Comparative Effects of Different Plant Essential Oils on In Vitro Rumen Fermentation and Methane Production (Postprint)

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Abstract

Under a substrate concentrate-to-forage ratio of 6:4, different doses of eugenol, D-limonene, anethole, cinnamaldehyde, thymol, or carvacrol were added to the substrate [resulting in plant essential oil concentrations of 0 (control), 50, 100, 200, and 400 mg/L in the fermentation fluid] to comparatively investigate the effects of different plant essential oils on in vitro rumen fermentation and methane (CH4) production using the in vitro gas production method. Each dose of each plant essential oil was set up with 3 replicates. In vitro rumen fermentation was simulated for 24 h, and gas production, CH4 content in the gas, as well as pH, volatile fatty acid (VFA) proportions, and ammonia nitrogen (NH3-N) concentration in the fermentation fluid were measured. The results showed that: 1) Except for thymol, the addition of various plant essential oils had no significant effect on the pH of the in vitro fermentation fluid (P>0.05). 2) The addition of eugenol, D-limonene, anethole, and cinnamaldehyde had no significant effect on total VFA concentration in the in vitro fermentation fluid (P>0.05), but total VFA concentration showed a quadratic change with increasing concentrations of thymol and carvacrol (PQ<0.01). Compared with the control group, the addition of 400 mg/L thymol and carvacrol significantly reduced total VFA concentration (P<0.01). The addition of D-limonene, anethole, thymol, and carvacrol altered the molar percentage of each VFA relative to total VFA. Compared with the control group, the addition of 50 mg/L D-limonene and anethole significantly increased the acetate proportion (P<0.05) and significantly decreased the propionate proportion (P<0.05); whereas the addition of 400 mg/L D-limonene and anethole significantly decreased the acetate proportion (P<0.05) and significantly increased the propionate and butyrate proportions (P<0.05). The addition of thymol and carvacrol had no significant effect on the acetate proportion (P>0.05); compared with the control group, 400 mg/L thymol and carvacrol significantly decreased the propionate proportion (P<0.05).

3) The addition of anethole, thymol, and carvacrol significantly affected NH3-N concentration in the in vitro fermentation fluid (P<0.05); compared with the control group, 400 mg/L thymol and carvacrol significantly reduced NH3-N concentration (P<0.05). 4) The addition of D-limonene, anethole, and cinnamaldehyde had no significant effect on 24 h gas production from in vitro fermentation (P>0.05). Compared with the control group, all concentrations of thymol and carvacrol significantly reduced 24 h gas production from in vitro fermentation (P<0.05), and showed a quadratic change with increasing concentrations of thymol and carvacrol (PQ<0.01). 5) The addition of D-limonene, anethole, and cinnamaldehyde had no significant effect on 24 h CH4 production from in vitro fermentation (P>0.05). Compared with the control group, 50 and 100 mg/L eugenol significantly increased 24 h CH4 production from in vitro fermentation (P<0.05), whereas 400 mg/L thymol and carvacrol reduced 24 h CH4 production by 84.7% (P<0.05) and 73.9% (P<0.05), respectively. Based on these experimental results, it can be concluded that different plant essential oils have different effects on in vitro rumen fermentation and CH4 production, which are also dose-dependent. Specifically, low doses of thymol and carvacrol promoted in vitro rumen fermentation, whereas high doses of thymol and carvacrol inhibited in vitro rumen fermentation and significantly reduced 24 h CH4 production.

Full Text

A Comparative Study on the Effects of Different Plant Essential Oils on Rumen Fermentation and Methane Production In Vitro

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Abstract

This study investigated the effects of different plant essential oils on rumen fermentation and methane (CH_4) production in vitro using the gas production technique. The substrate had a concentrate-to-forage ratio of 6:4, and different doses of eugenol, D-limonene, anethole, cinnamaldehyde, thymol, or carvacrol were added to achieve final concentrations of 0 (control), 50, 100, 200, and 400 mg/L in the fermentation fluid. Each dose of each essential oil had three replicates. After 24 hours of in vitro rumen fermentation, gas production and CH_4 content in the gas were measured, along with fermentation fluid pH, volatile fatty acid (VFA) proportions, and ammonia nitrogen (NH_3-N) concentration.

The results showed: (1) Except for thymol, the addition of various plant essential oils had no significant effect on fermentation fluid pH (P>0.05). (2)

The addition of eugenol, D-limonene, anethole, and cinnamaldehyde had no significant effect on total VFA concentration (P>0.05), but total VFA concentration showed a quadratic change with increasing thymol and carvacrol concentrations (PQ<0.01). Compared with the control group, 400 mg/L thymol and carvacrol significantly reduced total VFA concentration (P<0.01). The addition of D-limonene, anethole, thymol, and carvacrol altered the molar percentage of individual VFAs in total VFAs. Compared with the control, 50 mg/L D-limonene and anethole significantly increased acetate proportion (P<0.05) and significantly decreased propionate proportion (P<0.05); however, 400 mg/L D-limonene and anethole significantly decreased acetate proportion (P<0.05) and significantly increased propionate and butyrate proportions (P<0.05). Thymol and carvacrol addition had no significant effect on acetate proportion (P>0.05), but 400 mg/L thymol and carvacrol significantly decreased propionate proportion compared with the control (P<0.05). (3) The addition of anethole, thymol, and carvacrol significantly affected NH_3-N concentration (P<0.05); compared with the control, 400 mg/L thymol and carvacrol significantly reduced NH_3-N concentration (P<0.05). (4) The addition of D-limonene, anethole, and cinnamaldehyde had no significant effect on 24-hour gas production (P>0.05). Compared with the control, all concentrations of thymol and carvacrol significantly reduced 24-hour gas production (P<0.05), which showed a quadratic change with increasing concentrations (PQ<0.01). (5) The addition of D-limonene, anethole, and cinnamaldehyde had no significant effect on 24-hour CH_4 production (P>0.05). Compared with the control, 50 and 100 mg/L eugenol significantly increased 24-hour CH_4 production (P<0.05), while 400 mg/L thymol and carvacrol reduced CH_4 production by 84.7% (P<0.05) and 73.9% (P<0.05), respectively.

In conclusion, different plant essential oils have varying effects on in vitro rumen fermentation and CH_4 production, which are dose-dependent. Low doses of thymol and carvacrol promoted rumen fermentation, whereas high doses inhibited rumen fermentation and significantly reduced 24-hour CH_4 production.

Keywords: plant essential oil; rumen fermentation; methane production

Introduction

The greenhouse effect caused by increasing atmospheric concentrations of carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O), and other gases has attracted widespread global attention. Methane has 21 times the greenhouse effect of CO_2 and is currently considered the second most important gas contributing to global warming after CO_2. Ruminant livestock emit approximately 80 million tons of CH_4 annually, accounting for 33% of anthropogenic methane emissions [1]. Furthermore, CH_4 emissions represent a significant loss of dietary energy, generally comprising 2–12% of total feed energy [2] and up to 15% for low-quality forages. Therefore, research on measures to reduce ruminal CH_4

emissions has become a major focus.

While many chemical additives can effectively reduce CH_4 production in ruminants, growing scientific awareness of their negative effects has shifted interest toward natural, green additives. Plant essential oils are volatile, oil-like substances present in plants with aromatic odors that can be distilled with steam but are water-insoluble. Extracted from flowers, leaves, stems, roots, or fruits through steam distillation, pressing, cold maceration, or solvent extraction, they represent important active components of plant extracts. Studies have shown that plant essential oils not only possess antimicrobial properties but can also reduce CH_4 production [3-4] and decrease ruminal protein degradation, thereby reducing NH_3-N concentration [5-6]. However, different plant essential oils have varying effects on rumen fermentation and feed digestion [7-9]. This study selected three common monoterpenoid compounds (carvacrol, thymol, and D-limonene) and three phenylpropanoid compounds (cinnamaldehyde, anethole, and eugenol) to investigate their effects on in vitro rumen fermentation and CH_4 production, providing a theoretical basis for the application of plant essential oils in animal production.

1.1 Plant Essential Oils Tested

Six plant essential oils were selected for this experiment: eugenol (99% purity, Indonesia), thymol (99% purity, India), anethole (99% purity, Guangxi), carvacrol (99% purity, Nanjing), cinnamaldehyde (98% purity, Nanjing), and D-limonene (94% purity, Brazil).

1.2 Experimental Design

The in vitro culture substrate was formulated according to nutrient levels recommended in China' s *Feeding Standard of Meat Sheep* (NY/T 816–2004), using corn, soybean meal, and Chinese wildrye hay as raw materials, ground to pass a 0.5 mm sieve. The substrate composition and nutrient levels are shown in , with a concentrate-to-forage ratio of 6:4. A single-factor design was employed, with different doses of eugenol, D-limonene, anethole, cinnamaldehyde, thymol, or carvacrol added to achieve final concentrations of 0 (control), 50, 100, 200, and 400 mg/L in the fermentation fluid. Each dose of each essential oil had three replicates. A blank control group (for gas production correction) without substrate but with fermentation fluid only was also included, with three replicates.

1.3 In Vitro Rumen Fermentation Culture

The in vitro gas production method of Menke et al. [10] was used. Rumen fluid was collected from four fistulated sheep with permanent rumen fistulas and mixed with buffer at a 1:2 ratio to prepare artificial rumen fermentation fluid. Two hundred milligrams (dry matter basis) of substrate were weighed into the top of 100 mL culture tubes (syringes). Before adding the artificial rumen fermentation fluid, 0.5 mL of plant essential oil mixture (essential oils mixed with ethanol at different concentration gradients) was injected from the front of the culture tube, followed by 30 mL of artificial rumen fermentation fluid. According to the experimental design, the final essential oil concentrations in the fermentation fluid reached 0, 50, 100, 200, and 400 mg/L for different groups. Blank control tubes received 30 mL of artificial rumen fermentation fluid without substrate. All tubes were incubated at 39°C in a constant-temperature water bath for 24 hours, with scale readings recorded at different time points. Fermentation was terminated at 24 hours, and fermentation parameters and CH_4 production were measured.

The experiment was repeated in two batches.

1.4.1 Determination of Substrate Nutrient Content

Three parallel samples were used for nutrient content determination, with coefficient of variation controlled within 5%. Dry matter content was determined by oven drying at 105°C for 5 hours. Crude ash content was determined by combustion in a muffle furnace at 550°C for 5 hours, with organic matter content calculated as 100 minus crude ash content. Neutral detergent fiber (NDF) content was determined using the method of Van Soest et al. [11] with heat-stable α-amylase. Acid detergent fiber (ADF) content was determined according to AOAC (1991) [12]. Crude protein content (nitrogen × 6.25) was determined by the Kjeldahl method [12]. Calcium content was determined by potassium permanganate titration [13], and phosphorus content by molybdenum yellow colorimetry [13].

1.4.2 Gas Production Measurement

During in vitro incubation, culture tube scale readings were recorded at 0, 1, 2, 4, 6, 8, 10, 12, 16, 20, and 24 hours to calculate gas production at different time points and cumulative net gas production over 24 hours.

1.4.3 In Vitro Fermentation Parameter Determination

After gas collection, fermentation fluid was drained from the culture tubes and pH was measured immediately. The fermentation fluid was centrifuged (800×g, 15 min), and 1 mL of supernatant was added to a 1.5 mL centrifuge tube (previously stored with 200 µL of 25% metaphosphoric acid). Volatile fatty acid (VFA) concentrations were determined by gas chromatography (Agilent 6890N) with an HP-INNOwax capillary column (30.0 m × 320 µm × 0.5 µm, Catalog No.: 19091N-213), using 2-ethylbutyric acid (2EB) as an internal standard. Chromatographic conditions: flame ionization detector; carrier gas nitrogen (N_2) with a split ratio of 40:1; injection volume 0.4 µL at 220°C; constant flow mode at 2.0 mL/min with average linear velocity of 38 cm/s; temperature program:

120°C (3 min) → 10°C/min → 180°C (1 min); detector temperature 250°C; hydrogen (H_2) flow 40 mL/min; air flow 450 mL/min; column flow + makeup flow 45 mL/min. The molar percentage of each VFA in total VFAs was calculated.

Another 4 mL of supernatant was added to a 5 mL centrifuge tube (previously stored with 0.8 mL of 1% sulfuric acid), and NH_3-N concentration was determined by the phenol-hypochlorite colorimetric method [14].

1.4.4 CH_4 Production Determination

After 24 hours of in vitro incubation, culture tubes were removed and rapidly placed in an ice-water bath to stop fermentation. Gas from the tubes was extracted and immediately analyzed for CH_4 content using a TP-2060T gas chromatograph. Chromatographic conditions: thermal conductivity detector (TCD); TDX-01 packed column (1 mm × 3 mm × 2 mm); injector temperature 150°C; column temperature 120°C; detector temperature 150°C; carrier gas helium (He) at 50 mL/min; injection volume 0.1 mL. CH_4 production was calculated based on gas production and CH_4 content.

1.5 Statistical Analysis

Experimental data were analyzed using the General Linear Model (GLM) procedure of SAS 9.0. Variance analysis and multiple comparisons were conducted according to the single-factor design. The experiment was repeated in two batches, with six replicates per treatment. Linear (L) and quadratic (Q) comparisons were performed for the effects of different essential oil doses on in vitro rumen fermentation and CH_4 production. Significance was set at $P < 0.05$, and trend range at $0.05 \leq P \leq 0.10$.

2.1.1 Effects of Different Plant Essential Oils on Fermentation Fluid pH

As shown in , thymol addition significantly affected fermentation fluid pH ($P < 0.05$). When its concentration reached 400 mg/L, fermentation fluid pH was significantly higher than the control group ($P < 0.05$). Other plant essential oils had no significant effect on fermentation fluid pH ($P > 0.05$).

2.1.2 Effects of Different Plant Essential Oils on VFA Proportions in Fermentation Fluid

As shown in , thymol and carvacrol addition significantly affected total VFA concentration ($P < 0.05$), while other essential oils had no significant effect ($P > 0.05$). Compared with the control, 400 mg/L thymol and carvacrol significantly reduced total VFA concentration ($P < 0.05$), with total VFA concentration showing a quadratic change with increasing concentrations ($PQ < 0.01$).

D-limonene and anethole addition significantly affected acetate proportion ($P < 0.05$), while other essential oils had no significant effect ($P > 0.05$). Compared with the control, 50 mg/L D-limonene and anethole significantly increased acetate proportion ($P < 0.05$), while 200 and 400 mg/L D-limonene and 400 mg/L anethole significantly decreased acetate proportion ($P < 0.05$). Acetate proportion showed a linear decrease with increasing D-limonene ($PL < 0.01$) and anethole ($PL < 0.01$) concentrations.

Except for eugenol and cinnamaldehyde, D-limonene, anethole, thymol, and carvacrol addition significantly affected propionate proportion ($P < 0.05$). Compared with the control, 100, 200, and 400 mg/L D-limonene significantly increased propionate proportion ($P < 0.05$), showing a quadratic change ($PQ < 0.01$). Anethole at 50 and 400 mg/L significantly decreased propionate proportion ($P < 0.05$), also showing a quadratic change ($PQ < 0.01$). Propionate proportion showed a quadratic trend with increasing cinnamaldehyde concentration ($PQ = 0.04$). Thymol at 200 and 400 mg/L significantly decreased propionate proportion ($P < 0.05$), showing a linear decreasing trend ($PL = 0.02$). Carvacrol at 400 mg/L significantly decreased propionate proportion ($P < 0.05$), showing a quadratic change ($PQ = 0.04$).

D-limonene, anethole, and carvacrol addition significantly affected acetate-to-propionate ratio ($P < 0.05$). Compared with the control, 100, 200, and 400 mg/L D-limonene significantly decreased the ratio ($P < 0.05$), showing a quadratic change ($PQ < 0.01$). Anethole at 50 and 400 mg/L significantly increased the ratio ($P < 0.05$), while 200 mg/L significantly decreased it ($P < 0.05$), showing a quadratic change ($PQ < 0.01$). Carvacrol at 400 mg/L significantly increased the ratio ($P < 0.05$), showing a linear increase ($PL < 0.01$).

D-limonene and carvacrol addition significantly affected isobutyrate proportion ($P < 0.05$), showing quadratic ($PQ < 0.01$) and linear ($PL < 0.01$) decreases, respectively, with increasing concentrations. D-limonene, anethole, and carvacrol significantly affected butyrate proportion ($P < 0.05$), showing quadratic changes with D-limonene ($PQ < 0.01$) and anethole ($PQ = 0.02$), and a linear increase with carvacrol ($PL < 0.01$). Thymol and carvacrol significantly affected isovalerate proportion ($P < 0.05$), showing quadratic changes ($PQ < 0.05$) with increasing concentrations. D-limonene, anethole, thymol, and carvacrol significantly affected valerate proportion ($P < 0.05$), showing quadratic changes with D-limonene ($PQ = 0.01$), anethole ($PQ < 0.01$), and thymol ($PQ = 0.03$), and a linear decrease with carvacrol ($PL < 0.01$).

2.1.3 Effects of Different Plant Essential Oils on NH_3-N Concentration in Fermentation Fluid

As shown in , anethole, thymol, and carvacrol addition significantly affected NH_3-N concentration ($P < 0.05$), while other essential oils had no significant effect ($P > 0.05$). Compared with 100 mg/L anethole, 400 mg/L anethole significantly reduced NH_3-N concentration ($P < 0.05$), showing a quadratic change (PQ = 0.03). Compared with the control, 400 mg/L thymol significantly reduced NH_3-N concentration ($P < 0.05$), showing a linear decrease ($PL < 0.01$). Compared with the control, 400 mg/L carvacrol significantly reduced NH_3-N concentration ($P < 0.05$), showing a quadratic change ($PQ < 0.01$).

2.2 Effects of Different Plant Essential Oils on 24-Hour Gas Production and CH_4 Production

As shown in , eugenol, thymol, and carvacrol addition significantly affected 24-hour gas production ($P < 0.05$), while other essential oils had no significant effect ($P > 0.05$). Gas production showed a linear decrease with increasing eugenol concentration ($PL < 0.05$). All concentrations of thymol and carvacrol significantly reduced 24-hour gas production compared with the control ($P < 0.01$), showing quadratic changes with increasing concentrations ($PQ < 0.01$).

Cinnamaldehyde, thymol, and carvacrol addition significantly affected CH_4 content in fermentation gas ($P < 0.05$), while other essential oils had no significant effect ($P > 0.05$). All concentrations of cinnamaldehyde significantly increased CH_4 content compared with the control ($P < 0.05$), showing a quadratic change ($PQ = 0.02$). Thymol at 200 mg/L significantly increased CH_4 content ($P < 0.05$), while 400 mg/L significantly decreased it ($P < 0.05$), showing an overall quadratic change ($PQ < 0.01$). Carvacrol at 400 mg/L significantly decreased CH_4 content compared with 100 and 200 mg/L ($P < 0.05$), showing a quadratic change ($PQ < 0.01$). CH_4 content also showed a quadratic change with increasing D-limonene concentration ($PQ = 0.02$).

Eugenol, thymol, and carvacrol addition significantly affected 24-hour CH_4 production ($P < 0.05$), while other essential oils had no significant effect ($P > 0.05$). Compared with the control, 50 and 100 mg/L eugenol significantly increased 24-hour CH_4 production ($P < 0.05$). CH_4 production showed a linear decrease with increasing D-limonene concentration ($PL = 0.02$) and a quadratic change with increasing cinnamaldehyde concentration ($PQ = 0.04$). Compared with the control, 400 mg/L thymol and carvacrol significantly reduced 24-hour CH_4 production ($P < 0.05$), showing quadratic changes with increasing concentrations ($PQ < 0.01$).

3.1 Effects of Different Plant Essential Oils on In Vitro Rumen Fermentation

Plant essential oils are characterized by diverse composition, properties, and activities. Their most important active components include two chemical groups: terpenoids (monoterpenoids and sesquiterpenoids) and phenylpropanoid compounds. Both groups are nitrogen-free hydrocarbons derived from different metabolic precursors and synthesized through different pathways. Among the six essential oils used in this study, carvacrol, thymol, and D-limonene are monoterpenoid compounds, while cinnamaldehyde, anethole, and eugenol are phenylpropanoid compounds.

The effects of plant essential oils on rumen fermentation stem from their antimicrobial properties. Terpenoids and phenylpropanoid compounds act on bacterial cell membranes, with activity attributed at least partially to the hydrophobicity of cyclic hydrocarbons, allowing them to interact with and accumulate in bacterial lipid bilayers, occupying spaces between fatty acid chains. This interaction causes conformational changes in membrane structure, leading to its liquefaction and expansion. Loss of membrane stability results in ion leakage across the membrane, reducing the transmembrane ion gradient. Although bacteria can balance ion gradients using ion pumps, this process consumes substantial energy and slows bacterial growth [15-16]. Generally, oxygenated cyclic hydrocarbons exhibit the highest antimicrobial activity, particularly phenolic structures such as thymol and carvacrol, where hydroxyl groups and delocalized electrons allow interaction with water through hydrogen bridging as primary active sites, making their antimicrobial activity particularly potent [15-17]. When applied to ruminants, plant essential oils primarily affect rumen fermentation by influencing rumen microbial activity.

Rumen fluid pH reflects the rumen internal environment and is influenced by diet type, saliva secretion, and organic acid accumulation. In this study, only 400 mg/L thymol significantly increased fermentation fluid pH, while other essential oils at various concentrations had no significant effect. Since buffer solution plays an important role in maintaining constant pH in in vitro fermentation, the pH increase observed with 400 mg/L thymol may be attributed to its significant reduction of total VFA concentration.

Studies on the effects of dietary plant essential oils on total VFA concentration have shown inconsistent results. Some studies reported increased ruminal total VFA concentration with essential oil supplementation, potentially improving feed digestibility. However, most studies indicated that essential oils decreased total VFA concentration or had no significant effect. For example, Chaves et al. [18] found that dietary cinnamaldehyde (0.2 g/kg dry matter intake) increased total VFA concentration in the rumen. Castillejos et al. [19] maintained constant pH in a continuous culture system and found that a mixed essential oil blend at 1.5 mg/L increased total VFA concentration without simultaneously increasing organic matter digestibility. In contrast, two in vivo studies using the same essential oil blend in sheep (110 mg/d) and beef cattle (1 g/d) found no significant effects on total VFA concentration or individual VFA proportions [20-21].

Whether essential oils reduce total VFA concentration may depend on dose. Busquet et al. [5] investigated numerous essential oils at doses up to 3,000 mg/L in 24-hour in vitro rumen fermentation and found no significant effects on total VFA concentration except at the highest dose, when most treatments reduced total VFA concentration along with feed digestibility. Castillejos et al. [6] reported similar results with eugenol, guaiacol, limonene, thymol, and vanillin at concentrations up to 5,000 mg/L, where total VFA concentration was generally unaffected except at the highest dose, when all essential oils reduced total VFA concentration. This may be because high concentrations of essential oils inhibit rumen microbial fiber digestion, thereby reducing total VFA concentration. This could also explain why essential oils may better regulate rumen fermentation in high-concentrate diets or acidic rumen environments than in high-forage diets [22]. Lin et al. [23] used in vitro methods to study the effects of cinnamon oil, oregano oil, and their main components cinnamaldehyde and carvacrol at 0, 50, 200, 500, and 750 mg/L, finding that total VFA concentration decreased with increasing essential oil concentration, with significant inhibition of rumen fermentation at high concentrations (500, 750 mg/L).

In this study, different concentrations of eugenol, D-limonene, anethole, and cinnamaldehyde had no significant effect on total VFA concentration. However, all concentrations of thymol and carvacrol only significantly reduced total VFA concentration at the highest dose, which also significantly decreased 24-hour gas production, indicating that 400 mg/L thymol and carvacrol inhibited rumen fermentation. Overall, these results demonstrate that different essential oils have inconsistent effects on total VFA concentration, which are dose-dependent.

Some studies have found that certain essential oils and their components can alter VFA molar ratios, similar to monensin (e.g., decreasing acetate proportion and increasing propionate proportion), which are considered beneficial effects. Busquet et al. [24] used two doses of cinnamaldehyde and garlic oil (31.2 and 312 mg/L), finding that low-dose cinnamaldehyde and high-dose garlic oil decreased acetate proportion and increased propionate proportion. Lu et al. [25] added garlic oil at 30, 50, 100, 300, and 500 mg/L in vitro, finding that all concentrations except 30 mg/L significantly reduced acetate proportion, increased propionate and butyrate proportions, and decreased the acetate-to-propionate ratio. Other studies have reported undesirable changes in individual VFA proportions, such as decreased propionate proportion without affecting total VFA concentration [6].

In this study, D-limonene and anethole, which affected acetate proportion, increased it at low doses but decreased it at high doses, correspondingly decreasing propionate and butyrate proportions at low doses and increasing them at high doses. This shift from acetate to propionate can be considered a beneficial effect of essential oil addition. High-dose thymol and carvacrol significantly reduced propionate proportion while also significantly decreasing total VFA concentration, indicating inhibited rumen fermentation.

Ruminal NH_3-N concentration is an important indicator of rumen nitrogen metabolism, indirectly reflecting the balance between microbial protein degradation producing NH_3-N and microbial protein synthesis using NH_3-N. Most ruminal ammonia (over 50% of the total) is produced by a group of bacteria called "hyper-ammonia-producing" (HAP) bacteria [20], which constitute only about 1% of rumen bacterial populations [26] but exhibit very high deamination activity [27]. Inhibiting HAP bacteria can reduce ruminal ammonia production and improve nutrient utilization efficiency by increasing rumen protein utilization efficiency. Since plant essential oils can selectively inhibit HAP bacteria, they can significantly suppress ammonia production [28]. Different types of essential oil components require different optimal doses to affect rumen nitrogen metabolism. Castillejos et al. [6] found that guaiacol reduced ammonia concentration at 5 mg/L, limonene and thymol at 50 mg/L, while vanillin and eugenol had no significant effect even at 500 mg/L. In this study, anethole, thymol, and carvacrol affected NH_3-N concentration, with no significant effect at low doses but significant reduction at the highest dose (400 mg/L), indicating that high-dose essential oil addition inhibited rumen microbial activity and possibly HAP bacteria activity, consistent with the observed decrease in total VFA concentration and inhibition of rumen fermentation at high doses.

3.2 Effects of Different Plant Essential Oils on In Vitro Rumen CH_4 Production

Numerous studies have evaluated various additives, including plant essential oils, for reducing CH_4 production. Some essential oils can directly inhibit methanogens or indirectly reduce CH_4 production by inhibiting microbial metabolic processes that favor CH_4 generation [23]. Cobellis et al. [29] reviewed extensive in vitro studies summarizing the effects of essential oils on CH_4 production and rumen fermentation. Macheboeuf et al. [30] reported that 5 mmol/L oregano or cinnamon oil reduced CH_4 production by 98%, while the same dose of dill oil only reduced it by 12%, indicating different effects among essential oils. Patra et al. [31] used five different essential oils (from clove, eucalyptus, garlic, oregano, and peppermint) and found that 1 g/L oregano and garlic oils reduced CH_4 production the most, demonstrating dose-dependent effects.

Research results indicate that CH_4 reduction by essential oils is often accompanied by inhibited rumen fermentation, with negative effects depending on essential oil type, dose, and substrate [29]. Lu et al. [25] reported that adding 30, 50, 100, 300, and 500 mg/L garlic oil in vitro reduced CH_4 production by 65.0-98.3% but also decreased total VFA concentration. Migul et al. [32] added 100, 150, and 250 µL/L grape seed oil in vitro, finding 10.79% and 15.30% reductions in CH_4 content at 100 and 150 µL/L, respectively, but a 7.17% increase at 250 µL/L, indicating that appropriate concentrations can reduce CH_4 production, though all concentrations significantly reduced total VFA concentration. Lin et al. [23] reported that in vitro addition of different concentrations of oregano and cinnamon oils reduced CH_4 production with increasing concentration, with 50 mg/L reducing CH_4 production by 13.3% and 21.2%, respectively, while having minimal effect on total VFA concentration, suggesting ideal rumen modulation at this concentration.

In this study, different doses of thymol and carvacrol only significantly reduced CH_4 production at the highest dose (400 mg/L), decreasing it by 84.7% and 73.9%, respectively, while also significantly reducing total VFA concentration and 24-hour gas production, indicating that CH_4 reduction was accompanied by inhibited rumen fermentation. This is consistent with previous findings that CH_4 reduction effects are dose-dependent. One mechanism of CH_4 reduction by essential oils is inhibiting rumen protozoa activity, thereby inhibiting the activity of methanogens symbiotic with protozoa [30]. Other essential oils in this study (D-limonene, anethole, and cinnamaldehyde) had no significant effect on 24-hour CH_4 production, while 50 and 100 mg/L eugenol increased CH_4 production compared with the control, possibly because eugenol at these doses promoted rumen fermentation and microbial activity. These results also demonstrate that different essential oils have different effects on CH_4 production.

Currently, few in vivo studies on essential oils exist, and results are often contradictory due to different doses and types of essential oils. It is difficult to determine the dose requirements for effective ruminal concentrations due to variations in chemical composition, diet type, and feeding methods [33]. Wang et al. [34] fed sheep an oregano essential oil mixture (0.25 g/d) for 15 days and observed reduced CH_4 production, while Tomkins et al. [35] fed beef cattle a commercial essential oil blend (1 and 2 g/d) for 40 days with no reduction in CH_4 production. Future research needs to systematically evaluate essential oil effects in vivo, including optimal doses and combinations of different essential oils for better practical application.

Since most studies indicate that essential oils inhibit rumen fermentation and feed digestibility while reducing CH_4 production, few producers are interested in using them for CH_4 mitigation. Therefore, the main challenge remains finding essential oils that can reduce CH_4 production without decreasing feed digestibility or rumen fermentation.

Different plant essential oils have varying effects on in vitro rumen fermentation and CH_4 production, which are dose-dependent. In this study, low doses of thymol and carvacrol numerically increased total VFA concentration, while high doses (400 mg/L) significantly reduced total VFA concentration, NH_3-N concentration, 24-hour gas production, and CH_4 production.

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Note: Figure translations are in progress. See original paper for figures.