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Effects of Oregano and Clove Essential Oils on *in vitro* Ruminal Fermentation and Methane Production from Alfalfa Hay and Corn Stover

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ABSTRACT

Background: Livestock production faces challenges regarding nutrient utilization efficiency and environmental sustainability. Plant-derived essential oils represent a potential strategy for modulating ruminal fermentation and methane production. This study evaluated the effects of oregano (*Origanum vulgare*) and clove (*Syzygium aromaticum*) essential oils on *in vitro* ruminal fermentation and methane production, using alfalfa hay (high-nutritional-quality forage) and corn stover (low-nutritional-quality forage).

Methods: Essential oils were evaluated at concentrations of 0, 0.05 and 0.10 mL L^{-1}. Ruminal fluid was obtained from three ruminally fistulated steers, considered independent sources of biological inoculum and each treatment was incubated in triplicate for each inoculum source. Gas production kinetics were monitored over 96 h, while methane, carbon dioxide, ammonia nitrogen ($N\text{-}NH_3$) and volatile fatty acids were determined after 24 h.

Result: The inclusion of both oils generally reduced $N\text{-}NH_3$ and altered the volatile fatty acid profile, with an overall increase in propionate ($P<0.05$). Oregano did not significantly alter methane production at 0.05 mL L^{-1} but reduced it at 0.10 mL L^{-1} by 13.7% for alfalfa and 33.3% for corn stover. Clove reduced methane production by 29.3% and 26.6% at 0.05 mL L^{-1} and by 33.1% and 35.9% at 0.10 mL L^{-1}, respectively ($P<0.05$). Some treatments altered gas production kinetics and CO_2 levels. Essential oils modulated *in vitro* fermentation and methane production under the conditions evaluated. Additional *in vivo* studies are required before extrapolating these results to animal performance or production sustainability.

Key words: Essential oils, *in vitro* gas production, *in vitro* ruminal fermentation, Methane.

INTRODUCTION

Livestock production is an important contributor to anthropogenic greenhouse gas (GHG) emissions, with carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) being the main gases associated with agricultural activities. Among these gases, CH_4 is of particular environmental concern because of its strong warming effect and relatively high global warming potential. According to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change (IPCC), non-fossil or biogenic CH_4 has a global warming potential (GWP100) of approximately 27 times that of CO_2 over a 100-year time horizon (Forster *et al.*, 2021). In addition to its environmental implications, methane production represents an energy loss ranging from 2 to 12% of gross dietary energy consumed by ruminants, thereby reducing feed efficiency and animal productivity (Johnson and Johnson, 1995; Li *et al.*, 2019). Consequently, mitigating enteric methane emissions has become a priority for improving both the environmental sustainability and economic efficiency of livestock systems.

Several nutritional strategies have been proposed to reduce ruminal methanogenesis, including dietary manipulation through the use of plant-derived bioactive compounds (Bonilla and Lemus, 2012).

Among these compounds, essential oils have attracted considerable attention due to their antimicrobial properties and their ability to modify ruminal fermentation patterns and methane production; their effects can vary depending on the type of essential oil, inclusion level and characteristics of the fermentation substrate. These effects are associated with lower concentrations of ammonia nitrogen, methane and acetate and with an increase in the

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proportion of propionate and butyrate, which may favor alternative pathways for hydrogen utilization (Lakhani *et al.*, 2023).

Essential oils contain secondary metabolites capable of inhibiting specific ruminal microorganisms involved in methane formation, thereby improving fermentation efficiency and nutrient utilization (Burt, 2004). These compounds are composed mainly of terpenes and phenolic substances that possess antibacterial, antifungal, antioxidant, anti-inflammatory and insecticidal properties (Acamovic and Brooker, 2005). Due to these biological activities, essential oils have been increasingly investigated as natural feed additives capable of enhancing ruminal fermentation, improving animal health and reducing enteric methane emissions. Previous studies have demonstrated that essential oils extracted from oregano, clove, garlic, eucalyptus and peppermint can reduce methane production under *in vitro* conditions, although the magnitude of the response varies according to the oil source and dosage (Patra and Yu, 2012).

The rumen harbors a highly complex microbial ecosystem responsible for degrading feed components and converting them into nutrients available to the host animal. Through microbial fermentation, carbohydrates are transformed into volatile fatty acids (VFAs), which constitute the major energy source for ruminants. Therefore, alterations in ruminal microbial populations can significantly affect fermentation efficiency, nutrient digestibility, animal productivity and the quality of milk and meat products (Welkie *et al.*, 2010). Ruminal microbial activity is influenced by numerous factors, including diet composition, feed intake, passage rate, retention time and ruminal pH. These factors directly affect methane production by altering the availability of hydrogen and other substrates utilized by methanogenic archaea (Cardona-Iglesias *et al.*, 2017). Diet quality also plays a crucial role in methane production. High-fiber diets generally promote greater methane production because structural carbohydrates favor acetate formation and hydrogen release during fermentation. Conversely, diets rich in non-structural carbohydrates tend to reduce methane production by increasing propionate production, which competes for hydrogen utilization (Carro *et al.*, 2018).

However, comparative information on oregano and clove essential oils using forage substrates differing in nutritional quality remains limited. This represents an important research gap because differences in forage composition, particularly in crude protein and fiber concentrations, may influence microbial fermentation and the response to essential oil supplementation.

Oregano and clove essential oils were selected based on their recognized antimicrobial properties and their potential to modify ruminal microbial activity and fermentation patterns. Oregano essential oil contains bioactive compounds such as carvacrol and thymol, whereas eugenol is the main bioactive compound associated with clove essential oil. The inclusion levels of 0.05 and 0.10 mL L^{-1} were established as two increasing concentrations for experimental evaluation, while 0 mL L^{-1} was included as the control treatment. This experimental design allowed the effects of increasing concentrations of both essential oils on ruminal fermentation and methane production to be evaluated.

The study hypothesized that the inclusion of oregano and clove essential oils would modify ruminal fermentation characteristics and methane production and that the magnitude of these responses would differ between forage substrates with contrasting nutritional quality. Alfalfa hay was considered the higher-quality forage, whereas corn stover represented the lower-quality forage, based on their differences in chemical composition. It was further hypothesized that increasing essential oil concentrations would result in dose-dependent changes in fermentation characteristics and methane production. Therefore, the study aimed to evaluate the effects of oregano (*Origanum vulgare*) and clove (*Syzygium aromaticum*) essential oils at 0.05 and 0.10 mL L^{-1} on ruminal fermentation characteristics, gas production kinetics and methane production using alfalfa hay and corn stover as forage substrates differing in nutritional quality.

MATERIALS AND METHODS

The study was conducted at the Faculty of Veterinary Medicine and Animal Husbandry of the Juarez University of the State of Durango, located in Durango, Mexico from 2024.02.01 to 2025.03.20. Alfalfa hay and corn stover were purchased from a commercial forage supplier in the same locality.

The oregano oil used in this study was a commercial, natural, FCC-grade product (product no. W282812, lot no. 1416LHV, SAFC/Sigma-Aldrich®). Commercial clove oil (product no. C8392-100ML, lot no. MKCK0591) was also purchased from Sigma-Aldrich (Sigma Life Science); both oils were stored in their original, tightly sealed amber glass containers at 15-25°C and protected from light. The two essential oils were added to glass modules of the semi-automated Ankom system at levels of 0, 0.05 and 0.10 mL/L of ruminal buffer-liquid solution.

Chemical composition of the forages used

In this study, alfalfa hay and corn stover were selected as representative forages of high and low nutritional quality, respectively and analytical measurements of the forage samples were performed independently. Dry matter (DM), ash (A), ether extract (EE), organic matter (OM) and crude protein (CP) contents were analyzed following the procedures described by the AOAC (2005). Concentrations of neutral detergent fiber (NDF), acid detergent fiber (ADF) and lignin were determined using the method of Goering and Van Soest (1970), modified for the ANKOM fiber analysis system. The chemical composition of the experimental forages is presented in Table 1.

Kinetics of *in vitro* gas production

In vitro fermentation kinetics were evaluated using the ANKOM semi-automated gas production system following the procedure of Theodorou *et al.* (1994). Samples (1 g DM) of each experimental diet were placed in 250 mL glass fermentation bottles in triplicate. Each bottle received an incubation medium consisting of 80 mL of buffer solution and 40 mL of ruminal fluid (2:1 v/v ratio); Ruminal fluid was collected from multiple sites within the rumen and quickly stored in thermoses preheated with water at 39°C for transport to the laboratory prior to the morning feeding, from three rumen-fistulated Angus approximately 4 years of age, with an average live weight of 450±30 kg, which were housed in feedlots. Before the trial began, the animals were vaccinated, dewormed and given vitamin supplements and were adapted for 14 days to a diet composed of alfalfa hay and commercial concentrate in a 70:30 ratio respectively. These procedures were approved by the Committee for the Promotion and Protection of Animals of the State of Durango. Oregano and clove essential oils were added directly to the fermentation modules at concentrations of 0, 0.05 and 0.10 mL L^{-1}; the 0 mL L^{-1} treatments contained the corresponding forage and ruminal inoculum but no essential oil. The headspace of each bottle was flushed with CO_2 to ensure anaerobic conditions before sealing with ANKOM wireless pressure modules containing electronic pressure transducers (ANKOM Technology, Macedon, NY, USA). The fermentation bottles were incubated at 39°C in a Daisy II incubator. Cumulative gas production was monitored at 0, 3, 6, 12, 24, 36, 48, 72 and 96 h of incubation and pressure data were converted to gas volume according to the manufacturer's specifications.

Methane and carbon dioxide production

Fermentation was carried out for 24 hours using the ANKOM semi-automated gas production system, following the procedure described by Theodorou *et al.* (1994). At the end of the fermentation period, the flask valves were opened to release the accumulated gas and each module was connected *via* a tube to a GEM™5000 portable gas analyzer (LANDTEC, USA). The concentrations of CH_4 and CO_2 in the released gas were determined according to the manufacturer's instructions and expressed as percentages (%). The volumes of CH_4 and CO_2 were estimated by combining the gas production measured by the ANKOM system with the respective gas concentrations determined by the GEM™5000. For each sample, the volume of CH_4 was calculated using the following equation:

for methane

$$CH_4\ (mL) = \frac{Vgas\ (mL) \times (CH_4\ (\%)}{100}$$

for carbon dioxide,

$$CO_2\ (mL) = \frac{Vgas\ (mL) \times (CO_2\ (\%)}{100}$$

Where,

CH_4 (mL) and CO_2 (mL) = Estimated volumes of methane and carbon dioxide in mL, Vgas is the total gas production recorded by the ANKOM system (mL).

CH_4 (%) and CO_2 (%) = Respective concentrations measured by the GEM™5000 (%).

The resulting gas volumes were expressed on a dry matter basis (mL/g DM) when applicable.

Table 1: Chemical compositon of the experimental forages.

	Chemical composition (% Dry matter)	
	Alfalfa hay	Corn stover
DM	92.27	90.15
OM	95.81	88.98
CP	15.23	8.36
EE	1.10	1.38
NDF	52.0	61.80
ADF	26.5	32.64
Lignin	9.89	13.40

DM= Dry Matter; OM= Organic matter; CP= Crude protein; EE= Ether extract; NDF= Neutral detergent fiber; ADF= Acid detergent fiber.

Patterns of ruminal fermentation and *in vitro* methane production

One gram of each forage sample was weighed into ANKOM glass fermentation modules in triplicate. Subsequently, 120 mL of incubation medium composed of 80 mL of buffer solution and 40 mL of ruminal fluid in a 2:1 ratio was added to each module. Ruminal fluid was collected before the morning feeding from three ruminally fistulated steers receiving a diet composed of alfalfa hay and commercial concentrate (70:30, respectively). Immediately after inoculation, pure oregano and clove essential oils, without prior dilution, were added at inclusion levels of 0.05 and 0.10 mL L^{-1} of incubation medium. The 0 mL L^{-1} treatments consisted of untreated alfalfa hay or corn stover without essential oil addition. The fermentation modules were incubated at 39°C in a Daisy II incubator for 24 h. At the end of the incubation period, the fermentation fluid was filtered through four layers of cheesecloth.

Ammonia nitrogen ($N\text{-}NH_3$) concentration was determined by the colorimetric phenol-hypochlorite method using a CARY I-E spectrophotometer (Varian, USA) at 630 nm, according to Galyean (1980). A 100 mM $N\text{-}NH_3$ stock solution was prepared from ammonium sulfate, previously dried overnight at 100°C, in 100 mL of 0.1 N HCl. Working standards of 1, 2, 4, 6 and 8 mM were prepared by diluting aliquots of the stock solution. For the analysis, 0.05 mL (50 µL) of sample or standard was transferred to a test tube and 50 µL of distilled water was used as the blank. Then, 2.5 mL of phenol reagent and 2.0 mL of hypochlorite reagent were added and mixed thoroughly. The tubes were incubated in a water bath at 95°C for 5 min and allowed to

cool before absorbance was measured at 630 nm. $N\text{-}NH_3$ concentration was calculated from the calibration curve generated using the ammonia standards.

For volatile fatty acid (VFA) analysis, a 1.0 mL aliquot of the filtrate was centrifuged at 3000 rpm for 5 min and 0.5 mL of the resulting supernatant was mixed with 0.15 mL of 25% metaphosphoric acid (Galyean, 2010). Samples were stored frozen until analysis. Volatile fatty acid concentrations were determined by gas chromatography. Calibration standards were prepared according to Galyean (2010) at three concentration levels. A calibration curve was prepared for the quantification of volatile fatty acids (VFAs) using standard solutions containing acetic, propionic, butyric and branched-chain fatty acids at different concentrations.

The calibration curve consisted of three points: point 1 contained 20 mM acetic acid, 5 mM propionic acid, 2 mM butyric acid and 0.5 mM branched-chain fatty acids; point 2 contained 60, 20, 12 and 2.5 mM, respectively; and point 3 contained 120, 50, 30 and 6 mM, respectively. 2-Ethylbutyric acid was used as the internal standard for VFA quantification. For chromatographic analysis, 1 µL of each calibration solution and prepared sample was injected. VFA identification and quantification were performed using the ratio between the peak area of each analyte and that of the internal standard, based on the calibration curves obtained for each acid. Chromatographic separation was performed using an Agilent 6890 gas chromatograph equipped with an Agilent 7693 autosampler and coupled to a mass spectrometer. Separation of the main volatile fatty acids (VFAs) was carried out using an FFAP capillary column (30 m × 250 µm × 0.25 µm). High-purity helium was used as the carrier gas at a constant flow rate of 1 mL/min. The injector temperature was maintained at 180°C in split mode (70:1). Initially, the oven temperature was maintained at 40°C for 3 min; then, it was increased at a rate of 3°C/min to 52°C, which was held for 1 min; subsequently, it was increased at a rate of 10°C/min to 200°C, which was held for 15 min. The mass spectrometer operated at 230°C, with an ionization energy of 70 eV and an acquisition speed of 1.6 scans/s in SCAN mode. Identification of volatile fatty acids (VFAs) was based on comparison of the mass spectra with the NIST 2011 library and confirmation using the retention times of reference standards. Quantification was performed using the ratio of the peak area of each VFA to that of the internal standard (2-ethylbutyric acid), as well as the corresponding calibration curves. The concentrations of the quantified VFAs were converted to mmol/L and subsequently expressed as molar ratios (mol/100 mol) with respect to the total quantified VFAs.

Statistical analysis and fitting of *in vitro* gas production kinetics

The cumulative gas production kinetics were fitted to the model proposed by Gompertz:

$$GP = A\exp\{-\exp[-kd(t-Lag)]\}$$

Where:

GP = Cumulative gas production at time.

t (mL g^{-1} DM); A = Maximum gas production (mL g^{-1} DM); Lag = Latency phase (h).

e = Base of the natural logarithm (2.7182).

kd = Constant gas production rate (h^{-1}).

Data were analyzed using a 2 × 2 × 3 factorial arrangement in a completely randomized design, with forage type (alfalfa hay and corn stover), oil type (oregano and clove) and oil dose (0, 0.05 and 0.10 mL L^{-1}) as fixed effects. The statistical model included the main effects of forage, oil and dose and all their two- and three-way interactions. The residuals were evaluated for normality using the Shapiro-Wilk test and for homogeneity of variances using Levene's test. When significant effects or interactions were detected ($P \leq 0.05$), means were separated using Tukey's multiple-comparison test. Results are presented as least-squares means and the standard error of the mean (SEM) (SAS, 2002).

RESULTS AND DISCUSSION

Ruminal fermentation patterns

Table 2 shows that the response in ammoniacal nitrogen ($N\text{-}NH_3$) concentration depended on the specific combination of forage type, essential oil source and inclusion level, as indicated by the significant forage × oil × dose interaction ($P<0.01$). In the case of alfalfa hay supplemented with oregano oil, $N\text{-}NH_3$ decreased progressively from 9.44 to 8.58 and 7.99 mg dL^{-1} as the inclusion level increased from 0 to 0.05 and 0.10 mL L^{-1}, respectively. A similar dose-dependent decrease was observed with oregano oil in corn stover, although the reduction was more pronounced (9.05, 7.62 and 3.59 mg dL^{-1}). Clove oil also reduced $N\text{-}NH_3$ in alfalfa hay (9.23, 7.90 and 6.17 mg dL^{-1}), while in corn stover the greatest decrease occurred between 0 and 0.05 mL L^{-1} (from 8.99 to 3.85 mg dL^{-1}), with no further significant reduction observed at 0.10 mL L^{-1} (3.68 mg dL^{-1}). The observed reductions in $N\text{-}NH_3$ concentration may reflect a decrease in ruminal protein deamination and ammonia production, resulting from the antimicrobial activity of the essential oils. Rather than indicating a consistent response across all treatments, the significant three-way interaction suggests that the efficacy of each oil depended on the characteristics of the forage substrate and the inclusion level. Oregano essential oil is characterized by high concentrations of carvacrol and thymol, while eugenol is the main bioactive compound in clove essential oil. These phenolic compounds have been reported to inhibit proteolytic and ammonia-hyperproducing bacteria, thereby reducing ammonia formation during ruminal fermentation (Patra and Yu, 2012; Cobellis *et al.*, 2016). The lower $N\text{-}NH_3$ values observed in corn stover supplemented with clove oil (3.68-3.85 mg dL^{-1}) indicate that ammonia availability varied among the treatment combinations. Consequently, general conclusions regarding

the sufficiency of ammonia for microbial protein synthesis should be avoided, since the present study did not directly measure such synthesis and ammonia concentrations varied considerably among treatments. Instead, the results indicate that supplementation with essential oils altered nitrogen metabolism during fermentation, with the magnitude of the response depending on the combination of forage and oil. The ammonia concentrations recorded in the present study were lower than the values reported (15.27, 14.32 and 9.07 mg dL^{-1}) by Wang *et al.* (2025), who used an essential oil-based product with thymol and carvacrol as active ingredients. The antimicrobial activity of carvacrol, thymol and eugenol is associated with their ability to interact with bacterial cell membranes, increasing membrane permeability and causing disruption of cellular integrity, ultimately leading to cell lysis (Rodríguez-García *et al.*, 2016). Consequently, these compounds can modify ruminal microbial populations and alter fermentation patterns, including nitrogen metabolism.

Essential oils can reduce ruminal ammonia production by inhibiting hyper-ammonia-producing bacteria and decreasing amino acid deamination (Wallace, 2004; Patra and Saxena, 2009). In the present study, increasing doses of oregano and clove essential oils modified the volatile fatty acid (VFA) profile during *in vitro* fermentation. Acetic acid concentrations decreased as the dose of oregano oil increased in alfalfa hay and as the dose of clove oil increased in corn stover. At the 0.05 mL L^{-1} inclusion level, acetate concentrations were lower in treatments containing oregano oil than in those supplemented with clove oil, regardless of forage source.

In contrast, propionate concentration generally increased with increasing doses of both essential oils ($P<0.05$). The greatest relative increases occurred with oregano oil in alfalfa hay (13.6 to 16.6 mol/100 mol; +22%) and, to a lesser extent, with clove oil in corn stover (15.8 to 17.2 mol/100 mol; +9%), whereas oregano in corn stover (+7%) and clove in alfalfa hay (+10%) showed smaller increases at the 0.10 mL L^{-1} level. Because acetate concentration decreased concurrently in most treatment combinations (Table 2), these changes indicate a shift toward a more propionate-oriented fermentation pattern, consistent with increased hydrogen incorporation into propionate synthesis and a corresponding reduction in hydrogen availability for methanogenesis. The acetate: propionate ratio was not analyzed as a separate statistical outcome in this study but would be a useful metric to include in future work to quantify this shift more directly.

The reduction of acetate concentration to essential oil supplementation varied according to forage type, oil source and inclusion level. Oregano oil decreased acetate concentration in alfalfa hay, whereas the response in corn stover was less consistent; clove oil also produced forage-dependent responses. These changes may be related to the antimicrobial activity of phenolic compounds such as carvacrol and thymol in oregano oil and eugenol in clove oil, which can modify ruminal microbial activity and VFA production. In the present study, the inclusion of oregano oil in alfalfa hay significantly reduced the proportion of acetate from 75.7 mol 100 mol^{-1} to 66.7 mol 100 mol^{-1}; these values are similar to those reported by Budiman *et al.* (2024) using a matrix composed of peanut oil and whey protein isolate in sheep. The concurrent changes in acetate and propionate suggest a shift in fermentation patterns; however, these results should not be interpreted

Table 2: Least-squares means of the oil × forage × dose interaction of *in vitro* fermentation parameters.

			Doses (mL/L)				
	Oil	Forage	0	0.05	0.10	SEM	P<
$N\text{-}NH_3$ (mg dL^{-1})	Oregano	AH	9.44^{a}	8.58^{b}	7.99^{c}	0.214	0.005
		CS	9.05^{a}	7.62^{b}	3.59^{c}	0.214	0.005
	Clove	AH	9.23^{a}	7.90^{b}	6.17^{c}	0.214	0.005
		CS	8.99^{a}	3.85^{b}	3.68^{b}	0.214	0.005
Acetate, VFA mol/100 mol	Oregano	AH	75.7^{a}	68.6^{b}	66.7^{c}	0.427	0.005
		CS	69.9^{a}	67.6^{b}	68.9^{a}	0.427	0.005
	Clove	AH	73.9^{a}	76.3^{b}	73.2^{a}	0.427	0.005
		CS	71.1^{a}	70.1^{a}	69.2^{a}	0.427	0.005
Propionate mol/100 mol	Oregano	AH	13.6c	15.8^{b}	16.6^{a}	0.163	0.005
		CS	16.5^{a}	17.0^{b}	17.6^{b}	0.163	0.005
	Clove	AH	13.7^{a}	14.7^{b}	15.1^{b}	0.163	0.005
		CS	15.8^{c}	16.6^{b}	17.2^{a}	0.163	0.005
Butyrate mol/100 mol	Oregano	AH	5.1^{c}	7.0^{b}	9.0^{a}	0.132	0.001
		CS	6.3^{b}	6.6^{a}	6.4^{ab}	0.132	0.001
	Clove	AH	5.3^{b}	6.1^{a}	5.7^{ab}	0.132	0.001
		CS	6.0^{b}	6.5^{a}	6.8^{a}	0.132	0.001

^{abc}Values in rows with different letters indicate significant differences ($P<0.05$). AH=Alfalfa hay; CS=Corn stover; $N\text{-}NH_3$=Ammoniacal nitrogen. SEM=Standard error of the mean.

as direct evidence of improved substrate utilization or fermentation efficiency.

These findings are consistent with those reported by Busquet *et al.* (2005b), who observed alterations in ruminal fermentation following supplementation with essential oils under *in vitro* conditions. Likewise, Busquet *et al.* (2005a) reported that low doses of clove extract (2.2 mg L^{-1}) reduced the acetate-to-propionate ratio by decreasing acetate production and increasing propionate concentration. Similar responses were reported by Castillejos *et al.* (2008), who observed decreases in acetate and butyrate concentrations accompanied by a substantial increase in propionate concentration when clove oil was added to ruminal fluid cultures.

These changes suggest that essential oils modify ruminal fermentation kinetics and gas production patterns, although the magnitude and direction of these effects depend on the type of oil, inclusion level and forage source (Ungerfeld, 2015). These findings highlight the importance of determining optimal supplementation levels to maximize beneficial effects on ruminal fermentation while minimizing potential negative impacts on digestibility and microbial activity.

However, not all studies have reported consistent effects of essential oils on ruminal fermentation. Hristov *et al.* (2013) found that supplementation with oregano leaves in cattle diets did not significantly alter total VFA concentrations under *in vivo* conditions. Such discrepancies may be attributed to differences in essential oil composition, inclusion level, forage type, adaptation period and experimental conditions. Overall, the present results indicate that oregano and clove essential oils can modulate ruminal fermentation by altering VFA production patterns, favoring propionate formation while reducing acetate and ammonia concentrations.

In vitro gas and methane production parameters

Methane (CH_4) production showed differences in the observed results depending on the essential oil and the dose evaluated (Table 3). For oregano, the 0.05 mL/L dose did not significantly alter CH_4 production compared to the alfalfa treatment with no essential oil (15.9 and 15.3 mL g^{-1} DM) or the corn stover treatment (12.8 and 12.6 mL g^{-1} DM; P<0.005). A significant reduction was observed only at 0.10 mL/L, reaching 13.7% in alfalfa and 33.3% in corn stover compared to the

Table 3: Least-squares means of the oil × forage × dose interaction on *in vitro* gas, methane and carbon dioxide production parameters in two types of forage.

			Dose (mL/L)				
	Oil	Forage	0	0.05	0.10	SEM	P<
A (mL g^{-1} DM)	Oregano	AH	122.0^{a}	134.8^{b}	120.6^{c}	0.43	0.005
		CS	124.1^{b}	122.1^{c}	128.0^{a}	0.43	0.005
	Clove	AH	125.9^{c}	134.3^{b}	142.0^{a}	0.43	0.005
		CS	140.9^{a}	106.0^{c}	104.0^{b}	0.43	0.005
Kd (h^{-1})	Oregano	AH	2.1^{b}	2.8^{a}	2.0^{c}	0.10	0.001
		CS	1.5^{c}	2.0^{b}	2.2^{a}	0.10	0.001
	Clove	AH	3.2^{c}	3.5^{b}	3.7^{a}	0.10	0.001
		CS	1.8^{b}	1.5^{c}	1.6^{a}	0.10	0.001
Lag (h)	Oregano	AH	0.10^{c}	0.15^{b}	0.20^{a}	0.059	0.005
		CS	0.25^{b}	0.85^{a}	0.30^{b}	0.059	0.005
	Clove	AH	0.12^{b}	0.10^{c}	0.15^{a}	0.059	0.005
		CS	0.30^{c}	0.75^{b}	1.15^{a}	0.059	0.005
CH_4 (mL g^{-1} DM)	Oregano	AH	15.3^{a}	15.9^{a}	13.2^{b}	0.29	0.005
		CS	12.6^{a}	12.8^{a}	8.4^{b}	0.29	0.005
	Clove	AH	15.7^{a}	11.1^{b}	10.5^{c}	0.29	0.005
		CS	12.8^{a}	9.4^{b}	8.2^{c}	0.29	0.005
CO_2 (mL g^{-1} DM)	Oregano	AH	71.9^{b}	76.4^{a}	69.5^{b}	0.72	0.005
		CS	60.6^{c}	65.6^{a}	62.6^{b}	0.72	0.005
	Clove	AH	72.1^{b}	87.7^{a}	69.6^{c}	0.72	0.005
		CS	61.1^{b}	67.2^{a}	55.3^{c}	0.72	0.005
CO_2:CH_4	Oregano	AH	4.6^{a}	4.8^{ab}	5.1^{a}	0.07	0.001
		CS	4.7^{b}	5.1^{b}	7.3^{a}	0.07	0.001
	Clove	AH	4.5^{c}	8.0^{a}	6.5^{b}	0.07	0.001
		CS	4.7^{c}	7.1^{a}	6.6^{b}	0.07	0.001

^{abc} Values in rows with different letters indicate significant differences (P<0.05). AH=Alfalfa hay; CS=Corn stover. SEM=Standard error of the mean. A=Maximum gas production; Kd=Constant gas production rate; Lag=Pre-fermentation phase; CH_4=Methane; CO_2= Carbon dioxide, CO_2:CH_4 = CO_2 production divided by CH_4 production.

treatment with no oil. Clove oil, on the other hand, significantly reduced CH_4 production starting at a dose of 0.05 mL/L, with decreases of 29.3% and 26.6% in alfalfa and corn stover, respectively, while at 0.10 mL/L the reductions were 33.1% and 35.9% ($P<0.005$). Taken together, these results suggest a dose-dependent response for both oils, although clove oil exhibited a more pronounced inhibitory effect even at the low dose, whereas oregano oil required the high dose to significantly reduce methanogenesis. Essential oil supplementation generally increased the CO_2:CH_4 ratio, although the magnitude of the response depended on forage, oil and dose.

Lara *et al.* (2009) suggested that substrates rich in readily fermentable carbohydrates stimulate microbial growth and activity because of their higher concentration of fermentable organic matter. Enhanced microbial activity generally results in greater fermentation intensity and gas production. Therefore, the differences observed among forage sources and essential oil treatments may be attributed to variations in substrate availability, microbial adaptation and the selective antimicrobial effects of the bioactive compounds present in oregano and clove oils.

Overall, the results indicate that essential oils modify ruminal fermentation kinetics and gas production patterns, although the magnitude and direction of these effects depend on the type of oil, inclusion level and forage source. These findings highlight the importance of determining optimal supplementation levels to maximize beneficial effects on ruminal fermentation while minimizing potential adverse effects on ruminal fermentation.

Several studies have demonstrated the potential of essential oils to reduce enteric methane production during ruminal fermentation. In a comprehensive review, Patra and Yu (2012) reported methane reductions of 34.5%, 17.7%, 42.3%, 87.7% and 25.7% for clove, eucalyptus, garlic, oregano and peppermint essential oils, respectively, although responses varied considerably depending on oil composition, dose, substrate and experimental conditions. These reductions are frequently associated with changes in ruminal fermentation patterns, particularly decreases in acetate production and increases in propionate formation, which alter hydrogen utilization and reduce substrate availability for methanogenic archaea.

The asymptotic gas production (A) decreased as the inclusion level of oregano oil increased in alfalfa hay, whereas the highest A value was observed in corn stover supplemented with 0.10 mL L^{-1} oregano oil. In contrast, increasing doses of clove oil in alfalfa hay resulted in greater asymptotic gas production. These differences suggest that the effects of essential oils on fermentation kinetics are influenced by forage composition and the susceptibility of ruminal microorganisms to specific bioactive compounds.

The fractional rate of gas production (Kd) was generally greater in treatments supplemented with clove oil and alfalfa hay than in those containing oregano oil, indicating a faster fermentation process. Likewise, the lag phase was extended in corn stover supplemented with the highest dose of clove oil, suggesting a delay in microbial colonization and substrate degradation. Such effects may be associated with the antimicrobial activity of eugenol, which can temporarily suppress microbial populations before adaptation occurs.

Changes in gas production kinetics may be explained by the effects of essential oils on ruminal microbial activity. Essential oils can selectively inhibit microbial groups involved in carbohydrate fermentation, thereby altering substrate degradation and gas production patterns.

The results obtained in the present study agree with previous reports indicating that oregano essential oil can modify ruminal fermentation and reduce methane production. Wang *et al.* (2009) observed reductions in methane production following supplementation with a commercial oregano oil preparation, while Tekippe *et al.* (2011) and Jahani-Azizabadi *et al.* (2014) reported methane reductions exceeding 30% when oregano-derived compounds were included in experimental diets. Likewise, fennel and clove extracts have shown antimethanogenic effects under *in vitro* conditions (Patra *et al.*, 2010). Similar responses have been reported for garlic-derived compounds, with methane reductions ranging from 30% to 73.6% depending on the inclusion level and experimental conditions (Patra and Yu, 2012; Zafarian and Manafi, 2013).

Despite these promising results, responses to essential oil supplementation remain inconsistent. Beauchemin *et al.* (2009) reported that supplementation of beef cattle with a commercial blend of thymol, eugenol, vanillin and limonene at 1 g $animal^{-1}$ day^{-1} for 25 days did not affect methane production. Similarly, Hristov *et al.* (2013) noted that the efficacy of essential oils depends largely on the dose administered, the adaptation capacity of ruminal microorganisms and the chemical composition of the basal diet. These discrepancies highlight the complexity of ruminal microbial ecosystems and the need to evaluate essential oils under both *in vitro* and *in vivo* conditions.

According to Busquet *et al.* (2005b), the biological activity of essential oils depends not only on the concentration of active compounds but also on their chemical structure and interactions with ruminal microorganisms. Compounds such as thymol, carvacrol and eugenol can reduce populations of protozoa, bacteria and fungi, thereby modifying fermentation pathways and methane production. Evans and Martin (2000) reported that thymol consistently inhibited methane production *in vitro*; however, reductions in methane were accompanied by decreases in acetate and propionate concentrations, indicating that excessive inhibition of microbial activity may negatively affect fermentation efficiency.

Eugenol, the major bioactive compound in clove oil, possesses broad-spectrum antimicrobial activity against both Gram-positive and Gram-negative bacteria and has been associated with favorable modifications in ruminal fermentation (Calsamiglia *et al.*, 2007). Likewise, carvacrol

and thymol, the principal constituents of oregano oil, can alter microbial populations and redirect metabolic hydrogen toward alternative pathways such as propionate synthesis. Because propionate formation acts as a competitive hydrogen sink, an increase in propionate production reduces the amount of hydrogen available for methanogenesis (Ungerfeld, 2015).

The reduction in methane production observed in the present study may be associated with changes in ruminal fermentation pathways and, potentially, with an inhibitory effect of the essential oils on methanogenic microorganisms. However, direct inhibition of methanogenic archaea was not assessed in this study, as archaeal abundance, activity and microbial-community composition were not measured. Previous studies have suggested that bioactive compounds in essential oils may affect methanogenesis by altering microbial interactions and the availability of substrates such as H_2 and CO_2 (Murillo-Ortiz *et al.*, 2016). Therefore, the observed reduction in CH_4 production, together with the relatively stable CO_2 production, may be consistent with a potential modulation of methanogenic activity rather than providing direct evidence of archaeal inhibition.

The CO_2:CH_4 ratio observed in this study may provide a complementary indication of changes in fermentation gas partitioning following essential oil supplementation. Van Lier (2008) indicated that cellulolytic fermentation is generally associated with acetate production and greater hydrogen availability for methanogenesis, whereas propionate-oriented fermentation may redirect reducing equivalents away from methane formation. In the present study, the observed changes in VFA profiles and CH_4 production may therefore be consistent with a shift in fermentation pathways that reduced methane formation. However, the CO_2:CH_4 ratio should be interpreted cautiously because changes in the ratio may result from changes in either or both gas yields. Moreover, hydrogen metabolism and methanogen activity were not directly measured; therefore, these mechanisms should be considered potential explanations rather than demonstrated effects of the essential oils.

Although the available evidence indicates that essential oils possess considerable potential as natural methane-mitigating additives, most studies have been conducted under *in vitro* conditions. Consequently, further long-term in vivo research is required to determine optimal inclusion levels, evaluate animal responses and confirm the persistence of antimethanogenic effects under practical production conditions.

CONCLUSION

The results of this *in vitro* study indicate that oregano (*Origanum vulgare*) and clove (*Syzygium aromaticum*) essential oils modified the ruminal fermentation profile of alfalfa hay and corn stover. Increasing doses of both essential oils reduced ammonia nitrogen ($N\text{-}NH_3$) concentrations, which may indicate a reduction in protein deamination; however, nitrogen utilization efficiency was not directly assessed in this study. The effects on methane production differed between essential oils. Oregano essential oil reduced methane production significantly only at the highest inclusion level (0.10 mL L^{-1}), whereas the lower dose (0.05 mL L^{-1}) did not differ from the respective control in either forage. In contrast, clove essential oil reduced methane production at both 0.05 and 0.10 mL L^{-1} in both alfalfa hay and corn stover. These responses, together with the changes observed in the volatile fatty acid profile, indicate that the effects of the essential oils on ruminal fermentation and methane production were dependent on oil type and inclusion level.

Under the conditions of this *in vitro* screening system, oregano and clove essential oils show potential for modulating ruminal fermentation and reducing methane production. However, these findings should not be interpreted as evidence of improved animal performance or production-system sustainability. Further replicated *in vivo* studies are needed to evaluate effects on feed intake, nutrient digestibility, ruminal fermentation, methane production, animal performance, dose safety and palatability, as well as to characterize the chemical composition of the essential oils and relate their bioactive compounds to the observed responses.

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Disclaimer

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Ethical approval

All experimental procedures were approved by the Committee for the Foment and Animal Protection of Durango State, dated March 9, 2024, in Durango, Durango, Mexico.

Conflict of interest

The authors declare no conflicts of interest regarding this article's publication. No funding influenced the study design, data collection, analysis, publication decision, or manuscript preparation.

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