**Evaluation of different essential oils in modulating methane and ammonia production,
rumen fermentation, and rumen bacteria *in vitro*.**

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Abbreviations:

A/P, acetate/propionate; CCB, Ceylon cinnamon bark; CIB, cinnamon bark; CIL, cinnamon leaves; CTR,
control; DGGE, Denaturing gradient gel electrophoresis; DIL, dill seeds; DM, dry matter; DMD, dry matter
degradability; EO, essential oils; GHG, greenhouse gas; EUC, eucalyptus leaves; FID, flame ionization detector;
GC, gas chromatography; GC-MC, gas chromatography-mass spectroscopy; NDF, neutral detergent fiber;
NDFD, neutral detergent fiber degradability; ORE, oregano leaves; PCA, principal component analysis; RI,
linear retention indices; ROS, rosemary leaves; SEM, standard error means; VFA, volatile fatty acids.

Abstract

Various essential oils (EO) have been individually evaluated to mitigate methane and ammonia production by rumen microbiota. Interactions between EO can affect their potency but such interactions largely remain unexplored. In the present study, EO from oregano, rosemary, Ceylon cinnamon, cinnamon leaves, cinnamon bark, dill seeds, and eucalyptus were chemically characterized and then evaluated *in vitro*, both individually (at 1.125 ml/L culture) and in three-way EO combinations (at total EO 0.8 ml/L, equal ratio), for their effects on fermentation, methanogenesis, ammoniogenesis, and bacteria and archaea. All the EO and their combinations decreased production of total gas ($P<0.001$), methane ($P<0.001$), and ammonia (except eucalyptus EO; $P<0.001$), but they (except the Ceylon cinnamon-dill seeds-eucalyptus EO combination) also decreased dry matter digestibility ($P<0.001$). The EO individually decreased the abundances of *Prevotella* spp. ($P<0.001$) but only oregano EO reduced the abundance of archaea ($P<0.001$). The EO combinations significantly decreased the abundances of archaea ($P<0.001$), protozoa ($P<0.001$), and select groups or species of different rumen bacteria to different extents. Changes in bacterial and archaeal communities in response to several EO combinations were also shown by DGGE analyses. Combination of EO from Ceylon cinnamon, dill seeds, eucalyptus, and probably others, at low concentrations may be a practical approach to mitigate methane emission and nitrogen excretion from ruminant without adverse effect on feed digestion or fermentation.

Key words: Archaea; Essential oil; Methane; Rumen bacteria; Rumen fermentation.

1. Introduction

Ruminant livestock production contributes significantly to worldwide greenhouse gas (GHG) emissions. About 71% of the GHG emissions comes as methane produced from enteric fermentation (especially rumen fermentation), followed by nitrous oxide (25%) and methane (4%) from manure (FAO, 2013). In the coming years, the rapid growth of world population will increase the demand for milk and meat by 22% and 14%, respectively (FAO, 2013). This will inevitably increase GHG emissions from livestock production.

Numerous nutritional strategies have been evaluated in recent years to mitigate methane emission and nitrogen excretion from cattle by modulating the ruminal microbial fermentation processes. Some promising results have been obtained from natural feed additives (Hristov et al., 2013; Knapp et al., 2014). Among these natural rumen modifiers, plant secondary metabolites, such as saponins, tannins, and essential oils (EO), are of potential application.

Essential oils are synthesized by many plants as protectants against insect predation and microbial infection, and they are generally considered as safe. The major compounds identified in EO include monoterpene hydrocarbons (e.g. α -pinene, α -phellandrene, *p*-cymene, *m*-cymene, γ -terpinene, and limonene) and phenolic compounds (e.g. carvacrol, thymol, and eugenol). Owing to the phenolic ring and hydroxyl group, phenolic compounds have stronger antimicrobial activities than monoterpene hydrocarbons (Benchaar and Greathead, 2011; Cobellis et al., 2016). Generally, Gram-positive bacteria are more sensitive to EO than Gram-negative bacteria, but small EO compounds, such as carvacrol, are able to interact with cell membrane of Gram-negative bacteria, leading to loss of cell content and cell lysis (Benchaar and Greathead, 2011; Cobellis et al., 2016). Eugenol, a phenolic compound of cinnamon EO, can inactivate some microbial enzymes (Burt, 2004; Benchaar and Greathead, 2011). The strong antimicrobial activity of cinnamaldehyde, similar to that of

carvacrol, has been attributed to the presence of a carbonyl group, which was thought to be able to disrupt microbial cell membrane and inactivate microbial enzymes (Benchaa and Greathead, 2011; Cobellis et al., 2016). However, unlike the antimicrobial activity exhibited by their individual compounds, the antimicrobial potency of EO varies considerably depending on chemical composition (both components present and their proportions), chemical configurations of components, and interactions among EO components (Burt, 2004). For example, cinnamon EO is more anti-methanogenic than its individual compounds, which suggests a synergic activity among its components (Macheboeuf et al., 2008). Combinations of different EO could also bring about a greater antimicrobial efficiency due to additive and/or synergistic effects that can occur between components of different EO (Benchaa and Greathead, 2011).

Recently, many EO have been shown to positively affect starch and protein degradation, production of ammonia, volatile fatty acids (VFA), and methane due to their antimicrobial activities against some ruminal microorganisms, such as methanogenic archaea and hyper-ammonia producing bacteria (Patra, 2011). However, these positive effects are often accompanied with negative effects on fiber degradation (Patra and Yu, 2012). In fact, due to both the complexity of the rumen microbial ecosystem and EO composition, the effects of EO on different ruminal microbial populations and on their interactions with feed fermentation seem to be difficult to predict. Mixed responses of rumen microbiome to EO were also reported in different studies with respect to methane production, fermentation characteristics, and microbial populations. The chemical composition of EO, which affects their antimicrobial activities, is highly variable depending on many factors (e.g. plant species, stage of growth, parts of plant, extraction method). To date, only a few studies evaluated EO with a known chemical composition in modulating rumen microbiome and function.

In the present study, we determined the chemical composition of seven EO and comparatively evaluated their effects on methane and ammonia production, feed degradability, VFA production, rumen bacterial and archaeal communities, and abundance of common rumen microbial groups *in vitro*. This study showed that combinations of certain EO could potentially decrease methane emissions from cattle with little or no detrimental effect on feed digestion or fermentation.

2. Methods

2.1 Essential oils and experimental design

Essential oil from oregano leaves (ORE; *Thymus capitatus* L.), rosemary leaves (ROS; *Rosmarinus officinalis* L.), Ceylon cinnamon bark (CCB; *Cinnamomum zeylanicum*), cinnamon leaves (CIL; *Cinnamomum zeylanicum* Blume), cinnamon bark (CIB; *Cinnamomum zeylanicum* Blume), dill seeds (DIL; *Anethum graveolens* L.), and eucalyptus leaves (EUC; *Eucalyptus globulus* Labill.) were purchased from Essential Srl (Montopoli Val d'Arno, Italy). The composition of the EO was determined by gas chromatography (GC) and by gas chromatography-mass spectrometry (GC-MS) that were controlled by the HP ChemStation Software. The GC instrument (HP 6890) was equipped with a MS 5973 mass selective detector (Hewlett Packard, Palo Alto, CA), a fused silica capillary column (HP-5MS; 30 m x 0.25 mm i.d., 0.25 µm film thickness) and a flame ionization detector (FID). The oven temperature was programmed to hold at 40 °C for 7 min, ramp to 270 °C at 10 °C/min, and hold at 270 °C for 20 min. Injector and detector temperatures were maintained at 250 and 270 °C, respectively. Each of the EO samples was diluted in hexane to a final concentration of 0.125 µl/ml, and 1 µl was injected into the GC in the splitless mode using helium as carrier gas. Each component was calculated by dividing the peak area of each component by the total area of all the components detected. The values were the mean of 3

injections of each EO sample. All compounds were identified by comparison of their linear retention indices (RI) relative to the retention times of a homologous series of C5–C20 alkanes reported in the literature (Adams, 2007) and by comparison to the mass spectra from the NIST98 Mass Spectral Database.

Essential oils were evaluated first individually at the dose of 1.125 ml/L culture (trial 1) in 8 treatments: the 7 different EO and a control without EO (CTR). Because all the seven EO significantly decreased dry matter (DM) digestibility, neutral detergent fiber (NDF), and total VFA production at the dose tested, they were evaluated in three-way combinations (equal ratio) at a lower dose (0.8 ml total EO/L culture) in trial 2 using six treatments: control without EO (CTR); combination of DIL, ORE, and ROS (A); combination of CIL, ORE, and ROS (B); combination of CCB, EUC, and ORE (C); combination of CCB, DIL, and EUC (D); and combination of CIB, DIL, and ORE (E). These three-way EO combinations were chosen based on the different effects observed in trial 1. Each treatment of the two trials was done in triplicate.

2.2. *In vitro* fermentations

Fresh rumen content was collected from two rumen-cannulated lactating Jersey cows at 3 h post morning feeding. The animals were fed twice a day with a total mixed ration (TMR) composed of (DM basis) corn silage (390 g/kg), a mixture of alfalfa hay and grass hay (350 g/kg), and a concentrate mixture (260 g/kg) (crude protein, 162 g/kg; NDF, 351 g/kg; acid detergent fiber, 208 g/kg; lignin, 50 g/kg; DM basis). The cows were cared and handled following the protocols approved by The Ohio State University Animal Care and Use Committee. Rumen fluid was obtained by straining rumen content through four layers of cheesecloth into a bottle (500 ml) leaving no headspace. The fresh rumen fluid samples from the two cows were combined in an equal volume, kept at 39 °C, and immediately transported

to the laboratory within 15 min to be used as inoculum for the *in vitro* fermentations. The *in vitro* buffered medium (artificial saliva) was prepared anaerobically as described by Menke and Steingass (1988). Inside of an anaerobic chamber, 30 ml of the medium and 10 ml of the inoculum were dispensed into a 120 ml serum bottle already containing 400 mg of ground substrate as described by Patra and Yu (2014). The substrate was composed of alfalfa hay (388 g NDF per kg DM) and a concentrate mixture at equal ratio by weight. The concentrate mixture contained ground corn (730 g/kg), soy hulls (150 g/kg), soybean meal (80 g/kg), and a mineral vitamin mixture (40 g/kg). The serum bottles were sealed with butyl rubber stoppers and incubated at 39 °C in a water bath for 24 h in trial 1 and for 48 h in trial 2 with intermittent shaking. Trial 1 was a preliminary experiment and for this reason, a shorter time of incubation (24 h) was used. Further, in trial 2 the effects of EO combinations were evaluated in 48 h of incubation to verify the possibility of a microbial adaptation of these compounds in cultures.

2.3. Sampling and analyses

At the end of the incubation, fermentation was terminated by placing the bottles into ice-water. Gas pressure inside of each bottle was recorded using a manometer (Traceable[®], Fisher Scientific, USA) to determine total gas production. Headspace gas from each bottle was collected (4 ml) into a glass tube that was completely filled with distilled water by displacement, and its methane concentration was determined using gas chromatography (Patra and Yu, 2012). Samples of liquid cultures (1.5 ml from each bottle) were collected for microbial DNA extraction and analysis for VFA and ammonia. A pH meter (Fisher Scientific, Suwanee, USA) was used to measure the cultures pH. The remaining culture was then filtered using a filter bag (Ankom Technology, Macedon, USA; pore size 50 µm) to determine the residual DM (AOAC, 2012; Method 934.01) and NDF content (Van Soest et

al., 1991). Sodium sulphite was used in the NDF procedure without a heat stable amylase, and the results were expressed exclusive of residual ash. The VFA concentration of the culture was determined using GC as described by Patra and Yu (2014).

2.4. Microbial DNA extraction, quantitative real-time PCR, and denaturing gradient gel electrophoresis

Metagenomic DNA was extracted from microbial biomass pelleted from each culture following the method of Yu and Morrison (2004a). DNA quality was assessed by agarose gel (1.0%) electrophoresis. The concentration and purity of the DNA extracts were assessed using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, USA). The DNA samples were stored at -20°C until further analysis. The abundance of total bacteria, archaea, protozoa, and select rumen bacterial species (including fibrolytic and hyper-ammonia producing bacteria) were quantified using respective specific quantitative real-time (qPCR) assays using respective specific PCR primer pair (Table 1) as described by Patra and Yu (2014). The absolute abundance was expressed as $\log_{10}$ of *rrs* gene copies/ml of culture sample.

Denaturing gradient gel electrophoresis (DGGE) was used to profile the bacterial community and the archaeal community in the samples collected from trial 2. The V3 hypervariable region of the *rrs* gene was amplified using bacterium- and archaeon-specific primers (with a 40-bp GC clamp added to the 5' end of the forward primer; Table 1). The PCR and DGGE conditions and the gel image analysis were essentially the same as described previously (Yu et al., 2004b, 2008).

2.5. Statistical analysis

All data were analysed using the ANOVA procedure of SAS (SAS Institute Inc., 2010). The qPCR data (*rrs* gene copies/ml of culture) were log-transformed before statistical analyses to improve normality. Differences between treatment means were determined by Tukey's test. Data were reported as least squares means $\pm$ standard error. Differences were considered to be significant when $P \leq 0.05$, while $0.05 < P < 0.10$ were considered as a trend. The DGGE profile was analyzed using principal component analysis (PCA) using the PC-ORD program to assess the overall effect of EO on the bacterial community and the archaeal community.

3. Results

3.1. Essential oil chemical composition

The EO differ in their chemical compositions (Table 2). Overall, oxygenated monoterpenes were the major type of components of DIL, EUC, ORE, and ROS, whereas phenolic compounds were the major type of components of cinnamon EO (CIL, CIB, and CCB). Eight compounds were identified in DIL, representing 988 g/kg of this EO. The major compounds were carvone (389 g/kg), limonene (216 g/kg), α -phellandrene (210 g/kg), and dill ether (103 g/kg). The CIL, CIB, and CCB contained 20, 22, and 19 different identified compounds, which together accounted for 1000 g/kg, 991 g/kg and 988 g/kg of each EO, respectively. Although these three EO were derived from different types of cinnamon and/or different parts of the same type of plant, their compositions were similar, with *trans*-cinnamaldehyde (617 g/kg, 517 g/kg and 570 g/kg, respectively) as the main component. The other significant components were β -phellandrene (ranging from 43.0 to 63.2 g/kg), linalool (34.5 to 43.4 g/kg), and β -caryophyllene (62.6 to 92.8 g/kg). The CIB and CCB contained a higher amount of α -terpinene, linalool and *cis*-cinnamaldehyde than CIL, while CIB contained a higher amount of α -pinene, α -phellandrene, *p*-cymene, β -caryophyllene and

caryophyllene oxide but a lower proportion of eugenol than CIL and CCB. Nine compounds were identified in EUC, with 1,8-cineole (734 g/kg), *p*-cymene (146 g/kg), and α -pinene (66.5 g/kg) as the major constituents. Ten compounds were identified in ORE, representing 997 g/kg of this EO. The most abundant components were carvacrol (522 g/kg), *m*-cymene (228 g/kg), γ -terpinene (78.7 g/kg) and β -caryophyllene (65.9 g/kg). The ROS contained 16 compounds accounting for 997 g/kg of the whole oil. The main components were 1,8-cineole (356 g/kg), α -pinene (122 g/kg), camphor (103 g/kg), limonene (87.4 g/kg), and β -pinene (81.2 g/kg).

3.2. Effects of essential oils, individually or in three-way combinations, on production of total gas and methane, ammonia concentrations, and feed degradability

All the EO, either alone or in three-way combinations, decreased ($P<0.001$) total gas production compared with control (Tables 3 and 4). The most pronounced inhibition on total gas production was observed with CIB, CCB, ORE, and with the EO combinations DIL-ORE-ROS, CIL-ORE-ROS, and CIB-DIL-ORE. All the treatments also reduced ($P<0.001$) methane production (Tables 3 and 4). The EO combinations resulted in significant reduction (37.7% to 78.5%) in methane production, but the magnitudes of the reduction were smaller than that achieved by the EO individually (Table 3). All the EO, both individually (except EUC) and in the three-way combinations significantly decreased ($P<0.001$) ammonia concentrations, with the largest decreases achieved by CIB (by 43.9%; Table 3) and by the CIL-ORE-ROS combination (by 59.3%; Table 4). All the treatments, except the CCB-DIL-EUC combination, decreased ($P<0.001$) DM digestibility without affecting NDF degradability (Table 3 and 4). The CCB resulted in the most pronounced (by 42%) inhibition to DM digestibility.

246 3.3. *Effects of essential oils, individually or in three-way combinations, on ruminal pH and*
247 *volatile fatty acid production.*

248 The EO supplementations, either alone or in three-way combinations, had mixed effects
249 on ruminal pH and VFA concentrations (Tables 3 and 4). The three EO from cinnamon (i.e.,
250 CCB, CIL, and CIB), ORE, and the EO combinations DIL-ORE-ROS, CIL-ORE-ROS, and
251 CIB-DIL-ORE increased ($P<0.001$) culture pH. Total VFA production was significantly
252 ($P<0.001$) decreased (by up to 64%) by all EO except EUC (Table 3). Among the EO
253 combinations, DIL-ORE-ROS and CIB-DIL-ROS decreased VFA the most, while CCB-DIL-
254 EUC decreased VFA the least (Table 4). The molar proportion of acetate was increased by
255 DIL, CIL, and CCB but decreased by EUC and ROS ($P<0.001$). In contrast, none of the
256 three-way combinations affected the molar proportion of acetate. Supplementation with CIB,
257 CCB, or ORE did not change the molar proportion of propionate in the cultures, but the other
258 EO resulted in a decrease ($P<0.001$). Among the EO combinations, only CIL-ORE-ROS and
259 CIB-DIL-ORE affected (increased, $P<0.01$) the propionate proportion. All the EO showed
260 mixed effects ($P<0.001$) on the molar proportion of butyrate, with DIL, EUC, and ROS
261 increasing butyrate proportion, while CIB, CCB, and ORE decreased it (Table 3). On the
262 contrary, all the EO combinations, except CCB-DIL-EUC, increased ($P<0.001$) butyrate
263 proportion (Table 4). The molar proportion of isobutyrate was similar ($P<0.05$) among the
264 treatments (Table 3), except the EO combinations DIL-ORE-ROS, CIL-ORE-ROS, and CIB-
265 DIL-ORE, all of which reduced ($P<0.01$) that measurement (Table 4). The isovalerate
266 concentration was lowered by ROS ($P<0.001$) and the above three EO combinations
267 ($P<0.01$). The molar proportion of valerate was affected ($P<0.001$) only by ROS that
268 significantly increased it. Compared with that of the control, acetate to propionate ratio (A/P)
269 was higher ($P<0.001$) with DIL, CIL, and EUC. The EO combination CIL-ORE-ROS also
270 increased ($P<0.05$) A/P ratio.

3.4. Effects of essential oils, individually or in three-way combinations, on the abundance of selected microbes

The effects of individual EO on the abundance of total bacteria, archaea, and the genus *Prevotella* was evaluated using qPCR (Table 5). The CIB, CCB, and ORE reduced ($P<0.001$) the abundance of total bacteria, confirming their strong antimicrobial potency. Except ORE, none of the EO decreased ($P<0.001$) the abundance of archaea, the microbial population directly responsible for rumen methane production. All the treatments significantly reduced ($P<0.001$) the abundance of the genus *Prevotella*, which includes proteolytic bacteria. The three-way EO combinations also affected the abundance of total bacteria, archaea, protozoa, and several cellulolytic, proteolytic, and hyper-ammonia producing bacteria (Table 6). A tendency ($P=0.051$) in reducing the abundance of total bacteria was observed for the EO combinations DIL-ORE-ROS and CCB-EUC-ORE. The abundance of archaea and protozoa was markedly decreased ($P<0.001$) by all the EO combinations. The populations of both *Fibrobacter succinogenes* and *Ruminobacter amylophilus* were significantly decreased ($P<0.001$) by all the treatments. On the contrary, the abundance of *Ruminococcus albus* was reduced ($P<0.001$) only by the CIL-ORE-ROS combination and increased by the CCB-DIL-ORE combination, while the abundance of *Ruminococcus flavefaciens* was not affected by any of the treatments.

The three-way EO combinations showed mixed effects on the abundance of proteolytic and ammoniagenic bacteria. The abundance of *Prevotella ruminicola* was lowered ($P<0.001$) only by the CCB-EUC-ORE combination, while that of *Prevotella bryantii* showed a tendency ($P=0.08$) to be lowered by all the treatments. The abundance of *Selenomonas ruminantium* was reduced ($P<0.01$) with the DIL-ORE-ROS and the CCB-EUC-ORE combinations. However, none of the EO combinations affected the abundance of *Clostridium*

aminophilum, a hyper-ammonia producing bacterium. The EO combinations DIL-ORE-ROS, CIL-ORE-ROS, and CIB-DIL-ORE increased ($P<0.01$) the abundance of *Streptococcus bovis*, whereas all but the CCB-DIL-EUC combination reduced ($P<0.001$) the abundance of *Megasphaera elsdenii*.

3.5. Effects of three-way essential oil combinations on the bacterial and archaeal communities

When the bacterial and the archaeal communities of the *in vitro* cultures were profiled using DGGE, differences in banding patterns were noted between the EO combination treatments and the control and among the different EO combinations (Figure 1A and 2A). The PCA plots of the bacterial DGGE profiles indicated that the EO combinations resulted in apparent changes in some bacterial populations (Figure 1B and 1C). The first three principal components (PC's) together explained 63.19% of the total variation, with the first principal component (PC1) explaining 35.91% of the total variation (Figure 1B and 1C). The DIL-ORE-ROS and the CIB-DIL-ORE combinations resulted in the most noticeable changes in the bacterial communities. The PCA plots of archaeal DGGE profiles demonstrated that the archaeal communities apparently differed among the treatments (Figure 2B and 2C). The first three PC's explained 73.97% of the total variability, with the PC1 explaining 37.47% of the variability. The archaeal communities were separated into three clusters along PC1: combinations CCB-EUC-ORE and CCB-DIL-EUC, combinations DIL-ORE-ROS and CIL-ORE-ROS, and the control and combination CIB-DIL-ORE. The PC2, which explained 25.03% of the variability, separated combinations DIL-ORE-ROS and CIL-ORE-ROS from the other EO combinations. Both combinations DIL-ORE-ROS and CIL-ORE-ROS contained ORE and ROS, while combinations CCB-EUC-ORE and CCB-DIL-EUC both contained CCB and EUC.

4. Discussion

Essential oils are complex mixture of plant secondary metabolites with highly variable composition. For this reason, their mode of action against microorganisms or specific cellular targets is often difficult to determine and thus still remains poorly understood (Cobellis et al., 2016). In an attempt to link differences in mitigating methane production to specific component(s) of the EO, the chemical compositions of seven EO were determined. The seven EO differ in proportion in almost all the component identified, including monoterpene hydrocarbons (e.g. α -pinene, α -phellandrene, 1,8-cineole, carvone, *p*-cymene, *m*-cymene, limonene, camphor, and dill ether), phenolic compounds (e.g. carvacrol, *trans*-cinnamaldehyde, and eugenol). As discussed below, the anti-methanogenic potency of an EO is necessarily attributable to its major compound.

Methane production was completely inhibited by CIB, CCB, and ORE individually. Because DM and NDF digestibility, total gas production, and VFA were only partially inhibited, these three EO were more inhibitory to methanogens than to bacteria. However, the cinnamon oil from cinnamon leaves (CIL) decreased methane production to a lesser degree than the EO from cinnamon bark. Variable methane inhibition has been reported in the literature. Several studies have reported strong inhibition to methane production by cinnamon EO and oregano EO. For example, Macheboeuf et al. (2008) achieved 98% inhibition of methane production using 5 mM of oregano EO or cinnamon EO. While evaluating five different EO (EO from clove, eucalyptus, garlic, oregano, and peppermint), Patra and Yu (2012) recorded the greatest methane reduction (by 87%) by oregano EO at the dose of 1.0 g/L. Dose-dependent inhibition to methane production *in vitro* by Ceylon cinnamon bark EO was reported by Pawar et al. (2014), with a complete inhibition being noted at the dose of 833 μ l/L. In contrast, Roy et al. (2014) observed only slight inhibition (5.3%) of methane

production by cinnamon oil (its source was not reported) at 600 mg/L. It is tempting to speculate that *trans*-cinnamaldehyde, which is the major compound in CCB and CIB, and carvacrol, which is the major compound of ORE, may be primarily responsible for the observed methane inhibition. However, CIL and CCB and CIB have similar composition except a lower proportion of *cis*-cinnamaldehyde (6.90 vs. 25.0 g/kg), and CIL decreased methane to a less extent than CCB or CIB. These results suggest importance of some minor EO compounds in determining anti-methanogenic activities. Systematic studies are needed to evaluate each of the compounds in these EO to determine their potency to lower methane production.

The DIL, EUC, and ROS, all of which are EO rich in monoterpenes, especially oxygenated monoterpenes, exhibited much less inhibition to methane production than the cinnamon oil and ORE, with EUC being least inhibitory. These observations are in general agreement with those reported by other researchers, but greater inhibition was achieved in the present study than in other studies: 47.5% inhibition by DIL vs. 12% inhibition reported by Macheboeuf et al. (2008), 18.7% inhibition by EUC vs. 12% inhibition reported by Patra and Yu (2012), 43.5% inhibition by ROS vs. 24.0% inhibition reported by Roy et al. (2014). The discrepancy may be attributed to differences in the source and dose of the EO used. However, the lack of information of the source and chemical compositions of the EO used in the other studies makes it to pinpoint a specific cause for the discrepancy.

The magnitudes of methane reduction obtained with the EO combinations were smaller than that achieved by the EO individually. Because less EO, in total, was used when evaluated in combination than when evaluated individually (0.8 vs. 1.125 ml/L), the decreased inhibition to methane production might be attributed to the lower EO dose used in the EO combinations. However, potential antagonism among EO components from different EO should not be ruled out. Similarly, synergistic effect might not be excluded because of the

much lower dose used in the EO combinations. In fact, some EO compounds, even if present at low concentrations, may interact with the main compounds, affecting their biological activity. For example, Burt et al. (2004) reported a synergism between cinnamaldehyde and eugenol, both of which are present in cinnamon EO, and their interaction was thought to be responsible of the higher anti-methanogenic activity of cinnamon oil than that of cinnamaldehyde alone. Lin et al. (2012) tested mixtures of EO from thyme, oregano, cinnamon, and lemon in five different ratios *in vitro*. They observed that mixtures containing more phenol-based EO (EO from thyme and oregano) than aldehyde-based EO (EO from cinnamon and lemon) decreased methane production to a greater extent than the mixtures containing more aldehyde-based EO than phenol-based EO. A mixture of EO compounds (eugenol, carvacrol, citral, and cinnamaldehyde) was found more effective in reducing methane production (by 70.8%) in equal ratio than in unequal ratios (Lin et al., 2013a), suggesting that proportions among different EO components may affect their antimicrobial activity. Moreover, when a commercial mixture of EO (thymol, eugenol, vanillin, and limonene) was offered to beef cattle (1 and 2 g/d/animal), no effect on methane production were observed (Tomkins et al., 2015). The results from the present study and previous studies suggest complex interactions among EO components, between EO and feed, and between EO and rumen microbiota, and additional research is needed to determine the optimal EO combination, dose, and ratio to achieve most effective methane mitigation in livestock production and to avoid detrimental effect on feed digestion or fermentation.

The observed decrease in ammonia concentrations, with the largest decrease achieved by CIB, corroborates the study by Macheboeuf et al. (2008) who showed that *trans*-cinnamaldehyde and cinnamon EO lowered ammonia concentrations greater than other EO and EO compounds evaluated. It was proposed that EO decrease rumen ammonia concentration by inhibiting proteolysis, peptidolysis, and/or amino acid deamination,

396 especially deamination by hyper-ammonia producing bacteria (Patra, 2011). Several studies
397 showed that the effective dose of EO to decrease ammonia production was lower than that
398 needed to decrease methane production (Macheboeuf et al., 2008; Lin et al., 2012; Patra and
399 Yu, 2012; Cobellis et al., 2015). In the present study, however, methane production was
400 decreased to greater magnitudes compared to that of ammonia production, suggesting a
401 weaker dose effect on ammonia production than on methane production.

402 The observed adverse effects of EO on feed digestion could be a consequence of a broad
403 non-specific antimicrobial activity. Some authors suggested that EO could decrease protein
404 and starch degradation by inhibiting amylolytic and proteolytic bacteria without affecting
405 fiber degradation (Patra, 2011). Unfortunately, feed additives that decrease feed digestibility
406 will not result in net reductions in methane emissions per unit of animal product (Knapp et
407 al., 2014). For this reason, CCB-DIL-EUC is the most promising EO combination as it led to
408 effective inhibition to production of both methane and ammonia without adversely effecting
409 feed digestibility.

410 A number of studies showed that total VFA concentrations were not or only slightly
411 affected by EO at low doses but decreased at high doses (Patra, 2011; Cobellis et al., 2015).
412 Combinations of eugenol, carvacrol, citral, and cinnamaldehyde, in different ratios and at
413 different levels, showed similar effects on methane reduction, but combinations of aldehyde-
414 rich mixture led to greater inhibition to total VFA than combinations of phenol-rich mixture
415 (Lin et al., 2013a). The effects of EO on total VFA concentrations may also depend on other
416 factors, such as type of substrate and medium conditions. Data from a meta-analysis revealed
417 that concentrations of total VFA and propionate changed linearly and positively with
418 decreased methane production. Acetate production and A/P ratio increased linearly with
419 increasing inhibition of methane by EO (Patra, 2010). Unlike other phytochemicals, EO may
420 decrease methane production by directly inhibiting methanogens and by inhibiting hydrogen-

producing bacteria, such as *Lachnospira multiparus*, *R. albus* and *R. flavefaciens* and protozoa. Considering the effect on DM degradability, total VFA, and A/P ratio, the CCB-DIL-EUC combination may a promising EO that warrants further evaluation.

The observed decrease in abundance of archaea and protozoa by the EO combinations is consistent with the finding of a few previous studies, both *in vitro* and *in vivo* (Lin et al., 2012, 2013a, 2013b). It should be noted that individual EO markedly decreased methane production without decreasing the abundance of archaea after 24 h of incubation (except ORE), while all the three-way EO combinations lowered both methane production and the archaeal population after 48 h of incubation. This discrepancy might be attributable to the longer incubation time of the EO combinations and/or potential synergic anti-methanogenic activity between compounds of different EO. Archaea rely on methanogenesis for energy conservation, and thus decrease in methanogen population is expected with a reduced methane production during prolonged incubation. Besides, as suggested by Ohene-Adjei et al. (2008), EO may cause changes in archaeal community and/or in activity of methanogenesis pathway, eventually decreasing methanogen abundance and methane production. Besides, some methanogens are associated with protozoa, and protozoa-associated methanogens can contribute 9-37% of the rumen methane production (Szumacher-Strabel and Cieślak, 2010). Thus, EO might have decreased methanogen abundance and methanogenesis by decreasing abundance of protozoa. Similarly, the reduction of protozoal abundance by EO might have also contributed to the reduction of ammonia in the *in vitro* cultures.

Among the three major cellulolytic bacterial species, *F. succinogenes* appeared to be more sensitive to the EO combinations than the two *Ruminococcus* species (*R. albus* and *R. flavefaciens*). This observation is in general agreement with that reported by Patra and Yu (2012). The discord between significant decrease in the abundance of *F. succinogenes* and null effect on NDF degradability in all the treatments suggests that *F. succinogenes* had little

impact on the NDF degradability in the EO-treated cultures. Alternatively, other cellulolytic bacteria might have occupied the niche left by *F. succinogenes*.

Only one previous study in the literature examined the effects of a few EO (EO from clove, oregano, and peppermint) on some protein-degrading bacteria in mixed rumen cultures (Patra and Yu, 2014). That study showed that EO were effective in reducing the abundance of *P. ruminicola*, *P. bryantii* and *S. ruminantium*. The present study corroborates that previous study with respect to inhibiting the aforementioned proteolytic bacteria by EO. Hyper-ammonia producing bacterium *C. aminophilum* was also lowered by oregano EO (Patra and Yu, 2014). In the present study, oregano EO was not evaluated individually for its effects on hyper-ammonia producing bacteria, and the EO combinations containing oregano EO (from oregano leaves) did not decrease *C. aminophilum*. The stimulatory effect of EO on *S. bovis* is in general agreement with the low susceptibility of this species to EO in pure cultures (Evans and Martin, 2000) and with the increase of *S. bovis* by EO reported by Patra and Yu (2014). It was hypothesized that the increase of *S. bovis* population by EO was due to decrease in competing microbial populations.

Overall, the findings of DGGE analysis demonstrated that different bacterial and archaeal populations had different sensitivity to the three-way EO combinations used in the present study. In a recent study, Patra and Yu (2012) observed that different EO, when tested individually at different doses, changed the composition of both archaeal and bacterial communities to different extents in a dose-dependent manner. Evaluating the effects of some EO on archaeal communities in sheep rumen, Ohene-Adjei et al. (2008) also reported varying effects on archaeal community. The authors of that study attributed the EO effect to the effect of EO against rumen protozoa. Future research to determine the methanogens associated with rumen protozoa will help test that hypothesis.

5. Conclusions

The present study demonstrated that EO differ in chemical composition and have different effect on the same microbes. Different groups of rumen microbes also differ in sensitivity to the same EO. The EO effects may not necessarily be attributable to the major component in some EO. The EO containing a phenolic (e.g., carvacrol) or a carbonyl (e.g., cinnamaldehyde) compound demonstrated a stronger antimicrobial activity than EO that contain monoterpenes. Interactions among different EO and different EO components may affect their antimicrobial activity. Combinations of EO at moderate dose may be an effective approach to mitigate methane emission and nitrogen excretion from cattle with little or no adverse effect on rumen feed digestion or fermentation. Combination of EO from Ceylon cinnamon bark, dill seeds, and eucalyptus leaves may be a promising EO combination.

Conflict of interest

None of the authors has any financial or personal interest that would inappropriately influence or bias the contents of this paper.

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Figure captions

Figure 1. DGGE profile (A) and PCA plots (B and C) of bacterial community. CTR (●): control; A (■): combination of dill seeds, oregano leaves, rosemary leaves; B (▲): combination of cinnamon leaves, oregano leaves, rosemary leaves; C (◆): combination of Ceylon cinnamon bark, eucalyptus leaves, oregano leaves; D (□): combination of Ceylon cinnamon bark, dill seeds, eucalyptus leaves; E (+): combination of cinnamon bark, dill seeds, oregano leaves.

Figure 2. DGGE profile (A) and PCA plots (B and C) of archaea. CTR (●): control; A (■): combination of dill seeds, oregano leaves, rosemary leaves; B (▲): combination of cinnamon leaves, oregano leaves, rosemary leaves; C (◆): combination of Ceylon cinnamon bark, eucalyptus leaves, oregano leaves; D (□): combination of Ceylon cinnamon bark, dill seeds, eucalyptus leaves; E (+): combination of cinnamon bark, dill seeds, oregano leaves.

619 **Table 1.** Specific primers used for qPCR analysis and DGGE technique.

Organisms	Primers	Sequences (5'→3')	Annealing (°C)	Size (bp)	References
Real-time PCR					
Total bacteria	27f 1525r	AGA GTT TGA TCM TGG CTC AG AAG GAG GTG WTC CAR CC	55	1535	Lane 1991
Total bacteria	Eub358f Eub806r	TCC TAC GGG AGG CAG CAG T GGA CTA CCA GGG TAT CTA ATC CTG TT	60	448	Nadkarni et al. 2002
Archaea	TaqMan probe	6-FAM-5'-CGT ATT ACC GCG GCT GCT GGC AC-3'-TAMRA	70		
	ARC787f ARC1059r	ATT AGA TAC CCS BGT AGT CC GCC ATG CAC CWC CTC T	60	272	Yu et al. 2005
	316f 539r	GCT TTC GWT GGT AGT GTA TT CTT GCC CTC YAA TCG TWC T	54	223	Sylvester et al. 2004
<i>Fibrobacter succinogenes</i>	Fs219f Fs654r	GGT ATG GGA TGA GCT TGC GCC TGC CCC TGA ACT ATC	63	446	Koike and Kobayashi 2001
<i>Ruminococcus flavefaciens</i>	Rf154f Rf425r	TCT GGA AAC GGA TGG TA CCT TTA AGA CAG GAG TTT ACA A	55	295	Koike and Kobayashi 2001
<i>Ruminococcus albus</i>	Ra1281f Ra1439r	CCC TAA AAG CAG TCT TAG TTC G CCT CCT TGC GGT TAG AAC A	55	175	Koike and Kobayashi 2001
<i>Ruminobacter amylophilus</i>	Ram-F Ram-R	CAA CCA GTC GCA TTC AGA CAC TAC TCA TGG CAA CAT	57	642	Tajima et al. 2001
<i>Prevotella ruminicola</i>	P.rumi-F P.rumi-R	GGT TAT CTT GAG TGA GTT CTG ATG GCA ACT AAA GAA	53	485	Bekele et al. 2010
<i>Prevotella bryantii</i>	P.brya-F P.brya-R	ACT GCA GCG CGA ACT GTC AGA ACC TTA CGG TGG CAG TGT CTC	67	540	Bekele et al. 2010
<i>Prevotella</i> spp.	BAC303f BAC708r	GAA GGT CCC CCA CAT TG CAA TCG GAG TTC TTC GTG	56	418	Stiverson et al. 2011
<i>Clostridium aminophilum</i>	C.amin-57F C.amin-616R	ACG GAA ATT ACA GAA GGA AG GTT TCC AAA GCA ATT CCA C	57	560	Patra and Yu 2014
<i>Streptococcus bovis</i>	S. bovis-F S. bovis-R	CTA ATA CCG CAT AAC AGC AT AGA AAC TTC CTA TCT CTA GG	57	869	Bekele et al. 2010
<i>Megashaera elsdenii</i>	M. elsd-F M. elsd-R	GAC CGA AAC TGC GAT GCT AGA CGC CTC AGC GTC AGT TGT C	58	130	Bekele et al. 2010
<i>Selenomonas ruminantium</i>	S. rumi-F S. rumi-R	TGC TAA TAC CGA ATG TTG TCC TGC ACT CAA GAA AGA	53	513	Bekele et al. 2010
PCR-DGGE					
Total bacteria	GC-A357f 519r	CCC TAC GGG GCG CAG CAG GWA TTA CCG CGG CKG CTG	61→56°C	194	Yu and Morrison 2004b
Archaea	GC-RC344f 519r	ACG GGG YGC AGC AGG CGC GA GWA TTA CCG CGG CKG CTG	61→56°C	191	Yu et al. 2008

620 FAM: 6-carboxyfluorescein; TAMRA: 6-carboxytetramethylrhodamine

621 **Table 2.** Compounds identified in each EO.

Component ^a	Sources of essential oils (g/kg) ^b						
	DIL	CIL	CIB	CCB	EUC	ORE	ROS
α -Thujene	-	3.00±1.00	4.50±1.00	2.10±2.30	-	-	-
α -Pinene	14.4±2.10	13.0±1.70	21.3±3.70	12.0±1.20	66.5±2.50	20.6±4.40	122±5.00
Camphene	-	4.40±1.70	6.80±1.60	4.70±3.80	-	-	48.9±7.50
β -Pinene	-	4.90±1.10	7.60±1.10	5.20±3.50	14.9±0.90	11.9±3.10	81.2±6.30
Myrcene	-	-	-	-	-	26.8±2.90	27.7±2.20
β -Myrcene	-	-	-	-	13.9±0.80	-	-
3-Octanol	-	-	-	-	-	12.2±1.90	-
α -Phellandrene	210±9.60	22.8±2.00	38.9±0.20	20.3±1.80	5.10±1.50	-	-
Δ^3 -Carene	-	-	-	-	-	-	15.2±0.80
α -Terpinene	-	7.80±1.30	9.20±1.50	9.20±1.20	3.40±0.40	-	-
<i>p</i> -Cymene	21.5±1.60	22.8±1.80	31.9±1.80	26.4±2.80	146±2.70	-	17.2±3.10
<i>m</i> -Cymene	-	-	-	-	-	228±9.30	-
Limonene	216±3.00	-	-	-	-	-	87.4±3.10
β -Phellandrene	-	46.7±1.60	69.2±1.40	43.0±2.50	-	-	-
1,8-Cineole	-	-	-	-	734±3.20	-	356±13.0
γ -Terpinene	-	-	-	-	6.60±0.20	78.7±5.20	16.3±0.70
Linalool	-	34.5±2.90	42.5±2.70	43.4±1.80	-	6.40±4.30	16.9±2.80
Camphor	-	-	-	-	-	-	103±7.20
Borneol	-	-	-	-	-	-	15.2±1.40
Terpinene-4-ol	-	5.90±2.90	5.70±3.00	5.20±1.60	-	-	-
α -Terpineol	-	8.50±1.20	8.10±1.10	8.30±2.40	6.70±3.30	-	-
Bornyl acetate	-	-	-	-	-	-	18.6±1.00
Dill ether	103±6.80	-	-	-	-	-	-
<i>cis</i> -Dihydrocarvone	9.50±3.40	-	-	-	-	-	-
<i>trans</i> -Dihydrocarvone	25.7±4.10	-	-	-	-	-	-
<i>cis</i> -Cinnamaldehyde	-	6.90±2.00	25.0±2.00	25.9±1.70	-	-	-
Carvone	389±6.90	-	-	-	-	-	-
<i>trans</i> -Cinnamaldehyde	-	617±6.80	517±6.70	570±2.10	-	-	-
α -Humulene	-	-	-	-	-	-	16.7±3.00
β -Caryophyllene	-	62.6±9.70	92.8±9.70	69.6±2.00	-	65.9±5.10	46.0±2.90
Carvacrol	-	-	-	-	-	522±18.5	-
Eugenol	-	57.8±6.50	16.3±2.60	55.0±2.20	-	-	-
α -Copaene	-	13.1±2.60	18.9±5.60	13.4±3.20	-	-	-
β -Bourbonene	-	-	6.30±0.80	-	-	-	-
β -Elemene	-	-	6.10±0.90	-	-	-	-
<i>trans</i> -Cinnamyl acetate	-	31.4±7.50	12.7±2.50	39.4±1.20	-	-	-
Caryophyllene oxide	-	6.30±1.60	25.9±4.10	5.40±3.10	-	7.70±0.70	9.00±5.80
Humulene oxide	-	24.9±4.10	16.5±1.00	28.1±3.80	-	-	-
Benzyl benzoate	-	5.30±1.10	7.60±1.60	5.10±4.00	-	-	-
TOTAL	988	1000	991	993	997	997	997
Monoterpene hydrocarbons	462	125	189	124	257	382	416
Oxygenated monoterpenes	526	48.9	56.3	56.9	740	529	509
Sesquiterpenes	-	75.7	124	83.0	-	65.9	62.7
Oxygenated sesquiterpenes	-	31.2	42.4	33.5	-	7.70	9.00
Phenolics	-	718	579	691	-	-	-
Alcohols	-	-	-	-	-	12.2	-

622 ^a Compounds listed in order of their elution from GC. ^b Each value (mean±standard deviation) represents the
623 mean of three GC-MS runs. Sources of EO, DIL: dill seeds; CIL: cinnamon leaves; CIB: cinnamon bark; CCB:
624 Ceylon cinnamon bark; EUC: eucalyptus leaves; ORE: oregano leaves; ROS: rosemary leaves.

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Table 3. Effects of different essential oils (1.125 ml/L) on *in vitro* gas production and fermentation characteristics.

Parameters	Sources of essential oils*								SEM*	P-value
	CTR	DIL	CIL	CIB	CCB	EUC	ORE	ROS		
Total gas (ml)	127 ^a	109 ^c	97.9 ^d	83.4 ^e	83.0 ^e	120 ^b	81.5 ^e	111 ^c	1.38	<0.001
Methane (ml)	11.9 ^a	6.25 ^c	3.67 ^d	0.00 ^e	0.00 ^e	9.67 ^b	0.00 ^e	6.72 ^c	0.40	<0.001
Ammonia (mg/dL)	22.0 ^a	15.2 ^{bc}	13.5 ^{bc}	12.3 ^c	13.3 ^{bc}	17.8 ^{ab}	16.1 ^{bc}	14.7 ^{bc}	0.94	<0.001
DMD*	0.65 ^a	0.55 ^b	0.46 ^c	0.43 ^{cd}	0.38 ^d	0.57 ^b	0.41 ^{cd}	0.55 ^b	0.02	<0.001
NDFD*	0.71	0.70	0.71	0.68	0.66	0.69	0.69	0.71	0.02	0.164
pH	6.11 ^c	6.20 ^c	6.43 ^b	6.95 ^a	6.98 ^a	6.18 ^c	7.02 ^a	6.26 ^{bc}	0.04	<0.001
Total VFA* (mM)	42.1 ^a	25.0 ^c	21.1 ^d	15.1 ^e	15.4 ^e	41.5 ^a	15.4 ^e	36.9 ^b	0.74	<0.001
Acetate (%) [#]	55.2 ^c	58.7 ^{ab}	60.2 ^a	57.9 ^{abc}	58.3 ^{ab}	42.3 ^e	57.4 ^{bc}	45.1 ^d	0.56	<0.001
Propionate (%) [#]	20.8 ^a	14.6 ^{cd}	16.3 ^b	20.0 ^a	20.1 ^a	13.1 ^d	20.0 ^a	15.6 ^{bc}	0.31	<0.001
Isobutyrate (%) [#]	1.27 ^{ab}	1.39 ^{ab}	1.26 ^{ab}	1.58 ^{ab}	1.46 ^{ab}	1.25 ^{ab}	1.83 ^a	1.07 ^b	0.14	<0.05
Butyrate (%) [#]	18.0 ^d	21.4 ^c	18.4 ^d	15.8 ^e	15.7 ^e	38.0 ^a	15.8 ^e	32.1 ^b	0.41	<0.001
Isovalerate (%) [#]	2.03 ^{ab}	1.89 ^{abc}	1.84 ^{bc}	2.21 ^{ab}	2.16 ^{ab}	1.91 ^{abc}	2.42 ^a	1.50 ^c	0.11	<0.001
Valerate (%) [#]	2.61 ^{bc}	1.98 ^c	2.07 ^c	2.45 ^{bc}	2.31 ^c	3.38 ^b	2.54 ^{bc}	4.59 ^a	0.20	<0.001
A/P* ratio	2.66 ^c	4.03 ^a	3.70 ^a	2.89 ^{bc}	2.90 ^{bc}	3.22 ^b	2.87 ^{bc}	2.90 ^{bc}	0.07	<0.001

* DIL: dill seeds; CIL: cinnamon leaves; CIB: cinnamon bark; CCB: Ceylon cinnamon bark; EUC: eucalyptus leaves; ORE: oregano leaves; ROS: rosemary leaves; SEM: standard error means; DMD: dry matter degradability; NDFD: neutral detergent fiber degradability; VFA: volatile fatty acid; A/P: acetate/propionate.

[#] Molar proportion as % of total VFA.

Means (n=3) with different superscripts within a row differ significantly (P<0.05).

Table 4. Effects of three-way essential oil combinations (0.8 ml/L total) on *in vitro* gas production and fermentation characteristics.

Parameters	CTR	Three-way essential oil combinations*					SEM*	P-value
		A	B	C	D	E		
Total gas (ml)	147 ^a	120 ^b	119 ^b	126 ^b	129 ^b	120 ^b	2.43	<0.001
Methane (ml)	15.7 ^a	9.77 ^b	3.37 ^c	7.65 ^b	7.48 ^b	8.41 ^b	0.53	<0.001
Ammonia (mg/dL)	44.8 ^a	20.5 ^{bc}	18.3 ^c	25.9 ^b	22.7 ^{bc}	19.7 ^{bc}	1.44	<0.001
DMD*	0.74 ^a	0.54 ^c	0.56 ^c	0.61 ^b	0.71 ^a	0.54 ^c	0.01	<0.001
NDFD*	0.73	0.73	0.70	0.71	0.71	0.72	0.02	0.680
pH	6.99 ^b	7.17 ^a	7.20 ^a	7.10 ^{ab}	7.01 ^b	7.15 ^a	0.02	<0.001
Total VFA* (mM)	56.0 ^a	42.6 ^{bc}	46.9 ^{abc}	51.3 ^{ab}	48.7 ^{abc}	39.1 ^c	2.45	<0.01
Acetate (%) [#]	59.0	62.2	61.1	59.6	59.7	61.9	0.57	0.167
Propionate (%) [#]	19.4 ^a	14.7 ^{abc}	13.1 ^c	15.5 ^{abc}	18.5 ^{ab}	13.9 ^{bc}	0.86	<0.01
Isobutyrate (%) [#]	2.56 ^a	1.52 ^b	1.26 ^b	1.77 ^{ab}	1.57 ^{ab}	1.36 ^b	0.17	<0.01
Butyrate (%) [#]	12.5 ^c	17.0 ^{ab}	20.8 ^a	18.3 ^{ab}	15.6 ^{bc}	19.3 ^{ab}	0.73	<0.001
Isovalerate (%) [#]	4.07 ^a	2.35 ^b	1.97 ^b	2.71 ^{ab}	2.55 ^{ab}	1.81 ^b	0.26	<0.01
Valerate (%) [#]	2.59	2.14	1.78	2.10	2.13	1.63	0.21	0.127
A/P* ratio	3.05 ^b	4.28 ^{ab}	4.74 ^a	3.85 ^{ab}	3.23 ^{ab}	4.61 ^{ab}	0.35	<0.05

* CTR: control; A: combination of dill seeds, oregano leaves, rosemary leaves; B: combination of cinnamon leaves, oregano leaves, rosemary leaves; C: combination of Ceylon cinnamon bark, eucalyptus leaves, oregano leaves; D: combination of Ceylon cinnamon bark, dill seeds, eucalyptus leaves; E: combination of cinnamon bark, dill seeds, oregano leaves; SEM: standard error means; DMD: dry matter degradability; NDFD: neutral detergent fiber degradability; VFA: volatile fatty acid; A/P: acetate/propionate.

[#] Molar proportion as % of total VFA.

Means (n=3) with different superscripts within a row differ significantly (P<0.05).

Table 5. Effects of different essential oils (1.125 ml/L) on abundance ($\log_{10}$ *rrs* copies/ml) of total bacteria, archaea, and *Prevotella* spp.

	CTR	Source of essential oils*							SEM*	p-value
		DIL	CIL	CIB	CCB	EUC	ORE	ROS		
<i>Total Bacteria</i>	10.3 ^a	9.62 ^{ab}	9.49 ^{ab}	9.02 ^{bc}	8.91 ^{bc}	10.0 ^a	8.44 ^c	9.75 ^{ab}	0.20	<0.001
<i>Archaea</i>	7.89 ^{abcd}	7.28 ^{cde}	6.11 ^{de}	8.74 ^a	8.64 ^{ab}	8.10 ^{abc}	5.70 ^e	7.55 ^{bcd}	0.20	<0.001
<i>Prevotella</i> spp.	9.66 ^a	7.50 ^{bc}	7.04 ^{bc}	8.02 ^b	7.96 ^b	7.86 ^{bc}	6.90 ^c	7.32 ^{bc}	0.20	<0.001

* DIL: from dill seeds; CIL: from cinnamon leaves; CIB: from cinnamon bark; CCB: from Ceylon cinnamon bark; EUC: from eucalyptus leaves; ORE: from oregano leaves; ROS: from rosemary leaves; SEM: standard error means.

Means (n=3) with different superscripts within a row differ significantly ($p < 0.05$).

Table 6. Effects of three-way essential oil combinations (0.8 ml/L) on abundance ($\log_{10}$ rrs copies/ml) of total bacteria, archaea, protozoa and selected bacteria species.

	CTR	Three-way essential oil combinations*					SEM*	p-value
		A	B	C	D	E		
<i>Total Bacteria</i>	11.1	11.0	11.0	10.3	11.3	11.1	0.08	0.051
<i>Archaea</i>	8.32 ^a	7.53 ^b	7.47 ^b	7.68 ^b	7.85 ^b	7.59 ^b	0.10	<0.001
<i>Protozoa</i>	7.92 ^a	6.23 ^b	5.96 ^b	5.92 ^b	6.19 ^b	5.99 ^b	0.19	<0.001
<i>Fibrobacter succinogenes</i>	9.60 ^a	6.93 ^c	6.91 ^c	7.09 ^c	8.13 ^b	7.05 ^c	0.10	<0.001
<i>Ruminococcus albus</i>	9.20 ^b	8.82 ^{bc}	8.48 ^c	8.83 ^{bc}	9.90 ^a	8.86 ^{bc}	0.10	<0.001
<i>Ruminobacter amylophilus</i>	9.53 ^a	8.43 ^b	8.40 ^b	8.40 ^b	8.73 ^b	8.49 ^b	0.09	<0.001
<i>Ruminococcus flavefaciens</i>	9.42 ^{ab}	9.06 ^{ab}	8.67 ^b	9.80 ^a	10.1 ^a	9.06 ^{ab}	0.23	<0.01
<i>Prevotella ruminicola</i>	9.54 ^{ab}	9.22 ^{abc}	9.06 ^{bc}	8.69 ^c	9.69 ^a	9.00 ^{bc}	0.12	<0.001
<i>Prevotella bryantii</i>	8.91	7.22	7.01	8.10	7.27	7.33	0.45	0.080
<i>Selenomonas ruminantium</i>	9.39 ^a	8.92 ^b	9.10 ^{ab}	8.82 ^b	9.05 ^{ab}	9.08 ^{ab}	0.07	<0.01
<i>Clostridium aminophilum</i>	8.54	8.35	8.25	8.38	8.99	8.81	0.18	0.072
<i>Streptococcus bovis</i>	7.85 ^b	10.0 ^a	10.4 ^a	9.14 ^{ab}	9.24 ^{ab}	10.7 ^a	0.46	<0.01
<i>Megasphaera elsdenii</i>	0.48 ^a	-1.52 ^b	-1.33 ^b	-1.34 ^b	0.10 ^a	-1.11 ^b	0.11	<0.001

*CTR: control; A: combination of dill seeds, oregano leaves, rosemary leaves; B: combination of cinnamon leaves, oregano leaves, rosemary leaves; C: combination of Ceylon cinnamon bark, eucalyptus leaves, oregano leaves; D: combination of Ceylon cinnamon bark, dill seeds, eucalyptus leaves; E: combination of cinnamon bark, dill seeds, oregano leaves; SEM: standard error means.

Means (n=3) with different superscripts within a row differ significantly (p<0.05).

Figure 1.

Figure 2.