

quantify H₂S, CH₃SH and C₂H₆S, while the ODNA-P2 model was used to quantify NH₃ concentrations (Nissha Fis Inc., Osaka, Japan).

2.8. Volatile Fatty Acid Analysis

Volatile fatty acid analysis was performed using high-performance liquid chromatography (HPLC) as previously described [24], with slight modifications. Briefly, 1 mL of the serum bottle contents was centrifuged at 13,000 rpm for 10 min, and the supernatant was filtered with a 0.45 µm filter and measured using high-performance liquid chromatography (HPLC) (Agilent Technologies 12000 series, Waldbronn, Germany). An ultraviolet detector was used to analyze the organic acids at 210–220 nm, and the determination of the fermentation products was conducted using the MetaCarb 87H column (Agilent Technologies, Waldbronn, Germany) following the manufacturer's protocol. This assay was performed for 4 replicates (n = 4) at 0 and 12 h of incubation, while at 24 h of incubation, all 5 replicates were analyzed.

2.9. Genomic DNA Extraction

Total genomic DNA was extracted from samples that had been taken from three (n = 3) of the five replicates in each treatment group at the three time points. Extraction was performed using the QIAamp DNA stool Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. The concentrations and quantities of the genomic DNA were determined using a Nano-drop 1000 spectrophotometer (Thermo Scientific Inc., Willington, DE, USA) at the ratios 260–280 nm.

2.10. Quantitative Real-Time Polymerase-Chain Reaction Analysis

Extracted total genomic DNA was used for qPCR analyses using SYBR[®]Green and Taqman chemistries. The dye-based and hydrolysis probes with primers amplify the genes encoding the 16S rRNA from specific bacterial groups (Table 2). Gene cloning and quantification of the 16S rRNA from the isolated DNA were performed following a one-run PCR amplification reaction as previously described [25,26]. Briefly, a 20-µL final volume comprising 10 µL of the SYBR[®]Green mix and 0.4 µL Taqman[®]probe, 0.8 µL of each primer set (forward and reverse), and 8.4 µL of total genomic DNA extract were used in the PCR reaction performed using (CFX Maestro, BioRad, Singapore). The genomic DNA was diluted in series to generate standard curves, which were used as an additional quality control step for the analysis of the qPCR data to verify the specificity of the cloned products. The PCR reaction was conditioned to run 3 min of initial denaturation at 95 °C, followed by 30 cycles of denaturation at 95 °C for 30 s, at respective annealing temperatures (57–60 °C) for 30 s, extension at 72 °C for 1 min, and final extension for 5 min. We then calculated the copy numbers of 16S rRNA in each of the fermentation samples [27].

Table 2. Summary of primers used in the qPCR assays.

Target	Primer Name ¹	Primer Sequence (5' to 3')	Amplicon Length	Annealing Temp (°C)	Reference
All bacteria	Eub338 F	ACTCCTACGGGAGGCAGCAG	200	60	[28]
	Eub518 R	ATTACCGCGGCTGCTGG			
Firmicutes	Firm934 F	GGAGYATGTGGTTAATTCTGAAGCA	126	60	[29]
	Firm1060 R	AGCTGACGACAACCATGCAC			
<i>Bacteroides</i>	Bac303 F	GAAGGTCCCCACATTG	103	60	[30]
	Bfr-Fm R	CGCKACTTGGCTGGTTCAG			
<i>Lactobacillus</i>	LBLMA1 F	CTCAAACTAAACAAAGTTTC	210	57	[32]
	16-1 R	CTTGTACACCGCCCGTCA			
<i>Bifidobacterium</i>	xfp F	ATCTTCGGACCBGAYGAGAC	235	60	[33]
	xfp R	CGATVACGTGACGAAGGAC			
<i>Faecalibacterium prausnitzii</i>	FPR-2 F	GGAGGAAGAAGGTCTTCGG	248	60	[31]
	Fprau645 R	AATCCGCCTACCTCTGCACT			

Table 2. Cont.

Target	Primer Name ¹	Primer Sequence (5' to 3')	Amplicon Length	Annealing Temp (°C)	Reference
<i>Enterobacteriaceae</i>	ent-F	ATGGCTGTCGTCAGCTCGT	385	59	[34]
	ent-R	CCTACTTCTTTTGAACCCACTC			

¹ F, forward primer; R, reverse primer.

2.11. 16S rRNA Amplification and iSeq Sequencing

PCR amplification was performed using primers targeting the V3-V4 hypervariable regions of the 16S rRNA gene with gDNA. For bacterial amplification, primers of 341F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and 805R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'). All PCR reactions were performed with a TaKaRa Ex Taq™ DNA Polymerase (Kusatsu, Japan) using the manufacturer's protocol. The following were the reaction conditions: initial denaturation at 95 °C for 3 min, followed by 25 cycles of denaturation at 95 °C for 30 s, primer annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 5 min. And then, secondary amplification for attaching the Illumina NexTera barcode was performed with i5 forward primer (5'-AATGATACGGCGACCACCGAGATCTACAC-XXXXXXXXTCGTCGGCAGCGTC-3') and i7 reverse primer (5'-CAAGCAGAAGACGGCATACGAGATXXXXXXXXGTCTCTCGTGGGCTCGG-3'; X indicates the barcode region). The reaction conditions during secondary amplification were similar to those in primary amplification, except that the cycles were reduced to 8. Amplification products were confirmed by running gel electrophoresis for 30 min on 2% agarose gel and visualized under UV exposure by comparison with a molecular size standard (100 bp Plus DNA Ladder; Bioneer Inc., Daejeon, Republic of Korea). The amplified products were purified, and short fragments were removed using AMPure XP beads (Beckman Coulter, Indianapolis, IN, USA). The quality and product size was assessed on a Bioanalyzer 2100 (Agilent, Santa Clara, CA USA) using a High-Sensitivity DNA kit. The purified amplicons were then pooled at equimolar concentrations, and the final concentration of the library was determined using a Kapa qPCR Quantification Kit. Sequencing was performed using the Illumina iSeq 100 Sequencing system (Illumina, San Diego, CA, USA) according to the manufacturer's instructions.

Raw reads were analyzed using the steps described in the standard operating procedure of Mothur at MiSeq_SOP [35]. Briefly, 16S rRNA gene sequences were aligned against the 16S rRNA gene SILVA alignment using Mothur v1.33 [36]. Chimeric sequences were detected with UCHIME [37] and subsequently removed from the dataset. Taxonomic affiliation of 16S rRNA genes was performed with a Bayesian classifier [38] (80% bootstrap confidence score) against the 16S rRNA gene training set (v9) of the Ribosomal Database Project [39]. Unclassified sequences or sequences belonging to Eukaryota, Archaea, chloroplasts and mitochondria were discarded. Sequences were assigned to operational taxonomic units (OTUs) at 97% identity. Only abundant OTUs representing at least 0.1% of the library size were conserved for microbial community analyses. Taxonomic affiliation of these OTUs was performed using BLAST+ and RDP against the different databases: NCBI, BIBI and RDP [39–41].

2.12. Statistical Analysis

All statistical analyses were conducted in R version 4.1.3 (3 October 2022) [42] unless otherwise stated. For pH, total gas yield, concentration of odorous compounds in the gas, and concentration of volatile fatty acids, we used a one-way analysis of variance (ANOVA) with the general linear model for a randomized complete block design. This was followed by a post hoc Tukey honestly significant difference (HSD) test to define mean differences between specific treatments. Significance was considered at a corrected *p*-value < 0.05. Statistical differences in relative abundances of taxa were tested using the Kruskal–Wallis test, followed by pairwise comparisons using Wilcoxon rank-sum tests. To correct for

multiple hypothesis testing, the Benjamini–Hochberg (BH) FDR method was used with a cut-off of <0.20 at 12 h and <0.175 at 24 h. Correlation between genera and treatments was analyzed and visualized using the R package “pheatmap” v1.0.12 [43] on centered log-ratio (clr)- and z-transformed counts as implemented in “mia” v1.7.11 [44]. Other packages used in analysis included “ape” v5.7-1 [45], “phyloSeq” v1.38.0 [46], and “ggplot2” v3.4.2 within “tidyverse” v2.0.0 [47].

3. Results

3.1. Changes in Total Gas Production and pH in the In Vitro Fermentation

The pH and total gas production are considered important variables determining the maturity of fermentation [48]. In the present study, profiles of pH and total gas production are shown in (Figure 1) at the different time points of incubation. The pH measurements at 0 h of incubation revealed differences among the groups (p -value < 0.01); however, the pH remained generally within a narrow range. More interestingly, after 12 and 24 h of incubation, the differences were of a much greater magnitude and showed a particular pattern. At 12 h of fermentation, average pH values in T3, T4 and T5 (5.93, 5.94 and 5.95, respectively) were significantly lower than in groups C, T1 and T2 (6.39, 6.39 and 6.38) (p -value < 0.001). While the average pH values slightly increase across the board, the value pattern remains the same as at 12 h (Figure 1). Overall total gas yield increased over time, with significantly higher volumes measured among T3, T4 and T5 compared to C, T1 and T2 at both the 12 h time point (97.00 mL, 99.60 mL and 116.00 mL compared to 35.80 mL, 35.00 mL and 36.40 mL, respectively; p -value < 0.001) and at 24 h (143.00 mL, 130.00 mL, and 114 mL, compared to 48.20 mL, 49.20 mL, and 50.00 mL, respectively (p -value < 0.001)). The highest average volume of total gas measured was in T3 (143 mL) at 24 h of incubation. This was also significantly higher than the total gas yield from T4 and T5 at this time point (p -value < 0.001).

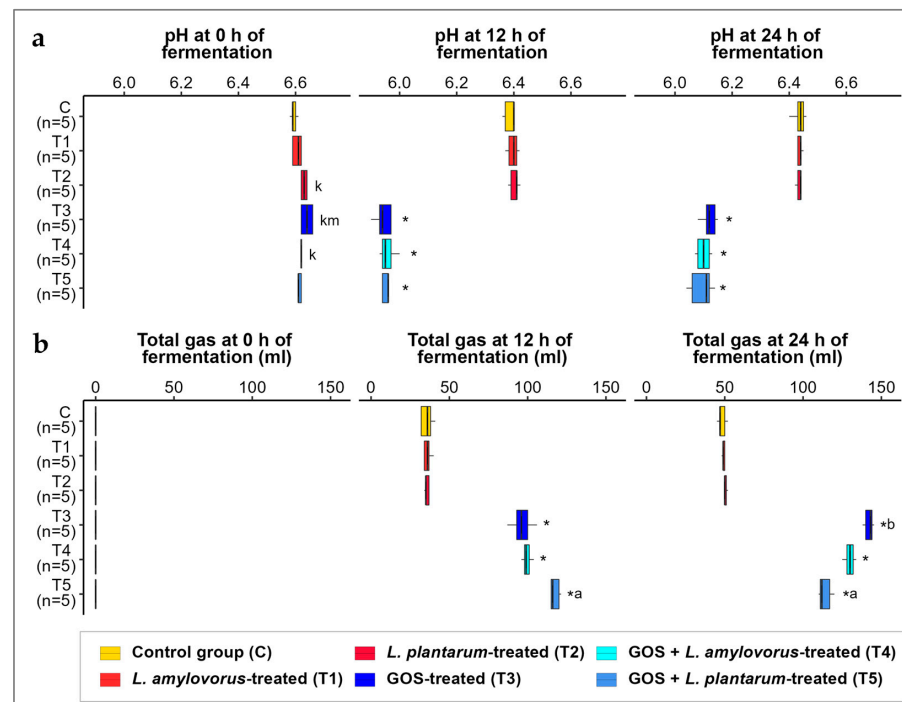


Figure 1. pH and total gas yield in the treatments. Box and range plots of measured pH (a) and total gas produced (b) in the treatments as measured at 0, 12 and 24 h of fermentation. “*” indicates that values were significantly different from C, T1 and T2 (controls and the probiotic-only treatments) (p < 0.05). “a” shows that values were significantly different from those in T3 and T4 (p < 0.05). “b” indicates that values were significantly different from those in T4 and T5 (p < 0.05). “k” indicates difference from C (p < 0.05). “m” indicates difference between T1 and T5 (p < 0.05). The whiskers represent $1.5 \times$ the interquartile range.

3.2. Changes of the Odorous Compounds in the In Vitro Fermentation

Figure 2 shows the odor gas compound profiles in the in vitro fermenter produced at 12 and 24 h of incubation in parts per billion (ppb), with mean values of the odor compounds produced showing the effect of the amendments on the in vitro fermentation. Among the amendments, T3, T4 and T5 showed significantly different emission patterns of ammonia and hydrogen sulfide throughout the incubation period. Conversely, methyl mercaptan was detected in significantly high concentrations in Controls (C) compared to T1 and T2 ($p < 0.0001$). Methyl mercaptan was not detected in T3, T4 or T5 at 12 h, and in none of the samples after 24 h of incubation. Concentrations of hydrogen sulfide increased with incubation across all treatments. However, the controls showed the highest concentration at 24 h, and together with T1 and T2, they had significantly higher concentrations than T3, T4 and T5 ($p < 0.0001$).

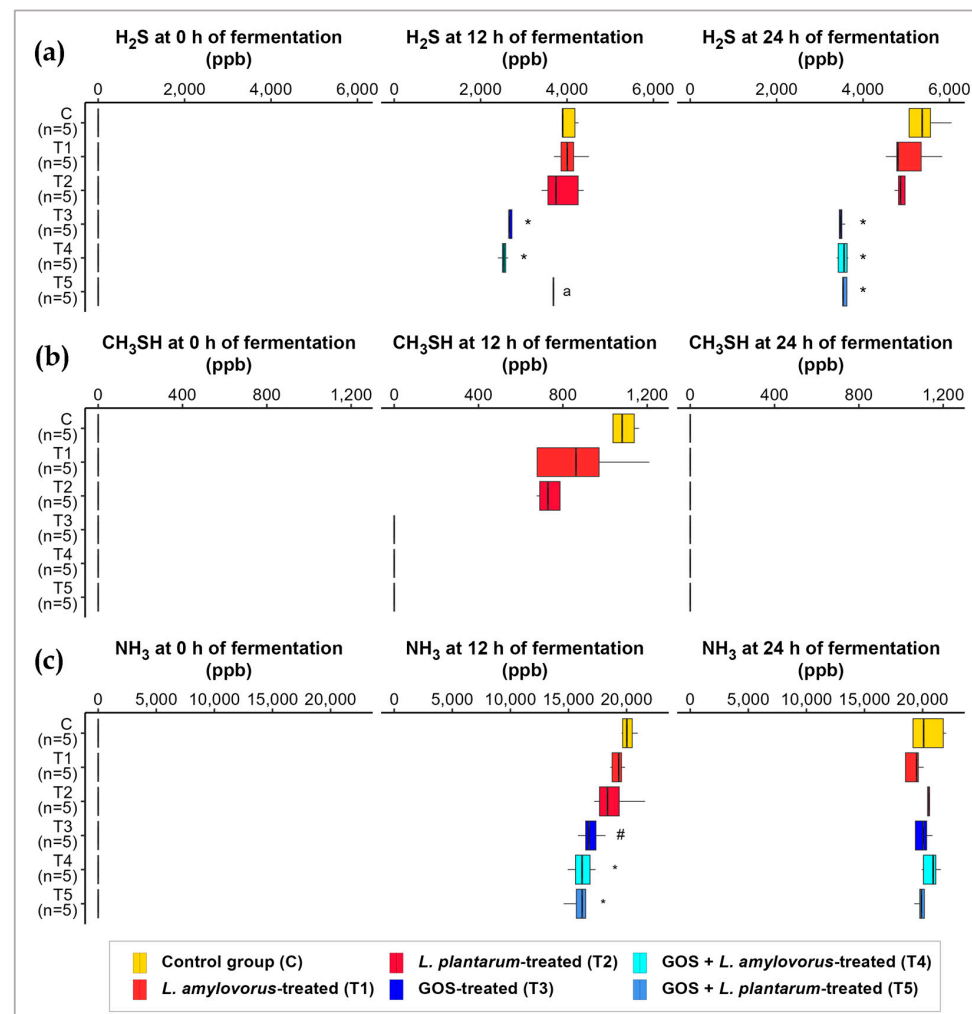


Figure 2. Concentration of odorous compounds in the gas produced. Box and range plots of the concentration of hydrogen sulfide (a), methyl mercaptan (b), and ammonia (c) measured in parts per billion (ppb) within the total gas produced among the treatments across the three time points (0, 12 and 24 h of fermentation). "*" indicates that values were significantly different from C, T1 and T2 (controls and the probiotic-only treatments) ($p < 0.05$). "a" shows that values were significantly different from those in T3 and T4 ($p < 0.05$). "#" indicates that values were significantly different from those in C and T1 ($p < 0.05$). The whiskers represent 1.5 × the interquartile range.

In addition, ammonia was found to be at its highest concentration in the controls at 12 h and decreased across the treatments (Figure 2c). Conversely, at 24 h of incubation, no significant difference in ammonia concentration was detected (Figure 2c).

3.3. Volatile Fatty Acid (VFA) Production after 24 h of In Vitro Fermentation

The yield of VFAs from in vitro fermentation of the hydrolyzed substrate after amending with the studied treatment is shown in Figure 3. At 0 h of incubation, both individual and total VFAs were detected in small and insignificant amounts in all treatments. However, the VFAs were detectable in high volumes after 12 and 24 h of fermentation. And this increase in the measured VFAs was highest in the treatments T3, T4 and T5. Interestingly, a sharp increment in the volume of acetate was recorded across all treatments by the 12th hour of fermentation compared to the other VFAs, and a dominant proportion of acetate was maintained at 24 h ($p < 0.0001$). After 24 h of incubation, the molar concentrations (mmol/kg) of acetate and propionate constituted the greatest proportion of the total VFAs in this in vitro fermenter.

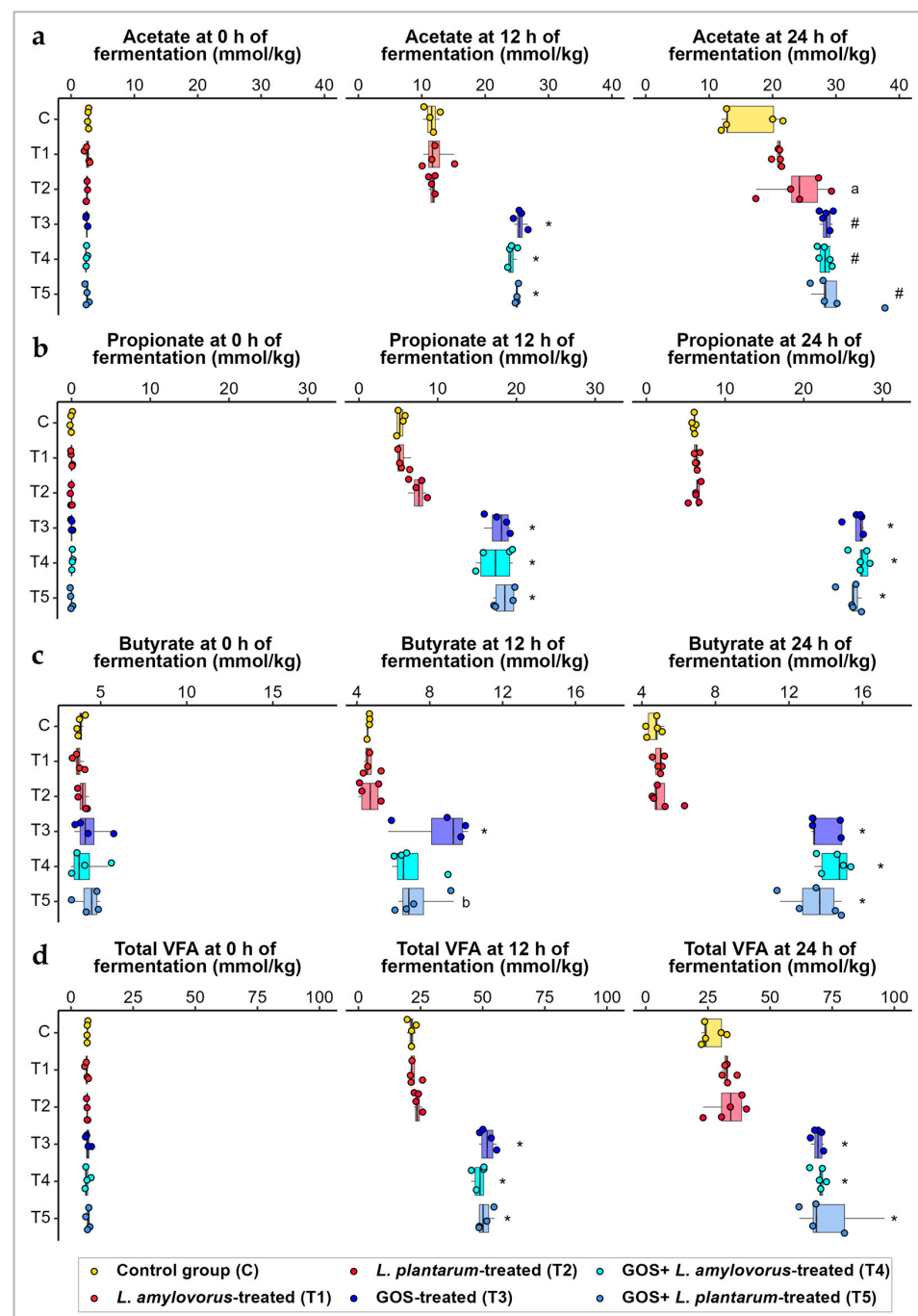


Figure 3. Volatile fatty acid yield from fermentation. Concentration of acetate (a), propionate (b), butyrate (c) and total volatile fatty acids (d) measured in millimoles per kilogram (mmol/kg).

At 0 and 12 h of fermentation, $n = 4$, while for comparisons after 24 h, $n = 5$. “*” indicates that values were significantly different from C, T1 and T2 (controls and the probiotic-only treatments) ($p < 0.05$). “#” indicates that values were significantly different from those in C and T1 ($p < 0.05$). “b” shows that values were significantly different from those in C and T2 ($p < 0.05$). “a” shows that values were significantly different from those in C ($p < 0.05$). The whiskers represent $1.5 \times$ the interquartile range.

3.4. Quantification of the Number of Bacterial Communities (Copies/g)

The copy numbers of selected bacterial strain populations after 24 h of incubation are presented in Table 3. Treatments T3 and T4 had significantly higher copy numbers of total bacteria ($p = 0.0085$) as well as *Firmicutes* ($p = 0.0092$) compared to controls. Treatment T4 also had significantly higher copy numbers of *Bacteroidetes* ($p = 0.0375$) and *Lactobacillus* ($p = 0.0260$) relative to the controls. On the other hand, there were significantly reduced copy counts of *F. prausnitzii* in T2 ($p = 0.0413$) and *Enterobacteriaceae* in T5 ($p = 0.0177$) compared to the controls. [Copy numbers of *Enterobacteriaceae* tended to be lower in T3, T4, and T5 compared to the controls and treatments T1 and T2]. Meanwhile, *Bifidobacterium* showed no significant difference in copy counts across treatments.

Table 3. Quantitative analysis of the bacterial population (log10 copies/g) of the in vitro fermentation content after 24 h of fermentation.

Time (24 h)	Treatment						SEM	p-Value
	Con	T1	T2	T3	T4	T5		
Total bacteria	10.00 ^c	10.25 ^c	10.13 ^c	10.73 ^{ab}	10.78 ^a	10.32 ^{bc}	0.126	0.0085
<i>Firmicutes</i>	9.33 ^c	9.54 ^c	9.43 ^c	9.94 ^{ab}	9.98 ^a	9.59 ^{bc}	0.106	0.0092
<i>Bacteroides</i>	8.32 ^b	8.63 ^b	8.28 ^b	9.28 ^{ab}	9.80 ^a	9.03 ^{ab}	0.265	0.0375
<i>Lactobacillus</i>	8.90 ^b	8.53 ^b	8.78 ^b	10.52 ^{ab}	11.69 ^a	9.34 ^b	0.555	0.0260
<i>Bifidobacterium</i>	6.93	6.97	6.86	6.97	7.18	7.11	0.084	0.3074
<i>Faecalibacterium prausnitzii</i>	7.22 ^a	7.27 ^a	7.04 ^b	7.24 ^a	7.29 ^a	7.28 ^a	0.050	0.0413
<i>Enterobacteriaceae</i>	8.80 ^{ab}	9.20 ^a	8.61 ^{abc}	8.18 ^{bc}	8.27 ^{bc}	8.04 ^c	0.189	0.0177

^{abc} Means without common superscripts along the same row show a statistical difference at $p < 0.05$. C = Control, T1 = *L. amylovorus*, T2 = *L. plantarum*, T3 = Galacto-oligosaccharide, T4 = Galacto-oligosaccharide + *L. amylovorus* and T5 = Galacto-oligosaccharide + *L. plantarum*.

3.5. Dynamics of the Bacterial Community during the Whole Fermentation

To understand the dynamics in bacterial communities during fermentation, we subjected the in vitro fermentation content to the 16S *rRNA* marker gene on the Illumina iSeq platform. Analysis of sequence data revealed changes in diversity (Figures 4 and S1) and microbial composition (Figure 5) following 12 and 24 h of in vitro fermentation across all treatment groups.

A comparison of alpha diversity and evenness among treatment groups did not reveal significant differences (Figure S1). On the other hand, in the exploration of patterns in beta diversity among the treatment groups, significant changes were observed. A principal coordinate analysis plot revealed a distinct separation of the bacterial communities across the treatments and incubation times, as shown in Figure 4. In this study, clustering in the bacterial community existed among the groups T3, T4 and T4, and Control, T1 and T2 at 12 and 24 h of incubation (PERMANOVA of weighted-UniFrac distances at 12 h; pseudo-F = 46.929049, p -value = 0.001; PERMANOVA of Weighted-UniFrac distances at all 24 h of fermentation; pseudo-F = 6.942514, p -value = 0.012).

Bacterial composition at the point of inoculation (0 h) shows a similar relative abundance profile at the phylum level across all treatments. At this point, *Proteobacteria* dominated the microbial communities, with relative abundances ranging from 59.4% to 100%. Through fermentation, changes in relative abundances were observed not only at the phylum level but also at the genus level in all groups after 12 and 24 h of incubation

(Figure 5 and Table 4). Overall, the phyla *Firmicutes* and *Bacteroidetes* dominated the bacterial communities (making up 25.40% ~ 83.90% and 7.86 ~ 38.32%, respectively) across the treatment groups after 12 and 24 h of incubation (Figure 5). The data also showed that *Firmicutes* had significantly higher relative abundances in treatments T3, T4 and T5 compared to the control and treatments T1 and T2. While, in contrast, *Proteobacteria* (and *Bacteroidetes* only at 12 h) occurred in significantly lower relative abundances among treatments T3, T4 and T5 compared to the controls and treatments T1 and T2. However, relative abundances of *Bacteroides* had increased in T5 by 24 h of fermentation (Figure 5 and Table 4).

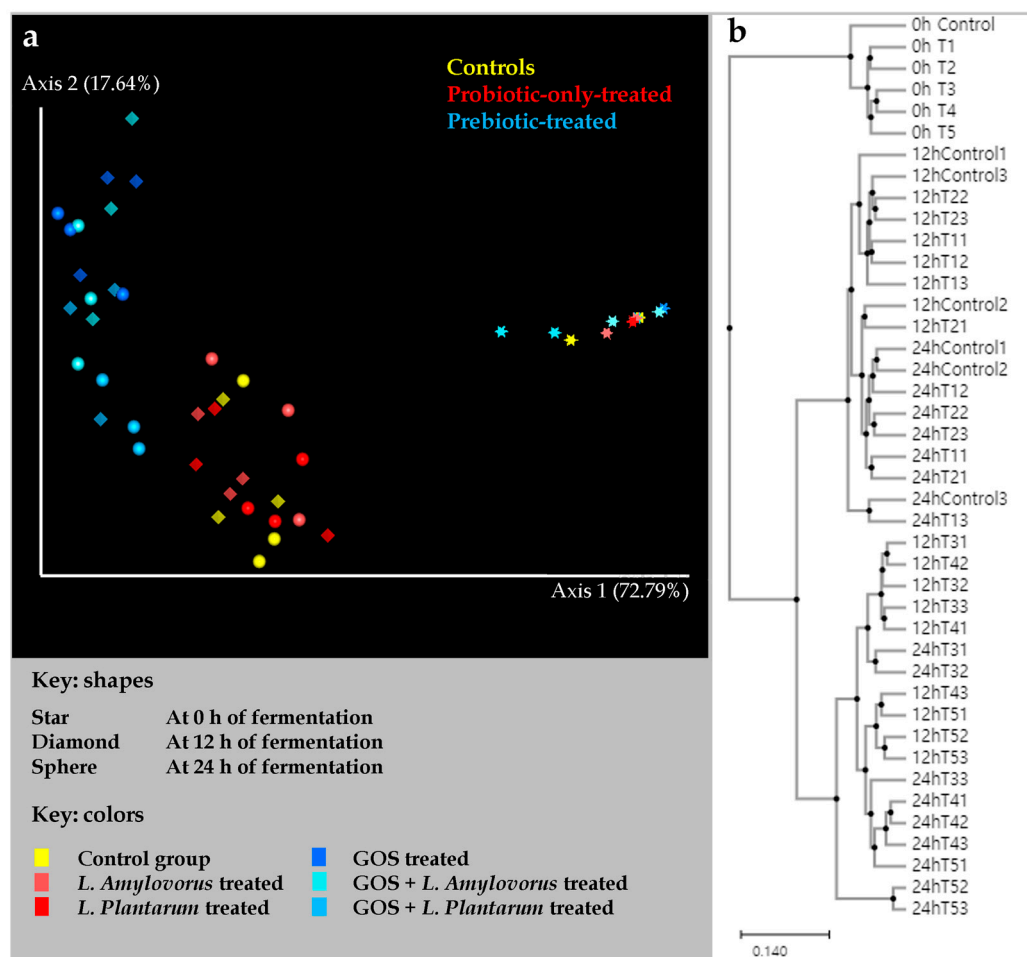


Figure 4. Microbial diversity. A Principal coordinate analysis (PCoA) plot based on weighted UniFrac distances (a) and a UPGMA cluster (b) of the in vitro fermentation microbial communities in all treatments across the three time points.

Shifts in relative abundances of genera within the microbial communities during in vitro fermentation are presented in Figure 6 and Table 4. Overall, *Bacteroides*, *Mitsuokella*, and *Megasphaera* represented the highest relative abundance across the treatments. Treatments T3, T4 and T5 differed from controls and treatments T1 and T2 by significantly higher abundances of *Mitsuokella*, *Megasphaera*, *Lactobacillus* and *Clostridium*, as well as significantly reduced abundances of *Escherichia*, *Serratia*, *Sporobacter*, *Cronobacter*, *Oscillibacter*, and *Desulfovibrio* (Table 4). However, *Bacteroides* appeared in significantly increased abundances in treatment T5 and decreased abundances in T3. Furthermore, T3 had significantly elevated relative abundances of unclassified genera of the orders *Selenomonadaceae* and *Veillonellaceae* while showing significantly reduced abundances of *Bacteroides*, *Prevotella*, and *Fusobacterium* (Table 4). The heatmap in Figure 6 (and Figure S2) shows additional analysis utilizing the correlation and clustering of treatment groups based on similarities in relative abundances of genera among them. The correlations observed suggest a close clustering of T4 and T5 as well

as a clustering of T1 and T2 at 12 h and more distinctly at 24 h of incubation. Again, the observed clustering is mainly explained by members of the following genera: *Prevotella*, *Succinivibrio*, *Mitsuokella*, *Megasphaera*, *Lactobacillus*, *Clostridium*, *Escherichia*, *Acidaminococcus*, *Phascolarctobacterium*, and *Fusobacterium*.

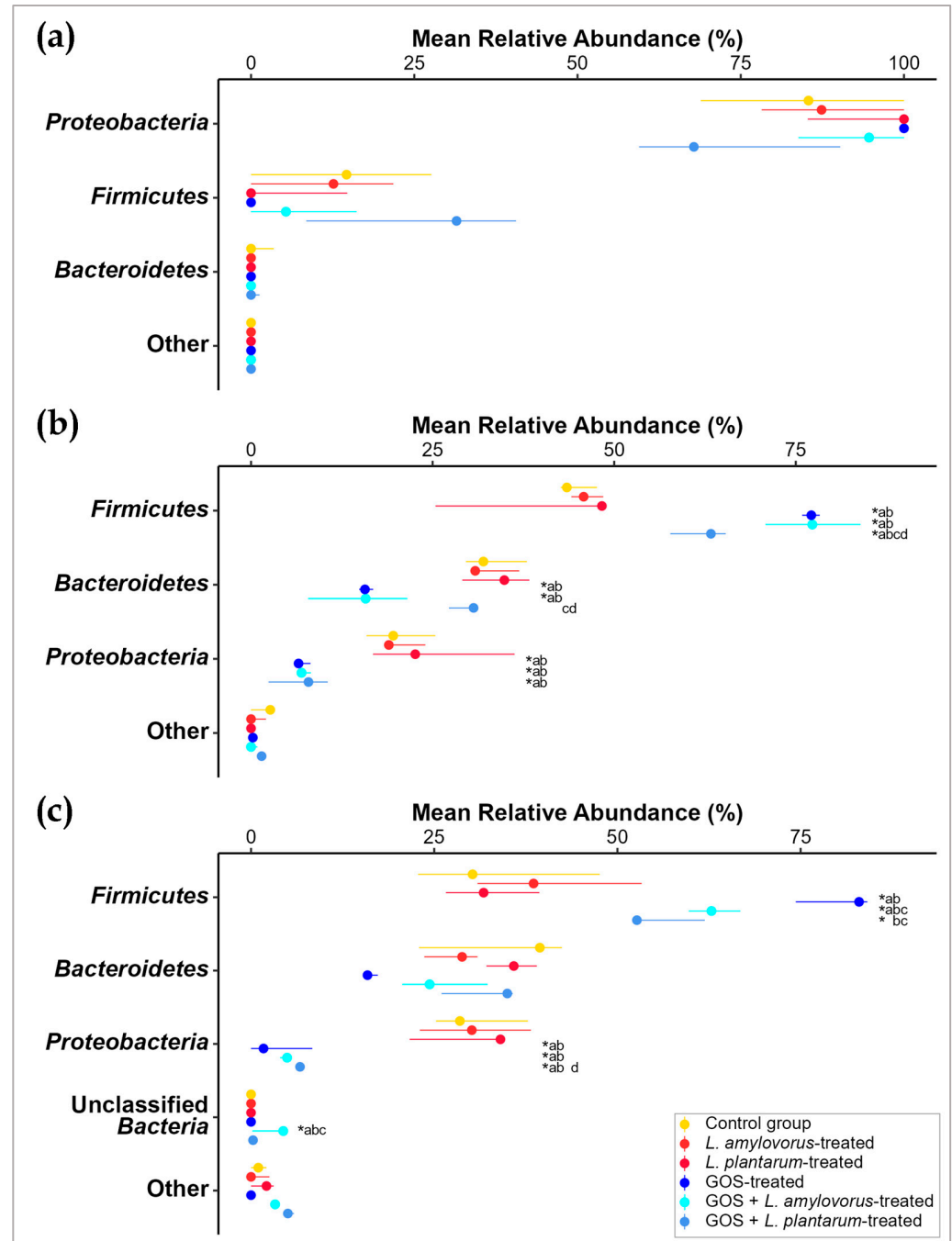


Figure 5. Most abundant prokaryotic phyla in the in vitro fermentation content at 0 h (a), 12 h (b) and 24 h (c). “*” indicates that values were significantly different from C (controls). “a” indicates that values were significantly different from those in T1. “b” shows that values were significantly different from those in T2. “c” shows that values were significantly different from those in T3. “d” indicates that values were significantly different from those in T4. Significance of differences was confirmed using the Kruskal–Wallis test, followed by pairwise comparisons using Wilcoxon rank-sum tests. To correct for multiple hypothesis testing, the Benjamini–Hochberg (BH) FDR method was used with a cut-off of <0.20 at 12 h and <0.175 at 24 h.

Table 4. Relative abundances (expressed as %) of bacterial communities in the in vitro fermentation, calculated based on Illumina iSeq read counts after 24 h of fermentation.

Taxon	Control	T1	T2	T3	T4	T5	SEM	p-Value
Phylum								
<i>Firmicutes</i>	42.49 ^c	44.14 ^c	37.76 ^c	77.79 ^a	70.41 ^a	55.84 ^b	2.877	<0.0001
<i>Bacteroidetes</i>	32.02 ^a	32.31 ^a	36.22 ^a	16.73 ^b	22.30 ^b	33.48 ^a	2.262	0.0005
<i>Proteobacteria</i>	20.92 ^a	19.53 ^a	21.13 ^a	3.83 ^b	3.97 ^b	5.71 ^b	1.178	<0.0001
<i>Fusobacteria</i>	1.26 ^{ab}	1.42 ^{ab}	2.14 ^a	0.27 ^c	0.80 ^{bc}	1.16 ^b	0.246	0.0095
<i>Actinobacteria</i>	0.75 ^c	0.36 ^c	0.40 ^c	0.84 ^c	1.54 ^b	2.43 ^a	0.198	<0.0001
Genus								
<i>Bacteroides</i>	17.07 ^{bc}	18.10 ^{ab}	21.09 ^{ab}	12.12 ^d	13.22 ^{cd}	22.50 ^a	1.433	0.0020
<i>Mitsuokella</i>	9.25 ^c	9.57 ^c	8.43 ^c	26.67 ^a	27.50 ^a	18.31 ^b	1.298	<0.0001
<i>Megasphaera</i>	6.70 ^c	6.25 ^c	5.81 ^c	27.39 ^a	25.39 ^a	18.93 ^b	1.634	<0.0001
<i>Prevotella</i>	8.91 ^a	9.24 ^a	8.83 ^a	2.79 ^b	7.26 ^a	8.44 ^a	0.686	0.0008
<i>Lactobacillus</i>	3.32 ^c	3.55 ^c	2.51 ^c	4.87 ^b	6.57 ^a	6.36 ^a	0.396	<0.0001
<i>Escherichia</i>	4.79 ^b	5.11 ^b	8.70 ^a	1.51 ^c	1.29 ^c	2.16 ^c	0.639	0.0002
<i>Serratia</i>	6.03 ^a	6.21 ^a	4.35 ^a	0.61 ^b	0.70 ^b	0.95 ^b	0.508	0.0001
<i>Sporobacter</i>	3.83 ^b	4.35 ^b	5.76 ^a	1.85 ^c	1.04 ^c	1.74 ^c	0.355	<0.0001
<i>Cronobacter</i>	4.77 ^a	4.47 ^a	2.58 ^b	0.73 ^c	0.40 ^c	0.44 ^c	0.276	<0.0001
<i>Acidaminococcus</i>	3.60	3.92	1.26	1.12	1.75	1.32	0.668	0.2040
<i>Succinivibrio</i>	2.71 ^a	1.25 ^{de}	2.28 ^{ab}	0.86 ^e	1.46 ^{cd}	1.97 ^{bc}	0.161	<0.0001
<i>Phascolarctobacterium</i>	1.31	1.19	1.35	1.25	0.97	1.14	0.250	0.9392
PAC001115_g	1.81 ^b	2.76 ^a	2.31 ^{ab}	0.89 ^c	0.59 ^c	0.71 ^c	0.212	0.0002
<i>Oscillibacter</i>	1.72 ^a	2.06 ^a	1.53 ^a	0.64 ^b	0.46 ^b	0.72 ^b	0.222	0.0037
<i>Fusobacterium</i>	1.26 ^{ab}	1.30 ^{ab}	2.04 ^a	0.27 ^c	0.80 ^{bc}	1.16 ^{abc}	0.251	0.0206
Unclassified <i>Selenomonadaceae</i>	1.36 ^b	0.84 ^b	0.42 ^b	3.83 ^a	0.14 ^b	0.16 ^b	0.295	0.0001
<i>Clostridium</i>	0.30 ^c	0.18 ^c	0.33 ^c	2.38 ^a	2.07 ^{ab}	1.39 ^b	0.209	0.0004
<i>Desulfovibrio</i>	2.21 ^a	1.83 ^a	1.92 ^a	0.04 ^b	0.04 ^b	0.05 ^b	0.157	<0.0001
Unclassified <i>Veillonellaceae</i>	0.65 ^b	1.19 ^b	0.54 ^b	3.58 ^a	0.04 ^b	0.03 ^b	0.334	0.0015

Bacterial phyla and genera were classified at a cut-off level of $\geq 0.5\%$ (phylum) and 1.0% (genus) relative abundance. The data represent iSeq 16S rRNA gene sequences at the phylum and genus level from in vitro samples. ^{abcde} Means without common superscripts along the same row show a statistical difference at $p < 0.05$. C = Control, T1 = *L. amylovorus*, T2 = *L. plantarum*, T3 = Galacto-oligosaccharide, T4 = Galacto-oligosaccharide + *L. amylovorus* and T5 = Galacto-oligosaccharide + *L. plantarum*.

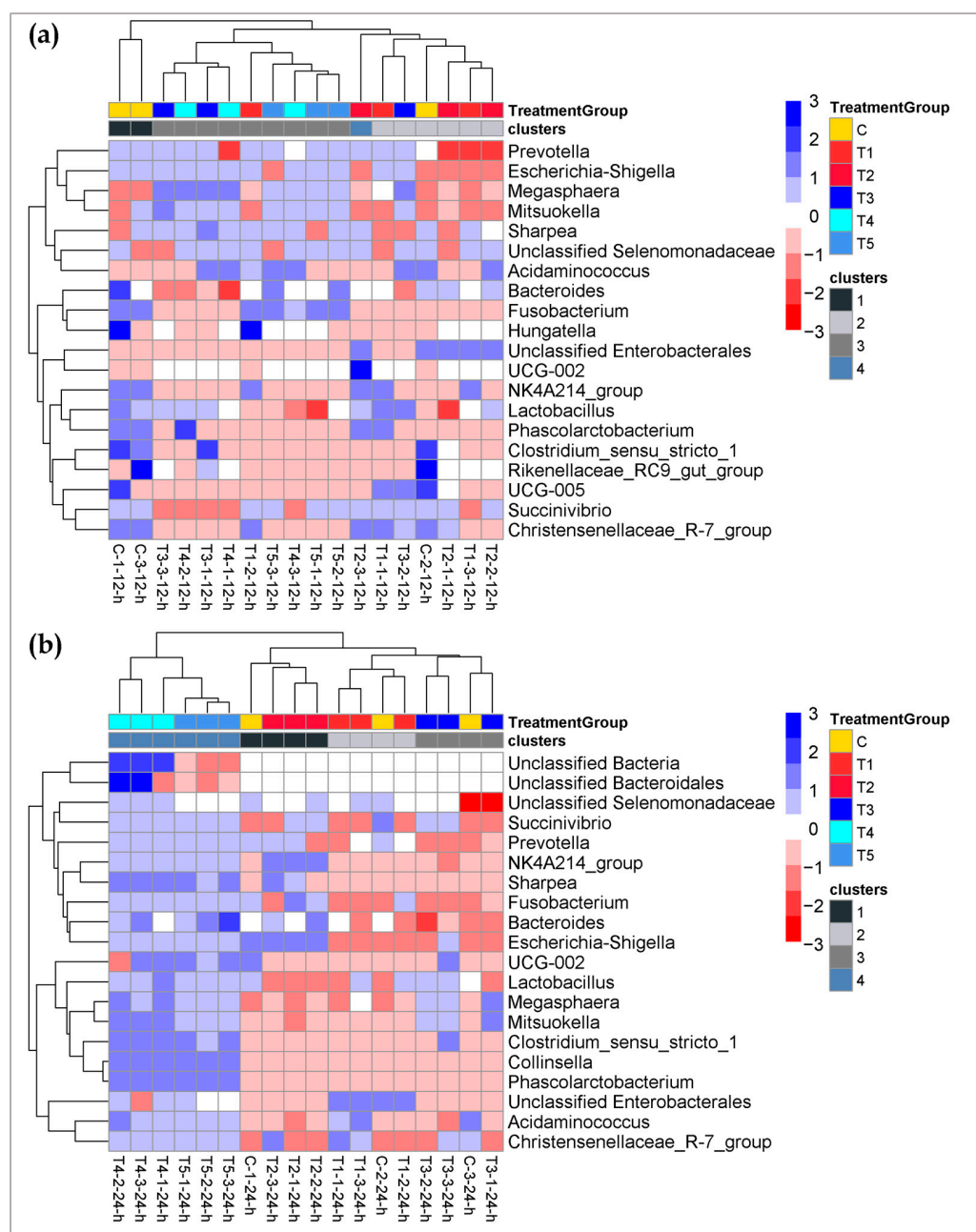


Figure 6. Clustering and correlation analysis of microbial genera among the treatments. Correlation heatmaps of the top 20 abundant genera in the treatment groups at 12 h (a) and 24 h of fermentation (b). To develop these heatmaps, first a centered log-ratio (CLR) transformation is performed on the counts among samples, followed by z-transformations on the features (genera).

4. Discussion

Ammonia, together with the sulfurous compounds hydrogen sulfide and methyl mercaptan, are the major sources of odor in pig farming systems [49]. Although volatile fatty acids also contribute to malodor, the odor associated with the short-chain VFA studied here is less unpleasant. In fact, some SCFA, such as butyrate, sustain gut health. In this study, we focused on changes in these fermentation metabolites along with shifts in microbial profiles in response to probiotic and prebiotic supplementation as an indicator of their potential for odor reduction and enhancement of gut health.

Overall, total gas yield increased with incubation time across all treatments. However, a low gas yield was observed among the control and probiotic-only groups. This might

be a result of a slow increase in the microbial population in these treatments, resulting in longer colonization times and overall low concentrations of active enzymes [50,51]. The dynamics of gas production are dependent on factors such as incubation time, condition, and nutritional value of the substrate [51]. In addition, the digestion rate of carbohydrates (starch, pectin and sugars), which constitute the main factor controlling the available energy for microbial growth, could have influenced the gas production in this study [52]. The general observation of declining pH values while total gas production increased during incubation is linked to the accumulation of organic acids in the fermentation reaction [53]. Indeed, the use of a protein substrate such as soybean meal has been shown to bring about a raise in pH during fermentation [54], largely due to an impaired yield of organic VFAs. Further, our data showed that the reduction in pH was more pronounced in T3, T4 and T5, which contained galacto-oligosaccharide, a rich fiber substrate associated with high organic acid production.

Compounds such as hydrogen sulfide, methyl mercaptan and ammonia are major contributors to the malodor generated during fermentation. We probed their concentration within the total gas produced and found that they appeared in significantly lower concentrations among the treatments relative to the controls. These compounds are products of amino acid degradation during microbial fermentation, and their reduced accumulation can therefore be linked to restrictions on microbial proteolysis. In this study, these odorous compounds were significantly reduced in the oligosaccharide-supplemented treatments compared to the controls and probiotic-only treatments. An explanation for this observation is that probiotics and prebiotics cause shifts in microbial communities towards a less proteolytic and more saccharolytic microbial composition, as has been shown in previous studies [55]. Our findings are also in agreement with Naidu and colleagues [56], who demonstrated a *Lactobacillus*-induced reduction in the yield of sulfur-containing and ammonia-nitrogen compounds during *in vitro* fermentation.

The mammalian gut microbiome is known to utilize dietary fiber, producing metabolites such as VFAs, which in turn actively influence physiological processes within the gut and throughout the host's body [57]. We quantified VFAs in our *in vitro* fermentation model and found a higher total yield across all treatments compared to the controls after 24 h. As anticipated, this increased yield was most significant among treatments supplemented with the galacto-oligosaccharide prebiotic [58]. The dominance of acetate and propionate among the VFAs in the treatments was consistent with a previous report [22]. The observed accumulation of butyrate in the GOS-containing treatments is probably due to the stimulation of butyrogenic microbiota within the microbiome by the GOS prebiotics [9]. In addition, the accumulating acetate might have favored the proliferation of members with the ability to metabolize it into butyrate and propionate [59,60], as observed in the GOS-treated groups between 12 and 24 h of incubation in our static *in vitro* fermentation model.

The composition and diversity of gut microbial ecosystems in pigs and most mammals are greatly influenced by diet and supplements such as probiotics and prebiotics. In this *in vitro* model, we confirm that galacto-oligosaccharides had a significant effect on diversity when administered as an only supplement or in synbiotic formulations along with probiotic *Lactobacillus* spp. Results obtained in this study indicate that galacto-oligosaccharides stimulated a relative proliferation of the phylum *Firmicutes* as well as its genera *Mitsuokella*, *Megasphaera*, *Lactobacillus* and *Clostridium*. The accumulation of members of these taxa among the galacto-oligosaccharide-supplemented treatments is consistent with previous reports on microbial fermentation of high-fiber diets in the monogastric gut [61,62]. The phylum *Firmicutes* consists of saccharolytic bacteria that are prolific producers of short-chain fatty acids, mainly butyrate [63]. Indeed, among the differentially increased genera were the mainly lactate-producing *Lactobacillus* and the acetate-producing *Mitsuokella*, along with the butyrate-producing *Megasphaera* and *Clostridium* [64,65]. It is worth pointing out that members of these taxa are also competent amino acid fermenters. However, they preferentially ferment saccharides in the presence of fermentable dietary fiber, and they continue to thrive as pH drops with the ensuing accumulation of SCFA metabolites [66]. Under these

conditions, overall amino acid catabolism greatly decreases within the microbial ecosystem, leading to a reduction in the release of odorous compounds [66].

We also noted a relative reduction in the abundance of the phyla *Proteobacteria* as well as the genera *Escherichia*, *Serratia*, *Sporobacter*, *Cronobacter*, *Oscillibacter*, and *Desulfovibrio* were among the galacto-oligosaccharide-treated ingesta relative to the controls and probiotic-only treatments. *Proteobacteria* have a diverse functional repertoire, with the widest coverage of amino acid metabolic pathways [67] as well as the reduction of sulfates and sulfites [68]. Therefore, the persistent dominance of *proteobacteria* in controls and probiotic-only supplemented treatments explains the observed high yield of odorous compounds compared to the prebiotic-supplemented fermentation reactions.

The inverse relationship observed between VFA concentrations and the genera *Escherichia*, *Serratia* and *Oscillibacter* in the oligosaccharide-supplemented treatments is explained by the inhibitory effect of acidity on these taxa [69,70]. Their proliferation as pH values started to rise, points to an increasing shift towards proteolytic activity in association with depleting concentrations of fermentable carbohydrates [71]. Increasing metabolism by *Serratia* contributes to raising the pH of the fermentation mixture by diverting the VFA intermediate, pyruvate, towards the synthesis of the neutral pH metabolite 2,3-butanediol [72]. *Oscillibacter* spp. are common members of mammalian gut microbiomes, including pigs, and their fermentation mainly yields valerate and some butyrate [70,73]. Valerate has an offensive smell, and its accumulation would therefore contribute to malodor [49]. The relative increase in abundance of the sulfate-reducing bacteria (SRB) and *Desulfovibrio* in the controls and the probiotic-only treated groups partly accounts for the observed higher yield of hydrogen sulfide (H₂S) in these fermentation samples. Hydrogen sulfide is produced by SRBs during the fermentation of sulfur-containing amino acids [74]. Further, it is believed that *Sporobacter* spp. degrade plant lignocellulose complexes in the presence of sulfides to produce acetate [75]. A co-accumulation of *Sporobacter* with the sulfate-reducing bacteria, *Desulfovibrio*, in these treatments suggests a buildup of sulfides from the degradation of sulfur-containing amino acids. *Cronobacter* spp. are inhibited by lactic acid from lactic acid bacteria, probably through interference with cell membrane integrity [76]. Therefore, lactic acid-producing bacteria can have a synergistic effect with many molecules that are toxic to *Cronobacter* spp., as has been demonstrated with copper [77]. Notably, our results indicated a more pronounced reduction of *Cronobacter* spp. among the galacto-oligosaccharide-supplemented treatments, particularly those combined with *L. amylovorus* and *L. plantarum*. Galacto-oligosaccharides have been reported to inhibit the adhesion of *Cronobacter* to host cells; however, the mechanism by which this effect occurs remains unknown [78]. The mechanism of inhibition in our in vitro fermentation model is also unclear but might be metabolic, for example, through stimulating the proliferation of microorganisms that outcompete them in accessing nutrients such as sialic acid [79].

5. Conclusions

Our study demonstrates that GOS administered alone or in synbiotic combination with the probiotic strains tested herein (*Lactobacillus amylovorus* or *Lactobacillus plantarum*) has the potential to lower emissions of malodorous compounds from swine manure. However, the reduction in ammonia (one of the major contributors to malodor) was not achieved. The results also indicate that such a measure would have the added advantage of promoting the production of beneficial VFAs that are important for swine nutrition and gastrointestinal health. Additionally, this study unraveled shifts in the microbial communities that are associated with the observed effects of GOS and probiotic supplementation.

However, the mammalian gut is a complex environment, and our findings might not be replicable in vivo. But it is also possible that the measured effect in our in vitro model might underestimate the effect of these supplements in vivo. Considering these limitations, in the future, animal feeding studies should be conducted to confirm the effect of this combination of GOS and probiotic strains on malodor emissions.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/fermentation9080716/s1>, Table S1: Feed formula and nutrient content of fermentation substrate (fattening diet) and gestating sow diet that was fed to the sample donating gestating sows; Figure S1: Microbial diversity across the treatment groups at the three time points. Shannon diversity (a) and Evenness (b) among the treatment groups. Pairwise comparisons were performed using Wilcoxon rank sum exact test combined with a correction for multiple hypothesis testing using the Benjamini–Hochberg method at a cut-off of 0.05. All pairwise comparisons were non-significant; Figure S2: Balance tree analysis of the ASVs in the treatments. Dendrogram heatmap (generated using the q2-gneiss plugin in qiime2) illustrating presence of various ASVs across treatments at 0 h (a); 12 h (b) and 24 h (c) of fermentation.

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