Original Article. January-December 2019; Vol. 9. Received: 26/02/2018 Accepted: 25/07/2018

http://dx.doi.org/10.21929/abavet2019.91

Differential evaluation of oregano extracts in the production of volatile fatty acids and methane during ruminal fermentation *in vitro*

Evaluación diferencial de extractos de orégano en la producción de ácidos grasos volátiles y metano durante fermentación ruminal *in vitro*

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ABSTRACT

The objective was to evaluate the effect of oregano extracts on ruminal fermentation in vitro in the production of gas, volatile fatty acids (VFA) and methane. Four preparations of oregano (*Lippia graveolens*) were obtained with different methodology; for an aqueous medium, were obtained two extracts (cooking and infusion), which were prepared with triple-destilled water. The alcoholic extract was prepared in ethanol: water (80:20, v/v) and finally the oily extract was obtained by hydrodistillation for one hour in a modified Clevenger equipment. The oregano extract with the best mitigation of methane was the oil extract by reducing the concentration of this gas three times with respect to the control 160.27 mM/L and 463.73 mM/L respectively. It can be concluded that according to the methodology used in the preparation of oregano extracts, it is the type of chemical structure and concentration of active ingredients that were found in each extract, so these differences are what marked the effects during the *in vitro* ruminal fermentation on the activity of ruminal microorganisms in the production of gas, VFA and methane.

Keywords: Volatile Fatty Acid, Extracts, Fermentation ruminal and Methane.

RESUMEN

El objetivo fue evaluar el efecto de extractos de orégano sobre la fermentación ruminal *in vitro* en la producción de gas, ácidos grasos volátiles (AGVs) y metano. Se realizaron cuatro preparaciones de orégano (*Lippia graveolens*) con diferente metodología; para un medio acuoso, se obtuvieron dos extractos (cocción e infusión), los cuales fueron preparados con agua tridestilada. El extracto alcohólico, fue preparado en etanol: agua (80:20, v/v) y finalmente el extracto oleoso se obtuvo por hidrodestilación durante una hora en un equipo clevenger modificado. El extracto de orégano con mejor mitigación de metano fue el oleoso al reducir tres veces la concentración de este gas con respecto al control; 160.27 mM/L y 463.73 mM/L respectivamente. Se puede concluir que, de acuerdo a la metodología empleada en la preparación de los extractos de orégano, es el tipo de estructura química y concentración de principios activos que se encontraron en cada extracto, por lo que, estas diferencias son las que marcaron los efectos durante la fermentación ruminal *in vitro* sobre la actividad de microorganismos ruminales en la producción de gas, AGVs y metano.

Palabras clave: Ácidos Grasos Volátiles, Extractos, Fermentación ruminal y Metano.

INTRODUCTION

Greenhouse gas (GHG) emissions and global warming are topical issues facing environmental organizations and the population in general. The gases emitted to the atmosphere by man include 82.5% carbon dioxide (CO_2), 9.5% methane (CH_4), 5.3% nitrogen oxides (NXO) and 2.7% halogenated gases. CH_4 emissions have generated great interest in organizations with environmental impact since it has a global warming potential 25 times greater than CO_2 (IPCC, 2015).

Within anthropogenic sources, agricultural activity is responsible for approximately 26% of total CH_4 emissions, due to the anaerobic enteric fermentation of food (USEPA, 2015). The methane produced by cattle (especially dairy and fattening) represents an energy loss of approximately 2 to 12% of the intake (Ingale *et al*., 2013, Vargas, 2012, Benchaar and Greathead, 2011), this being a of the most important inefficiencies in the nutrition systems of ruminants, therefore, the mitigation in the production of this gas can be of socio-economic benefit (adequate use of the energy efficiency of the food to obtain more meat and milk) and environmental (decrease in the emission of CH_4) (Moss *et al*., 2000).

In the rumen, organic matter (carbohydrates and proteins) is degraded by symbiotic microorganisms, where the final products are volatile fatty acids (VFAs) (acetic, propionic and butyric), as well as carbon dioxide (CO_2) and hydrogen (H_2). The formation of acetate and butyrate results in the production of H_2, a substrate that archaea bacteria (commonly called methanogens) use to reduce carbon dioxide and produce methane (Li *et al*., 2014; Attwood *et al*., 2008), on the other hand, the production of propionate serves as a competitive route for the utilization of H_2 which leads to a decrease in the total methane production (Li *et al*., 2014; Attwood *et al*., 2008), therefore , when different types of food are fermented in rumen, the molar proportion of the components of AGVs (acetate, propionate, butyrate) is different.

At present, there are methodologies and/or technologies that are used to reduce the amount of CH_4 produced by ruminants (Moumen *et al*., 2016); among them the implementation of chemical additives such as ionophores (eg monensin) in cattle feed (Crossland *et al*., 2017), however, the use of these chemicals has been limited by the residual presence of secondary metabolites in the products for human consumption or for the resistance generated by certain microorganisms due to the non-therapeutic use of antibiotics (ionophores); therefore, these facts led to its prohibition in some legislations such as the European Union since January 2006 (Estévez and Cutuli, 2011). These measures gave rise to the search for new strategies and alternatives aimed at the use of natural additives such as "plant extracts", which are an option in livestock feeding to modify their ruminal fermentation in a positive way and mitigate CH_4 emissions enteric (Wang *et al*., 2016; Kim *et al*., 2015; Durmic *et al*., 2014). The plant extracts contain bioactive compounds that have been investigated as an alternative in animal

nutrition to manipulate food degradability and fermentation, this effect is related to its chemical composition and / or antimicrobial properties (Irshaid *et al*., 2014; Ramdani *et al*. ., 2013; Patra and Saxena, 2010).

Investigations focused on exploring the effects of different essential oils, plant extracts and bioactive compounds in ruminal fermentation *in vitro* and *in vivo* (Friedman 2014, Khiaosa and Zebeli, 2013, Klevenhusena *et al*., 2012), have found that oregano (Origanum vulgare) can serve as an alternative plant because of its high antioxidant capacity and its antimicrobial potential. The main chemical constituents of oregano are carvacrol, ρ-cimeno, linalool, terpinene and thymol (Rodriguez-Garcia et al., 2015, Grondona *et al*., 2014, Acevedo *et al*., 2013, Teixeira *et al*., 2013); these compounds have antimicrobial effects that can affect the growth and growth of ruminal bacteria and inhibit methanogenesis; that is why oregano extract has been considered as an option to mitigate enteric methane emissions and improve ruminal fermentation with the production of AGVs (Durmic *et al*., 2014; Hristov *et al*., 2013; Kim *et al*., 2013 Bodas *et al*., 2008). In this work different extracts of oregano (aqueous, alcoholic and oily) were evaluated by means of the technique of *in vitro* gas production, as an alternative to reduce the emissions of ruminal CH_4, as well as the effect on the production of gas and AGVs

MATERIAL AND METHODS

The oregano plant (*Lippia graveolens*) belonging to the Verbenaceae family, it was collected in September 2015 in the municipality of Valparaíso, Zacatecas, Mexico, located at 23 ° 10 'north latitude and 104 ° 22' longitude west, located at an altitude between 700 and 3000 m a.s.l the average annual temperature is 12 to 24 ° C and an annual rainfall of 500 to 1000 mm (INEGI, 2015). The plant was allowed to dry at room temperature for 2 weeks after cutting. It was then dehydrated at 45 ° C in a Thermo Scientific® oven for 24 h to completely remove moisture.

The extracts were obtained from the dehydrated and crushed plant (mixture of leaves, flowers and stems). The extracts were prepared in three different media; watery, alcoholic and oily.

For an aqueous medium, two extractions were obtained: cooking and infusion. The ratio used was 25: 200 (grams of ground sample per 200 milliliters of triple-distilled water). For the determined cooking process, 250 g of the dehydrated plant together with 2 L of triple-distilled water were placed in a Pyrex glass flask and brought to a boiling point for 30 min in a Lab Tech® heating plate. At the end of this time the flask was removed from the heat source, allowed to stand for 10 minutes and filtered through Whatman paper no. 4. In the infusion extraction preparation, the triple-distilled water (2 L) was boiled for 5 minutes, the flask was removed from the heat source and immediately the oregano sample (250 g) was added, allowed to stand for 10 minutes and leaked on Whatman paper no. 4 (Martins et al., 2014;

Teixeira et al., 2013); during the whole process of preparation of both extracts, the flasks were kept covered to avoid the loss of water vapor. All the extracts were stored in amber flasks.

The alcoholic extraction was carried out using 250 g of milled sample in 2 L of absolute ethanol (J.T.Baker) and triple-distilled water (80:20, v / v); the mixture was placed in an amber flask and macerated for a month with shaking every third day, filtered through Whatman paper no. 4 and finally, 70% of the total volume of the solvent was vaporized in a Soxtec System HT type extractor (Fisher Scientific 1043) at 85 ° C for 45 min (Pesewu *et al*., 2008).

The oily extract was obtained from the dry sample (250 g) by hydrodistillation for one hour in a modified Clevenger system of Pyrex glass using triple-distilled water (Grondona *et al*., 2014). 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 dragged by the steam stream until it is condensed and finally it is recovered (Teixeira *et al*., 2013). Previously, hydrodistillations were carried out with lapses of different hours to establish the optimum time of operation, concluding that one hour was sufficient for a good extraction of the oil.

The concentration of active ingredients in each of oregano extracts was determined by gas chromatography (GC) on a Agilent Technologies series 6890N, using a polar column DB_WAX (J & W Scientific) Agilent Technologies with the following specifications: length 30 m , ID 0.320 mm, film 0.50 µm and temperature limits from 20 ° C to 240 ° C (250 ° C). Before testing, a calibration curve was performed with standard carvacrol, thymol, limonene, linalool and terpinene Sigma Aldrich reagent grade mark with 98, 99.5, 98, 97 and 85 % of purity respectively; from the chromatograms obtained by the CG analysis, the data were plotted using a linear regression for each of the standards used, observing a value of R> 0.999 for each of the compounds.

The *in vitro* gas production technique was performed as indicated by Theodorou *et al*., (1994); as a ground substrate, alfalfa was used, to maintain an anaerobic medium, CO_2 was used, the incubation temperature was 39 °C. The ruminal inoculum was obtained from two male sheep cannulated of Rambouillet race with one year old (weight 45 ± 3 kg) plus an adaptation 30 days which contained 83% hay (alfalfa and wheat straw) and 17% concentrate (ground corn, flouroline, macrominerals and microminerals). The treatment and care of the sheep was carried out according to the health and animal welfare protocols of the Academic Unit of Veterinary Medicine and Zootechnics of the Autonomous University of Zacatecas.

The extracts were added individually in each of the digestibility jars in different volumes (Agarwal *et al*., 2009); for a low and high volume, 0.1 and 1 mL of the oregano extract were

added respectively for each 100 mL of artificial ruminal medium, as a control the alfalfa substrate was used without the addition of additives. In the gas register produced, a Sper Scientific brand pressure meter was used. The gas pressure was cumulative and determined in units of pressure (Psi), the measurement time was at 3, 6, 9, 12, 24 and 48 h. For each volume of the different extracts, three repetitions were made.

The AGVs (acetic, propionic and butyric) were quantified by gas chromatography. The working conditions were: inlet temperature after injection of the sample 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 zero to two minutes with an increase of 10 ° C per minute until reaching 250 °C keeping this temperature constant for 5 minutes and then descending to 50 ° C keeping for two minutes with a He flow of 1.6 mL min^{-1} at a pressure of 12.13 psi and an average velocity of 25 cm s^{-1}. An ionizing flame detector (FID) was used at a temperature of 210 ° C with an H_2 flow of 40 mL min^{-1} and an air flow of 450 mL min^{-1}. Previously, a calibration curve was made with the Sigma Aldrich brand of acetic, propionic and butyric acid standards. Each of the determinations was made in triplicate.

The determination of methane was inferred from the concentration of AGVs, through the application of nonlinear mathematical models established by Moss et al. (2000), where they point out that the production of CH_4 can be calculated stoichiometrically, as shown in equation 1

$$CH_4 = 0.45 \quad C_2 \quad (acetate) - 0.275 \quad C_3 \quad (propionate) + 0.40 \quad C_4 \quad (butyrate) \quad (1)$$

The statistical analysis for the production of gas, volatile fatty acids and methane was carried out using a variance analysis using a completely randomized design and Tukey's means test, using the statistical package SAS 9.3 (SAS, 2011) to evaluate the differences statistics ($p < 0.05$). The source of variation considered was the extract of oregano (control, cooking, infusion, alcoholic and oily); for volatile fatty acids were considered as variables: acetic, propionic and butyric acid.

RESULTS AND DISCUSSION

From the mixture of leaves, flowers and stems of oregano (*Lippia gr*aveolens), the following yields were obtained shown in table 1. The yield for the four extracts (cooking, infusion, alcoholic and oily) was obtained from the volume ratio of the extract / volume of the solvent (Metoui *et al*., 2015). The highest yield was presented in the aqueous extracts; cooking and infusion with 75% and 87% respectively, followed by the alcoholic (33%). Pesewu *et al*. (2008), reported that the yields for aqueous extracts was 2.6 to 16.4%, while for alcoholics it is between 3.2 and 16%. Metoui *et al*. (2015), reported yields for aqueous and alcoholic extracts of 17.1 and 18.3% respectively, in table 1, yields are higher than those reported with the methodology used. For the oily extract, Aligiannis *et al*. (2001) reported yields for the Origanum plant of 0.6% using a modified Clevenger type apparatus for 3 hr and Metoui *et al*.

(2015), obtained 0.56% yield. For the oily extract by hydrodeslilation for one hour a yield of 0.33% was obtained.

The biological action of oregano extracts depends on the chemical composition and concentration of active principles, the type of plant and species, place of origin and the method of preparation thereof (Vokou *et al*., 1993). In table 2, it can be shown that according to the extraction method different concentrations of each of the analyzed standards were obtained, showing that the infusion was the method in which the least amount of active ingredients was extracted, with the presence of only two of them (limonene and thymol), however, the concentration of limonene (65,984 mg / mL) was higher in the infusion than in the rest of the other extracts. Carvacrol was dominant in the oil extract followed by the alcoholic, but there is no presence of this compound for the aqueous extracts. The highest active ingredient was terpinene with 15,700 mg / mL, presented in the alcohol extract.

Previous studies have reported that the major components in extracts of oregano (*Origanum vulgare*) are carvacrol and its thymol isomer, followed by its precursor terpinene (Rodríguez-García *et al.*, 2015 *;* Grondona *et al.*, 2014; Béjaoui *et al.*, 2013a). According to the results obtained, the highest amount of carvacrol was found in the oily extract with 165,201 mg / mL. Thymol was found in all preparations of oregano, showing the highest concentration of the alcoholic with 26.713 mg / mL, while the second highest was the oily with 24.741 mg / mL; this makes it clear once again that according to the extraction method different bioactive compounds as well as different concentrations will be obtained.

Acevedo *et al.* (2013), reported the chemical composition of the essential oil of the species *Origanum vulgare*, finding as a major compound thymol with 67.51%, followed by terpinene with 5.51%. In the present study, it was found that, for the oily extract, carvacrol was the compound with the highest concentration, being different from that reported by Acevedo et al. (2013), this variant may be due to the type of oregano plant used, since the species used for

Table 1. Performance of oregano extracts

Extract of oregano	Weight (g)	Solvent (mL)	Vol. extract (mL)	Percent Yield (%)
Cooking	250	2000	1500	75
Infusion	250	2000	1750	87.5
Alcoholic	250	2000	673	33.6
Oily	250	2000	6.5	0.33

Table 2. Concentration and chemical composition of oregano extracts (*Lippia graveolens*).

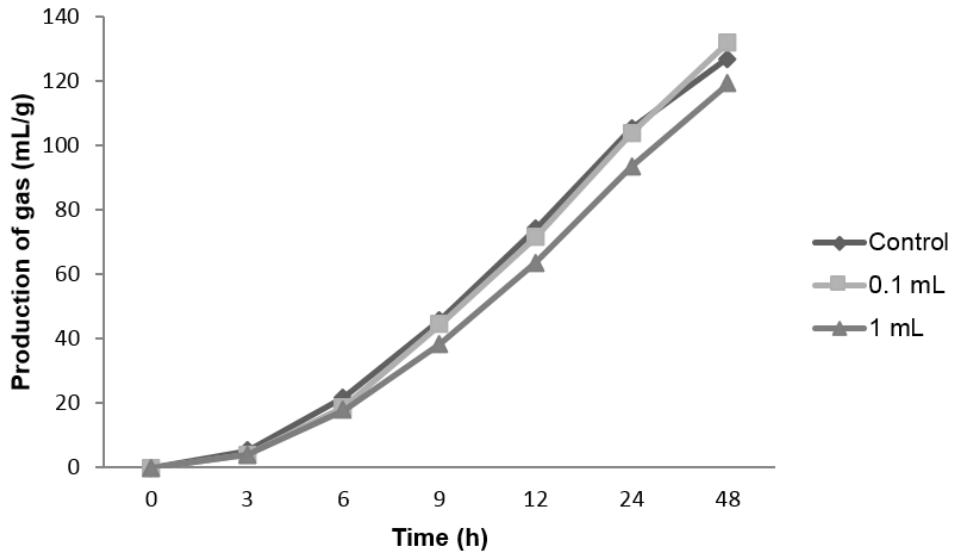
Extract	Terpinene (mg/mL)	Limonene (mg/mL)	Linalool (mg/mL)	Thymol (mg/mL)	Carvacrol (mg/mL)
Cooking	71.746	0	1.828	4.893	0
Infusion	0	65.984	0	8.749	0
Alcoholic	15,700	0	10.545	26.713	3.235
Oily	0	14.494	8.788	24.741	165.201

this research was *Lippia graveolens*. Wogiatzi *et al*. (2009), analyzed the species of *Origanum vulgare* collected from two different regions in Greece; finding that there is a difference in the composition of active ingredients according to the place of origin of the plant, since in the region of Leptokaria, the presence of carvacrol was high while in another place this compound was very low. In order to make a comparison with works that analyze the chemical composition of the same genus of oregano in the same country of origin, we have the work of Ortega et al. (2011), where they analyzed the essential oil of the species *Lippia palmeri* S. from the Hermosillo Sonora region, Mexico and found that the compound with the highest concentration was the p-cymene compound (22.3%), followed by thymol (21.3%).), carvacrol (8.8%), terpinene (6.7%), and limonene (1.2%), among other compounds. According to the results obtained from table 2, for *Lippia graveolens* collected in Valparaíso Zacatecas, Mexico; It was found that for the oily extract the main compound found was carvacrol, followed by thymol, limonene and linalool, with this it is clear that the chemical composition and concentration of active ingredients depends on the type of plant and species, place of origin and the method of preparation of the same.

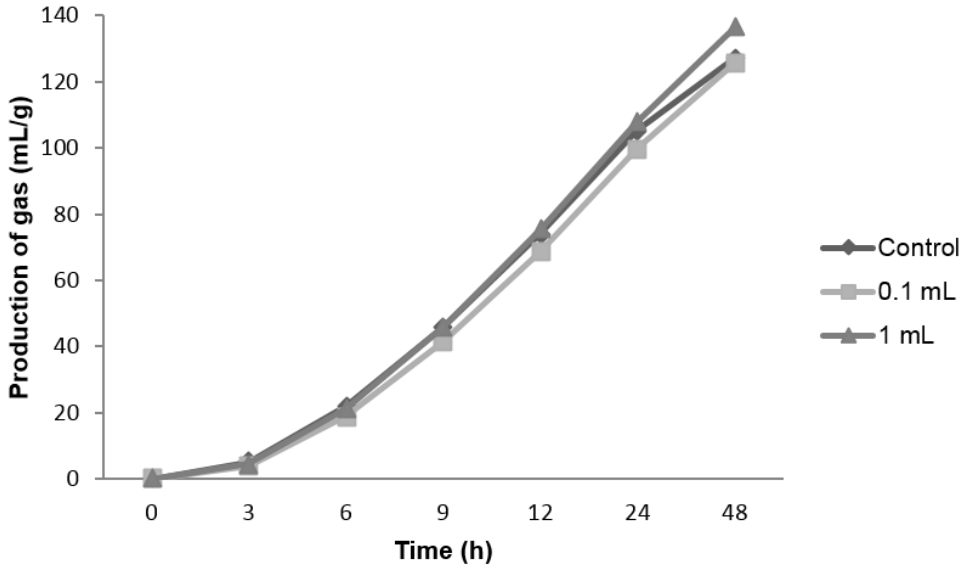
Once the technique of production of in vitro gas with each of the extracts of oregano (cooking, infusion, alcoholic and oily) in a ratio volume of the extract for each 100 mL of artificial ruminal medium (Agarwal *et al*., 2009), the production of gas, AGVs and CH4 was determined.

The effect of the different extracts of oregano in the production of gas (mL / g) *in vitro* in a period of 48 h, is shown in graphs 1, 2, 3 and 4; being observed that for the aqueous extracts; cooking (graph 1) and infusion (graph 2) has a similar behavior with the control. For the alcoholic (graph 3), with a high volume (1 mL of extract / 100 mL of ruminal medium) there was a small decrease at 12 h in the formation of gas, however, at the end of 48 h it reached the same gas production as the control. As regards the oily extract (graph 4), gas production was considerably reduced in both volumes (0.1 and 1 mL). In the extracts of cooking, infusion and alcoholic, it was observed that the exponential phase in the production of gas occurs from 6 h, while for the oily extract, this phase appears until 9 h in 0.1 mL of extract, however for 1 mL, and gas production is minimal, remaining so during 48 h.

The main bioactive compounds of oregano that have antimicrobial effects, are carvacrol and thymol, for which it is known that the mechanism of antimicrobial action, occurs mainly by incrusting in the bacterial cell membrane, which causes the disintegration of this structure favoring the escape of ions and causing cell lysis (Rodríguez-García *et al*., 2015; Friedman, 2014; García-García *et al*., 2011). As already reported in Table 2, the oil extract was the highest concentration of carvacrol and thymol exhibited, which is why Figure 4 shows a marked decrease in gas production, and it is inferred that the effect of these compounds as

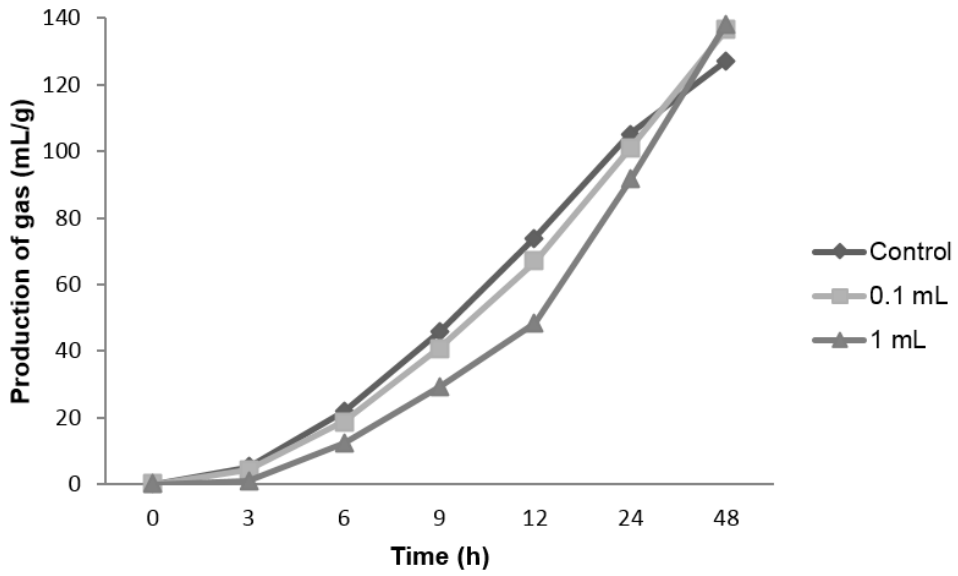


Graph 1. Gas production during *in vitro* fermentation by adding aqueous cooking extract.

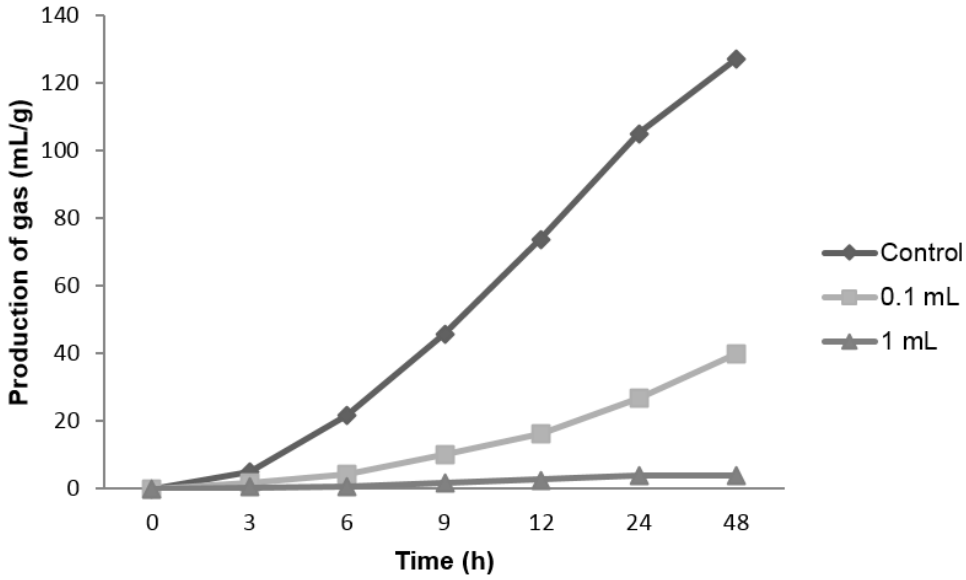


Graph 2. Gas production during *in vitro* fermentation by adding aqueous extract of infusion.

antimicrobial was to join the cell membrane of ruminal bacteria responsible for carrying out the fermentation, controlling the population of these microorganisms and with it the low production of gas. Cirino *et al*. (2014), evaluated the antibacterial effect of the oil of oregano (*Origanum vulgare*) and the active ingredients carvacrol and thymol individually, finding that the greatest antimicrobial effect was presented with the oil in comparison with the individual effect of the active principles, therefore There is synergy of the active compounds present in the oils, so that their antibacterial activity increases. For the aqueous extracts (graph 1 and 2), there is a trend in gas production similar to the control, this behavior was caused because the main antimicrobial compounds (table 2) are not present (carvacrol) or are in low concentration (thymol). Teixeira *et al*. (2013), evaluated the antibacterial activity of aqueous extracts, ethanolic and essential oil of *Origanum vulgare*, finding that the aqueous extracts



Graph 3. Gas production during *in vitro* fermentation adding alcohol extract.



Graph 4. Gas production during *in vitro* fermentation adding oil extract.

have no effect on the microorganisms evaluated since the concentration of active ingredients was lower in comparison with the ethanolic extract and essential oil. Therefore, based on the results obtained, it is further supported that carvacrol and thymol are the main compounds with the greatest antimicrobial effect on rumen bacteria, which could be due to their high concentrations.

At the end of the gas production technique or in vitro ruminal fermentation, the production of volatile fatty acids (VFAs) was quantified and the methane concentration was estimated. Table 3 shows the average gas production that was generated during in vitro fermentation at the end of 48 h in units of mg / mL and its standard deviation (SD), the average of the

production of AGVs (acetic, propionic and butyric) in mM / L and DE, as well as the methane concentration in mM / L and DE.

For the formation of gas, the aqueous (cooking and infusion) and alcoholic extracts, in each of their volumes, maintained a gas production similar to the control (127.24 mL / g). The oil preparation showed the lowest gas production; this was confirmed statistically ($p < 0.05$) since the gas (mL / g) that occurred did not show differences between aqueous and alcoholic preparations. For infusion in a volume of 0.1 mL there was no difference with respect to the control (127.24 mL / g); the oil, showed the lowest gas production, these values being different from the rest of the extracts. Wang et al. (2016), evaluated ethanolic extracts of medicinal plants where they report that there is no tendency to increase gas production (mL / g) when using extracts; since some of them decreased gas production and others increased it with respect to control (110.54 mL / g). As shown in Table 3, the different preparations of oregano were unequal in gas production, in addition to obtaining a control gas value (127.24 mL / g) very similar to that reported by Wang *et al*.

Regarding the concentration of volatile fatty acids, in Table 3, it is shown that the production of propionic acid was completely inhibited in both aqueous preparations, however, the production of butyric acid in infusion in a volume of 0.1 mL increased its concentration up to 10 times more with respect to the control. Oily, inhibited the production of AGVs. For the

Table 3. Gas production (mL / g) and volatile fatty acids (mM / L) *in vitro* in the different extracts of oregano.

Extract Oregano	Dose (mL)	Gas (mL/g) ± *DE	Volatile Fatty Acids (mM/L) ± *DE			Methane (mM/L) ± *DE
			Acetic	Propionic	Butyric	
Control	-----	127.24 ± 1.35^{bc}	1,346.30 ± 0.05^{d}	573.12 ± 0.18^{c}	38.77 ± 0.22^{b}	463.73 ± 0.06^{d}
Cooking	0.1	132.12 ± 1.49^{ab}	1,297.53 ± 0.06^{e}	0 ± 0^{f}	546.63 ± 0.13^{a}	802.54 ± 0.01^{a}
	1	119.31 ± 3.48^{d}	1,375.39 ± 0.05^{c}	590.18 ± 0.18^{b}	38.46 ± 0.22^{b}	472.01 ± 0.06^{d}
Infusion	0.1	125.53 ± 4.94^{c}	0 ± 0^{h}	0 ± 0^{f}	577.37 ± 0.29^{a}	230.95 ± 60.28^{e}
	1	136.41 ± 0.14^{a}	944.86 ± 0.30^{f}	0 ± 0^{f}	434.70 ± 0.15^{a}	599.07 ± 0.05^{c}
Alcoholic	0.1	136.38 ± 3.02^{a}	1,782.83 ± 0.02^{b}	601.27 ± 0.17^{a}	41.81 ± 0.22^{b}	653.65 ± 0.05^{bc}
	1	138.12 ± 3.20^{a}	1,806.41 ± 0.02^{a}	543.30 ± 0.18^{d}	24.44 ± 0.22^{b}	673.26 ± 0.04^{b}
Oily	0.1	40.07 ± 1.14^{e}	358.46 ± 0.11^{g}	3.77 ± 0.21^{e}	0 ± 0^{b}	160.27 ± 0.01^{f}
	1	3.81 ± 0.45^{f}	0 ± 0^{h}	0 ± 0^{f}	16.46 ± 0.22^{b}	6.59 ± 0.09^{g}

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

alcoholic extract, there was an increase in AGVs (acetic, propionic and butyric) with a low volume (0.1 mL), so this preparation has the best positive effect on ruminal fermentation *in vitro*. Therefore, a change in the proportions or growth of ruminal microorganisms generated by the addition of extracts of oregano, results in modifications in the profile of the fermentation for the production of AGVs. Statistically the production of volatile fatty acids; for the case of acetic there are no significant differences ($p > 0.05$) between infusion and oil. The alcoholic preparation was the best extract where the concentration of acetic acid was higher ($p < 0.05$), followed by cooking. The previous effect was also presented for the propionic fatty acid, since there were no statistical differences ($p > 0.05$) between the infusion and oil preparations, showing that the best extract was the alcoholic. With regard to the production of butyric fatty acid, it was better in infusion ($p < 0.05$) in both volumes (0.1 and 1 mL) followed by cooking in a volume of 0.1 mL. With the alcoholic and oily preparations there were no significant differences ($p > 0.05$) with respect to the control. In the case of this fatty acid, there were less differences between the extracts and the doses evaluated, there are researches where they report that the use of additives in the diet of ruminants does not negatively affect the production of volatile fatty acids, Hristov *et al*. (2013), reported that the concentration of AGVs was not affected after the addition of oregano leaves in vivo in the bovine diet. Rajabi *et al*. (2017), performed the technique of in vitro digestibility with aqueous extract of pomegranate shell, within the analyzed parameters are the AGVs, reporting that these acids were not affected ($p > 0.05$) by the addition of these extracts in the experimentation in vitro. Other studies report that the modification in the production of AGVs decreases when using additives from plants rich in tannins (*Leucaena leucocephala*, *Gliricidia sepium* and *Manihot esculenta*) in sheep (Rira *et al*., 2015). Therefore, there is no predisposition that indicates that the use of plant additives will increase or decrease the production of VFAs; everything depends on the experimental model, type of plant material, preparation of the extract and concentration, as well as the in vitro fermentation technique.

With respect to the determination of methane (CH_4), it was inferred from the concentration of volatile fatty acids; Table 3 shows the result of methane formation (mM / L) calculated from equation 1 proposed by Moss *et al*. (2000). The methane production was statistically different ($p < 0.05$) for the extracts of cooking, infusion (0.1 mL) and oily, the alcoholic and infusion extracts (1 mL) were statistically similar. The oil decreased considerably the CH_4 in the two volumes (0.1 and 1 mL), while for the dose of 1 mL the production of this gas (6.58 mM / L) was reduced up to 70 times with respect to the control (463.73 mM / L). The maximum production of CH_4 was with the cooking extract in a volume of 0.1 mL. Agarwal *et al*. (2009) reported that the addition of peppermint oil at a ratio of 0.1 µL / 1mL of ruminal fluid, with the technique of in vitro gas production, decreased the concentration of volatile fatty acids and methane; although in that work the plant used was mint (*Mentha piperita*), it has the same behavior as the oily extract of oregano (*Lippia graveolens*), since the latter also decreased

the AGVs and methane. The modifications in the total and proportion of the VFAs have an impact on the production profiles of methane, especially if it reduces the proportion of acetate and increases the proportion of propionate, therefore, changes in these will have a positive or negative effect on methane production. .

Oregano extract has been used for the purpose of decreasing methane emissions in cattle and improving ruminal fermentation. The results presented are similar to those reported by de Wang et al. (2009). They tested a commercial preparation of essential oils of oregano (ropadiar) used in veterinary medicine and compared it with a synthetic product (flavomycin), reporting that the average production of CH_4 expressed on the basis of digestible organic matter (OM) decreased compared to the control, however, the production of AGVs decreased as did the proportion of propionate in the ruminal fluid.

Evans and Martin (2000) observed that the thymol compound (0.4 mg / mL) derived from the Thymus and Origanum plants, is a strong methane inhibitor *in vitro*, but the concentrations of acetate and propionate also decreased. The concentrations obtained of thymol (table 2) for each of the extracts of oregano are higher than those used by these researchers, however, the alcoholic preparation did not show the behavior indicated by Evans and Martins since in this there was an increase of AGVs. Kumar *et al*. (2009) used eucalyptus oil to decrease ruminal methane, finding that methane production is reduced to 58% using a ratio of 1.66 mL of oil per 100 mL of ruminal fluid. For the infusion extract (0.1 mL / 100 mL of ruminal fluid) a methane decrease of 50.2% was found, while, in the oil for the same ratio, the methane decreased to 65.4% and in a ratio of (1 mL / 100 mL) the decrease was 98.5%. Ding *et al*. (2012) evaluated the effect of coconut oil on methane emissions and the microbial community in sheep, reporting that coconut oil was particularly effective in reducing emissions of this gas up to 61.2% with respect to control, in the results with oregano extract, a similar percentage (65.4%) is reported by Ding, which is registered in the oily extract (0.1 mL).

The results of the oregano extracts show a differential effect between them in each of the parameters evaluated (chemical composition, gas production, AGVs and methane), this attributed to the preparation methods, the in vitro technique used must also be considered, the dose administered and other biological factors, such as the pH of the ruminal fluid and the diet provided (Patra and Saxena, 2010). It is necessary to consider the acetic: propionic ratio and the production of each of the AGVs, according to the objective that is sought in animal nutrition for the improvement of cattle; either for meat production (the desired AGVs is propionic) and for milk production (the desired AGVs is butyric). This is why it is important to previously determine a method of preparation and an optimal dose in vitro to be used in vivo with improvements in ruminal fermentation and feeding efficiency.

CONCLUSIONS

The best extraction method to obtain the highest concentration of antimicrobial compounds was oily. The extract of oregano with the highest production of gas and AGVs was the alcoholic one, while the extract that presented the highest methane mitigation of 65.4% up to 98.5% was oily, these results make plant extracts alternatives to the use of additives chemicals to modify the fermentation in ruminants. Therefore, the use of new strategies in animal nutrition with the purpose of modifying ruminal fermentation in a positive way and decreasing the emissions of enteric CH_4, should be designed and analyzed *in vitro* before its application; since there is a large number of factors that can affect ruminal fermentation, so that the use of extracts allows their application in vivo models.

ACKNOWLEDGEMENT

The authors are grateful for the support and funding granted by PRODEP to the academic body in formation UAZ-CA18 to carry out this study.

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