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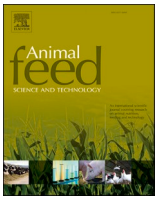

Circular use of supercritical CO_2-extracted Peppermint (*Mentha* × *piperita L.)* residues in ruminant nutrition: An *In Vitro* fermentation model

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ABSTRACT

The growing focus on circular economy strategies has encouraged the valorization of agro-industrial by-products as functional feed ingredients for ruminants. This study evaluated the effects of peppermint (*Mentha* × *piperita* L.) biomass and its post-distillation residues on in vitro rumen fermentation, volatile fatty acids (VFA), gas kinetics, methane production, and microbial composition. Four treatments were tested: control forage (*Lolium multiflorum Lam.*, CTRL), raw peppermint (Mp), residue after supercritical CO_2 extraction (rMp), and the same residue supplemented with peppermint extract (erMp). Compared to CTRL, rMp promoted gas production per gram of dry matter, while Mp and rMp increased acetate and decreased butyrate, indicating a shift of hydrogen flow from methanogenesis to acetogenesis. Methane concentration (CH_4 % v/v) was significantly reduced in Mp, confirming inhibition of methanogenesis, whereas erMp showed delayed fermentation, higher methane yield, and reduced microbial diversity, likely due to excess essential oil compounds. Microbial analysis revealed treatment-specific shifts: Mp and rMp promoted acetogenic and lactate-utilizing taxa (e.g., *Lachnospiraceae, Selenomonas ruminantium*) and suppressed *Prevotella*. Overall, peppermint-based substrates significantly altered microbial community structure (PERMANOVA, UniFrac). These results highlight that peppermint biomass and its extraction residues can beneficially modulate rumen fermentation, enhance acetate production

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and lower methane, if inclusion levels are properly managed. Post-extraction residues retain functional bioactivity, offering a sustainable strategy for waste valorization in circular livestock systems.

1. Introduction

In recent years, the growing demand for natural products, particularly essential oils, across many industries including cosmetics, pharmaceuticals, food processing, nutraceuticals, and aromatherapy has led to a substantial increase in essential oil production and, consequently, the generation of solid and liquid residues (Fallah et al., 2024; Sánchez-Vioque et al., 2013). The global essential oils market was valued at USD 23.74 billion in 2023 and is projected to grow at a compound annual growth rate (CAGR) of 7.6 % from 2024 to 2030 (Baser and Bonello, 2025).

Aromatic and medicinal plants have long been used in traditional medicine, cosmetics and food preservation due to their flavor-enhancing and therapeutic properties (Nieto, 2020). These effects are primarily attributed to essential oils, which are complex mixtures of terpenes, phenolic compounds, esters, and other bioactive molecules (Siddiqui et al., 2024). Peppermint essential oil is rich in monoterpenes, mainly menthol, menthone, isomenthone, and pulegone, with minor contributions from compounds such as carvone and limonene (Siddiqui et al., 2024). Among these, menthol is the predominant bioactive molecule and has been widely reported to exert antimicrobial activity against rumen microorganisms, particularly Gram-positive bacteria and protozoa, thereby modulating fermentation pathways (Patra and Yu, 2012; Calsamiglia et al., 2007). Menthol and menthone rich essential oils have been associated with shifts in volatile fatty acid profiles, reduced methane production, and changes in feed intake and digestibility (Khiaosa-ard and Zebeli, 2013). The phenolic constituents of the leaves include rosmarinic acid and several flavonoids, primarily eriocitrin, luteolin and hesperidin that can improve feed efficiency, enhance antioxidant status, support immune function, and improve meat/milk quality by fighting oxidative stress (Hudz et al., 2023; McKay and Blumberg, 2006). Essential oils are typically extracted from plant materials, through methods such as hydro-distillation, steam-distillation, and organic solvent extraction, using different plant parts including flowers, buds, seeds, leaves, barks, fruits, roots (Sánchez-Vioque et al., 2013). Although essential oil yields are typically below 8 %, the remaining solid residues still contain high levels of bioactive compounds. Studies have reported concentrations ranging from ~24 mg GAE/g (e.g., rosemary residues; Vella and Laratta, 2023) to ~100–130 mg GAE/g (e.g., thyme residues; Hcini et al., 2022).

Given the scale of waste generation, there is an urgent need to implement strategies aligned with the principles of the circular bioeconomy (Mishra et al., 2023). The circular economy model seeks to reduce resource consumption, waste production, and greenhouse gas emissions by recovering and repurposing *end of life* products (European Commission, 2020).

Agricultural and industrial residues rich in bioactive compounds offer sustainable alternatives to conventional feed additives, with potential antimicrobial and antioxidant effects able of modulating rumen fermentation (Cobellis et al., 2016; Calsamiglia et al., 2007; Cardozo et al., 2004). *Mentha* × *piperita* L. (peppermint), widely cultivated for its essential oil and used extensively in the food, cosmetic, and pharmaceutical sectors (Fallah et al., 2024), generates significant solid waste during extraction. If unmanaged, this waste can lead to environmental problems such as organic waste accumulation, greenhouse gas emissions, and potential soil or water pollution. Within a circular economy framework, this waste presents potential as a ruminant feed ingredient due to its residuals functional compounds (Khiaosa-ard and Zebeli, 2013). Research on the use of peppermint in ruminant diets remains limited. Gouda et al., (2025) evaluated the inclusion of dried peppermint leaves in a total mixed ration at 0.5 %, 1.0 %, 1.5 %, and 2 % of dry matter (DM), with 3.5 % essential oil on DM. Supplementation improved the degradability of crude protein, neutral detergent fiber (NDF), and acid detergent fiber (ADF), while methane reduction most pronounced at inclusion levels of 0.5–1.0 %. A concurrent increase in short-chain fatty acid production, mainly acetate and propionate, was also observed. Similarly, Hosoda et al., (2006) found that peppermint included as dried herbs (~5 % DM) had little impact on feed intake, milk yield, or fermentation, although minor effects on rumen pH and digestibility were noted. Peppermint in essential oil forms (especially in menthol rich blends combined with garlic) have shown greater efficacy, particularly in reducing methane emissions and, in some cases, improving production indicators (Rajkumar et al., 2022). However, the effects of peppermint essential oil extraction by products on rumen fermentation and animal performance vary depending on formulation, dosage, and duration of supplementation.

Before incorporating such by-products into livestock diets, it is essential to evaluate their effects on ruminal fermentation under controlled conditions using in vitro systems (Gouda et al., 2025).

In this study, essential oils from peppermint were extracted using Supercritical Fluid Extraction (SFE), an advanced technique employing CO_2 in its supercritical state. Under these conditions, above the fluid's critical temperature and pressure, CO_2 exhibits gas like diffusivity and liquid like solvating power, making it an efficient and selective solvent for extracting bioactive compounds (Nozari and Kander, 2025). This method is also environmentally safe, as CO_2 is inert, non-toxic, non-flammable, and has a relatively accessible critical point (31°C and 73.8 bar) compared to other solvents (Khalati et al., 2023; Goodarznia and Eikani, 1998).

The aim of this study was to compare the *in vitro* rumen fermentation characteristics of a common grass hay (*Lolium multiflorum*, Lam.) with peppermint biomass in three forms: the unprocessed plant (Mp), the residual biomass after SFE (rMp), and the reconstituted biomass composed of rMp supplemented with its extract (erMp). The evaluation focused on fermentation kinetics, chemical composition, gas and methane production, and the effects on microbial communities. These results offer preliminary insights into the potential application of peppermint extraction by-products as sustainable additives in ruminant nutrition.

2. Material and methods

2.1. Treatments preparation

The peppermint was collected in the fields of S.A.M. srl, located in Sospirolo Belluno (Italy). Taxonomic identification was performed by N. De Zordi and a voucher specimen was deposited at the Natural Product Lab of the Department of Pharmaceutical and Pharmacological Sciences, Padova University (ML2020A). The aerial part materials were dried in a cool desiccator (NWT-35, Italy). After drying at 35 °C until the peppermint reached 5.5 % of the residual humidity, the plant materials were stored in shade at 20 °C. The *Lolium multiflorum* Lam. was harvested at the beginning of the spiked stage.

Extract of *Mentha piperita* (eMp) and the related wastes (rMp) were produced at S.A.M. srl using the method previously described by Sut et al. (2021) by means of the TH22–10 × 2 supercritical CO_2 extraction apparatus (Toption Instrument Co. Ltd, YanTa District, Xi'an, China). Briefly, the plant was equipped with two 10 L extraction vessels and two 5 L separators. The carbon dioxide (Siad SpA, Trieste, Italy; 99.99 % purity, food grade) was carried with a high-pressure liquid pump (Toption Instrument Co. Ltd YanTa District, Xi'an, China). The extraction pressure was set at 150 bar, while the extraction temperature was set at 40°C.

Peppermint, the residual biomass, and the control test after their receiving were freeze-dried at the University of Padova laboratories (Legnaro, Padova, Italy). The freeze-drying process was conducted in a freeze-dryer (VirTis, Benchtop K, USA) at −80°C and between 0.1 and 0.2 mmHg for 72 ± 1 h. The dried samples were then ground using an ultra-centrifugal mill (Retsch ZM 200, Retsch GmbH, Germany) with a grinding grid of 1 mm. The ground samples were used for both *in vitro* fermentation and chemical analysis. The extract sample (eMp) was not subjected to proximate chemical analysis because it was obtained as a liquid substrate, which prevented accurate determination of dry matter-based components (such as CP, NDF, ADF) using standard methods designed for solid samples.

2.2. Chemical composition of treatments

The dry matter (DM) content of the treatments was determined by drying the samples overnight in an oven (Jointlab S.r.l., Italy) at 101–103 °C, following (AOAC, 2016) method 978.01. Ash content was assessed by heating the samples in a muffle furnace (Zetalab, Italy) at 550 °C for 4 h, then weighing them after cooling to room temperature in a desiccator, in accordance with (AOAC, 2016) method 942.05. Crude protein content (CP) was measured using the Kjeldahl procedure (AOAC, 2016), method 978.04, with a KjeltecTM 8400 analyzer (Foss Electric A/S, Hillerød, Denmark). Ether extract (EE) was determined following (AOAC, 2016) method 2003.05. Fibre content was analyzed according to the method of (Van Soest et al., 1991). Neutral detergent fibre (aNDF), acid detergent fibre (ADF), and acid detergent lignin (ADL) were measured using an Ankom fibre analyzer 200 (Ankom, Rochester, NY, USA). The aNDF was determined with heat-stable α-amylase and without sulphite, in accordance with (AOAC, 2016) method 2002.04. Acid detergent fiber (ADF) was measured following (AOAC, 2016) method 973.18, with the ADL content determined sequentially using the same method. The chemical compositions of the treatments and the control test (CTRL) are shown in Table 1.

2.3. Antibacterial activity

The antibacterial activity of Mp, rMp, and erMp was assessed against four microbial species: *Staphylococcus aureus* ATCC® 6538™ and Escherichia coli ATCC® 8739™ (Microbiologics, Inc., St. Cloud, MN, USA), *Lactococcus lactis* subsp. *lactis*, and *Brettanomyces bruxellensis* (obtained from the culture collection of Fondazione Edmund Mach-FEM, San Michele all'Adige, TN, Italy).

The strains were cultured under the following conditions to prepare the microbial suspension (inoculum): *S. aureus* in NB (Nutrient broth) at 37 °C for 48 h under agitation (250 rpm); *E. coli* in MTSB (Modified Tryptone Soya Broth) at 37 °C overnight; *Lc. lactis* in M17 broth at 30 °C for 48 h, and *B. bruxellensis* in 7 % YM broth (Yeast Malt) at 30 °C for five days. All media were purchased from Oxoid (Milan, Italy).

The optical density (OD) of all sample solutions was measured at 600 nm using a spectrophotometer (PowerWave 340, Bio-Tek Instruments, Winooski, VT, USA). In parallel, plate counts were performed for all microbial strains in order to quantify cell concentrations and validate spectrophotometric readings.

Table 1
Chemical composition of treatments (g/kg DM) and polyphenols content (g/kg DM).

Treatments	DM, g/kg	CP	EE	aNDF	ADF	ADL	Ash	AIA	NSC	Total polyphenols
Lolium multiflorum Lam.[a]	899.4	80.8	16.7	590.2	330.7	44.1	70.7	5.20	241.6	1.41
Mentha piperita L.	903.3	126.7	33.4	353.5	242.1	84.6	91.8	6.30	394.6	16.14
Mentha piperita residue	921.1	162.1	17.3	370.6	240.5	89.9	93.7	3.70	356.3	16.65
Extract of *Mentha piperita*[b]	nd	nd	nd	nd	nd	nd	nd	nd	nd	69.83[c]

DM: Dry Matter; CP: Crude Protein; EE: Ether Extract; aNDF: Neutral Detergent Fiber with heat stabile α-amylase and without sodium sulfite; ADF: Acid Detergent Fiber; ADL: Acid Detergent Lignin; AIA: Acid Insoluble Ash; NSC: Non-Structural Carbohydrates.

[a] *Lolium multiflorum* Lam. was used in the *in vitro* test as a control treatment.

[b] The extract of *Mentha piperita* was not analysed for chemical composition because it was a liquid matrix.

[c] The Total polyphenol content of *Mentha piperita* was expressed as g/kg of extract.

To evaluate the antibacterial activity, the agar well diffusion method described by Magaldi et al. (2004) was employed with minor modifications. Each plate was inoculated with 300 μL of the respective bacterial suspension, previously adjusted to approximately 10^7 CFU/mL, and spread over the surface of the respective agar medium. The culture conditions for each microorganism were as follows: *S. aureus* in BP agar (Baird Parker), with 5 % of egg yolk tellurite emulsion, at 37 °C for 48 h; *E. coli* in MTSA (Modified Tryptone Soya Agar) at 37 °C overnight in anaerobic conditions (in a jar with an AnaeroGen™ system); *Lc. lactis* in M17 agar at 30 °C for 48 h in anaerobic conditions and *B. bruxellensis* in WL agar (Wallerstein Laboratory) at 30 °C for six days. All media were purchased from Oxoid (Milan, Italy). Each assay was performed in duplicate.

Wells of 7 mm diameter were aseptically punched into the agar, and 50 μL of the natural extract solution (corresponding to 10 mg/mL of total polyphenols content) was applied to each well. Following incubation under the previously described conditions, inhibition zones were measured in millimeters. Chloramphenicol (50 μL, Oxoid, Milan, Italy) was used as the positive control for bacterial strains, while chitosan served as the positive control for yeast strain. The extraction solvent (acetone:water, 70:30 v/v) was also tested separately to ensure it did not exert antimicrobial activity on the tested microorganisms.

2.4. Experimental design and incubation procedure

During the *in vitro* fermentations trials, four different substrates were incubated: *Mentha* × *piperita L.* (peppermint) (Mp), residue of *Mentha* × *piperita L.* (peppermint) after SFE in CO_2 (rMp), extract of peppermint added to the residual biomass (erMp), and grass hay (CTRL). The purpose of incubating erMp was to assess whether any compositional changes occurred after SFE in CO_2 compared to the whole samples of Mp.

Each treatment was incubated in triplicate with rumen fluid from three different Simmental dairy cows, and this setup was repeated across three consecutive weekly *in vitro* sessions. In total, each treatment was tested with rumen fluid from all three donor cows in each of the three weeks. Additionally, 3 blanks bottles (containing only fermentation medium and rumen fluid without treatment samples) were incubated per each *in vitro* session. In total, 45 artificial rumens were tested (4 treatments × 3 rumen fluids × 3 *in vitro* sessions + 9 blanks). The rumen fluids were collected using an esophageal probe from fasting Simmental cows breed in the experimental farm "Lucio Toniolo" of the University of Padova (Legnaro, Padova, Italy). Approximately 1 L of rumen fluid per cow was collected, maintained at 39°C in thermos, and directly transported to the laboratory. Upon arrival, it was filtered through four layers of cheesecloth to remove coarse particles, ensuring anaerobic conditions were maintained using continuous CO_2 flow. The fermentation medium solution was prepared following the protocol of (Menke and Steingass, 1988) and maintained at 39°C under a CO_2 flux for at least 45 min to preserve anaerobic conditions. In each incubation bottle, 1.00 ± 0.01 g of Mp, rMp, and CTRL was weighed, while for erMp, 0.96 g of rMp was combined with 0.04 g of eMp. Each fermentation bottle (322 mL of total volume) was then filled with 100 mL of medium solution (Menke and Steingass, 1988) and 50 mL of filtered rumen fluid. To ensure strict anaerobic conditions and prevent interference with total gas and methane measurements, the bottle headspace (preheated at 39°C) was flushed with nitrogen (N_2) instead of CO_2 as recommended by Park and Lee (2022). The bottles were then closed with the Ankom^{RF} GP modules (Ankom Technology®, Macedon, NY, USA). These modules were used in order to monitor the gas production (GP) kinetics inside the bottles for 24 h of fermentation. Each bottle was fitted with a sensor capable of detecting pressure changes resulting from microbial fermentation within the 172-mL headspace (322-mL of total space in bottle, 100 mL of medium solution and 50 mL of rumen fluid). These sensors transmitted data wirelessly to a computer, where pressure values were continuously recorded using RF GPM software (version 11.4, Ankom Technology®, Macedon, NY, USA). Upon completion of the incubation period, the cumulative fermentation kinetics of each bottle were assessed by analyzing the gas production curves generated by the GP software (Cattani et al., 2014).

From the moment of rumen fluid collection to the start of the incubation process, all the procedures were completed within 40 min to preserve microbial activities.

2.5. Post-fermentation samples collection

At the end of the 24 h fermentation process, the gases accumulated in the bottle's headspace were extracted using a 10-mL syringe and transferred into a 10-mL vacutainer. These gas samples were promptly analyzed by a 490 Micro GC System (Agilent, California, USA) equipped with a thermal conductivity detector. The system operated with two distinct columns, each featuring different capillary properties and carrier gases (one using argon at 145°C and 30 psi, the other using helium at 100°C and 28 psi). The collected data were processed using the SOPRANE software (S.R.A. Instruments, France). This analysis allowed for the determination of the gas composition in each bottle at the end of the fermentation.

After the gas collection, the modules were taken out, and the pH of each bottle was measured with a pH METRE BASIC20 (Crison Instruments, Barcellona, Spain).

From the fermented rumen fluid in the bottle, two 4 mL aliquots were taken from each bottle and stored at −30°C after the addition of 1 mL of metaphosphoric acid (25 % w/v). These samples were preserved for the analysis of ammonia (N-NH_3) and volatile fatty acid (VFA). Ammonia nitrogen (N-NH_3) concentrations were measured using the Ammonia Rapid Assay Kit (K-AMIAR 02/20; Megazyme, Bray, Ireland). Volatile fatty acid (VFA) concentrations were analysed using a Jasco high-performance liquid chromatography (HPLC) system equipped with a PU-2080 pump (Tokyo, Japan), and RI-2031 refractive index detector, an AS-2055 autosampler, and a CO-2060 column oven. Separation was carried out on an Aminex HPX 87 H column (300 mm × 7.8 mm; Bio-Rad), with data processed using ChromNAV software (Version 2.0, Jasco). The mobile phase consisted of 0.005 N sulfuric acid at a flow rate of 0.6 mL/min, with an isocratic elution gradient and a sample injection volume of 20 μL. Calibration curves were generated using standard solutions of glacial acetic acid (Carlo Erba, CAS 64–19–7), propionic acid (Sigma-Aldrich, CAS 79–09–4), butyric acid (Fluka, CAS

107–92–6), iso-butyric acid (Sigma-Aldrich, CAS 79–31–2), valeric acid (Sigma-Aldrich, CAS 109–52–4), iso-valeric acid (Sigma-Aldrich, CAS 503–74–2), and caproic acid (Sigma-Aldrich, CAS 142–62–1).

Finally, the content of the bottles was filtered through glass crucibles (por.3), and the remaining dry matter (DMres) was extracted using a Fibertech FIWE Raw Fibre Extractor (Velp Scientifica, Monza e Brianza, Italy).

2.6. Calculation for rumen fluid traits

The non-structural carbohydrates (NSC) were calculated for each treatment using the formula proposed by (Bovera et al., 2004):

$$\boldsymbol{NSC}(\boldsymbol{g}/\boldsymbol{kgDM}) = \mathbf{1000} - (\boldsymbol{aNDF} + \boldsymbol{EE} + \boldsymbol{CP} + \boldsymbol{Ash})$$

where aNDF, EE, CP, and Ash are expressed as g/kg DM.

The *in vitro* degraded dry matter (dDM) was determined by subtracting the residual dry matter (DMres) remaining after 24 h of fermentation from the initial dry matter content of the incubated feed sample (DMfeed), with adjustments made for blank contributions (g). Dry matter degradability (DMd) was expressed as grams per kilogram of incubated dry matter (g/kg DM) following the method of Goering and Van Soest (1970):

$$dDM(g) = DMfeed - DMres$$

$$DMd(g/kgDM) = (dDM/DMfeed) \times 1000$$

The total gas production (GP) recorded after 24 h of incubation was adjusted for blank contributions and expressed in relation to both the incubated dry matter (mL/g DM) and the degraded dry matter (mL/g dDM).

The composition of gases (H_2 and CH_4) measured after 24 h was adjusted for blank contributions and expressed as volume proportions (% v/v), as well as the amounts produced per gram of incubated dry matter (mL/g DM) and per gram of degraded dry matter (mL/g dDM).

Ammonia nitrogen ($N\text{-}NH_3$) was reported in mMol/L, while individual VFAs were expressed as proportions of total VFA (g/100 g VFA).

Lastly, gas production (GP_{VFA}) and methane production (CH_{4VFA}) were estimated from the VFA profile (corrected for blank contributions) using the stoichiometric equations proposed by (Blümmel et al., 1999). These values were expressed as volume proportions (% v/v), per gram of incubated dry matter (mL/g DM), and per gram of degraded dry matter (mL/g dDM).

2.7. Total polyphenols quantification and phenolic profile from peppermint

In this study, we quantified only the total polyphenol content (TPC). Detailed profiling of volatile compounds (e.g., menthol, menthone, carvacrol) via gas chromatography was not conducted, which limits the interpretation of potential antimicrobial effects. This will be addressed in future studies.

Total polyphenols content (TPC) of peppermint was analysed using the Di Stefano and Guidoni (1989) method with some modification.

For the extract preparation, 2.5 g of CTRL, Mp, rMp and 0.5 g of erMp were taken and mixed in a solution composed by acetone: water (70:30 v/v). The mixture was homogenized, centrifuged at 10000 rpm for 10 min. The liquid phase was isolated from the solid residue and utilized for the analysis. A volume of 1 mL extract was dried with Rotavapor® R-300 and then resuspended with H_2SO_4 1 N in a 10 mL flask. The cartridge (Sep.pak C 18 0.5 g Waters) was activated with 2 mL of methanol and 5 mL of H_2SO_4 0.01 N. Loaded 1 mL of diluted sample onto the cartridge and washed with 2 mL of H_2SO_4 0.01 N. The sample was eluted in a 20 mL flask with 2 mL of methanol and 5 mL H_2O. For the reaction, was added 1 mL of Folin-Ciocalteau's reagent, waited 3 min and added 4 mL of 10 % Na_2CO_3 and make up to volume with H_2O. A control sample containing 2 mL of methanol and 5 mL of water was prepared and allowed to react concurrently with the other samples. After 90 min the solutions were filtered (Sartorius minisart 0.45 μm) and the measurements were taken using a spectrophotometer at 700 nm. Extracted polyphenols were measured using Folin-Ciocalteu (FC) reaction, a colorimetric method widely used for determining the total phenolic content in various samples. The principle of the method involves the reduction of the FC reagent, which is a mixture of molybdenum and tungsten oxides, by phenolic compounds present in the sample (Everette et al., 2010). It is one of the standard methods used in wine analysis, but it can also be applied to other food products, such as fruits and vegetables. Total polyphenols content is reported as mg of catechin per kg of DM, and as mg of catechin per kg for eMp due to its liquid condition.

2.8. Total DNA extraction from rumen samples

For total genomic DNA extraction, 1 mL of ruminal fluid were centrifuged at 4000 g for 10 min at + 4 °C and the supernatant was discarded. Genomic DNA was extracted from the pellet using the the QIamp® PowerFecal® DNA Kit (Qiagen, Milano, Italy) following the kit procedure. The quantification of yield and purity of the extracted DNA were determined by the Nanodrop 8800 fluorospectrometer (Thermo Scientific, Waltham, MA, USA).

2.9. Preparation of the MiSeq library

Amplicon library preparation, the determination of the quality and quantification of pooled libraries, and pair-end sequencing using the Illumina MiSeq system (Illumina, San Diego, CA, USA) were performed at the Sequencing Platform, Fondazione Edmund Mach (FEM, San Michele a/Adige, Italy).

PCR amplification was performed by targeting 16S rRNA gene V3-V4 variable regions (Claesson et al., 2010; Baker et al., 2003), with the bacterial primers 341 F (5′-CCTACGGGNGGCWGCAG-3′) and 806 R (5′-GACTACNVGGGTWTCTAATCC-3′). PCR amplification of each sample was carried out in 25 μL of reaction volume, with 12.5 μL of 2X KAPA Hifi HotStart Ready Mix (Kapa Biosystems Ltd., London, UK), 1 μM of each primer, 2 μL of DNA (10 ng/μL), and 9.5 μL of ddH20. All PCR reactions were carried out using a Verity™ 96-well Thermal Cycler, according to the following protocol: 95 °C for 5 min and 25 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 40 s, with a final elongation step of 72 °C for 5 min. PCR products were checked by gel electrophoresis and cleaned using an Agencourt AMPure XP system (Beckman Coulter, Brea, CA, USA), following the manufacturer's instructions. After seven PCR cycles (16S metataxonomic Sequencing Library Preparation, Illumina), Illumina adaptors were attached (Illumina Nextera XT Index Primer). Libraries were purified using Agencourt AMPure XP (Beckman Coulter, Brea, CA, USA) and then sequenced on an Illumina® MiSeq (Run Chemistry: 2 × 300 PE) platform (MiSeq Control Software 2.0.5 and Real-Time Analysis software 1.16.18, Illumina, San Diego, CA, USA).

2.10. Illumina data analysis and sequence identification by QIIME2

Raw paired-end FASTQ files were demultiplexed using idemp (https://github.com/yhwu/idemp/blob/master/idemp.cpp, last access 28/11/2024) and imported into Quantitative Insights in Microbial Ecology (Qiime2, version 2020.11). Sequences were quality-filtered, trimmed, de-noised, and merged using DADA2 (Callahan et al., 2016) chimeric sequences were identified and removed, using the consensus method, in DADA2. Representative sequences were aligned with MAFFT and used for phylogenetic reconstruction in FastTree, using plugin alignment and phylogeny (Katoh and Standley, 2013; Price et al., 2009). Taxonomic and compositional analyses were conducted by using the plugin feature-classifier (https://github.com/qiime2/q2-feature-classifier, last access 28/11/2024). A pre-trained Naive Bayes classifier based on the Greengenes 13_8 99 % operational taxonomic units (OTUs) database (http://greengenes.secondgenome.com/, last access 28/11/2024), which had previously been trimmed to the V4 region of 16S rDNA and bound by the 341 F/805 R primer pair, was applied to paired-end sequence reads to generate taxonomy tables. The data generated by Illumina sequencing were deposited in the NCBI Sequence Reads Archive (SRA), BioProject accession number PRJNA1143416.

2.11. Statistical analysis

Different statistical analyses were conducted to perform the appropriate model on the base of the data.

Regarding the ruminal parameters, the R software v.4.1.1. (R Core Team 2021) was used. The following linear mixed model was performed:

$$y_{ijklmn} = \mu + Treatment_i + Rumen\ fluid_j + Run_k + Position_l(Incubator_m) + e_{ijklmn}$$

where y_{ijklmn} is the observed traits; μ is the overall mean; $Treatment_i$ is the fixed effect of the ith treatment ($i = 4$ treatments; Mp, rMp, erMp, CTRL); $Rumen\ fluid_j$ is the random effect of the jth donor cow ($j = 3$ rumen fluids); Run_k is the random effect of the kth *in vitro* incubation run ($k = 3$ *in vitro* runs); $Position_l(Incubator_m)$ is the random effect of the lth position of the bottle ($l = 2$ position; up and down) in the mth incubator ($m = 2$ incubators); and e_{ijklmn} is the residual random error term. All the random effectd and residuals were assumed to have a normal distribution with a mean of zero and a variance of σ_n^2.

Orthogonal contrasts ($p < 0.05$) were built for all the investigated traits, and compared the effect of Mp compared to CTRL, and the effect of rMp and erMp compared to Mp.

Regarding Illumina Data analysis: Alpha-diversity determination was performed with the observed OTUs number and Shannon diversity index, and statistical significance between groups was evaluated by the Kruskal–Wallis H test in QIIME2; beta-diversities were calculated using the unweighted and weighted dissimilarity distance matrix in QIIME2. The beta-diversity distance matrix indicates differences in taxa composition between samples based on either presence–absence or quantitative species abundance data. Statistical significance of the beta-diversity distances between groups was assessed using PERMANOVA with 999 permutations in QIIME2.

Statistical significance of the concentrations of compounds of rumen fluid and the ratios of Firmicutes/Bacteroidetes between fermentations were tested using the Kruskal–Wallis *H*-test with the associated Dunn test, performed using XLSTAT version 2024.3.0. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Diet composition and total polyphenols content (TPC)

Table 1 shows the chemical composition and TPC of the experimental diets. The extract of *Mentha piperita* (eMp) was excluded from compositional analysis due to its liquid form.

Compared to the *Lolium multiflorum Lam.* (80.8 g/kg DM), both *Mentha piperita* and the peppermint residue after oil extraction showed a higher crude protein (CP) content, reaching 126.7 and 162.1 g/kg DM, respectively. Fiber content (aNDF and ADF) varied significantly among treatments, ranging from 353.5 to 590.2 g/kg DM, and from 240.5 to 330.7 g/kg DM, respectively. *Lolium multiflorum* Lam. exhibited the lowest values for acid detergent lignin (ADL), ash, and non-structural carbohydrates (NSC) as compared to the peppermint-based treatments. Total polyphenols content was substantially higher in the peppermint-based treatments. While *L. multiflorum* contained 1.41 g/kg DM of TPC, Mp and rMp contained 16.14 and 16.65 g/kg DM, respectively. The eMp exhibited a markedly concentrated TPC of 69.83 g/kg of extract.

3.1.1. Diets degradability and fermentation's products

Table 2 summarizes the *in vitro* rumen fermentation traits after 24 h of incubation. Among the measured parameters, pH showed significant differences across treatments, while dry matter degradability (dDM), total gas production (GP), gas per unit of degraded DM (GP/dDM), and ammonia nitrogen (N-NH_3) did not show significant overall treatment effects. Final pH was lower in CTRL and erMp than in Mp and rMp ($P < 0.01$). dDM was similar in CTRL, Mp, and rMp (42.7–44.8 g/100 g DM) but reduced in erMp (36.6 g/100 g DM), although not statistically significant.

Total GP ranged from 164 mL/g DM (CTRL) to 185 mL/g DM (rMp). The difference between rMp and CTRL was significant ($P < 0.05$), indicating enhanched gas production from the peppermint residue. N-NH_3 values ranged from 9.76 to 10.38 mMol/L, and did not differ significantly among treatment.

3.1.2. Volatile fatty acid (VFA) profiles

Table 3 presents the VFA composition of the fermentation fluid after 24 h of incubation. Significant treatment effects were observed for most VFAs except iso-butyric, caproic, and total branched-chain VFAs (sum of iso-butyric, iso-valeric, and n-valeric acids). Acetic acid proportions were significantly higher in Mp (57.9 %), rMp (57.1 %), and erMp (57.0 %), than in CTRL (52.7 %). By contrast, n-butyric acid was significantly lower in all peppermint-based treatments (14.9–15.7 %) than in CTRL (19.5 %). Propionic acid was slightly lower in peppermint-based treatments, (17.6–19.2 %), than in CTRL (19.9 %), with rMp showing a significant reduction. Iso-valeric acid and n-valeric acids increased moderately in rMp and erMp. No significant differences were detected for iso-butyric, caproic, or the combined branched-chain VFAs.

3.1.3. Rate and cumulative kinetics of gas production (GP)

The effects of the dietary treatments on gas production and composition after 24 h of *in vitro* rumen fermentation are presented in Tables 4 and 5. Methane yield per gram of DM ranged from 24.8 mL/g (Mp) to 30.4 mL/g (erMp) and only Mp produced a lower amount of methane compared to erMp ($P < 0.05$). Hydrogen production, expressed both as mL/g DM and % v/v, was not significantly affected by treatment. Methane concentration in the gas phase (CH_4 % v/v) was significantly influenced by treatment. The Mp treatment showed the lowest CH_4 proportion compared to CTRL (18.0 %, $P < 0.001$) and the erMp (17.3 %; $P < 0.01$) while rMp displayed an intermediate CH_4 concentration (16.4 %) that did not differ significantly from CTRL. Methane production per gram of degraded dry matter (CH_4 mL/g dDM) was lowest in Mp (56.3 %) compared to erMp (91.7 %, $P > 0.05$).

Estimates of gas production and methane derived from VFA stoichiometry (Table 5) do not support this trend and instead suggest an opposite effect of Mp on methane production. Methane calculated as a percentage of total gas (CH_{4VFA} %, v/v) was significantly higher in all peppermint treatments compared to CTRL, with values ranging from 17.7 % (Mp) to 18.1 % (erMp) vs. 16.3 % in CTRL. The contrasts confirmed significant differences between CTRL and both Mp and rMp, while no difference was observed between Mp and erMp. In terms of volume, CH_{4VFA} mL/g DM was significantly higher in rMp than CTRL, while erMp and Mp were numerically higher but not statistically different. Methane volume per degraded DM (CH_{4VFA} mL/g dDM) peaked in erMp (96.2 mL/g dDM), with rMp (79.7 mL/g dDM) and CTRL (67.5 mL/g dDM) in the intermediate range; however, treatment effects for this variable were not significant. Total gas production estimated by VFA (GP_{VFA}) did not differ significantly across treatments.

Table 2
In vitro rumen fermentation traits (at 24 h) of treatments.

Treatments	pH	DMd, g/100 g DM	GP, mL/g DM	GP, mL/g dDM	N-NH_3, mMol/L
Lolium multiflorum Lam. (CTRL)[a]	6.75	42.7	164	412	10.13
Mentha piperita L. (Mp)	6.84	44.8	168	374	9.76
Mentha piperita residue (rMp)	6.81	44.8	185	415	10.21
Extract + Mp residue (erMp)	6.75	36.6	183	514	10.38
SE[c]	0.03	2.81	16	41.3	0.22
P-value[b]	**	ns	ns	ns	ns
Contrasts					
CTRL *vs Mentha piperita*	***	ns	ns	ns	ns
CTRL *vs* rMp	**	ns	*	ns	ns
Mentha piperita vs erMp	**	ns	ns	ns	ns

DMd: Dry Matter degradability; GP: Gas Production; N-NH_3: ammonia nitrogen; SE: Standard Error.
ns: non-significant; * *P*-value < 0.05; ** *P*-value < 0.01; *** *P*-value < 0.001.
[a] *Lolium multiflorum* Lam. was used in the *in vitro* test as a control.
[b] The *P*-value refers to the significance of the treatments in the statistical model.
[c] SE: Standard Error.

Table 3
Volatile fatty acid proportions (g/100 g VFA) after 24 h of incubation of treatments.

Treatments	Acetic acid	Propionic acid	iso-butyric acid	n-butyric acid	iso-valeric acid	n-valeric acid	Caproic acid	Branched[a]
Lolium multiflorum Lam. (CTRL)[b]	52.7	19.9	2.19	19.5	2.63	1.72	1.36	6.55
Mentha piperita L. (Mp)	57.9	19.2	2.17	15.0	2.51	1.44	1.94	6.15
Mentha piperita residue (rMp)	57.1	18.4	2.62	14.9	3.06	1.66	2.37	7.35
Extract + Mp residue (erMp)	57.0	17.6	2.28	15.7	3.39	1.79	2.07	7.44
SE[c]	0.75	0.49	0.43	0.42	0.28	0.13	0.55	0.70
P-value[d]	***	**	ns	***	*	*	ns	ns
Contrasts								
CTRL *vs Mentha piperita*	***	ns	ns	***	ns	*	ns	ns
CTRL *vs* rMp	***	*	ns	***	ns	ns	ns	ns
Mentha piperita vs erMp	ns	*	ns	ns	*	**	ns	ns

ns: non-significant; * *P*-value < 0.05; ** *P*-value < 0.01; *** *P*-value < 0.001
[a] Branched: iso-butyric acid + iso-valeric acid + n-valeric acid.
[b] *Lolium multiflorum* Lam. was used in the *in vitro* test as a control.
[c] SE: Standard Error.
[d] The *P*-value refers to the significance of the treatments in the statistical model.

Table 4
In vitro gas composition and production (hydrogen, H_2; methane, CH_4) at 24 h of incubation of treatments.

Treatments	CH_4, mL/g DM	H_2, mL/g DM	CH_4, % v/v	H_2, % v/v	CH_4, mL/g dDM	H_2, mL/g dDM
Lolium multiflorum Lam. (CTRL)[a]	29.2	0.87	18.0	0.54	71.3	2.15
Mentha piperita L. (Mp)	24.8	0.97	14.7	0.60	56.3	2.21
Mentha piperita residue (rMp)	30.2	0.77	16.4	0.42	70.5	1.79
Extract + Mp residue (erMp)	30.4	1.01	17.3	0.56	91.7	3.13
SE[b]	2.87	0.15	0.56	0.07	9.37	0.47
P-value[c]	ns	ns	**	ns	ns	ns
Contrasts						
CTRL *vs Mentha piperita*	ns	ns	***	ns	ns	ns
CTRL *vs* rMp	ns	ns	ns	ns	ns	ns
Mentha piperita vs erMp	*	ns	**	ns	*	ns

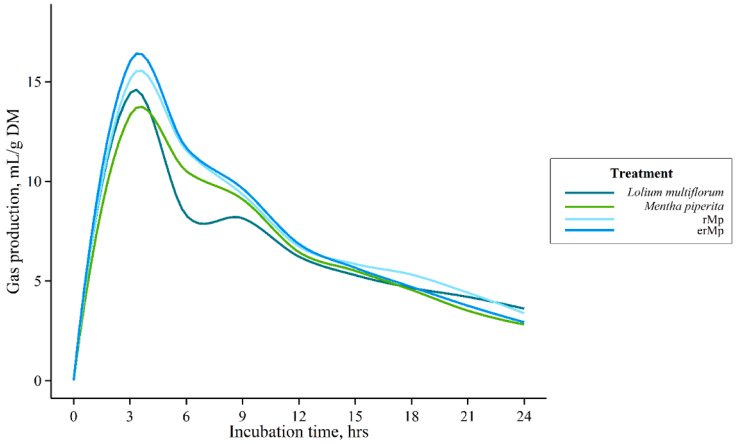
ns: non-significant; * *P*-value < 0.05; ** *P*-value < 0.01; *** *P*-value < 0.001
[a] *Lolium multiflorum* Lam. was used in the *in vitro* test as a control.
[b] SE: Standard Error.
[c] The *P*-value refers to the significance of the treatments in the statistical model.

Table 5
In vitro gas production (GP_{VFA}) and methane production (CH_{4VFA}) computed from volatile fatty acid production at 24 h of incubation of treatments (Blümmel et al., 1999).

Treatments	GP_{VFA}, mL/g DM	GP_{VFA}, mL/g dDM	CH_{4VFA}, % v/v	CH_{4VFA}, mL/g DM	CH_{4VFA}, mL/g dDM
Lolium multiflorum Lam. (CTRL)[a]	162	414	16.3	26.5	67.5
Mentha piperita L. (Mp)	167	375	17.7	29.6	66.6
Mentha piperita residue (rMp)	194	445	17.9	34.7	79.7
Extract + Mp residue (erMp)	188	529	18.1	34.1	96.2
SE[b]	18.2	53.8	0.13	3.22	9.55
P-value[c]	ns	ns	***	*	ns
Contrasts					
CTRL *vs Mentha piperita*	ns	ns	***	ns	ns
CTRL *vs* rMp	ns	ns	***	*	ns
Mentha piperita vs erMp	ns	ns	ns	ns	ns

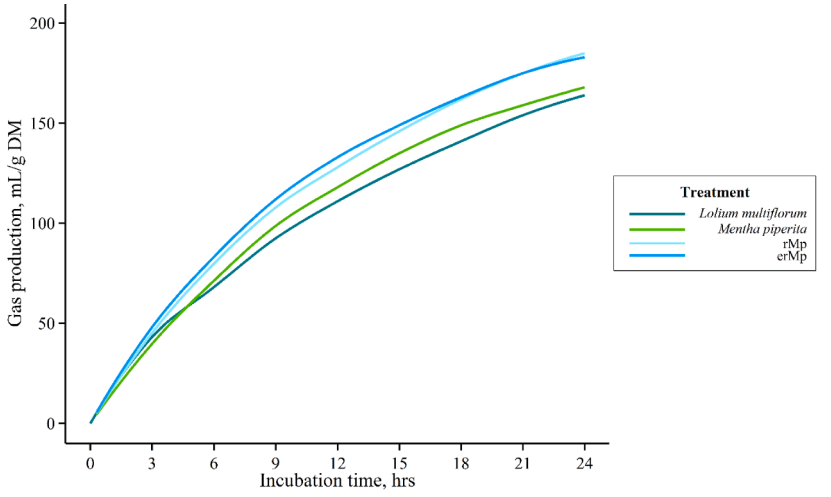
ns: non-significant; * *P*-value < 0.05; ** *P*-value < 0.01; *** *P*-value < 0.001
[a] *Lolium multiflorum* Lam. was used in the *in vitro* test as a control.
[b] SE: Standard Error.
[c] The *P*-value refers to the significance of the treatments in the statistical model.

Figs. 1 and 2 present the gas production kinetics over 24 h of *in vitro* rumen fermentation across the four dietary treatments. In Fig. 1, which reports gas production rate (mL/g DM/h), the Mp and rMp treatments exhibited notably higher fermentation activity compared to CTRL, especially during the initial fermentation phase. Compared to the CTRL, the gas production rate of Mp was greater



Contrasts	3h	6h	9h	12h	15h	18h	21h	24h
CTRL *vs Mentha piperita*	ns	***	*	ns	ns	ns	*	***
CTRL *vs* rMp	ns	***	**	ns	ns	ns	ns	ns
Mentha piperita vs erMp	*	ns	ns	ns	ns	ns	ns	ns

Fig. 1. *In vitro* kinetics of gas production per h of incubation (mL/g DM/h) of treatments. In the table below are reported the contrasts. The significances are reported by 3-hours interval of incubation. The asterisks reported in the figure represent the level of significance for the results of the contrasts (absence: non-significant difference; * *P*-value < 0.05; ** *P*-value < 0.01; *** *P*-value < 0.001).



Contrasts	3h	6h	9h	12h	15h	18h	21h	24h
CTRL *vs Mentha piperita*	ns	ns	ns	ns	ns	ns	ns	ns
CTRL *vs* rMp	ns	*	*	*	*	*	*	*
Mentha piperita vs erMp	*	*	*	ns	ns	ns	ns	ns

Fig. 2. *In vitro* kinetics of cumulated gas production (mL/g DM) of treatments measured for 24 h. In the table below are reported the contrasts. The significances are reported by 3-hours interval of incubation. The asterisks reported in the figure represent the level of significance for the results of the contrasts (absence: non-significant difference; * *P*-value < 0.05; ** *P*-value < 0.01; *** *P*-value < 0.001).

at 9 and 21 h (P < 0,05), and lower at 12 and 24 h of incubation (P < 0,05). rMp also showed a higher gas production rate at 6 and 9 h, while the erMp was significantly lower than Mp at 3 h (P < 0.05).

Cumulative gas production data, shown in Fig. 2, further supports these observations. rMp had the highest cumulative gas volumes across most of the incubation, with significantly greater gas production than CTRL beginning from 6 h and continuing through the 24-hour period (P < 0.05 to P < 0.001). Mp showed a numerically higher cumulative gas production than CTRL throughout the fermentation, although these differences did not reach statistical significance. erMp showed lower cumulative gas production than Mp

during the early stages (3–9 h, $P < 0.05$), suggesting delayed fermentation activity; however, these differences diminished later in the incubation.

3.1.4. Taxonomic analysis of the in vitro rumen microbiota

MiSeq sequencing yielded 1756,020 quality-filtered reads. The taxonomic profile of the *in vitro* rumen fermentation bacterial microbiome revealed that at the phylum level (Table 6), The *in vitro* microbiota was dominated by Bacteroidetes (47–50 %) and Firmicutes (34–42 %). In peppermint-based fermentations (Mp, rMp, and erMp), the relative abundance of *Tenericutes* increased in peppermint treatments, especially in rMp and erMp (2.2 and 2.0 %, respectively). Verrucomicrobia remained below 0.2 %. Archea, Actinobacteria and Chloroflexi composed a smaller amount of the microbial population in all the samples. *Fibrobacteres* (2–4 %) were found in CTRL, Mp and rMp samples. *Fibrobacteres*.

The dominant taxa included *Prevotella, Streptococcus, Butyrivibrio, Lachnospiraceae, Ruminococcaceae* and *Succiniclasticum* (Fig. 3) aligned with previous studies (Zhao et al., 2025). Notably the Mp fermentation showed an increased relative abundance of *Lachnospiraceae, Selenomonas ruminantium* and *Tenericutes*.

3.1.5. Microbial diversity in Vitro rumen fermentation

To evaluate differences in the bacterial microbiota among the *in vitro* rumen fermentations, microbial richness (observed OTUs), diversity (Shannon index), and evenness were compared between treatments (Table 7). Observed OTUs were significantly affected by the substrate ($P = 0.041$), with CTRL showing the highest richness (79 ± 26) compared to Mp (59 ± 9), while rMp (68 ± 25) and erMp (64 ± 22) displayed intermediate values. The Shannon index also differed significantly across treatments ($P = 0.0025$), being highest in Mp (0.528 ± 0.016) and lowest in erMp (0.491 ± 0.017). Similarly, evenness was markedly influenced by treatment ($p < 0.001$), with CTRL exhibiting the highest value (0.932 ± 0.008) and erMp the lowest (0.638 ± 0.041). Overall, erMp showed the most pronounced reduction in both diversity and evenness compared to the other groups.

Beta diversity analysis of microbial communities, based on both weighted and unweighted UniFrac distances, revealed statistically significant differences between fermentation treatments (Table 8). Significant dissimilarities were observed between the CTRL and rMp samples (*weighted* Pseudo-F = 4.182, $P = 0.002$; *unweighted* Pseudo-F = 1.740, $P = 0.004$). CTRL also differed significantly from rMp (*weighted* Pseudo-F = 4.965, $P = 0.002$; *unweighted* Pseudo-F = 1.878, $P = 0.008$). Differences with erMp were weaker but still significant for unweighted UniFrac ($P = 0.043$), indicating shifts in presence/absence patterns.

Considering the Pairwise Comparisons among Mp treatments, rMp vs erMp was significantly different in both weighted ($P = 0.034$) and unweighted ($P = 0.028$) UniFrac metrics and rMp vs. erMp, were also significantly distinct (*weighted* Pseudo-F = 2.732, $P = 0.037$; *unweighted* Pseudo-F = 2.307, $P = 0.002$). These comparisons highlight that even among the Mp-based fermentations, the extraction process and re-addition of the essential oil induced marked community shifts.

3.1.6. Correlation between microbial taxa and in vitro ruminal fermentation parameters

Pearson's correlation (Fig. 4) revealed significant associations between bacterial taxa and fermentation parameters. *Lachnospiraceae* positively correlated with CH_{4VFA} %, and CH_4 (mL/g DM), suggesting a role in methane production. *Succiniclasticum* negatiively correlated with CH_4 per unit of VFA suggesting a contribution to more efficient fermentation. *Tenericutes* were also positively associated with CH_{4VFA} % and total methane. *Selenomonas ruminantium* was positively associated with hydrogen production (H_2 mL/g DM), in agree with its known hydrogen-producing role (Scheifinger et al., 1975). *Paraprevotellaceae* was positively correlated with pH, indicating a role in pH stability. Strong negative correlations ($r \leq -0.5$) were observed between *Lachnospiraceae* and ammonia nitrogen ($N\text{-}NH_3$), suggesting improved nitrogen assimilation. [Other Bacteroidales] were negatively correlated with acetic acid, indicating

Table 6
Phylum composition (expressed as percent) in *in vitro* rumen samples as revealed by high-throughput sequencing analysis. Grey squares represent the relative abundances in the range 1–10 %, dark grey squares represent the relative abundances in the range 11–100 %.

Sample	Rumen fluid	CTRL	Mp		0.350
Archaea	0.483	0.604	0.137	0.078	0.350
Actinobacteria	n.d.	0.100	n.d.	n.d.	0.111
Bacteroidetes	73.022	49.950	47.397	47.613	47.939
Chloroflexi	n.d.	0.294	0.084	0.199	0.132
Cyanobacteria	0.126	n.d.	n.d.	0.067	n.d.
Fibrobacteres	0.183	3.834	4.081	2.134	0.565
Firmicutes	19.711	33.597	40.274	41.291	42.179
Fusobacteria	n.d.	n.d.	0.111	n.d.	n.d.
Lentisphaerae	0.128	0.044	n.d.	0.062	n.d.
Planctomycetes	0.973	1.934	0.465	0.617	0.694
Proteobacteria	1.879	6.231	3.250	3.729	3.050
SR1	2.274	0.823	0.609	0.802	0.750
Spirochaetes	0.219	1.693	0.891	1.029	1.374
Synergistetes	n.d.	n.d.	n.d.	n.d.	0.091
TM7	0.498	0.842	0.227	n.d.	0.474
Tenericutes	0.504	0.053	1.965	2.215	2.074
Verrucomicrobia	n.d.	n.d.	0.509	0.165	0.215

n.d. not detected or < than 0.001

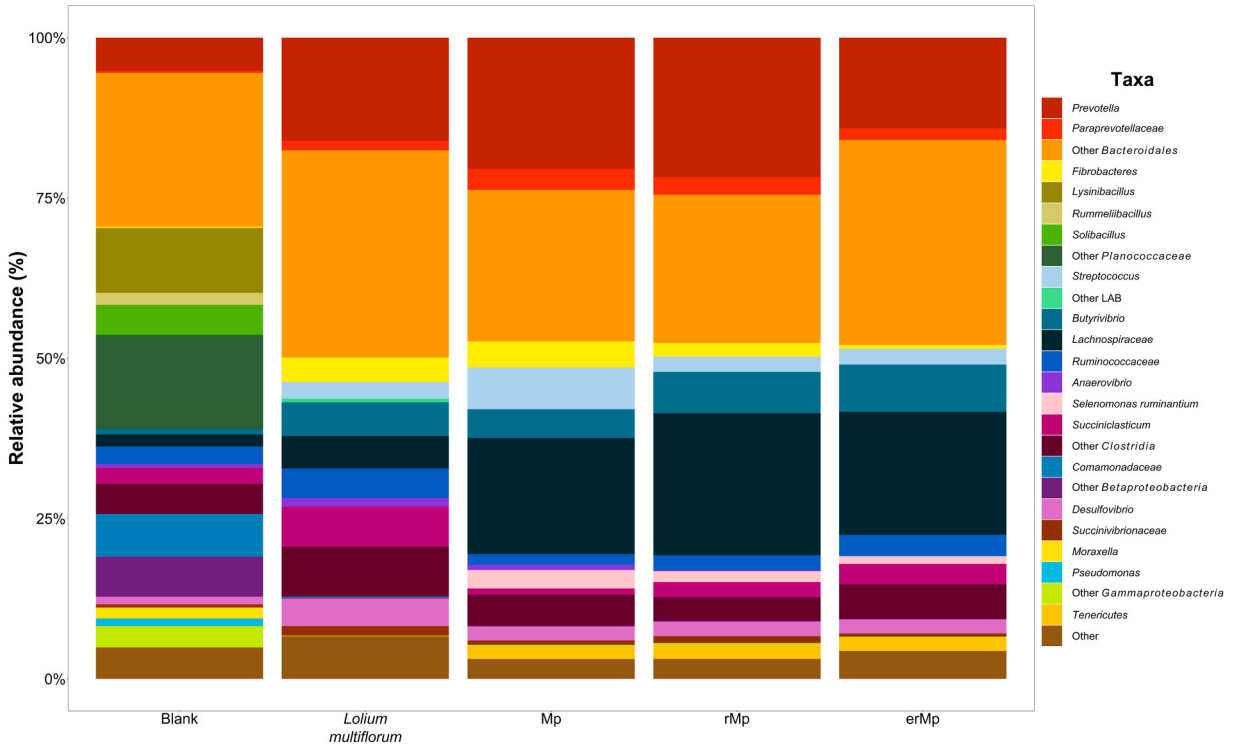


Fig. 3. Relative abundances (%) of bacterial taxa identified by MiSeq Illumina in *in vitro* rumen fermentation samples collected.

Table 7
Richness expressed as observed OTU number (Obs OTUs) and diversity expressed by Shannon, and Evenness indices of the bacterial communities identified by 16S amplicon sequencing of the peppermint-based and CTRL in vitro rumen fermentations. Results are shown as mean values and standard deviations (SD) of 9 values.

	Observed OTUs	Shannon	Evenness
CTRL	79 ± 26^{b}	0.517 ± 0.191^{ab}	0.932 ± 0.008^{c}
Mp	59 ± 9^{a}	0.528 ± 0.016^{b}	0.808 ± 0.027^{b}
rMp	68 ± 25^{ab}	0.501 ± 0.019^{a}	0.820 ± 0.027^{b}
erMp	64 ± 22^{ab}	0.491 ± 0.017^{a}	0.638 ± 0.041^{a}

For each variable (Obs OTUs, Shannon, and Evenness), values with different letters are significantly different ($p < 0.05$, one-way Anova with post hoc Tukey HSD).

Table 8
PERMANOVA analysis (999 permutations) results for bacterial communities based on weighted and unweighted UniFrac distances.

Pairwise Comparisons for peppermint-based Fermentation	Weighted UniFrac		Unweighted UniFrac	
	Pseudo-F	*P*-value	Pseudo-F	*P*-value
CTRL vs. Mp	4.182	0.002**	1.740	0.004**
CTRL vs. rMp	4.965	0.002**	1.878	0.008**
CTRL vs. erMp	1.619	0.112	1.490	0.043*
Mp vs. rMp	1.971	0.088	2.172	0.002**
Mp vs. erMp	3.298	0.034*	1.736	0.028*
rMp vs. erMp	2.732	0.037*	2.307	0.002**

Significance levels: * P-value < 0.05; ** *P*-value < 0.01

competition role with acetate producing pathways. *Streptococcus* and *Succiniclasticum* were both negatively associated with N-NH_3, that may indicate competition with acetogenic taxa or shifts toward alternative fermentation pathways such as propionate or branched-chain VFA formation. *Streptococcus* and *Succiniclasticum* were also negatively correlated with N-NH_3, supporting their involvement in nitrogen use.

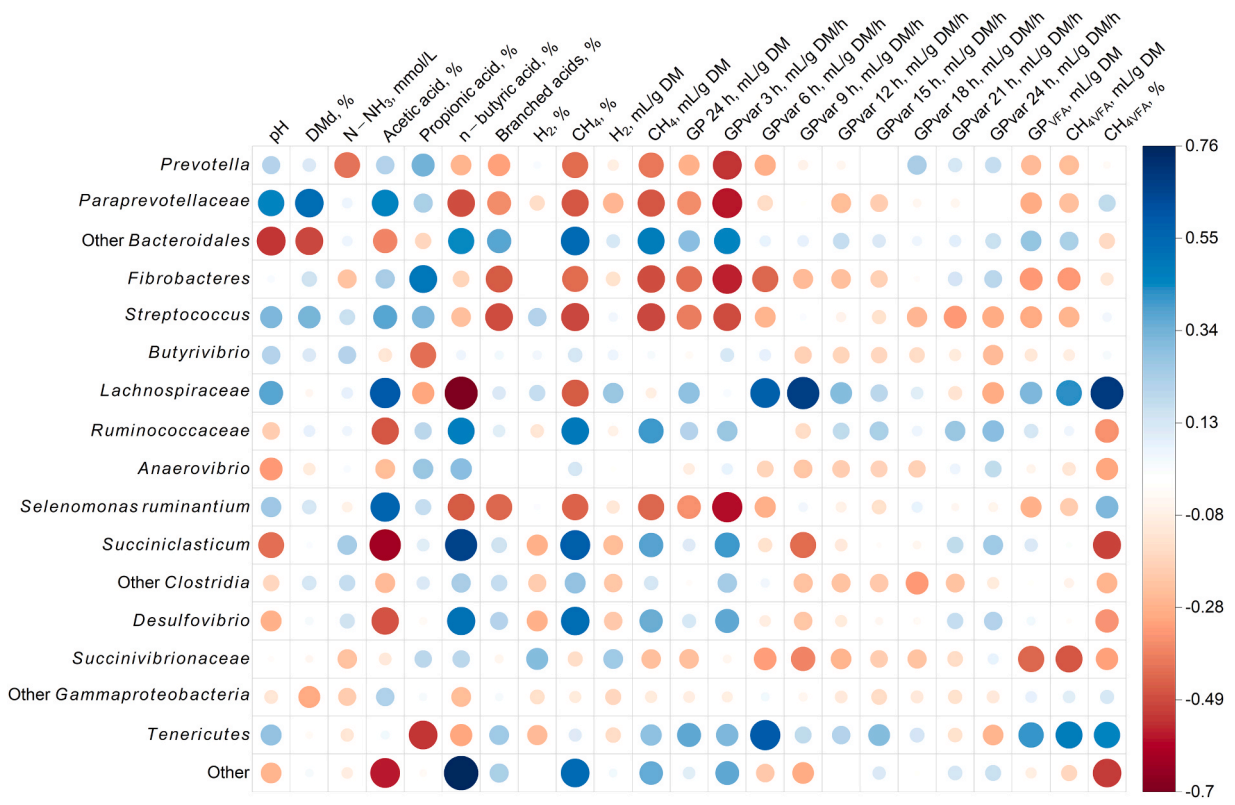


Fig. 4. Pearsons' correlation between taxa and ruminal parameters.

4. Discussion

The pH values observed in this study indicate a moderate but significant modulation of fermentation conditions by peppermint-based substrates. The significantly higher pH in the *Mentha piperita* (Mp) treatment compared to *Lolium multiflorum* Lam. (CTRL) suggests a buffering effect of the unprocessed peppermint biomass. This effect may be attributed to its content of alkaline minerals as well as polyphenols and essential oil components, which are known to influence microbial metabolism and substrate fermentation (Patra and Yu, 2012; Calsamiglia et al., 2007). The rMp residue also maintained a slightly elevated pH compared to CTRL, supporting the idea that even after supercritical CO_2 extraction, the biomass retains functional compounds able of modulating rumen fermentation conditions. In contrast, the erMp treatment, where essential oil extract was reintroduced into the residue, resulted in pH values similar to CTRL, suggesting a loss of buffering capacity or enhanced acidogenesis, possibly due to the antimicrobial and acidogenic properties of concentrated essential oil compounds (Cobellis et al., 2016). Despite differences in pH, dry matter degradability (DMd) did not differ significantly among treatments. Both mint plant (Mp) and residual mint plant (rMp) showed degradability comparable to the CTRL, indicating that neither the unprocessed Mp nor its extracted residue impaired microbial breakdown of the substrate. This finding supports the feasibility of incorporating peppermint biomass and its extraction residues as a partial forage replacement without compromising rumen degradability, even though their lignin (ADL: 8 vs. 4 % DM, respectively; Table 1) and total polyphenol contents are higher compared to of grass hay.

However, gas production dynamics revealed subtle but meaningful differences. The rMp residue produced significantly higher total gas per gram of DM than the CTRL, indicating enhanced fermentability. This effect is likely attributable to the physical alterations of the plant's soluble and fibrous structures caused by the supercritical CO_2 extraction process. The erMp also produced relatively high gas volumes although not significantly different from CTRL or Mp, maybe due to the reduced dDM observed in this group, which may have limited the availability of fermentable substrate. The higher gas production per unit of degraded DM (GP, mL/g dDM) observed in erMp suggests that, despite lower substrate degradation, the fermented fraction yielded proportionally more gas, potentially reflecting a shift in microbial fermentation pathways toward more gas-intensive end products, although this trend did not reach statistical significance.

Ammonia nitrogen ($N\text{-}NH_3$) concentrations remained stable across all treatments, indicating that neither peppermint biomass nor its derivatives adversely affected nitrogen metabolism or microbial protein synthesis under the *in vitro* conditions tested. This is an important consideration for the inclusion of plant-based by-products in ruminant diets, as excessive ammonia accumulation generally reflects inefficient protein utilization. Although essential oils and tannins extracted from medicinal plants are often reported to exert antimicrobial effects that can inhibit ruminal microbial activity and fiber degradation, such effects were not evident in the present

study (Patra and Yu, 2012).

The volatile fatty acid (VFA) shifts observed align closely with the fermentation dynamics described by Massaro et al. (2025) who demonstrated that aromatic alpine herbs redirected hydrogen (H_2) away from methanogenesis toward alternative sinks such as acetate and branched-chain VFAs. In the present study, inclusion of Mp, rMp and erMp led to a consistent and significant increase in acetic acid proportions ($P < 0.001$ vs. CTRL), supporting the hypothesis that acetogenesis becomes a more prominent hydrogen sink in the presence of plant secondary metabolites, particularly polyphenols and terpenoids, both abundant in peppermint. The concomitant reduction in n-butyric acid, in particular in rMp (14.9 % vs. 19.5 % in the CTRL; $P < 0.001$), further reinforces this shift, because butyrate formation is also a hydrogen consuming pathway, and its suppression suggests inhibition of butyrate-producing microbial populations or a redirection of energy flow toward acetate production (Fagundes et al., 2020). Similar reductions in butyrate have been reported by Busquet et al. (2006) when rumen fluid was incubated with herb-rich substrates, highlighting a functional trade-off between butyrogenesis and acetogenesis under phytochemical pressure. In contrast to studies reporting increased propionate production, (Phupaboon et al., 2025) our results showed a slight but significant decrease in propionic acid, particularly in rMp and erMp. This discrepancy may reflect differences in herb composition and phytochemical profiles as mint residues may selectively influence microbial groups less associated with propionate formation. Nevertheless, both studies demonstrate a restructuring of fermentation pathways away from methane and toward alternative VFA profiles. The increased iso-valeric and n-valeric acid concentrations observed in erMp further suggest enhanced amino acid fermentation, potentially as a microbial adaptation to the antimicrobial action of the reintroduced essential oil extract. Massaro et al. (2025) similarly reported increased minor and branched-chain VFAs as indicators of metabolic flexibility under altered microbial selection pressure.

Gas production and composition demonstrate that Mp, particularly in its whole plant form, can alter rumen fermentation patterns in ways that reduce methane output. This effect is accompanied by an apparent contradictory increase of methane estimated from VFA stoichiometry. Several mechanisms may explain this response. First, changes in CO_2 solubility may have altered the proportion of CH_4 within total gas production. The increase in pH observed with peppermint could have promoted greater CO_2 release from the fermentation medium; however, the absence of differences between measured GP and gas production predicted from VFA profiles suggests that this effect was quantitatively limited.

Second, direct inhibition of methanogenic archaea by bioactive compounds could explain the lower measured CH_4 despite VFA stoichiometry predicting greater H_2 production. Nevertheless, the lack of H_2 accumulation in fermentation gases indicates that this mechanism likely played a minor role. Finally, the assumptions underlying VFA stoichiometry may have been violated due to the activation of alternative H_2 sinks. The shift in the fermentation pattern, characterized by an accumulation of acetate relative to propionate and butyrate, together with changes in the microbial profile, suggests that enhanced acetogenesis may represent a relevant alternative pathway for H_2 utilization (Ungerfeld, 2020). This interpretation is supported by the increased acetate production observed in Mp fermentations (Table 3), indicating enhanced acetogenesis, a known competitive sink for hydrogen sink in the rumen (Li et al., 2025). This functional redirection mirrors the metabolic adaptations described by Hundal et al. (2021), where herbs shifted fermentation toward acetate and branched-chain VFAs, away from methane and butyrate. Interestingly, the rMp treatment preserved a portion of this functionality, producing methane levels comparable to CTRL.

The rMp treatment retained part of this functionality, producing methane levels comparable to CTRL, suggesting that although residual biomass remains fermentable, its ability to suppress methanogenesis is partially diminished after supercritical CO_2 extraction. However, its capacity to promote acetate production supports its potential as a sustainable feed ingredient within circular economy models. In contrast, reintroduction of the mint extract (erMp) resulted in the highest methane per unit of degraded DM and the least favorable CH_4 profile, despite relatively high total gas production. This pattern may reflect overexposure to bioactive compounds, such as menthol and menthone, which, at elevated concentrations, can exert antimicrobial stress, selectively inhibiting fibrolytic and acetogenic populations. Huang et al., (2023) similarly, warned that phytochemical rich treatments may become counterproductive when not properly dosed or balanced, leading to partial restoration of methanogenesis or microbial inefficiencies. Hydrogen concentrations remained stable across treatments, with no significant accumulation. This supports the conclusion, also emphasized by (Massaro et al., 2025), that effective redirection of hydrogen does not necessarily result in measurable H_2 buildup, when alternative sinks such as acetogenesis or amino acid fermentation are active. Thus, the reduced methane observed in Mp, despite unchanged H_2, suggests that hydrogen redirection rather than hydrogen sparing.

Gas production kinetics further demonstrate that both the Mp and rMp were fermented more rapidly than grass hay, particularly during the most active phases of microbial metabolism. The early and sustained increase in gas production observed in Mp suggests a rapid microbial colonization, likely due to its higher content of non-structural carbohydrates (NSC) and readily fermentable fiber. Similarly, the rMp performance indicates that structural modifications induced by CO_2 extraction may have improved substrate accessibility, resulting in higher cumulative gas output.

In contrast, erMp showed delayed gas production during early incubation, suggesting transient microbial inhibition. This effect is likely associated with essential oil compounds with antimicrobial properties; while this inhibitory effect is absent in rMp (residual after oil extraction) reintroduction of the purified extract (erMp) may have altered microbial activity due to changes in compound concentration or structure following SFE.

Taken together, fermentation kinetics indicate that peppermint-based substrates can modify gas production dynamics depending on their form and processing. Compared to whole Mp, rMp stimulate a faster and more extensive fermentation, potentially supporting shifts toward acetate production and away from methanogenesis. In contrast, the extract-supplemented residue appears to delay microbial activity and may impair fermentation kinetics when not properly dosed. These findings emphasize the importance of both substrate composition and the balance of bioactive compounds in shaping rumen microbial responses and fermentation performance.

4.1. In vitro rumen fermentation microbiota composition

The observed microbial community shifts indicate that peppermint biomass and its extraction residues modulate the rumen ecosystem by selectively promoting or suppressing specific taxa. The increased abundance of *Lachnospiraceae* in peppermint treatments is consistent with their role as acetogenic fiber degraders (Gagen et al., 2015). Some members of this family convert H_2 and CO_2 to acetate via the acetyl-CoA pathway, contributing to the elevated acetate levels observed (Table 3). The higher abundance of *Selenomonas ruminantium* supports its role in lactate and sugar fermentation, producing acetate, CO_2, and H_2 (Scheifinger et al., 1975). The increased abundance of *Tenericutes* in Mp treatments is noteworthy, although not classical acetogens, these taxa may participate in hydrogen flux regulation or acetate metabolism, under conditions that suppress dominant microbial taxa (Wang et al., 2020).

Prevotella, a dominant genus involved in proteolysis and carbohydrate degradation (Deusch et al., 2017), was promoted in mint treatments compared to the grass hay due to the higher content of readily fermentable carbohydrates but was suppressed in erMp, suggesting inhibitory effects of concentrated essential oil extracts. This observation aligns with the known antimicrobial activity of peppermint terpenoids such as menthol and menthone (Camele et al., 2021). While TPC provides an overall estimate of antioxidant potential, a detailed GC–MS profiling of individual volatile compounds (e.g. menthol, menthone, carvacrol) would be required to directly link specific bioactive molecules to the observed microbiota shifts. Future studies will address this aspect to strengthen mechanistic interpretation. Overall, these findings indicate that the efficacy of essential oils depends on their physical association with the feed: when added separately from the plant matrix (erMp vs. Mp), their antimicrobial action may be stronger, whereas when embedded within the plant structure (Mp vs. rMp), they may act as a barrier to microbial colonization and fiber degradation, but with a milder antimicrobial effect. The taxonomic shifts observed support a functional realignment of the rumen microbiota under Mp treatments showing: a shift toward acetate and methane-oriented pathways, enhancing presence of acetogens and hydrogen producers and the suppression of some sensitive fibrolytic or proteolytic taxa including *Fibrobacteres, Prevotella spp.,* and *Butyrivibrio spp.*

Such realignments are consistent with ecological responses to phytochemical pressure, where resilient or specialized taxa become dominant (Shabat et al., 2016). While this selective adaptation may enhance short-term fermentation efficiency, it can simultaneously reduce microbial diversity, an important driver of functional redundancy and ecosystem stability, ultimately compromising rumen resilience. This trade-off between efficiency and stability was also evident in the present study (Guo et al., 2024).

4.2. Microbial diversity of in vitro rumen fermentation

Based on both the number of observed OTUs and evenness, the highest microbial richness was found in the CTRL fermentations, while peppermint-based treatments, particularly erMp, resulted in a marked reduction in microbial richness, diversity, and evenness. This decline in alpha diversity, most pronounced in the extract-supplemented treatment, is likely attributable to the antimicrobial activity of essential oil constituents, including menthol, menthone, and other monoterpenes. These compounds are known to exert bacteriostatic and bactericidal effects, especially against Gram-positive bacteria, and have been shown to inhibit various rumen microbial populations in a concentration and exposure dependent manner (Patra and Yu, 2012, 2015).

Reduced richness and evenness imply a loss of microbial complexity and ecological balance, potentially limiting functional redundancy and resilience of the microbial community (Weimer, 2015). This can lead to a more selective community structure dominated by certain taxa, as reflected in our taxonomic analysis. Treatments with peppermint, particularly Mp, showed increased relative abundances of Lachnospiraceae and *Selenomonas*, taxa often associated with acetate production and alternative hydrogen sinks (Gagen et al., 2015; Sawanon et al., 2011). These findings are consistent with (Schären et al., 2018) and further supported by (Andersen et al., 2025), who demonstrated that alpine herbs rich in secondary metabolites induced similar shifts *in vitro*, promoting acetogenic and lactate-utilizing bacteria while reducing overall diversity. Moreover, our findings directly align with those of Massaro et al., (2025) who observed that herbs altered microbial composition in ways that diverted hydrogen away from methanogenesis, favoring functional groups involved in acetogenesis and amino acid fermentation.

Despite lower diversity, some studies suggest that such simplified microbial ecosystems may be more efficient under specific conditions, particularly when dominant taxa are functionally aligned with substrate characteristics (Shabat et al., 2016). This trade-off between functional efficiency and ecological resilience, is evident in our results: peppermint-based treatments, while potentially promoting more streamlined fermentation (e.g., higher acetate, lower butyrate), may also make the microbial ecosystem more sensitive to perturbation or less adaptive to dietary changes.

The PERMANOVA analysis confirmed that the overall microbial structure was significantly altered in all Mp-based treatments compared to CTRL. These changes are characteristic of selective microbial suppression and niche opening, as induced by phytochemicals like monoterpenes and polyphenols. Essential oils are known to suppress sensitive taxa, particularly fibrolytic and methanogenic populations, while allowing resistant or functionally specialized microbes to proliferate (Patra and Saxena, 2010; Calsamiglia et al., 2007). The significance observed in both weighted and unweighted UniFrac distances confirms that peppermint treatments affected not only species abundance but also the presence/absence of lower-abundance taxa indicative of deep restructuring of the rumen microbial community.

These compositional shifts were functionally consistent with fermentation outcomes. For example, increased relative abundances of *Lachnospiraceae* and *Selenomonas ruminantium* in Mp and rMp corresponded with elevated acetate levels, suggesting enhanced acetogenesis. *Tenericutes*, also elevated in these treatments, may play a syntrophic or hydrogen-regulating role, indirectly supporting acetogenic processes, although they are not classical acetogens (Wang et al., 2020). This microbial profile closely parallels the community configurations reported by both Andersen et al. (2025) and Massaro et al. (2025), who found that herb-rich substrates promoted taxa involved in non-methanogenic hydrogen use and fermentation plasticity. These findings underscore a shared microbial

response to phytochemicals that support hydrogen redirection, methane reduction, and increased acetate output.

The Pearson correlation matrix further supports these functional links. *Lachnospiraceae* showed strong positive correlations with CH_4-related traits, consistent with their dual role in acetate and hydrogen production, which can be utilized by methanogens. Their negative correlation with ammonia ($N\text{-}NH_3$) suggests involvement in nitrogen assimilation or suppression of proteolysis. *Succiniclasticum* correlated negatively with $N\text{-}NH_3$, indicating a role in efficient microbial protein synthesis, likely via the succinate-propionate pathway. *Tenericutes* also showed positive correlations with methane and acetate, suggesting involvement in syntrophic or redox-sensitive metabolic roles. The strong correlation of *Selenomonas ruminantium* with hydrogen production is consistent with its function as a lactate- and sugar-fermenting organism producing H_2 and acetate (Sawanon et al., 2011). Finally, the negative correlation between other Bacteroidales and acetate proportion may reflect competition with acetogenic groups or shifts toward alternative fermentation products such as propionate (Zeng et al., 2019).

In summary, the integration of alpha- and beta- diversity metrics, taxonomic shifts, and correlation analyses clearly demonstrates that peppermint-based substrates, particularly those rich in essential oil compounds, restructure the rumen microbiota in functionally meaningful ways. These changes promote taxa involved in acetate- and gas-centered fermentation, often at the expense of diversity and stability, a dynamic consistent with the broader microbial shifts reported in herb-based rumen fermentation models (Andersen et al., 2025; Massaro et al., 2025). This supports the growing consensus that phytogenic feed additives can selectively steer rumen microbial metabolism toward more sustainable outcomes, though achieving the right balance of efficacy and ecological stability remains critical.

5. Conclusions

This study demonstrates the potential of peppermint (*Mentha* × *piperita L.*) by-products from supercritical CO_2 extraction as sustainable feed components in ruminant nutrition. Despite compositional alterations post-extraction, the residual biomass (rMp) supported *in vitro* rumen fermentation comparable to the unprocessed plant (Mp), showing similar dry matter degradability and enhanced gas production. Peppermint-based treatments, particularly Mp and rMp, increased acetate concentrations and altered gas composition, with Mp significantly reducing methane % v/v. Microbiota analysis revealed distinct community shifts under peppermint supplementation, including increases in *Lachnospiraceae*, *Selenomonas ruminantium*, and *Tenericutes*, taxa linked to acetogenesis, lactate metabolism, and hydrogen flux. These changes were accompanied by reduced microbial diversity, especially in the erMp treatment, suggesting selective microbial pressure from essential oil compounds.

These findings contribute to the understanding of how plant-derived by-products can redirect rumen fermentation pathways toward acetate production and potentially reduce methane emissions. They also support the application of circular economy principles in livestock production by converting peppermint extraction residues into valuable feed substrates. This approach aligns with EU bioeconomy strategies and offers a dual benefit: reducing agro-industrial waste and improving the environmental efficiency of ruminant systems. However, the reintroduction of concentrated essential oil extracts (erMp) may impair microbial diversity and fermentation dynamics if not properly managed. Further *in vivo* research is needed to confirm these effects on animal performance, nitrogen use efficiency, and enteric methane mitigation under practical feeding conditions.

Data availability

The raw data and the model supporting the conclusion of this manuscript will be made available by the authors, upon reasonable request. The model was not deposited in an official repository.

CRediT authorship contribution statement

Elena Franciosi: Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Franco Tagliapietra:** Writing – review & editing, Visualization, Validation, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Jonas Andersen:** Writing – review & editing, Methodology. **Selene Massaro:** Writing – review & editing, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Giulia Dallavalle:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Giorgia Secchi:** Writing – review & editing, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Nicola De Zordi:** Writing – review & editing, Methodology. **Urska Vrhovsek:** Writing – review & editing. **Andrea Angeli:** Writing – review & editing, Methodology.

Ethics approval

All procedures and experimental tests were conducted according to Italian legislation law relating to animal welfare. All practices for obtaining rumen fluid for the *in vitro* trials were approved by the Ethics Committee by the University of Padova (Italy). The approval number for this experiment is OPBA 1312041/2022 approved the 26 July 2022.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT (OpenAI) in order to assist with English language editing and

improving clarity of the manuscript, in line with Elsevier's and Animal's editorial policies. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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