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Essential Oils as In Vitro Ruminal Fermentation Manipulators to Mitigate Methane Emission by Beef Cattle Grazing Tropical Grasses

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Abstract: There is increasing pressure to identify natural feed additives to mitigate methane emissions from livestock systems. Our objective was to investigate the effects of essential oils (EO) extracts star anise (Illicium verum), citronella (Cymbopogon winterianus), clove bud (Eugenia caryophyllus), staigeriana eucalyptus (Eucalyptus staigeriana), globulus eucalyptus (Eucalyptus globulus), ginger (Zingiber officinale), ho wood (Cinnamomum camphora), melaleuca (Melaleuca alternifolia), oregano (Origanum vulgare) and white thyme (Thymus vulgaris) on in vitro methane emissions from four rumen-cannulated Nellore cattle grazing a tropical grass pasture as inoculum donors. The semi-automated gas production technique was used to assess total gas production, dry matter degradability, fermentation and suppress rumen methanogenesis without negative effects on feed digestibility, indicating promise as alternatives to ionophores for methane reduction in beef cattle.

Keywords: greenhouse gases; methanogenesis; oregano; ruminants; thyme; NH3-N

1. Introduction

In 2020, the Brazilian cattle herd was the largest in the world, representing 14.3% of the international herd, with 217 million head [1]. Despite the crisis caused by the new coronavirus pandemic, Brazil was the largest exporter of meat in the world, with 2.2 million tons and 14.4% of the international market [1].

However, despite the recognized importance of livestock to food production and income generation, there is currently much debate about the environmental impact of the activity, mainly related to climate change. Brazilian livestock is singled out for its greenhouse gas (GHG) emissions [2]. Such criticism has been based on low animal performance indexes, verified in animal production systems, based on degraded pastures which are below their production potential [3,4]. Low exploration model efficiency has generated greater amounts of GHG per kg of meat and/or milk produced [3]. In 2019, total emissions from agriculture were 10.7 billion tons of carbon dioxide equivalent, of which methane (CH4) from ruminant livestock was the largest contributor (2.8 Gt CO2...
This production is directly related to the efficiency of ruminal fermentation and consequently to the loss of energy in the production systems [6]. Brazil has the largest world emissions from agriculture followed by Indonesia and China [5], and only CH$_4$ by beef herd contributes to 80% of Brazil emissions [2].

Ruminant contribution to climate change has led to the search for natural alternatives that can be added to grazing cattle diets to mitigate GHG emissions without compromising livestock productivity [7]. Some natural plant products, including essential oils (EO), possess antimicrobial properties capable of manipulating rumen microbiomes [8]. However, data on EO acceptability in cattle feed are scarce. Thus, the objective of our study was to investigate the effects of 10 EO on in vitro rumen methane emissions and degradability in Nelore beef cattle.

2. Material and Methods

2.1. Essential Oils

Ten essential oils used in this study were commercially obtained from Brazilian industry (Ferquímica®, Vargem Grande Paulista, SP, Brazil): star anise (*Illicium verum*), citronella (*Cymbopogon winterianus*), clove bud (*Eugenia caryophyllus*), staigeriana eucalyptus (*Eucalyptus staigeriana*), globulus eucalyptus (*Eucalyptus globulus*), ginger (*Zingiber officinale*), ho wood (*Cinnamomum camphora*), melaleuca (*Melaleuca alternifolia*), oregano (*Origanum vulgare*), and white thyme (*Thymus vulgaris*). The chemical composition of EO was carried out in order to identify the main bioactive compounds according to Wiley Library (Version 8), using a gas chromatograph coupled to a mass spectrometer (GC/MS QP2010 Plus, Shimadzu, Kyoto, Japan), with a diphenyl-dimethyl-polysiloxane capillary column (5% diphenyl and 95% dimethyl polysiloxane) with 30 m × 0.25 mm, 0.25 µm, model Rtx©-5MS (Bellefonte, PA, USA) at the Multi-User Laboratory for Biochemistry and Instrumental Analysis of the Department of Agribusiness, Food and Nutrition at ESALQ/USP (Piracicaba, Brazil).

2.2. Methanogenesis Bioassay

The study was approved by the ethics committee of the Department of Animal Science, College of Animal Science and Food Engineering, University of São Paulo (2416120916).

The in vitro bioassay used a pressure transducer according to methodology proposed by Theodorou et al. (1994) [9] and modified by Mauricio et al. (1999) [10]. Four male Nelore cattle, rumen cannulated, were donors of rumen content. The animals were kept in a tropical grass pasture with free access to water and mineral supplements.

All EO were tested in four dose rates (0, 50, 250 and 500 mg/L) in a randomized block design, arranged with four blocks, 10 treatments (EO), four dose rates and two replicates. The substrate used was a coast-cross Bermudagrass (*Cynodon dactylon*) grass hay, ground in a Willey mill with a 1-mm sieve. Substrates were analyzed (Table 1) for dry matter (DM; ID 930.15), mineral matter (ash; ID 942.05), crude protein (CP; ID 954.01), ether extract (EE; ID 920.39), neutral detergent fiber (NDF; ID 973.18) and acid detergent fiber (ADF; ID 973.18) according to AOAC (1998) [11]. NDF and ADF were determined sequentially with the addition of a-amylase and included residual ash. The collection and preparation of the inoculum followed the recommendations of Bueno et al. (2005) [12]. Glass bottles with a capacity of 160 mL were used; to each bottle, 500 mg of substrate, 25 mL of rumen inoculum and 50 mL of buffered medium were added. After 4, 8, 12, 16, 20, 24 h of incubation, the pressure in the head space was measured. Total gas production after 24 h of fermentation was determined by summing the volume of gases produced in each pressure measurement of the bottles. The pressure inside each flask was measured, and 1.5-mL gas samples were collected in Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) for CH$_4$ characterization by gas chromatography [13].
Table 1. Chemical composition of substrate used in the in vitro bioassay (g/kg DM).

| Substrate         | DM  | OM  | MM  | CP  | NDF | ADF |
|-------------------|-----|-----|-----|-----|-----|-----|
| Coast cross hay   | 880 | 927 | 73  | 165 | 743 | 42.1|

ADF, acid detergent fiber; DM, dry matter; CP, crude protein; MM, mineral matter; OM, organic matter; NDF, neutral detergent fiber.

After 24 h of incubation, samples of ruminal liquid (2 mL) contained in each bottle were collected for the determination of short-chain fatty acid (SCFA) and NH$_3$-N. In vitro DM degradability (IVDMD) and in vitro organic matter degradability (IVOMD) were then determined. Analysis of both were made to correct any differences in mineral content among samples. IVDMD was determined by filtering the fermentation residues in sintered crucibles (porosity n. 1) lined with glass wool and then dried in an oven (105 °C) to determine the residue. Using the difference in residues (ash) of the muffle burning (550 °C), IVOMD was estimated. The partitioning factor (PF), calculated by relating DM degradation to total gas production (mL) in 24 h, was used to compare microbial efficiency (Blummel et al., 1997) [14].

2.3. Methane Determination

The CH$_4$ quantification was performed by a gas chromatograph (GC-2014, Shimadzu, Kyoto, Japan), equipped with a micro-packed column (ShinCarbon®, Restek corporation, Bellefonte, PA, USA) at 60 °C and a dual direct injector and dual FID detector at 120 °C, according to the method described by Santos et al. (2020) [13]. A calibration curve (0, 30, 60 and 90 mL/L) was generated using methane (99% purity) as the standard. The operational conditions were column, injector and detector temperatures at 100 °C and 120 °C, respectively.

2.4. Short Chain Fatty Acid Determination

An SCFA profile in ruminal liquid was performed by a gas chromatography (GC-2014; Shimadzu, Kyoto, Japan) split-injector, a flame ionization detector and a capillary column (Stabilwax®, Restek, Bellefonte, PA, USA) at 145 °C (isothermal), as described by Bueno et al. in 2020 [15]. Acetic, propionic, isobutyric, butyric, isovaleric and valeric acid (99.5% purity, Chem Service, West Chester, PA, USA) were used as quantitative external standards. The operational conditions were: injector and detector temperatures at 250 °C; helium as the carrier gas at 8.01 mL/min; hydrogen flow to the flame jet at 60 kPa; and synthetic air at 40 kPa. The samples were thawed at room temperature and centrifuged at 14,500 × g for 10 min. The supernatant (800 µL) was transferred to a dry and clean flask with 200 µL formic acid (98–100%) and 100 µL of the internal standard (100 mM 2-ethyl butyric acid, Chem service, West Chester, PA, USA).

2.5. NH$_3$-N Determination

NH$_3$-N concentrations in the ruminal liquid were determined by colorimetry according to the method described by Kulasek (1972) [16] and adapted by Foldager (1977) [17]. In each sample, 10% sodium tungstate was added and then centrifuged at 2000 × g for 15 min. The supernatant was then transferred to another tube with 1 mL of the salicylic acid buffer and 1 mL of sodium hypochlorite oxidizing solution. Finally, the tubes were placed in water bath (37 °C) until they became green.

2.6. Experimental Design and Statistical Analysis

The experimental design was a randomized complete block with two factors (10 essential oils plus four doses) and four blocks (inoculum) with two replications inside each block. Regression analysis performed by the SAS 9.3 program (Institute Inc., Cary, NC, USA) [18] was used for measuring correlations among all investigated parameters.
3. Results

3.1. Ruminal Degradability

IVDMD, IVOMD and partition factor (PF) of the evaluated EO are shown in Tables 2 and 3. Among all EO, only white thyme linearly reduced IVDMD.

Table 2. Effects of essential oils on the in vitro dry matter (IVDMD) and organic matter (IVOMD) degradabilities after 24 h of incubation.

| Essential Oils   | Doses (mL/g) | M EPM | p-Value |
|------------------|--------------|-------|---------|
|                  | 0 | 50 | 250 | 500 | L | Q |
| Star anise       | 24.67 | 28.76 | 21.05 | 20.08 | 23.64 | 4.31 | 0.192 | 0.411 |
| Citronella       | 24.67 | 28.07 | 21.17 | 31.86 | 26.44 | 5.10 | 0.441 | 0.680 |
| Clove bud        | 24.67 | 33.20 | 25.11 | 21.17 | 26.04 | 5.81 | 0.276 | 0.561 |
| Globulus         | 24.67 | 28.47 | 27.79 | 26.09 | 26.76 | 4.74 | 0.849 | 0.958 |
| Staigeriana      | 24.67 | 28.22 | 24.23 | 23.19 | 25.08 | 5.27 | 0.551 | 0.840 |
| Ginger           | 24.67 | 33.92 | 26.09 | 24.64 | 27.33 | 4.61 | 0.365 | 0.674 |
| Ho wood          | 24.67 | 27.48 | 26.99 | 27.68 | 26.71 | 4.58 | 0.851 | 0.792 |
| Melaleuca        | 24.67 | 28.44 | 31.25 | 23.29 | 26.91 | 5.93 | 0.864 | 0.882 |
| Oregano          | 24.67 | 25.45 | 25.47 | 27.07 | 25.66 | 5.52 | 0.438 | 0.736 |
| White thyme      | 24.67 | 36.28 | 21.17 | 18.54 | 25.16 | 4.78 | 0.041 | 0.136 |

L = linear effect; M = means; Q = Quadratic effect; SEM = Standard error of the mean. * Equation: $y = 32.24 - 0.037x$.

Table 3. Effects of essential oils on the dry matter partition factor (PF<sub>DM</sub>) and organic matter (PF<sub>OM</sub>) after 24 h of in vitro incubation.

| Essential Oils   | Doses (mL/g) | M SEM | p-Value |
|------------------|--------------|-------|---------|
|                  | 0 | 50 | 250 | 500 | L | Q |
| Star anise       | 25.82 | 27.60 | 21.29 | 19.04 | 23.44 | 3.24 | 0.074 | 0.202 |
| Citronella       | 25.82 | 27.66 | 21.20 | 19.28 | 23.49 | 3.71 | 0.130 | 0.312 |
| Clove bud        | 25.82 | 31.71 | 25.04 | 20.73 | 25.82 | 4.81 | 0.203 | 0.453 |
| Globulus         | 25.82 | 28.35 | 27.73 | 24.02 | 26.48 | 3.78 | 0.524 | 0.746 |
| Staigeriana      | 25.82 | 28.99 | 24.18 | 23.41 | 25.60 | 4.12 | 0.411 | 0.712 |
| Ginger           | 25.82 | 33.01 | 26.84 | 25.01 | 27.67 | 3.47 | 0.298 | 0.588 |
| Ho wood          | 25.82 | 28.33 | 31.04 | 24.39 | 27.40 | 3.33 | 0.574 | 0.487 |
| Melaleuca        | 25.82 | 28.83 | 31.56 | 24.98 | 27.80 | 4.74 | 0.790 | 0.830 |
| Oregano          | 25.82 | 26.30 | 26.34 | 22.71 | 25.29 | 4.27 | 0.162 | 0.324 |
| White thyme      | 25.82 | 34.58 | 21.05 | 20.62 | 25.52 | 3.72 | 0.099 | 0.229 |

PF<sub>DM</sub> (mL DMS/g Produced Gases)
Table 3. Cont.

| Essential Oils   | Doses (mL/g) | M   | SEM | p-Value |
|------------------|--------------|-----|-----|---------|
|                  | 0            | 50  | 250 | 500     |
| **PFOM (mL OMD/g Produced Gases)** |              |    |     |         |
| Star anise       | 2.73         | 2.91| 2.15| 2.64    | 2.61 | 0.57 | 0.733| 0.730 |
| Citronella       | 2.73         | 2.83| 2.04| 2.99    | 2.65 | 0.60 | 0.721| 0.554 |
| Clove bud        | 2.73         | 3.85| 2.57| 2.73    | 2.97 | 0.78 | 0.547| 0.822 |
| Globulus         | 2.73         | 2.76| 2.54| 2.58    | 2.65 | 0.50 | 0.762| 0.941 |
| Staigeriana      | 2.73         | 2.98| 2.16| 2.25    | 2.53 | 0.52 | 0.347| 0.578 |
| Ginger           | 2.73         | 3.08| 2.09| 2.14    | 2.51 | 0.43 | 0.167| 0.325 |
| Ho wood          | 2.73         | 2.76| 2.81| 3.65    | 2.99 | 0.55 | 0.263| 0.491 |
| Melaleuca        | 2.73         | 2.51| 2.49| 2.15    | 2.47 | 0.43 | 0.840| 0.953 |
| Oregano          | 2.73         | 2.35| 3.20| 11.21   | 4.87 | 0.79 | 0.010| 0.016 |
| White thyme      | 2.73         | 3.26| 2.09| 7.97    | 4.01 | 1.00 | 0.093| 0.038 |

L = Linear effect; M = Means; Q = Quadratic effect; SEM = Standard error of the mean. * Equations:

1. \( y = 2.21 + 0.008x \)
2. \( y = 15.47 + 0.0005x \)
3. \( y = 3.36 - 0.017x + 0.00005x^2 \)

The PF is a variable that relates degradability and gas production and is expressed by mg of DM (PFDM) or OM (PFOM) degraded per mL of gas produced during the fermentation process. Oregano EO had a positive linear effect on PFDM and PFOM (Table 3). That means that, with increasing doses of this EO, larger amounts of degraded DM and OM were needed to generate 1 mL of gas. The gas produced represents the partial loss of energy from fermented foods (Mauricio et al., 1999) \[10\] and, therefore, the increase in the partitioning factor would mean more efficient energy use. White thyme EO showed a quadratic relationship between dose and PFOM.

3.2. Short-Chain Fatty Acids and N-NH3 Quantifications

The NH3-N concentrations and short chain fatty acids in the different treatments and levels evaluated are shown in Tables 4–9. Citronella and ho wood EO showed a positive linear relationship between NH3-N and levels of inclusion. However, the average levels of NH3-N collected after 24 h of incubation showed numerically little variation among all EO and tested levels, suggesting a stabilization of the environment and the fermentation process.

Table 4. Effects of essential oils on the concentration of ammonia nitrogen (NH3-N, in mg/dL) after 24 h of in vitro incubation.

| Essential Oils | Doses (mg/dL) | M   | SEM | p-Value |
|----------------|--------------|-----|-----|---------|
|                | 0            | 50  | 250 | 500     |
| Star anise     | 17.08        | 15.59| 16.44| 16.67   | 16.45| 0.63 | 0.439| 0.747 |
| Citronella     | 17.08        | 16.00| 17.92| 18.99   | 17.50| 0.72 | 0.010| 0.041 |
| Clove bud      | 17.08        | 16.19| 17.14| 17.27   | 16.92| 0.60 | 0.232| 0.476 |
| Globulus       | 17.08        | 16.10| 14.72| 14.51   | 15.60| 1.46 | 0.355| 0.616 |
| Staigeriana    | 17.08        | 15.67| 14.91| 17.41   | 16.27| 0.86 | 0.391| 0.192 |
| Ginger         | 17.08        | 17.72| 16.37| 14.08   | 16.31| 1.17 | 0.083| 0.192 |
| Ho wood        | 17.08        | 15.93| 16.32| 18.77   | 17.03| 0.64 | 0.009| 0.039 |
| Melaleuca      | 17.08        | 14.68| 15.55| 16.06   | 15.84| 1.48 | 0.911| 0.960 |
| Oregano        | 17.08        | 16.80| 16.60| 16.33   | 16.70| 1.02 | 0.698| 0.903 |
| White thyme    | 17.08        | 15.22| 15.57| 15.81   | 15.92| 1.10 | 0.847| 0.857 |

L = Linear effect; M = Means; Q = Quadratic effect; SEM = Standard error of the mean. * Equations:

1. \( y = 16.10 + 0.006x \)
2. \( y = 15.47 + 0.0005x \)
Table 5. Effects of essential oils on the total concentration of short chain fatty acids (SCFA, in mM) after 24 h of in vitro incubation.

| Essential Oils     | Doses (mL/g) | M  | SEM  | p-Value |
|--------------------|--------------|-----|------|---------|
|                    | 0  | 50 | 250 | 500 L  | Q  |
| Star anise         | 11.52 | 11.59 | 20.66 | 12.44 | 14.05 | 4.49 | 0.767 | 0.377 |
| Citronella         | 11.52 | 9.60 | 24.66 | 12.80 | 14.64 | 5.60 | 0.698 | 0.250 |
| Clove bud          | 11.52 | 14.34 | 26.95 | 14.76 | 16.89 | 5.61 | 0.690 | 0.221 |
| Globulus E.        | 11.52 | 15.98 | 13.02 | 19.35 | 14.97 | 4.07 | 0.357 | 0.598 |
| Staigeriana E.     | 11.52 | 13.41 | 19.38 | 18.79 | 15.77 | 3.43 | 0.170 | 0.274 |
| Ginger             | 11.52 | 14.35 | 15.96 | 24.60 | 16.61 | 4.06 | 0.055 | 0.159 |
| Ho wood            | 11.52 | 16.69 | 18.98 | 17.32 | 16.13 | 3.59 | 0.494 | 0.702 |
| Melaleuca          | 11.52 | 13.58 | 16.05 | 26.22 | 16.84 | 3.78 | 0.297 | 0.582 |
| Oregano            | 11.52 | 11.66 | 16.00 | 3.99  | 10.79 | 3.91 | 0.437 | 0.291 |
| White thyme        | 11.52 | 10.68 | 16.28 | 17.57 | 14.01 | 7.06 | 0.229 | 0.488 |

L = Linear effect; M = Means; Q = Quadratic effect; SEM = Standard error of the mean.

Total SCFA concentrations were not affected by any of the analyzed oils. With the exception of the ginger EO that suffered a positive linear effect only for the C₂ concentration, no other EO changed with concentrations of C₂, C₃ and C₄ (respectively, Tables 6–8). However, citronella, globulus, staigeriana, ginger, melaleuca, oregano and white thyme EO induced a positive linear effect for the C₂:C₃ ratio (Table 9), thus making the rumen less energetically efficient. The observed effects were dependent on the doses used. The thymol 500 mg/L dose reduced the total concentration of SCFA (−28.5%), the proportion of propionate (−18.4%) and the concentration of NH₃-N (−31.9%) while increasing the proportion of acetate (C₂) (+1.8%) and C₂:C₃ ratio (+35.5%).

Table 6. Effects of essential oils on acetic acid (mM) production after 24 h of in vitro incubation.

| Essential Oils     | Doses (mL/g) | M  | SEM  | p-Value |
|--------------------|--------------|-----|------|---------|
|                    | 0  | 50 | 250 | 500 L  | Q  |
| Star anise         | 7.54 | 7.90 | 14.92 | 8.58  | 9.74 | 3.35 | 0.734 | 0.337 |
| Citronella         | 7.54 | 6.62 | 18.61 | 9.54  | 10.58 | 4.29 | 0.410 | 0.211 |
| Clove bud          | 7.54 | 10.36 | 20.21 | 9.49  | 11.90 | 4.13 | 0.775 | 0.155 |
| Globulus           | 7.54 | 10.50 | 9.32  | 14.09 | 10.36 | 2.93 | 0.240 | 0.476 |
| Staigeriana        | 7.54 | 9.48 | 14.72 | 14.13 | 11.47 | 2.55 | 0.111 | 0.166 |
| Ginger             | 7.54 | 10.32 | 11.75 | 18.07 | 11.92 | 3.01 | 0.043 | 0.134 |
| Ho wood            | 7.54 | 11.49 | 14.08 | 11.28 | 11.10 | 2.60 | 0.587 | 0.639 |
| Melaleuca          | 7.54 | 9.83 | 11.93 | 19.52 | 12.21 | 2.88 | 0.239 | 0.490 |
| Oregano            | 7.54 | 7.98 | 10.77 | 2.27  | 7.14  | 2.56 | 0.376 | 0.222 |
| White thyme        | 7.54 | 7.60 | 11.98 | 12.69 | 9.95  | 5.18 | 0.209 | 0.466 |

L = Linear effect; M = Means; Q = Quadratic effect; SEM = Standard error of the mean. * Equation: \( y = 8.19 + 0.019x \).

Table 7. Effects of essential oils on propionic acid (mM) production after 24 h of in vitro incubation.

| Essential Oils     | Doses (mL/g) | M  | SEM  | p-Value |
|--------------------|--------------|-----|------|---------|
|                    | 0  | 50 | 250 | 500 L  | Q  |
| Star anise         | 2.42 | 2.17 | 3.34 | 1.97  | 2.48 | 0.69 | 0.856 | 0.487 |
| Citronella         | 2.42 | 1.71 | 3.45 | 1.37  | 2.24 | 0.82 | 0.617 | 0.325 |
| Clove bud          | 2.42 | 2.46 | 4.11 | 2.47  | 2.87 | 0.94 | 0.155 | 0.445 |
| Globulus           | 2.42 | 3.17 | 2.09 | 2.99  | 2.67 | 0.70 | 0.918 | 0.793 |
| Staigeriana        | 2.42 | 2.36 | 2.66 | 2.59  | 2.51 | 0.55 | 0.766 | 0.939 |
| Ginger             | 2.42 | 2.35 | 2.50 | 3.85  | 2.78 | 0.66 | 0.143 | 0.281 |
| Ho wood            | 2.42 | 3.08 | 2.88 | 3.62  | 3.00 | 0.65 | 0.346 | 0.643 |
| Melaleuca          | 2.42 | 2.31 | 2.35 | 3.46  | 2.64 | 0.57 | 0.873 | 0.977 |
| Oregano            | 2.42 | 2.20 | 2.46 | 0.43  | 1.88 | 0.68 | 0.149 | 0.220 |
| White thyme        | 2.42 | 1.84 | 2.24 | 2.05  | 2.14 | 0.92 | 0.605 | 0.824 |

L = Linear effect; M = Means; Q = Quadratic effect; SEM = Standard error of the mean.
Table 8. Effects of essential oils on butyric acid (mM) production after 24 h of in vitro incubation.

| Essential Oils     | Doses (mL/g) | M   | SEM | p-Value | L  | Q  |
|--------------------|--------------|-----|-----|---------|----|----|
| Star anise         | 1.14         | 1.14| 1.80| 1.51    | 1.40| 0.39|
| Citronella         | 1.14         | 0.96| 2.01| 1.45    | 1.39| 0.44|
| Clove bud          | 1.14         | 1.15| 1.85| 1.91    | 1.51| 0.42|
| Globulus           | 1.14         | 1.37| 1.23| 1.77    | 1.38| 0.36|
| Staigeriana        | 1.14         | 1.19| 1.51| 1.63    | 1.37| 0.31|
| Ginger             | 1.14         | 1.28| 1.30| 2.04    | 1.44| 0.35|
| Ho wood            | 1.14         | 1.57| 1.58| 2.05    | 1.59| 0.37|
| Melaleuca          | 1.14         | 1.11| 1.37| 2.65    | 1.57| 0.35|
| Oregano            | 1.14         | 1.12| 2.10| 1.23    | 1.40| 0.66|
| White thyme        | 1.14         | 0.93| 1.52| 2.68    | 1.57| 0.93|

L = Linear effect; M = Means; Q = Quadratic effect; SEM = Standard error of the mean.

Table 9. Effects of essential oils on the ratio of acetic and propionic acids after 24 h of in vitro incubation.

| Essential Oils     | Doses (mL/g) | M   | SEM | p-Value | L  | Q  |
|--------------------|--------------|-----|-----|---------|----|----|
| Star anise         | 3.09         | 3.49| 4.21| 4.40    | 3.80| 0.47|
| Citronella         | 3.09         | 3.62| 5.23| 6.76    | 4.67| 0.51|
| Clove bud          | 3.09         | 4.08| 4.83| 3.60    | 3.90| 0.50|
| Globulus           | 3.09         | 3.28| 4.60| 4.58    | 3.89| 0.46|
| Staigeriana        | 3.09         | 4.10| 5.32| 5.44    | 4.49| 0.46|
| Ginger             | 3.09         | 4.09| 4.82| 4.65    | 4.16| 0.42|
| Ho wood            | 3.09         | 3.75| 4.87| 3.27    | 3.74| 0.51|
| Melaleuca          | 3.09         | 4.24| 4.93| 5.43    | 4.42| 0.59|
| Oregano            | 3.09         | 4.76| 4.41| 4.79    | 4.26| 0.48|
| White thyme        | 3.09         | 3.26| 4.86| 5.03    | 4.06| 0.59|

L = Linear effect; M = Means; Q = Quadratic effect; SEM = Standard error of the mean; * Equations: 1 \( y = 3.10 + 0.008x \); 2 \( y = 3.07 + 0.004x \); 3 \( y = 3.50 + 0.005x \); 4 \( y = 3.52 + 0.003x \); 5 \( y = 3.51 + 0.004x \); 6 \( y = 3.15 + 0.004x \).

3.3. Total Gas Production and Methane Quantifications

Essential oils influenced total gas production (TGP) in the rumen, which had a negative linear relationship \( (p < 0.05) \) in citronella, ho wood, oregano and white thyme (Table 10). At a dose of 500 mg/L, citronella and ho wood EO decreased TGP by approximately 25%. Oregano and thyme EO caused a sharp decrease in TGP of up to 75%, showing that these substances have high antimicrobial activity in in vitro ruminal conditions.

Table 10. Effects of essential oils on the production of total gases (mL/g DM) after 24 h of in vitro incubation.

| Essential Oils     | Doses (mL/g) | M   | SEM | p-Value | L  | Q  |
|--------------------|--------------|-----|-----|---------|----|----|
| Star anise         | 96.65        | 101.19| 102.05| 81.66    | 95.39| 7.95|
| Citronella         | 96.65        | 102.41| 103.87| 73.11    | 94.01| 6.37|
| Clove bud          | 96.65        | 88.06| 98.55| 82.87    | 91.53| 7.20|
| Globulus           | 96.65        | 104.99| 110.60| 99.63    | 102.97| 7.42|
| Staigeriana        | 96.65        | 101.44| 116.40| 107.23   | 105.43| 6.21|
| Ginger             | 96.65        | 111.48| 134.76| 120.39   | 115.82| 10.21|
| Ho wood            | 96.65        | 104.36| 111.84| 72.86    | 96.43| 6.46|
| Melaleuca          | 96.65        | 119.49| 129.24| 118.27   | 115.91| 9.64|
| Oregano            | 96.65        | 114.39| 87.00| 23.93    | 80.49| 10.32|
| White thyme        | 96.65        | 108.92| 104.20| 26.55    | 84.08| 8.60|

L = linear effect; M = means; Q = Quadratic effect; SEM = Standard error of the mean; TGP = Total gas production. * Equations: 1 \( y = 110.49 - 0.064x \); 2 \( y = 107.56 - 0.057x \); 3 \( y = 112.70 - 0.151x \); 4 \( y = 114.65 - 0.128x \).
The production of methane under various EO doses, expressed in net production (net CH\textsubscript{4}) or per gram of degraded dry matter (CH\textsubscript{4} g DDM), are shown in Table 11. Variations in the amount of CH\textsubscript{4} can occur even when DM disappearance of the diet does not change with the addition of different EO, with lower CH\textsubscript{4} production associated with lower TGP.

Table 11. Effects of essential oils on methane production after 24 h of in vitro incubation.

| Essential Oils     | Doses (mL/g) | M   | SEM | p-Value |
|--------------------|--------------|-----|-----|---------|
|                    | 0  | 50 | 250 | 500 |
| Net CH\textsubscript{4} (%) |     |     |     |     |
| Star anise         | 6.92 | 8.13 | 9.01 | 7.52 | 7.90 | 0.64 | 0.699 | 0.344 |
| Citronella         | 6.92 | 7.96 | 10.3 | 7.26 | 8.11 | 0.42 | 0.584 | 0.001 1 |
| Clove bud          | 6.92 | 7.90 | 9.50 | 6.40 | 7.68 | 0.78 | 0.332 | 0.082 |
| Globulus           | 6.92 | 8.55 | 9.87 | 9.57 | 8.73 | 0.62 | 0.120 | 0.141 |
| Staigeriana        | 6.92 | 8.45 | 10.93 | 11.43 | 9.43 | 0.45 | 0.001 2 | 0.001 |
| Ginger             | 6.92 | 8.16 | 10.79 | 10.89 | 9.19 | 0.45 | 0.001 3 | 0.001 |
| Ho wood            | 6.92 | 8.05 | 9.42 | 5.18 | 7.39 | 0.59 | 0.046 4 | 0.003 |
| Melaleuca          | 6.92 | 9.13 | 11.30 | 9.95 | 9.32 | 0.50 | 0.171 | 0.006 5 |
| Oregano            | 6.92 | 9.23 | 6.63 | 0.17 | 5.74 | 0.93 | 0.001 6 | 0.001 |
| White thyme        | 6.92 | 9.05 | 8.53 | 0.57 | 6.27 | 0.48 | 0.003 7 | <0.0001 |

CH\textsubscript{4} (mL/g IVDMD)

| Essential Oils     | Doses (mL/g IVDMD) | M   | SEM | p-Value |
|--------------------|-------------------|-----|-----|---------|
|                    | 0  | 50 | 250 | 500 |
| Star anise         | 67.81 | 81.04 | 177.24 | 102.40 | 107.12 | 52.52 | 0.645 | 0.440 |
| Citronella         | 67.81 | 69.39 | 125.12 | 117.62 | 94.99 | 35.16 | 0.350 | 0.542 |
| Clove bud          | 67.81 | 65.73 | 113.40 | 92.28 | 84.81 | 25.39 | 0.471 | 0.540 |
| Globulus           | 67.81 | 84.53 | 85.02 | 101.79 | 84.79 | 19.11 | 0.418 | 0.722 |
| Staigeriana        | 67.81 | 79.98 | 129.02 | 131.32 | 102.03 | 31.04 | 0.196 | 0.373 |
| Ginger             | 67.81 | 54.12 | 130.35 | 109.89 | 90.54 | 30.75 | 0.250 | 0.350 |
| Ho wood            | 67.81 | 66.03 | 128.45 | 72.47 | 83.69 | 23.75 | 0.957 | 0.995 |
| Melaleuca          | 67.81 | 107.53 | 106.01 | 82.60 | 90.99 | 33.20 | 0.925 | 0.855 |
| Oregano            | 67.81 | 86.84 | 131.71 | 3.92 | 72.57 | 39.70 | 0.426 | 0.184 |
| White thyme        | 67.81 | 57.19 | 118.88 | 35.75 | 69.31 | 31.47 | 0.873 | 0.388 |

L = Linear effect; M = Means; Q = Quadratic effect; SEM = Standard error of the mean. * Equations: \(1 y = 7.63 + 0.021x - 0.0004x^2; \) \(2 y = 8.32 + 0.007x; \) \(3 y = 8.18 + 0.006x; \) \(4 y = 8.66 - 0.005x; \) \(5 y = 7.98 + 0.024x - 0.00004x^2; \) \(6 y = 9.31 - 0.016x; \) \(7 y = 9.53 - 0.013x. \)

The same EO that demonstrated negative linear behavior for TGP also showed a drop in the net production of CH\textsubscript{4} including citronella, ho wood, oregano and white thyme. White thyme and oregano showed a higher potential for mitigating CH\textsubscript{4} emissions, in doses above 250 mg/L, producing 0.17 and 0.57 mL, respectively, in the highest dose (500 mg/L) against 6.92 mL without the use of any EO.

CH\textsubscript{4} expressed in mL/g IVDMD represents the relationship between the production of CH\textsubscript{4} net and IVDMD; the greater the value attributed to this variable implies a greater participation of CH\textsubscript{4} net production per gram of in vitro degraded dry matter (IVDMD) during the in vitro incubation process. Although the presence of EO changed CH\textsubscript{4} (mL/g IVDMD) production, variability was such that no statistical relationship was observed between the production of CH\textsubscript{4} and IVDMD.

4. Discussion

Although there is already research on some EO using the in vitro technique of gas production, our work investigated additional EO and variables that were not previously considered. The effects of EO tend to be influenced by their majority variable components (including diet composition), making it difficult to parse out their effect on ruminant nutrition [7]. The possible synergic or antagonistic interactions are difficult to measure and interpret [7].

A reduction of TGP in animals consuming diets with \textit{Lippia turbinate} and \textit{Tagetes minuta} was observed by Garcia et al. (2020) [19]. Those authors observed a reduction in in vitro
gas production, without affecting the digestibility of the substrates in intermediate doses. In our study, oregano and white thyme EO caused a drastic reduction in TGP of up to 75%, indicating that these substances have high antimicrobial activity in ruminal conditions. To explain this relationship, Lambert et al. (2001) [20] stated that oregano EO has molecules of thymol and carvacrol, and that these would be most responsible for its antimicrobial effect. These same molecules were observed in the chemical characterization of oregano and thyme EO.

García-García et al. (2011) [21] showed that thymol alone may not be as effective at reducing CH₄ as the combination between it and carvacrol. When testing the isolated effects of carvacrol or thymol on *Listeria innocua*, they obtained stronger effects for the first compound, while the binary effect of carvacrol plus thymol was more effective in inactivating bacterial growth, evidencing the synergistic effect between the components. Garcia et al. (2020) [19] observed that increasing doses of EO had progressively inhibitory action in substrates digestibility, with almost total inhibition when using 300 µL/L. In contrast, Khorrami et al.’s (2015) [22] in vivo study observed that thyme and cinnamon essential oils (500 mg/kg DM) did not decrease feed intake and nutrient digestion.

Several factors can influence the TGP and consequently the production of CH₄ by the animals through rumen fermentation [19]. One of the most important factors in this process is the extent of feed degradation. According to Bueno et al. (2005) [12], the in vitro gas production system estimates the degradation of the feed (substrate). Previous studies also suggest that in vitro gas production assays should be complemented with residue determination at the end of the incubation process, since the gas measurements alone provide an incomplete explanation. The determination of the final residue indicates the amount of substrate that was effectively used during the in vitro fermentation process (Getachew et al., 1998) [23].

Castilejos et al. (2006) [24] and Fraser et al. (2007) [25], when testing 500 ppm thymol (the primary compound of thyme EO) and cinnamaldehyde, also observed a reduction in the disappearance of IVDMD with these substances, as we also found. Other studies using EO showed a reduction in feed degradation [26,27], which would be a disadvantage for animal production in the use of this additive.

The search for EO that reduce ruminal CH₄ emissions without affecting feed degradation is important to improve feed efficiency and thus contribute to innovation in green technologies [19]. Feeds with low degradation may make it difficult for rumen microorganisms to extract substrates and, consequently, result in nutrient limitation [28].

In our study, some EO, such as citronella, ho wood, oregano and thyme, likely induced hydrophobic characteristics on the soluble portions of the feed substrate, preventing microbial attack and its metabolism. This was evidenced when analyzing the TGP drop and the maintenance in the degradability of the substrate with increasing inclusion levels.

The partition factors for both DM and OM in oregano and white thyme EO in our research were superior to the other EO when evaluated at a dosage of 500 mg/L. This may be explained by the low production of gases with a concurrent lower substrate disappearance in the presence of white thyme EO and constant substrate disappearance when oregano was added.

The quantification of ammonia concentration is an important indication of nitrogen use efficiency during in vitro bioassays, since 60% to 80% of the nitrogen incorporated by rumen microorganisms comes from ammonia [29]. Its concentration may be affected both by the degradability content of CP present in the substrate and by rumen microorganism use. Feed CP functions as a source of rumen NH₃-N for ruminal microorganisms so it is essential to have sufficient energy available. Thus, rumen NH₃-N is a direct result of the quantity generated and the quantity used by rumen microorganisms [30].

All the EO evaluated presented little variation in NH₃-N content; however, the EO of citronella and ho wood had a positive linear relation between NH₃-N and inclusion levels, suggesting a stabilization of the microbiota environment. The concentrations were always sufficient to support microbiota growth, all being well above the minimum value reported.
by Satter and Slyter (1974) [31], of 5 mL/dL. In addition, they were above 13 mg/dL—the minimum value necessary to avoid compromising the availability of N for microorganisms, and the ingestion and digestibility of fiber [28].

Benchaar et al. (2007) [32] did not observe an effect of cinnamon (400 mg/L), oregano (200 mg/L) and thymol (200 mg/L) EO on rumen NH$_3$-N concentration in relation to the treatment without additives. Cardozo et al. (2005) [33], when testing 0.3 mg/L of garlic, cinnamon and oregano EO, also did not observe a difference compared to the control diets without EO. However, garlic EO doses of 30 and 300 mg/L reduced ammonia concentration by 32.7% and 55.4%, for cinnamon EO by 31.6% and 61.9%, and for oregano EO by 26.9% and 64.5%, respectively. According to those authors, either EO decreased the deamination or microorganisms used the peptides and amino acids as a source of nitrogen, with a consequent reduction in NH$_3$-N.

More important than analyzing the effect of each EO on each variable related to ruminal fermentation is to analyze the influence and consequences that each exerts on the other [34]. The absence of effects on total concentrations of SCFA can also be seen as a positive if it is accompanied by increased feed degradability as well as decreases in C$_2$:C$_3$ ratios and the production of CH$_4$.

A secondary metabolite can have a beneficial effect against methane-producing microorganisms but can also reduce the total concentration of SCFA and/or the degradability of feed in ruminants [35]. There is an inverse relationship between C$_3$ and CH$_4$ production; the metabolic route of C$_3$ production, besides serving as a drain of H$^+$, still generates less of this ion compared to the C$_2$ or C$_4$ route [28,36]. By increasing the C$_3$ production, there is more competition with methanogenic archaea for substrate, generating less CH$_4$ production.

Citronella, globulus, staigeriana, ginger, melaleuca, oregano and white thyme EO had a positive linear relationship with C$_2$:C$_3$ ratios, indicating that the rumen was less energy efficient. However, the C$_2$:C$_3$ ratio and the maintenance of the degradability of coast-cross hay suggests that the addition of EO led to a decline in selectivity in cellulolytic bacteria, responsible for the degradation of fibrous substrates and the main producers of C$_2$.

Diet rich in concentrate are more prone to low ruminal pH, enhancing the effects of EO. According to Cardozo et al. (2005) [33], cinnamon EO and its main component, cinnamaldehyde, increase C$_2$:C$_3$ ratios in a rumen incubation medium with pH 7.0 while in a medium with pH 5.5, it causes a reduction in the C$_2$:C$_3$ ratio. Calsamiglia et al. (2007) [37] also found that thymol EO was more effective at pH 5.5 than at 6.5.

Castillejos et al. (2006) [24] in a 24 h in vitro assay using a substrate with 60% forage evaluated eugenol, guaiacol, limonene, thymol and vanillin EO at doses of 5, 50, 500 and 5000 mg/L. The effects observed by these authors depended on the doses used. The dose of 500 mg/L of thymol EO reduced the total concentration of SCFA (−28.5%), the proportion of propionate (−18.4%) and the concentration of NH$_3$-N (−31.9%) while there was an increase in the proportion of C$_2$ (+1.8%) and in the C$_2$:C$_3$ ratio (+35.5%).

In an experiment testing thyme, oregano and cinnamon EO, and their respective pure compounds—thymol, carvacrol and cinnamaldehyde—on rumen activity, Macheboeuf et al. (2008) [38] observed results similar to those found in our experiment; however, cinnamon EO and its main compound cinnamaldehyde in concentrations lower than 3 mmol/L did not affect the production of CH$_4$ at 5 mmol/L. There was no production of CH$_4$ due to hydrogen accumulation in the headspace of the vial. Oregano, thyme, thymol and carvacrol EO were more toxic to ruminal microbiota, due to the lower dose needed (1, 1.5, 2.0 and 2.5 mmol/L, respectively) to inhibit the ruminal production of CH$_4$. This result reinforces the theory previously discussed that EO effects on ruminal microbiota depend on the dose used.

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The variable CH$_4_e$, expressed in mL/g IVDMD, represents the relationship between the production of net CH$_4$ and IVDMD. The greater the value attributed to this variable, the greater the participation of net CH$_4$ produced per gram of degraded dry matter (IVDMD) during the in vitro incubation process. Although the EO in our trial changed CH$_4$ (mL/g IVDMD), there were no relationships observed between the production of CH$_4$ and IVDMD.
This can be attributed to the high standard error of the mean, since CH$_4$, expressed as mL/g IVDMD, associates variability in gas measurements, CH$_4$ liquid concentration, and residual non-degradable DM.

5. Conclusions

Our study showed that oregano and white thyme EO had the greatest reduction on CH$_4$ production compared with other EO in vitro when using rumen liquid from cattle consuming high-fiber grass diets. However, these EO did not affect substrate disappearance and provided the lowest net CH$_4$ production. Taken together, these preliminary data lead us to believe that these two EO effectively suppress rumen CH$_4$ emissions without negatively affecting digestibility. However, additional research, especially in vivo, is needed with these two EO looking at a greater variety of diets and substrates with sufficient replication to reduce standard errors. We further recommend testing these EO in vivo trials.

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