Evaluating the effects of essential oils on methane production and fermentation under in vitro conditions

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ABSTRACT
The effects of adding essential oils (EO) on rumen fermentation and methane production were examined. The aim of experiment one was to screen the effects of four different EO (clove oil (CLO), white thyme oil (WTO), citronella oil (CTO) and anise oil (ANO)) at 500 mg/L of culture fluid on methane production under in vitro conditions. Rumen contents collected from a cannulated Holstein dairy cow was used in a 24-hour batch culture experiment. Treatments were a control (CON) or CON plus EO at 500 mg/L. Results showed that all EOs, except CTO, decreased (p<0.05) methane production. The aim of experiment two was to test the effects of three different dose levels of CLO, WTO, and ANO on methane production and fermentation in 24-h batch culture experiments. Treatments were CON or CON plus EO supplemented at 125, 250, and 500 mg/L. Relative to CON, methane production decreased (p<0.05) with the three EO at the 500 mg/L dose. At the 250 mg/L dose, ANO and CLO decreased (p<0.05) methane production and at the 125 mg/L dose, only CLO decreased methane production. Relative to CON, total VFA concentration declined (p<0.05) in cultures incubated with WTO and with ANO at 500 mg/L dose. Relative to CON, the addition of CLO, WTO and ANO at 500 mg/L decreased (p<0.05) dry matter (DM) digestibility. In conclusion, our results showed that EO effects on methane production depend on EO source and dose level. Although the addition of ANO and WTO at the high doses resulted in lower methane production, they had negatively impacted on rumen microbial fermentation. Clove oil on the other hand reduced methane production without negatively impacting rumen fermentation.

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Introduction
There has been an increasing concern over methane emission by ruminant animals due to its contribution to global warming and energy loss (2–12% of gross energy intake; Johnson & Johnson 1995). Feed additives that are capable to modify rumen fermentation to enhance the efficiency of feed energy utilisation while decreasing rumen methanogenesis are of great interest. Additives such as antibiotics have been proven to be very effective in reducing methane production in the rumen (Chen & Wolin 1979; Tomkins et al. 2009), however, the development of resistant microbes and appearance of residues in food productions such meat and milk have discouraged the use of antibiotics in animal feed. Therefore, there has been a growing interest in exploring natural modifiers of rumen microbes, such as plant secondary metabolites, to improve livestock productivity. Essential oils (EOs) are one of the secondary metabolites present in majority of plants. Different studies (Deans & Ritchie 1987; Chao et al. 2000) have demonstrated the antimicrobial effects of EO against a wide range of microbes including bacteria, protozoa and fungi. Essential oils EOs are recognised as safe a rumen modifier feed additives and thus may be used as a safe alternative to antibiotics (Wallace et al. 2002). Although different in vitro studies have been conducted on EOs potential to modify rumen fermentation; the results from those studies were in consistent (Busquet et al. 2006). Discrepancies among the studies were attributed to different types and doses of EO and diet composition (Busquet et al. 2006; Castillejos et al. 2006; Calsamiglia et al. 2007). Although EOs have shown inhibitory effects on methanogenic Archaea and methane production in the rumen (Patra & Yu 2012, 2014), adverse effects on feed digestibility and fermentation have
also been reported. Our hypothesis is that EO may reduce methane production without adversely affecting rumen fermentation and therefore, the aim of this experiment was to study the effects of four EO on methane production and microbial fermentation under in vitro rumen conditions.

Materials and methods

Essential oil sources

The four EO used in this experiment were: citronella oil (Cymbopogon winterianus), white thyme oil (Thymus vulgaris), clove oil (Eugenia caryophyllus) and anise oil (Illicium verum). Essential oils were acquired from NOW Foods Essential Oil Company (Bloomingdale, IL). All EO were extracted by steam distillation.

Experiment 1

Four different EO were screened in this experiment for their effects on methane production using in vitro batch culture. Treatments were either a control without EO (CON) or control with citronella oil (CTO), white thyme oil (WTO), clove oil (CLO), or anise oil (ANO). A 500 mg/L of each EO, dissolved in 1 ml of ethanol, was added to the culture fluid. Controls were also dosed with the same amount of ethanol.

A ruminally fistulated Holstein cow was used to collect ruminal contents 4 h after morning feeding. The cow was fed a total mixed ration (TMR) containing alfalfa hay (550 g/kg) ground corn (300 g/kg), soy hulls (100 g/kg), and soybean meal (50 g/kg; all on DM basis). The rumen contents were strained through 2 layers of cheesecloth and used within approximately 15 min after collection. ANKOM gas jars containing finely grounded diet (3 g), strained ruminal fluid (40 ml), media (160 ml), and reducing solution (8 ml) according to Goering and Van Soest (1970) were used as batch rumen cultures. The diet contained (on a DM basis) alfalfa hay (250 g/kg), corn silage (250 g/kg), ground corn (300 g/kg), soybean meal (100 g/kg), soy hulls (80 g/kg), and mineral-vitamin mix (20 g/kg). Each jar was gassed with CO₂ before sealing then connected to a Tedlar gas collection bag (CEL Scientific Corp., Santa Fe Springs, CA). Jars were placed in an incubator maintained at 38°C for 24 h. Gasses from jars were programmed to be released into connected bags when the psi exceeded 1.0. Every four hours, the jars were shaken by hand for approximately 20 s. After 24 h, gas bags were disconnected from jars and stored at room temperature until gas analysis. Each treatment was run in triplicate.

From each gas jar, three separate gas samples were collected, using a 1 ml gas tight needle syringe (27G 1 1/4; Fisher Scientific, Chicago, IL) and analysed for gas composition using gas chromatography (SRI 8610C, Torrance, CA) equipped with TCD detector (6” × 1/8” S.S. ShinCarbon) and ST 80/800 column (2 m × 2 mm ID). The oven temperature was programmed at 38°C for five min, then increased at 5°C/min to 270°C and held for five min. The carrier gas was argon. Samples gas peaks (CO₂, H₂, N₂, O₂ and CH₄) were identified by comparing the retention times with those of the corresponding standard (Scotty Analyzed Gases 14, Sigma-Aldrich, St. Louis, MO).

The relative proportion of each gas in collected gas bags was calculated using the response factor (RF) equation:

\[
RF = \frac{CC_i \times \text{Area}_{i \text{ref}}}{\text{Area}_{i \text{ref}}} 
\]

where RF is the response factor, CCᵢ is the proportion of gas i in the sample of the gas being tested, Areaᵢ is the area of gas i peak, CCᵢref is the proportion of the reference gas (helium) in the internal standard, and Areaᵢref is the area of the peak of the reference gas.

To calculate the relative proportion of each gas in the collected gas bags, Avogadro’s Law was used

\[
N = P(V/RT)
\]

where N is the amount of gas produced in moles, P is the pressure in kilopascal, V is the head–space volume in the gas jars in litres, T is Kelvin temperature and R is the gas constant.

The effects of EO (500 mg/L) on DM and NDF digestibility were also estimated by placing a 3 g of the EO in a mobile nylon bag (5 × 10-cm Dacron bags that had a 50-μm pore size; Ankom Inc., Fairport, NY) and incubating in the Ankom Jars as described in experiment 1. After 24 h incubation, the bags were removed and rinsed in cold water for a total of six rinses. The bags were then dried in an oven at 55°C for 48 h, placed in a desiccator for 3 h, and weighed. The samples and diets were then analysed for DM (AOAC 1990) and NDF (Van Soest et al. 1991) and digestibility was measured using as the difference between the contents in the initial samples and the residues remaining after incubation.

Experiment 2

Based on the results from experiment 1, three EO (anise oil, clove oil and white thyme oil) were selected and evaluated at 125, 250 and 500 mg/L of the culture fluid for their effects on fermentation and methane production using the same jars as in experiment 1.
Treatments for each EO were control (no EO); or control with 125, 250 or 500 mg EO/L.

As with the first experiment, EOs were dissolved in ethanol and a total of 1 ml was added to the culture jars. Controls were also dosed with the same amount of ethanol. As with the first experiment, ruminal fluid, diet and buffers were added to each ANKOM gas jars and incubated at 38 °C for 24 h. After 24 h, gas bags were removed and stored at room temperature until gas analysis as described previously. Each treatment was run in triplicate.

After 24 h of incubation, two samples were collected from each culture jar for ammonia-N and volatile fatty acids (VFA) determination. For ammonia-N analysis, the samples centrifuged (IEC Centra GBP8R, Needham Heights, MA) at 20,000 × g for 10 min at 4 °C. The supernatant was acidified with 0.5 ml 0.1 N HCl and then analysed for ammonia-N (Cotta & Russell 1982).

Volatile fatty acid samples added with 1 ml of 25% meta-phosphoric acid and the centrifuged at 20,000 × g for 20 min at 4 °C. The supernatant was collected and the volatile fatty acid of samples determined as described by Jenkins (1987).

### Statistical analysis

Data from the three experiments were analysed using the MIXED procedures of SAS (Statistical Analysis System, Version 9.1, 2003). The fixed effect was treatment and the random effect was the replicate. The PDIF was used to test means difference and significance was declared at $p < .05$.

### Results

The effects of EO on methane production and individual gases are presented in Table 1. Addition of ANO, CLO and WTO to rumen cultures decreased methane production by 32, 37 and 76%, respectively relative to CON. The addition of CTO had no effect ($p > .05$) on methane production. From experiment one, three EO (ANO, WTO and CLO) were selected for experiment two.

In experiment two, the three EO selected from experiment one were tested at different levels (125, 250, and 500 mg/L of culture fluid) for their effects on methane, VFA, ammonia-N, and cultures pH (Tables 2–4). The effects of CLO dose levels on gasses are presented in Table 2. Relative to CON, total gas and CO2 production decreased ($p < .05$) with the 250 and 500 mg/L doses and were least with the 500 mg/L dose. The reduction in total gas and CO2 production averaged 15 and 19% for the 250 mg/L dose and 23 and 26% with the 500 mg/L dose, respectively.

| Table 2. Effect of clove oil (CLO) dose level on gasses production and fermentation parameters. |
| --- |
| **Treatments, mg/L** |
| Item | CON | 125 | 250 | 500 | SEM | p Value |
| --- | --- | --- | --- | --- | --- | --- |
| pH | 6.15<sup>a</sup> | 6.01<sup>ab</sup> | 5.75<sup>b</sup> | 5.89<sup>c</sup> | 0.057 | .01 |
| Gas production, ml | | | | | | |
| Total | 168.88<sup>a</sup> | 156.01<sup>b</sup> | 142.99<sup>b</sup> | 129.59<sup>c</sup> | 4.875 | .01 |
| CH4 | 27.07<sup>a</sup> | 22.87<sup>b</sup> | 22.07<sup>b</sup> | 18.96<sup>c</sup> | 1.519 | .03 |
| CO2 | 120.7<sup>a</sup> | 108.46<sup>b</sup> | 97.49<sup>b</sup> | 89.20<sup>c</sup> | 4.667 | .01 |
| H2 | 0.08 | 0.07 | 0.05 | 0.07 | 0.012 | .46 |
| NH3-N, mg/dL | 26.48 | 26.40 | 26.57 | 26.77 | 0.246 | .53 |
| Total VFA, mM | 88.13 | 75.74 | 74.06 | 70.99 | 7.774 | .23 |
| VFA, mole/100 mole | | | | | | |
| Acetate | 46.58 | 40.88 | 41.32 | 44.46 | 3.768 | .43 |
| Propionate | 30.72 | 33.27 | 33.22 | 30.85 | 1.872 | .33 |
| Butyrate | 16.35 | 18.20 | 18.02 | 17.63 | 1.470 | .62 |
| Iso-butyrate | 1.30 | 1.54 | 1.55 | 1.34 | 0.361 | .85 |
| Valerate | 2.26 | 2.50 | 2.52 | 2.45 | 0.204 | .59 |
| Iso-valerate | 2.77 | 3.16 | 3.37 | 3.27 | 0.288 | .28 |
| Acetate/propanone | 1.55 | 1.24 | 1.30 | 1.44 | 0.203 | .46 |
| VFA: volatile fatty acids; NH3-N: ammonia N; CH4: methane; CO2: carbon dioxide; H2: hydrogen; SEM: standard error mean; CON: control. <sup>a,b,c</sup> Rows with different superscripts are statistically different at $p < .05$. |

| Table 3. Effect of white thyme oil (WTO) dose level on gasses production and fermentation parameters. |
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| **Treatments, mg/L** |
| Item | CON | 125 | 250 | 500 | SEM | p Value |
| --- | --- | --- | --- | --- | --- | --- |
| pH | 6.48 | 6.49 | 6.48 | 6.46 | 0.021 | .64 |
| Gas production, ml | | | | | | |
| Total | 163.40<sup>a</sup> | 159.78<sup>b</sup> | 147.63<sup>b</sup> | 78.94<sup>c</sup> | 4.160 | .01 |
| CH4 | 22.79<sup>a</sup> | 21.82<sup>b</sup> | 21.13<sup>b</sup> | 19.76<sup>b</sup> | 0.865 | .01 |
| CO2 | 118.10<sup>a</sup> | 111.15<sup>ab</sup> | 105.10<sup>b</sup> | 89.18<sup>c</sup> | 3.041 | .01 |
| H2 | 0.07 | 0.05 | 0.05 | 0.05 | 0.039 | .11 |
| NH3-N, mg/dL | 27.88<sup>a</sup> | 27.85<sup>b</sup> | 26.97<sup>b</sup> | 21.50<sup>b</sup> | 0.636 | .01 |
| Total VFA, mM | 87.51 | 68.25 | 54.64 | 54.10 | 4.774 | .23 |
| VFA, mole/100 mole | | | | | | |
| Acetate | 46.92 | 36.53 | 43.24 | 42.24 | 3.948 | .17 |
| Propionate | 27.45 | 33.26 | 32.93 | 32.47 | 2.316 | .13 |
| Butyrate | 18.10<sup>ab</sup> | 20.63<sup>a</sup> | 15.22<sup>b</sup> | 17.90<sup>ab</sup> | 1.319 | .04 |
| Iso-butyrate | 1.42<sup>b</sup> | 1.92<sup>a</sup> | 1.54<sup>b</sup> | 1.58<sup>a</sup> | 0.143 | .05 |
| Valerate | 2.91<sup>ab</sup> | 3.77<sup>a</sup> | 3.51<sup>a</sup> | 2.27<sup>b</sup> | 0.366 | .02 |
| Iso-valerate | 2.26 | 3.90 | 3.56 | 3.55 | 0.209 | .08 |
| Acetate/propanone | 1.77 | 1.15 | 1.32 | 1.30 | 0.251 | .18 |
| VFA: volatile fatty acids; NH3-N: ammonia N; CH4: methane; CO2: carbon dioxide; H2: hydrogen; SEM: standard error mean; CON: control. <sup>a,b,c</sup> Rows with different superscripts are statistically different at $p < .05$. |

CH4: methane; CO2: carbon dioxide; H2: hydrogen; SEM: standard error mean.

<sup>a,b,c</sup> Columns with different superscripts are statistically different at $p < .05$.
Clove oil had a dose-dependent effect on methane production. Compared to CON, methane production decreased (p ≤ .05) by 16, 18, and 30% as CLO dose increased. Relative to CON, CLO treatments had no effect (p > .05) on H2 production, ammonia-N, total VFA concentration, or VFA profile.

The effects of WTO dose levels on gases are presented in Table 3. Compared to CON, total gas and CO2 production decreased (p ≤ .05) with the 250 and 500 mg/L doses and were least with 500 mg/L dose. The reduction in total gas and CO2 production averaged 10 and 52% for the 250 mg/L dose and 11 and 53% with the 500 mg/L dose, respectively. Relative to CON, only the 500 mg/L had a significant effect (p ≤ .05) on methane production reducing it by 78%. Treatment diets had no effect (p > .05) on H2 production compared to CON. Compared to CON, total VFA concentration decreased (p ≤ .05) with each level of WTO. Ammonia-N concentration decreased (p ≤ .05) only in cultures incubated with the 500 mg/L dose compared to CON.

The effects of ANO dose levels on gases are presented in Table 4. Compared to CON, total gas and CO2 production decreased (p ≤ .05) with the 250 and 500 mg/L doses and were least with the 500 mg/L dose. The reduction in total gas and CO2 production averaged 13 and 17% for the 250 mg/L dose and 14 and 18% with the 500 mg/L dose, respectively. Compared to CON, methane production decreased (p ≤ .05) by 17 and 19% with the 250 and 500 mg/L doses, respectively. Treatment diets had no effect (p > .05) on H2 production compared to CON. Compared to CON, total VFA concentration decreased (p ≤ .05) with the 500 mg/L dose. Compared CON, the proportions of acetate and acetate to propionate ratio increased (p ≤ .05) while the proportions of propionate decreased with the 250 mg/L. Compared to CON, each treatment diet decreased (p ≤ .05) butyrate proportions. The 125 and 250 mg/L doses increased (p ≤ .05) ammonia-N concentration. Relative to CON, the addition of CLO, WTO and ANO to rumen cultures at 500 mg/L decreased (p ≤ .05) DM digestibility, but had no effect on NDF digestibility (Table 5).

Table 4. Effect of anise oil (ANO) dose level on gasses production and fermentation parameters.

| Item                  | CON   | ANO  | WTO  | CLO   | SEM  | p Value |
|-----------------------|-------|------|------|-------|------|---------|
| pH                    | 6.14  | 6.15 | 6.17 | 6.21  | 0.003| .01     |
| Gas production, ml    |       |      |      |       |      |         |
| Total                 | 149.06| 147.47| 130.42| 123.51| 6.457| .02     |
| CH4                   | 20.77 | 20.30| 17.31| 16.53 | 1.183| .03     |
| CO2                   | 110.8 | 107.88| 94.91| 90.35 | 5.642| .03     |
| H2                    | 0.07  | 0.06 | 0.05 | 0.09  | 0.010| .04     |
| NH3-N mg/dL           | 26.01 | 26.80| 26.89| 25.67 | 0.228| .01     |
| VFA, mM               | 75.11 | 81.51| 97.13| 47.74 | 4.482| .02     |
| VFA, m mole/100 m mole|       |      |      |       |      |         |
| Acetate               | 39.91 | 45.86| 52.88| 43.16 | 3.237| .03     |
| Propionate            | 32.61 | 29.99| 26.05| 35.31 | 1.881| .01     |
| Butyrate              | 19.85 | 16.39| 14.04| 14.16 | 1.050| .01     |
| Iso-butyrate           | 1.52  | 1.96 | 1.57 | 1.38  | 0.183| .08     |
| Valerate              | 2.78  | 2.54 | 2.33 | 2.96  | 0.146| .10     |
| Iso-valerate           | 3.33  | 3.25 | 3.13 | 3.53  | 0.133| .11     |
| Acetate:propionate     | 1.25  | 1.54 | 2.03 | 1.23  | 0.180| .01     |

VFA: volatile fatty acids; NH3-N: ammonia N; CH4: methane; CO2: carbon dioxide; H2: hydrogen; SEM: standard error mean; CON: control. a,b,cRows with different superscripts are statistically different at p ≤ .05.

Table 5. Effect of essential oils on dry matter and neutral detergent fibre digested.

| Item                  | CON   | ANO  | WTO  | CLO   | SEM  | p Value |
|-----------------------|-------|------|------|-------|------|---------|
| Digestibility, %       |       |      |      |       |      |         |
| Dry matter             | 56.29 | 52.20| 48.44| 50.76 | 1.594| .01     |
| Neutral detergent fibre| 53.05 | 51.91| 53.59| 51.46 | 1.854| .41     |

CON: control; ANO: anise oil; WTO: white thyme oil; CLO: clove oil. a,b,cRows with different superscripts are statistically different at p ≤ .05.

Discussion

Essential oils extracted from different plant species may vary in their chemical structures and bioactive components (Burt 2004). Essential oils added to ruminant diets have altered digestion and fermentation of diets, microbial populations, and methanogenesis in the rumen (Calsamiglia et al. 2007; Cieslak et al. 2013). Results from this study showed that except for CTO, all EO tested in this study decreased rumen methane and total gas production. The addition of 250 and 500 mg/L doses of all EO to cultures resulted in significant reductions in total gas and CO2 production. These reductions suggest that the rumen fermentation and microbial activity were negatively impacted at these levels.

Clove oil contains the phenolic compound eugenol at 85% that has been reported to have a wide spectrum of antimicrobial effects against both Gram-negative and Gram-positive bacteria (Deans & Ritchie 1987). Clove oil doses tested in this study had no effect on total VFA suggesting had no detrimental effect on rumen fermentation. In the current study, the proportions of individual VFAs were not affected by CLO in agreement with the findings of Castillejos et al. (2006) who reported no changes in the proportions of individual VFAs when eugenol was added to rumen cultures at incremental doses (5 to 5000 mg/L). The addition of CLO to cultures caused significant reductions in the production of methane without altering VFA concentrations. The production of methane decreased in a dose-dependent manner with the addition of CLO to rumen cultures is in agreement with previous findings (Chaves et al. 2008; Patra et al. 2010). Patra and
Yu (2012) reported addition of CLO at increasing doses (0.25, 0.50, and 1.0 g/L) reduced methane production by 11, 17, 34.4%, respectively. Chiquette and Benchaar (2005) reported that eugenol (500 to 600 mg/L) decreased methane production by 42% under *in vitro* conditions. It is well reported that the effect of EOs on methane production may result from either toxicity to methanogens, a reduction in H₂ production due to decreased acetate and butyrate production (i.e. reduced fibre degradation) or a reduction in organic matter digestion (Cieslak et al. 2013; Kumar et al. 2014). Additionally, EOs may have also stimulated propionate production. An increase in propionate production can negatively affect methanogenesis by increasing competition for H₂ (Newbold et al. 2005). The reductions in methane production with the 250 and 500 mg/L CLO diets may be also attributed in part to the reductions in substrate fermentation (decreased total gas and CO₂ concentration). This suggestion is also supported with the observed reduction in DM digestibility with the CLO diet. García-González et al. (2008) showed that phenolic compounds decreased methanogenesis by reducing digestibility. Although total gas and CO₂ and propionate production were not affected by 125 mg/L dose, the reduced methane concentration suggests that CLO has an inhibitory effect on methanogens. Essentials oils may affect methane production by directly inhibiting the growth and activity of methanogenic microbes or indirectly by decreasing the number of protozoa associated with methanogens (Cieslak et al. 2013). The decreases in pH values with the 250 and 500 mg/L CLO diets may have resulted from numerical increases in propionate and butyrate production. All doses of CLO did not affect ammonia-N concentration. A study by Castillejos et al. (2008) showed that ammonia-N concentrations increased when CLO was added at 50 mg/L but when CLO was added at 500 mg/L ammonia-N concentration did not change.

According to Lawrence and Reynolds (1984); thymol and carvacrol are considered the two main active phenolic compounds in WTO accounting for up to 60% of the total identified compounds. Thymol and carvacrol have demonstrated strong antimicrobial activity against bacteria (Dorman & Deans 2000; Evans & Martin 2000) and according to Davidson and Naidu (2000); this inhibitory activity is due to the presence of the phenolic group in these EO compounds. White thyme oil doses tested in this study decreased total VFA concentration and DM digestibility suggesting that WTO had detrimental effects on rumen fermentation. Castillejos et al. (2006) reported no effects of thymol at 5 and 50 mg/L on VFA profiles and production, but total VFA production decreased at 500 mg/L. The VFA are produced during rumen microbial fermentation and are considered the main energy source for ruminant animals (Van Soest 1982). Therefore, any reduction in total VFA concentration would be nutritionally unfavourable for the animal. Addition of 500 mg/L WTO to cultures decreased methane production suggesting that WTO was only effective at high dose. Similarly, Evans and Martin (2000) observed no effects on methane concentration when thymol was used at 50, 100 and 200 mg/L of culture fluid in 24 h incubations of mixed rumen bacteria. However, at high concentration (400 mg/L), thymol strongly decreased methane concentration. It is possible that microbial populations adapted to and/or degraded plant secondary metabolites when EO administered at low doses as suggested by Cardozo et al. (2004: 0.22 mg/L) and Busquet et al. (2005b: 2.2 mg/L) but not at higher doses (Busquet et al. 2005a: 300 mg/L). A lack of significantly effect on methane emission with lower doses in the present experiment might also be related to basic nutrient components of the diets or the selectivity of essential oil against to some microorganisms. Some essential oils at lower doses may suppress the colonisation and/or digestion of non-structural carbohydrates by amylolytic without affecting fibre digestion (Wallace et al. 2002). McEwan et al. (2002) observed the attachment of rumen microbes to starch rich grains and protein was lower when a blend of essential oil was added to sheep ration. Evans and Martin (2000) found that thymol selectively inhibited at 90 mg/L, the growth of *Selenomonas ruminantium* but not *S. bovis* whilst at 400 mg/L all rumen microorganisms tended to be inhibited. The tendency for increases in H₂ concentration with the addition of 500 mg/L WTO to rumen cultures suggests that WTO affected rumen methanogens. WTO or its components may inhibit methanogenic Archaea, or decrease the utilisation of H₂ by these microorganisms, resulting in the accumulation of molecular H₂ in the medium. The tendency for greater proportions of propionate in cultures incubated with the high dose of WTO in the current study may partly be related in reduction in methane production. The reduction in methane production in the rumen is usually associated with an increase in propionate production as propionate formation functions as a H₂ sink in the rumen when less H₂ is directed toward methane production. Other studies (Russell 1998; Tekippe et al. 2012) have also reported negative association between methane production and propionate formation in the rumen. The decreased ammonia-N concentrations in the culture of 500 mg/L WTO may be due to the interaction between
the phenolic compounds (thymol and carvacrol) and proteins and/or WTO effects on rumen ammonia-producing bacteria and protozoa. The addition of thymol to rumen cultures at 500–1000 mg/L was also associated with significant reductions in ammonia-N concentrations (Borchers 1965; Castillejos et al. 2006).

Davidson and Naidu (2000) reported that anethol, the major compound of ANO (up 80–90%), has strong antimicrobial activity against bacteria. The supplementation to rumen culture at 500 mg/L dose decreased total VFA and DM digestibility suggesting that ANO had detrimental effects on rumen fermentation in high dose. Busquet et al. (2006) reported a decrease in VFA concentration when ANO was added to rumen batch cultures at 3000 mg/L but no effects on VFA concentration when ANO was added at up to 300 mg/L. The increase in pH value with the 500 mg/L ANO diet may be due to decrease in substrate fermentation (decreased VFA concentration). The lower butyrate concentration in cultures incubated with doses of ANO suggests that this EO or its components have detrimental effect on main butyrate producers such as protozoa, Butyrivibrio fibrisolvens. The addition of ANO at 250 and 500 mg/L to cultures decreased methane production. Although, \( H_2 \) concentration tended to increase by 500 mg/L dose (Tables 1 and 4), the increased pH value suggests that the inhibitory effects of ANO on rumen methanogens was less pronounced than substrate degradation. The observed increases in ammonia-N concentrations with 125 and 250 mg/L doses may have resulted from either an increase in bacterial lysis and/or an increase in amino acids deamination by ammonia-producing bacteria (Wallace et al. 2002). McIntosh et al. (2003) reported that the growth of the amylolytic bacterium Ruminobacter amylophilus was not affected when a blend of EO was used but its lysis greatly increased in the stationary growth phase. Previous in an in vitro study (Gunal et al. 2014) observed similar increases in ammonia-N concentrations with the addition of ANO to culture at 125, 250 and 500 mg/L. The observed greater ammonia-N concentration with 125 and 250 mg/L cultures may suggest that the addition of this EO reduced rumen efficiency as more nitrogen would be escaping as ammonia instead of amino acids for absorption.

Conclusions

Results from the present study showed that except for CTO, EO tested in this study had effects on rumen methane and total gas production. Although the addition of the WTO and ANO reduced methane production at the high doses, they also negatively impacted rumen microbial fermentation. However, CLO reduced methane production without negatively affecting rumen fermentation. The reductions in methane production with the tested EO most probably the results of methanogens inhibition and the negative effects of EO on diet fermentation.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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