Italian Journal of Animal Science

ISSN: (Print) (Online) Journal homepage: www.tandfonline.com/journals/tjas20

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To cite this article: Sara Glorio Patrucco, Alessandro Lotto, Martina Dorigo, Rita Fornaciari, Antonio Sagliano, Nicola Martinelli, Alessandra Cosani, Khalil Abid, Salvatore Barbera, Hatsumi Kaihara & Sonia Tassone (2025) Influence of lemongrass and oregano essential oils and their combination on *in vitro* ruminal fermentation and greenhouse gas emissions in total mixed ration for dairy cows, Italian Journal of Animal Science, 24:1, 266-280, DOI: 10.1080/1828051X.2025.2450509

To link to this article: https://doi.org/10.1080/1828051X.2025.2450509

Published online: 19 Jan 2025.

ITALIAN JOURNAL OF ANIMAL SCIENCE
2025, VOL. 24, NO. 1, 266–280
https://doi.org/10.1080/1828051X.2025.2450509

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RUMINANTS NUTRITION AND FEEDING

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Influence of lemongrass and oregano essential oils and their combination on *in vitro* ruminal fermentation and greenhouse gas emissions in total mixed ration for dairy cows

Sara Glorio Patrucco[a], Alessandro Lotto[b], Martina Dorigo[b], Rita Fornaciari[b], Antonio Sagliano[b], Nicola Martinelli[b], Alessandra Cosani[b], Khalil Abid[a], Salvatore Barbera[a], Hatsumi Kaihara[a] and Sonia Tassone[a]

[a]Dipartimento di Scienze Agrarie, Forestali e Alimentari (DISAFA), Università di Torino, Grugliasco, Italia; [b]Nutristar S.p.A, Reggio Emilia, Italia

ABSTRACT

Ruminants play a crucial role in the food chain, but are also considered contributors of greenhouse gas (GHG) emissions . Essential oils (EOs) are emerging as natural feed additives in ruminants' nutrition to enhance animal health, performance and reduce environmental footprint. Among EOs, lemongrass (*Cymbopogon winterianus*) and oregano (*Origanum vulgare*) EOs (LEO and OEO) have attracted attention as modulators of ruminal fermentations, but their role needs to be clarified. The experiment was designed using a randomised setup to assess the effects of LEO and OEO on *in vitro* ruminal fermentation and GHGs, using total mixed ration (TMR) as substrate (incubation time 24h). Experimental treatments included (doses as % of TMR on DM basis): 1) control only TMR (0% EOs) 2) 0.07% LEO 3) 0.07% OEO 4) 0.035% LEO + 0.035% OEO 5) 0.07% LEO + 0.07% OEO. Each treatment was repeated three times in two experimental runs. Only EO combinations reduced total gas (−9%, p=0.001). All EOs decreased CO_2 emissions by −5 to −12% with no significant differences between treatments (p<0.001), although anti-methanogenic effects were not observed (p=0.192). Volatile fatty acids were slightly affected only by EOs blend at the highest dose, resulting in a reduction of propionate (−1.3%, p=0.02), an increase in acetate:propionate (+0.16%, p=0.04) and isovalerate (+0.7%, p=0.03). LEO reduced pH (−0.6%, p=0.004), while OEO increased oxidation capacity (+4.2%, p=0.004), but both parameters remained within physiological ranges. Canonical discriminant analysis confirmed distinct EOs effects, highlighting their potential as natural additives for improving ruminal fermentation and mitigating ruminant environmental footprint.

HIGHLIGHTS

1. Lemongrass and oregano essential oils (LEO and OEO) alone and in blends demonstrated to reduce greenhouse gases, in particular carbon dioxide.
2. Only the blend of LEO and OEO at the highest dosage slightly influenced volatile fatty acids.
3. LEO and OEO did not modify, out of ruminant physiological ranges, pH, oxidation reduction potential and conductivity of rumen fluid.

ARTICLE HISTORY
Received 12 September 2024
Revised 23 December 2024
Accepted 2 January 2025

KEYWORDS
Lemongrass and oregano essential oils; *in vitro* ruminal fermentation; greenhouse gas emissions; methane; carbon dioxide

Introduction

Ruminant production sector plays a crucial role in the world food chain, significantly contributing to the provision of meat and dairy products (Godde et al. 2021; Michalak et al. 2021). As the global population is projected to reach 9.7 billion by 2050, the demand for these animal products is expected to rise accordingly (Henchion et al. 2021). However, livestock are a climate change driver, generating 14.5% of total anthropogenic greenhouse gas (GHG) emissions in the world (Giamouri et al. 2023). According to the Italian Institute for Environmental Protection and Research (ISPRA), agriculture in Italy accounted for 7.4% of GHGs in 2022, with 79% of these emissions originating from livestock (Vitullo et al. 2024). The main GHGs in

CONTACT Sonia Tassone sonia.tassone@unito.it

livestock are methane (CH_4) and nitrous oxide (N_2O), followed by carbon dioxide (CO_2). CH_4 and CO_2 are primarily emitted through enteric fermentation and anaerobic manure decomposition, while N_2O is released through denitrification during manure management (Moran 2005; Sejian et al. 2016; Alonso-Moreno et al. 2018). The challenge lies in balancing the need to increase livestock production with the necessity to reduce climate impacts and GHGs (Michalak et al. 2021). One promising approach in livestock sector is the inclusion of feed additives in ruminant diets (Kataria 2016).

Essential oils (EOs) have gained increasing interest as natural feed additives in livestock farming. They are volatile aromatic bioactive compounds originating from the secondary metabolism of plants (Cobellis, Trabalza-Marinucci, and Yu 2016; Caroprese et al. 2023). EOs are studied and applied in ruminant diets especially for their potential to modulate ruminal fermentation and consequently GHGs by enhancing or inhibiting specific rumen microbiota populations (Kholif and Olafadehan 2021; Brice et al. 2022; Caroprese et al. 2023). Some EOs can directly inhibit methanogenic archaea and/or indirectly decrease CH_4 production by depressing some microbial metabolic processes contributing to methanogenesis (Michalak et al. 2021). EOs can also reduce CO_2 production in the rumen due to their antimicrobial proprieties (Alabi et al. 2023; Bokharaeian et al. 2023; Ike et al. 2024). EOs' studies and applications in animal nutrition have significantly increased over the years, largely as a result of the ban imposed by European Union on the use of antibiotics as feed additives, due to the transmission and proliferation of resistant bacteria *via* the food chain (European Union 2003; Zeng et al. 2015). The Food and Drug Administration categorised EOs as generally recognised as safe (GRAS) for human and animal consumption, making them an attractive alternative for enhancing animal health, performance and reducing their environmental footprint (Benchaar et al. 2008; Food and Drug Administration 2024). In fact, EOs in ruminant nutrition are studied not only for their direct impacts on ruminal fermentations, but also for their beneficial effects on milk yield, digestion, oxidative stress, immune system and feed palatability (Tudor 2023).

Lemongrass (*Cymbopogon* spp.) and oregano (*Origanum vulgare*) essential oils (LEO and OEO) have attracted considerable attention in animal nutrition research due to their potential modulation of ruminal fermentation. LEO has been the focus of studies exploring this property (Nanon et al. 2014; Singh et al. 2018; Soares et al. 2023), and OEO has similarly been investigated for the same purpose (Zhou et al. 2020; Zhang et al. 2021; Cui et al. 2024). They have promising abilities to decrease total gas production, reduce CH_4 emissions and influence the quantity of total volatile fatty acids (tVFA) (Cobellis, Trabalza-Marinucci, Marcotullio, et al. 2016; Singh et al. 2018; Zhou et al. 2020), but their roles and dosages are not completely defined (Cobellis, Trabalza-Marinucci, and Yu 2016; Soares et al. 2023; Luo et al. 2024). Moreover, ruminal fermentation modulation of EOs can be enhanced combining them (Cobellis, Trabalza-Marinucci, Marcotullio, et al. 2016), but to the best of our knowledge no research investigated the interaction effects of LEO and OEO blend. Furthermore, measuring often-overlooked parameters such CO_2 (Hernandez et al. 2017), hydrogen sulphide (H_2S) (Shah et al. 2020), hydrogen (H_2) (McCauley et al. 2020), oxidation reduction potential (ORP) (Huang et al. 2018) and conductivity (Fang 2023) could offer crucial insights into LEO's and OEO's effects on ruminal fermentation and GHG emissions.

Based on the previous assumptions the aim of this study was to investigate the *in vitro* influence of LEO and OEO, both individually and in combination, on ruminal fermentation and GHGs, hypothesising that LEO and OEO would individually reduce GHG emissions in ruminal fermentation, and that their combination would produce a synergistic effect, significantly enhancing fermentation efficiency and offering a sustainable strategy to mitigate the environmental impact of ruminant production.

Materials and methods

Experimental design

This study was conducted to investigate the influence of LEO and OEO additives on *in vitro* ruminal fermentation of the total mixed ration (TMR) of dairy cows for 24 h. A completely randomised design was applied, incorporating five experimental treatments and a blank (no EOs and no TMR) needed to correct for the effect of the *inoculum*. The experimental treatments included (doses as % of dry matter TMR): (1) control only TMR (0% EOs), (2) LEO at 0.07% (L), (3) OEO at 0.07% (O), (4) a combination of LEO (0.035%) and OEO (0.035%) (LO1) and (5) a combination of LEO (0.07%) and OEO (0.07%) (LO2). The 0.07% dosage used in this study was determined through the company's extensive R&D experience. Initial dosages selection was based on studies investigating EOs as ruminal fermentation modulators (e.g. Hart et al. 2008; Kolling et al.

2018; Zhou et al. 2020). Building on this foundation, the company further refined the dosage through years of trial-and-error, ultimately identifying 0.07% EOs in the dry matter of TMR as the optimal concentration, which provided the best performance and proved practical for large-scale farm applications.

The parameters measured were total gas, ruminal gases (CO_2, CH_4, H_2S, H_2), pH, ORP, Cond and VFA. The obtained data (except pH, ORP and Cond) were corrected by subtracting the blank. The experiment was conducted in triplicate across two runs, resulting in a total of six replicates for each treatment ($N = 6$), as was done by Sahebi Ala et al. (2021). The overall experimental design is summarised in Figure 1.

Rumen fluid collection

Rumen fluid collection was conducted following the standardised method of Fortina et al. (2022). Briefly, for each run, the rumen fluid was collected in a slaughterhouse from the rumen of three clinically healthy, multiparous dairy cows at the end of their productive lifespan. At the time of slaughter, the cows were not lactating and exhibited no clinical signs of metabolic disorders, infectious diseases, or nutritional deficiencies, confirming their overall health and ensuring that the rumen microbiota was not affected by any underlying health conditions. For each experimental run, 3 L of rumen fluid were collected from the rumen of each of three dairy cows, resulting in a total of 9 L, which were then thoroughly mixed. The rumen fluid was sampled immediately after slaughter and delivered to the laboratory within 30 min, maintaining it at 39 °C. It was then strained and kept at 39 °C under a CO_2 atmosphere. Filtered rumen fluid was mixed with the Menke et al. (1979) buffer (ratio 1:2, vol/vol).

Total mixed ration

The fermentation substrate was a TMR for dairy cows. The proportions of the ingredients and the chemical composition are detailed in Table 1. The chemical composition of the TMR was conducted according to AOAC standard procedures (AOAC 2023). Dry matter (DM) (method 930.15) and ash content (method 942.05) using a Leco TGA801 gravimetric oven (Leco Instruments UK Ltd., Cheshire, UK); crude protein (CP) by Dumas combustion (method 990.03) using a LECO CN928 N Analyser (Leco Instruments UK Ltd., Cheshire, UK); ether extract (EE) was measured using Soxhlet extraction (method 920.39) (Fat Ex-tractor E-800, Büchi

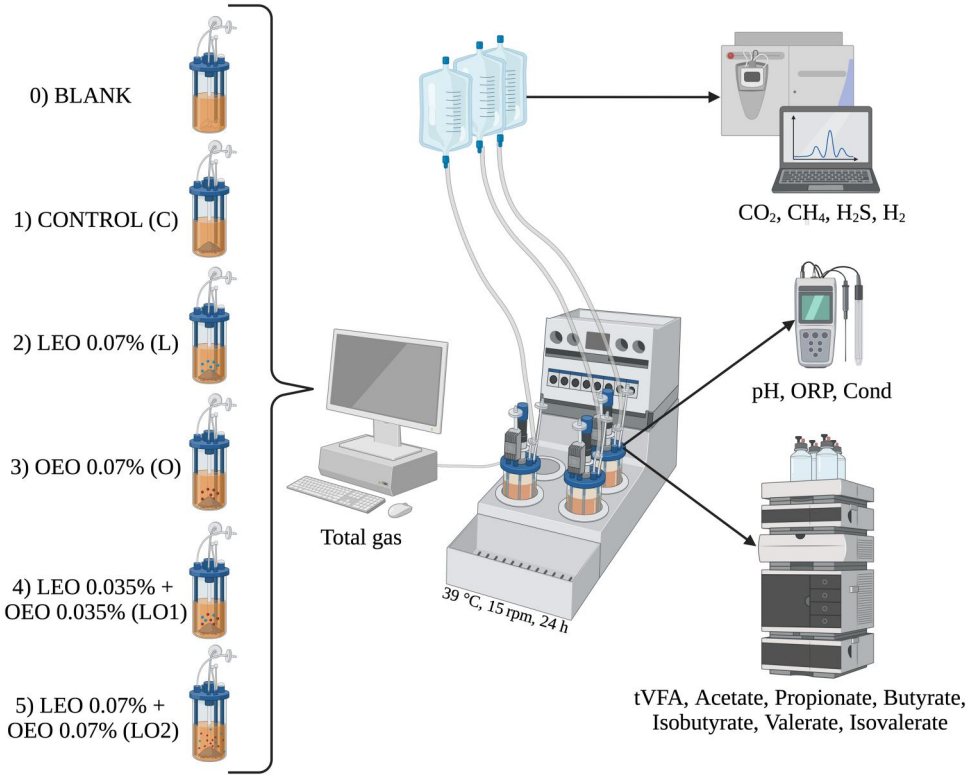

Figure 1. Summary of the experimental design (ORP: oxidation reduction potential; Cond: conductivity; tVFA: total volatile fatty acids) created with BioRender.com.

Table 1. Ingredients and chemical composition of total mixed ration used in the experiment.

Ingredient	g kg^{-1} DM
Corn flour	282
Meadow hay	260
Alfalfa hay	219.3
Wheat bran	130
Beet pulp	49.2
Soybean meal	43.5
Minerals and probiotics mix	14
Monocalcium phosphate	1.5
Ammonium sulphate	0.5
Chemical composition	**(% of DM)**
DM (% fresh matter)	90.30
CP	14.80
EE	2.81
Ash	8.05
NDF	35.76
ADF	20.99
Total starch	22.79

Note: DM: dry matter; CP: crude protein; EE: ether extract; NDF: neutral detergent fibre; ADF: acid detergent fibre.

Table 2. Lemongrass and oregano essential oils (LEO and OEO) origin and composition of main bioactive compounds.

	LEO	OEO
Species	*Cymbopogon winterianus*	*Origanum vulgare*
Family	Poaceae	Lamiaceae
Origin	Indonesia	Spain
Bioactive compounds	Citronellal (35 – 50%) Geraniol (15–25%) Citronellol (5–20%)	Carvacrol (60–75%) p-Cymene (4–15%) γ-Terpinene (4–10%) Thymol (0–5%)

Labortechnik AG, Flawil, Switzerland); total starch content was determined using a total starch assay kit (Megazyme, International Ireland Ltd., Bray, Ireland) (method 996.11). Neutral detergent fibre (NDF) following the procedure of Mertens (2002) with the use of alpha amylase; acid detergent fibre (ADF), was determined following the procedure of Van Soest et al. (1991). NDF and ADF were analysed using Ankom Delta Fibre Analyser (Ankom Technology Corporation, Fairport, NY). NDF and ADF content included ash.

Feed additives

The feed additives used were the purified extracts of EOs derived from lemongrass (*Cymbopogon winterianus*) (LEO) and oregano (*Origanum vulgare*) (OEO). LEO and OEO are produced in leaves and flowers (Missopolinou et al. 2012; Mukarram et al. 2021). EOs yields as % of DM plant weight are 1–2% for LEO and 1–5% for OEO (Missopolinou et al. 2012; Ranade and Thiagarajan 2015). These natural EOs were formulated into a powder by being absorbed onto a solid support comprising zeolite and silicon dioxide. Origin and main bioactive compounds composition are detailed in Table 2. Additional information about the composition and formulation of LEO and OEO cannot be disclosed due to business confidentiality. These additives were incorporated into TMR, as detailed in the experimental design section, immediately before the incubation.

In vitro fermentation and total gas production measurement

The *in vitro* fermentation was conducted according to the method of Braidot et al. (2023). Briefly, the apparatus used was the RITTER Biogas Batch Fermentation System composed of 18 RITTER PMMA fermentation bottles, each with 1 L of total available capacity. Each bottle was closed with an airtight cap equipped with a mixing system (rotation rhythm: 40 s of rotation followed by a 20 s stop at a speed of 15 xg), connected with a flexible plastic tube (inner diameter 4 mm) to RITTER MilliGascounters and uniformly tempered at 39 °C in heating oven (Type BBFS-18). The total gas volume was automatically acquired from Biogas Batch Fermentation Systems through real time data logged in to Windows® software RIGAMO. In addition, each bottle was also connected with gas sampling bag (GSB-P/10, Dr.-Ing. Ritter Apparatebau GmbH & Co. KG, Germany) to collect and quantify ruminal fermentation gases. Each bottle was filled with 750 mL of buffered rumen fluid plus 5.81 g of DM a combination of TMR and LEO or/and OEO additives at the percentages reported in the experimental design section and in Figure 1.

Ruminal gases quantification: CO_2, CH_4, H_2S, H_2

After the *in vitro* fermentation, each gas sampling bag was analysed to quantify ruminal fermentation gases, in particular: CO_2, CH_4, H_2S and H_2, using the mass spectrometer (MS) (Hiden Analytical HPR-20 R&D). Calibration gases (single point calibration) consisting of CO_2 (69.78%), H_2 (1.96%), CH_4 (14.85%) and H_2S (0.101%) in argon were used to calibrate the MS using the software QGA (2.0.4) (Figure 1).

pH, oxidation reduction potential and conductivity measurements

After the incubation, the following parameters were measured: pH, oxidation reduction potential (ORP) and conductivity (Cond) using the XS pH 8 PRO Stirrer - Bench pHmeter with Electrode Standard DHS for pH

and ORP and the XS COND 7 Vio portable conductivity metre with Cell 2301 T for Cond (Figure 1).

Volatile fatty acids quantification

After the incubation, 5 mL of buffered rumen fluid were collected and mixed with 5 mL of 0.01 N H_2SO_4. The samples were then stored at −20 °C until analysis.

To analyse VFA was followed the method applied by Spanghero et al. (2023). In brief, the samples were thawed at room temperature, centrifuged at 9880 xg for 30 min at 10 °C and filtered through syringe filters (RC 0.45 μm, 25 mm; GVS North America Sanford, ME 04073 – USA). The filtrate was transferred into the autosampler vials and 20 μL were injected into high-performance liquid chromatography (HPLC) system. The system used was the Jasco Extrema LC-4000 including a Jasco PU-4180–HP pump, a Jasco UV-4075 UV-visible detector, a Jasco AS-4550 autosampler (20 μL loop) and a Jasco C0-4061 column oven (set at 40 °C). The HPLC separations were achieved using an Aminex HPX-87H column (300 mm × 7.8 mm) with a precolumn (Bio-Rad). Sulphuric acid 0.008 N was used as the mobile phase at a flow rate of 0.6 mL/min. Full spectra were recorded in the range of 190–400 nm and the optimum wavelength detection for all VFA was found to be 220 nm. Peaks of analytes were compared with the retention times of a standard mixture and quantification was based on the external standard method. VFA standards of acetate, propionate, butyrate, isobutyrate, isovalerate and valerate were obtained from Merck (Figure 1) (Wang et al. 2021; Spanghero et al. 2023).

Statistical analysis

Data from the two runs (total 6 repetitions for each treatment) were elaborated with RStudio 2022.07.2 and SAS 9.4. Statistical analyses were performed to evaluate the effects of feed additives (C, L, O, LO1, LO2) on gas production variables (total gas, CO_2, CH_4, H_2S, H_2), pH, ORP, Cond and VFA (tVFA, acetate, propionate, butyrate, isobutyrate, valerate, isovalerate, acetate:propionate) using linear mixed-effects models (LME) with the 'lme' function from the 'nlme' package in R (version 3.1–161). The statistical model used was as follows: $Yij = \mu + Tj + Ri + eij$, where Yij is each observation of the dependent variable (total gas, CO_2, CH_4, H_2S, H_2, pH, ORP, Cond or VFA), μ is the overall mean, Tj is the fixed effect of the feed additive treatment (C, L, O, LO1, LO2), Ri is the random effect of the run and eij is the residual error term. To verify the significance of the differences between feed additive treatments, post-hoc tests were conducted using the 'emmeans' and 'cld' functions from the 'emmeans' package (version 1.8.3). Estimated marginal means (EMMs) for each feed additive level were calculated, and pairwise comparisons were performed with Sidak adjustment for multiple comparisons. Experimental data were shown in the tables by means and standard deviation. Differences among means with $p < 0.05$ were represented as statistically significant differences.

To visualise and comprehensively analyse the effects of the tested additives, we employed Canonical Discriminant Analysis (CDA). This multivariate technique reduces dimensionality by finding linear combinations of quantitative variables that maximise separation between groups. Using feed additive levels as the classification variable, CDA generated canonical variables that captured the essential patterns of between-group variation. Prior to the CDA, we conducted Stepwise Discriminant Analysis (SDA) to identify the most significant parameters, streamlining the subsequent canonical analysis.

Results

Total gas and ruminal gases quantification: CO_2, CH_4, H_2S, H_2

Results of the total gas and ruminal gases quantification, as detailed in Table 3, indicate significant effects of the various treatments applied. Specifically, the combination of the two EOs at both dosages, LO1 and LO2, resulted in a substantial reduction in total gas production compared to the C ($p = 0.001$). CO_2

Table 3. Total gas and ruminal gases quantification: CO_2, CH_4, H_2S, H_2 (mL/g of feed dry matter) after 24 h of *in vitro* fermentation ($N = 6$).

	Treatment						
	C	L	O	LO1	LO2	SEM	*p* Value
Total gas	207 ± 21.8^{a}	194 ± 18.1^{ab}	200 ± 18.1^{ab}	188 ± 11.0^{b}	189 ± 13.6^{b}	6.6	.001
CO_2	165 ± 22.0^{a}	151 ± 17.9^{b}	157 ± 19.2^{b}	147 ± 13.8^{b}	147 ± 15.4^{b}	5.4	<.001
CH_4	40 ± 1.6	42 ± 0.9	42 ± 2.6	40 ± 3.3	41 ± 2.1	2.0	.192
H_2S	0.5 ± 0.32	0.4 ± 0.36	0.7 ± 0.19	0.6 ± 0.12	0.6 ± 0.13	0.25	.361
H_2	0.2 ± 0.04^{a}	0.2 ± 0.06^{ab}	0.1 ± 0.02^{b}	0.2 ± 0.02^{ab}	0.2 ± 0.01^{ab}	0.04	.022

Notes: C = control; L = lemongrass 0.07%; O = oregano 0.07%; LO1 = lemongrass 0.035% and oregano 0.035%; LO2 = lemongrass 0.07% and oregano 0.07%. Different superscripts within a row indicate significant differences ($p < .05$). Results are expressed as mean ± SD.

Table 4. Fermentation parameters: pH, oxidation reduction potential (mV) and conductivity (mS/cm) after 24 h of *in vitro* fermentation ($N = 6$).

	Treatment						
	C	L	O	LO1	LO2	SEM	*p* Value
pH	6.80 ± 0.060^{a}	6.76 ± 0.064^{b}	6.78 ± 0.076^{a}	6.81 ± 0.078^{a}	6.80 ± 0.082^{a}	0.004	.004
ORP	-360 ± 6.3^{b}	-354 ± 5.8^{ab}	-345 ± 8.9^{a}	-349 ± 1.9^{ab}	-349 ± 6.2^{ab}	1.2	.004
Cond	12 ± 0.52	12 ± 0.68	12 ± 0.60	12 ± 0.42	12 ± 0.46	0.04	.05

Notes: ORP = oxidation reduction potential; Cond = conductivity; C = control; L = lemongrass 0.07%; O = oregano 0.07%; LO1 = lemongrass 0.035% and oregano 0.035%; LO2 = lemongrass 0.07% and oregano 0.07%. Different superscripts within a row indicate significant differences ($p < .05$). Results are expressed as mean ± SD.

Table 5. The quantity of volatile fatty acids after 24 h *in vitro* fermentation (tVFA as mg/mL; individual VFAs as % of tVFA) ($N = 6$).

	Treatment						
	C	L	O	LO1	LO2	SEM	*p* Value
tVFA	2.7 ± 0.41	2.9 ± 0.13	3.0 ± 0.11	3.2 ± 0.37	3.2 ± 0.46	0.06	.07
Acetate	55.2 ± 2.28	55.8 ± 2.20	55.2 ± 1.82	55.3 ± 2.47	55.4 ± 2.28	0.09	.50
Propionate	21.1 ± 1.2^{a}	20.4 ± 0.88^{ab}	20.4 ± 0.89^{ab}	20.1 ± 0.23^{ab}	19.8 ± 0.23^{b}	0.10	.02
Butyrate	18.4 ± 1.53	17.9 ± 1.08	18.1 ± 0.71	18.2 ± 1.58	18.4 ± 1.50	0.16	.79
Isobutyrate	0.98 ± 0.297	1.07 ± 0.158	1.16 ± 0.183	1.16 ± 0.152	1.16 ± 0.262	0.038	.39
Valerate	2.90 ± 0.498	3.09 ± 0.441	3.03 ± 0.46	3.12 ± 0.495	3.12 ± 0.491	0.015	.05
Isovalerate	1.43 ± 0.885^{b}	1.76 ± 0.832^{ab}	2.10 ± 0.883^{ab}	2.07 ± 1.056^{ab}	2.13 ± 1.096^{a}	0.087	.03
Acetate:Propionate	2.63 ± 0.252^{b}	2.74 ± 0.217^{ab}	2.71 ± 0.203^{ab}	2.75 ± 0.154^{ab}	2.79 ± 0.136^{a}	0.013	.04

Notes: C = control; L = lemongrass 0.07%; O = oregano 0.07%; LO1 = lemongrass 0.035% and oregano 0.035%; LO2 = lemongrass 0.07% and oregano 0.07%; tVFA = total Volatile Fatty Acids. Different superscripts within a row indicate significant differences ($p < .05$). Results are expressed as mean ± SD.

emissions were significantly lower than that of the C for all the feed additives tested individually and in combination (L, O, LO1 and LO2) ($p < 0.001$). In contrast, CH_4 emissions were not significantly affected by any of the EOs treatments ($p = 0.192$). Furthermore, the production of H_2S remained unchanged across all the feed additive treatments ($p = 0.361$), while H_2 was reduced only by O when compared to C ($p = 0.022$).

pH, oxidation reduction potential and conductivity measurements

Table 4 summarises the fermentation parameters after 24 h of the *in vitro* fermentation. In detail, pH was not affected by O, LO1 and LO2, while it was reduced by L ($p = 0.004$). Only O caused an increase of ORP which indicates greater oxidising capacity ($p = 0.004$). No significant differences in Cond were observed among the treatments ($p = 0.05$).

Volatile fatty acids quantification

Table 5 reports the quantity of VFA measured after 24 h of the *in vitro* fermentation. No significant differences were observed for tVFA, acetate, butyrate, isobutyrate and valerate ($p > 0.05$). However, compared to C, LO2 caused a reduction in propionate ($p = 0.02$) and consequently an increase of the acetate:propionate ratio ($p = 0.04$). Moreover, with the same treatment, an increased production of isovalerate was recorded ($p = 0.03$).

Canonical discriminant analysis of selected ruminal fermentation parameters

CDA was performed on 9 key ruminal fermentation parameters, selected from an initial set of 16 through Stepwise Discriminant Analysis (SDA). The selected parameters were pH, ORP, CO_2, H_2, tVFA, propionate (P), isobutyrate (IB), acetate:propionate ratio (AP) and isovalerate (IV).

Individual univariate analyses of these parameters showed limited discriminatory power, with R^2 values ranging from 0.05 to 0.45, with ORP showing the highest significance ($p < 0.01$). However, multivariate analysis revealed significant combined effects ($p < 0.0001$, Wilks' Lambda Test), suggesting strong interactions among parameters.

The first two canonical variables (Can1 and Can2) demonstrated robust discriminatory power, with squared canonical correlations of 0.89 and 0.83, respectively. Can1 accounted for 60.4% of the between-group variation, primarily distinguishing control (C) from lemongrass (L) treatments, while positioning the OEO treatments intermediately. Can2 explained an additional 36.3% of variation, separating both C and L groups from the OEO treatments. Together, these two canonical variables captured

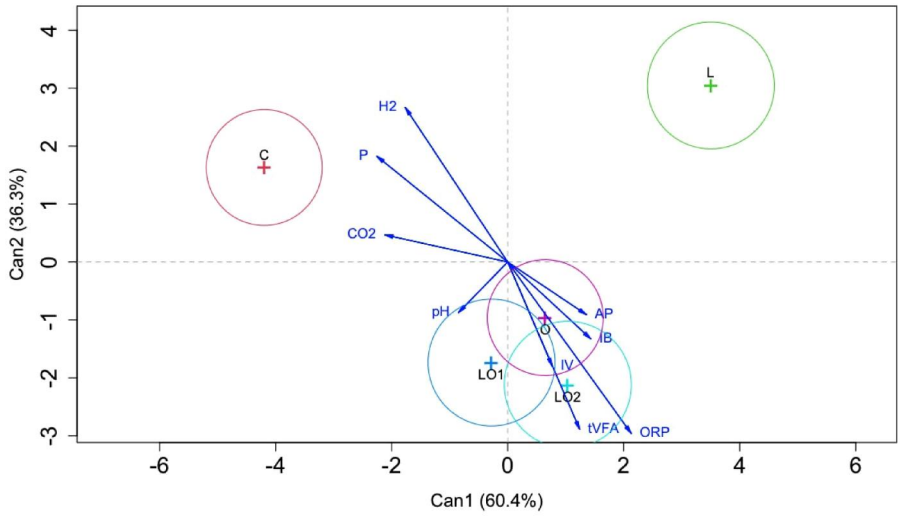

Figure 2. Canonical discriminant analysis (CDA) of the nine selected ruminal fermentation parameters by a stepwise discriminant analysis: pH, ORP (oxidation reduction potential), CO_2, H_2, tVFA (total volatile fatty acids), P (propionate), IB (isobutyrate), AP (acetate:propionate), IV (isovalerate). C = control, L = lemongrass 0.07%, O = oregano 0.07%, LO1 = lemongrass 0.035% and oregano 0.035%, LO2 = lemongrass 0.07% and oregano 0.07%.

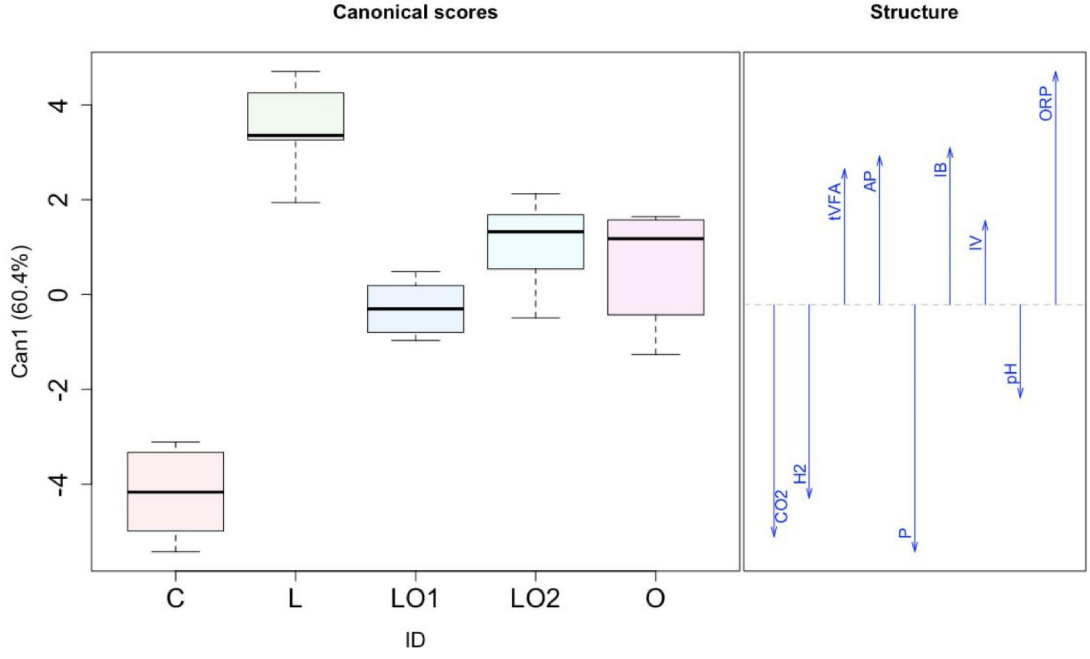

Figure 3. Contribution of the 9 ruminal fermentation parameters to the first canonical variable (Can1). pH, ORP (oxidation reduction potential), CO_2, H_2, tVFA (total volatile fatty acids), P (propionate), IB (isobutyrate), AP (acetate:propionate), IV (isovalerate). C = control, L = lemongrass 0.07%, O = oregano 0.07%, LO1 = lemongrass 0.035% and oregano 0.035%, LO2 = lemongrass 0.07% and oregano 0.07%.

96.7% of the total variation, providing a comprehensive representation of treatment effects.

The most influential discriminating parameters for Can1 were propionate, ORP, CO_2 and H_2, with pH also making a substantial contribution. As illustrated in Figures 2 and 3, the multivariate relationships among these parameters revealed clear treatment-specific patterns. Notably, the ORP parameter showed that oregano's effects masked those of lemongrass, suggesting a dominant influence of oregano in the combined treatments.

Discussion

In this study we investigated, for the first time, the combination of LEO and OEO at two dosages (LO1 and LO2) and we considered some generally

overlooked ruminal fermentation parameters (CO_2, H_2S, H_2, ORP and Cond) to better understand the roles of LEO, OEO and their combination in the modulation of ruminal fermentation.

Total gas, ruminal gases: CO_2, CH_4, H_2S, H_2 and volatile fatty acids quantification

Total gas production was negatively affected by the combination of LEO and OEO at the two dosages LO1 and LO2, resulting in a decrease in total gas by about 9% compared to C, while no effect was observed for the additives used alone (L and O). A similar trend of reduction in total gas was showed by Joch et al. (2019) using, in an *in vitro* experiment, a blend of EOs (a mix of cresols, thymol, limonene, vanillin, guaiacol, eugenol and salicylates) at the dosage of 1000 mg/L (corresponding to 0.08% addition to TMR in each serum bottle), while no reduction was detected at lower doses. Also, Rossi et al. (2022) found a similar reduction in total gas (10%) caused by a combination of EOs (from cloves, coriander seed and geranium), tannins from chestnuts and flavonoids from olives at the dosage of 0.0025% on DM substrate (glucose) in an *in vitro* incubation. This result, obtained with lower dose at 0.0025% compared to our study at 0.07% (LO1) and 0.14% (LO2), could be due to the addition of tannins and flavonoids, both knows for their suppressive effects on ruminant GHG emissions (Min et al. 2020; Tedeschi et al. 2021). Alabi et al. (2023) tested, *in vitro,* four EO blends composed of four or five EOs at the dosage of 0.02% on TMR and registered a greater decrease of total gas ranging from 12.7% (clove, oregano, peppermint, eucalyptus EOs) to 32% (garlic, lemongrass, cumin, lavender and nutmeg EOs). The higher reduction compared to our results could be due to the higher number of EOs used in the formulation of the blends (two in our study *vs* four/five in their work). Coşkuntuna et al. (2023) evaluated similar dosages of lavender essential oils: 0, 0.05 and 0.1% on dairy cow concentrate feed. However, in contrast to our findings, they did not observe a reduction in total gas production after 24 h of *in vitro* fermentation.

Both feed additives, whether used alone or in combination at two different dosages, demonstrated suppressive effects on CO_2 emissions compared to the C. Specifically, reductions averaged 9% for L, 5% for O, 11% for LO1 and 12% for LO2. These results indicate that combining EOs was more effective than using them individually. Doubling the dosage of the combination resulted in a slight increase in CO_2 reduction. Despite this tendency the differences among the four treatments were not statistically significant. The reduction in CO_2 emissions contributes to lowering the carbon footprint of livestock production, which is a critical aspect in efforts to mitigate climate change (Kinley et al. 2020). The integration of these EOs is well aligned with the increasing consumer demand for sustainable and eco-friendly livestock production practices (Kamra et al. 2012). Similar, Coşkuntuna et al. (2023) observed reduction of 17% in CO_2 production at dosage 0.1% of lavender EOs on dairy cow concentrate feed. By contrast, in the same study, it did not find any CO_2 suppressive effect at the dosage of 0.05%. No CO_2 variation were found in the *in vitro* study of Günal et al. (2017) with LEO at the dosage of 500 mg/L of buffered rumen fluid (corresponding to 3.5% of LEO in diet) and in the *in vivo* study of Olijhoek et al. (2019) with OEO at dosages from 0.7% to 5.3% of DM dietary. The discrepancies in CO_2 suppression effects among these studies could be attributed to variations in experimental condition (*in vitro vs in vivo*), animal diet (differences in TMR composition), EOs form and dosages.

LO1 and LO2 contribute to the reduction of total gas and CO_2 production. The potential mechanisms caused by the synergic effect by LEO and OEO bioactive compounds could be: (i) inhibition of rumen microbiota: the hydrophobicity that is typical of EOs is responsible for the disruption of bacterial structures that leads to increased permeability due to an inability to separate the EOs from the bacterial cell membrane (Calsamiglia et al. 2007; Nazzaro et al. 2013); (ii) alteration of fermentation pathways: the bioactive compounds in LEO and OEO can interfere with enzymes involved in the decarboxylation reactions that lead to CO_2 production (Patra 2011; Caroprese et al. 2023); (iii) selective bacterial activity: LO1 and LO2 may promote the activity of specific ruminal bacteria that produce less CO_2 and consequently reduce total gas (Alabi et al. 2023; Bokharaeian et al. 2023; Ike et al. 2024).

In our study, the EOs tested, both individually and in combinations, did not exhibit anti-methanogenic effects. These findings align with previous studies that evaluated the effect of LEO at dosages of 0.02, 0.04, 0.06% of TMR (Soares et al. 2023), 0.01, 0.02, 0.03% of TMR (Nanon et al. 2014) in *ex situ* and *in vitro* trials respecitivily; OEO at dosages of 0.005% of TMR *in vivo* trial (Benchaar 2020). In contrast, other studies have reported anti-methanogenic *in vitro* effects with LEO at 10, 20, 40, 80 μL/40 mL of buffered rumen fluid (corresponding to 0.002, 0.004, 0.009, 0.018% of DM diet, assuming the ISO 3217:2015 relative density of LEO = 0.872 – 0.897 g/mL) (Singh et al. 2018), 167, 333, 500,

667, 833 μL/L of buffered rumen fluid (corresponding to 0, 2, 5, 7, 10, 12% of DM diet, assuming the ISO 13171:2015 relative density of OEO = 0.930 – 0.955 g/mL) (Pawar et al. 2014); OEO at 91,130 mg/L of buffered rumen fluid (corresponding to 1.1% and 1.6% of OEO in feed) (Zhou et al. 2020) and 1.0, 1.5, 2.0 g/L of buffered rumen fluid (corresponding to 5, 10, 15, 20% of OEO in feed) (Cobellis et al. 2015). These differences suggest that the effectiveness of EOs in reducing CH_4 production could vary depending on different factors such as EOs form (liquid *vs* solid) and doses, animal diet (different composition of TMR or different substrate TMR *vs* concentrate), experimental conditions.

Monitoring the effects of feed additives on H_2S production is crucial, as high levels can cause toxic effects as polioencephalomalacia (Lévy 2020). None of the tested additives showed an increase in H_2S levels compared to the C. Similarly, Coşkuntuna et al. (2023) did not observe any significant change in H_2S level with 0.05% lavender essential oil, while a reduction was observed at 0.1%. By contrast Alabi et al. (2023) found an increased production of H_2S in some EOs blends (mix of garlic, lemongrass, cumin, lavender and nutmeg with and without fumaric acid at the dose of 0.02% of TMR), comparing to the control. The variation in H_2S production highlights the complexity of interactions between EOs and rumen microbial populations. Moreover, other parameters such as dietary sulphur levels, pH and dietary NDF content can also affect H_2S production (Shah et al. 2020).

EOs could inhibit hydrogen-producing bacteria such as *Lachnospira multiparus*, *Ruminococcus albus* and *Ruminococcus flavefaciens* and protozoa, resulting in a decreased production of H_2 and a consequent decrease of CH_4 (Cobellis, Trabalza-Marinucci, Marcotullio, et al. 2016). In our study, only O influenced H_2 production, resulting in a 27% reduction compared to C. However, this reduction was not associated with changes in CH_4 emissions. The lack of correlation between reduced H_2 production and CH_4 emissions could be due to the complex and adaptive interactions within the rumen microbiota, where alternative metabolic pathways and microbial compensatory mechanisms can use H_2 as sink (i.e. reductive acetogens, sulphate-reducing bacteria, propionate producers, nitrate reducing bacteria) (Lan and Yang 2019). The ineffectiveness of lemongrass in modulating H_2 is in agreement with the work of Günal et al. (2017) which used a dose of 500 mg/L of buffered rumen fluid (corresponding to 3.5% of LEO in diet).

In general, ruminal fermentation gases are closely linked to the production of total volatile fatty acids (tVFA) (Judd and Kohn 2018). However, in our study, we observed a reduction in total gas and CO_2 production due to the inclusion of EOs, but this was not accompanied by a corresponding decrease in tVFA levels, which remained unchanged. These findings align with previous researches on the role of EOs in ruminal fermentation. For example, Günal et al. (2017) reported that clove EOs reduced CO_2 and total gas production without affecting tVFA concentrations. Similarly, Alabi et al. (2023) found that a blend of EOs (including anise, clove, oregano, cedarwood and ginger) reduced total gas production without causing a decrease in tVFA. Additionally, in a study by Patra and Yu (2012), five EOs—clove, eucalyptus, garlic, oregano and peppermint—led to a reduction in fermentation gases, but had mixed effects on tVFA, with decreases observed for clove and oregano, no changes for garlic and peppermint and an increase for eucalyptus. These findings emphasise the complex interactions between EOs and ruminal fermentation, highlighting that more research is needed to fully understand the underlying mechanisms and the varied effects of different EOs.

Individual VFA were not affected by any of the feed additives, with the notable exception of LO2. The ineffectiveness of EOs (except LO2) in modulating VFA concentrations indicate that they did not negatively impact ruminal fermentation. This observation aligns with the overall lack of impact on VFA described in the review of Cobellis, Trabalza-Marinucci, and Yu (2016), in which similar results were observed with various EOs at the dosage of 0.1 g/L of buffered rumen fluid (0.8% of EOs in feed) including: *Ocimum basilicum*, lemongrass, *Eucalyptus citriodora*, *Ocimum gratissimum*, *Laurus nobilis*, *Mentha piperita*, etc.

By contrast at the highest dosage, lemongrass and oregano EOs blend (LO2) significantly reduce propionate levels and increased isovalerate compared to the control. However, similar trends for propionate and, though not statistically significant, are also observed in the individual EOs (L and O) and in the blend at the lowest dosage (LO1). This suggests that both EOs have the potential to inhibit propionate production, with the synergistic effect at the highest dose being significant compared to C. The reduction of propionate can be attributed to the selective antimicrobial effects of the essential oil compounds against bacteria that are key propionate producers, as *Selenomonas ruminantium* and *Megasphaera elsdenii* (Benchaar et al. 2007; Wang et al. 2012). Phenolic compounds, as thymol and caravacrol can disrupt the outer membranes of these Gram-negative bacteria, as demonstrated by Helander et al. (1998) and Dorman and Deans (2000),

thereby impeding their growth and metabolism. Additionally, thymol has been shown to suppress the growth of *Streptococcus bovis* (Evans and Martin 2000), a significant amylolytic bacterium involved in propionate production through starch fermentation (Benchaar et al. 2007). The increase in isovalerate levels reflects a shift in fermentation pathways. Isovalerate is produced during the breakdown of branched-chain amino acids (decarboxylation and deamination) and the inhibition of propionate-producing microbes likely allows proteolytic bacteria to thrive (Allison 1978; Wallace et al. 1997; Apajalahti et al. 2019).

According to our results, Patra and Yu (2012) observed a decrease in propionate and an increase in AP with oregano and peppermint EOs at the dosage of (0.25, 0.5, 1 g/L of buffered rumen fluid, corresponding to 2.5, 5, 10% EOs in feed).

These changes in the VFA profile could have important implications for dairy cattle. The higher AP could improve the fat content of dairy products (National Research Council (U.S.) 1988). However, the reduction in propionate levels, observed with LO2 supplementation, may impair glucose availability, potentially leading to decreased milk yield (García-Roche et al. 2021). Therefore, dairy nutritionists should carefully evaluate the inclusion of LO2 in feed formulations, aiming to balance the benefits for milk composition with the potential impacts on overall milk production.

pH, oxidation reduction potential and conductivity measurements

To assess rumen health and ensure proper metabolic function, monitoring pH, ORP and Cond is essential. Despite their importance, few studies have thoroughly examined these parameters in relation to EOs (Fang 2023).

In our study, rumen pH ranged from 6.76 to 6.80, consistently within the ideal range of 5.5 to 7.0 for optimal rumen functions (Fregulia et al. 2021). Only LEO caused a slight reduction in pH, but the change was less than 1%. This slight reduction is unlikely to cause adverse effects on rumen health, as it remains well within the physiological range required for maintaining microbial balance and preventing acidosis (Fregulia et al. 2021). Our findings are consistent with Soares et al. (2023), who found similar pH values with LEO (0.02, 0.04, 0.06% of TMR), ranging between 6.68 to 6.77 without any statistical differences in comparison to the control. Benchaar (2020), also stated that OEO and its main bioactive compound, carvacrol, (0.005% of TMR) did not affect ruminal pH. The absence of LEO effect on pH suggests that these additives do not disrupt the rumen's acid-base balance, which is critical for ensuring efficient fermentation (Riond 2001).

ORP values of rumen fluid typically shows negative readings, reflecting the strong reducing power of the rumen environment caused by microbial fermentation activity (Dijkstra et al. 2020). In our study ORP values ranged from −360 mV in the C to −345 mV in the O treatment, remaining within the physiological range for ruminants (−430 mV to −115 mV) according to the review of Huang et al. (2018). These values reflect a stable reducing environment conducive to effective microbial activity, which is important for maintaining the health of rumen microorganisms and supporting overall digestive function (Huang et al. 2018).

Cond of rumen fluid is rarely investigated despite its potential role in better understanding the nutritional elements and ion activities during the fermentation process (Fang 2023). In our study Cond was 12 mS/cm and did not differ among the treatments. This value was in the range found in few previous studies from 11.4 to 18.3 mS/cm (Mayberry et al. 2009; Peñafiel and Ticona 2015).

Canonical discriminant analysis of selected ruminal fermentation parameters

CDA revealed distinct clustering patterns, clearly separating the treatments into three groups: C, L and a cluster containing O, LO1 and LO2. The key parameters driving this discrimination were propionate (P), ORP, CO_2 and H_2. Each of these parameters reflects changes in microbial activity and fermentation pathways induced by the EOs.

The C group represents typical ruminal fermentation, with balanced microbial activity and normal fermentation profiles. In contrast, treatments involving LEO, OEO and their combinations (LO1, LO2) displayed distinct shifts in fermentation patterns. Specifically, oregano-based treatments were particularly effective in reducing propionate production, lowering CO_2 and H_2 emissions and altering ORP. These effects are likely due to the antimicrobial properties of bioactive compounds in oregano, such as carvacrol and thymol, which target specific ruminal microbes involved in CO_2 and propionate production. This suggests that OEO treatments influence fermentation pathways by modulating the microbial community, particularly those microbes responsible for the production of these gases and fermentation end-products.

Previous studies have applied multivariate techniques as CDA and Canonical Correspondence Analysis (CCA) primarily to explore the effects of different diets on various parameters, including fermentation kinetics (Gannuscio et al. 2024), the relationship between bacterial community structure and ruminal fermentation (Belanche et al. 2019), dimethylacetals in rumen fluid (Cappucci et al. 2018), volatile organic compounds in beef meat (Vasta et al. 2011) and the fatty acid profile of muscle (Cama-Moncunill et al. 2021). These studies highlight the growing importance of multivariate approaches in understanding complex biological processes. However, to the best of our knowledge, our study is the first to apply CDA specifically to examine the impact of EOs on ruminal fermentation parameters.

Conclusions

LEO and OEO, both individually and in combinations significantly modulated ruminal fermentation and reduced GHGs, specifically CO_2, although their antimethanogenic activity was not confirmed. The VFA profile was slightly affected only by the combination of the EOs at the highest dose, resulting in a reduction in propionate and an increase in AP and isovalerate. Moreover, the addition of EOs did not influence pH, ORP and Cond, that remained within the physiological range for ruminants. The CDA confirmed distinct effects of the EOs, highlighting their potential as natural feed additives for improving ruminal fermentation and mitigating the environmental impact of ruminant livestock production. Based on these outcomes, we would recommend LO2 as the most promising treatment for future studies aiming to further investigate EOs as natural feed additives for reducing the environmental impact of ruminant livestock production. Future studies should include feed degradability and microbiota analysis to gain a deeper understanding of the effects of LEO and OEO on ruminal fermentation. Furthermore, investigating potential synergies with other feed additives and assessing the economic feasibility of incorporating these EOs into ruminant diets would be highly beneficial.

Acknowledgements

The authors would like to acknowledge the whole team of the laboratory Rumilab of Nutristar S.p.A for providing the facilities and equipment to conduct the experiment and for the ruminant nutrition expertise.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

SGP was funded by Ph.D. PROGRAMS, 37th CYCLE (PON - D.M. 1061/21) - GREEN TOPICS.

ORCID

Sara Glorio Patrucco http://orcid.org/0000-0003-2040-7708
Khalil Abid http://orcid.org/0000-0002-4070-5238
Salvatore Barbera http://orcid.org/0000-0002-0897-7962
Hatsumi Kaihara http://orcid.org/0009-0001-0636-904X
Sonia Tassone http://orcid.org/0000-0002-0227-1969

Data availability statement

The data presented in this study are available on request from the corresponding author.

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