Modulation of milking performance, methane emissions, and rumen microbiome on dairy cows by dietary supplementation of a blend of essential oils

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ABSTRACT

Cattle represent a high contribution of the livestock's greenhouse gas emissions, mainly in the form of methane. Essential oils are a group of plant secondary metabolites obtained from volatile fractions of plants that have been shown to exert changes in the rumen fermentation and may alter feed efficiency and to reduce methane production. The objective of this study was to investigate the effect on rumen microbial population, CH_4 emissions and milking performance of a mixture of essential oils (Agolin Ruminant, Switzerland) incorporated daily in the ration of dairy cattle. Forty Holstein cows (644 ± 63. 5 kg of BW producing 41.2 ± 6.44 kg/d of milk with 190 ± 28.3 DIM) were divided into two treatments (n = 20) for 13 wk and housed in a single pen equipped with electronic feeding gates to control access to feed and monitor individual DM intake (**DMI**) on a daily basis. Treatments consisted of no supplementation (**Control**) or supplementation of 1 g/d of a blend of essential oils (**BEOs**) fed in the TMR. Individual milk production was recorded using electronic milk meters on a daily basis. Methane emissions were recorded using sniffers at the exit of the milking parlour. At day 64 of the study, a sample of rumen fluid was collected from 12 cows per treatment after the morning feeding using a stomach tube. There were no differences in DMI, milk yield, or milk composition between the two treatments. However, cows on BEO exhaled less CH_4 (444 ± 12.5 l/d) than cows on Control (479 ± 12.5 l/d), and exhaled less ($P < 0.05$) CH_4/kg of DM consumed (17.6 vs 20.1 ± 0.53 l/kg, respectively) from the first week of study, with no interaction with time, which suggests a fast action of BEO of CH_4 emissions. Rumen relative abundance of *Entodonium* increased, and those of Fusobacteria, Chytridiomycota, *Epidinium,* and *Mogibacterium* decreased in BEO compared with Control cows. Supplementing 1 g/d of BEO reduces CH_4 emissions on absolute terms (l/d) and diminishes the amount of CH_4 produced by unit of DM consumed by cows relatively soon after the first supplementation, and the effect is sustained over time without impacting intake or milking performance.

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Implications

This study was conducted with the aim of evaluating the potential medium-term effects of supplementing a combination of eugenol, geranyl acetate, and coriander essential oils on milking performance, methane emissions, and rumen microbiome of dairy cows. Supplementing this combination of essential oils in a ration containing no silages slightly reduces feed efficiency, but does not affect milk production. Daily methane emissions both in absolute terms and per unit of feed consumed are reduced, and the rumen microbiome changes slightly. Feeding this combination of essential oils is effective in reducing methane emissions from dairy cattle-fed rations with no silages.

Introduction

According to the Intergovernmental Panel on Climate Change report, greenhouse gas (**GHG**) emissions from livestock represent 6.3% of total anthropogenic GHG emissions (IPCC, 2022). Among livestock, cattle represent a high contribution to the sector's GHG emissions (∼35%), mainly in the form of methane (Aan den Toorn et al., 2020), especially in Asia as the main continent responsible

for CH_4 emissions from both enteric fermentation and rice production (IPPC, 2022). However, one must keep in mind that the GHG emissions from livestock, including ruminants, mostly originate from CO_2 that was already in the air and was captured by the feed (i.e., corn) and consumed by the animals; thus, their net contribution to GHG, even though CH_4 is thought to be ∼23 times more potent than CO_2 as a greenhouse gas (Wuebbles and Hayhoe, 2002) should be considered, somehow, differently than anthropogenic emissions derived from fossil fuels, whose carbons are extracted directly from underground and were not previously present in the atmosphere (at least recently).

Essential oils (**EOs**) are a group of plant secondary metabolites obtained from volatile fractions of plants (Patra and Saxena, 2010) and have been studied as potential modifiers of ruminal fermentation with the aim of improving the efficiency of nutrient utilisation and to reduce methane production (Tekippe et al., 2013; Pirondini et al., 2015; Elcoso et al., 2019); and in fact, some EOs have been shown to exert negative effects on Gram-positive bacteria (Carrazco et al., 2020) and reduce methane production (Santos et al., 2010; Hart et al., 2019; Zhou et al., 2019). However, in terms of animal performance, the response of EO *in vivo* has been highly variable. Several authors (Benchaar et al., 2007; Tassoul and Shaver, 2009; Sgoifo Rossi et al., 2022) have reported positive effects on DM intake (**DMI**) and milk performance, but others (Spanghero et al., 2009) found no impact of EO on milk production or total tract digestibility, and Blanch et al. (2016) described improvements in milk yield of multiparous, but not primiparous cows. Differences in animal response to EO can be attributed to a number of factors including the type and dose of EO used, the proportion of each EO within the mixture, the stage of lactation, and the type of ration fed, among many other aspects. A common blend of EO used in dairy cows is a combination of eugenol, coriander essential oil, and geranyl acetate (Santos et al., 2010; Castro-Montoya et al., 2015; Elcoso et al., 2019). Eugenol (4-allyl-2-methoxyphenol) is a phenolic monoterpene which has been shown to have antimicrobial activity against both Gram-positive and Gram-negative bacteria (Walsh et al., 2003). Similarly, the EO from geranium has been associated with anti-bacterial effects (Lis-Balchin and Deans, 1997). Lastly, the EO from coriander has been reported to potentially alter *in vitro* digestion and methane production when feeding high-forage diets (Jahani-Azizabadi et al., 2011). Taken together, it could be hypothesised that the combination of eugenol, coriander EO, and geranyl acetate has the potential to modify rumen fermentation and decrease methane production in the rumen by modulating the growth of some rumen microbes. However, there is a lack of studies addressing changes in microbial population *in vivo* when feeding this combination of EO. Hence, the aim of the study was to evaluate the potential medium-term effects of supplementing a combination of eugenol, geranyl acetate, and coriander EO (Agolin® Ruminant, Agolin SA, Bière, Switzerland) on milking performance, methane emissions, and rumen microbiome. Preliminary results from this study were first published in abstract form elsewhere (Bach et al., 2022).

Material and methods

Animals, experimental design, diets, and management

This study was conducted under the supervision and approval of the Animal Care Committee of IRTA (Barcelona, Spain; expedient number: 11045). A total of 40 lactating (32 multiparous and eight primiparous) Holstein cows were blocked by parity (primiparous or multiparous) randomly split into two treatments (n = 20) following a complete randomised design. Randomisation was conducted using a random generator script in Python. Sample size

was defined for milk yield as main outcome variable (as it was expected to collect the potential effects on DMI and rumen changes) based on a power analysis for repeated measures conducted with GLIMMPSE (https://www.glimmpse.samplesizeshop.org) and a Hotelling lawley trace test (Akbari et al., 2013) with power = 0.80, and α = 0.05. Cows in both treatments received the same diet, with the only difference being that half of the cows were supplemented with 1 g/d of a blend of EO (**BEO**) containing eugenol, geranyl acetate, and coriander EO (Agolin® Ruminant, Agolin, Bière, Switzerland), whereas the other half received no supplementation (**Control**). The BEO blend was prepared several times during the study by mixing the EO, corn, and soybean meal at 0.125, 62.49, and 37.39%, respectively. A similar premix was prepared by mixing corn and soybean meal at 62.60 and 37.40%, respectively, to be fed to the Control cows. The premixes were then included in the TMR on a daily basis along with the rest of ingredients. The rations consumed by cows are depicted in Table 1. Animals were on their respective diets for 13 weeks (91 d). The week before the study onset, all cows were fed the same TMR (i.e., the Control diet).

All grains were included in the TMR ground between 2 and 3 mm, and forages were chopped at a theoretical length of cut of 30 mm. Samples of TMR were taken at weekly intervals and stored at −20 °C for subsequent nutrient analyses. Nutrient analyses followed Bach et al. (2021) and included DM (ID 934.01), ash (ID 942.05), ether extract (ID 920.39), and N (ID 984.13) content following the Association of Official Analytical Chemists (AOAC International, 2000), and for NDF and ADF according to Van Soest et al. (1991) using an ANKOM220 Fiber Analyzer unit (ANKOM Technology Corporation, Fairport, NY, USA) with sodium sulfite and a heat-stable amylase. Non-fibre carbohydrates were calculated as 100 minus CP, NDF, ether extract, and ash.

Measurements and sample collection

Individual feed intake was recorded on a daily basis using electronic feed bins (MooFeeder, MooSystems, Cortes, Spain) that controlled the access of cows in the same pen to specific bins containing the different dietary treatments. Individual milk production at every milking was determined using electronic milk meters (AfiMilk, Afikim Ltd., Israel), and milk fat and milk protein content were determined also electronically every milking using the AfiLab system (Afikim Ltd., Israel), which was calibrated fortnightly. All cows were body weighed twice daily at the exit of the milking parlour using an electronic scale. Individual methane exhalation was determined on all cows (20 cows per treatment) during 20 minutes (10 in the morning and 10 min in the afternoon) coinciding with the exit of the milking parlour for a visit duration of 15.7 ± 2.2 min from Monday to Thursday on alternate days, to obtain 52 sets of measures for every cow in the study. Methane and carbon dioxide recordings were performed using the sniffer technique with an electronic meter (NDIR, Guardian NG Edinburg Instruments Ltd., Livingston, UK) validated by Rey et al. (2019) and previously used by Garnsworthy et al. (2019) to assess the CH_4 production of dairy cows in commercial farms. There was a total of five measurement stations consisting of a feed bin and two electronic meters each (one for CH_4 and one for CO_2). These stations were placed at the exit of the milking parlour, and cows were offered 100 g of soybean meal to ensure that their head was placed inside the recording station. The stations had a gas sampling tube from the front of a cow's head pumped air into the electronic gas analyser to continuously measure CH_4 and CO_2. Air was sampled at a rate of 1 L/min through an 8-mm polyamide tube, using approximately 2 m of tube from the analyser to cow's nostrils. Each day before starting measurements, the NDIR analyser was verified using standard mixtures of CH_4 or CO_2 and nitrogen (0.0, 0.25, 0.50, 0.75 and 1.0%; MESA International Technologies

Table 1
Ingredient and nutrient composition (DM basis) of the dairy cattle experimental diets.

Item	Treatment	
	Control	BEO
Ingredient, % of DM		
Alfalfa hay	7.71	7.70
Fescue hay	11.00	11.00
Ryegrass hay	20.2	20.2
Straw	0.77	0.77
Soybean meal	11.90	11.90
Mineral/Vitamin premix[1]	7.35	7.35
Corn grain	19.92	19.89
Wheat grain	9.72	9.72
Soybean hulls	9.72	9.72
Palm oil	1.71	1.71
Agolin Ruminant	-	0.04
Nutrients (DM basis)		
CP, % of DM	16.5	16.4
NDF, % of DM	36.3	36.8
ADF, % of DM	21.6	21.2
Ash, % of DM	7.4	7.2
Ether extract, % of DM	3.7	3.9
NFC, % of DM	36.1	35.7
NE_L, Mcal/kg of DM[2]	1.68	1.68

Abbreviations: BEOs = Blend of essential oils; NFCs = Non-fibre carbohydrates; NE_L = Net energy of lactation.

[1] Contained: 81.6 mg/kg of Zn; 11.5 mg/kg of Cu; 57.6 mg/kg of Mn; 9.86 mg/kg of Co; 1.92 mg/kg of I; 0.34 mg/kg of Se; 58 mg/kg of S; 120 000 IU/kg of vitamin A; 28 800 IU/kg of vitamin D; and 1 920 IU/kg of vitamin E.

[2] NE_L was estimated using NRC (2001) equations.

INC, Santa Ana, CA, USA). Methane and CO_2 concentrations were recorded at 1-s intervals and persisted in a database. Baseline or ambient CH_4 and CO_2 concentration were calculated as mean CH_4 and CO_2 concentrations before starting the measurements and subtracted from the subsequent measured data. Before the start of the study, four measures (2 d) of CH_4 and CO_2 exhalations were collected for each animal to later serve as covariates in the statistical analyses.

At day 64 of the study, a sample of rumen fluid was collected from 12 cows per treatment (randomly selected before the onset of the study) 2 h after the morning feeding using a stomach tube. Samples were frozen at −80 C until subsequent DNA extraction and processing following (López-García et al, 2022). Briefly, the DNA from rumen fluid was extracted and purified using the Qiagen DNeasy Powersoil Pro isolation kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The DNA concentrations and their purity were measured by spectrophotometry using a NanoDrop ND-1000 UV/Vis Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE) and the Qubit Fluorometer (Life Technologies, Grand Island, NY). One microgram of DNA was analysed following the 1D Native barcoding genomic DNA (with EXP-NBD104 and EXP-NBD114) and ligation sequencing kit (SQK-LSK109) protocol from Oxford Nanopore Technologies (UK), using the GridION sequencer (Oxford Nanopore Technologies, UK) and R9.4.1 flow cells. Guppy toolkit (Version 6.1 ONT) was used for basecalling and demultiplexing. A quality control was then applied removing sequences with QS < 7 and length < 150 bp. Sequence analysis was performed using SqueezeMeta pipeline for long reads (Tamames and Puente-Sánchez, 2019), which performs Diamond Blastx against GenBank nr-NCBI (downloaded May 12, 2021) taxonomic database, then identifying and annotating open reading frames using the LCA method. Reads were processed in Blastx by SQM long reads pipeline. All sequences mapped as non-microbial (i.e., virus, animals, and plants) were discarded. Microbial sequences were then filtered by prevalence to reduce data sparsity and sequencing errors.

Calculations and statistical analysis

Alpha-diversity (i.e. diversity within a community from an animal sample) at the genus level was calculated as the Shannon index using the phyloseq (McMurdie and Holmes, 2013) function "estimate_richness()" on R (version 4.1.3; R Core Team, 2022). Beta diversity (i.e., diversity between communities of different animal samples) across all samples was calculated using the Bray-Curtis dissimilarity index (Bray and Curtis, 1957), and a gradient analysis for each treatment (CS or SDL) was performed using non-metric multidimensional scaling also at the genus level. The experimental unit was the animal because treatments were applied at the animal level. Data from the 7-d baseline period, preceding the study onset, were averaged within cow and used as a covariate. Data were averaged within cow and week and submitted to a mixed-effects model that included the 7-d baseline period as a covariate, the fixed effects of treatment, week, and their 2-way interactions, and the random effects of cow and block (parity). Repeated structure was modelled using animal within treatment as a subject and an autoregressive order one covariate-variance matrix structure, which yielded the smallest Bayesian criterion among the tested covariate-variance structures. Differences in ß-diversity between treatments were assessed by PERMANOVA on Bray-Curtis dissimilarity matrices performed with 1,000 permutations. Differential relative abundance (**RA**) between treatments was assessed using a multivariate ANOVA (with no repeated measures) with the Limma package (Ritchie et al., 2015) of R (version 4.1.3; R Core Team, 2022) using centred log-transformed data as input and correcting for false discovery rates (Benjamini and Hochberg, 1995). False discovery rate-adjusted *P*-values are shown throughout the manuscript.

Results

Performance and methane exhalation

Performance results are depicted in Table 2. There were no differences in any of the parameters recorded between Control and BEO cows, except for feed efficiency, which was lower ($P < 0.05$) in BEO than in Control cows throughout the study.

Animals on BEO exhaled less ($P = 0.05$) methane than cows on Control, and exhaled less ($P < 0.05$) methane per kg of DM consumed, right from the first week of study, with no interaction with time (Table 2). Furthermore, the amount of methane produced by kilogram of DM consumed was reduced ($P < 0.01$) by 8.8% in BEO compared with Control cows. However, when CH_4 emissions were expressed per unit of milk production, there were no differences between treatments (Table 2).

Rumen microbiome

The alpha-diversity of the rumen microbiome, measured as Shannon index, was lower ($P < 0.05$) in BEO than in Control cows (Fig. 1). The rumen microbiome at all evaluated taxa levels below class (until genus) was more similar (i.e., lower ß diversity) among BEO than Control cows (Table 3). At the kingdom level, BEO cows tended (adj. *P*-value = 0.06) to have a lower RA (0.9 ± 0.13%) of Eukaryota than Control cows (1.28 ± 0.13%), but RA of Bacteria and Archaea did not differ between treatments (Fig. 2). The rumen RA (expressed relative to Eukaryote population) of the phylum Chytridiomycota tended (adj. *P*-value = 0.08) to be lower in BEO (25.3 ± 0.5%) than in Control (26.6 ± 0.05%) cows; whereas that of the phylum Evosa was greater (adj. *P*-value < 0.001) in BEO (1. 18 ± 0.001%) than in Control (0.82 ± 0.001%) cows. Within the Eukaryote kingdom, the rumen RA of unclassified *Neocalli-*

Table 2
Performance and estimated methane exhalation of Holstein dairy cows as affected by treatments.

Item	Treatment			P-value[1]		
	Control	BEO	SE	T	W	TxW
BW, kg	635	629	4.57	0.32	<0.01	0.54
DMI, kg/d	24.8	25.2	0.26	0.38	<0.01	0.95
Milk yield, kg/d	38.8	38.7	0.53	0.93	<0.01	0.89
Milk fat, %	3.64	3.68	0.03	0.31	<0.01	0.36
Milk fat, kg/d	1.40	1.41	0.02	0.90	<0.01	0.72
Milk protein, %	3.23	3.27	0.01	0.54	<0.01	0.89
Milk protein, kg/d	1.27	1.26	0.02	0.86	<0.01	0.96
ECM,[2] kg/d	40.0	40.1	0.54	0.86	<0.01	0.93
Feed efficiency[3]	1.67	1.57	0.03	0.04	<0.01	0.79
CH_4, l/d	479	444	12.5	0.05	0.01	0.96
CH_4, l/kg of Milk	12.43	11.54	0.43	0.15	<0.01	0.92
CH_4, l/kg of DMI	20.1	17.6	0.53	<0.01	0.02	0.94

Abbreviations: Control = Unsupplemented; BEO = supplemented with 1 g/d of a blend of essential oils; DMI = DM intake; ECM = Energy-corrected milk.
[1] T: Effect of treatment; W: Effect of week; TxW: Effect of the interaction between treatment and week.
[2] Energy-corrected milk was calculated following Erdman (2011).
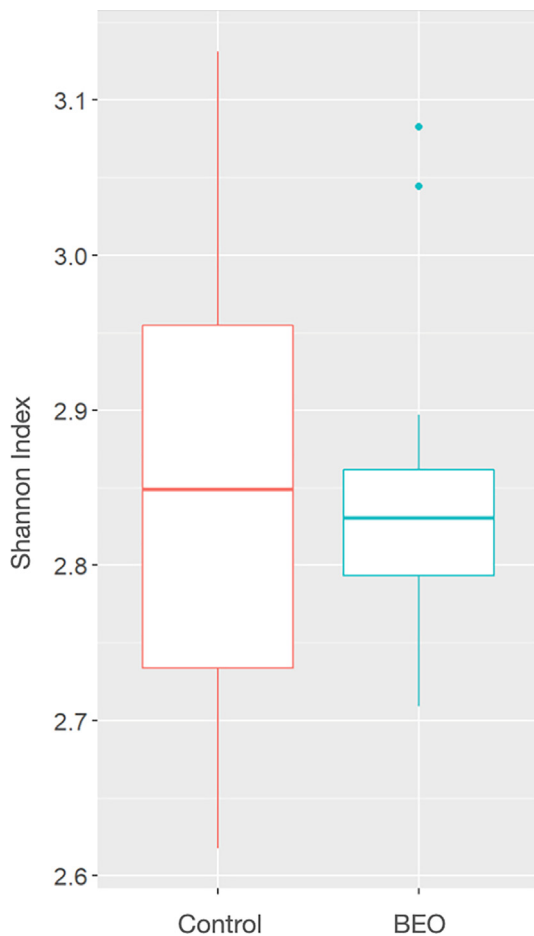[3] Calculated as energy-corrected milk/DM intake.

Fig. 1. Alpha-diversity (Shannon index) of the rumen microbiome of Holstein cows as affected by treatment. Control = unsupplemented, BEO = supplemented with 1 g of a blend of essential oils/d.

Table 3
ß-diversity (average distance to centroid) of the rumen microbiome of Holstein cows calculated as distance from group centroids of cows as affected by treatments.

Taxa	Treatment		P-value
	Control	BEO	
Phylum	1.49	1.37	0.16
Class	2.11	2.02	0.18
Order	4.15	3.80	0.01
Family	5.56	5.08	<0.01
Genus	7.96	7.28	0.03

Abbreviations: Control = Unsupplemented; BEO = supplemented with 1 g/d of a blend of essential oils.
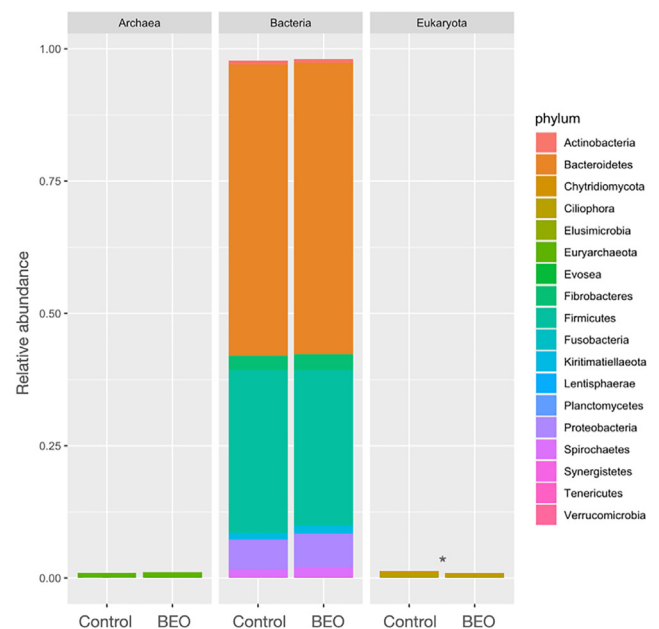

Fig. 2. Relative abundance of kingdoms in the rumen of Holstein cows as affected by dietary treatments. Asterisk denotes differences at Adjusted P-value < 0.05 between treatments. Control = unsupplemented, BEO = supplemented with 1 g of a blend of essential oils/d.

mastigomycetes tended (adj. P-value = 0.09) to be lower in BEO than in Control cows (Fig. 3). Similarly, at the phylum level, the rumen RA of Chytridiomycota was lower (adj. P-value < 005) in BEO than in Control cows (Fig. 4). The rumen RA abundance of *Entodinium* was greater (adj. P-value < 0.05) in BEO than in Control cows, whereas those of *Epidinium* and Ciliphora were lower (adj. P-value < 0.05) in BEO than in Control (Figs. 3 and 4, respectively).

Within the Bacteria kingdom, at the phylum level, the rumen RA of Fusobacteria was lower (adj. P-value < 0.05), and those of Spirochaetes greater (adj. P-value < 0.04) in BEO than in Control cows (Fig. 4). Lastly, at the genus level, the rumen RA of *Mogibacterium* of
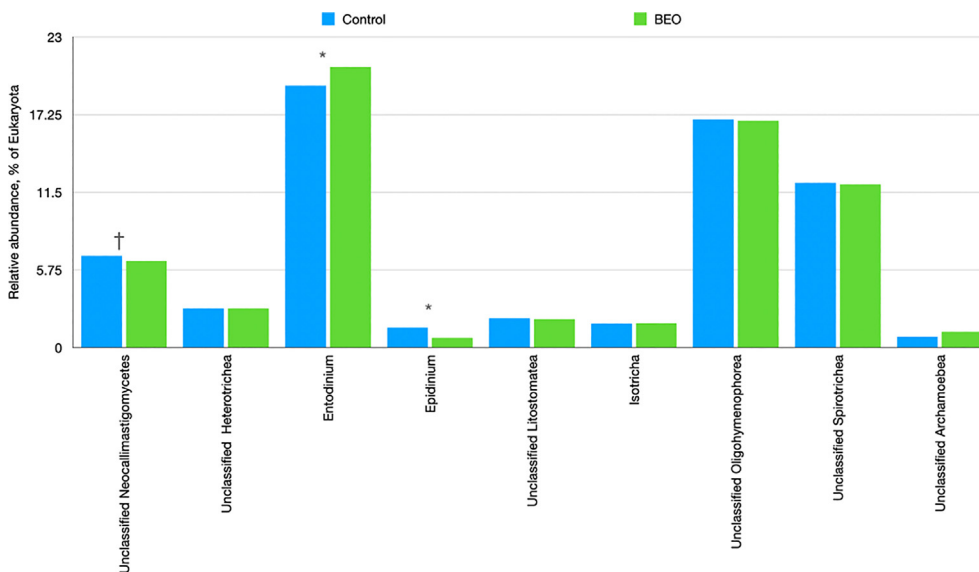
Fig. 3. Relative abundance (with respect to total Eukaryote) of genera and unclassified classes in the rumen of Holstein cows as affected by dietary treatments. \dagClass tends to differ at Adjusted *P*-value < 0.10. *Genus differs at Adjusted *P*-value < 0.05. Control = unsupplemented, BEO = supplemented with 1 g of a blend of essential oils/d.
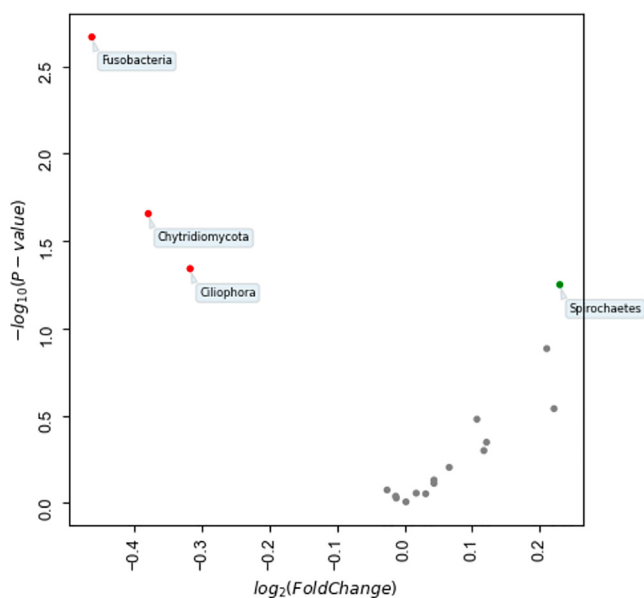

Fig. 4. Volcano plot of relative abundance of phyla in the rumen of Holstein cows unsupplemented or supplemented with a blend of essential oils.

BEO cows was lower (adj. *P*-value < 0.02) than in Control cows (data not shown).

Discussion

Performance and methane exhalation

Feed efficiency was lower in BEO than in Control cows throughout the study. This result would indicate that BEO had a relatively fast negative (but moderate) effect on feed efficiency (as there was no interaction with time). This reduction was likely the result of the non-significant increase in DMI observed in BEO compared with Control cows (Table 2). In the literature, animal responses, in terms of feed efficiency, when feeding EO are inconclusive. Elcoso et al. (2019) reported an increase in feed efficiency when feeding the same EO as in the current study to dairy cows. The different response in these two studies could be linked to the type of rations fed. Elcoso et al. (2019) fed a ration containing grass and corn silages, whereas herein, the ration had no silages. Belanche et al. (2020) conducted a meta-analysis of 23 *in vivo* studies and reported that feed efficiency increased when feeding the same mix of EO used herein for periods > 4 wk. However, a former meta-analysis (involving a variety of EO and doses) reported no effects on performance or feed efficiency in dairy cattle (Khiaosa-Ard and Zebeli, 2013). Reasons for discrepancies in the outcomes in feed efficiency could be linked to the type of diet. Studies involving a synthetic compound with CH_4-inhibiting effects have reported that its effectiveness is lower in diets containing high proportion of forages (Schilde et al., 2020) or non-grain silages (van Gastelen et al., 2022) than in high-concentrate rations. Thus, it seems likely that CH_4 responses to feeding CH_4-inhibiting compounds may depend on the ingredient composition of the diet.

Methane can represent a loss between 2 and 12% of gross energy intake (Johnson and Johnson, 1995); thus, it could be expected that a reduction in methane exhalation would improve feed efficiency. However, if DMI increases as a response to feeding EO, digesta passage rate may also increase and this may hinder the ability of the animal to extract all the nutrients and energy from the diet, hence reducing feed efficiency. For example, Melgar et al. (2020) described a non-significant increase in DMI (about one additional kg; similar to herein) and a concomitant non-significant decrease in feed efficiency when supplementing dairy cows with 40 mg of a methane-inhibiting compound. Similarly, Reynolds et al. (2014) tested different doses of a methane-inhibitor in dairy cows, and although feed efficiency was not reported, the efficiency of N utilisation (i.e., N produced in milk/ N consumed) tended to decrease as methane inhibition in the rumen increased. Another potential indication that feed utilisation may be impaired when feeding methane-inhibitors is the fact that, in most occasions, when supplementing some types of methane-inhibiting compounds, such as 3-nitrooxypropanol, feed intake increases (Hristov et al., 2022). A plausible explanation could be that cows are trying to compensate a potential decrease in the amount of energy they are able to extract from the diet (due to some inhibition of the digestibility of the diet) with more feed consumption, as intake is greatly dictated by energy demand

(Allen and Bradford, 2012). This is a hypothesis contrary to the belief that while reducing CH_4 emissions, more energy should be available to the animal to sustain milk yield (because more carbons are potentially retained by the animal), and thus increase feed efficiency, but if more energy was available, cows would most likely not increase intake. Also, as discussed below, if rumen microbial population within the rumen is shifted when feeding EO, fibre degradation may be impaired, and in turn hamper feed efficiency.

Cows on BEO exhaled less methane than cows on Control and there was no interaction with time, which suggests a relatively fast action of Agolin® on methane emissions in the current study. The decrease in methane exhalation herein (9.2%) is in line with the observations in a former meta-analysis (Belanche et al., 2020) conducted using similar methane-inhibitors than in the current study. As mentioned before, however, the magnitude of CH_4 reduction seems to depend on diet composition. Also, the lack of an interaction between treatment and time in the reduction of methane produced by kilogram of DM consumed by BEO compared with Control cows further supports that the action of Agolin® was relatively fast after being supplemented in the diet.

The reductions (~8>%) in CH_4 emissions expressed per kg of DM consumed in BEO cows herein are a little below of those reported in the meta-analysis from Belanche et al. (2020), who concluded that the blend of EO used in the current study resulted in a decrease of ~13% of CH_4 emitted per kg of feed consumed. Furthermore, the lack of differences in CH_4 emissions when expressed relative to milk production herein is in disagreement with the meta-analysis from Belanche et al. (2020). Again, these differences may be linked with the type of diet fed in the current study, which had no wet forages (i.e., silages).

Rumen microbiome

The alpha-diversity of the rumen microbiome was lower in BEO than in Control cows (Fig. 1), and the rumen microbiome at all evaluated taxa levels below class (until genus) was more similar (i.e., lower ß diversity) among BEO than Control cows (Table 3). Herein, ß-diversity values (calculated as distance from the group centroid) in the rumen microbiome indicate a low degree of similarity in specific taxa between individuals within the dietary treatment. Alpha-diversity has been negatively associated with milk yield (Xue et al., 2018), although in the current study, cows on Control and BEO produced the same amount of milk despite BEO cows had a lower alpha-diversity. Monteiro et al. (2022) reported no differences in alpha-diversity in the rumen of cows classified as either highly or poorly efficient, but found that ß-diversity in dairy cows classified as having a low residual feed intake (thus highly efficient) was lower than in those with high residual feed intake, but no differences were observed when feed efficiency was assessed as the ratio between milk yield and DMI.

Methanobacteria are within the Archaea domain, and the lack of differences in rumen RA of this domain observed herein would make it, a priori, difficult to explain the observed differences in methane exhalation, as these bacteria have been negatively associated with feed efficiency in ruminants (Delgado et al., 2019). However, ciliate protozoa and fungi (both within the kingdom of Eukaryote) are the main rumen microbes that have been previously associated with rumen methane emissions (López-García et al., 2022), and herein, the rumen RA (expressed relative to Eukaryote population) of the phylum Chytridiomycota tended (adj. *P*-value = 0.08) to be lower in BEO (25.3 ± 0.5%) than in Control (26.6 ± 0.05%) cows, which could partially explain the reduction in CH_4 exhalation in BEO cows. Similarly, the tendency towards lower rumen RA of *Neocallimastigomycetes* observed herein may partially explain the reduction in CH_4 exhalations recorded in the current study. The role of fungi in methane production in the rumen has been previously described. For instance, *Neocallimastigomycetes* contains the genus *Neocallimastix*, and this class and genus have been linked with improved cell wall degradation of fibrous ingredients (Fontes and Gilbert, 2010), which may lead to a concomitant increase in methane production (Boots et al., 2013; Aydin et al., 2017).

It has been shown that methanogenic bacteria are closely associated with protozoa in the rumen (Krumholz et al., 1983) and protozoa counts and types have been associated with CH_4 emissions, both in vivo (Dai et al., 2022) and in vitro (Spanghero et al., 2022). Herein, rumen RA of Ciliphora were lower (adj. *P*-value < 0.05) in BEO than in Control cows (Fig. 4), and rumen RA of *Entodonium* increased, whereas those of *Epidinium* decreased in BEO compared with Control. Khiaosa-Ard and Zebeli (2013) conducted a meta-analysis and concluded that EO may affect protozoa numbers, but with different patterns: at high EO doses (>0.20 g/kg DM) protozoa numbers are reduced, whereas at lower EO doses, they are increased. Interestingly, not all ciliated protozoa may have the same effect on rumen methane production. Newbold et al. (1995) evaluated the effect of different protozoal population on rumen methane production, and reported that methane production was lowest in rumen fluids containing either *Isotricha prostoma* plus *Entodinium* or *Polyplastron multivesiculatum*, and greatest in rumen fluids containing *Entodinium*, *Eudiplodinium* and *Epidinium*. Interestingly, *Entodinium* have been shown to harbour a large number of intracellular bacteria but no methanogens (Kittelmann and Janssen, 2011). Thus, the greater rumen RA of *Entodinium* in BEO cows herein is in line with previous results showing a reduction in rumen methane production. On the other hand, *Epidinium* have been shown to harbour intra- and extracellular methanogenic archaea (Lloyd et al., 1996) and they have been linked with increased methane production in the rumen (Newbold et al., 1995). Therefore, the lower rumen RA of *Epidinium* herein would also support the observed reduction in CH_4 exhalation in BEO compared with Control cows. In fact, this change in protozoal RA in the rumen could be one of the main potential mechanisms responsible for the observed changes in CH_4 emissions observed herein. A recent review (Hegarty et al., 2021) concluded that evidence about the potential ability of EO to alter enteric emissions is inconsistent, but noted that the mechanisms by which EO could elicit reductions in CH_4 emissions could be linked to a decrease in the rumen protozoa and methanogenic populations resulting in greater productions of propionate in the rumen.

The RA of Fusobacteria was lower and those of Spirochaetes greater in the rumen of BEO than that of Control cows. Bacteria within Fusobacteria have been reported to be able to can penetrate the blood and infect the body of cows with the development of liver abscesses and hoof lesions (Tadepalli et al., 2009). On the other hand, bacteria within the phylum Spirochaetes have been associated with low-forage diets (Pandit et al., 2018), but to our knowledge, no association between this phylum and feed efficiency or methane exhalation has been established. Lastly, the RA of *Mogibacterium* in the rumen of BEO cows was lower than in Control cows. This genus has been associated with increased feed efficiency in ruminants (McGovern et al., 2020; McLoughlin et al., 2020), but also with higher methane-emitting steers (Wallace et al., 2015).

Limitations of the study

The number of biological replicates enrolled herein was determined based on milk production as a primary outcome. Thus, it is possible that a different sample size would be required to detect the potential effects of BEO on other parameters measured in this study. Another potential limitation is that methane was measured

at exact times of the day (after milking), and thus, they may not fully represent the dynamics of methane emissions throughout the day. However, alternative systems such as GreenFeed (C-Lock Inc., Rapid City, SD, USA) collect data when animals visit a station to consume feed, but visits are not regular across days and they do not fully represent the entire dynamics of methane emissions either. Lastly, rumen samples were collected at a single day and 2 h after feeding. Further studies are needed to assess potential changes in the microbiol population over different periods of time and at different hours relative to feeding.

Conclusion

The results from this study indicate that feeding 1 g/d of a blend of essential oils containing eugenol, geranyl acetate, and coriander reduces methane emissions either on absolute terms or expressed per kilogram of feed consumed with no consequences on milk production or composition. However, feed efficiency is slightly reduced. These changes are likely induced by shifts in the rumen microbial population, which experienced an increase in relative abundances of *Entodonium* and a decrease in relative abundances of Fusobacteria, Chytridiomycota, *Epidinium,* and *Mogibacterium* when cows are supplemented with the blend of essential oils used herein.

Ethics approval

This study was conducted under the supervision and approval of the Animal Care Committee of IRTA (Barcelona, Spain; expedient number: 11045) and was compliant with the guidelines established by European Union Directive 2010/63/EU on the protection of animals used for scientific purposes.

Data and model availability statement

None of the data were deposited in an official repository. However, they are freely available from the corresponding author upon request.

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Alex Bach: Methodology, Formal Analysis, Conceptualisation, Investigation, Writing – Original draft.
Guillermo Elcoso: Carrying out the experiment.
Miguel Escartín: Carrying out the experiment.
Katrin Spengler: Review and Editing.
Arnaud Jouve: Review and Editing.

Declaration of interest

Alex Bach, Guillermo Elcoso, and Miguel Escartín declare no conflict of interest. Katrin Spengler and Arnaud Jouve are employees of Agolin SA.

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