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Lactic Acid Bacterial Consortia Modulate Fermentation and Microbial Communities in a Dynamic in Vitro Ruminant Gastrointestinal Model

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Article

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Posted Date: November 11th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11258627/v1

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Additional Declarations: No competing interests reported.

LACTIC ACID BACTERIAL CONSORTIA MODULATE FERMENTATION AND MICROBIAL COMMUNITIES IN A DYNAMIC *IN VITRO* RUMINANT GASTROINTESTINAL MODEL

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Keywords: lactic acid bacteria, dynamic *in vitro* digestive system, maize silage, short-chain fatty acids, branched-chain fatty acids, metagenomics, metabolomics

Abstract

Optimization of microbial fermentation throughout the ruminant gastrointestinal tract is important for efficient nutrient utilization and animal health. The aim of this study was to evaluate the effect of lactic acid bacteria (LAB) consortia applied to a total mixed ration (TMR) and maize silage on microbial community structure and fermentation profiles in a dynamic *in vitro* digestion system of the ruminant gastrointestinal tract. The model was established using fresh cow rumen fluid as the source of the microbial inoculum. Importantly, subsequent metabolite and microbiota analyses were performed on samples collected from reactors representing the respective intestinal compartments of the model, rather than on donor rumen fluid. Bacterial inoculants containing *Lentilactobacillus buchneri, Pediococcus acidilactici, Lentilactobacillus parakefiri, Lentilactobacillus diolivorans* and *Furfurilactobacillus kisonensis* were tested. A control variant receiving non-inoculated feed was compared with an experimental variant supplemented with LAB. Lactic acid, short-chain fatty acids (SCFAs), and branched-chain fatty acids (BCFAs) were quantified in the collected reactor samples. The experimental variant showed higher propionate, valerate, and lactic acid concentrations together with lower BCFA concentrations. Metagenomic and metabolomic analyses indicated that LAB supplementation was associated with distinct temporal dynamics and greater preservation of selected bacterial groups, including *Bacteroidota*, reduced abundance of selected taxa with opportunistic potential, and distinct metabolic profiles associated with the experimental treatment and feeding phase. These findings suggest that the selected LAB consortia were associated with altered microbial community succession and fermentation dynamics during dietary transitions within the in vitro gastrointestinal ecosystem. Further in vivo validation is required to determine whether these effects are reproduced under physiological conditions.

Introduction

Optimizing microbial fermentation and establishing a stable, metabolically active gastrointestinal ecosystem are fundamental to efficient nutrient utilization and productivity in ruminants. During transitions to solid feed, microbial communities must adapt to changing substrates and physicochemical conditions. Short-chain fatty acids (SCFAs) produced by microbial fermentation are major metabolic products of carbohydrate utilization and contribute substantially to energy metabolism in ruminants [1, 2].

In modern ruminant nutrition, total mixed rations (TMRs) containing maize silage are widely used to provide a balanced supply of fermentable starch and structural fiber. However, variation in silage fermentation quality and TMR stability can alter the substrates entering the gastrointestinal tract and consequently affect microbial fermentation. Poorly controlled fermentation may favor proteolytic pathways and increase the formation of ammonia, biogenic amines, and other products of protein degradation, potentially reducing nitrogen-use efficiency and digestive performance [3, 4, 5].

The use of specifically selected lactic acid bacteria (LAB) consortia is a promising strategy for improving feed preservation and modulating subsequent microbial fermentation. Multi-species consortia may act synergistically within TMR and silage matrices and may also influence microbial interactions after the treated feed enters the gastrointestinal tract. Nevertheless, the relationships between LAB-treated feeds, rumen-derived microbial communities, and metabolic responses in downstream gastrointestinal compartments remain incompletely understood [6].

Direct evaluation of these interactions *in vivo* is associated with ethical, financial, and logistical constraints, particularly when repeated sampling from multiple gastrointestinal compartments is required. Dynamic *in vitro* digestion systems offer a controlled and reproducible alternative by reproducing selected features of gastrointestinal passage, pH, anaerobiosis, and microbial fermentation. Such models can be established with mature rumen fluid from donor cows as a diverse microbial inoculum. However, the origin of the inoculum must be distinguished from the site of subsequent analysis: once rumen-derived microorganisms adapt to individual reactors, samples collected from intestinal reactors represent the microbial communities and metabolic activity of those simulated compartments, not the donor rumen itself [7, 8].

Aim of the study

This study aimed to investigate the effects of selected LAB consortia applied to a total mixed ration and maize silage on microbial communities and fermentative metabolites in a dynamic *in vitro* digestive system of the ruminant gastrointestinal tract. Fresh cow rumen fluid was used as the microbial inoculum to establish the model, whereas metabolomic, metagenomic, and fermentation analyses were performed on samples collected from reactors representing the respective intestinal compartments. Particular attention was given to short-chain fatty acids (SCFAs), branched-chain fatty acids (BCFAs), and lactic acid, and to their relationships with changes in microbial community structure and the broader metabolomic profile across successive experimental phases.

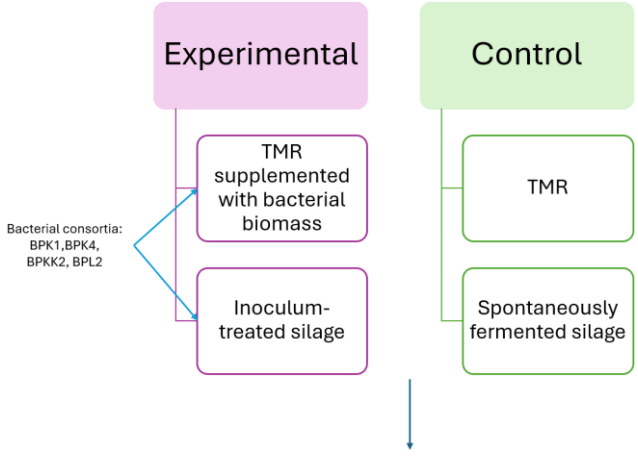
Experimental overview

The study was designed to evaluate whether selected LAB consortia applied to TMR and maize silage could improve the stability of microbial fermentation in a dynamic *in vitro* digestive system. The approach combined physicochemical and microbiological characterization of the feeds with targeted organic-acid measurements, metagenomics, and metabolomics [9].

The first stage of the study involved preparing a lyophilized Total Mixed Ration (TMR) and TMR supplemented with bacterial biomass consisting of specific consortia. The study utilized strain consortia deposited in the Polish Collection of Microorganisms: BPK1 (*Lentilactobacillus buchneri, Pediococcus acidilactici*), BPK4 (*L. buchneri, P. acidilactici, Lentilactobacillus parakefiri*), BPKK2 (*L. buchneri, P. acidilactici*), and BPL2 (*Lactobacillus diolivorans, P. acidilactici, Furfurilactobacillus kisonensis*). Additionally, whole-plant corn silage was prepared at the dough/early-flint maturity stage. One portion was ensiled spontaneously (41% dry matter, DM), while the second was ensiled with the addition of the aforementioned bacterial inoculants (38% DM).

The experiment used a multi-stage dynamic *in vitro* digestion system designed to reproduce selected compartments of the ruminant gastrointestinal tract. Fresh rumen fluid from a donor cow was used to establish a rumen-derived microbial community within the model. Subsequent analytical samples were collected from reactors representing the relevant intestinal compartments. Reactor-specific operating conditions, including temperature, pH, retention time, mixing, and anaerobiosis, were controlled according to the compartment being simulated; the detailed operating conditions are described below (Figure 1) [10].

Fresh rumen fluid collected from a donor cow served as the microbial inoculum for establishment of the model. Over 14 weeks, the system was supplied with model medium during the adaptation period (weeks 1–4), followed by TMR during weeks 5–8 and maize silage during weeks 9–12; model medium was reintroduced during weeks 13–14. Two parallel experiments were conducted: an experimental variant in which selected bacterial consortia were incorporated into the TMR and used during maize ensiling, and a control variant using corresponding TMR and maize silage without bacterial inoculation. Samples were collected weekly from the relevant intestinal reactors to determine selected SCFAs (formic, acetic, propionic, butyric, and valeric acids), BCFAs (isobutyric and isovaleric acids), and lactic acid. Selected reactor samples were also subjected to metagenomic and metabolomic analyses (Table 1) [10].



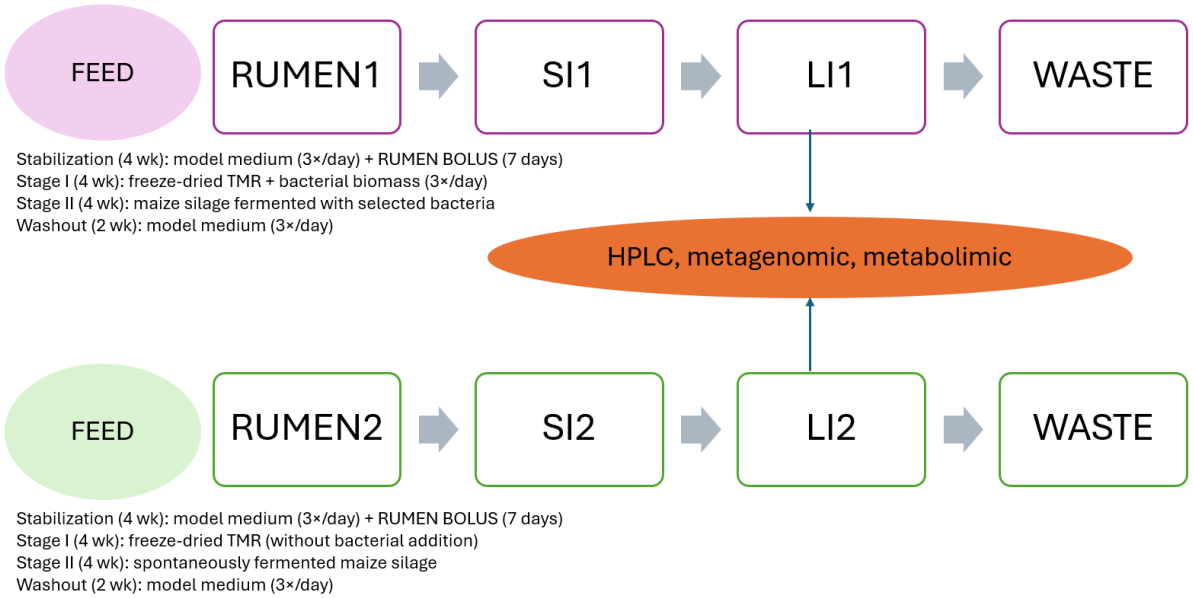


Figure 1. Schematic overview of the second part of the experimental design. RUMEN – rumen-simulating compartment; SI – small-intestine compartment; LI – large-intestine compartment; 1 – experimental trial; 2 – control trial [10].

Table 1. Experimental design, feeding phases, and sample coding for metagenomic and metabolomic analyses

Phase	Sampling week	Feed	Experimental variant	Sample code for metagenomic/metabolomic analyses
Adaptive	2 and4	Model medium and RUMEN BOLUS	Control	UP1C
			Experimental	UP2P
			Control	UP3C
			Experimental	UP4P
TMR testing	6 and 8	TMR	Control	UP5TMR
		LAB-inoculated TMR	Experimental	UP6PTMR
		TMR	Control	UP7TMR
		LAB-inoculated TMR	Experimental	UP8PTMR
Silage testing	10 and 12	Spontaneously fermented maize silage	Control	UP9CS
		LAB-inoculated maize silage	Experimental	UP10PS
		Spontaneously fermented maize silage	Control	UP11CS
		LAB-inoculated maize silage	Experimental	UP12PS
Wash-out	14	Model medium	Control	UP13CT
			Experimental	UP14PT

Materials and methods

Ethical approval and sample collection

The experimental protocol was approved by the Second Local Ethics Committee for Animal Experimentation in Warsaw (Permit No. WAW2/157/2021). All animal procedures were conducted in accordance with the applicable national and institutional guidelines for the care and use of animals for scientific purposes [11].

Rumen fluid was obtained from three healthy, non-lactating, two-year-old Jersey heifers fitted with permanent rumen cannulas and having an average body weight of approximately 350 kg. The animals were maintained at the Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, Jabłonna, Poland. Each heifer received a daily diet consisting of 6.0 kg of meadow hay, 0.8 kg of barley meal, 0.2 kg of soybean meal, and 0.04 kg of a mineral–vitamin premix. The animals were fed twice daily and had unrestricted access to drinking water and salt licks.

Rumen fluid was collected through the cannulas before the morning feeding from different regions of the rumen. Equal volumes were obtained from each donor animal. The fluid collected from each heifer was mixed separately to ensure sample homogeneity and then filtered through four layers of sterile surgical gauze under continuous CO_2 flushing to maintain anaerobic conditions. Equal volumes of the filtered rumen fluid from the three donor animals were subsequently pooled to reduce individual-animal variation and obtain a composite microbial inoculum.

The pooled rumen fluid was immediately transferred to pre-warmed, CO_2-flushed airtight containers and transported to the laboratory at approximately 39°C. The freshly prepared pooled rumen fluid was used as the microbial inoculum for establishment of the dynamic in vitro digestive system; samples used for subsequent metabolomic, metagenomic, and fermentation analyses were obtained from the relevant model reactors.

Biological material

- Maize biomass (*Zea mays*) with a dry matter content of 35%, total protein content of 8.98 ± 0.025%, and crude fiber content of 17.45 ± 0.015% was used. The maize biomass was obtained from the Michał Glonek Agricultural Farm, Michałów 2, Rokiciny, Poland.
- The dry TMR consisted of 26% soybean meal, 21% maize meal, 13% molasses, 10% alfalfa hay, 10% triticale, 10% wheat, and 10% mineral-vitamin premix.
- The study utilized four bacterial consortia, which are the property of the Department of Environmental Biotechnology at the Lodz University of Technology: BPK1 (*Lentilactobacillus buchneri, Pediococcus acidilactici*), BPK4 (*L. buchneri, P. acidilactici, L. parakefiri*), BPKK2 (*L. buchneri, P. acidilactici*), and BPL2 (*Lentilactobacillus diolivorans, P. acidilactici, Furfurilactobacillus kisonensis*). These consortia exhibited no antagonistic interactions with one another. The bacterial strains were originally isolated from spontaneously fermented maize and lupine silages using MRS medium (de Man, Rogosa, and Sharpe), which supports the growth of lactic acid bacteria [12]. After spontaneous fermentation, silage samples were diluted and cultured under anaerobic conditions. The detailed compositions and functional properties of the consortia have been disclosed in filings to the Polish Patent Office under the following application numbers: BPK1–P.

452894, BPK4–P. 452900, BPKK2–P. 452895, and BPL2–P. 452896. These consortia are also components of the invention entitled “Probiotic Feed Preparation and Its Use,” filed with the Polish Patent Office (UPRP) on 26 August 2025, under application number P. 453014 [13].

Reagents and growth media

- MRS broth, selective for LAB, was used for the cultivation of bacteria, with the following composition (g/liter): enzymatic digest of casein 10.0; meat extract 8.0; yeast extract 4.0; D(+)Glucose 20.0; di-Kaliumhydrogen-phosphate 2.0; Tween ®80 1.0; di-Ammonium hydrogen citrate 2.0; sodium acetate 5.0; magnesium sulfate heptahydrate 0.2; manganese sulfate Monohydrate 0.04 (Merck, Darmstadt, Germany). The bacteria were cultured at a temperature of 30 ± 1°C for 48 h under anaerobic conditions using an AnaeroGen Sachet (Oxoid™ AnaeroGen™ 2.5L Sachet, Thermo Scientific™).
- Digestive system model medium: The basic model medium was prepared by mixing and sterilizing the following ingredients (in grams per liter): arabinogalactan (1.0 g), pectin (2.0 g), xylan (1.0 g), starch (4.0 g), glucose (0.4 g), yeast extract (3.0 g), peptone (3.0 g), mucin (1.0 g), cysteine (0.5 g), and distilled water [13].
- Pancreatic juice: The composition of pancreatic juice included (in grams per liter): NaHCO3 (12.5 g), bile salts (6.0 g), and pancreatin (0.9 g) [14].

Preparation of freeze-dried TMR with bacterial biomass

The preparation of the bacterial inoculum and the inoculation of the experimental diets were conducted in a multi-stage process, utilizing a portion of the Total Mixed Ration (TMR) as a protective matrix during freeze-drying [15, 16]. First, four selected lactic acid bacteria (LAB) consortia were multiplied individually. Each consortium was cultured in liquid MRS medium under anaerobic conditions at 30 ± 1^{0}C for 24 h to reach the logarithmic growth phase, adjusting the initial bacterial concentration of each individual liquid culture to 10^4 CFU/mL. To prepare the protected solid inoculum, the liquid bacterial cell suspensions of each consortium were centrifuged (4000 rpm for 10 min) to separate the supernatant. The obtained cell pellets were washed with sterile physiological saline solution (0.85% NaCl). Following the removal of the water phase, the isolated bacterial biomass from the four consortia was combined in equal proportions (25% share each) and blended with an appropriate amount of fresh TMR, which served as a protective carrier to enhance bacterial survival during thermal and vacuum stress. The mixture was thoroughly homogenized and subjected to freeze-drying using a Labconco™ Triad freeze dryer (model 794001030) equipped with an nXDS Edwards vacuum pump. The process was conducted in manual mode, with an initial freezing temperature set to -15^{0}C. After collector stabilization, the frozen samples were placed under a pressure of 0.15 Pa at a temperature of -10^{0}C for 24 hours. This was followed by a secondary drying step a 30^{0}C for another 24 hours to minimize moisture absorption from the environment. Due to water sublimation and biomass concentration, the final lactic acid bacteria count in this lyophilized TMR-protective preparation reached approximately 10^9 CFU/g of dry product. This concentrated, freeze-dried preparation was subsequently used to inoculate the final experimental diets (the main TMR matrix and the maize silage) based on fresh matter (FM). The final target inoculation dose was fixed at 10^6 CFU per gram of fresh matter (10^6 CFU/g FM) for both experimental diets.

To determine bacterial viability and cell recovery after the freeze-drying process, the lyophilized preparation was subjected to three successive subcultures (passages) in liquid MRS broth, followed by serial decimal dilutions and pour-plating on MRS agar. Plates were incubated under anaerobic conditions at 30±1^{0}C for 48 h to enumerate viable colony-forming units.

Silage preparation

The ensiling process was carried out under strictly controlled laboratory conditions. The maize crop was harvested at the dough-to-dent stage and chopped using a commercial self-propelled forage harvester to a theoretical length of cut (TLC) of approximately 10–15 mm. To determine the precise volume of the liquid-reconstituted lyophilized inoculum required, the dry matter (DM) content of the fresh chopped maize was determined beforehand. The reconstituted multi-strain LAB inoculant was applied at a ratio of 5% (v/w) and meticulously sprayed onto the maize biomass to ensure an even and homogeneous distribution of the bacteria, achieving the target concentration of 10^6 CFU/g of fresh matter.

For the fermentation process, the inoculated maize biomass was divided into replicates, with each experimental vacuum bag (polyethylene/polyamide, high-barrier bags) packed with exactly 2.0 kg of the fresh material. To ensure strict anaerobic conditions conducive to lactic acid fermentation and to completely eliminate oxygen, the packed bags were sealed using a professional chamber vacuum packaging machine. A total of 10 replicate bags were prepared for the experimental variant. The ensiling process lasted for eight weeks (56 days) and was conducted in a temperature-controlled environment maintained at 21 – 23^{0}C [14]. At designated sampling intervals and upon completion of the 8-week fermentation period, individual bags were opened via a destructive sampling method (where specific bags were sacrificed at each time point) to obtain representative silage samples for subsequent in vitro digestion analyses.

Physicochemical and microbiological analyses

- Microbial abundance was determined by serial tenfold dilutions of 1 g silage in 99 mL of 0.85% NaCl, followed by deep seeding and incubation under anaerobic conditions at 30 ± 1 °C for 48 h [13].
- Dry matter content was determined by drying approximately 1 g of each sample at 130°C for 3 h. Protein content was analyzed using the Kjeldahl method as described by Dygas and Berłowska (2023). Crude fiber was determined with a FOSS Fibertec™ 8000 system (Hilleroed, Denmark) according to the same reference. The pH was measured potentiometrically (METTLER TOLEDO Five Easy Plus FP20), and total acidity was determined by titration with 0.1 M NaOH to pH 9 using an automatic titrator (TitroLine 500, SI Analytics) [14].

Dynamic *in vitro* digestion system

A dynamic *in vitro* digestion system based on consecutive glass fermenters (Prodigest, Ghent, Belgium) was used to simulate selected compartments of the ruminant gastrointestinal tract. The system included a rumen-simulating stage and downstream intestinal reactors. Fresh rumen content from a donor cow was used to establish the microbial community and therefore served as the source inoculum rather than as the analytical sample. The reactors were maintained at 37 °C in a thermostatic water bath, continuously stirred, and flushed with nitrogen every 24 h

to maintain anaerobiosis. In the intestinal fermentation module, pH was automatically regulated using HCl or NaOH within the ranges of 6.20–6.80, 3.60–4.00, and 5.80–6.20 for the rumen, small-intestine, and large-intestine compartments, respectively.

The experimental design comprised a two-week stabilization period, an eight-week treatment period, and a two-week wash-out phase. During adaptation, the reactors were supplied three times daily with model medium containing arabinogalactan, pectin, xylan, starch, glucose, yeast extract, peptone, mucin, and cysteine [17]. RUMEN BOLUS (Topro Animal Health) was administered during the first seven days to support establishment of the rumen-derived microbial community within the model.

After stabilization, the experimental system was supplied three times daily with freeze-dried TMR enriched with bacterial biomass for four weeks, followed by four weeks of feeding with maize silage fermented using the selected LAB consortia. Model medium was then reintroduced for two weeks. A parallel control experiment was conducted under the same conditions using freeze-dried TMR without bacterial supplementation and spontaneously fermented maize silage. Artificial pancreatic juice containing $NaHCO_3$, Oxgall, and pancreatin was used to reproduce the relevant digestive conditions. Samples for SCFA, BCFA, lactic acid, metabolomic, and metagenomic analyses were collected from the appropriate intestinal reactors according to the experimental sampling scheme.

Determination of SCFAs, BCFAs, and lactic acid

The concentrations of short-chain fatty acids (SCFAs: formic, acetic, propionic, butyric, and valeric acids), branched-chain fatty acids (BCFAs: isobutyric and isovaleric acids), and lactic acid were determined using high-performance liquid chromatography (HPLC). Samples were collected from the relevant intestinal reactors of the dynamic *in vitro* digestion system. Prior to analysis, samples were deproteinized using the Carrez method. Briefly, samples were centrifuged (4000 rpm, 10 min), and 3.5 mL of supernatant was mixed with 0.2 mL of Carrez I solution. After 2 min, 0.2 mL of Carrez II solution was added, followed by 3.1 mL of distilled water. After a second centrifugation (4000 rpm, 10 min), the supernatant was filtered through a 0.22 µm PTFE syringe filter. Chromatographic separation was performed using an HPLC system (Shimadzu, Japan) equipped with a photodiode array (PDA) detector and a Repromer H column (300 × 8 mm, 9 µm particle size; Dr. Maisch GmbH, Germany). The mobile phase consisted of 5 mM sulfuric acid delivered isocratically at a flow rate of 0.5 mL/min. The column temperature was maintained at 60 °C, the injection volume was 20 µL, and the total run time was 60 min [18].

Quantification was based on external standard calibration curves. Linearity for the identified metabolites was confirmed from regression equations (e.g., for lactic acid: [missing equation], [missing coefficient]). The limits of detection (LOD) and quantification (LOQ) were determined at signal-to-noise ratios of 3:1 and 10:1, respectively. Method accuracy was assessed using recovery tests (target range 95–105%), and repeatability was evaluated by calculating the relative standard deviation (RSD) from replicate injections). Final concentrations were expressed in mg/mL [18].

Metabolomic analysis

- Samples for metabolomic analysis were collected weekly from the relevant intestinal reactors of the dynamic in vitro digestion system. The microbial community in the model originated from cow rumen fluid used as the initial inoculum, whereas the metabolomic profiles reported here reflect metabolic activity within the intestinal compartments from which the samples were collected.
- Prior to UHPLC-UHRMS analysis, samples were diluted with LC-MS-grade acetonitrile (1:3, v/v) to precipitate proteins, centrifuged again, and the final supernatant was transferred to autosampler vials. The procedure followed the optimized protocol described by Liszkowska-Walisiak et al. [18].
- Preparation of extracts for LCMS. Acetone (900 µL) was added to the reactor effluent (300 µL) and the mixture was shaken on a vortex shaker for 1 minute. Then, sample was incubated at -20 °C for 60 minutes, centrifuged for 5 min (4 °C, 12000xg) and supernatant was transferred to previously weighed Eppendorf vial. Content of vial was dried evaporated under vacuum using a speed vac (2 mbar) for approximately 15 hours. The dry extract was weighed and then dissolved methanol (0.13 mg per 80 µl of methanol). The dissolved extract was centrifuged (4 °C, 12000xg, 5 min) and transferred to HPLC vial.
- UHPLC-QToF-UHRMS and MS/MS analysis of extracts. Mass spectrometry-liquid chromatography analyses were performed on a Bruker Elute UHPLC system operated by Hystar 3.3 software and an ultrahigh resolution (60000+) mass spectrometer Bruker Impact II (Bruker Daltonik GmbH) ESI QTOF-MS equipped with Data Analysis 4.2 (Bruker Daltonik GmbH), TASQ (2022b) and Metaboscape (2022b). The column used for AutoMSMS measurements was a reverse phase Bruker Intensity Solo C18 with 2-µm particles and dimensions of 2.1×50 mm. Eluent A was water with 0.1% HCOOH (Sigma-Aldrich, USA) and eluent B was acetonitrile (Sigma-Aldrich, USA) with 0.1% HCOOH. Volume of 5 µL of extract was loaded on the column at a flow rate of 200 µLmin-1, using 4% B. Eluent B percentage was changed with time as follows: 0 min – 1%, 0.56 min - 1% B, 4.72 min - 99%, 5.56 min - 99%, 5.6 min – 1%, 9.45 min – 1%. Solvent flow was 450 µLmin-1. The column was held at 40 °C. The column exit was connected to the ESI source. AutoMSMS measurements were conducted in both positive and negative ion modes in the m/z range 50–1200. Collision-Induced Dissociation (CID) was performed with the following settings: an absolute area threshold of 5000 counts, active exclusion after 2 spectra, and release after 0.3 minutes. The isolation mass settings were as follows: for m/z = 100, the width was 4; for m/z = 300, the width was 5; for m/z = 500, the width was 6; and for m/z = 1000, the width was 8. The collision energy was set to 30 eV. Internal calibration on 10 mM sodium formate (Sigma-Aldrich, USA; in 1 : 1 = water : isopropanol V/V) ions was performed automatically in Metaboscape using a syringe pump at an infusion flow rate of 1.5 µL/min, in high precision calibration (HPC) mode. The untargeted annotations were performed in Metaboscape (ver. 2022) with a criterion of mass deviation (Δm/z) under 2 ppm and mSigma value under 20 as the maximum acceptable deviations of the mass of the compound and the isotopic pattern, respectively [19, 20, 21]. All the molecular formulas were obtained using the Smart Formula tool and the C, H, N, O, P, S, Cl, Br, I and F elements. MSMS spectra were automatically matched against MSMS libraries: the Bruker HMDB 2.0 library (spectral data with retention times), MassBank of North America (MoNA) library (MassBank of North America, 2022), and NIST ver. 2020 MSMS library (Mass Spectrometry Data Center, 2022).

- Statistical analysis. All metabolite datasets exported using Metaboscape ver. 2022b were examined utilizing MetaboAnalyst 6.0 [22]. Prior to statistical evaluation, the data were log-transformed and auto-scaled. Unsupervised multivariate analysis was performed using Principal Component Analysis (PCA) to assess overall clustering patterns and global differences between samples. Enrichment analysis was conducted in MetaboAnalyst 6.0 utilizing the Small Molecule Pathway Database (SMPD) to identify the major chemical structures of chemicals that most distinguished both groups. To identify metabolites differentiating the two experimental groups: gut microbiota samples from models maintained on a diet with the fermented preparation and gut microbiota samples from models maintained on a diet with the non-fermented preparation: fold changes (FC) were calculated at each time point as the ratio of metabolite intensities in the fermented-preparation group relative to the non-fermented-preparation group. Metabolites with FC > 1.5 (higher in the fermented-preparation group) or FC < 0.67 (higher in the non-fermented-preparation group) and meeting the criteria of p-value < 0.05 (unpaired two-sample t-test) and false discovery rate (FDR) < 0.05 (Benjamini–Hochberg correction) were considered significantly altered.

DNA isolation and metagenome sequencing

DNA was isolated from the samples using the TGuide S96 Magnetic Universal DNA Kit (Tiangen Biotech Co., Ltd., Beijing, China) on the TGuide S96 Automated Nucleic Acid Extractor by weSEQ.IT company (Rybnik, Poland). The homogenization was performed using TGrinder H24 Tissue Homogenizer (Tiangen Biotech Co., Ltd., Beijing, China). DNA concentration and purity were evaluated using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) and a QubitTM3 Fluorometer (Invitrogen). Sequencing libraries were subsequently constructed using the VAHTS Universal Plus DNA Library Prep Kit for Illumina V4 (Vazyme Biotech Co., Ltd., Nanjing, China) according to the manufacturer's protocol. Briefly, genomic DNA was subjected to enzymatic fragmentation, end-repair, and A-tailing using the FEA Enzyme Mix. Adapter ligation was performed using Rapid DNA Ligase in Rapid Ligation Buffer 3. The post-ligation products were purified with VAHTS DNA Clean Beads (Vazyme). Finally, library enrichment was carried out via PCR amplification using VAHTS HiFi Amplification Mix and PCR Primer Mix 3, followed by a final purification step.

The libraries were sequenced on the Illumina NovaSeq X Plus platform (Illumina, San Diego, CA) using the NovaSeq X Series 25 B Rgt Kit. An average of 18.4 M ± 0.9 M (mean + standard deviation) pairs of 150 bp reads were generated per library. The raw reads were deposited in the NCBI database under the BioProject accession: PRJNA1470393 and BioSample accessions: SAMN60411663–SAMN60411676.

Metagenomic assembly, binning and MAG reconstruction

Metagenome-assembled genomes (MAGs) were recovered from shotgun metagenomic sequencing data using the metaWRAP pipeline (v. 1.3.2; [23]). Raw sequencing reads were quality-controlled using the metaWRAP Read_QC module, including quality and adapter trimming with Trim Galore! (v. 0.5.0; [24]) with Cutadapt (v. 1.18; [25]), and removal of human-derived reads using BMTagger against the human hg38 reference genome [26]. FastQC [14] was used to assess read quality before and after quality control. MultiQC (v. 1.35; [28]) was used to generate summary reports from the results of FastQC analyses.

Reads from all samples were co-assembled using metaSPAdes (SPAdes v. 3.13.0; [29]), and the resulting assembly quality was assessed using QUAST (v. 4.1; [30]). The resulting contigs were binned using three independent binning algorithms: metaBAT 2 (v. 2.12.1; [31]), MaxBin 2.0 (v. 2.2.6; [32]) and CONCOCT (v. 1.0.0; [33]). The resulting bin sets were consolidated and refined using the metaWRAP Bin_refinement module with a minimum completeness of 70% and maximum contamination of 5% (-c 70 -x 5). The refined bins were subsequently reassembled using the metaWRAP Reassemble_bins module using the same thresholds (-c 70 -x 5). The quality of the resulting MAGs was assessed using CheckM [34]. MAGs with 70% completeness and 5% contamination were retained for subsequent analyses. A total of 91 MAGs meeting these criteria were recovered.

The MAGs were taxonomically classified using GTDB-Tk (v.2.5.2) against the Genome Taxonomy Database release 226 (GTDB r226) [25] using default parameters on the Galaxy Europe platform [36].

The abundance of the MAGs across individual metagenomic sequencing libraries was estimated using CoverM (v. 0.4.0) [37]. Paired-end sequencing reads from each library were mapped to the reassembled MAGs using the coverm genome command. The minimum covered fraction threshold was set to 0.

Processing of MAGs information was done using custom-made scripts written in Perl v. 5.26.2 or Python v. 3.7.9. The majority of calculations mentioned in this paper ware performed using the BlueOcean computational cluster which is part of TUL Computing & Information Services Centre infrastructure.

Experimental design, replication, and statistical analysis

To ensure the reliability and reproducibility of the results at both biological and technical levels, the study was designed with a distinction between biological, technical, and analytical replicates:

- Silage Preparation (technical/biological replicates of feed): The ensiling process for both spontaneously fermented and LAB-inoculated maize silages was performed in n=10 independent replicate vacuum bags (2.0 kg of fresh material each) stored under identical, temperature-controlled conditions. Analytical measurements on silage samples (such as pH, dry matter, and total acidity) were performed in triplicate (n=3 analytical replicates) for each opened bag.
- Dynamic *in vitro* digestion system (reactor-level replication): The dynamic *in vitro* digestion system was operated using two parallel, completely independent experimental lines running simultaneously (Figure 1):
    - A control line (fed with conventional TMR and spontaneously fermented maize silage).
    - An experimental line (fed with TMR and maize silage supplemented with the selected LAB consortia). Each line consisted of a sequence of dedicated independent bioreactors representing the rumen (RUMEN1/RUMEN2), small intestine (SI1/SI2), and large intestine (LI1/LI2).

- Sampling Strategy and Statistical n for ANOVA: Samples for high-performance liquid chromatography (HPLC) metabolite quantification (SCFAs, BCFAs, and lactic acid), metagenomic sequencing, and metabolomic profiling were collected weekly from the respective intestinal reactors (specifically the large-intestine compartments, LI1 and LI2).
    - To account for temporal dependency resulting from repeated weekly sampling within the same continuous reactor lines, data from each distinct feeding phase (adaptation: weeks 2 and 4; TMR testing: weeks 6 and 8; silage testing: weeks 10 and 12; wash-out: week 14) were evaluated using a Repeated Measures ANOVA or by calculating phase-integrated mean values representing n=3 independent analytical batches per phase.
    - Where one-way or factorial ANOVA was applied, the number of independent observations per variant represented analytical determinations performed on weekly pooled reactor effluent samples, with the effective sample size clearly designated as n=3 independent time-points/replicates per experimental phase.
- Statistical Testing: All statistical analyses were performed using Statistica v.14.0.1 (TIBCO Software Inc., Palo Alto, CA, USA). Significant differences between the control and experimental variants within corresponding experimental weeks or phases were determined using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test, with statistical significance set at $p<0.05$. Data are expressed as mean ± standard deviation (SD) derived from independent analytical determinations.

Results

Physicochemical and microbiological characteristics of spontaneously fermented and LAB-inoculated maize silage

The microbiological and physicochemical characteristics of fresh maize, TMR, spontaneously fermented maize silage, and LAB-inoculated maize silage are presented in Table 2.

Table 2. Physicochemical and microbiological parameters of fresh maize and maize silages obtained by spontaneous and LAB-inoculated fermentation

Sample	Parameters					
	LAB [CFU/g]	**pH**	**Dry matter [%]**	**Protein content [%]**	**Crude fiber content [%]**	**Total acidity [%]**
TMR	3.5×10^5	6.20 ± 0.022*	88.50 ± 0.210	18.40 ± 0.120	9.80 ± 0.045*	1.19 ± 0.023*
Fresh maize	1.2×10^6	6.43 ± 0.034*	35.00 ± 0.002*	8.98 ± 0.045*	17.45 ± 0.015*	0.30 ± 0.044*
Spontaneously fermented maize silage	4.7×10^8	4.01 ± 0.002*	41.00 ± 0.032*	7.54 ± 0.057	7.23 ± 0.033*	6.86 ± 0.004*
LAB-inoculated maize silage	6.7×10^9	3.90 ± 0.011*	38.00 ± 0.005*	7.12 ± 0.003*	7.20 ± 0.086	6.12 ± 0.053

*$p<0.05$

LAB counts increased significantly ($p < 0.05$) from 1.2×10^6 CFU/g in fresh maize to 4.7×10^8 CFU/g after spontaneous fermentation and 6.7×10^9 CFU/g after LAB-inoculated fermentation. The higher LAB abundance in the inoculated variant indicates greater growth of

lactic acid bacteria under the applied fermentation conditions. Fermentation reduced pH to 4.01 in the spontaneously fermented silage and 3.90 in the LAB-inoculated silage and was accompanied by total acidity values of 6.86% and 6.12%, respectively. Dry matter content was 41% after spontaneous fermentation and 38% after LAB-inoculated fermentation. Protein content decreased relative to fresh maize (8.98%), reaching 7.54% and 7.12%, respectively. Crude fiber content varied only slightly (7.20–7.45%), indicating limited changes in this parameter during fermentation.

Effects of LAB supplementation on fermentation metabolites in the dynamic *in vitro* digestive system

Short-chain fatty acid profile in the large-intestine compartment

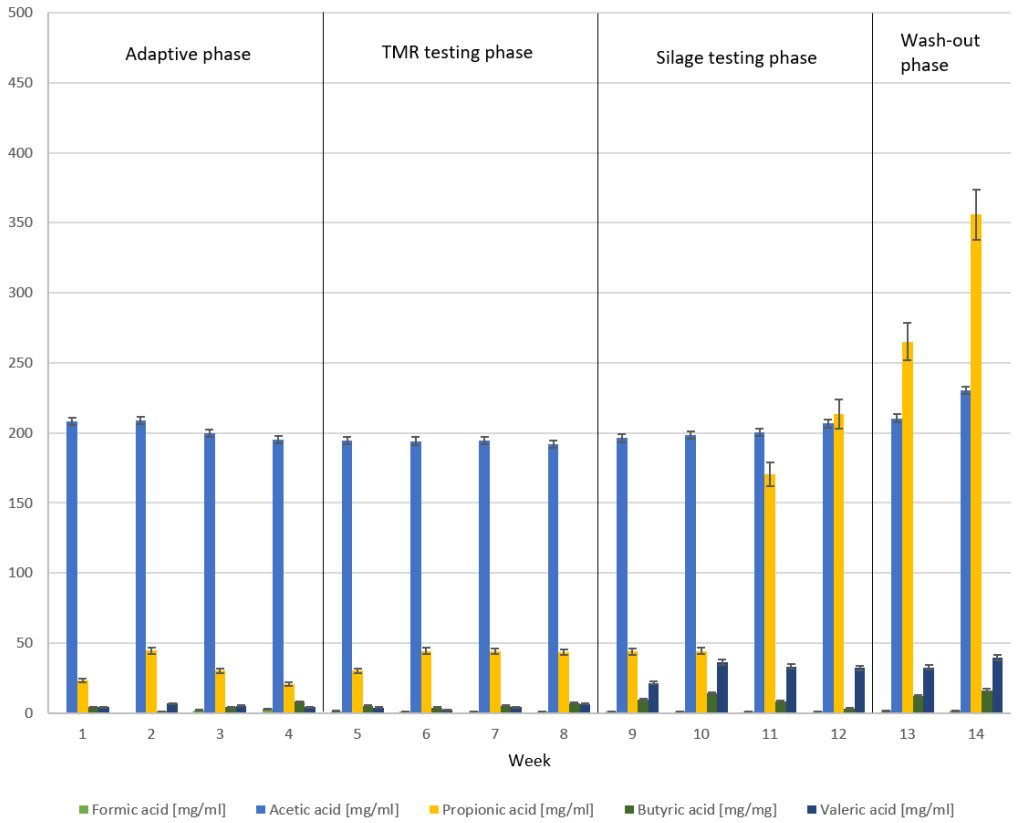
Analysis of short-chain fatty acids (SCFAs) in samples collected from the large-intestine compartment of the dynamic *in vitro* digestion system revealed clear differences between the control and experimental variants, particularly in propionate, butyrate, and valerate concentrations (Figure 2a,b). Acetate remained the dominant SCFA in both systems, with cumulative concentrations (calculated as the sum of concentrations across all sampling weeks) of 3038.000 ± 142.000 mg/mL in the control and 3085.000 ± 128.000 mg/mL in the experimental variant. The comparable acetate concentrations indicate that the principal acetate-producing fermentation pathways remained active in both variants.

Propionate reached a cumulative concentration of 1382.000 ± 78.000 mg/mL in the control and 1895.000 ± 92.000 mg/mL in the experimental variant. The higher propionate concentration coincided with greater stability of *Bacteroidota* in the corresponding microbial profiles, including members of *Prevotellaceae*. This association is consistent with a treatment-related shift in carbohydrate fermentation, although the *in vitro* data do not establish a direct causal contribution of individual taxa.

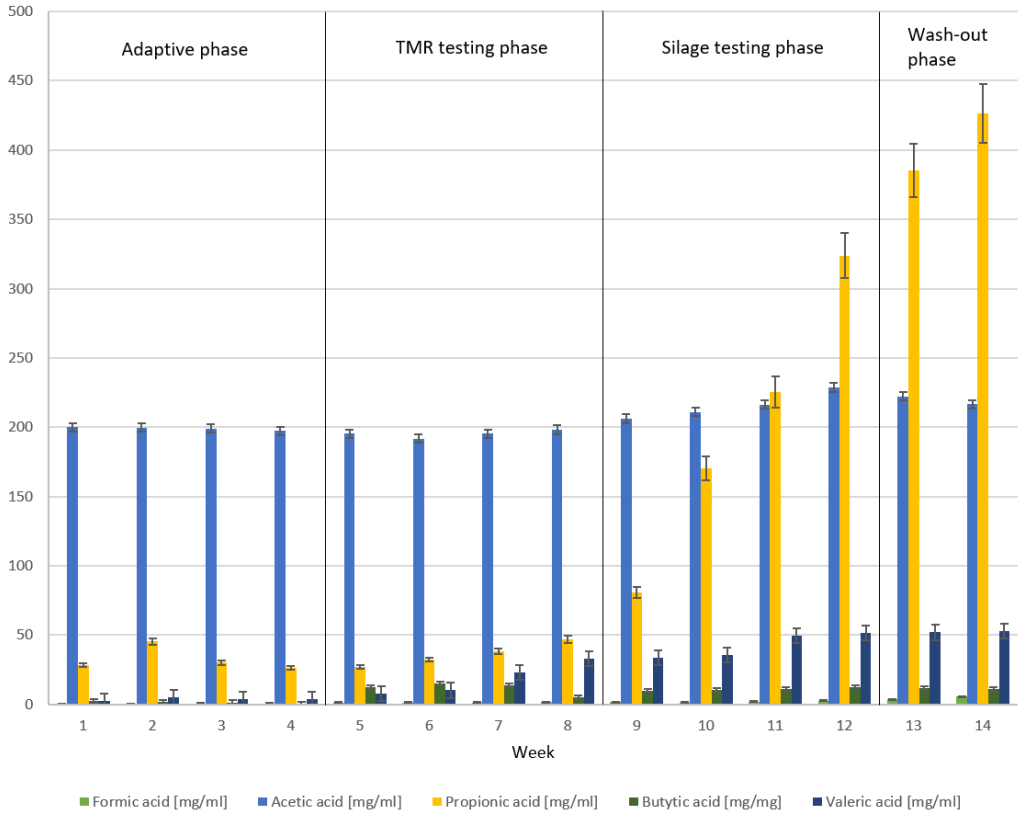
Butyrate showed temporal variability, with cumulative concentrations of 113.000 ± 14.000 mg/mL in the control and 129.000 ± 12..000 mg/mL in the experimental variant. The higher value in the experimental system coincided with a more balanced *Bacillota* community that included *Lachnospiraceae* alongside the supplemented LAB.

Valerate also differed between variants, reaching 236.000 ± 22.000 mg/mL in the control and 367.000 ± 31.000 mg/mL in the experimental system. Because valerate may arise through several microbial metabolic routes, this difference should be interpreted together with the BCFA profile and the observed changes in microbial community composition rather than as a standalone indicator of a specific fermentation pathway.

Formate concentrations were 18.400 ± 1.900 mg/mL in the control and 24.900 ± 2.100 mg/mL in the experimental variant. Overall, LAB supplementation was associated with a distinct SCFA profile in the analyzed large-intestine compartment. These concentrations describe fermentation within the sampled reactor and should not be interpreted as direct measurements of ruminal SCFAs in the donor animal.



488 a)



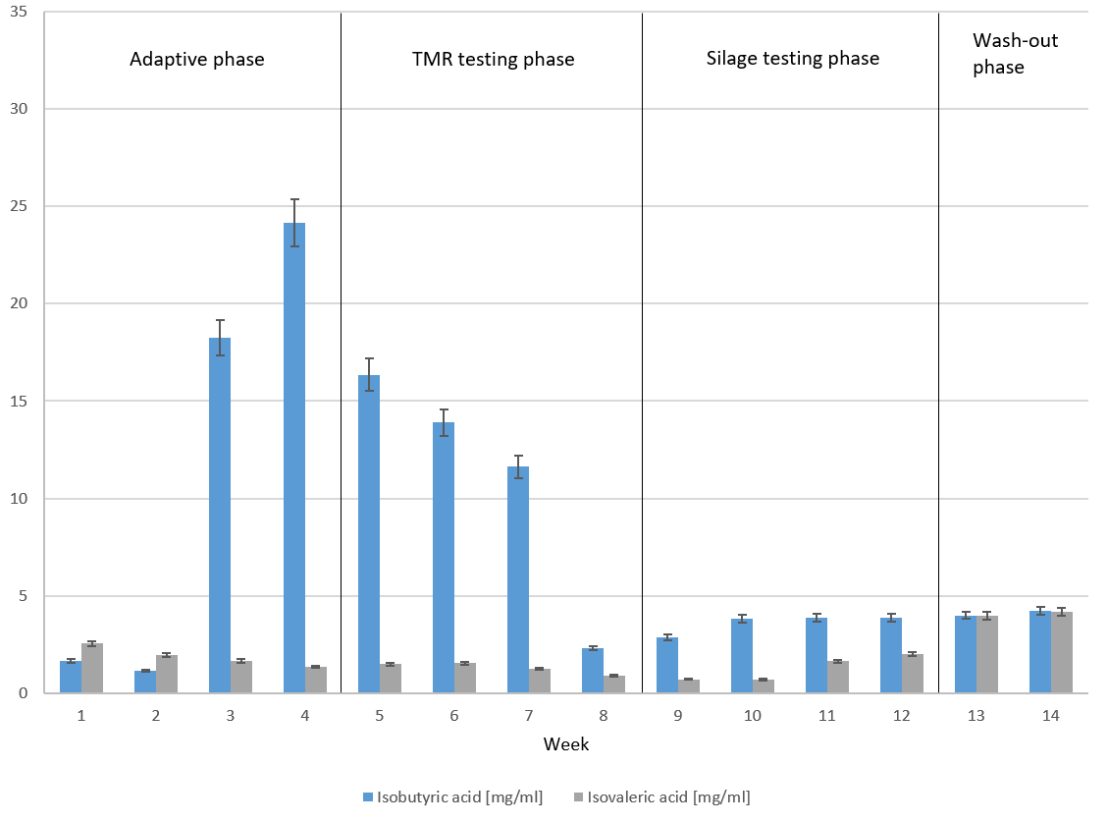
489 b)

490 Figure 2. Short-chain fatty acid profile in samples collected from the large-intestine compartment
491 of the dynamic *in vitro* digestive system: (a) control variant; (b) experimental variant.

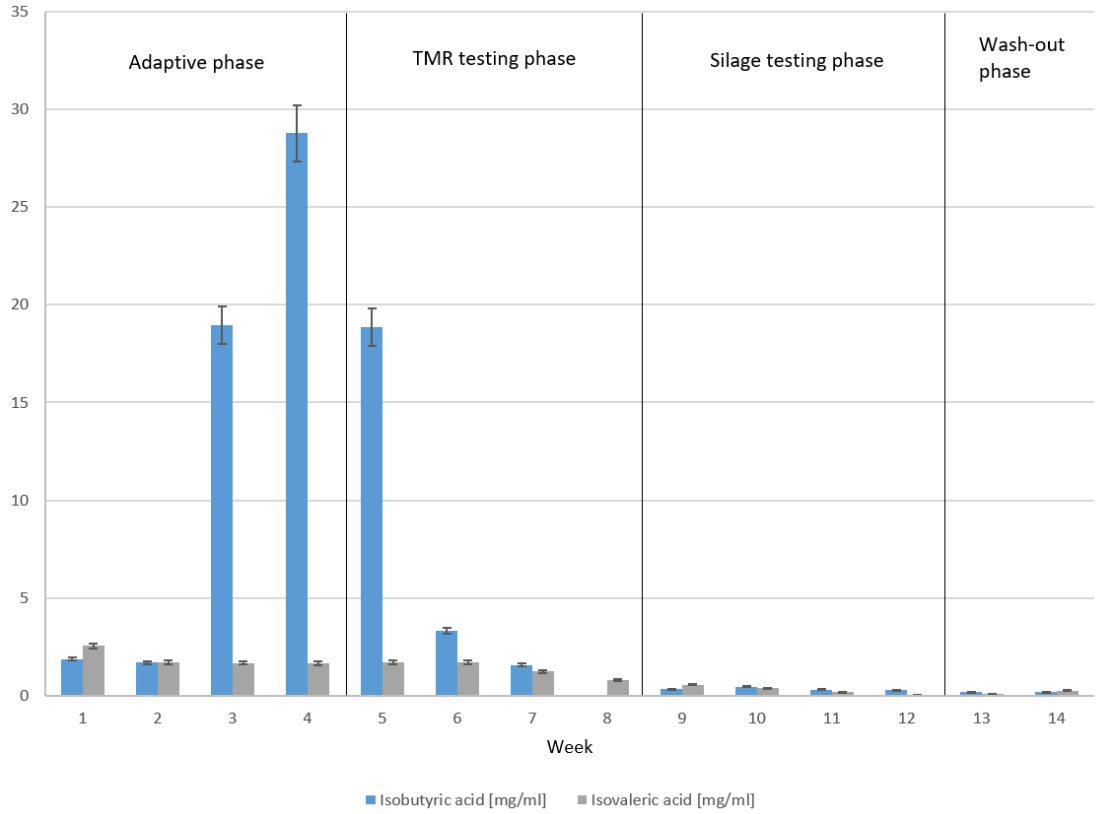
Branched-chain fatty acid profile in the large-intestine compartment

The branched-chain fatty acid (BCFA) profile in samples collected from the large-intestine compartment differed clearly between the control and experimental variants (Figure 3a,b). Isobutyric and isovaleric acids were consistently lower in the experimental system, suggesting a reduced contribution of branched-chain amino acid fermentation. The cumulative concentration of isobutyric acid was 114.090 ± 8.400 mg/mL in the control and 78.850 ± 5.200 mg/mL in the experimental variant ($p = 0.012$). The greatest divergence occurred between weeks 3 and 7. Isovaleric acid showed a similar pattern, with cumulative concentrations of 29.680 ± 2.800 mg/mL in the control and 18.440 ± 1.600 mg/mL in the experimental variant ($p = 0.0240$), with the largest differences observed from week 7 onward.

The lower BCFA concentrations in the experimental variant are consistent with a shift away from amino-acid-associated proteolytic fermentation toward greater carbohydrate utilization. This metabolic pattern coincided with greater stability of *Bacteroidota* and lower abundance of selected taxa, including *Fusobacteriota*, in the corresponding reactor samples. The association should be interpreted within the context of the *in vitro* intestinal compartment and does not by itself demonstrate altered protein metabolism *in vivo*.



a)



b)

Figure 3. Branched-chain fatty acid profile in samples collected from the large-intestine compartment of the dynamic *in vitro* gastrointestinal model: (a) control variant; (b) experimental variant.

Lactic acid dynamics in the large-intestine compartment

The lactic acid profile differed clearly between the variants. Cumulative concentrations were higher in the experimental system (947.850 ± 62.400 mg/mL) than in the control (605.870 ± 48.200 mg/mL). Initial values were comparable, but lactic acid increased systematically in the experimental system from week 5 onward. Importantly, the higher lactic acid concentration was not accompanied by uncontrolled acidification of the analyzed model compartment, which is compatible with changes in lactate production and/or utilization within the microbial network. The experimental variant maintained *Lachnospiraceae* and other taxa with potential lactate-utilizing capacity in the corresponding microbial profiles.

The increase in lactic acid occurred in parallel with higher propionate concentrations and lower BCFA concentrations, supporting a treatment-related shift in fermentation pattern toward greater carbohydrate utilization. The data are consistent with microbial cross-feeding between lactate producers and consumers within the *in vitro* system. However, because the measurements were obtained from the large-intestine reactor, they should not be used to infer protection from or risk of acute rumen acidosis *in vivo*.

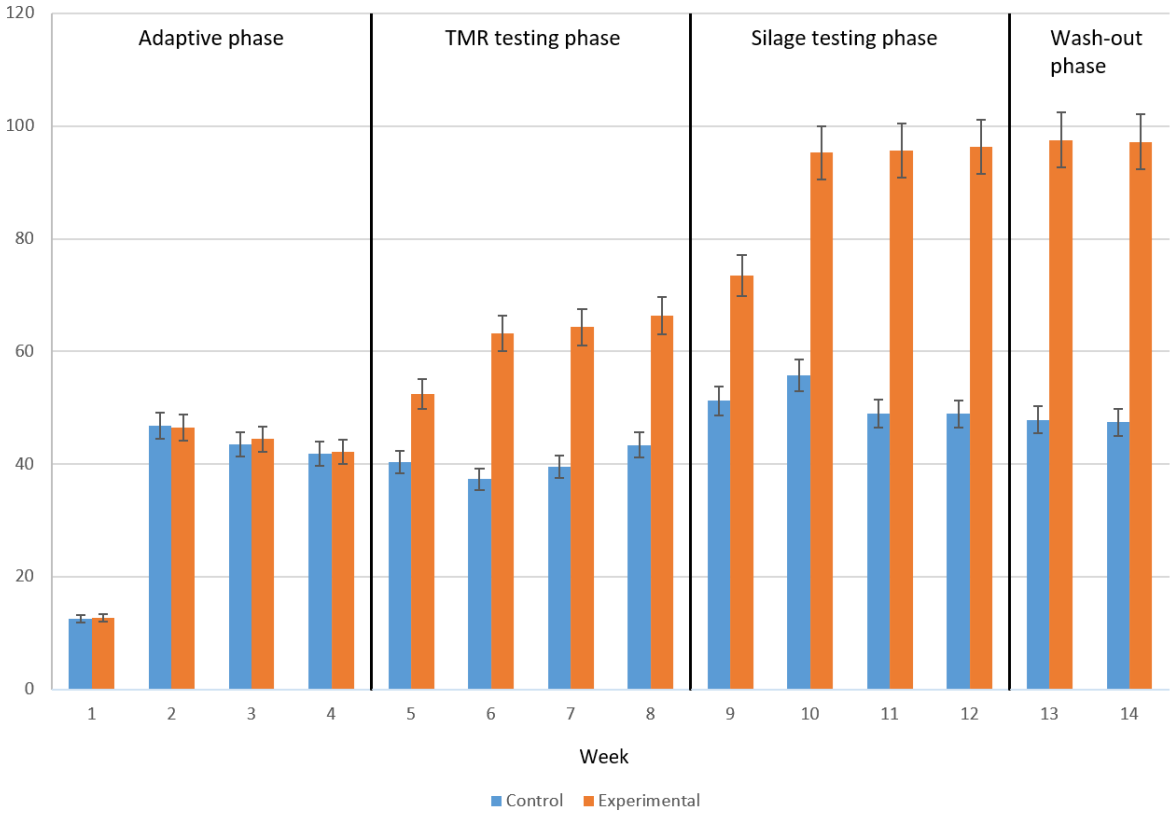


Figure 4. Lactic acid concentration in samples collected from the large-intestine compartment of the dynamic *in vitro* gastrointestinal model.

Effects of LAB supplementation on microbial community structure in the dynamic *in vitro* digestive system

Taxonomic profiles at the phylum level revealed substantial changes in microbial community structure across the experimental phases. The model was established using cow rumen fluid as the source inoculum; therefore, the initial community reflected the rumen origin of the microorganisms. However, the profiles reported during the experiment were generated from samples collected from the relevant model reactors and represent rumen-derived microbial communities adapted to the physicochemical conditions of those compartments. During the adaptation phase, both variants were dominated by *Bacteroidota* and *Bacillota*. A pronounced divergence emerged during silage feeding: *Bacteroidota* declined to below 3% in the control, whereas the experimental variant maintained approximately 22–24% (Figure 5).

The experimental variant also showed a more stable *Bacillota/Bacteroidota* balance and lower sustained abundance of *Pseudomonadota* and *Fusobacteriota* during key transition periods. These patterns are consistent with greater structural stability of the microbial community under LAB supplementation. Because the analysis was performed on reactor samples, these findings describe the modeled gastrointestinal ecosystem rather than direct changes in the donor cow rumen microbiota.

Differences between variants persisted into the wash-out phase after LAB supplementation had ceased. This persistence may represent an ecological memory effect within

the *in vitro* system; however, it should be interpreted cautiously and does not demonstrate long-term microbial programming in calves or other *in vivo* effects.

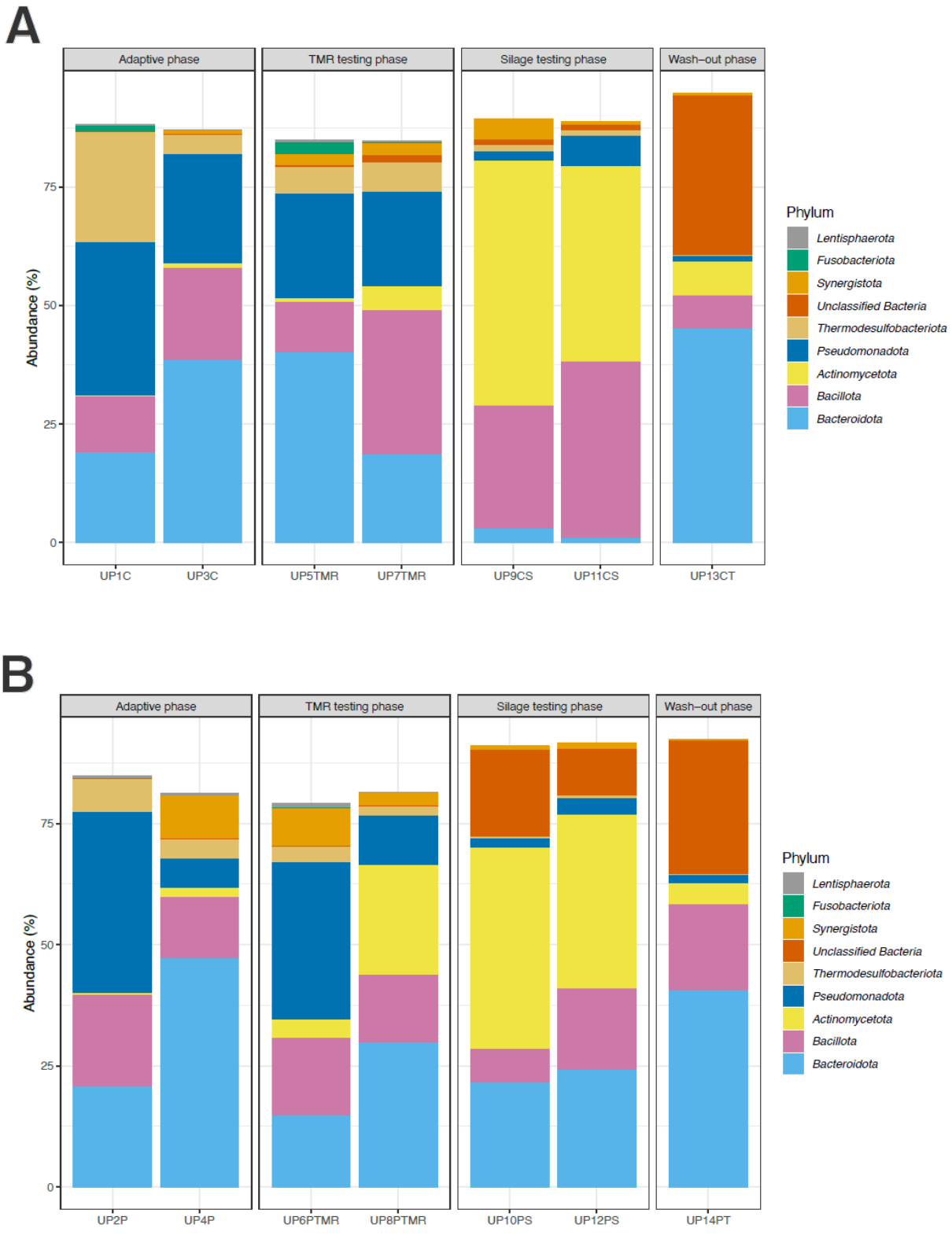


Figure 5. Taxonomic composition of microbial communities at the phylum level during different experimental phases in the dynamic *in vitro* gastrointestinal model. (A) Control variant; (B) experimental variant supplemented with LAB consortia. Phases: adaptation, TMR testing, silage testing, and wash-out.

Microbial community dynamics during the adaptation phase

During the initial adaptation phase, the control (C) and experimental (P) variants exhibited broadly comparable microbial profiles dominated by *Bacteroidota* and *Bacillota*. In the experimental variant, *Bacteroidota* reached approximately 45% at UP4P and included unclassified *Rikenellaceae* and *Parabacteroides*, consistent with retention of carbohydrate-degrading members of the rumen-derived inoculum [38, 39]. *Bacillota* remained at approximately 13–19% in the experimental variant and included unclassified *Lachnospiraceae* and members of *Clostridia*. *Actinomycetota* remained a minor component in both variants (<2%), represented mainly by unclassified *Bifidobacteriaceae*.

Pseudomonadota initially exceeded 30% in both systems and included *Enterobacteriaceae* and *Klebsiella spp*. Their abundance declined more rapidly in the experimental variant, reaching approximately 6% by the end of adaptation, whereas the control remained at approximately 23% [40, 41]. *Fusobacteriota*, represented by *Fusobacterium varium*, also declined during adaptation, and both variants converged to approximately 0.2%. Together, these observations indicate progressive adaptation of the rumen-derived inoculum to the conditions of the model reactors.

Microbial response to TMR

During the TMR phase, the experimental variant retained a relatively high abundance of *Bacteroidota*, reaching approximately 30% at UP8PTMR, and showed a more balanced *Bacillota* community. The control variant exhibited larger taxonomic fluctuations. The experimental treatment also limited the increase in *Fusobacteriota: Fusobacterium varium* reached approximately 2.6% at UP5TMR in the control but only 0.33% in the corresponding experimental phase.

Pseudomonadota remained at approximately 20–22% in the control during the TMR phase. In the experimental system, a transient increase to approximately 32%, associated with *Acinetobacter nosocomialis*, was followed by a decline to approximately 10%. These results indicate a dynamic but comparatively rapid restructuring of the microbial community under LAB treatment.

Microbial response to maize silage

Silage feeding produced the clearest divergence between the variants. *Bacteroidota* declined to below 3% in the control but remained at approximately 22–24% in the experimental system, with unclassified *Prevotellaceae* becoming prominent. *Bacillota* increased to more than 35% in the control, largely through expansion of unclassified *Lactobacillaceae*, whereas the experimental variant remained at approximately 17%, with *Lactobacillaceae* coexisting with unclassified *Lachnospiraceae* and *Lentilactobacillus buchneri*.

Actinomycetota also increased during the silage phase. The control showed a peak above 50%, dominated by unclassified *Bifidobacteriaceae*, whereas the experimental variant reached a more moderate peak of approximately 40% with contributions from *Bifidobacteriaceae* and *Atopobiaceae*. By the silage phase, *Pseudomonadota* fell below 5% in both systems. Overall, the experimental variant showed greater preservation of major bacterial groups during the dietary transition.

Microbial community - persistence during the wash-out phase

During the wash-out phase, reintroduction of the baseline model medium was accompanied by recovery of *Bacteroidota* to approximately 40–45% in both variants, with unclassified *Prevotellaceae* dominating the post-intervention community. *Bacillota* remained at approximately 18% in the experimental variant but declined to approximately 7% in the control, indicating different recovery trajectories between the two systems.

Actinomycetota declined to approximately 5–7% in both variants, whereas *Fusobacteriota* became nearly undetectable and *Pseudomonadota* remained below 2%. These observations demonstrate substantial plasticity of the reactor-associated microbial communities and suggest that treatment-related structural differences persisted after removal of the experimental feeds.

Overall, LAB supplementation was associated with greater compositional stability during TMR and silage transitions and with distinct recovery dynamics during wash-out. These effects should be interpreted as properties of rumen-derived microbial communities within the dynamic *in vitro* gastrointestinal model, not as direct evidence of changes in the rumen microbiome of living animals.

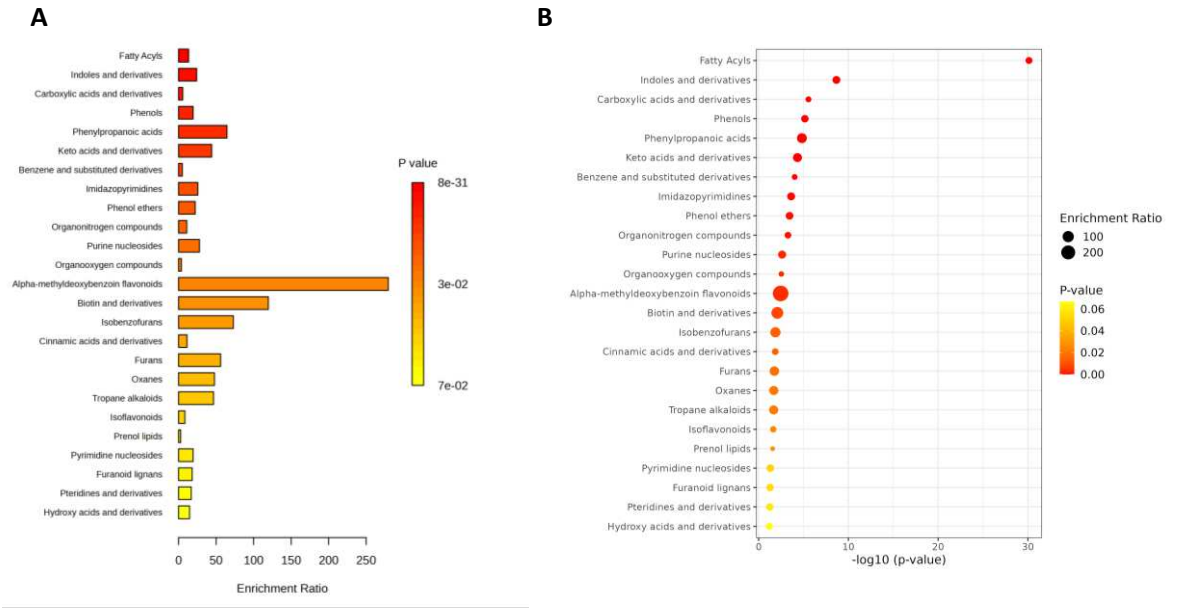
Metabolomic profiles across experimental feeding phases

Untargeted metabolomic profiling of samples collected from the relevant intestinal reactors showed clear separation according to experimental phase and treatment variant; the adaptation period was used to establish baseline metabolic activity before introduction of the experimental feeds.

Metabolomic response to TMR supplementation

Introduction of TMR produced the most pronounced divergence between metabolomic profiles. The biopreparation variant (P TMR) showed increased representation of metabolites associated with protein and nitrogen metabolism, including mesoporphyrin IX and tyramine in the complementary analytical dataset. Enrichment analysis (Figure 6) highlighted differences between P TMR and conventional TMR (C TMR), whereas the reverse comparison is shown in Figure 7. These profiles represent metabolic activity in the sampled intestinal reactor communities rather than the donor rumen.

Figure 6. Chemical main-class enrichment analysis of metabolites elevated in intestinal-reactor samples from models receiving TMR supplemented with the biopreparation (P TMR) compared with conventional TMR (C TMR). The bar plot shows the top enriched metabolite classes based on enrichment ratio and p-value; the bubble plot represents the same classes, with point size indicating enrichment ratio and color indicating statistical significance.

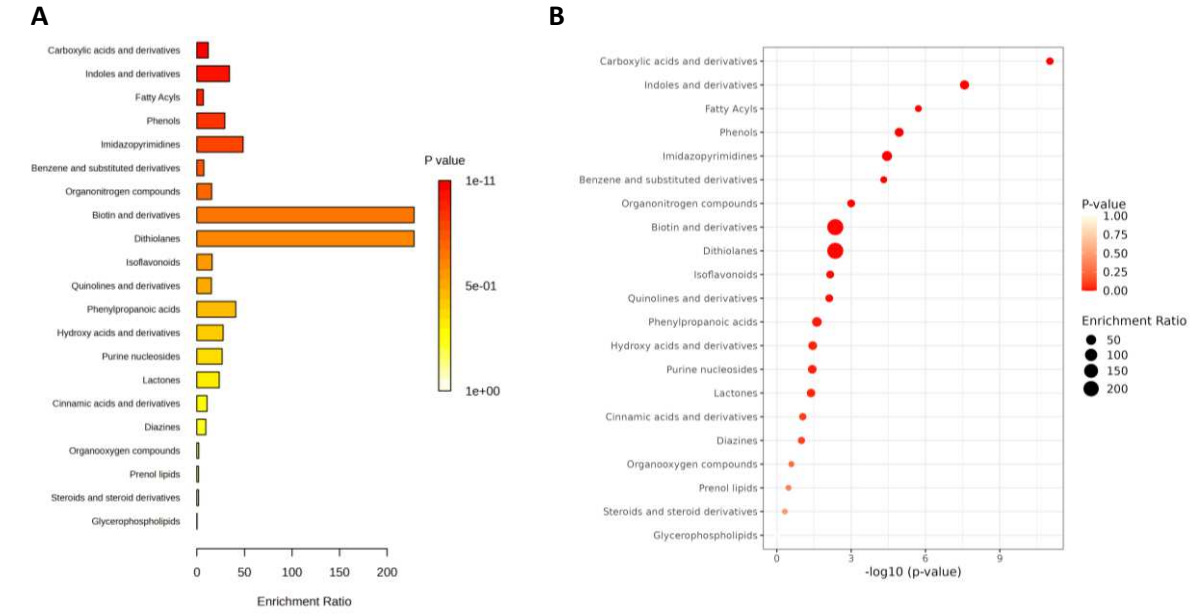


633

634 Figure 7. Chemical main-class enrichment analysis of metabolites elevated in intestinal-reactor
635 samples from models receiving conventional TMR (C TMR) compared with TMR supplemented
636 with the biopreparation (P TMR). The bar plot shows the top enriched metabolite classes based
637 on enrichment ratio and p-value; the bubble plot represents the same classes, with point size
638 indicating enrichment ratio and color indicating statistical significance.

639 *Metabolomic response to LAB-inoculated maize silage*

640 During the silage phase, metabolomic signatures reflected treatment-specific adaptation to
641 fermented forage. Enrichment profiles for the experimental silage variant (PS) indicated increased
642 representation of metabolites associated with anaerobic carbohydrate fermentation, consistent
643 with the higher lactic acid concentration measured in this variant. The corresponding comparison
644 with conventional silage (CS) is shown in Figures 8 and 9.



645

Figure 8. Chemical main-class enrichment analysis of metabolites elevated in intestinal-reactor samples from models receiving silage supplemented with the biopreparation (PS) compared with conventional silage (CS). The bar plot shows the top enriched metabolite classes based on enrichment ratio and p-value; the bubble plot represents the same classes, with point size indicating enrichment ratio and color indicating statistical significance.

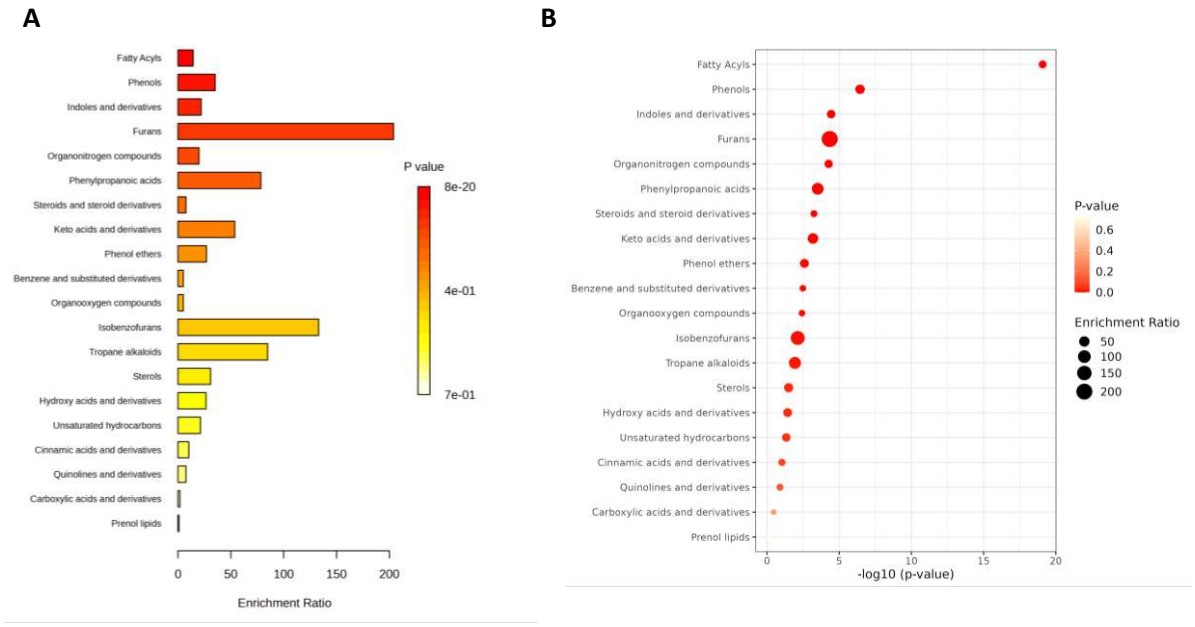


Figure 9. Chemical main-class enrichment analysis of metabolites elevated in intestinal-reactor samples from models receiving conventional silage (CS) compared with silage supplemented with the biopreparation (PS). The bar plot shows the top enriched metabolite classes based on enrichment ratio and p-value; the bubble plot represents the same classes, with point size indicating enrichment ratio and color indicating statistical significance.

Persistence of metabolic differences during the wash-out phase

During the wash-out phase, metabolomic differences between the experimental (PT) and control (CT) variants persisted after supplementation had ceased. Enrichment analysis showed differences in carboxylic acids and other metabolite classes (Figures 10 and 11). Together with the reported Hotelling T^2 trajectory analysis, this persistence is consistent with a treatment-associated metabolic memory effect within the *in vitro* reactor ecosystem, although it does not establish persistent effects *in vivo*.

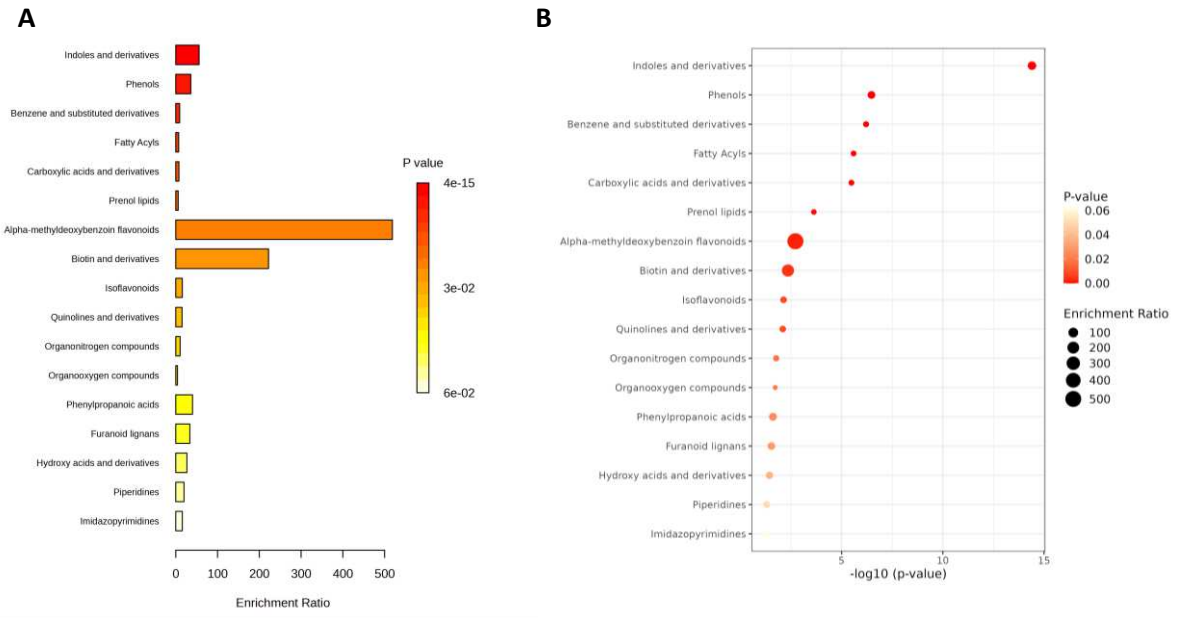


Figure 10. Chemical main-class enrichment analysis of metabolites elevated in intestinal-reactor samples from the experimental variant (PT) compared with the control variant (CT) during the wash-out phase. The bar plot shows the top enriched metabolite classes based on enrichment ratio and p-value; the bubble plot represents the same classes, with point size indicating enrichment ratio and color indicating statistical significance.

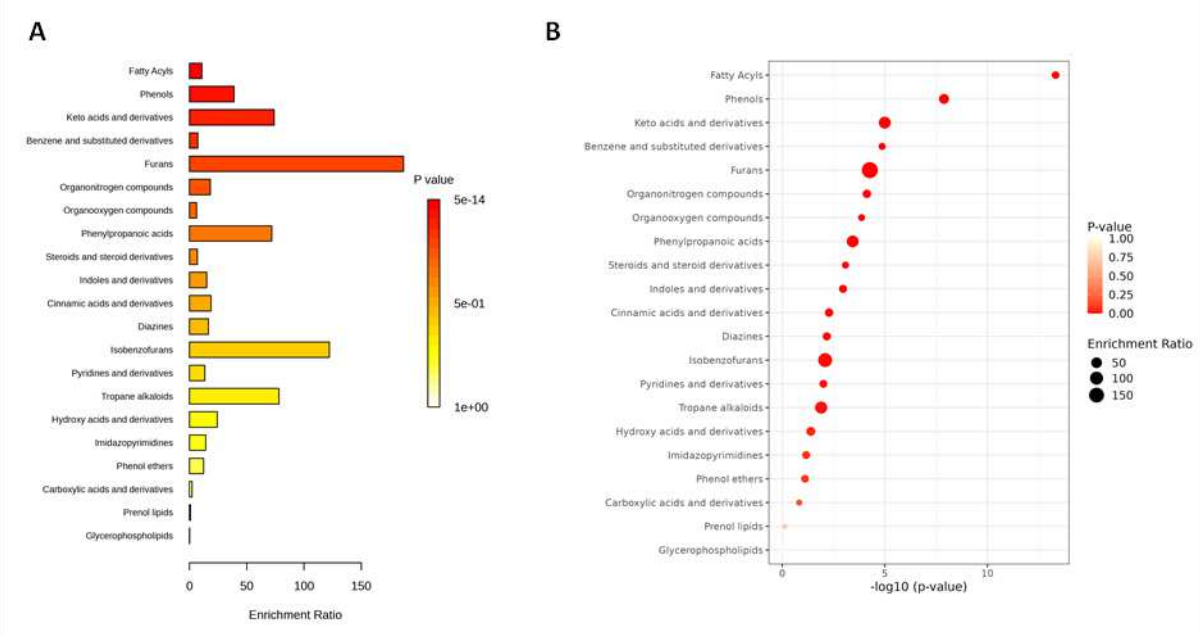


Figure 11. Chemical main-class enrichment analysis of metabolites elevated in intestinal-reactor samples from the control variant (CT) compared with the experimental variant (PT) during the wash-out phase. The bar plot shows the top enriched metabolite classes based on enrichment ratio and p-value; the bubble plot represents the same classes, with point size indicating enrichment ratio and color indicating statistical significance.

Discussion

The present study evaluated the effects of selected lactic acid bacteria (LAB) consortia on fermentation profiles, microbial community composition and metabolomic signatures in a dynamic *in vitro* digestive system inoculated with fresh bovine rumen fluid. Establishment and development of a diverse gastrointestinal microbiota are closely associated with gastrointestinal function and host development in young ruminants [42, 43]. The transition from milk to solid feed represents a substantial change in substrate availability and is accompanied by pronounced changes in rumen microbial community structure and fermentation activity [39, 44, 45]. In the present study, the LAB treatment was associated with distinct changes in fermentation products, bacterial community composition and metabolomic profiles across successive experimental phases. However, these findings should be interpreted in the context of the experimental design. LAB inoculation was performed during preparation of the maize silage and consequently altered the ensiling process and the physicochemical characteristics of the substrate subsequently introduced into the gastrointestinal model. LAB inoculants are known to modify the dynamics of silage fermentation and microbial succession, although the magnitude and direction of these effects depend on the inoculant composition, forage characteristics and ensiling conditions [41, 46, 47]. Thus, direct effects of the inoculated microorganisms cannot be unequivocally distinguished from effects mediated by changes in silage fermentation and substrate composition. Furthermore, the metagenomic, metabolomic and organic-acid analyses were performed on samples collected from the experimental reactors rather than directly from the donor rumen fluid. Accordingly, the results characterize rumen-derived microbial communities adapted to the defined *in vitro* conditions and should not be interpreted as direct measurements of the donor rumen microbiome.

The most pronounced differences between the experimental variants concerned the profile of fermentation products. The LAB-treated system exhibited higher concentrations of propionate and valerate, together with a marked increase in lactate, whereas the increase in acetate was comparatively small. Propionate is an important end product of ruminal carbohydrate fermentation and represents a major precursor for hepatic gluconeogenesis in ruminants. Its formation is closely related to the utilization of fermentable carbohydrates by the ruminal microbial community [45, 48]. In the present study, propionate concentration was approximately 37.1% higher in the LAB-treated system, whereas acetate differed by only approximately 1.5%. This disproportionate response does not indicate a generalized increase in the production of all major fermentation acids and may instead reflect a change in the relative distribution of fermentation end products.

The higher propionate concentration may be considered compatible with altered carbohydrate utilization within the reactor microbial community. The retention of *Bacteroidota* during the silage phase, including members assigned to *Prevotellaceae*, provides a plausible ecological context for this observation. Members of the *Bacteroidota*, particularly *Prevotellaceae*, are frequently detected as abundant components of rumen-associated microbial communities and contribute to the degradation of plant-derived carbohydrates [38, 41]. *Prevotella*-associated metabolic pathways may also contribute to succinate formation and subsequent propionate production. However, the present metagenomic analysis was based on relative taxonomic abundance and did not quantify transcriptional activity, pathway abundance or metabolic flux. Therefore, the observed association between the persistence of *Prevotellaceae* and increased

propionate concentration should not be interpreted as evidence that these taxa were responsible for the additional propionate.

The LAB-treated system also exhibited a marked increase in cumulative lactate concentration, which was approximately 56.4% higher than in the control. This finding is compatible with the expected contribution of LAB to lactic acid production during fermentation of the silage substrate [36, 41]. However, lactate is not necessarily a terminal product in ruminal microbial ecosystems. It may function as an intermediate metabolite and can be utilized by secondary fermenters, thereby contributing to the formation of other short-chain fatty acids. In particular, *Megasphaera elsdenii* has been extensively characterized as a lactate-utilizing ruminal bacterium capable of converting lactate into several fermentation products, including propionate and butyrate [17]. The simultaneous increase in lactate and propionate observed in the present study is therefore compatible with changes in lactate production and/or utilization within the microbial network. Nevertheless, lactate turnover was not directly measured, and the microorganisms responsible for potential lactate utilization were not experimentally identified. Consequently, microbial cross-feeding represents a plausible interpretation rather than a demonstrated process. In addition, pH in the intestinal reactors was actively regulated within predefined ranges. The absence of uncontrolled acidification in this system therefore cannot be used to infer a reduction in the risk of ruminal acidosis *in vivo*.

The observed changes in butyrate and valerate further indicate that LAB-associated treatment was accompanied by modification of the fermentation profile. Butyrate represents an important microbial fermentation product in the rumen and is involved in epithelial metabolism and ruminal development [49]. Nevertheless, the physiological significance of the relatively modest changes observed in the present in vitro system cannot be established without direct measurements of epithelial metabolism, host responses or animal performance. Similarly, increased valerate concentration indicates altered microbial fermentation but does not by itself demonstrate an improvement in ruminal function.

A contrasting metabolic response was observed for the branched-chain fatty acids isobutyrate and isovalerate, whose concentrations decreased by approximately 30.9% and 37.9%, respectively. Branched-chain fatty acids are generated predominantly during microbial catabolism of branched-chain amino acids, including valine, leucine and isoleucine, and their formation is therefore associated with amino-acid-derived fermentation processes [50]. The lower concentrations observed in the LAB-treated system are consistent with a reduced relative contribution of branched-chain amino-acid fermentation under the experimental conditions. However, this interpretation should remain restricted to the fermentation profile. The present data do not permit conclusions regarding nitrogen retention, microbial protein synthesis or nitrogen-use efficiency, because microbial biomass production, ammonia flux and nitrogen incorporation were not directly quantified.

The metagenomic analysis demonstrated pronounced phase-dependent changes in microbial community structure. During the initial adaptation period, both experimental systems underwent substantial compositional changes, including a marked decline in *Pseudomonadota*. Such changes are consistent with adaptation of the rumen-derived inoculum to the physicochemical conditions and substrate composition of the experimental reactors. Rumen microbial communities are strongly influenced by dietary substrate availability and host-associated environmental conditions, and substantial differences in community composition may occur following dietary transitions [38, 41, 51]. Thus, part of the temporal variation observed

766 in the present experiment was likely related to reactor adaptation rather than exclusively to the
767 LAB treatment.
768 Differences between the experimental variants became more pronounced during
769 exposure to TMR and, particularly, maize silage. During the silage phase, *Bacteroidota*
770 represented approximately 22–24% of the bacterial community in the LAB-treated system,
771 whereas their relative abundance decreased to below 3% in the control. In parallel, the control
772 system exhibited a marked increase in *Bacillota*, including *Lactobacillaceae*, and
773 *Actinomycetota*, including *Bifidobacteriaceae*. The LAB-treated system displayed a different
774 taxonomic trajectory, with persistence of *Bacteroidota* and a different distribution of *Bacillota* and
775 *Actinomycetota*. Such treatment-associated changes are consistent with previous observations
776 that LAB inoculation can modify microbial succession during ensiling [41, 47]. However, because
777 the present treatment altered both the microbial inoculum and the chemical characteristics
778 of the silage, the relative contribution of these factors cannot be resolved experimentally.
779 The divergent shifts of the bacterial phyla indicate that the LAB-associated silage
780 treatment was accompanied by a different pattern of microbial community succession following
781 exposure to the fermented substrate. One possible explanation is that differences in silage-
782 derived substrates and fermentation metabolites modified the selective environment within the
783 reactors and consequently affected the relative representation of microbial populations.
784 Although the experimental and control communities exhibited distinct temporal patterns,
785 ecological stability was not directly quantified using formal measures of resistance, resilience, or
786 recovery following a defined perturbation. The present results therefore demonstrate differences
787 in community composition and temporal trajectories rather than providing direct evidence that
788 LAB increased ecological stability [52].
789 The lower relative abundance of selected *Pseudomonadota* and *Fusobacteriota* during
790 some experimental phases may also indicate differences in community succession. However,
791 these observations should not be directly interpreted as evidence of improved animal health.
792 Taxonomic abundance does not necessarily reflect metabolic activity or pathogenic potential,
793 and the present in vitro model does not contain the epithelial, immunological and systemic
794 components required to evaluate host–microbiome interactions. Consequently, reductions in
795 individual bacterial taxa cannot be translated directly into conclusions regarding disease risk or
796 host health [53].
797 The silage phase is particularly important for interpretation of the observed microbial
798 differences because LAB inoculation substantially modified the ensiling process. In the present
799 experiment, culturable LAB counts were markedly higher in the inoculated silage than in the
800 spontaneously fermented control, and differences were also observed in pH, dry matter, protein
801 content and total acidity. Similar effects of LAB inoculation on silage fermentation characteristics
802 and microbial community succession have been described previously [41, 46, 47]. Consequently,
803 the subsequent response of the gastrointestinal reactor community may have resulted from both
804 the microbial contribution of the inoculant and the altered physicochemical properties of the
805 treated silage. These mechanisms are intrinsically linked under the present experimental design
806 and cannot be experimentally separated. The results should therefore be interpreted as effects
807 associated with the LAB-treated fermentation regime rather than as evidence of a direct effect of
808 the inoculated strains on the gastrointestinal microbial community.
809 The untargeted metabolomic analysis provided an additional level of evidence that the
810 metabolic environment of the reactor differed according to treatment and experimental phase.

The observed separation of metabolomic profiles indicates that treatment-associated effects extended beyond the targeted organic acids. However, the metabolomic response should not be reduced to a single metabolic transition from proteolytic to saccharolytic metabolism. Untargeted metabolomics interrogates a substantially broader chemical space than targeted analysis of SCFAs and BCFAs, and individual molecular features may have uncertain annotation or multiple potential biological origins. Accordingly, the metabolomic data provide evidence of treatment-associated differences in the metabolic state of the system but do not, by themselves, establish specific metabolic pathways or their microbial origin. Integration of metabolomic and metagenomic datasets may generate hypotheses concerning potential taxon–metabolite relationships, but confirmation of such relationships would require targeted metabolite identification, functional analyses and, ideally, measurements of metabolic flux.

Some treatment-associated differences remained detectable during the subsequent wash-out phase. This observation indicates that the microbial community and metabolomic profile did not immediately converge to an identical state following removal of the treatment-associated substrate. Such persistence may result from differences in community succession, residual substrate effects or ecological interactions established during the preceding experimental phases. The persistence of treatment-associated differences can therefore be regarded as evidence of a carry-over effect within the experimental reactor system.

Overall, the present findings demonstrate that exposure to LAB-treated feed was associated with coordinated differences in fermentation products, bacterial community composition and metabolomic profiles within the dynamic *in vitro* digestive system. The principal metabolic features included increased concentrations of propionate and lactate and decreased concentrations of isobutyrate and isovalerate, accompanied by phase-dependent differences in the relative abundance of several bacterial groups. The persistence of *Bacteroidota* during the silage phase in the LAB-treated system was particularly notable and coincided with a distinct trajectory of other major bacterial phyla. Collectively, these observations indicate that the LAB-associated ensiling regime influenced the subsequent metabolic environment and temporal development of the rumen-derived microbial community within the experimental model. However, the present data do not establish direct causal relationships between the inoculated LAB strains, individual microbial taxa and specific metabolites, nor do they demonstrate improved ruminal function or host health. The findings should therefore be considered evidence of treatment-associated microbial and metabolic responses under controlled in vitro conditions. Further in vivo studies incorporating measurements of ruminal fermentation, microbial activity, host responses and animal performance are required to determine whether the observed responses are reproducible under physiological conditions and whether they have functional relevance for ruminant nutrition and health.

Conclusions

The selected LAB consortia modified fermentation and microbial community dynamics in a dynamic in vitro digestive system established with rumen-derived inoculum. Metabolomic, metagenomic, and organic-acid analyses were based on samples collected from the relevant intestinal reactors, and the observed responses therefore characterize microbial communities adapted to those modeled compartments rather than the donor rumen. LAB supplementation was associated with higher propionate and lactic acid concentrations, lower BCFA concentrations, and distinct temporal patterns of microbial community composition during

dietary transitions, while altering microbial community dynamics within the modeled gastrointestinal ecosystem. These findings indicate that LAB supplementation was associated with specific shifts in community succession and fermentation profiles, but their physiological relevance requires confirmation *in vivo*.

Conflicts of Interest

The authors declare no conflict of interest.

Data availability

All data generated or analysed during this study are included in this published article.

The raw metagenomic sequencing data generated from the isolated DNA samples are available in the NCBI database under BioProject accession number PRJNA1470393 and BioSample accession numbers SAMN60411663–SAMN60411676.

Author contributions

B.P.-K. prepared the methodological section, wrote the initial draft of the manuscript, and coordinated revisions; I.M. and J.B. provided conceptualization and critical intellectual supervision of the text; M.W. designed and performed the metagenomic analysis; A.W., T.R., and J.N. developed the methodology and performed the metabolomic analyses; G.B. contributed to the inoculum collection. All authors reviewed and approved the final manuscript.

Acknowledgements

This research was completed while the first author was a doctoral candidate at the Interdisciplinary Doctoral School of Lodz University of Technology, Poland.

The work was financed under the grant “Opracowanie innowacyjnego sposobu żywienia cielaków poprawiającego efektywność produkcji” (Grant ID 00072.DDD.6509.00169.2022.05; European Agricultural Fund for Rural Development, RDP 2014–2020).

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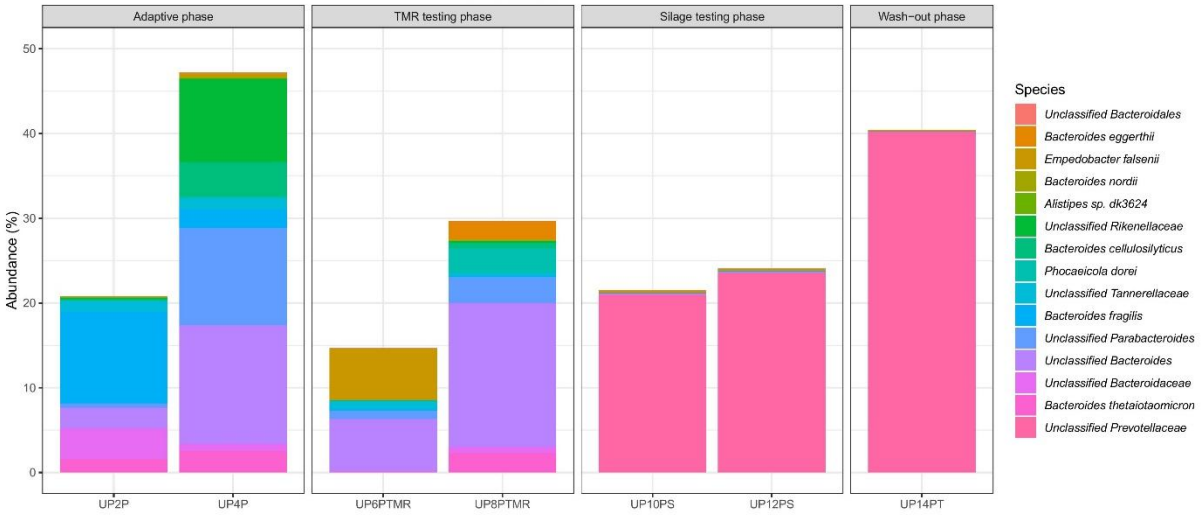
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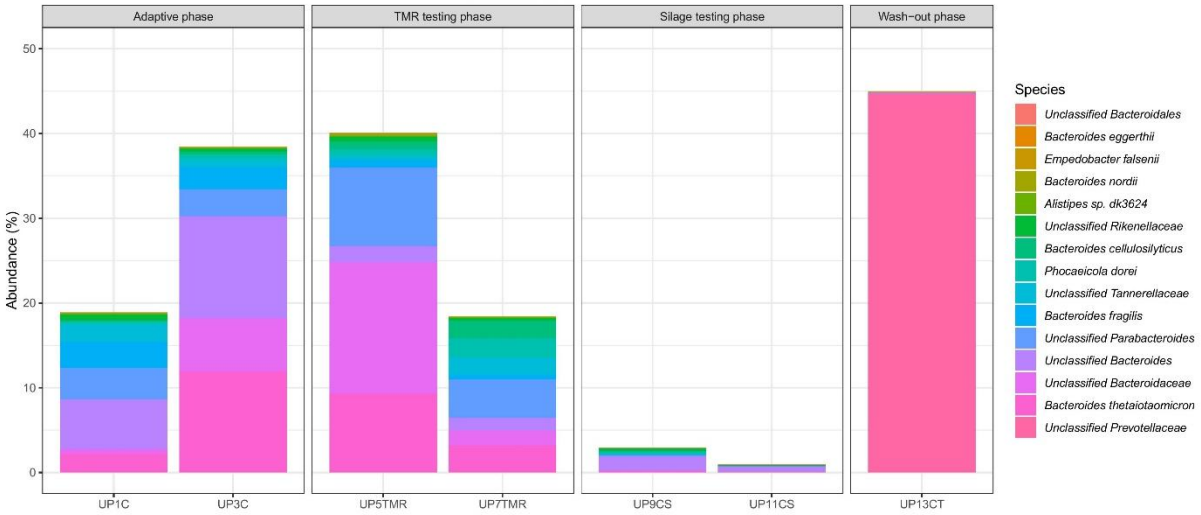
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Supplementary Materials

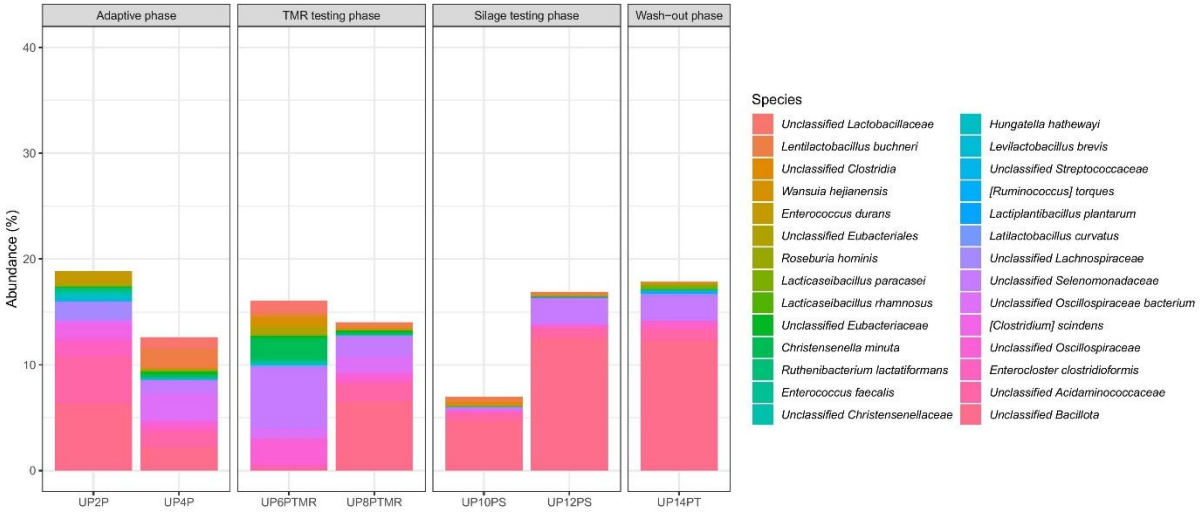


a) Experimental group

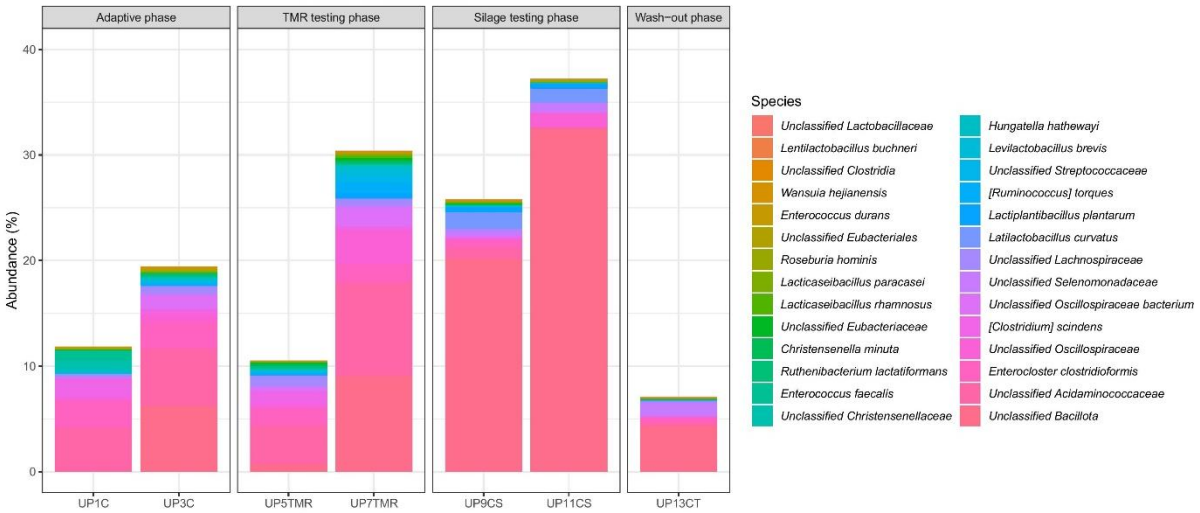


b) Control group

Appendix 1. Dynamics of *Bacteroidota* relative abundance at the species level in the experimental (P) and control (C) groups across the experimental phases: adaptation, TMR testing, silage testing, and wash-out.

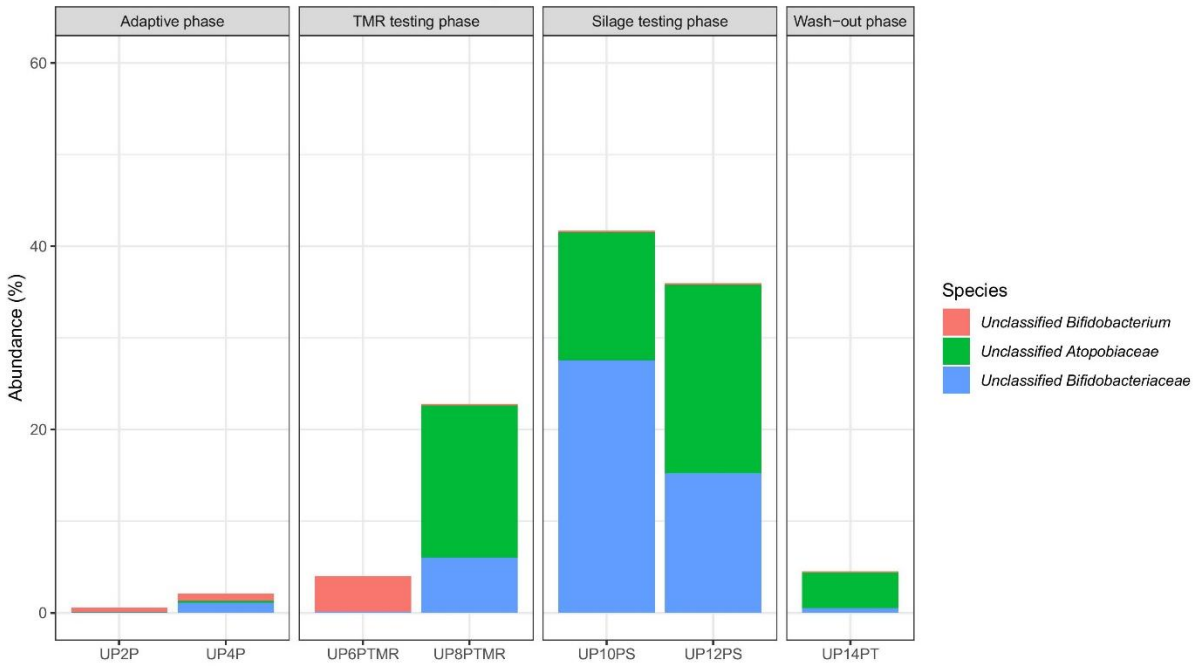


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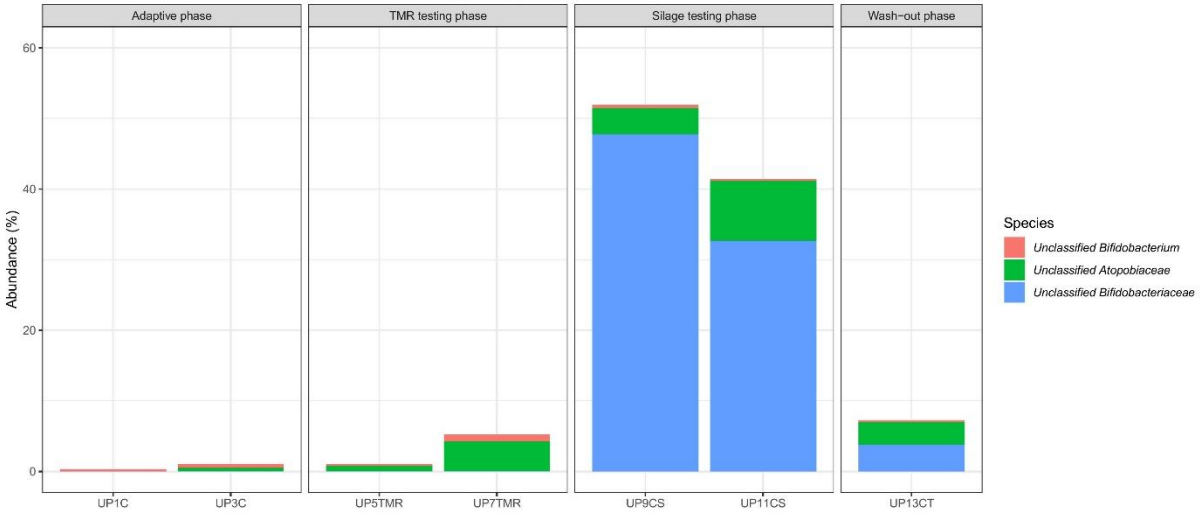


b) Control group

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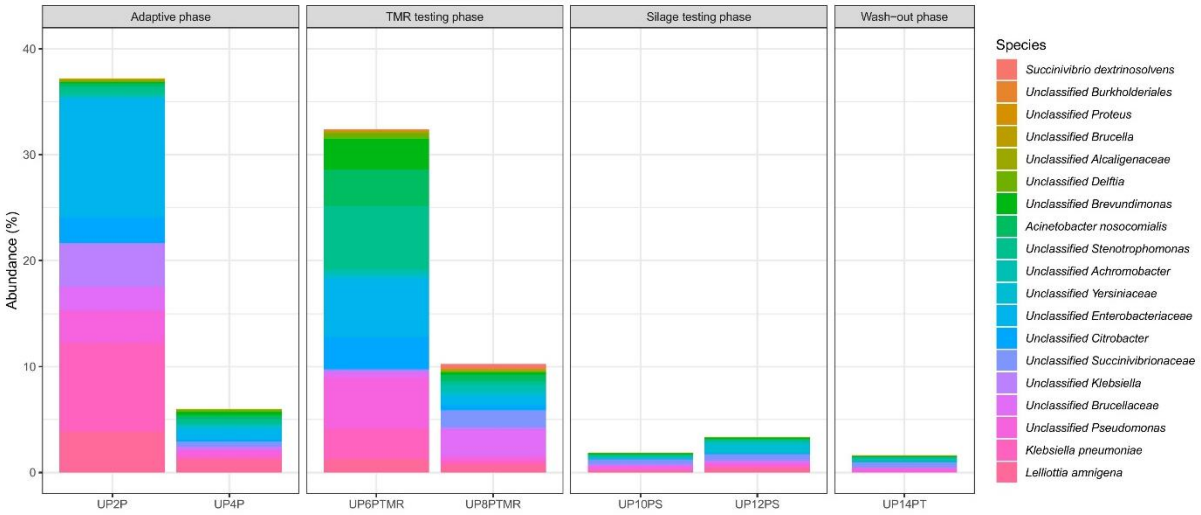


a) Experimental group

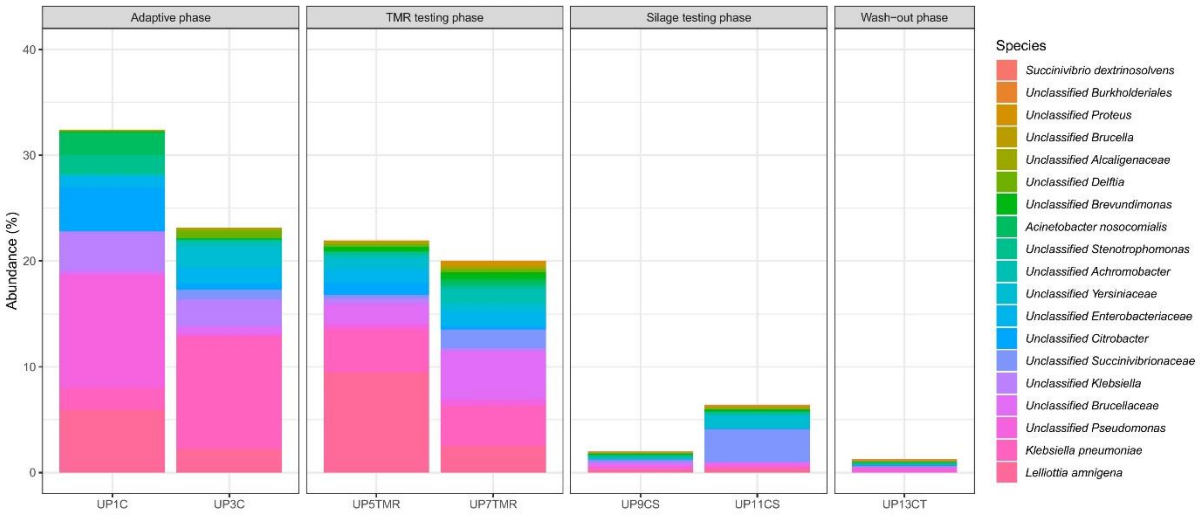


b) Control group

Appendix 3. Dynamics of *Actinomycetota* relative abundance at the species level in the experimental (P) and control (C) groups across the experimental phases: adaptation, TMR testing, silage testing, and wash-out.

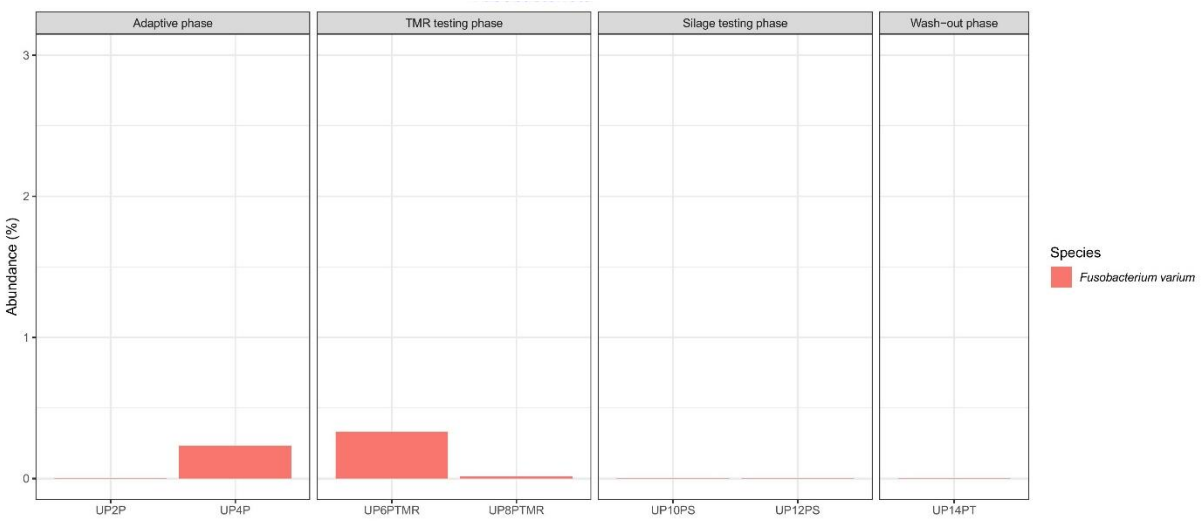


a) Experimental group



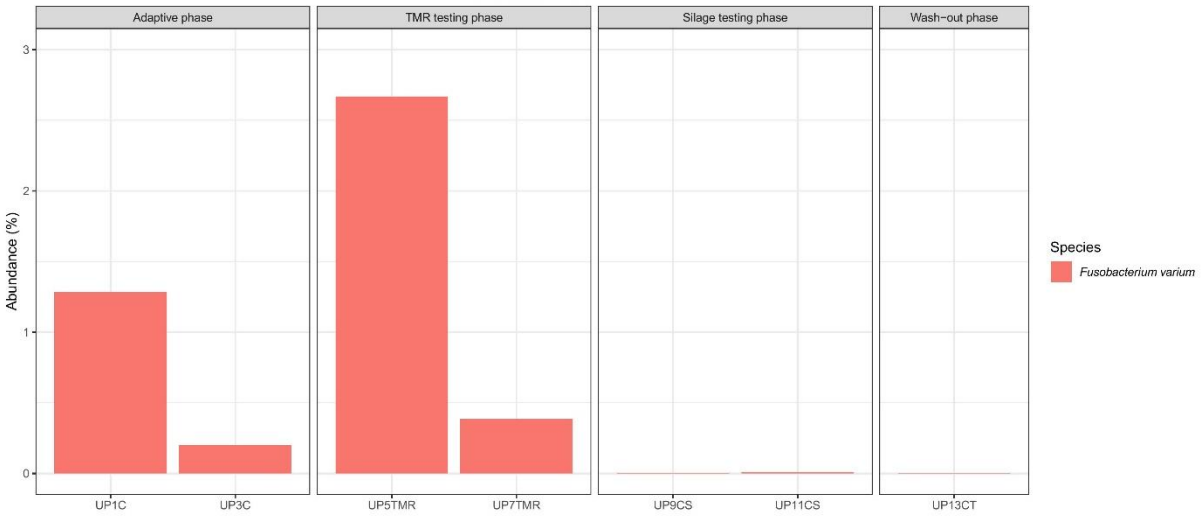
b) Control group

Appendix 4. Dynamics of *Pseudomonadota* relative abundance at the species level in the experimental (P) and control (C) groups across the experimental phases: adaptation, TMR testing, silage testing, and wash-out.



1070

1071 a) Experimental group



1072

1073 b) Control group

1074 Appendix 5. Dynamics of *Fusobacteriota* relative abundance at the species level in the
1075 experimental (P) and control (C) groups across the experimental phases: adaptation, TMR testing,
1076 silage testing, and wash-out.