Original Research Article

Evaluation of dietary addition of 2 essential oils from *Achillea moschata*, or their components (bornyl acetate, camphor, and eucalyptol) on in vitro ruminal fermentation and microbial community composition

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ABSTRACT

This study investigated the effects of 2 *Achillea moschata* essential oils extracted from plants collected in 2 different valleys of the Italian Alps and 3 pure compounds of oils — bornyl acetate (BOR), camphor (CAM), and eucalyptol (EUCA) — on in vitro ruminal fermentation and microbiota. An in vitro batch fermentation experiment (Exp. 1) tested the addition of all of the substances (2 essential oils and 3 compounds) in fermentation bottles (120 mL) at 48 h of incubation, whereas a subsequent in vitro continuous culture experiment (Exp. 2) evaluated the pure compounds added to the fermenters (2 L) for a longer incubation period (9 d). In both experiments, total mixed rations were incubated with the additives, and samples without additives were included as the control (CTR). Each treatment was tested in duplicate and was repeated in 3 and 2 fermentation runs in Exp. 1 and 2, respectively. Gas production (GP) in Exp. 1 was similar for all of the treatments, and short chain volatile fatty acid (SCFA) production was similar in both experiments except for a decrease of SCFA produced ($P = 0.029$) due to EUCA addition in Exp. 2. Compared to CTR, BOR and CAM reduced the valerate proportion ($P = 0.04$) in Exp. 1, and increased ($P < 0.01$) the acetate proportion in Exp. 2. All treatments increased ($P < 0.01$) total protozoa counts ($+36.7\%$ and $+48.4\%$ compared to CTR on average for Exp. 1 and 2, respectively). In Exp. 1, all of the treatments lowered the Bacteroidetes and Firmicutes and increased the Proteobacteria relative abundances ($P < 0.05$), whereas in Exp. 2, the EUCA addition increased ($P = 0.012$) the *Ruminococcus*. In Exp. 1, methane (CH_4) as a proportion of the GP was lowered ($P = 0.004$) by the addition of CAM and EUCA compared to CTR, whereas in Exp. 2, EUCA reduced the amount of stoichiometrically calculated CH_4 compared to CTR. Overall, essential oils extracted from *A. moschata* and the pure compounds did not depress in vitro rumen fermentation, except for EUCA in Exp. 2. In both experiments, an increase of the protozoal population occurred for all the additives.

1. Introduction

Methane (CH_4) is a greenhouse gas produced by ruminal micro-organisms as a consequence of enteric fermentation. Although CH_4 is an inevitable product of fermentation, its emission can be decreased by supplementing ruminant diets with specific additives and ingredients. Plants produce a wide variety of secondary metabolites to prevent disease, pest, and predator attacks (Jouany and Morgavi, 2007). Among secondary metabolites, essential oils have been widely evaluated as feed additives for improving microbial

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metabolism in the rumen and inhibiting methanogenesis (Calsamiglia et al., 2007; Patra and Yu, 2012; Pirondini et al., 2015). Essential oils are obtained from the steam distillation of plants and include a variable mixture of different compounds, like terpenes and phenylpropanoid derivatives (Aziz et al., 2018), characterized by different properties (Bakkali et al., 2008). Many of the active compounds in essential oils have antimicrobial activity. Antimicrobial activity should be evaluated for the selection of additives that can decrease CH_4 production without compromising overall fermentation in the rumen. Only a few individual active compounds of essential oils have been tested for their effects on rumen fermentation and CH_4 production (Joch et al., 2018). In recent studies (Joch et al., 2016, 2018), compounds such as bornyl acetate (BOR) and camphor (CAM) decreased CH_4 production without adverse effects on short chain fatty acid (SCFA) production during short-term in vitro incubations. Together with eucalyptol (EUCA), CAM and BOR represent the main components of *Achillea moschata* essential oil (Vitalini et al., 2016). *A. moschata* is a plant that has antimicrobial properties (Vitalini et al., 2016), and which has traditionally been used to treat human digestive disorders as well as animal ones (Vitalini et al., 2015). The antibacterial properties of the essential oil from *Achillea* spp. are likely to be due to its high concentration of CAM (about 27% of the oil) and EUCA (about 11% of the oil) (Si et al., 2006). Furthermore, in recent studies (Fidan et al., 2019; Zerkani et al., 2019; Baali et al., 2019), essential oils having BOR among their main constituents also showed antimicrobial activity.

Another issue in the search for feed additive-based mitigation strategies is that the rumen's microbial ecosystem may adapt to the inclusion of feed additives to the diet. In that case, only a transient reduction of CH_4 emissions may be achieved (Klop, 2016). Cardozo et al. (2004) reported a transient effect of plant extracts on fermentation characteristics that disappeared after 6 d, indicating that microbial adaptation can occur after short-term exposure. Hence, the evaluation of the additive by a continuous rumen fermenter is another step that should be performed before conducting an in vivo study. This study hypothesized that the use of *A. moschata* essential oils and their main pure compounds could affect rumen fermentation patterns because of a change in the rumen microbiome communities resulting in lower CH_4 production without negatively affecting fermentability. The objective of the current study was to investigate the effect of *A. moschata* essential oils collected from 2 different locations in Italy and their main pure compounds (BOR, CAM, and EUCA) on the rumen fermentation and the microbiota. In vitro batch fermentation systems were used to screen the essential oils and pure compounds. In contrast, a continuous culture system was used to evaluate the potential adaptation of microbiota to the oils' pure compounds.

2. Materials and methods

The study consisted of 2 in vitro rumen fermentation experiments: a batch fermentation at the Department of Agricultural and Environmental Sciences in Milano (Exp. 1) and a continuous culture fermentation at the Department of Agricultural, Food, Environmental, and Animal Sciences in Udine (Exp. 2). Experiment 1 used fistulated animals, which were handled as outlined by the Directive 2010/63/EU on animal welfare for experimental animals, according to the University of Milan Welfare Organism (OPBA) and with authorization number 904/2016-PR from the Italian Ministry of Health.

2.1. Plant material, essential oils, and pure compounds

A. moschata was collected during the blossom period in July 2017 in 2 different (in terms of altitude, geomorphology, lithology, and temperature influencing the essential oil composition) valleys

of the Rhaetian Alps (located in Sondrio Province, Northern Italy). Specifically, the samples were harvested at 2,400 m in Valfurva and 2,000 m in Valchiavenna. Two voucher specimens (No. AMVF 104 and No. AMVC105, respectively) were deposited at the Department of Agricultural and Environmental Sciences, Milan State University (Milan, Italy) after their identification, according to the morphological traits described in Flora d'Italia (Pignatti, 1982). The air-dried aerial parts (50 g) of *A. moschata* were subjected to steam distillation for 1 h in a Clevenger-type apparatus. The obtained distillates were dried over anhydrous sodium sulfate and were concentrated with a rotary evaporator at 30 °C to produce pale blue yellow oils. The 3 main compounds of *A. moschata* essential oil, BOR, CAM, and EUCA, were purchased from Sigma–Aldrich (Milan, Italy).

2.2. Experiment 1: in vitro batch fermentation

Ruminal fluid was collected from 2 fistulated dry Italian-Friesian dairy cows that were fed a total mixed ration (TMR) composed of corn silage, grass hay, cornmeal, and soybean meal (434, 323, 105, and 136 g/kg DM, respectively); the diet chemical analysis was CP 145, NDF 420, NFC 330, and ash 650 g/kg DM. The cows were fed the TMR twice daily (07:00 and 19:00) to achieve DM intake of 8 kg/d.

Rumen liquor was collected 2 h after the morning feeding. The liquor was strained through 4 layers of cheesecloth and poured into a flask, pre-warmed at 39 °C, and purged with carbon dioxide (CO_2). The buffer solution was prepared according to Menke and Steingass (1988). The fermentation substrates were 2 TMR and the main ingredients were corn silage, cornmeal, solvent extracted soybean meal, alfalfa and grass hays (352, 172, 170, 145 and 133 g/kg DM on average, respectively) and contained 155 and 348 g/kg DM of CP and NDF, respectively. Approximately 200 mg of each TMR was weighed in duplicate in serum bottles (120 mL), and each bottle was inoculated with 30 mL of rumen inoculum and the experimental additives (2 *A. moschata* essential oils, BOR, CAM, and EUCA), following the procedure of Menke and Steingass (1988). A control sample (CTR; i.e., sample without additive) was also incubated. Each compound was dissolved in ethanol, and the compound concentration used was at 200 mg/L of inoculum. The concentration of ethanol in the final inoculum was 0.67% (vol/vol), in agreement with the findings of Benchaar et al. (2007a), where the final concentration of ethanol in culture fluid was less than 2% (vol/vol). The same amount of ethanol was added to CTR.

For each additive (2 *A. moschata* essential oils, BOR, CAM, and EUCA), a corresponding blank (inoculum + ethanol + additive) was incubated, and each additive was tested against the CTR (TMR + inoculum + ethanol).

Three incubation runs were conducted in a shaking water bath at 39 °C for 48 h. At 24 and 48 h of incubation, headspace pressure was recorded using a digital manometer (model 840082, Sper Scientific, Scottsdale, AZ, USA), and a sample of air was collected from the bottle headspace using a gas-tight syringe (Hamilton, USA) and stored in gas-tight vials (Labco Exetainer Vials, UK). The gas pressure data recorded at each time-point were converted to the volume of gas produced (GP) using the ideal gas law. At the end of the incubation, pH was recorded, and 3 samples of liquor were collected: 10 mL for rumen microbiota characterization, 5 mL for the protozoa count, and 5 mL for SCFA determination. For protozoa analysis, 5 mL of 50% formalin solution was added to the samples, whereas for SCFA analysis the samples were acidified with 5 mL of 25% metaphosphoric acid. The samples for microbiota and SCFA were immediately frozen.

Gas samples were analyzed for CH_4 concentration using an Agilent 3000A GC gas chromatograph (Agilent Technologies, Santa

Clara, CA, USA) equipped with a thermal conductivity detector (170 °C) using a stainless steel column (Carboxen 1000, 60/80 mesh Supelco, USA) with helium as the carrier gas (30 ml/min, isothermal oven temperature: 120 °C). Gas calibration was completed using a standard mixture of CO_2 and CH_4 (SAPIO, Italy) with 4 points of calibration. The SCFA concentrations were determined using a Varian 3800 gas chromatograph (Varian Chromatography Systems, Walnut Creek, CA, USA), following the guidelines of Pirondini et al. (2012).

2.3. Experiment 2: in vitro continuous fermentation

Eight 2,000-mL single-flow continuous fermenters (1,500 mL of effective volume) were used, as described in Mason et al. (2015), to perform 2 fermentation runs, which lasted 9 d each with 6 d of adaptation and 3 d of sampling. In each fermentation, the rumen fluid was collected in the same slaughterhouse from 4 culled dairy cows, which were fed a TMR based on corn silage, grass hay, cornmeal, and soybean meal and were slaughtered in healthy conditions. The rumen fluid was collected immediately after the slaughter of cows (in the morning, after about 12 h from the last feeding of animals) and transported to the laboratory within half an hour in airtight glass bottles, refluxed with CO_2 and maintained at 39 °C. The fermentation substrate was a TMR composed of corn silage, cornmeal, hay, soybean meal and a micromineral and vitamin premix (370, 260, 180, 170 and 20 g/kg DM, respectively), which was dried at 60 °C (48 h) and then coarsely ground; it contained 150 and 350 g/kg of CP and NDF on DM, respectively. The treatments consisted of the daily addition of the 3 pure compounds—BOR, CAM, and EUCA—to the fermentation fluid (100 mg/L) of the fermenters (2 fermenters per treatment within each fermentation run) as well as using 2 fermenters with only ethanol (CTR) added. The pure compounds and the standard diet were provided to each fermenter twice a day in equal doses (at 09:00 and 17:00) for a total of 18 g/d of DM. Artificial saliva (Slyter et al., 1966) was continuously infused using a peristaltic pump at 1.3 mL/min. During the last 3 d before morning feeding, the pH was directly measured (GLP 22, Crison Instruments, S.A. Barcelona, Spain), whereas samples for the ammonia-nitrogen, SCFA, protozoa and bacterial DNA (10, 5, 5, and 1 mL sample, respectively) were collected for later analysis. The samples for the ammonia-nitrogen were stored at −20 °C, thawed at room temperature, and then analyzed using an ammonia electrode (Ammonia Gas Sensing Combination Electrode, Hach Company, 2001). Samples for SCFA analysis were mixed with 5 mL of 0.05 mol/L H_2SO_4 and were stored at −20 °C; after thawing, they were centrifuged at $20,000 \times g$ for 30 min at 20 °C and filtered by a polypore filter (0.45 μm, Agilent Technologies, Milan, Italy).

The SCFA concentration was measured as described by Martillotti and Puppo (1985).

The CH_4 yield was estimated by the equation of Moss et al. (2000), considering a hydrogen recovery of 90% (default):

$$CH_4 \ (mmol/L) = 0.45 \times Acetic \ (mmol/L) - 0.275 \times Propionic \ (mmol/L) + 0.40 \times Butyric \ (mmol/L)$$

2.4. Protozoa and microbiome analysis

Protozoa were counted as described by Dehority (2003). For the extraction of the DNA from the rumen microbiota, 350 μL of rumen fluid was stored at −80 °C pending extraction. Particular attention was devoted to this operation; 350 μL were taken immediately after shaking, as the rumen fluid has rapid precipitation. In this way, all

of the analyzed samples had the same characteristics. The DNA from the rumen fluid was extracted using the NucleoSpin Soil kit (Macherey−Nagel, Germany) following the procedures and using the reagents suggested by the kit manufacturer.

2.5. PCR amplification of 16S gene, PCR products sequencing, and bioinformatics analyses

In order to identify the bacterial community present in the rumen fluid, a portion of the 16S gene was used, as described by Takahashi et al. (2014). For the amplification, the following primers were used: Pro341F: 5′-CCTACGGGNBGCASCAG -3′ and Pro805R: 5′-GACTACNVGGGTATCTAATCC -3. The amplifications were performed using 5 μL of the extracted DNA in a final reaction volume of 25 μL using Platinum Taq DNA polymerase high fidelity (Thermofisher, MA, USA), following the manufacturer instructions. The amplifications were performed for 27 cycles using 55 °C as the annealing temperature. The libraries were purified with Beads Amplure XP 0.8X, amplified with Indexes Nextera XT Illumina; they were normalized, mixed, and loaded on Miseq using the 2×300 bp (paired-end) approach to generate a minimum of 50,000 sequences (±20%). The raw sequences R1 and R2 (raw reads) were verified and filtered by quality, trimmed by the primers, and fused by Qiime2 v8 software. DADA2 (Qiime2) software isolated the amplicon sequence variants (formerly operational taxonomic units), whose sequences were compared against the Greengenes v13-8 to obtain the taxonomic assignment.

2.6. Statistical analyses

The data from Exp. 1 and 2 were statistically analyzed by the proc mixed procedure of SAS 9.4 (SAS Institute Inc., Cary, NC USA), with the following model: $Y_{ijk} = \mu + \alpha_i + \beta_j + \varepsilon_{ijk}$, where Y_{ijk} is the dependent variable; μ is the overall mean; α_i is the random effect of the fermentation run ($i = 1$ to 3 in Exp. 1 and $i = 1$ to 2 in Exp. 2); β_j is the fixed effect of the dietary treatment ($j = 1$ to 6 in Exp.1 and $j = 1$ to 4 in Exp. 2); and ε_{ijk} is the random error. The least square means were reported. For all of the statistical analyses, significance was declared at $P \leq 0.05$ and trends at $P \leq 0.10$.

The linear regression analysis between CH_4 production (% of the GP total) and the main bacterial phyla and protozoa was performed by the proc reg procedure of SAS 9.4. Correlation analysis between the main bacterial phyla and protozoa was performed using the Pearson correlation method and the proc corr procedure of SAS 9.4.

3. Results

3.1. Experiment 1: gas and CH_4 productions, and rumen fermentation parameters

The results of the GP are shown in Table 1. The GP (mL/200 mg DM) was not affected by the additive. The CH_4 production as a percentage in the total GP at 48 h was lower for EUCA (22.5% in total GP) and CAM (22.4% in total GP), as compared to the CTR (24.2% in total GP) ($P = 0.044$). Among the other parameters, tendencies ($P < 0.10$) were found at 24 and 48 h for CH_4 production (mL/200 mg DM) and for the CH_4 percentage in the total GP at 24 h, with EUCA and CAM being the most promising compounds. The 2 essential oils did not affect any of the parameters evaluated.

The pH and total SCFA were not affected by the treatments (Table 2). The CTR had the highest values (% of the SCFA) for iso-butyrate (1.74) ($P = 0.003$) and for iso-valerate (3.11) ($P < 0.001$), as compared to the other treatments (on average 1.62 and 2.79, respectively, for iso-butyrate and iso-valerate). Bornyl acetate and CAM reduced valerate (on average 1.69, % SCFA) as compared to CTR

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Table 1
Gas production (GP) and CH$_4$ production of diets with the different additives in the Exp.1 (mL/200 mg DM unless otherwise stated).

Item	CTR[1]	Pure compound[2]			Essential oil[3]		SEM	P-value
		BOR	CAM	EUCA	OIL 1	OIL 2		
GP 24 h	31.1	30.2	30.3	30.3	30.5	30.0	2.90	0.772
GP 48 h	46.3	45.2	44.7	44.7	45.2	44.8	2.97	0.545
GP 24 to −48 h	15.2	15.0	14.4	14.4	14.6	14.8	1.05	0.334
CH$_4$ 24 h	7.84	7.44	7.02	7.06	7.97	7.52	1.18	0.063
CH$_4$ 48 h	11.3	10.9	10.2	10.2	11.1	10.5	1.54	0.075
CH$_4$ 24 to 48 h	3.59	3.68	3.35	3.35	3.32	3.31	0.747	0.744
CH$_4$ 24 h, %	22.9	22.1	20.9	21.0	23.6	22.5	1.03	0.082
CH$_4$ 48 h, %	24.2^a	23.8^{ab}	22.4^b	22.5^b	24.4^a	23.2^{ab}	2.06	0.044

^{a,b} Means in the same row with different superscripts are statistically different at $P < 0.05$.
[1] CTR, control.
[2] BOR, bornyl acetate; CAM, camphor; EUCA, eucalyptol.
[3] OIL 1, *Achillea moschata* essential oil derived from sample 1; OIL 2, *Achillea moschata* essential oil derived from sample 2.

(1.81) ($P = 0.04$). The acetate to propionate ratio was not affected by the treatment ($P = 0.299$).

3.2. Protozoa count and relative abundance of the main bacterial phyla and euryarchaeota

The results of the protozoa count and the relative abundances of the main bacterial phyla and Euryarchaeota are shown in Table 3. All of the compounds increased the total number of protozoa ($P = 0.006$) compared to the CTR. The genus *Entodinium* increased (on average 91.8% vs. 83.0% of the total protozoa, respectively, for samples treated with additives and CTR; $P < 0.001$), whereas all of the experimental treatments decreased ($P = 0.001$) the genus *Diplodinium* (13.6% vs. 6.6% of the total protozoa, for the CTR and treatments with additives, respectively). The percentage of the other protozoa (Ophryscolecinae, *Isotricha*, and *Dasytricha*) was lower ($P = 0.018$) in BOR, CAM, and EUCA (on average 0.91) compared to the CTR (3.38). There was a negative relationship ($P < 0.001$) between the total protozoa and CH$_4$ production (% in total GP), although Ophryscolecinae were quadratically correlated ($P = 0.002$) to CH$_4$ production (Fig. 1).

The additives affected the relative abundance of Bacteroidetes, Proteobacteria, and Firmicutes ($P = 0.003$, 0.014, and 0.014, respectively) as compared to CTR. Bacteroidetes and Firmicutes

Table 2
Rumen fermentation parameters of diets with the experimental additives in the Exp. 1

Item	CTR[1]	Pure compound[2]			Essential oil[3]		SEM	P-value
		BOR	CAM	EUCA	OIL 1	OIL 2		
pH	6.74	6.68	6.70	6.73	6.72	6.77	0.096	0.207
SCFA, mmol/L	63.8	62.4	60.6	59.3	59.2	57.7	5.29	0.189
SCFA percentage								
Acetate (A)	63.1	64.5	64.2	63.3	63.5	63.4	1.61	0.286
Propionate (P)	17.2	16.7	17.0	17.3	17.2	17.3	0.870	0.594
iso-butyrate	1.74^a	1.58^c	1.59^{bc}	1.65^b	1.65^b	1.64^{bc}	0.060	0.003
Butyrate	13.0	12.7	12.8	13.1	13.1	13.0	0.480	0.481
iso-valerate	3.11^a	2.83^b	2.72^b	2.79^b	2.83^b	2.80^b	0.045	<0.001
Valerate	1.81^a	1.69^b	1.69^b	1.75^{ab}	1.80^a	1.78^{ab}	0.187	0.040
A-to-P ratio	3.68	3.89	3.81	3.67	3.70	3.68	0.287	0.299

$^{a, b, c}$ Means in the same row with different superscripts are statistically different at $P < 0.05$.
[1] CTR, control.
[2] BOR, bornyl acetate; CAM, camphor; EUCA, eucalyptol.
[3] OIL 1, *Achillea moschata* essential oil derived from sample 1; OIL 2, *Achillea moschata* essential oil derived from sample 2.

abundances decreased, whereas the Proteobacteria abundance increased for all treatments compared to CTR. No effect related to the additive was observed for the Euryarchaeota abundance ($P = 0.189$). The Euryarchaeota relative abundance was positively associated with CH$_4$ (% in total GP at 48 h) as follows:

Euryarchaeota relative abundance (%) = 0.0582 × CH$_4$ production (% in total GP) - 0.658; ($R^2 = 0.313$); (root mean square error [RMSE] = 0.146; $P = 0.059$).

The correlations among the protozoa and the main bacterial phyla and Euryarchaeota are reported in Appendix Table. The most significant correlations showed that Euryarchaeota was positively correlated with Ophryscolecinae ($r = 0.697$; $P < 0.05$), but negatively correlated with *Entodinium* ($r = −0.584$; $P < 0.05$). In contrast, Proteobacteria was positively correlated with *Entodinium* ($r = 0.658$; $P < 0.05$). The regressions among the main bacterial phyla and the CH$_4$ emissions (% in total GP) are shown in Fig. 1. Firmicutes and Bacteroidetes were positively related to CH$_4$ emission, whereas Proteobacteria and Spirochaetes were negatively correlated.

3.3. Experiment 2: continuous rumen fermenter system

The results of the continuous rumen fermenter are shown in Tables 4 and 5. The inclusion of BOR and EUCA increased the pH in the fermentation liquid ($P < 0.001$). The production of SCFA showed a decrease of about 20% when EUCA was added compared to CTR ($P = 0.029$), whereas this compound did not modify the proportions of different SCFA, except for an increase in the valerate ($P = 0.001$) in comparison with the other treatments. Bornyl acetate and CAM increased the proportion of acetate in the fermentation liquid by about 10% compared to the CTR (61.26%, 58.44%, and 54.34% for BOR, CAM, and CTR, respectively). Valerate was modified by BOR inclusion, resulting in a lower value ($P < 0.05$) than CTR and EUCA. The stoichiometrically calculated CH$_4$ was less for EUCA than for the other treatments (7.40 vs. 8.87 mmol/L on average, $P = 0.025$).

Protozoa were affected by the addition of the 3 compounds compared to CTR, with an increase of about 50% ($P < 0.001$). No modification for the protozoa groups was detected. The relative abundance of bacterial phyla and genera was affected by the addition of EUCA, which increased ($P = 0.011$) the presence of *Ruminococcus* (Table 5). Moreover, the addition of EUCA tended to increase Firmicutes and decrease Bacteriodetes ($P = 0.090$ and $P = 0.084$, respectively). The relative abundance of *Anaereovibrio* was lower ($P < 0.05$) for CAM and BOR compared to EUCA.

4. Discussion

A. moschata is a medicinal plant, which has been traditionally used in ethnomedicine to treat various digestive disorders in humans and animals. For this plant, Vitalini et al. (2016) showed a broad spectrum of antimicrobial activity against some food pathogen bacteria, such as *Bacillus cereus*, *Staphylococcus aereus*, *Escherichia coli*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*. The present study aimed to evaluate the effects of the main pure compounds of *A. moschata* essential oil and the essential oil on in vitro rumen fermentation and the microbiome. To the best of our knowledge, the role of *A. moschata* essential oil in modifying rumen metabolism and enhancing nutrient utilization by animals has never been investigated. The main compounds of *A. moschata* essential oil are BOR, CAM, and EUCA. Interestingly, these compounds gave promising results for lower enteric CH$_4$ production in short-time in vitro incubations without decreasing SCFA production (Joch et al., 2016, 2018). In Exp. 1 (48 h of incubation), the GP and SCFA did not decrease with the additives, whereas in Exp. 2 (9 d of incubation), the addition of EUCA decreased SCFA production,

Table 3
Rumen protozoa count and relative abundance of main bacterial phyla in the Exp. 1

Item	CTR[1]	Pure compound[2]			Essential oil[3]		SEM	P-value
		BOR	CAM	EUCA	OIL 1	OIL 2		
Total protozoa, $\times 10^3$ cell/mL	68.0[b]	92.0[a]	104.0[a]	92.3[a]	89.5[a]	86.9[a]	26.3	0.006
Total protozoa, %								
Entodinium	83.0[b]	93.6[a]	92.4[a]	93.8[a]	89.8[a]	89.4[a]	1.81	<0.001
Diplodinium	13.6[a]	5.85[b]	6.69[b]	4.92[b]	7.30[b]	8.24[b]	1.31	0.001
Other protozoa	3.38[a]	0.577[c]	0.900[c]	1.24[bc]	2.88[ab]	2.35[ab]	0.953	0.018
Phyla, % (total observations)								
Firmicutes	30.7[a]	25.8[b]	27.8[b]	25.1[b]	26.9[b]	26.0[b]	0.802	0.031
Bacteroidetes	37.1[a]	32.8[b]	27.7[d]	28.3[cd]	30.9[bcd]	31.8[bc]	1.11	0.014
Proteobacteria	22.5[b]	32.8[a]	36.7[a]	39.7[a]	36.5[a]	34.4[a]	1.95	0.014
Spirochaetes	3.59	4.67	4.61	3.77	2.98	4.52	0.375	0.107
Euryarchaeota	0.71	0.39	0.41	0.45	0.46	0.51	0.077	0.189
Others	5.31	3.52	2.82	2.71	2.35	2.80	1.01	0.445

[a, b, c, d] Means in the same row with different superscripts are statistically different at $P < 0.05$.
[1] CTR, control.
[2] BOR, bornyl acetate; CAM, camphor; EUCA, eucalyptol.
[3] OIL 1, *Achillea moschata* essential oil derived from sample 1; OIL 2, *Achillea moschata* essential oil derived from sample 2.
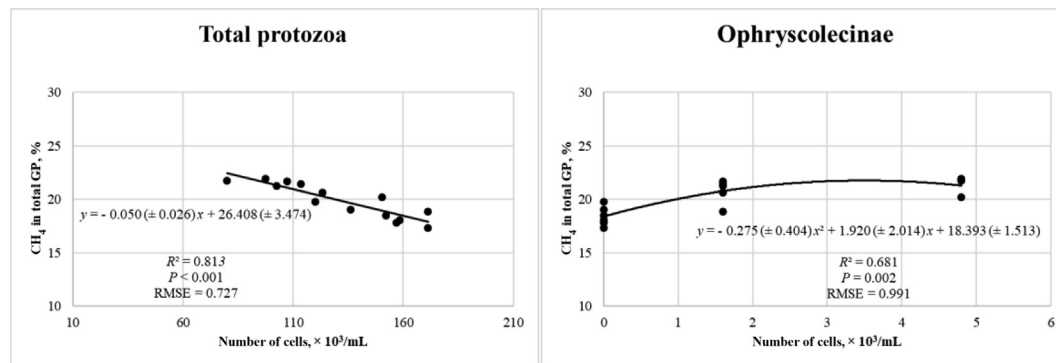

Fig. 1. Linear and quadratic regressions between total protozoa count and Ophryscolecinae and CH_4 emission (% in total GP 48 h). GP = gas production; RMSE = root mean square error.

Table 4
Rumen fermentation parameters and protozoa count in the Exp. 2

Item	CTR[1]	Pure compound[2]			SEM	P-value
		BOR	CAM	EUCA		
pH	5.98[b]	6.15[a]	6.06[b]	6.20[a]	0.032	<0.001
Ammonia, mg/dL	13.3	13.9	13.9	12.6	0.543	0.356
SCFA, mmol	33.3[a]	31.0[ab]	32.3[a]	26.8[a]	1.36	0.029
SCFA, % (total SCFA)						
Acetate (A)	54.3[b]	61.3[a]	58.4[a]	51.5[b]	1.29	0.001
Propionate (P)	15.4[ab]	16.0[ab]	17.3[a]	12.5[b]	1.04	0.048
iso-butyrate	0.441	0.255	0.312	0.159	0.071	0.092
Butyrate	17.2	13.8	13.9	19.8	1.55	0.055
iso-valerate	0.917	0.802	0.917	0.905	0.110	0.853
Valerate	11.7[b]	7.99[c]	9.21[bc]	15.2[a]	0.874	0.001
A-to-P ratio	3.55	4.04	3.42	4.15	0.285	0.250
Calculated CH_4[3], mmol/L	9.02[a]	8.75[a]	8.85[a]	7.40[b]	0.344	0.245
Total protozoa, $\times 10^3$ cell/mL	54.9[b]	84.1[a]	80.7[a]	79.6[a]	2.76	<0.001
Total protozoa, %						
Entodinium	95.3	95.5	95.1	95.4	0.911	0.990
Diplodinium	3.52	3.10	3.93	3.08	0.883	0.889
Other protozoa	1.20	1.38	1.00	1.52	0.599	0.934

[a, b, c] Means in the same row with different superscripts are statistically different at $P < 0.05$.
[1] CTR, control.
[2] BOR, bornyl acetate; CAM, camphor; EUCA, eucalyptol.
[3] CH_4 was calculated according to the equation described by Moss et al. (2000).

which is possibly related to lower digestibility of the diet supplemented with EUCA. The difference between the 2 experiments might be due to several reasons, such as different doses used, the different donor animals, and the possible adaptation of rumen bacteria to the additives. The selected compounds were oxygenated monoterpenes, which degrade little in the rumen; for example, Malecky et al. (2012) showed that after 24 h of incubation with the caprine rumen inoculum, oxygen-containing terpenes were less degraded than linear and monocyclic terpenes. Hence, it can be speculated that in the short trial (Exp. 1), the additives were slowly degraded, whereas in Exp. 2, the rumen microbiome was probably more adapted to the additives. Similarly, Cardozo et al. (2004), using a continuous fermenter, observed a different SCFA profile due to essential oil supplementations at 2 d of fermentation, whereas these differences disappeared at longer fermentation lengths (e.g., 6 d) due to rumen microbial adaptation.

The SCFA profile was affected by the additive. In Exp. 2, EUCA increased the valerate proportion. Ungerfeld (2015) suggested that an increase in ruminal hydrogen availability, following methanogenesis inhibition, enhances the fermentation pathways that consume hydrogen, such as the formate, valerate, and caproate biosynthesis process. The estimated CH_4 production was lower for EUCA than for other treatments. On the other hand, CAM and BOR increased the acetate proportion (without affecting the acetate to propionate ratio) compared to CTR, which was likely related to a better fiber digestibility. According to this hypothesis, the pH value of BOR was higher than that of CTR.

In both experiments, the 3 dominant bacterial phyla were Bacteroidetes, Firmicutes, and Proteobacteria, but with a different ratio in the CTR samples (i.e., ethanol) between the 2 experiments (e.g., 31:37:23 in Exp.1 and 55:27:11 in Exp. 2, for Bacteroidetes,

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Table 5
Relative abundance of main bacterial phyla and genera in the Exp. 2

Item	CTR[1]	Pure compound[2]			SEM	P-value
		BOR	CAM	EUCA		
Operational taxonomic unit	404	323	398	351	65.4	0.788
Phyla, % (total observations)						
Firmicutes	55.1	54.2	54.8	62.8	2.43	0.090
Bacteroidetes	26.6	30.8	31.0	22.1	2.48	0.084
Proteobacteria	10.8	9.28	7.83	6.48	1.11	0.088
Spirochaetes	2.93	1.98	2.18	3.05	0.410	0.224
Actinobacteria	2.70	2.43	2.65	2.78	0.381	0.923
Euryarchaeota	0.985	0.680	0.743	0.885	0.222	0.765
Others/Unclassified	1.93[ab]	1.33[b]	1.60[ab]	2.85[a]	0.251	0.040
Genera, % (total observations)						
Anaeroplasma	0.110	0.162	0.310	0.143	0.064	0.113
Anaerovibrio	0.520[ab]	0.333[b]	0.290[b]	0.575[a]	0.737	0.028
Bifidobacterium	2.08	1.60	2.19	2.08	0.377	0.675
Butyrivibrio	4.87	3.39	5.93	4.53	0.714	0.118
Clostridium	0.885	0.489	1.09	1.44	0.423	0.419
Fibrobacter	0.288	1.34	0.043	0.233	0.555	0.361
Megasphaera	7.21	5.55	8.55	6.09	1.36	0.366
Methanobrevibacter	0.585	0.740	0.550	0.830	0.264	0.707
Oscillospira	1.28	0.880	1.61	2.29	0.617	0.378
Prevotella	20.2	15.7	22.4	15.8	3.79	0.441
Pseudobutyrivibrio	0.758	1.15	1.98	1.93	0.521	0.330
Ruminococcus	1.18[b]	0.813[b]	1.37[b]	2.60[a]	3.72	0.011
Shuttleworthia	5.11	3.52	3.32	9.19	2.33	0.214
Succiniclasticum	4.10	4.70	5.03	4.67	1.19	0.993
Succinivibrio	0.623	1.95	2.37	1.17	0.972	0.496
Treponema	2.96	1.75	1.77	3.09	0.829	0.437
YRC22	0.613	0.510	0.808	0.530	0.224	0.708

[a, b] Means in the same row with different superscripts are statistically different at $P < 0.05$.
[1] CTR, control.
[2] BOR, bornyl acetate; CAM, camphor; EUCA, eucalyptol.

Firmicutes, and Proteobacteria, respectively). Other studies (Jami et al., 2014; Li et al., 2009) reported a considerable variation between animals concerning the abundance of the main bacterial phyla. In the present study, the rumen inoculum for the 2 experiments was collected from different animals fed different diets and reared in different conditions, which probably caused the observed difference.

In Exp. 1 (48 h), all of the treatments increased the relative abundance of Proteobacteria and decreased that of Firmicutes and Bacteroidetes. Wallace et al. (2015) found out that in beef cattle, there was a 4-fold abundance of Proteobacteria in animals with lower CH_4 emissions compared to those with higher emissions. Similarly, Danielsson et al. (2017) reported a higher abundance of Proteobacteria in low-CH_4 emitting cows than in high-CH_4 emitting ones. These results appear to agree with the results for the EUCA and CAM treatments in Exp. 1.

Although the effects were less marked, in Exp. 2, there was a higher Firmicutes-to-Bacteroidetes ratio for EUCA than for CTR, and the Firmicutes-to-Bacteroidetes ratio was found to be strongly correlated with daily milk-fat yield (Jami et al., 2014). However, Delgado et al. (2019) found that more efficient cows presented a higher relative abundance of Bacteroidetes and a lower, but not significant, relative abundance of Firmicutes. A lower amount of Bacteroidetes in the digestive microbiota was also associated with an impaired feed conversion rate, residual feed intake (Jami et al., 2014), and an increased fat deposition in mice (Turnbaugh et al., 2006). Overall, a lower amount of Bacteroidetes in the rumen might redirect energy intake, resulting in an increased fat deposition at the expense of lowering milk production per unit of feed intake (Delgado et al., 2019). Hence, the possible use of EUCA as a feed additive should be carefully evaluated. Another effect observed in Exp. 2, due to the EUCA treatment, was the increase in

the relative abundance of *Ruminococci*. The bacteria belonging to this genus played a fundamental role in cellulose degradation (Christopherson et al., 2014). However, the increase of the abundance of *Ruminococci* was not associated with a change in the fermentative pattern (i.e., acetate proportion); on the contrary, a decrease in the SCFA concentration was observed with EUCA supplementation.

The additives had no effect on the relative abundance of Euryarchaeota in the present study. Shi et al. (2014) suggested that the number of Euryarchaeota was not as important for the CH_4 yield as the metabolic activity of individual methanogenic species. However, in the present study, there was a positive correlation between Euryarchaeota and CH_4 production, expressed as a percentage of the total gas.

Supplementation with the additives markedly increased the total number of protozoa (i.e., 30-50%) in both experiments, despite the different experimental conditions (e.g., inoculum donor animals and length of incubation and doses). In agreement with these results, a study from Broudiscou et al. (2000) showed that *Achillea millefolium* increased large ruminal protozoa. The effect of essential oils on the protozoal population varies in the literature. Some studies reported a lack of effect (Benchaar et al., 2007b; Newbold et al., 2004), whereas others found that essential oils had a stimulatory effect on the protozoa (Patra et al., 2006), although the mechanism was not elucidated (Patra and Saxena, 2010). An opposite trend between the in vitro CH_4 yields and the protozoa counts was found in our experiments, which was unexpected given the assumed role protozoa plays in the rumen methanogenesis process (Newbold et al., 2015). A meta-analysis by Guyader et al. (2014) concluded that methanogenesis is also regulated by other mechanisms besides protozoa numbers because, in several experiments, a variation in CH_4 emission was observed without corresponding changes in the protozoa numbers. In addition, Sarnataro et al. (2020) changed the concentration of the in vitro protozoa population by adding secondary plant compounds; however, the authors did not measure a variation in the CH_4 yield. Similarly, Wenner at al. (2020), in a continuous culture experiment, found that CH_4 yield was not decreased by defaunation.

Different protozoa may have differing effects on rumen CH_4 production. Belanche et al. (2014) demonstrated that holotrich protozoa have a different endosymbiotic correlation with methanogens than entodiniomorphids. These differences may explain the more significant impact of holotrich protozoa on rumen methanogenesis than the entodiniomorphids (Belanche et al., 2015). The results of Exp. 1 demonstrated a quadratic relationship between CH_4 and Ophryscolecinae, which were also negatively correlated to Proteobacteria. To the best of our knowledge, this relationship has never been reported in the literature.

The role of protozoa in the rumen metabolism is not yet well defined, but some concerns arise from their contribution to CH_4 and ammonia yields in the rumen. However, protozoa are a relevant fraction of the natural microbiota of the rumen and have some beneficial effects on the nutrition of the host. Protozoa engulf starch granules and are, therefore, considered able to attenuate the risks of rumen acidosis. Moreover, protozoa may contribute to fiber digestion. Newbold et al. (2015) showed that the elimination of rumen protozoa significantly decreased NDF (-20%) and ADF digestibility (-16%), probably as a result of the loss of protozoal fibrolytic activity. Recent investigations also suggested possible positive effects related to protein nutrition because protozoa contributed 10 to 30% of the duodenal microbial protein flow (Sylvester et al., 2005) and were characterized by a higher lysine content than the rumen bacteria (Sok et al., 2017). Finally, the greater proportion of unsaturated fatty acids and CLA in the protozoa organisms could increase the supply of beneficial fatty acids

for lower gut absorption by the ruminants (Or-Rashid et al., 2011). Given these considerations, a dietary additive that can increase rumen protozoa may have interesting applications that should be further evaluated.

5. Conclusions

The results of the present study indicated that the essential oils from *A. moschata* did not depress in vitro rumen fermentation, except for EUCA, and may have some interesting effects on rumen microbiota. Some of the compounds (EUCA and CAM) were able to reduce in vitro CH_4 yield, but this effect was not associated with the evident modifications of the methanogenic bacteria. In addition, there was a clear and relevant stimulation of all the essential oils studied in the protozoal population, which was also maintained during long-lasting incubations without adaptive phenomena. The lack of correspondence between the total protozoa counts and the CH_4 yield in the in vitro conditions requires further research efforts to elucidate the relationship between methanogens and protozoa better.

Author contributions

Stefania COLOMBINI: writing original draft, review and editing, conceptualization, data elaboration, funding acquisition; Andrea ROTA GRAZIOSI: 48 h in vitro analysis, writing review and editing, data elaboration; Pietro PARMA: microbiome analysis, data elaboration; Marcello IRITI: selection and proposal of *Achillea moschata* plant material, oil extraction and processing, writing review and editing; Sara VITALINI: selection of plant material and oil extraction and processing, writing review and editing; Chiara SARNATARO: continuous fermenter analysis, writing review and editing, data elaboration. Mauro SPANGHERO: conceptualization, data elaboration, writing review and editing, funding acquisition.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2020.11.001.

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