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Modification of ruminal fermentation *in vitro* for methane mitigation by adding essential oils from plants and terpenoid compounds

Modificación de la fermentación ruminal *in vitro* para mitigación de metano mediante la adición de aceites esenciales de plantas y compuestos terpenoides

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ABSTRACT

Essential oils from plants are volatile aromatic compounds, mainly terpenoids, phenylpropanoids; monoterpenes, sesquiterpenes, and alcohols. These present a wide range of antimicrobial and antioxidant activity, so the addition of essential oils of clove, eucalyptus, peppermint, rosemary, oregano, and cinnamon can modify ruminal fermentation by reducing bacteria population producing methane and thus have a reduction in this gas production. The objective of this work was to evaluate different essential oils and terpenoid compounds to improve ruminal fermentation and the volatile fatty acid production, attenuating methane generation. The chemical composition (terpenoids) of the oils, as well as volatile fatty acids (VFAs), were determined by gas chromatography. For *in vitro* digestibility, the *in vitro* gas production technique was used, and the ruminal liquid was used. Methane was inferred from VFA concentration. It was found that all the essential oils presented each one of terpenoids in different concentrations, reporting the highest carvacrol concentration in clove essential oil (303 mg mL^{-1}) and oregano (1.20 mg mL^{-1}). Terpinene was presented in greater quantity in peppermint essential oil (4.83 mg mL^{-1}); for peppermint and rosemary oil, linalool was higher and for limonene, the highest concentration was in eucalyptus oil (449 mg mL^{-1}) and rosemary (12.42 mg mL^{-1}). For gas production in digestibility, eucalyptus essential oil at a dose of 0.3 presented 176 mL g^{-1} DM. For *in vitro* digestibility, rosemary oil in high dose (0.6 mL) presented the best ruminal fermentation since it had better methane mitigation (716.83 mM/L) without negatively affecting the VFA concentration (acetate, 1892.2; propionic, 526.14; butyric, 24.99 mM/L), as well as terpenoids thymol, linalool, and limonene in high doses. It is concluded that the best *in vitro* ruminal fermentation with methane mitigation was observed with rosemary oil and terpenoid compounds were thymol, linalool, and limonene in the high dose.

Keywords: plants, terpenoid compounds, volatile fatty acids, and methane.

RESUMEN

Los aceites esenciales de plantas son compuestos aromáticos volátiles, principalmente terpenoides, fenilpropanoides; monoterpenos, sesquiterpernos y alcoholes, estos presentan una amplia gama de actividad antimicrobiana y antioxidante, por lo que la adición de aceites esenciales de clavo, eucalipto,

menta, romero, orégano y canela pueden modificar la fermentación ruminal al disminuir la población de bacterias productoras de metano y así tener una reducción de la producción de este gas. El objetivo fue evaluar diferentes aceites esenciales y compuestos terpenoides para mejorar la fermentación ruminal y la producción de ácidos grasos volátiles, atenuando la generación de metano. Se determinó la composición química (terpenoides) de los aceites, así como ácidos grasos volátiles (AGVs) por cromatografía de gases. Para la digestibilidad *in vitro*, se empleó la técnica de producción de gas *in vitro* y se utilizó líquido ruminal. El metano se infirió a partir de la concentración de AGVs. Se encontró que todos los aceites esenciales presentaron cada uno de los terpenoides en diferentes concentraciones, reportando la mayor concentración de carvacrol en el aceite esencial de clavo (303 mg mL^{-1}) y en orégano (1.20 mg mL^{-1}); el terpineno se presentó en mayor cantidad en el aceite esencial de menta (4.83 mg mL^{-1}); para el aceite de menta y romero, linalol fue más elevado y para el limoneno la mayor concentración fue en el aceite de eucalipto (449 mg mL^{-1}) y romero (12.42 mg mL^{-1}). Para la producción de gas en las digestibilidades el aceite esencial de eucalipto a dosis de 0.3 presentó 176 mL g^{-1} MS. Para digestibilidad *in vitro* el aceite de romero en dosis alta (0.6 mL), presentó la mejor fermentación ruminal ya que tuvo mejor mitigación de metano (716.83 mM/L) sin afectar de manera negativa la concentración de AGVs (acetato, 1892.2; propiónico, 526.14; butírico, 24.99 mM/L), así como los terpenoides timol, linalol y limoneno en dosis alta. Se concluye que la mejor fermentación ruminal *in vitro* con mitigación de metano se observó con el aceite de romero y para los compuestos terpenoides fueron timol, linalol y limoneno en la dosis alta.
Palabras clave: Plantas, Compuestos terpenoides, Ácidos grasos volátiles y Metano.

INTRODUCTION

Aromatic or shrub plants have been widely used empirically in traditional medicine to treat different health conditions (Cruz *et al.,* 2017; Yuan *et al.,* 2016), however, the effects of these have generated interest in livestock production systems, since with the implementation of plant additives, rumen fermentation can be effectively modified by inhibiting deamination and methanogenesis that results in an enteric methane reduction (CH_4), NH_3-N and acetate. Therefore, a higher concentration of propionate and butyrate will be produced; as well as a decrease in enteric CH_4, which is an important greenhouse gas (Kurniawati *et al.,* 2020; Wang *et al.,* 2016; Kim *et al.,* 2015).
The essential oils of plants (EOP) are volatile aromatic compounds, constituted by a mixture of secondary metabolites (SM); mainly terpenic compounds, phenylpropanoids; monoterpenes, sesquiterpenes and alcohols, aldehydes, ethers, esters, ketones and phenols; which are primarily responsible for aroma (Bakkali *et al.,* 2008). EOPs have a wide range of antimicrobial and antioxidant activity (Gallegos-Flores *et al*., 2019), which is why they have generated interest as a natural alternative using chemical compounds to modify ruminal fermentation, since synthetic additive implementation has been limited by residue appearance in products for human consumption, or by the resistance generated by certain microorganisms due to the non-therapeutic use of antibiotics (ionophores) in ruminants (Brown *et al.,* 2017; Estévez and Cutuli, 2011). Some of the more common main EOP compounds include: thymol and carvacrol (thyme and oregano), eugenol (cloves), pinene (juniper), limonene (dill), 1,8-cineole (eucalyptus), cinnamaldehyde (cinnamon), capsaicin (hot peppers), terpinene (tea tree), allicin (garlic), and anethol (anise) (Kurniawati *et al.,* 2020).

There are aromatic plants that have been used as edible spices, and from which their essential oils are rich in terpenoid compounds with strong antimicrobial activity, which can affect the development and ruminal bacteria growth and inhibit methanogenesis. Among the oils are: cinnamon (*Cinnamomum zeylanicum*), clove (*Syzygium aromaticum*), eupcaliptus (*Eucalyptus* spp), peppermint (*Mentha spicata*), oregano (*Origanum vulgare*) and rosemary (*Salvia rosmarinus*) (Condo *et al.,* 2018; Firmino *et al.,* 2018; Dhakad *et al.,* 2017) Given the concern about the production of greenhouse gases that contribute to global warming, mainly methane emitted by ruminants, it is necessary to investigate the essential oil use, since they have an antibacterial effect, it is deduced that they influence the rumen microbiota, and therefore modify fermentation and decrease methane concentration.

The objective of the present research was to evaluate different essential oils and terpenoid compounds, to increase ruminal fermentation and volatile fatty acid production, attenuating methane generation.

MATERIAL AND METHODS

Biological material

Plants of cinnamon (*Cinnamomum zeylanicum*), cloves (*Syzygium aromaticum*), eucalyptus (*Eucalyptus* spp), peppermint (*Mentha spicata*), oregano (*Origanum vulgare*) and rosemary (*Salvia rosmarinus*) were used.

Obtaining essential oils from plants

The essential oil samples were obtained from the dried sample by hydrodistillation for 2 h, using a modified Clevenger system. During the boiling process, the dry material absorbs the water and the essential oil diffuses through the cell walls by means of osmosis, then it is vaporized and carried away by the evaporator current (Teixeira *et al.,* 2013).

Chemical composition determined by gas chromatography

It was determined by means of a gas chromatograph (CG; Agilent Technologies 6890N series), using the polar column DB_WAXetr. The working conditions were; temperature after injection 250 °C at a pressure of 12.13 psi with a flow of He 36.5 mLmin-1. The conditions for the column were; initial temperature 50 °C from 0 to 2 minutes, with an increase of 10 °C until reaching 250 °C, using an ionizing flame detector (IFD) at a temperature of 210 °C with an H_2 flow of 40 $mLmin^{-1}$ and an air flow of 450 $mLmin^{-1}$; previously a calibration curve was made. The standards used were reagent grade Sigma Aldrich brand: carvacrol, thymol, limonene, linalool and terpinene, with a purity percentage of 98, 99.5, 98, 97 and 85% respectively. Each of the determinations was carried out in triplicate Bañuelos *et al*. (2018).

Preparation of terpenoid compounds

The terpenoid compounds used for *in vitro* digestibility were those used as standards in CG reagent grade Sigma Aldrich brand: carvacrol, thymol, limonene, linalool and terpinene prepared with 50% ethanol.

Determination of *in vitro* gas production

The sheep feeding for *in vitro* gas production was used ruminal fluid from two hair sheep, cannulated and fed a diet containing 83% hay (50% alfalfa and 50% wheat straw) and 17% of concentrate (63% ground corn, 25% of flour, 5.5% of calcium carbonate, 5.5% of mono-calcium phosphate, 0.5% of pre-mix of vitamins A, D and E and 0.5% of microminerals). Food was provided daily at 08:00 and 16:00 with free access to water. The sheep were fed for 30 days before the extraction of the ruminal fluid, as time to adapt to the ration.

***In vitro* gas production**

The oils were added individually in each of the digestibility jars in different volumes (Ugbogu *et al*., 2019). The alfalfa substrate was used as a control without additive addition. *In vitro* gas production was determined using the method proposed by Theodorou *et al*. (1994) for which fermentation units (UF) of 120 mL were used for each sample. In recording the gas produced, a Sper Scientific brand pressure gauge was used. The gas pressure was cumulative and determined in pressure units (Psi); the measurement time was at 3, 6, 9, 12, 24 and 48 h; for each volume of the different oils, performing three repetitions.

Determination of volatile fatty acids in ruminal fluid

The VFAs (acetic, propionic and butyric) were quantified by gas chromatography. The working conditions were; inlet temperature after sample injection is 50 °C at a pressure of 12.13 psi with a flow of He 36.5 mL min^{-1}. The conditions for the column were; initial temperature 50 °C, from 0 to 2 minutes with an increase of 10 °C per minute until reaching 250 °C, keeping this temperature constant for 5 minutes, and then dropping to 50 ° C maintaining for two minutes with a flow of I have 1.6 mL min^{-1} at a pressure of 12.13 psi and an average speed of 25 cm s^{-1}. An ionizing flame detector (IFD) was used at a temperature of 210 °C with a H_2 flow of 40 mL min^{-1} and an air flow of 450 mL min^{-1}. A calibration curve was previously made. The standards used were Sigma Aldrich brand reagent grade: acetic, propionic and butyric, with a purity percentage of 99.5, 98 and 99% respectively. Each of the determinations was carried out in triplicate.

Methane determination

Methane was inferred from the VFA concentration, by applying non-linear mathematical models established by Moss *et al*. (2000), where it is pointed out that CH_4 production can be calculated stoichiometrically, using the following equation:

$$[CH_4] = 0.45\ C_2(acetate) - 0.275\ C_3(propionate) + 0.4\ C_4\ (butyrate)$$

Statistical analysis

The statistical analysis for gas production, volatile fatty acids and methane was carried out through variance analysis, using a completely random design and the Tukey's test of means; using the SPSS® statistical package to evaluate statistical differences ($p < 0.05$) (Cytel Software, 2010). The source of variation considered were essential oils and terpenoid compounds; for volatile fatty acids, the following variables were considered: acetic, propionic and butyric acid.

RESULTS AND DISCUSSION

For the essential oils of cinnamon (*Cinnamomum zeylanicum*), clove (*Syzygium aromaticum*), eucalyptus (*Eucalyptus* spp), peppermint (*Mentha spicata*), oregano (*Origanum vulgare*) and rosemary (*Salvia rosmarinus*), it was observed that the highest carvacrol and thymol concentration is present in clove oils (carvacrol 303 mgmL^{-1}), and oregano one (carvacrol, 1,652 mg mL^{-1}; thymol, 0.247 mg mL^{-1}) (Table 1). These two compounds have been reported to have an antibacterial effect against gram negative and positive bacteria, for which it is known that the action mechanism is to embed themselves in the bacterial cell membrane, causing this structure disintegration, followed by cell lysis (Rodríguez-García *et al*., 2015; Friedman, 2014; Béjaoui *et al*., 2013; García-García *et al*., 2011). Therefore, this antibacterial effect can influence microbiota ruminal population and therefore modify ruminal fermentation.

Table 1. Terpenoid compounds present in essential oils analyzed by gas chromatography

OIL	Carvacrol mg mL^{-1}	Thymol mg mL^{-1}	Linalool mg mL^{-1}	Terpinene mg mL^{-1}	Limonene mg mL^{-1}
Cinnamon essential oil	0.0375	0.0108	0.047	0.1431	2.5167
Clove essential oil	303	0.0068	0.0383	0.2753	1.5496
Eucalyptus essential oil	0.07	0.0142	0.4621	0.8725	499
Peppermint essential oil	0.0169	0.025	3.9401	4.8388	9.56
Oregano essential oil	1.652	0.2474	0.0878	0	0.1449
Rosemary essential oil	0.0524	0.0753	8.865	0.3725	12.425

Nile *et al*. (2017) report that essential oils are rich in terpenes (carvacrol, citral, linalool and geraniol); and phenolic compounds coinciding in the present work, since both compounds were found. Albado *et al*. (2001) reported the presence of terpenoid compounds, phenols and compounds metabolically related to carvacrol in essential oils of oregano; therefore, this research coincides with the present study, since the terpenoids in oregano oil (carvacrol, thymol and linalool), although in different concentrations. Bañuelos *et al*. (2018) mention that terpenoids constituted 11.2% of the oil with α-pinene (1.3%), limonene (3%) and 1,8-cineole (2.9%), as the main monoterpenes in the essential oil of oregano and *R. graveolens*. The presence of limonene in the present investigation coincided with these results.

The identified compounds are important for their pharmacological activity; for example, limonene is antibacterial, antifungal, antiseptic, and antiviral; thymol is antibacterial, antifungal, anti-inflammatory, antioxidant, antirheumatic and antiseptic; carvacrol is antibacterial, antifungal, anti-inflammatory, antiseptic, antispasmodic, and expectorant (Sorentino and Landmesser, 2005).

In the *in vitro* digestibility technique, the highest obtaining of gas in the *in vitro* digestibility technique (Table 2) was observed in the essential oil of eucalyptus in its three doses (0.1 = 157.59 ± 3.62 mL g^{-1} DM, 0.3 = 176.86 ± 1.10 mL g^{-1} MS and 0.6 = 175.30 ± 3.62 mL g^{-1} DM), Wang *et al*. (2016) reported that when using medicinal plant extracts there is not always a tendency to increase the ruminal gas concentration (mL g^{-1} DM); as some of them may have the opposite effect.

In terpenoid compounds, a low gas production is observed for the 0.6 mL dose, registering 48.24 ± 3.41, 29.77 ± 4.87, 28.63 ± 0.50, 28.98 ± 3.09 and 29.80 ± 3.12 mL g^{-1} DM ± SD (thymol, carvacrol, linalool, limonene and terpinene respectively). Chouhan *et al*. (2017) and Gallegos-Flores *et al*. (2019) report that the secondary metabolites (terpenoids) of plants are recognized as antimicrobial agents that act against bacteria, protozoa and fungi. Therefore, this effect is reflected in the low gas production, because it inhibits the growth of ruminal methanogenic bacteria, and therefore acetic, propionic and butyric acids are those that are generated in greater quantity during the fermentation of the substrates in the rumen.

Gas concentration (total), VFAs and CH_4 are presented in (table 3); propionic acid production was completely inhibited in the essential oils of eucalyptus and oregano (dose 0.1); while in the terpenoid compounds, gas production was inhibited in thymol, dose 0.3; carvacrol 0.6; linalool 0.3; limonene 0.1 and 0.3 and terpinene 0.1; except for rosemary essential oil (dose 0.1). All doses decreased propionic acid production compared to the alfalfa control. The production of butyric acid was inhibited in the essential oil of cloves and cinnamon in doses of 0.1 and 0.3; while the highest production of butyric was presented in limonene at the dose of 0.1 (684.93 ± 0.09 mM/L±SD), but acetic and propionic production is inhibited.

Sejian *et al*. (2015) point out that 40 to 60 % of the total greenhouse gases (GHG) from livestock come from enteric fermentation, manure management and the different activities related to obtaining food for animals. Therefore, terpenoid compounds show a decrease in methane; such is the case of limonene in its 0.1 dose, presenting high production of butyric acid, but inhibition of acetic and propionic.

Table 2. Gas production with the different doses of oils and terpenoids

Sample	Dose (mL)	Total gas production in mL g^{-1} DM±SD					
		3 h	6 h	9 h	12 h	24 h	48 h
Rosemary essential oil	0.1	11.67±0.14	28.52±0.46	51.13±1.44	75.38±2.29	108.57±0.16	144.94±1.44
	0.3	11.87±1.85	27.87±3.56	49.09±6.15	72.13±9.32	108.80±10.17	146.98±5.55
	0.6	9.26±0.04	22.84±6.01	40.39±15.97	58.95±28.24	94.41±10.17	139.13±3.73
Clove essential oil	0.1	6.39±5.12	11.42±5.90	17.91±1.07	22.64±1.35	28.22±10.49	35.76±6.15
	0.3	9.15±1.96	15.39±2.81	19.42±1.07	20.72±1.35	21.58±4.69	22.48±9.39
	0.6	9.31±0.11	14.34±0.75	17.81±1.14	19.01±1.21	19.77±1.28	20.72±1.24
Eucalyptus essential oil	0.1	13.63±2.31	33.90±4.23	61.06±6.26	88.38±7.61	122.18±10.49	157.59±3.62
	0.3	16.90±0.96	39.89±2.28	69.92±3.98	99.14±4.45	137.02±3.09	176.86±1.10
	0.6	15.54±5.69	36.67±7.86	64.28±5.39	92.85±4.45	132.64±13.00	175.30±3.62
Peppermint essential oil	0.1	8.50±3.45	18.01±2.19	27.26±0.59	33.50±3.72	45.97±11.79	65.29±4.49
	0.3	9.26±0.53	14.64±2.38	20.93±4.48	23.29±7.22	24.85±4.94	26.26±7.60
	0.6	7.49±1.24	11.42±2.28	14.24±4.73	15.39±5.58	16.20±6.12	16.80±6.69
Oregano essential oil	0.1	13.38±0.71	21.10±0.82	26.43±0.92	28.24±0.84	29.30±0.78	30.66±0.82
	0.3	14.39±1.64	22.26±2.40	27.74±2.60	29.43±2.38	30.41±2.15	31.82±1.96
	0.6	12.07±2.28	18.86±3.79	24.07±4.41	26.06±4.52	27.36±4.48	29.05±4.68
Cinnamon essential oil	0.1	14.11±3.22	24.47±8.41	31.71±2.72	34.58±37.90	35.91±3.68	37.88±9.66
	0.3	14.18±0.05	22.01±1.74	27.87±2.72	29.83±3.36	30.73±3.66	32.39±3.88
	0.6	15.29±0.78	24.22±1.56	30.31±1.73	32.44±1.85	33.70±2.10	35.66±2.31
Thymol	0.1	1.95±0.37	5.03±0.53	10.04±1.20	33.74±4.08	41.26±4.49	47.99±4.92
	0.3	2.34±0.04	5.37±0.06	9.89±0.46	31.84±2.53	39.17±3.13	45.83±3.55
	0.6	2.14±0.14	4.79±0.70	9.28±1.16	31.82±3.22	40.19±3.91	48.24±3.41
Carvacrol	0.1	2.17±0.04	5.01±0.12	9.68±0.43	14.97±0.46	22.28±0.15	28.47±0.38
	0.3	2.52±0.01	5.38±0.04	9.70±0.25	14.86±0.38	21.73±0.39	29.06±0.85
	0.6	2.03±0.08	4.72±0.51	9.07±0.48	13.90±1.21	21.67±2.74	29.77±4.87
Linalool	0.1	2.06±0.04	5.61±0.04	10.80±0.04	16.31±0.04	22.79±0.11	30.37±1.90
	0.3	2.09±0.29	5.47±0.33	10.31±0.45	16.54±1.89	23.65±3.07	31.98±1.86
	0.6	2.04±0.03	5.27±0.14	9.88±0.33	15.05±0.46	21.64±0.33	28.63±0.50
Limonene	0.1	1.97±0.04	5.35±0.03	10.31±0.05	15.63±0.06	21.97±1.43	29.18±0.09
	0.3	2.16±0.02	5.59±0.01	10.48±0.00	15.86±0.06	22.36±0.01	30.68±2.00
	0.6	1.95±0.02	4.60±0.03	8.70±0.27	13.50±0.63	20.10±0.82	28.98±3.09
Terpinene	0.1	2.02±0.06	5.39±0.18	10.19±0.31	15.37±0.51	21.41±0.07	28.71±2.30
	0.3	2.04±0.12	5.22±0.11	9.73±0.04	14.60±0.03	21.04±0.31	28.92±1.67
	0.6	2.24±0.19	4.90±0.28	8.87±0.43	13.48±0.69	20.64±0.87	29.80±3.12
Alfalfa	0	2.19±0.04	4.96±0.41	8.86±0.96	13.53±0.92	22.12±1.13	32.30±5.41

* DM: dry matter. SD: Standard deviation.

Table 3. Total gas production (mL g^{-1} DM), volatile fatty acids (mM/L) and *in vitro* methanoid in the different essential oils and terpenoids used

Sample	Dose (mL)	Total gas production (mL g^{-1} DM) ± *SD	Volatil fatty acids (mM/L) ± *SD			Methane mM/L
			Ácetic acid	Propionic acid	Butyric acid	
Rosemary essential oil	0.1	144.94±1.44[a]	2380.5±0.02[a]	782.20±0.15[a]	43.62±0.19[b]	873.57±0.06[bc]
	0.3	146.98±5.55[a]	2154.3±0.03[a]	664.61±0.16[a]	32.88±0.19[b]	799.85±0.07[bc]
	0.6	139.13±3.73[a]	1892.2±0.04[a]	526.14±0.16[a]	24.99±0.19[b]	716.83±0.07[bc]
Clove essential oil	0.1	35.76±6.15[c]	435.87±0.13[b]	39.15±0.19[b]	0.00±0.00[c]	185.37±0.01[a]
	0.3	22.48±9.39[c]	312.6±0.13[b]	40.46±0.19[b]	0.00±0.00[c]	129.54±0.01[a]
	0.6	20.72±1.24[cd]	322.25±0.13[b]	40.85±0.19[b]	15.16±0.19[bc]	139.85±0.12[a]
Eucalyptus essential oil	0.1	157.59±3.62[a]	2343.2±0.02[a]	0.00±0.00[c]	617.94±0.10[a]	1301.62±0.07[c]
	0.3	176.86±1.10[a]	2291.8±0.02[a]	676.56±0.16[a]	37.97±0.19[b]	860.46±0.06[bc]
	0.6	175.30±3.62[a]	2514.2±0.01[a]	764.87±0.43[a]	46.61±0.19[b]	939.72±0.06[bc]
Peppermint essential oil	0.1	65.29±4.49[b]	244.9±11.77[b]	51.76±25.88[a]	18.39±3.82[bc]	103.32±33.26[a]
	0.3	26.26±7.60[c]	868.6±12.01[ab]	51.00±25.69[a]	106.3±44.19[ab]	419.42±33.55[b]
	0.6	16.80±6.69[d]	234.92±4.36[b]	52.84±0.19[b]	21.46±0.19[b]	99.77±2.74[a]
Oregano essential oil	0.1	30.66±0.82[c]	121.7±0.14[b]	0.00±0.00[c]	10.32±0.19[bc]	58.89±0.20[a]
	0.3	31.82±1.96[c]	117.0±0.15[b]	21.72±0.19[b]	17.95±0.19[bc]	53.88±0.13[a]
	0.6	29.05±4.68[c]	105.6±0.15[b]	25.85±0.19[b]	17.56±0.19[bc]	47.48±0.13[a]
Cinnamon essential oil	0.1	37.88±9.66[bc]	473.2±0.12[b]	108.35±0.19[ab]	0.00±0.00[c]	183.15±0.01[a]
	0.3	32.39±3.88[c]	249.48±0.14[b]	47.47±0.19[b]	0.00±0.00[c]	99.21±0.01[a]
	0.6	35.66±2.31[c]	323.43±0.13[b]	72.03±0.19[b]	39.63±0.19[b]	141.59±0.12[a]
Thymol	0.1	47.99±4.92[bc]	1884.20±0.04[a]	720.35±0.15[a]	39.87±0.19[b]	665.74±0.08[b]
	0.3	45.83±3.55[bc]	1874.53±0.24[a]	0.00±0.00[c]	551.46±0.11[a]	1064.12±0.21[c]
	0.6	48.24±3.41[bc]	1824.96±0.05[a]	696.55±0.15[a]	34.59±0.19[b]	643.52±0.08[b]
Carvacrol	0.1	28.47±0.38[c]	1933.54±0.23[a]	0.00±0.00[c]	603.55±0.10[a]	1111.51±0.20[c]
	0.3	29.06±0.85[c]	1826.95±0.05[a]	724.29±0.15[a]	35.87±0.19[b]	637.30±0.08[b]
	0.6	29.77±4.87[c]	1436.98±0.01[a]	0.00±0.00[c]	438.03±0.13[a]	821.85±0.08[bc]
Linalool	0.1	30.37±1.90[c]	1868.50±0.05[a]	731.91±0.15[a]	38.56±0.19[b]	654.98±0.08[b]
	0.3	31.98±1.86[c]	1891.33±0.04[a]	0.00±0.00[c]	582.39±0.11[a]	1084.05±0.09[c]
	0.6	28.63±0.50[c]	1908.58±0.04[a]	713.00±0.15[a]	33.15±0.19[b]	676.05±0.08[b]
Limonene	0.1	29.18±0.09[c]	1990.96±0.02[a]	0.00±0.00c	603.49±0.10[a]	1137.33±0.07c
	0.3	30.68±2.00[c]	1909.62±0.04[a]	0.00±0.00[c]	567.93±0.11[a]	1086.50±0.09[c]
	0.6	28.98±3.09[c]	1755.33±0.05[a]	625.03±0.34[a]	35.26±0.19[b]	632.12±0.27[b]
Terpinene	0.1	28.71±2.30[c]	0.00±0.00[c]	0.00±0.00[a]	684.93±0.09[a]	273.97±0.05[ab]
	0.3	28.92±1.67[c]	1990.91±0.04[a]	762.28±0.15[a]	40.87±0.19[b]	702.63±0.07[bc]
	0.6	29.80±3.12[c]	1886.46±0.27[a]	0.00±0.00[c]	583.48±0.10[a]	1082.30±0.23[c]
Alfalfa	0	32.30±5.41[c]	1673.52±0.06[a]	775.33±0.15[a]	43.50±0.19[b]	557.27±0.08[b]

* SD: Standard deviation, mean values with different letters in the same column differ statistically ($p<0.05$).

CONCLUSIONS

Methane mitigation was observed with rosemary oil at its maximum dose in *in vitro* ruminal fermentation; since it presents increased concentrations of VFAs (acetic, propionic and butyric). Terpenoid compounds with the best ruminal fermentation *in vitro* were thymol, linalool and limonene in the maximum dose. It is suggested to deepen the use of essential oils of plants, because they could be an alternative in the search for organic products with greater sustainability.

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