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Effects of level and type of essential oils on rumen methanogenesis and fermentation: A meta-analysis of *in vitro* experiments

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ABSTRACT. The aim of this study was to evaluate the effects of the level and type of essential oils (EOs) derived from oregano (ORO), thyme (THO), clove (CLO), and cinnamon (CIO), when used as feed additives, on *in vitro* rumen fermentation and methanogenesis. This was achieved by conducting a meta-analysis that integrated data from relevant studies. A total of 17 articles were included comprising 154 data points. The collected data were subsequently analysed using a mixed model methodology implemented in a SAS software version 9.4. The findings showed that the level of EOs exerted a significant ($P < 0.01$) linear effect, resulting in increased pH, decreased volume of total gas, CH_4, and NH_3, reduced total volatile fatty acids (VFA) levels, dry matter digestibility (DMD), organic matter digestibility (OMD), and bacterial population. A quadratic effect was also observed with respect to reduced ($P < 0.01$) CO_2 and propionate (C_3) levels. Additionally, the protozoan population demonstrated a linear decrease ($P < 0.05$), while butyrate (C_4) concentration exhibited a quadratic increase ($P < 0.05$). However, increasing levels of EOs did not affect the acetate(C_2)- and methanogen-producing bacterial populations. With respect to different EO types, ORO and THO significantly reduced ($P < 0.05$) total gas (30–41%) and CH_4 production (38–39%) compared to the control. ORO and CLO in turn significantly decreased ($P < 0.05$) C_3 generation (5–7%), while ORO increased C_4 levels (14%) compared to the control. Moreover, CLO, CIO, and THY administration led to a decrease in OMD (8–23%). Due to the limited dataset on microbial population, the diverse types of EOs showed no significant impact on bacterial, protozoan, or methanogen populations. In conclusion, while specific doses of EOs can suppress ruminal methane emissions, they can also inhibit rumen fermentation processes. Among the EOs examined, ORO demonstrated the most potent antimicrobial ability against methanogenesis.

Introduction

Application of feed additives is one of the measures utilised to enhance ruminant productivity (Hundal et al., 2019). These additives, integral to animal nutrition, serve to optimize nutrient utilisation by refining feed quality (McDonald et al., 2022). Recent focus has shifted towards investigating natural feed additives sourced from plant extracts as viable alternatives. These additives, derived from plant secondary metabolites, are able to modulate rumen microbial activity and fermentation dynamics (Şahan et al., 2021). Among such additives, essential oils (EOs) are plant secondary metabolites, which contain bioactive substances with antimicrobial properties. Additionally, EOs can modify rumen fermentation products, control pathogenic microbes, and coat feed (Daning et al., 2020).

Research on the utilisation of EOs as feed additives, particularly in mitigating ruminal methane gas (CH_4) emission, has been extensively carried out. Ruminal CH_4 production constitute approx. 30% of global methane emissions (Embaby et al., 2019), contributing significantly to greenhouse gas emissions, and thus global warming (Laabori et al., 2017). In addition, ruminal CH_4 production is responsible for a substantial loss of dietary energy (2–12%) (Johnson and Johnson, 1995). These factors underscore the importance of mitigating enteric methane gas production. One of the effective methods to reduce gastrointestinal methane generation is through the use of EOs (Embaby et al., 2019).

Some sources of EOs, such as clove oil, cinnamon oil, oregano oil, and thyme oil, have been recurrently examined for their efficacy in mitigating CH_4 and improving *in vitro* rumen fermentation. Previous *in vitro* studies reported that certain levels and types of EOs could favourably modify rumen microbial fermentation and CH_4 production (Embaby et al., 2019). However, contradictory findings have been documented in other studies, indicating negative impacts of EOs on rumen fermentation parameters (Benchaar et al., 2007; Şahan et al., 2021). These discrepancies may be reconciled through further analysis using approaches like meta-analysis, which integrates data from many related studies to derive aggregated conclusions (St-Pierre, 2001). The objective of the present work was therefore to evaluate the effects of the levels and types of EOs on rumen methanogenesis and fermentation by integrating data from various related *in vitro* studies using meta-analysis.

Material and methods

Literature search and database development

The search included the keywords 'essential oils' and 'ruminant' in the Scopus, Google Scholar, ScienceDirect, and ResearchGate databases. Initially, a total of 381 articles were obtained spanning the publication dates from 1996 to 2022. These works were screened based on several inclusion criteria: (a) publication in English, (b) experimentation involving ruminants (cattle, beef cattle, buffalo, sheep, and goats), (c) experiments utilising *in vitro* rumen fermentation system, (b) application of EOs in the form of extracts (mg/kg), (c) documentation of EO types and dietary levels, and (d) reporting rumen fermentation, digestibility, and CH_4 emission parameters. Ultimately, 18 *in vitro* studies yielding 154 data observations were included in the final selection (Table 1).

The extracted data were organized and entered into an Excel spreadsheet (Microsoft Co., Redmon, WA, USA), and general information was summarised in a database. The types of EOs included oregano oil (ORO), thyme oil (THO), clove oil (CLO), and cinnamon oil (CIO). The studies supplemented different levels of EOs, ranging from 0 to 2 000 (mg/kg). Various *in vitro* techniques were employed, such as

Table 1. Summary of articles used for meta-analysis of the effects of levels and types of essential oils (EOs) in ruminants *in vitro*

No.	Reference	*In vitro* method	Type of livestock	Concentrate: forage ratio	Type of EOs	Level of Eos, mg/kg
1	Benchaar et al. (2007)	BC	Dairy cow	50:50	ORO, CLO, CIO, THO	0–400
2	Castillejos et al. (2008)	BC	Dairy cow	10:90	ORO, CLO, THO	0–500
3	Chaves et al. (2012)	HGT	Dairy cow	0:100	ORO, CIO	0–120
4	Patra and Yu (2012)	BC	Dairy cow	60:40	ORO, CLO	0–1000
5	Gunal et al. (2013)	BC	Dairy cow	30:70	CLO, THO	0–500
6	Gunal et al. (2014)	BC	Dairy cow	30:70	CIO	0–500
7	Pawar et al. (2014)	HGT	Buffalo	50:50	CLO, CIO	0–883
8	Roy et al. (2014)	HGT	Buffalo	50:50	ORO, CIO	0–600
9	Cobellis et al. (2015)	HGT	Sheep	60:40	ORO	0–2000
10	Nanon et al. (2015)	BC	Beef cattle	50:50	CLO, CIO	0–200
11	Nanon et al. (2015)	BC	Beef cattle	50:50	CLO, CIO	0–1600
12	Pinski et al. (2015)	BC	Dairy cow	40:60	CIO	0–500
13	Roy et al. (2015)	HGT	Buffalo	50:50	CLO, THO	0–600
14	Cobellis et al. (2016)	HGT	Dairy cow	30:70	ORO, CIO	0–1125
15	Gunal et al. (2017)	BC	Dairy cow	40:60	CLO, THO	0–500
16	Hundal et al. (2019)	HGT	Sheep	50:50	CIO	0–200
17	Embaby et al. (2019)	HGT	Dairy cow	40:60	ORO	0–500
18	Fandino et al. (2020)	BC	Beef cattle	90:10	ORO, CLO, THO	0–400

CLO – clove oil, CIO – cinnamon oil, ORO – oregano oil, THO – thyme oil, BC – batch culture, HGT – Hohenheim gas test incubation

the Hohenheim gas test incubation (HGT; Menke and Steingass, 1988) and batch culture incubation (BC; Tilley and Terry, 1963). CH_4 concentration in this *in vitro* meta-analysis study was determined using gas chromatography (GC). To ensure data integrity, each quantitative data point was standardised to the same units of measurement. An outlier test was performed using z-scores to identify and address any anomalous data observations.

Statistical analysis

The present meta-analysis utilised a mixed model methodology (St-Pierre, 2001; Sauvant et al., 2008). Statistical analysis was conducted using the PROC MIXED procedure implemented in the SAS 9.4 software (SAS Institute, 2014). Individual studies included in the analysis were treated as random effects, while the level and type of EOs were considered as fixed effects. Two statistical models were applied: the continuous predictor variable consisted of EO levels, and the mathematical model was as follows (Equation 1):

$$Y_{ij} = B_0 + B_1 X_{ij} + B_2 X^2_{ij} + s_i + b_i X_{ij} + e_{ij} \quad (1),$$

where: Y_{ij} – dependent variable; B_0 – overall intercept across all studies (fixed effect); B_1 – linear regression coefficient of Y on X (fixed effect); B_2 – quadratic regression coefficient of Y on X (fixed effect); X_{ij} – value of the continuous predictor variable (level of EOs); s_i – value of the random effect of study i; b_1 – random effect of study on the regression coefficient of Y on X in study i; and e_{ij} – unexplained residual error. The number of replicates in the studies was used to weight these models, following the approach by Jayanegara et al. (2014). The significance of the model was determined at a threshold of $P < 0.05$. The linear model was applied in cases where the mixed model analysis was not significant in the quadratic model.

The mathematical model used to analyse the effect of EO types on parameters with a discrete predictor variable was as follows (Equation 2):

$$Y_{ij} = \mu + s_i + \tau_j + s\tau_{ij} + e_{ij} \quad (2),$$

where: Y_{ij} – dependent variable; μ – overall mean; s_i – random effect of the ith study; τ_j – fixed effect of the jth levels of the factor τ; $s\tau_{ij}$ – random interaction between the ith study and the jth level of the factor τ, and e_{ij} – unexplained residual error. This model structure has been described by Jayanegara et al. (2014). When a variable showed a significant difference at $P < 0.05$, least square means and Tukey's post-hoc test were used to compare differences between means.

Results

Effects of EO levels on rumen methanogenesis and fermentation

The levels of EOs linearly reduced total gas and CH_4 production ($P < 0.01$), while also quadratically reducing CO_2 production ($P < 0.05$; Table 2). In addition, elevated EO doses resulted in a linear increase in pH ($P < 0.01$), accompanied by decreases in NH_3, total volatile fatty acids (VFA), and OMD contents, as well as reduced total bacterial ($P < 0.01$), and protozoan populations ($P < 0.05$). Moreover, the higher EO levels quadratically decreased C_3 ($P < 0.01$), while simultaneously increasing C_4 production ($P < 0.01$). No significant effects of EO administration were observed regarding dry matter digestibility (DMD), neutral detergent fibre digestibility (NDFD), and the abundance of methanogen bacteria.

Table 2. Effect of essential oil supplementation levels on rumen methanogenesis and fermentation *in vitro*

Response variables	Unit	n	M	INT	SE INT	Slope	SE Slope	*P*-value
Gas production								
total gas	ml	112	L	116	14.5	-0.03	0.01	<0.01
CH_4	ml	105	L	19.6	1.87	-0.01	0.001	<0.01
CO_2	ml	19	Q	97.1	19.1	-0.07	0.02	<0.01
						0.02	0.09	0.02
Rumen fermentation								
pH		68	L	5.94	0.13	0.002	0.008	<0.01
NH_3	mM	142	L	3.69	1.06	-0.001	0.002	<0.01
Total VFA	mM	149	L	80.5	7.24	-0.015	0.002	<0.01
C_2	%	142	L	55.4	2.19	-0.004	0.006	0.94
C_3	%	141	Q	21.7	0.81	-0.003	0.001	<0.01
						0.001	0.00	<0.01
C_4	%	108	Q	14.3	1.06	0.002	0.009	0.02
						-0.002	0.00	<0.01
C_2/C_3	%	147	Q	2.65	0.19	0.001	0.003	<0.01
						-0.003	0.001	<0.01
In vitro digestibility								
DMD	%	79	L	63.2	2.19	-0.006	0.002	<0.01
OMD	%	35	L	66.1	3.41	-0.02	0.002	<0.01
NDFD	%	64	L	41.5	6.15	-0.006	0.002	0.50
Microorganism								
bacteria	log_{10}	10	L	11.0	0.59	-0.001	0.003	<0.01
protozoa	log_{10}	7	L	8.34	0.55	-0.003	0.009	0.03
methanogen	log_{10}	10	L	7.14	0.59	-0.005	0.007	0.50

n – number of observations, M – models, INT – intercept, SE – standard error, L – linear, Q – quadratic, CH_4 – methane, CO_2 – carbon dioxide, NH_3 – ammonia, total VFA – total volatile fatty acids, C_2 – acetate, C_3 – propionate, C_4 – butyrate, DMD – dry matter digestibility, OMD – organic matter digestibility, NDFD – neutral detergent fibre digestibility

Effects of EO types on rumen methanogenesis and fermentation

ORO and THO significantly decreased ($P < 0.05$) total gas production (30–41%) and

CH_4 emissions (38–39%) compared to the control (Table 3). However, none of the EOs significantly affected CO_2 levels. In contrast, ORO and CLO significantly decreased ($P < 0.05$) C_3 concentration (5–7%), while ORO caused an increase in C_4 levels (14%) compared to the control. Additionally, CLO, CIO, and THY oils decreased OMD (8–23%). There were no significant differences observed between individual EO types in terms of pH, NH_3, and C_2 production, as well as the C_2/C_3 ratio. Due to the limited data available concerning microbial populations, no significant effects of EO types on bacterial, protozoan, or methanogen populations were recorded.

Table 3. Effects of types of essential oils on rumen methanogenesis and fermentation *in vitro*

Response variables	Control	CLO	CIO	ORO	THO	SEM	*P*-value
Gas production, ml							
total gas	114 ± 52.1^{a}	110 ± 37.5^{ab}	112 ± 45.7^{ab}	67.6 ± 62.9^{c}	80.0 ± 60.3^{bc}	4.69	0.04
CH_4	20.4 ± 8.97^{a}	21.5 ± 6.59^{a}	20.9 ± 7.53^{a}	12.4 ± 8.48^{b}	12.6 ± 7.16^{b}	0.79	0.03
CO_2	100 ± 44.8	98.4 ± 9.66	107 ± 8.12	23.6 ± 28.1	90.7 ± 30.4	10.1	0.17
Rumen fermentation							
pH	5.93 ± 0.44	5.95 ± 0.45	6.09 ± 0.26	5.78 ± 0.35	5.96 ± 0.59	0.05	0.07
NH_3, mM	2.25 ± 2.29	1.62 ± 0.85	2.83 ± 3.58	3.02 ± 3.65	2.10 ± 2.67	0.22	0.13
total VFA, mM	84.5 ± 30.2^{ab}	81.6 ± 33.8^{ab}	76.0 ± 37.0^{c}	80.8 ± 39.6^{bc}	86.3 ± 35.6^{a}	2.85	<0.01
C_2, %	57.2 ± 7.95	54.5 ± 9.32	55.6 ± 6.84	59.0 ± 3.27	52.0 ± 9.80	0.67	0.55
C_3, %	21.3 ± 3.18^{ab}	19.8 ± 3.12^{c}	20.6 ± 1.89^{bc}	20.4 ± 3.67^{bc}	22.5 ± 2.10^{a}	0.24	0.02
C_4, %	12.6 ± 4.04^{c}	11.3 ± 5.22^{cd}	13.5 ± 4.56^{bc}	14.7 ± 2.70^{a}	13.8 ± 4.35^{bc}	0.40	<0.01
C_2/C_3	2.82 ± 0.68	2.73 ± 0.84	2.83 ± 0.76	3.14 ± 0.75	2.35 ± 0.76	0.06	0.09
Digestibility, %							
DMD	62.8 ± 7.44	62.6 ± 6.65	57.5 ± 8.49	60.1 ± 7.00	62.3 ± 6.24	0.86	0.25
OMD	63.5 ± 3.70^{a}	49.2 ± 10.7^{b}	55.1 ± 11.5^{b}	60.3 ± 6.07^{ab}	58.4 ± 6.17^{b}	1.77	0.03
NDFD	35.2 ± 7.13	37.3 ± 7.67	37.2 ± 11.2	37.6 ± 19.3	-	1.41	0.89
Microorganism, log_{10}							
bacteria	10.9 ± 0.82	11.3 ± 0.27	9.26 ± 0.33	10.5 ± 0.71	-	0.28	0.19
protozoa	8.10 ± 0.25	7.42 ± 1.25	-	6.10 ± 1.08	-	0.44	0.70
methanogen	7.60 ± 0.41	6.77 ± 0.34	6.85 ± 1.65	6.00 ± 0.26	-	0.31	0.63

CLO – clove oil, CIO – cinnamon oil, ORO – oregano oil, THO – thyme oil, SEM – standard error of the mean, CH_4 – methane, CO_2 – carbon dioxide, NH_3 – ammonia, total VFA – total volatile fatty acids, C_2 – acetate, C_3 – propionate, C_4 – butyrate, DMD – dry matter digestibility, OMD – organic matter digestibility, NDFD – neutral detergent fibre digestibility; $^{a-d}$ – means with different superscripts within a row are significantly different at $P < 0.05$

Discussion

Effects of EO levels on rumen methanogenesis and fermentation

In the present study, the decrease in total gas emissions following the addition of EOs coincided with reductions in CO_2, total VFA, DMD, and OMD levels. This trend aligns with findings from prior meta-analysis studies (Klevenhusen et al., 2012; Susanto et al., 2023), indicating lower rumen fermentation activity. A decrease in total gas production coupled with decreased digestibility signifies low fermentation activity (Roy et al. 2015). The decrease in total gas production indicates inhibition of rumen microbial growth due to the strong antimicrobial properties of EOs (Benetel et al., 2022).

The addition of EOs led to a linear decrease in CH_4 production, as indicated by the reduced number of protozoa in the rumen (Table 2). The inhibition of CH_4 production can be attributed to the decline in the protozoan population, resulting in lower H_2 generation, and consequently inhibiting the interspecies transfer of H_2 to CH_4 (Malik et al., 2017). H_2 is utilised as a substrate in methanogenesis, thus the lower abundance of protozoa results in substrate shortage for this process (Malik et al., 2017). A decline in protozoan populations is expected because the anti-protozoan activity of EOs inhibits their growth. Patra and Yu (2012) noted that the antimicrobial activity of EOs was mainly related to their potent action against protozoa. A possible mechanism likely involves the disruption of cell membranes due to the lipophilic nature of bioactive compounds, leading to the loss of cell contents and cell lysis (Benchaar et al., 2007).

Ruminal CH_4 emissions represent a significant loss of energy for the animal, thus inhibiting CH_4 production could improve ruminant productivity (Ungerfeld, 2020). Suppression of methanogenesis

redirects H_2 utilization towards C_3 synthesis, potentially leading to increased C_3 synthesis (Malik et al., 2017). However, our study revealed a significant decrease in C_3 levels, followed by an increase in the C_2:C_3 ratio, and a reduction in size of bacterial populations (Table 2). These outcomes were anticipated due to the antimicrobial effects of EOs, which inhibit the growth of propionate-forming Gram-negative bacteria. These findings are consistent with the observations of Castillejos et al. (2006), who reported that the addition of EOs inhibited the growth of Gram-negative bacteria. The hydrophobic properties of EOs enable them to penetrate the barrier layer of Gram-negative bacteria through outer membrane porin proteins (Nikaido, 2003).

The addition of EOs resulted in an increase in rumen pH, which was consistent with previous meta-analysis studies (Makmur et al., 2023; Susanto et al., 2023). These reflects the ability of EOs to maintain rumen pH stability, albeit indicating a reduction in fermentation activity (Şahan et al., 2021). Correspondingly, Benchaar et al. (2007) reported that an elevation in rumen pH was associated with a decrease in total VFA. The results presented here also demonstrated a decrease in NH_3 levels, likely attributed to the potent antimicrobial activity of EOs and/or their capacity to coat proteins in the feed. The antimicrobial properties of EOs were shown to inhibit the growth of hyperammonia-producing bacteria (Castillejos et al., 2006). The decline in NH_3 levels could also be associated with the ability of phenolic compounds to bind dietary proteins, increasing the proteins' resistance to microbial degradation (McSweeney et al., 2001; Niderkorn and Jayanegara, 2021).

Effects of EO types on rumen methanogenesis and fermentation

The present study has demonstrated that ORO and THO decrease total gas production (30 and 40%, respectively) compared to the control. Benetel et al. (2022) similarly reported that ORO and THO suppressed total gas emissions, suggesting their potent antimicrobial activity, which could inhibit rumen microbial growth and subsequently reduce feed fermentation. Consistent with these findings, Benchaar et al. (2007) observed reduced total gas production after carvacrol and thymol addition, both of which are key constituents of ORO and THO EOs (Hyldgaard et al., 2012).

This study revealed that only ORO significantly suppressed CH_4 production (39%) compared to the control group. These results have suggested that ORO exhibits the most significant effect in mitigating CH_4 emissions. Embaby et al. (2019) reported that ORO contained phenolic compounds such as carvacrol, known for its broad-spectrum antimicrobial activity. The antimicrobial action of carvacrol involves diffusion into the lipid layer of the microbial cell membrane, thereby increasing its permeability (Hyldgaard et al., 2012). However, despite the reduction in CH_4 production, ORO addition did not influence the rumen microbial population in this study. This discrepancy might be attributed to the limited number of observations (Yanza et al., 2021), as previous research suggests that essential oils exert a direct toxic effect on methanogens, thereby modulating either the rumen methanogen population or its activity, and subsequently decreasing methane production (Cieslak et al., 2013).

ORO and CLO supplementation resulted in a lower C_3 concentrations compared to the levels recorded in the control group (5 and 7%, respectively). CLO contains eugenol, while ORO contains carvacrol as its main bioactive substance (Hyldgaard et al., 2012). Benchaar et al. (2007) reported that high doses of carvacrol and eugenol significantly reduced C_3 generation, indicating that medium to high doses of oregano and clove oil could inhibit the growth of propionate-forming bacteria. However, it is worth noting that the decrease in C_3 observed in this study remained within the normal range typically found in ruminants, which ranges from 20–26% (McDonald et al., 2022). Interestingly, only ORO addition resulted in an increased proportion of C_4 compared to the control group. ORO exhibited the highest C_4 levels, reaching 14.70%. However, it should be noted that this value falls within the typical range of rumen C_4 concentration, which is approx. 15% (McDonald et al., 2022). In the present study, despite ORO inhibitory effect on fermentation, it did not significantly alter the overall composition of the fermented products.

CLO, CIO, and THO supplementation resulted in a decrease in OMD, which was similar to the findings of Roy et al. (2015). Supplementation with high doses of CLO and THO (600 mg/kg) led to decreased OMD. This effect is likely attributable to the presence of phenolic compounds in CLO and THO, which may disturb the membrane cell integrity of certain ruminal bacteria (Hyldgaard et al., 2012), ultimately inhibiting OMD.

Conclusions

Increasing levels of essential oils (EOs) administered in the rumen *in vitro* demonstrate the ability

to reduce ruminal CH_4 production; however, they also tend to inhibit the overall rumen fermentation. Among different types of EOs, oregano oil (ORO) appears to possess the most potent antimicrobial activity against methanogenesis. ORO supplementation also leads to reductions in both C_3 and C_4 levels, although these concentrations remain within the normal range.

Conflict of interest

The Authors declare that there is no conflict of interest.

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