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Effect of essential oils and aqueous extracts of plants on *in vitro* rumen fermentation and methane production

Aarón Alejandro Molho-Ortiz[a] | **Atmir Romero-Pérez**[a] | **Efrén Ramírez-Bribiesca**[b] | **Claudia Cecilia Márquez-Mota**[a] | **Francisco Alejandro Castrejón-Pineda**[a] | **Luis Corona**[a]*

[a]Departamento de Nutrición Animal y Bioquímica, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México, 04510, Mexico.
[b]Colegio de Postgraduados, Montecillo, 56230, Mexico.

*Corresponding author: gochi@unam.mx

Abstract The objective of this study was to evaluate *in vitro* rumen fermentation and methane production under the influence of two sources of phytochemicals: essential oils (EOs) and aqueous extracts (AEs). Treatments were set up in a completely randomized block design, with 4×2+1 factorial arrangement of four species, S (garlic, G; cinnamon, C; rosemary, R; eucalyptus; EU) × two types of presentation, P (essential oil, EO; aqueous extract, AE) and a basal diet, BD (50% concentrate, 20% alfalfa and 30% corn silage). Rumen fermentation was evaluated using the *in vitro* gas production technique. All experimental units were incubated with 500 mg of BD for 72 hours. Treatments were added at a single dose of 900 mg/L of rumen inoculum. Gas pressure was recorded at 0, 2, 4, 6, 8, 10, 14, 18, 24, 30, 36, 42, 48, 60 and 72 h post-incubation. There was an interaction effect (P × S) between plant extract presentation (P) and plant species (S) for all variables. Treatments GEO, CEO, REO decreased volatile fatty acids (mmol/200 mg), microbial mass production (mg/g), CH_4 production (mL/g), *in vitro* dry matter digestibility ($P < 0.05$), and total gas production at 24 and 72 h post-incubation ($P < 0.05$; mL/g DM, mL/g OM). No differences ($P > 0.05$) were observed between AEs and BD. In conclusion, the use of EOs negatively affected rumen fermentation parameters and the production of CH_4. Garlic and cinnamon EOs effectively reduced methane emissions; however, they also reduced *in vitro* dry matter digestibility.

Keywords: cinnamon, garlic, greenhouse gas, phytochemicals, sheep

1. Introduction

The world's population will reach 9.6 billion people in 2050, with twice the purchasing power for the consumption of meat and dairy products (FAO 2016), so it has been estimated that greenhouse gas (GHG) emissions from livestock activities will increase as the demand for these products increases (O'Mara 2011). The agriculture, forestry, and other land-use sectors contribute approximately 25% (10–12 Gt CO_2 equivalent/year) of the net anthropogenic emissions of GHGs (IPCC 2014b), the most significant ones being carbon dioxide (CO_2), nitrous oxide, and methane (CH_4). Methane has a warming potential 21–28 times greater than CO_2 (IPCC 2014a) and is released by enteric fermentation, slurry management, and rice crops; together, these add a total of 5–5.8 Gt CO_2 equivalent/year (IPCC 2014b). Some studies estimate that the livestock supply chain contributes 14.5% of all human emissions (Gerber et al 2013). Methane formation during ruminal fermentation is considered a loss of energy of 2–12% of the gross energy intake (Kobayashi 2010). Multiple natural strategies have been investigated to reduce enteric methane production. Alternatives based on the use of natural compounds are attractive due to regulation in the use of antibiotics (European parliament 2003). Essential oils (EOs) (Cobellis et al 2016), tannins (Poornachandra et al 2019), and saponins (Jafari et al 2016) can modulate ruminal activity and, in some cases, increase animal productivity; however, their effectiveness on *in vivo* and *in vitro* experiments has not been consistent and conclusive (Patra and Saxena 2009), so there is a window of opportunity for research these substances, particularly EOs and plant extracts (Wencelová et al 2015). It has been shown that different effects could be observed according to the type of solvent used in the extraction; for example, plant extracts in ethanol, methanol, and water (*T. chebula*, *T. belerica*, *E. officinalis,* and *A. indica)* all decreased *in vitro* dry matter digestibility (IVDMD). In contrast, only *T. chebula* methanol extract decreased CH_4 production close to zero (Patra et al 2006a). Otherwise, garlic's aqueous extract (AE) caused higher gas production *in vitro*. However, ethanol and methanol extracts of the same plant reduced gas and methane production (Patra et al 2006b), and AEs of olive leaves increased methanogenesis (Aggoun et al 2017). Meanwhile, Sirohi et al (2009) contrasted acetone, methanol, and water extracts of garlic and eucalyptus, finding a decrease in CH_4 production in acetone and methanol garlic extracts, but an increase when it was extracted with water

(2.61 mL/g DM higher than control). Moreover, eucalyptus acetone extract reduced methanogenesis (37.34 mL/g DM lower than control), eucalyptus water extract, decreased CH_4 4.79 mL/g DM compared to control (Sirohi et al 2009). Essential oils (CEO, GEO, and REO) have shown potential methane inhibiton (Cobellis et al 2016). It has also been concluded that there are differences in metabolites present in AEs and EOs (Salzer and Furia, 1977). We hypothesize that depending on the type of extraction, different secondary metabolites are obtained in the aqueous or oily extracts, and their effect on CH_4 production will be different. The objective of this study was to evaluate the effects of AEs and EOs of garlic (*A. sativum)*, cinnamon (*C. verum)*, rosemary (*R. officinalis*), and eucalyptus (*E. globulus*) on ruminal fermentation and CH_4 production, using the *in vitro* gas production technique.

2. Materials and Methods

The experimental protocol (n° 553) for the use of experimental animals (three rumen fistulated male ovine, 55 ± 0.5 kg) as rumen liquid donors was approved by the Institutional Committee for the Care and Use of Experimental Animals, according to official animal care normativity (NOM-062-ZOO-1999) (Diario Oficial de la Federación 2001). Experiments were carried out at the Center for Practical Teaching and Research in Animal Production and Health. Analysis of all samples (residual DM, gas samples, and rumen inoculum) was carried out in the Department of Animal Nutrition and Biochemistry of the Faculty of Veterinary Medicine and Animal Science of the National Autonomous University of Mexico.

2.1. Experimental design and treatments

Treatments were set up in a completely randomized block design, with 4×2+1 factorial arrangement of four species, S (Garlic, G; cinnamon, C; rosemary, R; eucalyptus; EU) × two types of presentation, P (essential oil, EO; aqueous extract, AE) and a basal diet, BD, consisting of 50% commercial concentrate (Purina®, Ovina Engorda 15, México), 20% alfalfa and 30% corn silage on a dry matter (DM) basis. The basal diet was used as a control. The blocking criterion was incubation run (4), using four repetitions in the first, second, and third repetitions and two in the final (n = 14). All phytochemicals were added at a dose of 900 mg/L of rumen inoculum, as Joch et al (2017) reported.

2.2. Phytochemicals

Essential oils of garlic (GEO), cinnamon (CEO), rosemary (REO), eucalyptus (EEO), and the aqueous extract of cinnamon (CAE) were donated by the Rosa Helena Dueñas™ laboratory. Aqueous extracts of eucalyptus (EAE) and rosemary (RAE) were isolated in our laboratory, using an extraction technique (Fernández-Agulló et al 2015) with a plant material/solvent ratio of 1:10. The solvents used were ethanol and distilled water in a 50:50 ratio. The extraction conditions were 50°C, shaking speed at 200 rpm for 90 min on a digital orbital shaker (Heathrow Scientific® HS120460, USA). After the extract was obtained, the plant material was vacuum filtered with a Büchner funnel and a Kitasato flask. The filtered extract was then evaporated to remove ethanol on a rotary evaporator (Büchi™ Rotavapor® R-200, Switzerland) with the following conditions: 60°C, 70 rpm for approximately 30 min, until evaporation of half the initial volume. The aqueous garlic extract (GAE) was prepared from a dry garlic concentrate (ADEGERMEX™ laboratory). Garlic concentrate (0.9 g) was dissolved in 100 mL of water, heated at 50 °C, and shaking speed was 200 rpm for 30 min (Tocmo et al 2016).

2.3. Chemical analysis

The chemical composition of each ingredient in the BD (commercial sheep concentrate, alfalfa, and corn silage) was determined using different AOAC (2016) methods: The neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were also analyzed (Van Soest et al 1991). Organic matter (OM) was determined by subtracting the weight of ashes after ignition and reported as percentage. The proportion of each ingredient was used to calculate the chemical composition of the diet (Table 1).

2.4. In vitro gas production technique

Rumen fluid was collected through the rumen cannula from three male Pelibuey sheep (55 ± 1 kg, 1-year-old) fed a diet of the same composition as the BD and water *ad libitum*. Rumen fluid was collected in the morning before animals were fed, filtered to eight layers of cheesecloth, and preserved under anaerobic conditions and temperature (39°C). Subsequently, rumen fluid was mixed with reduced and mineral solutions (Menke et al 1979) in a ratio of 1:9 v/v to obtain the rumen inoculum (Theodorou et al 1994). Previously, 500 mg DM of BD (ground in a Model 4 Wiley® mill, through a 1-mm screen) was placed in 125-mL amber glass bottles, used as experimental units. All phytochemicals were added at a dose of 900 mg/L of rumen inoculum (Joch et al 2017). Then, 90 µL of each treatment was applied inside the amber glass bottles using a micropipette. Subsequently, 100 mL of rumen inoculum were added and continuously flushed with CO_2 to maintain anaerobic conditions. Bottles with rumen inoculum and no substrate were also incubated as blanks to adjust gas production values. Bottles were hermetically sealed with rubber stoppers and aluminum caps and then placed in a water bath with lateral oscillation (30/min) at 39 °C. Gas pressure (kg/cm^2) was recorded at 0, 2, 4, 6, 8, 10, 14, 18, 24, 30, 36, 42, 48, 60 and 72 h post-incubation, using a digital manometer (Traceable®, Fisher Scientific, USA).

2.5. Gas sampling and analysis

The total gas in the headspace of the glass bottles was completely removed using a 60-mL syringe with a needle. Produced gas was collected at 6, 12, 18, 24, 48, and 72 h. Subsequently, the gas collected was rapidly injected into

sealed flasks containing 38 mL of a saturated NaCl solution pH 2 (350 g of NaCl, 5 mL of 0.1% methyl orange) (Torres-Salado et al 2017). Gas samples were analyzed for CH_4 by gas chromatography in an Autosystem XL Perkin Elmer® chromatograph equipped with an HP AL/S column (Agilent Technologies, part 1902P-S21; 15 m × 0.53 mm × 15 µm), and N_2 carrier gas was injected at 6.5 mL/min flow and 4 psi pressure. The oven temperature was set to 40 °C for 3 min with an increase of 20°C/min until 175°C and held for 3 min. The flow of H_2 was 400 mL/min, and airflow was 40–45 mL/min. The injector temperature was 200 °C, and an FID detector (200 °C) was used. Volume injection was 1 µl.

Table 1 Ingredients and chemical composition of the basal diet.

Item	%
Ingredient	
Corn silage	30
Alfalfa hay	20
[2]Commercial concentrate	50
Chemical composition	
Dry Matter	99.3
Crude protein	12.4
Ether extract	4.4
Organic matter	90.8
Crude fiber	15.5
NDF	43.9
ADF	21.8
NFE	58.5
TDN	68.8
[3]DE (Kcal/kg)	3033.4
[4]ME (Kcal/kg)	2487.4
[5]NEm (Kcal/kg)	1595.5
[6]NEg (Kcal/kg)	996.6

[1]Dry matter basis. [2] Purina®, Ovina Engorda 15. AOAC methods: Dry matter (method 934.01), crude protein (method 2001.11) ether extract (method 920.39), ashes (method 942.05) crude fiber (method 962.09). NDF = Neutral detergent fiber. ADF = Acid detergent fiber. NFE = Nitrogen free extract. TDN = Total digestible nutrients. DE = Digestible energy. ME = Metabolizable energy, NEm = Net energy for maintenance. NEg = Net energy for growth. NFE was calculated as: 100 – (%CP + % EE + %CF +% Ashes). TDN was calculated as: %TDN = ((CP × 0.75) + (EE × 0.9) × 2.25 + (CF × 0.5) + (NFE × 0.75)) [3]DE, [4]ME, [5]NEm and [6]NEg were estimated based on NRC (2001) following equations:
DE = TDN * 4.409. EM = DE × 0.82. NEm = [(1.37 × ME) - (0.138 × ME^2) + (0.0105 × ME^3)] - 1.12. NEg = [(1.42 × ME) - (0.174 × ME^2) + (0.0122 × ME^3)] - 1.65

2.6. pH and in vitro digestibility of dry matter

After 72-h incubation, fermentation was stopped by placing the amber glass bottles in an ice bath. Rumen pH was then determined with a portable potentiometer (pH Tester model 30 Double Function®). Residual DM was used to estimate the IVDMD as described by Theodorou et al (1994). Contents of all bottles were filtered individually using filter paper discs (Whatman No. 41), a Büchner funnel, and a vacuum pump. After filtering, the filter paper discs were placed in a forced-air oven at 55 °C for 48 h and weighed (Ohaus-Explorer® model AX12478, México) to determine residual DM.

2.7. Ruminal kinetics

The gas pressure readings (kg/cm^2) were transformed to gas volume (GasVol; mL) with a linear regression equation:

GasVol = Pressure / 0.019; R^2 = 0.988 (1)

Data was adjusted with pressure values registered in the blanks; these were subtracted from the pressure readings of the treatments on each incubation time. After adjusting data, the gas volume and incubation time obtained from the previously linear regression equation were averaged and grouped to obtain cumulative gas volume per hour and total gas production at 24 h and 72 h (mL/g). Cumulative gas values were used to fit the model of France et al (1993).

2.8. Estimation of volatile fatty acids (VFA) and production of microbial mass (MM)

The VFA production (mmol/200 mg DM) was calculated using gas production (Gp), according to Getachew et al (2002), using the following equation:

VFA (mmol/200 mg DM) = 0.0222 Gp - 0.00425 (2)

The production of MM was calculated according to the methodology of Blümmel et al (1997), with modifications (Salem 2012), using the following equation:

MM (mg/g DM) = APS (mg DM) - (mL gas × 2.2 mg/mL) (3)

where APS = apparently degraded substrate and 2.2 mg/mL is a stoichiometric factor that expresses the mass (mg) of C, H, and O required by the VFA associated with the production of 1 mL of gas.

2.9. Statistical analysis

The variables were analysed with the MIXED procedure of SAS. Treatment means were compared with a Tukey analysis, using the following contrasts: EO *vs* AE, BD *vs* EO and BD *vs* AE, according to the following model (Cochran and Cox 1992):

$Yijk = \mu + \alpha i + \beta j + (\alpha\beta)ij + \gamma k + \varepsilon ijk$ (4)

In the model, Yijk is the response variable in the i-th species, the j-th presentation of extract, in the k-th repetition of the γ-th run, µ is the general mean, αi is the effect of the species at level i, βj is the effect of the presentation of extract at level j, (αβ) ij is the effect of the interaction species × presentation of extract at level ij, γk is the block effect and εijk is the random error. Contrasts were considered significant when the *P*-value was ≤0.05.

3. Results

3.1. Fermentation parameters

The interaction between phytochemicals presentation and species (P × S) was significant ($P < 0.001$) for all fermentation parameters (pH, IVDMD, VFA, and MM; Table

2). Rumen pH (Table 2) was lower ($P < 0.001$) for CEO, GAE, and RAE concerning BD. Mean pH for GEO, EEO, REO, CAE, and EAE was no different ($P > 0.05$) from BD.

The VFA production (Table 2) was lower ($P < 0.001$) for REO, GEO and CEO compared to BD ($P < 0.001$) by 13, 22 and 47%, respectively. Furthermore, CEO and GEO decreased ($P < 0.001$) VFA production compared to EEO, AE, and BD. The MM production was decreased ($P < 0.001$) by REO, GEO, and CEO (Table 2) by 11, 19, and 57%, respectively, compared to BD. The lowest ($P < 0.001$) MM production was obtained with CEO, whose mean was 63% lower compared to EEO, which was similar ($P > 0.05$) to AE and BD.

Table 2 Effect of essential oils and aqueous extracts on fermentation parameters: pH, *in vitro* dry matter digestibility, volatile fatty and microbial mass production.

	Species	pH	IVDMD %$^{72h-1}$	VFA (mmol/200 mg DM)	MM (mg/g DM)
Essential oils	GEO	6.55^{abc}	50.5^{c}	2.95^{c}	192.20^{c}
	CEO	6.41^{c}	47.7^{c}	1.99^{d}	102.50^{d}
	EEO	6.51^{abc}	61.7^{b}	4.05^{a}	278.23^{a}
	REO	6.66^{a}	56.8^{bc}	3.28^{bc}	211.95^{bc}
Aqueous extracts	GAE	6.46^{bc}	70.6^{a}	3.86^{a}	241.31^{abc}
	CAE	6.63^{ab}	68.1^{a}	3.90^{a}	251.62^{ab}
	EAE	6.53^{abc}	67.9^{a}	3.97^{a}	257.82^{ab}
	RAE	6.46^{bc}	68.4^{a}	4.00^{a}	260.09^{ab}
Control	BD	6.63^{a}	69.2^{a}	3.79^{ab}	237.62^{abc}
	SEM	0.17	1.1	0.06	5.5
Contrasts	Block	<0.001	<0.001	<0.001	<0.001
	Presentation	0.55	<0.001	<0.001	<0.001
	Specie	0.38	<0.001	<0.001	<0.001
	P × S	<0.001	<0.001	<0.001	<0.001
	EOs *vs* AEs	0.59	<0.001	<0.001	<0.001
	BD *vs* EOs	0.01	<0.001	<0.001	0.002
	BD *vs* AEs	0.006	0.71	0.26	0.26

Abbreviations: IVDMD = *in vitro* dry matter digestibility; VFA =volatile fatty acids (mmol/200 mg DM); MM= microbial mass production (mg/g DM); Presentation = essential oil (EO) or aqueous extract (AE); Specie; garlic (G); cinnamon (C); eucalyptus (E); rosemary (R); BD = basal diet; Block = experimental block; P × S = interaction between presentation and species. Contrasts: EOs vs AEs = essential oils vs aqueous extracts; BD vs EOs = basal diet vs essential oils; BD vs AEs = basal diet vs aqueous extracts.

3.2. In vitro dry matter digestibility

All EOs decreased ($P < 0.001$) IVDMD compared to all AEs and BD (21.2% and 21.7%, respectively). Treatments GEO and CEO decreased IVDMD ($P < 0.001$) by 18% and 22% compared to EEO and by 27% and 31% concerning BD.

3.3. Total gas and CH_4 production

Total gas and CH_4 were expressed as mL/g of DM, OM, and dry-matter digestibility (DMD; Table 3). Interaction between phytochemical presentation and species (P × S) was found for all total gas and CH_4 variables (Table 3; $P < 0.001$).

Inclusion of REO, GEO, and CEO decreased (Table 3; $P < 0.001$) the total gas production at 24 h and 72 h (mL/g of DM and mL/g of OM) when compared to BD (13%, 22%, and 47% at 24h and 14%, 19% and 40% at 72h, respectively). Addition of GEO and CEO decreased (Table 3; $P < 0.001$) the methane production (mL/g DMD ^{72h}) when compared to EEO, REO, AE, and BD by 64.7% on average.

As observed in Figure 1, GEO, CEO, and REO showed lower cumulative gas production than the other treatments. Cumulative gas production for AEs did not differ from that of BD.

4. Discussion

4.1. Fermentation parameters

Their chemical nature can explain the inhibitory effect on rumen fermentation caused by EOs: a complex mixture of secondary plant metabolites with a highly variable composition. Their action mechanism against rumen microorganisms is still poorly understood (Cobellis et al 2016). However, it is speculated their mechanism involves membrane disruption of microorganisms (Griffin et al 1999). Decreases in pH are often associated with reductions in gas production, DM disappearance, and total VFA concentration (Fondevila and Pérez-Espés 2008). Negligible changes in pH due to GEO (Mateos et al 2013), REO (Castillejos et al 2008), and EEO (Cobellis et al 2016) have previously been reported. Decreases of pH were previously observed for CEO *in vivo* (Chaves et al 2008) and *in vitro* (Amin et al 2021). However, decreases are often related to VFA increases, not observed in the present study. Decreases on pH are also related to incubation times longer than 24 hours and characteristics of the *in vitro* technique, like lack of end-products removal (Williams et al 2010). To our knowledge, the effect of GAE on ruminal pH has not been reported, but the presence of highly

degradable carbohydrates can lower pH (Hoover, 1986). The detrimental effects on rumen microbial fermentation in the present study (decrease in VFA production) when GEO, CEO, and REO were used have been previously reported (Doreau et al 2017). Macheboeuf et al (2008) used cinnamaldehyde (main active compound in CEO) and GEO at 5 mM/L in a 25:75 F/C diet. Reductions up to 60% of VFA production were observed by the CEO.

Table 3 Effect of essential oils and aqueous extracts on gas production parameters: Total gas production and CH_4 production.

	Species	Total gas					CH_4		
		(mL/g $DM^{24\,h}$)	(mL/g $DM^{72\,h}$)	(mL/g $OM^{24\,h}$)	(mL/g $OM^{72\,h}$)	(mL/g $DMD^{72\,h}$)	(mL/g $DM^{72\,h}$)	(mL/g $OM^{72\,h}$)	(mL/g $DMD^{72\,h}$)
Essential oils	GEO	333.09^{b}	485.47^{b}	325.35^{b}	474.21^{b}	1932.1^{a}	4.60^{c}	4.49^{c}	22.71^{b}
	CEO	224.92^{c}	361.87^{c}	219.89^{c}	353.77^{c}	1770.6^{ab}	7.02^{c}	6.86^{c}	33.37^{b}
	EEO	456.44^{a}	637.65^{a}	443.54^{a}	619.63^{a}	2083.6^{a}	28.22^{a}	27.42^{a}	93.53^{a}
	REO	370.03^{b}	521.22^{b}	360.42^{b}	507.66^{b}	1848.3^{a}	21.31^{b}	20.76^{b}	75.36^{a}
Aqueous extracts	GAE	434.72^{a}	622.11^{a}	420.62^{a}	601.94^{a}	1508.7^{b}	27.65^{a}	26.76^{a}	74.44^{a}
	CAE	440.72^{a}	632.19^{a}	426.94^{a}	612.42^{a}	1861.9^{a}	23.09^{ab}	22.38^{ab}	68.78^{a}
	EAE	447.31^{a}	640.77^{a}	433.38^{a}	620.81^{a}	1900.4^{a}	28.97^{a}	28.07^{a}	83.99^{a}
	RAE	451.21^{a}	645.28^{a}	437.03^{a}	625.00^{a}	1893.5^{a}	27.79^{a}	26.92^{a}	81.51^{a}
Control	BD	427.43^{a}	603.78^{a}	413.84^{a}	584.61^{a}	1773.7^{ab}	26.18^{ab}	25.35^{ab}	77.99^{a}
	SEM	7.77	7.680	7.490	7.506	33.667	6.08	5.91	25.11
Contrasts	Block	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.005
	Presentation	<0.001	<0.001	<0.001	<0.001	0.708	<0.001	<0.001	<0.001
	Specie	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	P × S	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	EOs *vs* AEs	<0.001	<0.001	<0.001	<0.001	0.797	<0.001	<0.001	<0.001
	BD *vs* EOs	<0.001	<0.001	<0.001	<0.001	0.403	<0.001	<0.001	<0.001
	BD *vs* AEs	0.278	0.099	0.279	0.101	0.318	0.639	0.639	0.895

Abbreviations: Total gas = total gas production at 24 and 72 h (mL/g); DM = dry matter; OM = organic matter; DMD= dry matter digestibility; Presentation = essential oil (EO) or aqueous extract (AE); Specie = garlic (G), cinnamon (C), eucalyptus (E), rosemary (R); BD = basal diet; Block = experimental block; P × S = interaction between presentation and species; Contrasts: EOs vs AEs = essential oils vs aqueous extracts; BD vs EOs = basal diet vs essential oils; BD vs AEs = basal diet vs aqueous extracts.

Additionally, GEO decreased acetate production by 15% after 24 h. In the present study REO; CEO and GEO decreased gas production; however, gas production does not consider the quantity of substrate converted in microbial biomass, so gas measurement can't be considered an estimate of apparent rumen digestibility (Blümmel and Ørskov, 1993). A positive correlation between gas production and IVDMD has been previously reported (Apori et al 1998). Moreover, a positive correlation between gas production and VFA production has been reported with high R^2 values (R^2 = 0.94; n = 94; $P < 0.001$) (Getachew et al 2002), which might explain reduction of VFA production observed. Gas production is an indicator of quantitative VFA production, since digested substrate is partitioned among VFA gas and microbial biomass (Getachew et al 2002). This approach has also been used and validated in different publications (Amanzougarene and Fondevila, 2020). However, gas production does not reflect the substrate utilized for microbial growth (Getachew et al 2004). As established by Ørskov (1994), MM production is limited to the number of units of carbohydrate fermented; moreover, it has been established that microbial biomass can be substantially altered by two means, bacterial lysis and turnover of protein within the rumen. In the present study, decreases in MM production caused by REO, GEO, and CEO could be explained because of the non-specific antibacterial properties of these compounds. The relationship between *in vitro* gas production and microbial biomass was established by Blümmel et al (1997), which found a significant negative relationship between gas produced and microbial biomass (R^2 = 0.67). This methodology has been used previously to estimate microbial mass (Sahli et al 2018).

4.2. In vitro dry matter digestibility

In the present work, IVDMD was decreased ($P < 0.001$) for GEO CEO, EEO, and REO treatments. These results are expected as previous studies observed similar decreases when high doses of essential oils were used. Foskolos et al (2015) evaluated propyl-propane thiosulfinate (one of the active compounds on GEO) on continuous culture fermentators, it reduced 33% true organic matter digestibility when a high dose was used (300 mg/L *vs.* 30 mg/L). Similarly,

Cobellis et al (2016) used 1.125 mg/L of REO, EEO, and CEO and observed a decrease in IVDMD. Righi et al (2017) evaluated different essential oils (cinnamon, clove, thyme, oregano, carvacrol) and their active compounds on different feedstuffs (soybean meal, maize meal, lucerne hay, and a total mixed ration) on *in vitro* rumen fermentation. Interestingly EOs have different effects depending on the fermented substrate; for example, the addition of most EOs depressed DMD of soybean meal. Adverse effects of some EOs on feed digestion could be a consequence of their broad and non-specific antimicrobial activity (Cobellis et al 2016).

The antimicrobial activity of garlic has been attributed to its organosulfur compounds, particularly to allicin (Ankri and Mirelman 1999). Intact garlic bulbs contain the sulphur compounds S-alkenyl-L-cysteine sulfoxide and S-allylcysteine sulfoxide, which are present at between 1 and 5% of the dry weight of the plant (Patra 2012). When cells are damaged, the enzyme allinase releases and converts these compounds into other volatile and reactive components called thiosulfates, with allicin as the most abundant compound (Patra and Yu 2012a). Although, pure allicin is a volatile compound that is difficult to mix in aqueous solution and is reactive, turns quickly to other compounds under different conditions (Cardozo and Kamel 2008; Lawson and Gardner 2005). Cinnamon's most active compound is cinnamaldehyde, present at 60–75% in the EO, together with 4–10% of phenols, mainly eugenol and other hydrocarbons (Gopu et al 2008). The bioactive compounds of rosemary are more complex; they could contain in their leaves a cocktail of phenolic compounds and di-terpenes (carnasol, carnosic acid, rosmanol, epirsonmanol, isorosmanol, methyl carnosate and rosmarinic acid), and their activity as rumen modifiers and antioxidant compounds has been well documented (Cobellis et al 2015). Also, it has been observed that major components of REO (such as monoterpenes α-pinene, β-pinene, camphene, 1,8-cineol, camphor, borneol, bornyl acetate and verbenone) are known for their antimicrobial properties against gram-positive and gram-negative bacteria (Jiang et al 2011). The presence of a phenolic moiety and the position of a hydroxyl group in the phenolic structure of the EO (e.g., EOs containing thymol or eugenol) can influence the antimicrobial potency of the EO (Ultee et al 2002).

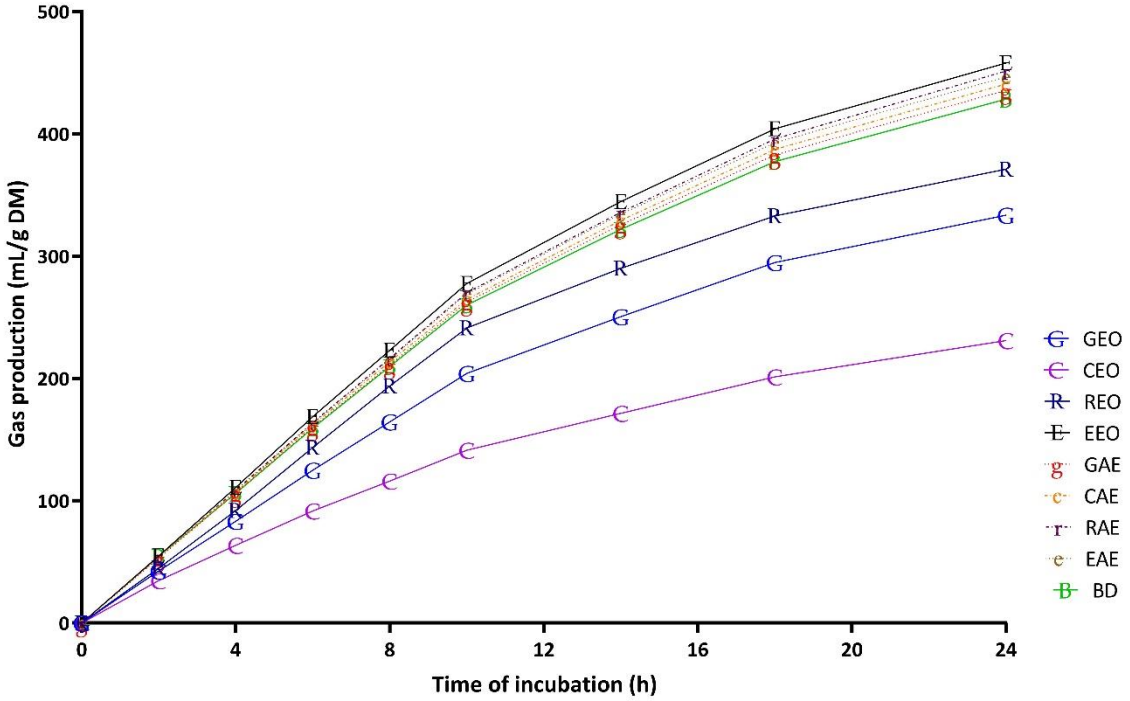

Figure 1 Effect of essential oils and aqueous extracts on cumulative gas volume kinetics. Abbreviations: First letter corresponds to Specie = garlic (G), cinnamon (C), eucalyptus (E), rosemary (R); second and third letter correspond to Presentation = essential oil (EO) or aqueous extract (AE). BD = basal diet. Curves were fitted from the model of France et al (1993).

4.3. Total gas and methane production

In the present study, total gas was reduced ($P < 0.001$) by GEO, CEO, and REO. This is a response of the antibacterial properties EOs and has been previously reported. Jahani-Azizabadi et al (2011) evaluated several EOs in a high-forage diet (80% alfalfa and 20% concentrate) where GEO was the most potent compound in reducing total gas and CH_4 production. Patra and Yu (2015) evaluated different phytochemicals, including GEO, nitrate, and saponin (*Q. saponaria*), and their combinations in two types of diet: 70:30 and 30:70 F/C. Treatment GEO reduced total gas production concerning control treatment in a high-concentrate diet; methane was decreased by GEO in both diets. Doreau et al (2017) conducted an *in vitro* trial using 8 mg of GEO in a 30:70 F/C diet and found no differences in total gas production after

5 h of incubation but a reduction in total gas production at 24 h of incubation. The reduction in total gas and CH_4 production by EOs indicates that these compounds could affect the fermentation of OM at 24 and 72h. Additives included at higher doses exhibit more biological activity and tend to decrease gas production at digestibility expenses. In the present study, REO decreased ($P < 0.001$) total gas (mL/g DM, mL/g OM), which is comparable to previously published data by O'Grady et al (2006), where *in vitro* fermentation trials were conducted in the presence of barley grain (1 g) as substrate. Using a low dose of REO (0.1 g) decreased gas production relative to the control treatment, indicating that fermentation and gas production were inhibited. A decrease in total gas was registered for CEO and REO and a decrease in IVDMD for CEO, REO, and EEO. These findings correspond with previous study by Cobellis et al (2016), who showed that EOs containing compounds such as cinnamaldehyde, present in CEO, have stronger antimicrobial activity than those that contain monoterpenes and phenols, for example, EEO and REO. Adverse effects of EOs on feed digestibility could also be a consequence of their non-specific antimicrobial activity.

Suppressing CH_4 production without the negative effects on digestibility or fermentation is challenging. An ideal feed additive should improve rumen fermentation characteristics without adversely affecting feed intake or digestibility (Cobellis et al 2016), which might be possible with the optimal dose of compounds, specific action against methanogens, and specific substrate characteristics. The most effective treatment to reduce CH_4 emission in the present study was GEO, which has already been reported as a potential modifier of rumen methanogenic communities. Ferme et al (2004) identified in GEO a particular compound, diallyl disulfide, which was the first plant extract to act selectively against methanogens and protozoa (Anassori et al 2011). It has been suggested that the organosulfur compounds found in GEO can directly inhibit rumen methanogenic archaea, inhibiting the enzyme 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase (Busquet et al., 2005; Patra and Saxena, 2010). As a result, the synthesis of the isoprenoid unit is inhibited, the *Archaea* membrane becomes unstable, and the cells die (Roy et al 2014).

In a similar manner to the present study, Patra et al (2006b) evaluated aqueous, ethanolic, and methanolic extracts of garlic, fennel (*F. vulgare*), clove (*S. aromaticum*), onion (*A. cepa*) and ginger (*Z. officinalis*) in a 50:50 F/C diet. Their results suggested that AEs were not effective in reducing methane production, indicating that antimethanogenic factors are extracted only into alcohol and methanol. Methanolic extractions were more effective in reducing CH_4 production (65–83% inhibition) than ethanolic extracts (26–52% inhibition). Due to the volatile nature of the compounds present in EOs, those that are extracted with solvents or at low temperatures may have a higher bioactivity than those extracted with steam or water (Hart et al 2008). Another *in vitro* study conducted by the same research group (Patra et al 2009) suggested that the bioactive compounds present in garlic were more soluble in organic solvents such as ethanol and methanol, making GAE less effective than GEO in reducing the production of methane.

The positive effects of GEO and CEO in the reduction of CH_4 production come with negative effects on several fermentation variables (total gas, IVDMD, VFA, and MM). Therefore, it is important to determine proper doses, where the addition of the phytochemical allows a decrease in CH_4 without inhibiting ruminal fermentation. A suitable alternative product is one that reduces CH_4 but not negatively affect other variables. The latter was not found in this research. However, contrary to our results, some authors reported positive effects (Yang et al 2007) or no effects (Meyer et al 2009) on feed intake or rumen feed degradability. As previously discussed, this depends on EOs composition, extraction method, diet composition, and selected dose. Several experiments have been conducted to determine suitable doses. Based on previous *in vitro* studies (Jahani-Azizabadi et al 2011; Patra and Yu 2012a), it appears that effective concentrations of GEO range from 300 to 500 mg/L. However, lower doses (135 mg/g of the substrate) could also reduce CH_4 production (up to 20%), with no effect on gas production and marginal reductions in IVDMD (García-González et al 2008). Recently, Dey et al (2021) observed that a low dose of GEO (33.33 µL/L) in an *in vitro* trial with buffalo rumen fluid could reduce CH_4 production by 38.35% without impairing feed digestion. Besides that, the four major components of garlic were tested individually, evaluating their effect on *in vitro* methanogenesis. It was then understood that only garlic oil, diallyl disulfide, and allyl mercaptan could inhibit CH_4 emission.

In contrast, allicin was ineffective (Kamel et al 2008). It also has been demonstrated that the effect of the inclusion of garlic oil on *in vitro* methane and VFA production is diet- and dose-dependent (Kamel et al 2009). On the other hand, the use of a higher dose (1.125 mL/L culture) of CEO almost reduced to zero the production of CH_4 in a trial where it was used individually, and by 37.7– 78.5% when it was combined with other sources of EO, such as oregano, rosemary and eucalyptus leaves (Cobellis et al 2016). It is challenging to escalate doses from *in vitro* to *in vivo* trials because sometimes the functional levels of phytochemicals *in vitro* experiments are too high to be achieved in practice (Sharma and McNeill, 2009). However, some experiments have used doses from 1 g CEO/cow/d (Benchaar et al 2008) to 5 g GEO/cow/d (Yang et al 2007), increasing true ruminal digestibility (6.5%) and milk fat (0.26%, or 104 g/d) with the use of higher doses. Unfortunately, commercial EOs are expensive, so their beneficial effects on animal welfare and performance should be demonstrated before actual use in farms (Cobellis et al 2016).

Moreover, many aspects are still unknown, such as the synergistic and/or antagonistic interaction among EOs active compounds, such as trials that evaluated CEO and GEO combinations in small doses (300 mg/cow/d) (Blanch et al 2016) decreased CH_4 production. In contrast, a combination of different micro-encapsulated EOs had no effect (Alemu et

al 2019). A meta-analysis conducted by Ungerfeld (2018) showed no association between methanogenesis inhibition and improvement in ruminants productivity. Further investigation might be necessary to understand the conditions under which methane mitigation and animal productivity enhancement can be achieved.

5. Conclusions

Overall, the use of garlic, cinnamon, and rosemary EOs negatively affected ruminal fermentation. Garlic and cinnamon EOs decreased methane production by 64.7% on average when compared to the other treatments. These three species are apparently better rumen modulators than eucalyptus. However, digestibility was also reduced; therefore, dose adjustment and *in vivo* studies have to be evaluated. On the other hand, AEs of all species showed negligible effects on ruminal fermentation, suggesting that water extraction methods may be inappropriate to obtain those secondary compounds present in the different species that impact rumen fermentation parameters, such as gas and methane production.

Conflict of Interest

The authors declare that there is no conflict of interest.

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