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Research article

Mentha piperita (Peppermint) leaves as feed additive: *In vitro* ruminal gas, methane and carbon dioxide productions, nutrient degradability, and ruminal fermentation kinetics

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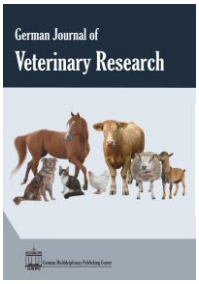

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Abstract

This study determined how phytochemicals in dried peppermint (*Mentha piperita*) affect *in vitro* ruminal gas production (GP), methane (CH_4) and carbon dioxide (CO_2) production, fermentation parameters, and nutrient degradability of a total mixed ration containing (per kg DM): 500 g concentrate feed mixture, 400 g berseem hay, and 100 g rice straw. Peppermint leaves were included at 0 (control), 0.5, 1.0, 1.5, and 2% of the diet, and the experiment lasted for 48 h. Each treatment was incubated in triplicate bottles across two independent runs, with two blank bottles (inoculum only) per run, to establish a baseline for fermentation GP. Peppermint ($p=0.002$) decreased the asymptotic CH_4 in a dose-dependent manner, and the lag was prolonged with an increasing level of peppermint in the diet. The diet containing 0.5% peppermint had the highest DM degradability, and the diet containing 2% peppermint had the highest neutral detergent fiber (NDF). In comparison, the diet with 1.5% peppermint had the highest acid detergent fiber (ADF) degradation. Diets containing 1.5% peppermint produced the highest ($p<0.05$) short-chain fatty acids (SCFAs), while 0% peppermint produced the lowest. The addition of peppermint (0.5-2%) improved GP, reduced CH_4, and increased the degradability of DM, NDF, and ADF, concentrations of total SCFAs, acetate, and propionate. The best-performing dose that is environmentally friendly and improves digestive parameters is 0.5-1% peppermint in the diet, but further *in vivo* studies are warranted.

Keywords: Degradability, *In vitro* fermentation, Methane, Phytogenics

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Introduction

Feed additives, such as essential oils, plant extracts, plant seeds, herbs, and spices, can improve the nutritional benefits derived from animal diets (Kholif, 2023; Kholif et al., 2024a; Morsy et al., 2024). This shift became necessary due to the ban on the use of medically important antibiotics in animal feed as growth promoters. The shift from antibiotic-based feed additives to phytogenic feed additives, with a focus on not only production but also efficient and sustainable production, is supported by various studies (Hosoda et al., 2005; Beyzi, 2020; Chesson, 2023; Kholif et al., 2024c). Given this, studies have ascertained the efficacy of phytochemicals as natural feed additives, especially those of phytogenic origin, as capable of enhancing rumen

fermentation efficiency, reducing methane (CH_4) and gas production (GP), and improving rumen ecology. Different essential oil blends (Ike et al., 2024), *Moringa oleifera* leaves (Morsy et al., 2022), *turmeric rhizomes* (Kholif et al., 2024b), and lupin seeds (Morsy et al., 2024) have been shown to improve nutrient degradability, enhance rumen fermentation, and reduce the production of biogases such as CH_4 and carbon dioxide (CO_2). These effects are linked to the presence of bioactive compounds in these additives, which modulate the composition and metabolic activity of microorganisms in the rumen (Benchaar et al., 2008; Kholif and Olafadehan, 2021; Kholif, 2023). Beyond their nutritional value, the environmental footprint of animal feeds has become an increasingly important factor in feed evaluation. A good feed additive should not only improve production but also reduce greenhouse gas (GHG) emissions from enteric fermentation (Gomaa et al., 2018).

A literature review conducted by Curabay et al. (2020) on the use of essential oil in alfalfa hay diets revealed that 1200 mg/L of peppermint oil reduced CO_2 and CH_4 production. However, it also resulted in decreased levels of dry matter (DM), organic matter (OM), crude protein (CP) digestibility, and metabolizable energy (ME). In an *in vivo* study with lambs, it was demonstrated that lambs given 2.5% dried *Origanum vulgare*, *Rosmarinus officinalis*, and peppermint exhibited the least digestibility, protozoa, and ammonia-N (NH_3-N) levels, but growth performance was not affected (Farghaly and Abdullah, 2021).

Aguiar et al. (2023) reported that 0.6 g/kg peppermint essential oil increased the activities of digestive enzymes (i.e., amylase, protease, and lipase) and antioxidant enzymes. Zhu et al. (2019) showed that 15 mL of peppermint oil possesses antioxidant and antimicrobial properties and improves digestive ability in the human stomach and intestine. In Nile tilapia, other studies have indicated that 3.3 g/kg of peppermint essential oil exhibits antimicrobial actions, which can contribute to maintaining a healthy gut microbiota (Zaminhan-Hassemer et al., 2022), improving feed palatability, and stimulating feed intake, thereby enhancing nutrient utilization (Cardoso et al., 2021). While various studies, including those by Tawfeeq et al. (2019) and Hosoda et al. (2005) have reported different effects of peppermint essential oil or extracts on ruminant digestibility, health, and CH_4 reduction, which may be explained by differences in the administered dosages, plant parts used, and the extraction methods employed.

Peppermint (*Mentha piperita*) has probiotic as well as antimicrobial activities, antioxidant properties, and immunomodulation functions (Raut and Karuppayil, 2014; Pavlić et al., 2021). This suggests that the exhibited effects may be a question of dosage or species. It has metabolites such as menthol, menthone, menthofuran, and pulegone (Barros et al., 2013). According to Riachi and De Maria (2015), peppermint leaves contain about 1.2–3.9% essential oil on a dry matter basis. The quantity and chemical profile of the oil are highly influenced by the plant's geographic origin, growth stage, specific peppermint species, genotypes (Lu et al., 2022), agronomic practices (Machiani et al., 2018), and environmental factors (Verma et al., 2016) can influence the concentration of plant metabolites in each herb, thereby eliciting varying responses and discrepancies in results. Given the various factors that can influence its yield and composition, more studies must take place, using different dosages and extraction methods to derive the best nutritional practices before they are fed to ruminants.

It was hypothesized that the bioactive compounds present in dried peppermint leaves could influence rumen fermentation patterns, lower GHG emissions, and enhance nutrient degradation. Accordingly, this study aimed to evaluate the impact of incorporating varying levels of dried peppermint leaves into the diet on in vitro gas production, including CH_4 and CO_2 emissions, as well as ruminal fermentation characteristics.

Material and methods

Ingredients and treatment

A basal total mixed ration (TMR) was formulated as the substrate, consisting of (per kg DM): 500 g concentrate feed mixture, 400 g berseem hay, and 100 g rice straw. This substrate mirrored the control diet utilized in earlier research (Kholif et al., 2024c; Morsy et al., 2024). The nutrient compositions of peppermint leaves, ingredients, and TMR are shown in Table 1.

Table 1: Nutrient composition (g/kg DM) of peppermint (*Mentha piperita*) leaves, individual feed components, and the experimental diet used for incubation.

Parameters[1]	*Mentha piperita*	CFM[2]	Berseem hay	Rice straw	Diet[3]
DM	935.7	903	890	940	893
OM	792.2	923	884	851	819
CP	100.3	165	128	42	136
EE	73.2	47	54	19	62
NFC	211	414	224	166	359
NDF	407.8	297	478	624	379
ADF	248.8	175	381	394	240

[1]Parameters: DM refers to dry matter, OM to organic matter, CP to crude protein, EE to ether extract, NFC to non-fibrous carbohydrates, NDF to neutral detergent fiber, and ADF to acid detergent fiber. [2]The concentrate feed mixture (CFM) per kilogram of DM included: 170 grams of soybean meal, 395 grams of wheat bran, 395 grams of maize, 20 grams of limestone, 10 grams of a vitamin and mineral premix, and 10 grams of salt. [3]Each diet, on a DM basis, consisted of 500 grams of concentrate mixture, 400 grams of berseem hay, and 100 grams of rice straw per kilogram

Mature peppermint whole leaves were sourced from a local supplier (Harraz, Bab El-Khalq, Cairo, Egypt), then cleaned and air-dried at 37 °C. Prior to essential oil analysis and the in vitro experiment, the peppermint leaves were ground in a blender to a 1 mm particle size and thoroughly mixed. Essential oil content was determined at the Central Laboratory of the National Research Centre (Egypt) using a Perkin Elmer Auto System XL gas chromatograph-mass spectrometer (GC-MS) equipped with a ZB-5 capillary column (60 m × 0.32 mm internal diameter; Agilent, USA). The injector temperature was initially held at 50 °C for 1 min, then increased at a rate of 3 °C per min until reaching 240 °C. Helium served as the carrier gas at a constant flow rate of 1 mL/min, with a split injection ratio of 1:10. The GC column effluent was introduced directly into the mass spectrometer (MS) source. Mass spectra were acquired in electron ionization (EI) mode at 70 eV. The sector mass analyzer was programmed to scan a mass range of 40–300 amu with a scan time of 1 s. Tentative identification of the compounds was achieved by comparing their relative retention times and mass spectra with those in the NIST and WILEY spectral libraries integrated into the GC-MS system.

In vitro fermentation and biodegradation

The *in vitro* fermentation medium was prepared following the method of Goering and Van Soest (1975). A reducing solution containing sodium sulfide (2 mL) was added to the buffer shortly before the addition of rumen fluid. Each 250 mL bottle was filled with 80 mL of buffer solution and 20 mL of ruminal inoculum.

Rumen fluid was obtained from three male Barki sheep (average body weight: 42 ± 0.6 kg; age: 25 ± 3 weeks) that had been raised for fattening and sourced from a slaughter facility in Cairo, Egypt. The animals were confirmed to be healthy by veterinary inspection prior to slaughter and exhibited no signs of disease or digestive disorders. Before slaughter, sheep were maintained under the same management conditions and fed the same diet for at least 30 days. The animals had unrestricted access to clean water and were offered a diet composed of concentrate feed, berseem hay, and rice straw in a dry matter ratio of 500:400:100. Rumen content was sampled following the standardized protocol for collection, handling, and utilization of ruminal fluid as outlined by Fortina et al. (2022). At the abattoir, rumen fluid was harvested within 10 min post-mortem. Approximately 250 g of rumen digesta was manually collected and pressed through a sieve into a plastic container using a colander. This procedure was repeated until a total volume of 1000 mL was obtained. The ruminal fluid was then passed through a double layer of cheesecloth to eliminate coarse feed particles. The retained solids were further squeezed to extract microorganisms adhering to the fibrous material. The initial pH of the collected inoculum was recorded at 6.5.

Approximately 1 g (±10 mg) of the total mixed ration (TMR) was accurately weighed and sealed in ANKOM F57 filter bags (Ankom Technology, Macedon, NY, USA). The prepared bags were then inserted into 250 mL fermentation vessels equipped with pressure sensors and connected to the ANKOMRF Gas Production System, an automated wireless in vitro gas production module (Ankom Technology, Macedon, NY, USA). Peppermint leaves were included at 0 (control), 0.5 (0.005 g), 1 (0.01 g), 1.5 (0.015 g), and 2% (0.02 g) of the diet on a DM basis. The levels were chosen based on ranges commonly used in *in*

vitro studies with phytogenic additives that demonstrated biological efficacy without causing inhibitory effects on fermentation (Kholif et al., 2024a; Morsy et al., 2024). Precise amounts of peppermint leaves were weighed and placed into filter bags using a Luna Analytical Balance (LAB 124e, Adam Scales & Balances, Thetford, UK). To determine baseline gas production during fermentation, two blank bottles containing only the inoculum, without any feed substrate, were included in each incubation batch. Each run consisted of five treatments with three replicates each, along with two blank controls. This procedure was conducted over two separate incubation runs on consecutive weeks.

Pressure readings were automatically logged at 10 min intervals over a 48 h incubation period, and cumulative pressure values were used to calculate total GP. These pressure readings were then converted to gas volumes (mL) under standard conditions (0 °C and 1 bar). To determine net GP, the gas volume measured in the blank control bottles was subtracted from the total values. At specific time points (2, 4, 6, 8, 10, 12, 24, 36, and 48 h) 5 mL gas samples were collected from the sampling ports and injected into a Gas-Pro detector (Gas Analyzer CROWCON Model Tetra3, Abingdon, UK) for the quantification of CH_4 and CO_2 concentrations. The entire procedure was replicated in two independent incubation runs conducted in successive weeks.

Collection and evaluation of fermentation parameters

Following 48 h of incubation, fermentation was halted by placing the bottles on ice for approximately 5 min. Immediately afterward, the pH of each bottle was measured using a digital pH meter (Orion Star™ A121, Thermo Scientific, Beverly, MA, USA). The ANKOM F57 filter bags were then rinsed thoroughly and dried in a forced-air oven at 55 °C for 48 h. The extent of DM, NDF, and ADF degradation was determined by comparing the weight of the dried residues with the initial weight of the substrate before incubation. Cumulative production of total gas, CH_4 and CO_2 was standardized based on the amount of degraded DM (*d*DM), NDF (*d*NDF), and ADF (*d*ADF) after 48 h of fermentation.

Supernatant samples (5 mL) of the fermented fluid were collected from each bottle into glass tubes for the analysis of ammonia nitrogen (NH_3-N) and short-chain fatty acids (SCFAs), both total and individual. A 3 mL aliquot was mixed with an equal volume of 0.2 M hydrochloric acid to preserve the sample for NH_3-N analysis, which was conducted in accordance with AOAC (1997) (method ID 954.01). For NH_3-N quantification, approximately 100–200 mg of the sample was digested with 5 mL of concentrated H_2SO_4 for 1.5 h using a micro-Kjeldahl apparatus. After digestion, the contents were diluted to a final volume of 50 mL and subjected to steam distillation. The resulting distillate was collected in a flask containing 50 mL of 4% boric acid solution along with Tashiro's indicator. The amount of NH_3-N was determined by titration with 0.1 M hydrochloric acid.

A volume of 0.8 mL from each bottle was combined with 0.2 mL of a 250 g/L metaphosphoric acid solution for the analysis of short-chain fatty acids (SCFAs). The samples were then analyzed using high-performance liquid chromatography (HPLC) with an Inert Sustain column, following the method described Abdel-Nasser et al. (2023), with slight alterations. An Eclipse AQ-C18 HP column (4.6 mm × 150 mm i.d., 3 µm) was employed for the chromatographic separation. The mobile phase used was 0.005 N sulfuric acid, delivered with a linearly programmed gradient flow rate as follows: 0–4.5 min (0.8 mL/min); 4.5–4.7 min (1 mL/min); 4.7–4.71 min (1 mL/min); 4.71–8.8 (1.2 mL/min); 8.8–9 (1.3 mL/min); 9–23 (1.3 mL/min); 23–25 (0.8 mL/min). The diode array detector (DAD) was monitored at 210 nm. The injection volume was 5 µL for each of the sample solutions. The column was kept at a constant temperature of 55 °C during the analysis. Calibration of the integrator was carried out using a standard solution containing known concentrations of individual short-chain fatty acids (SCFAs), sourced from Sigma Chemie GmbH (Steinheim, Germany). All chromatographic procedures were conducted at the Chromatography Laboratory of the Central Laboratories Network, National Research Centre, Egypt.

Chemical analysis

Ash content in peppermint leaves, individual feed components, and the total mixed ration (TMR) was assessed by incinerating the samples in a muffle furnace at 550 °C for 12 h, in accordance with method 942.05. Crude protein (CP) was measured using the Kjeldahl technique (method 954.01), while ether extract (EE) was determined through Soxhlet extraction with diethyl ether,

following method 920.39, as outlined by AOAC (1997) methods. The NDF content was measured using a protocol that included α-amylase and sodium sulfite, based on the approach described by Van Soest et al. (1991). Acid detergent fiber was quantified using method 973.18 from AOAC (1997), with results reported excluding residual ash. Non-fiber carbohydrates (NFC) were estimated using the formula NFC = 1000 − NDF − CP − EE − ash, and organic matter (OM) was calculated as 100 − ash.

Calculations and statistical analysis

To estimate the kinetics of gas production (GP), methane (CH_4), and carbon dioxide (CO_2), the recorded volumes (expressed as mL/g dry matter) were modeled using the NLIN procedure in SAS (Version 9.4; SAS Institute Inc., Cary, NC). The data were fitted to the non-linear model described by France et al. (2000) model as: $y = A \times [1 - e^{-c\,(t-Lag)}]$ where y represents the cumulative volume of GP, CH_4 or CO_2 production at time t (h); *A* is the maximum (asymptotic) GP, CH_4 or CO_2 (mL/g DM); *c* is the fractional rate constant of GP, CH_4 or CO_2 (/h), and Lag (h) denotes the latency period (h) before the onset of measurable GP, CH_4 or CO_2 release.

The partitioning factor after 48 h of incubation (PF_{48}), expressed as mg degraded DM per mL of gas produced, was calculated following the method of Blümmel et al. (1997). Gas yield at 24 h (GY_{24}), based on 200 mg of DM, was determined using the formula: GY_{24} = mL of gas produced per gram of DM divided by the amount of apparent degraded substrate (ADS). The ME content was estimated using the equation proposed by Menke et al. (1979): 2.20 + 0.136 × GP + 0.057 × CP. Microbial CP production (MCP) synthesis was computed using the following formula from Blümmel et al. (1997): MCP = mg ADS - (mL gas × 2.2mg/mL).

Data were analyzed using the mixed procedure of SAS in a randomized block design. Each run formed a block, and the experimental unit was specified as the additive level within each block. The model: $Y_{ijk} = \mu + L_i + R_j + \varepsilon_{ijk}$ was employed, where: Y_{ijk} is the observation, μ is the population mean, L_i is the peppermint level effect, R_j is the run (block) effect, $(L\times R)_{ij}$ is the interaction between run and additive level, and ε_{ijk} is the residual error. The results from the two replicates of each substrate sample were averaged before statistical analysis, and the resulting mean was treated as the experimental unit. Linear and quadratic contrasts were employed to determine the level responses (increasing peppermint levels). Significance was declared at a level of $p<0.05$.

Results

Mentha piperita volatile compounds

Peppermint leaves contained 3.35% essential oil on a DM basis. Table 2 summarizes the volatile compounds detected in peppermint leaves through GC-MS analysis. Fourteen compounds were identified, including 1,8-cineole (known as eucalyptol), camphor, estragole, α- and β-pinene, and α-terpineol, among others. The analysis revealed that the compound with the highest concentration was 1,8-cineole, constituting 66.39%, followed by camphor with 6.66%, while the lowest was α-terpineol, constituting 0.45%.

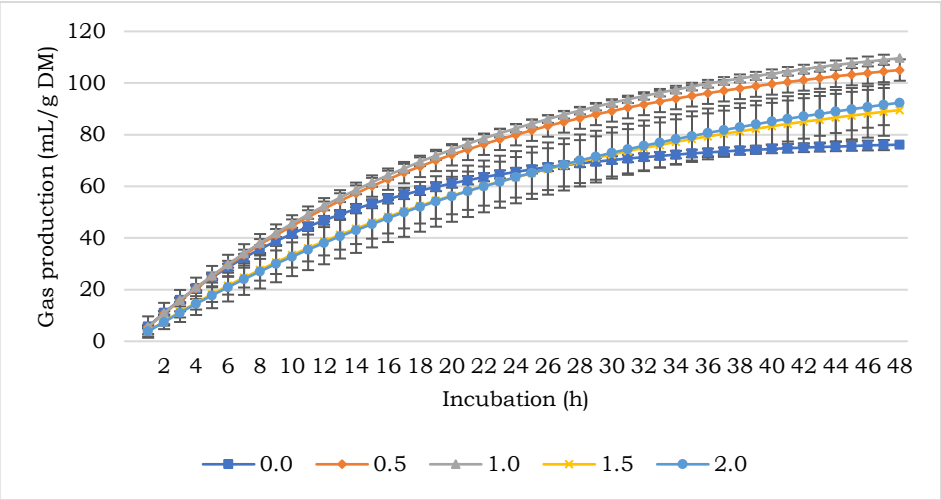
Effect on biogases production

Figures 1, 2, and 3 show GP (mL), CH_4 (mL), and CO_2 (mL), respectively, per g DM, *d*DM, *d*NDF, and *d*ADF. The general trend in the graphs shows that all gases (GP, CH_4, and CO_2) increased with the digestion time. Figure 1 shows that throughout the experimental period, diets containing 1.0% peppermint produced the highest GP, while the 0% peppermint produced the least GP, with variations in DM (Figure 1A), *d*DM (Figure 1B), *d*NDF (Figure 1C), and *d*ADF (Figure 1D). Figure 2 shows that peppermint at various levels decreased CH_4 production per gram of DM (Figure 2A), *d*DM (Figure 2B), *d*NDF (Figure 2C), and *d*ADF (Figure 2D), while no effects were detected in CH_4 production per gram of DM. Figure 3 shows that 1.0% of peppermint produced the highest CO_2 gases, and 0% of peppermint produced the least. However, looking at the CO_2 produced per gram DM (Figure 3A), *d*DM (Figure 3B), the 1.0% peppermint maintained the lead in producing the highest CO_2. In contrast, the diet without peppermint supplementation produced the lowest CO_2, following the same pattern for the gas produced per gram of *d*NDF and *d*ADF (Figure 3C and D).

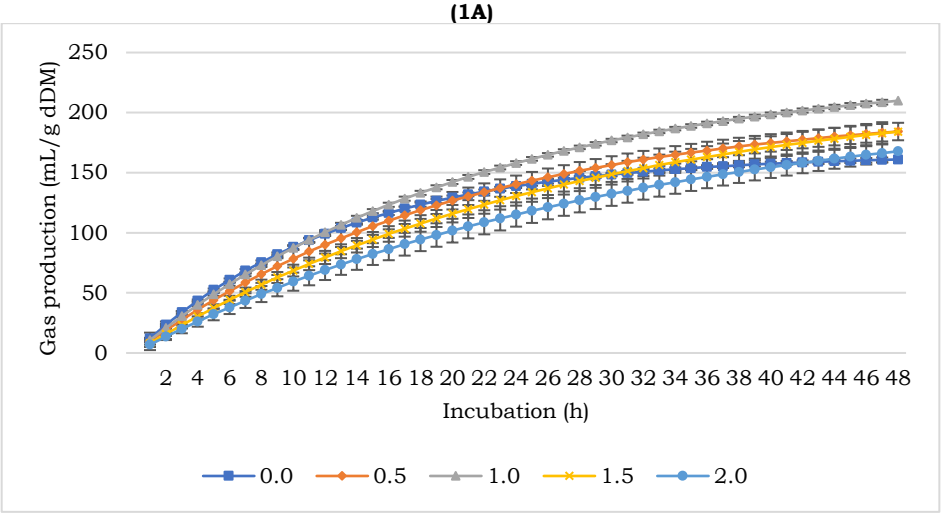
Table 2: Identification of volatile compounds in peppermint (*Mentha longifolia*) leaves using GC-MS analysis.

Peak	Compound[1]	Formula	RT[2]	Concentration[3] (%)	Concentration (mg/100 g DM)
1	α-Pinene	$C_{10}H_{16}$	3.702	2.28	0.08
2	β-Pinene	$C_{10}H_{16}$	4.786	1.79	0.06
3	1,8-Cineole	$C_{10}H_{18}O$	6.781	66.39	2.22
4	L-Fenchone	$C_{10}H_{16}O$	8.536	3.68	0.12
5	Camphor	$C_{10}H_{16}O$	9.939	6.66	0.22
6	Citronella	$C_{10}H_{18}O$	10.276	1.5	0.05
7	Isomenthone	$C_{10}H_{18}O$	10.524	1.44	0.05
8	Sabinene hydrate	$C_{10}H_{18}O$	10.847	1.15	0.04
9	α-terpineol	$C_{10}H_{18}O$	11.227	0.45	0.02
10	Estragole	$C_{10}H_{12}O$	11.41	4.78	0.16
11	2,4,6-trimethylcyclohex-3-ene-1-carbaldehyde	$C_{10}H_{16}O$	12.171	1.32	0.04
12	Carvone	$C_{10}H_{14}O$	12.293	3.25	0.11
13	Terpinolene	$C_{10}H_{16}$	14.17	2.93	0.10
14	Trans-caryophyllene	$C_{15}H_{24}$	15.229	2.39	0.08

[1]Compounds were identified using authentic standards, comparison with NIST library spectra, and references from published literature, [2]RT is the retention time (min), [3]Concentration were calculated based on the summed peak areas of the identified compounds



(1A)



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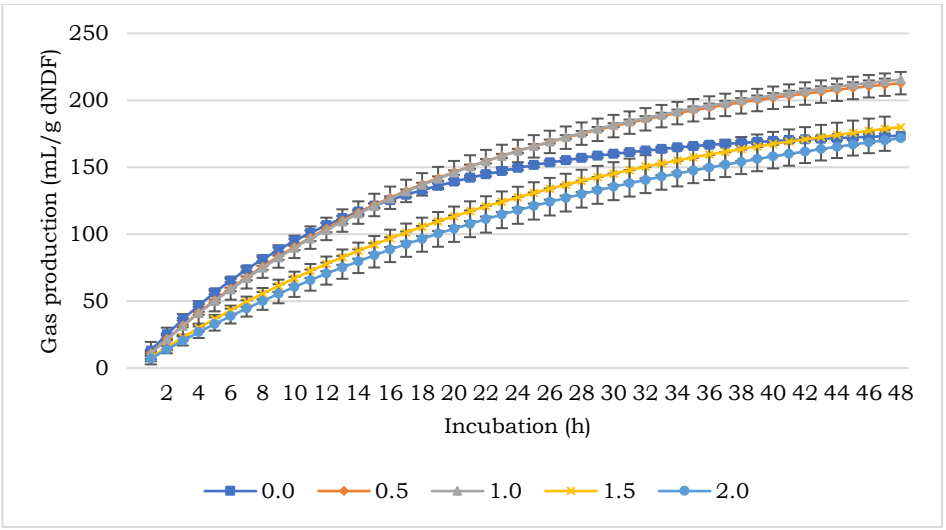

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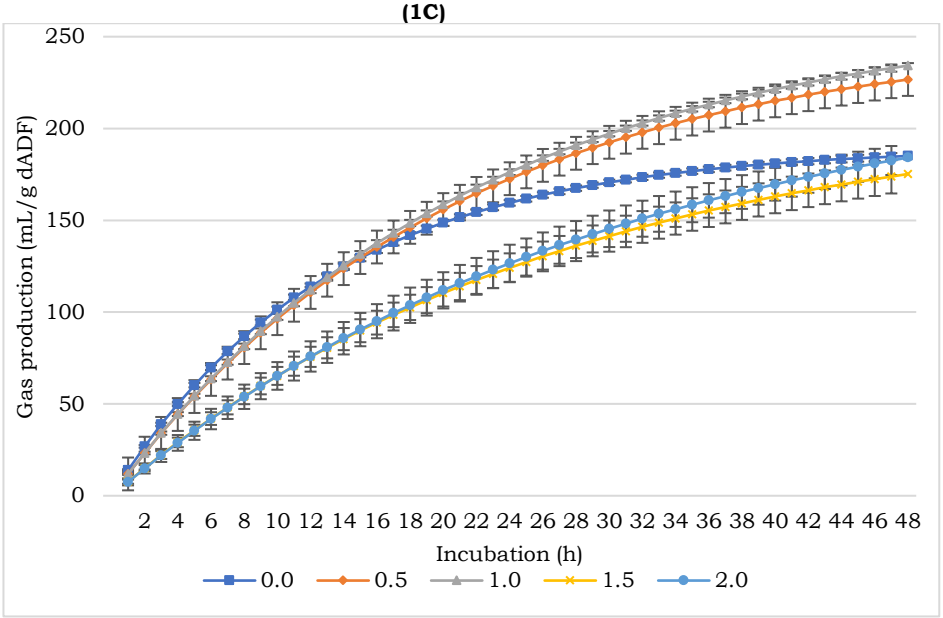
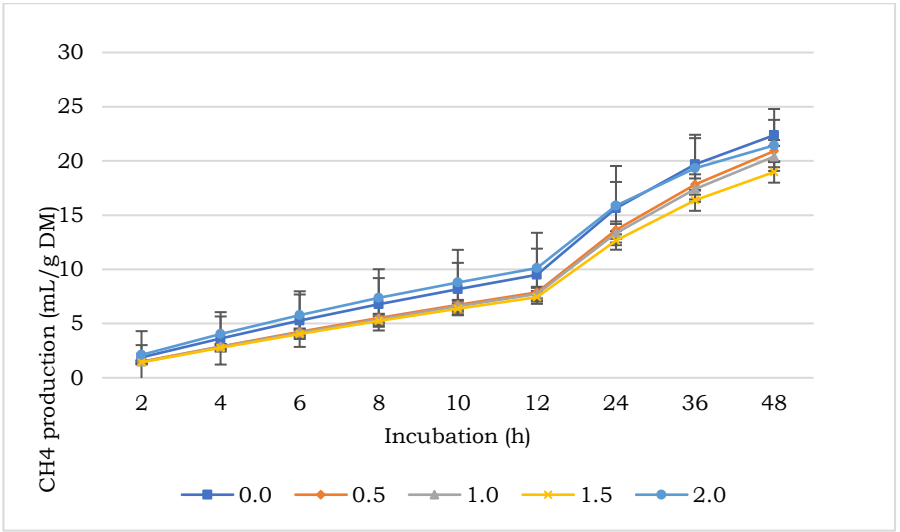


(1D)

Figure 1: *In vitro* ruminal gas production expressed as (1A) mL/g of incubated DM, (1B) mL/g of *d*DM, (1C) mL/g of *d*NDF, and (1D) mL/g of degradable *d*ADF from a total mixed ration supplemented with varying levels of peppermint leaves. dDM: degradable dry matter; dNDF: degradable neutral detergent fiber; dADF: degradable acid detergent fiber.



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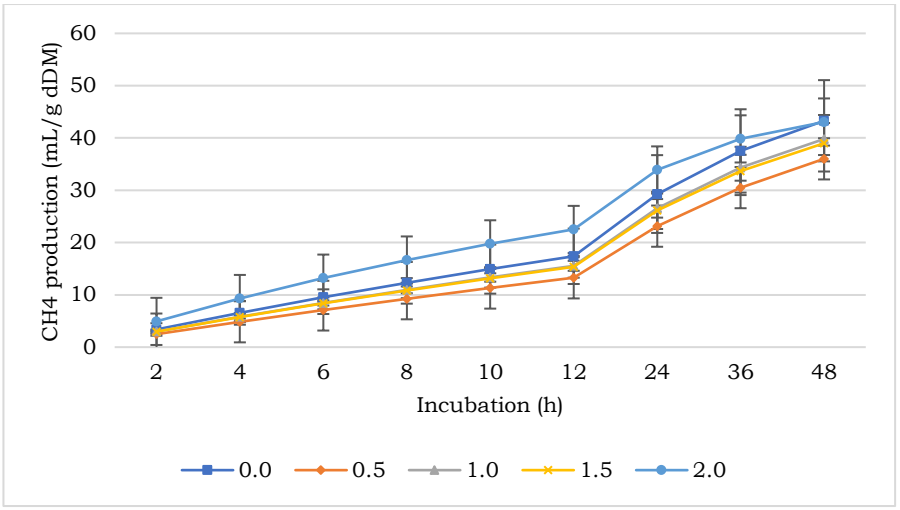

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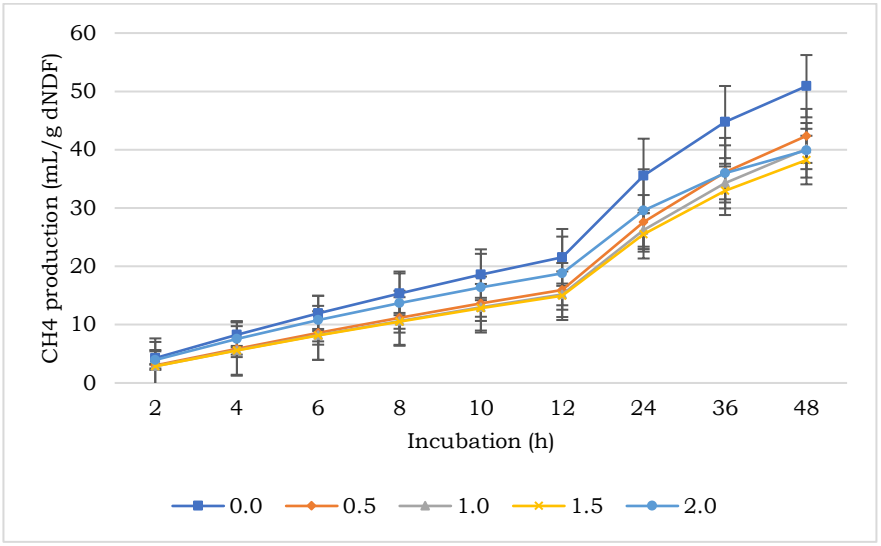

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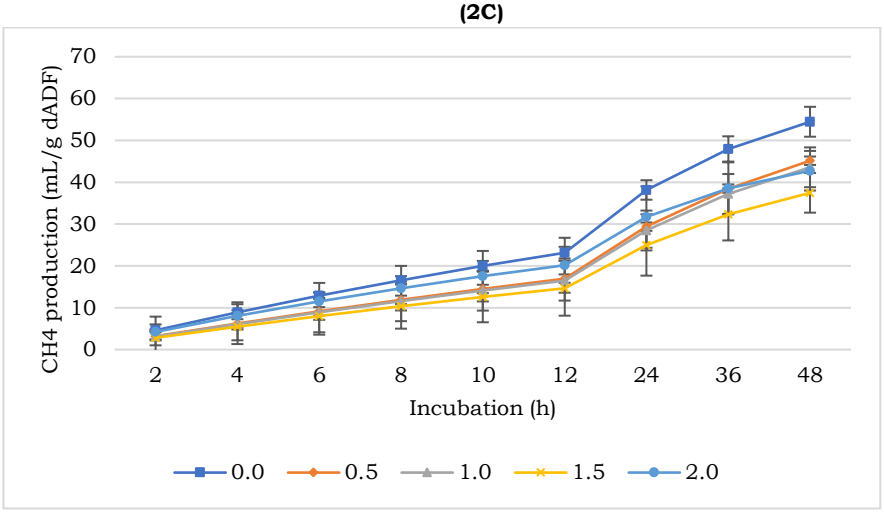
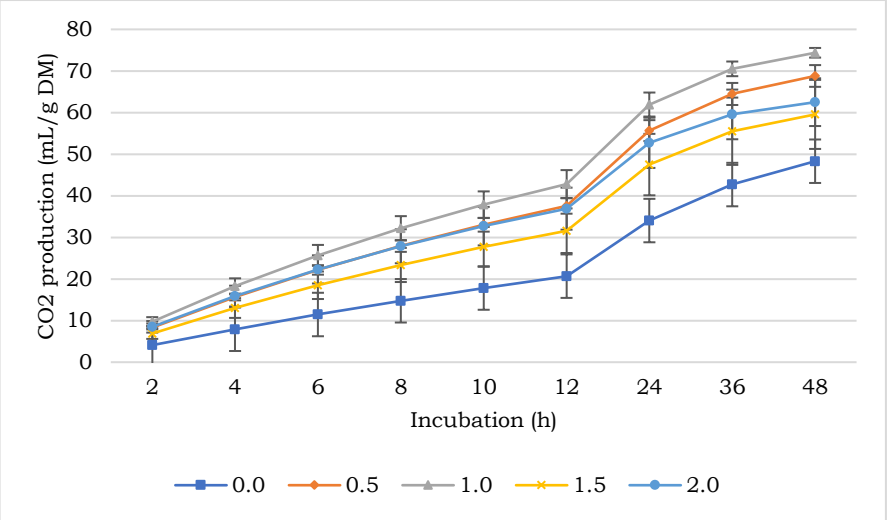
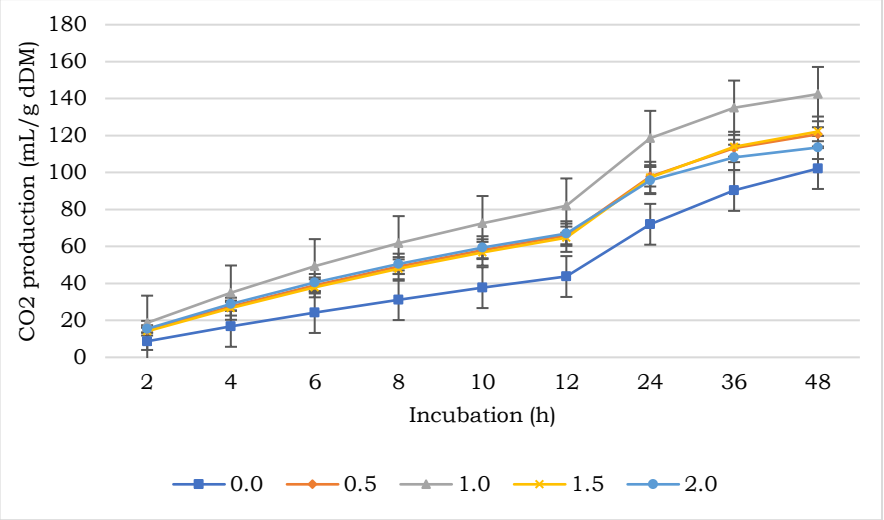


(2D)

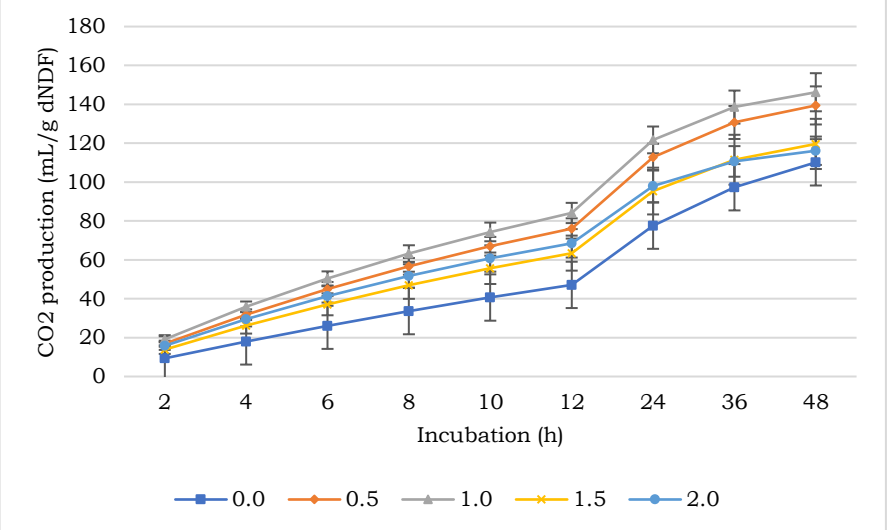
Figure 2: *In vitro* ruminal methane (CH_4) production expressed as (2A) mL/g of incubated DM, (2B) mL/g of *d*DM, (2C) mL/g of *d*NDF, and (2D) mL/g of *d*ADF from a total mixed ration supplemented with varying levels of peppermint leaves. dDM: degradable dry matter; dNDF: degradable neutral detergent fiber; dADF: degradable acid detergent fiber.


80
70
60
50
40
30
20
10
0
CO2 production (mL/g DM)
2
4
6
8
10
12
24
36
48
Incubation (h)
0.0
0.5
1.0
1.5
2.0

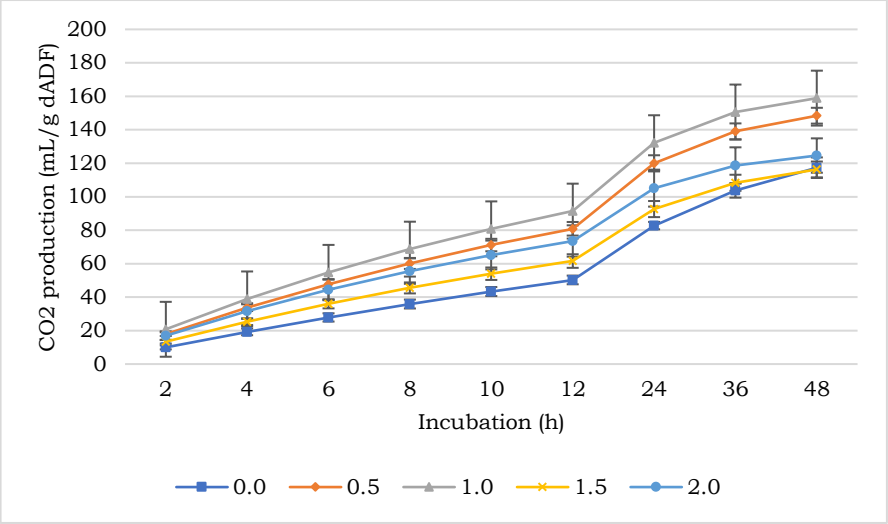
(3A)


180
160
140
120
100
80
60
40
20
0
CO2 production (mL/g dDM)
2
4
6
8
10
12
24
36
48
Incubation (h)
0.0
0.5
1.0
1.5
2.0

(3B)


180
160
140
120
100
80
60
40
20
0
CO2 production (mL/g dNDF)
2
4
6
8
10
12
24
36
48
Incubation (h)
0.0
0.5
1.0
1.5
2.0

(3C)



(3D)

Figure 3: *In vitro* ruminal carbon dioxide (CO_2) production expressed as (3A) mL/g of incubated DM, (3B) mL/g of *d*DM, (3C) mL/g of *d*NDF, and (3D) mL/g of *d*ADF from a total mixed ration supplemented with varying levels of peppermint leaves. dDM: degradable dry matter; dNDF: degradable neutral detergent fiber; dADF: degradable acid detergent fiber.

Table 3 presents the *in vitro* rumen gas, CH_4, and CO_2 kinetics with varying levels of peppermint leaves. Gas production parameters showed that the diet containing 1.0% peppermint had the highest ($p<0.001$) asymptotic GP (mL/g DM), produced at a high rate (GP/h), while the diet containing 0.0% had the least GP at a slow rate. While the asymptotic GP increased with higher doses, the rate of GP (per hour) showed a decline. Peppermint linearly ($p=0.002$) decreased the asymptotic CH_4 in a dose-dependent manner, and it was observed that the rate of CH_4 production and the Lag were prolonged with increasing levels of peppermint in the diet. Carbon dioxide parameters (asymptotic (mL/g DM) and the rate of production (/h) increased ($p<0.05$) in a dose-dependent manner. The proportion of CH_4 showed that CH_4 from diets that contained 1% peppermint leaf had the lowest ($p=0.002$) CH_4, while the diet with no peppermint produced the highest. Furthermore, peppermint did not significantly ($p>0.05$) affect the Lag and proportion of CO_2 at the end of the experiment. However, it linearly ($p=0.021$) increased the CO_2 proportion of CO_2 at 48 h of incubation.

Table 3: Kinetics of *in vitro* rumen gas, methane (CH_4), and carbon dioxide (CO_2) production in response to increasing levels of peppermint leaves (% on a DM basis).

	Gas parameters[1]			**CH_4 parameters[2]**			**CO_2 parameters[3]**		
Level	*A*	*c*	Lag	*A*	*c*	Lag	*A*	*c*	Lag
0	78.1c	0.076a	1.55	28.6ab	0.035	1.42c	58.8c	0.036b	2.47
0.5	116.4ab	0.048b	1.21	29.4a	0.026	1.67ab	73.1ab	0.061a	2.19
1	123.2a	0.046b	1.60	28.5ab	0.026	1.81a	77.6a	0.067a	2.18
1.5	107.7b	0.037bc	1.84	25.3b	0.029	1.57b	63.7bc	0.057a	2.04
2	119.0ab	0.033c	1.59	25.3b	0.044	1.61b	64.8bc	0.070a	2.26
SEM	3.02	0.0029	0.141	0.84	0.0061	0.083	2.76	0.0039	0.183
				p value					
Treatment	<0.001	<0.001	0.104	0.013	0.264	0.032	0.004	0.009	0.586
Linear	<0.001	<0.001	0.149	0.002	0.287	0.047	0.774	0.004	0.346
Quadratic	<0.001	0.004	0.941	0.243	0.054	0.014	0.001	0.024	0.235

Different letters indicate a difference in means ($p<0.05$) within the same column. P-value represents the significance level obtained from the F-test for treatment effects; SEM denotes the standard error of the mean; [1]GP parameters: *A* represents the asymptotic GP (mL/g DM), *c* is the fractional GP rate (/h), and Lag denotes the lag time before GP starts (h); [2]Methane (CH_4) production parameters: *A* indicates the asymptotic CH_4 production (ml/g DM), *c* is the CH_4 production rate (/h), Lag represents the delay period before CH_4 production starts (h); [3]Carbon dioxide (CO_2) production parameters: *A* denotes the asymptotic CO_2 production (mL/g DM), *c* represents the CO_2 production rate (/h), Lag is the lag time before CO_2 production initiates (h).

Following 48 h of incubation, diets supplemented with 0.5% and 1% peppermint exhibited the greatest GP per gram of DM (quadratic effect, p=0.002), with the 1% level yielding the highest GP per gram of *d*DM (quadratic effect, p<0.001), *d*NDF (quadratic effect, p=0.002), and *d*ADF (quadratic effect, p=0.004) (Table 4). Peppermint did not impact CH_4 production per gram of DM; however, it did result in a linear decrease in CH4 production per gram of *d*DM (p=0.032), *d*NDF (p=0.004), *d*ADF (p=0.009), and as a percentage of total GP (p=0.034). The lowest CH_4 to SCFA ratio was observed at the 1.5% peppermint level (p=0.044). After 48 h of incubation, CO_2 production per gram of dry matter was elevated in diets containing 0.5%, 1%, and 2% peppermint. However, the 0.5% and 1% levels showed the highest CO_2 production per gram of *d*DM, *d*NDF, and *d*ADF. Peppermint at all levels also linearly decreased the CH_4 to CO_2 ratio compared to the control treatment.

Effect on degradability and fermentation

Table 5 presents the *in vitro* ruminal fermentation parameters for diets supplemented with varying concentrations of peppermint leaves. Peppermint supplementation increased *d*DM (p=0.028), *d*ADF (p=0.001), and *d*NDF (p=0.011) in a dose-dependent manner. The diet supplemented with 0.5% peppermint exhibited the greatest *d*DM, whereas the 2% peppermint diet showed the highest *d*NDF. The highest *d*ADF was observed in the diet containing 1.5% peppermint. Furthermore, total SCFAs, including acetate (C_2) and propionate (C_3) concentrations, were maximized in the 1.5% peppermint diet, while the control diet (0% peppermint) recorded the lowest values. Consequently, fermentation parameters showed that the diet containing 1% peppermint had the highest (p<0.01) NH_3-N, ME (p=0.008), and GY_{24} (p=0.004) and the lowest MCP (p=0.021).

It was observed that the control had the highest values (p=0.005) for PF_{48}. However, peppermint supplementation did not affect butyrate (C_4). While peppermint addition did not significantly affect pH (p=0.086; linearly p=0.554), it had a quadratic (p=0.027) reduction effect. Furthermore, peppermint affected PF_{48} and linearly increased it, but 1% peppermint had the lowest value.

Discussion

***Mentha piperita* composition and bioactive components**

For centuries, plants have been recognized as valuable sources of medicinal compounds, owing to the diverse arrays of bioactive chemicals found in them. However, the chemical makeup of plants differs depending on the specific part examined (Weinhold et al., 2022) and the time of harvesting (Paulauskienė et al., 2021). In the present study, 14 compounds were identified, comprising 13 with a C10 carbon skeleton and one featuring a C15 structure. The primary volatile constituents detected were 1,8-cineole, camphor, and estragole. Some of the main compounds (1,8-cineole and isomethone), identified in our study, were similar to the findings of Beigi et al. (2018). In this study, it was observed that in the leaves, 1,8-cineole had the highest concentration, while α-terpineol had the lowest. Other compounds, such as menthone and menthol, have been identified in other studies (Beigi et al., 2018; Štrbac et al., 2023), but were not detected in our study. The variation in the results could be attributed to the plant's source, drying methods, and the growth conditions, including environmental factors and agricultural practices, as well as the detection methods.

Table 4: *In vitro* ruminal production of gas, methane (CH_4), and carbon dioxide (CO_2) at 48 h of incubation expressed per gram of incubated DM, dDM, dNDF, and *d*ADF, influenced by varying peppermint leaves supplementation levels (% on a DM basis).

Level	Gas production (mL/g)				CH_4 production (mL/g)						CO_2 production (mL/g)					CH_4:CO_2
	DM	*d*DM	*d*NDF	*d*ADF	DM	*d*DM	*d*NDF	*d*ADF	%	CH_4:SCFA[1]	DM	*d*DM	*d*NDF	*d*ADF	%	
0	76.1^{c}	161.0^{b}	173.5^{b}	185.1^{b}	22.4	47.3^{a}	50.9^{a}	54.4^{a}	29.3^{a}	0.96^{a}	48.3^{c}	102.2^{c}	110.2^{c}	117.3^{b}	63.5	0.46^{a}
0.5	105.0a^{b}	184.3^{b}	212.8^{a}	226.7^{a}	20.9	36.7^{b}	42.4^{b}	45.1^{ab}	19.9^{b}	0.85^{ab}	68.8^{ab}	120.7^{b}	139.4^{ab}	148.4^{a}	65.5	0.30^{b}
1	109.6^{a}	209.9^{a}	215.5^{a}	234.2^{a}	20.4	39.1^{b}	40.1^{b}	43.6^{ab}	18.6^{b}	0.80^{ab}	74.4^{a}	142.4^{a}	146.2^{a}	158.9^{a}	67.8	0.27^{b}
1.5	89.5b^{c}	184.2^{b}	179.9^{b}	175.2^{b}	19.0	39.4^{b}	38.2^{b}	37.5^{b}	21.4^{b}	0.73^{b}	59.6^{bc}	122.2^{b}	119.6^{bc}	116.3^{b}	66.4	0.32^{b}
2	92.4b^{c}	167.9^{b}	171.6^{b}	184.2^{b}	21.4	39.1^{b}	39.9^{b}	42.8^{ab}	23.4^{ab}	0.85^{ab}	62.5^{ab}	113.6^{bc}	116.1^{c}	124.6^{b}	67.6	0.35^{b}
SEM	3.52	5.16	6.12	6.76	1.07	1.67	2.18	3.02	1.34	0.04	2.71	3.51	5.00	3.97	1.05	0.02[5]
								p value								
Treatment	0.004	0.005	0.007	0.002	0.301	0.027	0.015	0.031	0.002	0.049	0.005	0.002	0.002	<.001	0.083	0.003
Linear	0.158	0.423	0.088	0.032	0.278	0.032	0.004	0.009	0.034	0.044	0.050	0.053	0.627	0.193	0.021	0.021
Quadratic	0.002	<0.001	0.002	0.004	0.114	0.094	0.029	0.054	0.003	0.023	0.003	<.001	0.004	<.001	0.206	0.007

Different letters indicate a difference in means (p<0.05) within the same column. P-value indicates the significance level from the F-test for treatment effects; SEM refers to the standard error of the mean.

Table 5: *In vitro* ruminal fermentation characteristics of diet supplemented with varying peppermint leaves supplementation levels (% on a DM basis).

Level	Degradability[1]			SCFA[2]					Fermentation[3]					
	*d*DM	*d*NDF	*d*ADF	Total	C_2	C_3	C_2:C_3	C_4	pH	NH_3-N	ME	PF_{48}	MCP	GY_{24}
0	473^{b}	439^{b}	412^{b}	23.4b	11.4^{b}	7.90^{b}	1.47^{a}	4.08	6.27	10.4^{b}	4.68^{ab}	6.22^{a}	328^{b}	139^{ab}
0.5	571^{a}	494^{a}	464^{ab}	24.7^{ab}	11.5^{b}	9.48^{a}	1.22^{c}	3.71	6.30	11.7^{a}	4.95^{ab}	5.44^{bc}	395^{ab}	140^{ab}
1	523^{ab}	510^{a}	468^{ab}	25.5^{a}	12.2^{ab}	9.28^{a}	1.33^{b}	3.98	6.33	11.8^{a}	5.02^{a}	4.77^{bc}	342ab	158^{a}
1.5	487^{ab}	497^{a}	511^{a}	25.9^{a}	12.7^{a}	9.48^{a}	1.34^{b}	3.72	6.40	11.5^{a}	4.50^{b}	5.43^{c}	347^{ab}	130^{b}
2	550^{ab}	538a	501^{a}	25.1^{a}	12.7^{a}	9.14^{a}	1.42ab	3.23	6.17	10.8^{ab}	4.50^{b}	5.97^{ab}	410^{a}	115^{b}
SEM	19.9	11.0	16.1	0.32	0.36	0.483	0.026	0.305	0.052	0.38	0.096	0.160	16.4	5.5
							p value							
Treatment	0.028	0.001	0.011	0.003	0.039	0.019	0.045	0.380	0.086	0.023	0.008	0.005	0.021	0.004
Linear	0.294	0.002	0.001	0.001	0.008	0.013	0.950	0.111	0.554	0.631	0.024	0.006	0.015	0.009
Quadratic	0.441	0.193	0.189	0.004	0.856	0.086	0.037	0.510	0.027	0.014	0.011	0.005	0.428	0.003

Different letters indicate a difference in means (p<0.05) within the same column represents the significance level from the F-test for treatment effects; SEM is the standard error of the mean; [1]*d*DM refers to dry matter degradability (g/kg incubated), *d*NDF donates neutral detergent fiber degradability (g/kg incubated), and *d*ADF represents acid detergent fiber degradability (g/g incubated); [2]SCFA refers to short chain fatty acids (mmol/g DM), C_2 represents acetate (mmol/g DM), C_3 donates propionate (mmol/g DM), C_4 is butyrate (mmol/g DM); [3]NH_3-N donates ammonia-N (mg/g DM), GY_{24} stands for gas yield at 24 h (mL gas/g *d*DM), ME is metabolizable energy (MJ/kg DM), PF_{48} indicates the partitioning factor at 48 h of incubation (mg *d*DM: mL gas), MCP is microbial crude protein production (mg/g DM).

Furthermore, volatile compounds such as 1,8-cineole and camphor possess significant bioactive properties, such as antibacterial, antifungal, and anti-inflammatory effects, which have been demonstrated in several studies (Beigi et al., 2018; Štrbac et al., 2023). 1,8-cineole, in particular, has been shown to be effective in inhibiting pathogenic bacteria, suggesting that it could help in modulating the gut microbiome of ruminants when used as a feed additive. Likewise, camphor has been recognized for its potential to reduce microbial growth, which may contribute to reducing methane production in the rumen, as fewer methanogens would be available to convert hydrogen (H_2) and CO_2 into CH_4. These findings highlight the variability in raw peppermint leaf materials, which can lead to considerable differences in extract composition and potentially produce varying effects on functional targets *in vivo* (Inarejos-Garcia et al., 2023). In other studies, these plant materials were subjected to different processes, such as fresh leaves, shade drying, microwaving, and hot air drying. Alternatively, the fact that the same analytic method was used could mean that sources and forms of peppermint affect its composition. Notwithstanding, the numerous bioactive ingredients suggest that it can be useful as a feed additive, but *in vivo* studies are warranted to accompany our findings.

Plant volatile compounds that alter the rumen microbiome can provide various dietary effects, including effects on ruminal bacteria (Woodward et al., 2001; Kholif and Olafadehan, 2021). For example, terpenoids like 1,8-cineole have been reported to significantly inhibit the growth and metabolic activity of rumen microbes (Benchaar et al., 2008; Kholif and Olafadehan, 2021). As noted by de Sousa et al. (2023). These compounds also possess antibacterial properties against bacteria, protozoa, and fungi. They affect enzyme activity, signal transduction pathways, bacterial colonization, and cell membrane integrity. However, the ruminal fermentation profile can vary based on the source and concentration of volatile compounds, which influence ruminal bacteria in different ways. Therefore, identifying the most active volatile compounds in the leaves and determining their optimal doses could help mitigate such variations, ensuring consistent and predictable outcomes when included in animal diets.

Effect on gas production

In vitro GP is a powerful, tested, and trusted technique used in animal nutrition to ascertain the potential of any sample for certain characteristics before they are used for live animals. Therefore, GP from anaerobic fermentation is evidence of degradability, suggesting the availability of rapidly degradable substrates such as degradable carbohydrates (Getachew et al., 1998). It often indicates the microbial degradability of samples. However, samples showing high capacity for GP also correlate with high CH_4 production (Kholif et al., 2024a). In the present study, the observed increase in total GP, reduction in CH_4 production, and corresponding rise in CO_2 level in the peppermint-supplemented group suggest that peppermint may serve as a promising eco-friendly feed additive. Peppermint supplementation increased GP, indicating enhanced microbial digestive activity (Spirling and Daniels, 2001).

Our findings on GP align with previous studies where peppermint oil was applied at concentrations of 1.5 and 3 µL/mL (Beyzi, 2020), or 0, 0.33, 1.0, and 2.0 µL/mL of incubation medium (Agarwal et al., 2009). These studies reported an initial increase in rumen microbial activity with lower concentrations of peppermint oil, followed by a decline in microbial activity as the concentration increased, eventually falling below the control level. The CH_4 production showed that peppermint decreased CH_4, indicating its antimethanogenic properties. Our findings agree with those reported by Agarwal et al. (2009) and Beyzi (2020) on the ability of peppermint leaves to reduce CH_4 due to the presence of peppermint essential oils. Diets with 1.0% peppermint had one of the highest total CH_4 production, despite producing one of the highest total gases, too. However, the volume represents the lowest percentage of the total CH_4 produced.

The observed reduced CH_4 production with 2% peppermint inclusion (~3.3 mL/g DM) corresponds to approximately 33 liters less methane produced per cow per day, assuming a daily intake of 10 kg DM. This equates to a reduction of ~0.024 kg CH_4/day, or ~0.66 kg CO_2-equivalent emissions per day. On an annual basis, this represents a potential mitigation of approximately 242 kg CO_2-eq per cow, highlighting the role of peppermint as a natural

feed additive in lowering the carbon footprint of ruminant production systems. Furthermore, the lower CH_4 meant increased CO_2, suggesting there was a shift in the conversion of CO_2 and H_2 gases to CH_4. The symbiotic relationship between methanogens and protozoa is well known. Therefore, the reduction in CH_4 and increase in CO_2 suggest that there was a disruption in the symbiosis. The study of Ando et al. (2003) indicates that peppermint decreases the total number of protozoa (*Entodinium, Isotricha,* and *Diplodinium)*, while another study (Agarwal et al., 2009) informed that at a lower dose, peppermint can increase methanogens. Methanogens can reduce CO_2 to CH_4 by converting several substrates during fermentation (Robinson and Buan, 2018). They achieve this by localizing on the outer layers of the bacterial biofilm, where they capture H_2 diffusing from carbohydrate fermentation sites and subsequently combine it with CO_2 to generate CH_4 (Song et al., 2005). Based on previous experiments and the absence of protozoal count measurements in the current experiment, it can be speculated that the reduced CH_4 and elevated CO_2 productions observed in the peppermint-supplemented groups, relative to the control, may be attributed to peppermint's potential to suppress ruminal protozoa. This suppression likely results in decreased methanogenic activity in proportion to the total gas produced.

Effect on degradability

Dry matter degradability is a crucial measure of a feed or forage's nutritional value and its capacity to supply energy to livestock. It represents the proportion of dry matter in a feed that is broken down and absorbed by the animal's digestive tract. Dry matter degradability is positively correlated with the energy content of the feed. Feeds with higher *d*DM provide more digestible energy to the animal. Feeds with higher *d*DM are typically linked to improved animal growth rates and production performance. Therefore, from this study, peppermint, though it affected degradability, was neither linear nor quadratic. This implies an individual dose impact rather than a general trend. They suggest that a low level of peppermint leaves was the only one that had a positive effect on the rumen microbes to lead to improved microbial activities, especially the amylolytic microbes, while a dose-dependent trend was observed in *d*NDF and *d*ADF. This suggests that the peppermint leaves might have favored the proliferation of fibrolytic microbes.

Ahmed et al. (2014) reported that higher GP was found to be associated with improved *d*DM in the presence of exogenous. Saad et al. (2016) also reported that peppermint improved feed digestibility in sheep. The gas produced may be a result of increased substrate or the ability of additives to increase enzymes or rumen microbes. Agarwal et al. (2009) reported decreased carboxymethylcellulase and xylanase activities at a higher level of peppermint oil and increased fungi and *Ruminococcus flavefaciens* at a lower dose.

Both NDF and ADF are important indicators of the nutritional quality of ruminant animals' feed. Highly digestible ADF and NDF can be beneficial for maintaining rumen health, preventing acidosis, and promoting the growth of beneficial rumen microorganisms. Therefore, the observed linear increase in *d*ADF and *d*NDF may be attributed to the fiber-degrading microbes' activity. Our result is similar to the result of Rashid et al. (2019), who reported that adding peppermint oil to the diet reduced CH_4 and led to a significant improvement in *in vitro* degradability of DM, OM, and ME. It showed that it supported the growth of fibrolytic microbes, which might favor fibrolytic enzymes.

Indeed, the reason why our result differed from Agarwal et al. (2009) and Beyzi (2020) despite increasing doses may be because of the materials we used. In our study, we used the leaves, while in other studies, many used peppermint oil, which might have increased the concentration of bioactive ingredients per gram of peppermint used. The reduced digestibility when peppermint was used was reported by Hosoda et al. (2005), where 5% peppermint was added to the diet.

Effect on fermentation

Gas production is directly proportional to SCFAs, and the higher the gas produced, the higher the SCFAs production (Alam and us Saqib, 2017). Short-chain fatty acids are metabolic by-products produced during microbial fermentation under anaerobic conditions. Ruminants and pseudo-ruminants are absorbed through the rumen wall or hindgut wall to supply energy. The rate of digestibility and the type of feed can influence the proportion of each major SCFA (C_2, C_3, and C_4). Thus, the elevated SCFAs levels are linked to increased GP and enhanced degradability. The observed raise in C_3 levels in

this experiment implies that peppermint leaves favored the production of gluconeogenic acid. Therefore, the increased C_2 and C_3 in a dose-dependent manner suggests increased availability. Butyrate stimulates epithelial proliferation and enhances the rate of crypt cell production (Peng et al., 2007). The peppermint reduced its production and seemed to shift the production in favor of C_3 production. The concentrations of SCFAs reflect energy availability and can supply up to 80% of the animal's daily energy needs (Fellner, 2004). It's directly proportional to ME and OM degradability (Menke et al., 1979). Our results revealed that beyond the 1% peppermint inclusion level, the ME level started to decrease compared to the control diet. This indicates that a higher dose of peppermint reduces ME availability.

Although peppermint did not significantly influence the pH, the increase in pH value above the control suggests that peppermint can help mitigate the incidence of acidosis and provide an optimal environment for microbial functioning. Furthermore, the detected pH in our study was within the optimal range (6.00 and 6.80), as reported for optimal rumen function by Kamra (2005) and Ososanya et al. (2013).

Notwithstanding, the supplementation favored microbial protein production, which can be made available to the lower part of the gut for amino acid availability. This also relates to an increase in NH_3-N, which gut microbes can use in combination with carbohydrates for their proliferation. Thus, in our study, peppermint leaves did not decrease NH_3-N, which was contrary to Khamisabadi et al. (2016), where it was reported. The high GY_{24} reported in 0.5 and 1% supplementation suggests that within 24 h of intake, there is the potential for increased digestibility and nutrient availability *in vivo*. The PF_{48} reflects the efficiency of converting degraded substrate into microbial biomass. Therefore, a decreased PF_{48} would reflect a decreased efficiency in converting degraded substrate into microbial biomass (Elghandour et al., 2016). However, the lower PF_{48} in the present study for 1% peppermint suggests that for every gram of DM degraded, more nutrient was extracted compared to other treatments. This indicates that 1% peppermint facilitated the breakdown of feed samples, which gave microbes access and improved the surface area for microbes to derive more nutrients. This implies that the treatment with higher PF_{48} did not produce more gas per gram degraded, which implies less digestive efficiency.

Conclusion

This study demonstrated that incorporating 0.5 to 2% peppermint leaves (on a DM basis) into the diet improved total and individual SCFAs levels, particularly C_2 and C_3, as well as nutrient degradability (*d*DM, *d*NDF, and *d*ADF) and MCP. The optimal supplementation doses, which are both environmentally friendly and beneficial for digestive parameters, were found to be 0.5–1.5% peppermint inclusion. Adding peppermint leaves into a diet composed of equal parts of concentrate and roughages enhanced GP and reduced *in vitro* ruminal CH_4 production per g DM, *d*NDF, and *d*ADF. These effects suggest peppermint supplementation could reduce the environmental footprint of ruminant production and support sustainable livestock management. Further research is needed to explore the effects of higher peppermint leaf supplementation *in vivo* on ruminant production performance and alterations in rumen microbiota. Such research will help optimize peppermint use in animal feeding strategies to promote both productivity and ecological sustainability. Additionally, methods for harvesting peppermint leaves should be evaluated to create a more consistent product capable of addressing potential challenges related to widespread distribution.

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