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In vitro screening of selected essential oils from medicinal plants acclimated to Benin for their effects on methane production from rumen microbial fermentation

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Enteric methane production lowers the efficiency of feed utilization in ruminants and contributes to greenhouse gas emissions which are responsible for global climate change. This study examined the effects of nine essential oils (EO) from *Citrus aurantifolia, Cymbopogon citratus, Eucalyptus citriodora, Laurus nobilis, Lippia multiflora, Mentha piperita, Ocimum basilicum, Ocimum gratissimum* and *Zingiber officinalis* on enteric methane production in *in vitro* batch cultures screening experiments using *Andropogon gayanus* grass. Two *in vitro* batch culture incubation runs were conducted independently on separate days at two different ranges of dosages: 0 (control), 150, 300, 600 and 1200 mg/L inoculum and 0 (control), 25, 50, 100 and 150 mg/L inoculum. The effects of EO on *in vitro* gas production, methane production and apparent dry matter disappearance (DMD) were assessed relative to the control containing no additive. *O. basilicum, E. citriodora, O. gratissimum* and *C. aurantifolia*, significantly inhibited (Z' > 0 and relative decrease ≥ 15%) enteric methane production (g DM incubated) relative to control at dosages of 300-1200 mg/L and *L. nobilis, C. citratus* and *M. piperita* significantly decreased it at 600 and 1200 mg/L. A substantial decrease (Z' > 0 and relative decrease ≥ 15%) in methane production per g DM incubated was apparent for *Z. officinalis* and *L. multiflora* at dosage of 1200 mg/L. Most EO had globally negligible effects on methane production (Z' ≤ 0 and relative decrease < 15%) at dosages of 25 to 150 mg/L. Substantial decrease in apparent DMD together with gas production (g DM) incubated was observed relatively to the control with *Z. officinalis* and *L. multiflora* at 1200 mg/L and with the remaining EO at 600 and 1200 mg/L. Overall, this screening investigation demonstrated that addition of assayed EO (except *Z. officinalis* and *L. multiflora*) at dosages close to 300 mg/L seem to potentially decrease enteric methane production with limited negative effects on dry matter digestibility of forage grass *in vitro*.

Key words: Essential oil, *in vitro*, rumen, digestibility, methane production.

INTRODUCTION

Methane is known as a potent greenhouse gas and its accumulation in the atmosphere is thought to be a key factor in global anthropogenic warming besides carbon dioxide and nitrous oxide (Intergovernmental Panel on Climate Change, 2013). One of the significant contributions to the increase of methane in the atmospheric

Table 1. List of medicinal plants (scientific name, common name and registration number) and plant parts from which the essential oils were extracted.

Scientific name of plants	Common name	Registration number	Plant part	Yield (%)
Citrus aurantifolia (Christm). Swing	Lime	AP 2086 HNB	Fruit peel	0.8
Eucalyptus citriodora Hook	Lemon scent	AAC 181 HNB	Leaves	2.57
Laurus nobilis L.	Laurel	AP 2065 HNB	Leaves	0.25
Mentha piperita L.	Peppermint	AAC177 HNB	Leaves	0.7
Ocimum gratissimum L.	African basil	AAC 176 HNB	Leaves	0.7
Zingiber officinalis Rosc.	Ginger	AP 2095 HNB	Rhizomes	1.0

concentration is by the rumen microbial fermentation in domestic ruminant livestock (Lassey, 2007). Therefore, the interest in modulating the rumen microbial fermentation occurring in ruminant has been increasing with reduction in enteric methane production being the ultimate target. To this end, a number of strategies were recently explored using products with antimicrobial properties including plant secondary metabolites of enteric fermentation which is mediated by microorganism activity in the rumen (Ribeiro et al., 2015; Satyanagalakshmi et al., 2015). Most plant extracts are considered as safe in animal production owing to their natural source unlike chemical additives and antibiotics. As a result, it has been demonstrated that essential oils (EO), among other researched natural products, can favorably alter rumen microbial fermentation and reduce enteric methane production owing to their antimicrobial activity (Bodas et al., 2012).

Antimicrobial properties of EO depend on their chemical composition, which is a function of plant species, harvesting seasons, geographical origin, analytical methodology and the plant organ used (Burt, 2004). Many EO have been shown to inhibit methane production in *in vitro* incubations at high doses together with a decrease in total volatile fatty acid concentrations and feed digestion (Benchaar and Greathead, 2011). There is a challenge in identifying EO that could potentially benefit ruminal fermentation and lower methane production. Therefore, this study aimed to screen the effects of EO from medicinal plants acclimated to Benin on methane production from rumen microbial fermentation of *Andropogon gayanus* grass using *in vitro* batch cultures. The plants were selected because their EO are edible and commonly used in medicinal pharmacopoeia in Benin.

MATERIALS AND METHODS

In vitro experimental design and treatment

The EO were evaluated for their effects on methane production, gas production and apparent dry matter disappearance (DMD) relative to the controls (inoculums plus substrate without EO) in *in vitro* batch cultures using *A. gayanus* grass. Essential oils were tested in an initial screening in a single run as suggested by Secundino et al. (2010) at 4 dosages of 150, 300, 600 and 1200 mg/L inoculum (run 1) as used in a previous study (Benchaar and Greathead, 2011), which observed that most EO inhibited methane production at dosages above 300 mg/L. Based on the results from this experiment where apparent DMD and gas production decreased with most EO at dosages between 300-1200 mg/L, all EO were evaluated in a second incubation run at lower dosages of 25, 50, 100 and 150 mg/L (run 2) to limit their adverse effect on apparent DMD.

Incubation sets containing only inoculum served as blanks and were used to correct fermentation residues, gas and methane production resulting from the inoculum. In the preliminary and second screening assays, a single incubation run was carried out as suggested by Secundino et al. (2010) and each treatment as well as control and blank samples were tested in triplicate.

Essential oils

Six EO were prepared by processing various parts (leaves, rhizomes or fruit peel) of six medicinal plants (Table 1) as previously described by Baba-Moussa et al. (2012). The plants were collected by the departments of Ouémé (Porto Novo, Sèmè, Djérègbé), Plateau (Kétou) and Zou (Setto) in Benin and were botanically identified by the National Herbarium of Benin where their voucher specimens were deposited. For each EO, appropriate plant fractions to obtain the desired volume were collected from many plants with combination of all collected samples. The EO were extracted from the collected plant materials after 72 h of air-drying by steam-distillation for 180-240 min using a Clevenger-type apparatus (Clevenger, 1928) and EO composition was analyzed by gas chromatography coupled to mass spectrometry (GC-MS). In steam distillation, vapours of the volatile components were carried from a plant material by steam, which was produced from water contained in a heated round-botton flask. The resultant mixture of steam flow and oil vapour was condensed in a refrigeration tube, and then collected in a Florentine flask where water and oil were separated. A gas chromatograph (DELSI GC 121 G, Type 300 N°464, DELSI instruments: 92, Surenes, France) equipped with a flame ionization detector and a capillary column (CP WAX 52 CB made by Chrompack, Middelburg, Netherland; length 25 m × 0.25 mm interior diameter, 0.25 µm film thickness) was used. GC-MS was performed (model 5970, Hewlett-Packard, Palo Alto, CA, USA)

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using a DB-5 non-polar capillary column (length 25 m × 0.25 mm interior diameter) with ionization energy of 70 eV. The column temperature was kept at 50°C for 5 min and programmed to subsequently increase at a rate of 5°C/min to 300°C. Essential oils constituents were identified on the basis of their Kovats retention indices and mass spectral fragmentation, using standards, literature data (Adams, 2001) and an established laboratory data bank (Laboratory of Pharmacognosy and Essential Oils at the University of Abomey-Calavi, Benin).

An additional three EO extracted by hydrodistillation and analyzed by GC-MS as reported in Baba-Moussa et al. (2012) were obtained from the Laboratory of Pharmacognosy and Essential Oils at the University of Abomey-Calavi (UAC), Benin. The main components in these EO were (%): estragole, 84.98 for *O. basilicum*; geranial 7.17, thymol 8.46, eucalyptol 10.09, and p-cymene 21.89 for *L. multiflora*; and myrcene 10.78, neral 30.75, and geranial 39.42 for *C. citratus.*

Fermentation substrate

The aerial part of *A. gayanus* grass was collected by cutting plants 10 cm above the soil surface at the flowering stage during July and August 2012 at the pilot farm of the Faculty of Agronomic Science, UAC, Benin (longitudes 1° and 30°40' East). The grass was sun-dried over a period of 3 days followed by oven drying at 60°C for 48 h. Grass was then ground through a 1 mm screen with combination of all the collected samples before use as the fermentation substrate.

Ruminal inoculum and *in vitro* batch incubations

Two ruminally fistulated non-lactating cows fed barley grain and barley silage in a DM ratio of 1:3 were used as donors of rumen fluid for the entire study. Cows were cared for in accordance with standards set by the Canadian Council on Animal Care (CCAC, 1993). Rumen fluid was collected 2 h after the morning feeding by straining rumen contents from four sites within the rumen through four layers of sterilized cheesecloth into pre-warmed insulated containers. Collected rumen fluid was transported immediately to the laboratory under anaerobic conditions, pooled in equal portions and mixed with a mineral buffer (Menke et al., 1979) in 1:2 ratio to prepare the rumen microbial inoculum.

The *in vitro* batch incubations were conducted as described by Wang et al. (2000). Incubations were performed in 125-mL serum vials pre-loaded with 500 mg of substrate and warmed to 39°C. Inoculum (40 mL) was dispensed under a stream of O_2-free CO_2 and an appropriate amount of each EO was added into the inoculum by using a pipette to obtain the final desired concentration. Vials were immediately sealed and affixed to a rotary shaking incubator (120 revolutions per minute) at 39°C for 48 h.

Sampling and analysis

Gas production from each of the three culture vials used for each treatment as well as control and blank was measured at 6, 12, 24 and 48 h of incubation using a water displacement technique (Fedorak and Hrudey, 1983). Prior to measuring of gas production at each time point, 10 mL of headspace gas was sampled for methane analysis (Chaves et al., 2006; Wang et al., 2008).

After 48 h of incubation, each vial was removed from the incubator and its content was transferred into a pre-weighed 50-mL centrifuge tube and centrifuged at 500 ×g (4°C, 10 min) to obtain a solid fraction (fermentation residue) containing undegraded substrate and residual feed particle-associated microbial biomass (Narvaez et al., 2013). The fermentation residue from each vial was washed with dH_2O and centrifuged (500 ×g, 4°C, 10 min) two times, then dried at 50°C and weighed to determine apparent DMD (Narvaez et al., 2013).

The substrate was analyzed for dry matter (DM) and organic matter (OM) content as described by Association of Official Analytical Chemists (AOAC, 2003), neutral detergent fiber (NDF) and acid detergent fiber (ADF) as described by Van Soest (1991). Heat-stable α-amylase and sodium sulphite were used in NDF procedure and expressed inclusive of residual ash. Combustion analysis (NA2100, Carlo Erba Instruments, Rodano, Milan, Italy) was used to determine N.

Calculations and statistical analysis

At each incubation time point, cumulative methane produced was calculated (López et al., 2007) and total net gas production per g of DM incubated (considering the 10 mL sampled for methane analysis) or methane production per g of DM incubated was estimated by subtracting the mean values of blanks from that of the control and test vials. Net cumulative methane and gas production per g of DM incubated were estimated after 48 h of incubation. The apparent DMD was calculated as the difference between incubated weight of the substrate and the dry weight of the fermentation residue corrected for residue weight in the blank (Narvaez et al., 2013).

Because the incubation run was not repeated in each screening trial, the statistical evaluation of results from this study was limited. Therefore, the statistical Z' (Z-prime) factor was calculated as 1-3 $(\sigma_{test} + \sigma_{control})/|\mu_{test} - \mu_{control}|$, where σ indicates standard deviation and μ indicates mean, and it was used to assess the separation between test (EO treatment) and control values regarding net cumulative methane production, net cumulative gas production and apparent DMD after 48 h of incubation (Secundino et al., 2010). Test and control values were declared overlapping at $Z' \leq 0$, whereas both values are different when $Z' > 0$ (Secundino et al., 2010). As the acceptance criterion required for Z' to be confirmed as a sample with a substantial decrease in methane production was not established as observed by Secundino et al. (2010), the effect of each treatment on methane production relative to the control was expressed as the percentage of induced change (increase or decrease) in methane production as compared to the control using the equation $R = (\mu_{test} - \mu_{control}) \times 100/\mu_{control}$ where R (%) = relative effect of EO on methane production, μ_{test} = net cumulative methane production after 48 h of incubation with a given treatment; $\mu_{control}$ = net cumulative methane production after 48 h of incubation from the control (Secundino et al., 2010). Relative decrease (R) in methane production (per g of DM incubated) with addition of EO was considered as significant when $Z' > 0$ and R value was 15% or higher as Secundino et al. (2010) observed from previous laboratory studies which shows that a plant causing a relative decrease of 15% or higher can be considered a promising plant additive deserving further investigation.

RESULTS

Chemical composition of fermentation substrate and essential oils

The substrate used in this study had nutrient composition (g /kg in DM) of OM 920, NDF 679, ADF 347 and CP 105.

The main components in EO analyzed by GC-MS in the

Table 2. Main volatile components (≥ 1%) in plant extracts analyzed by GC-MS.

Scientific name of plants	Number of identified compounds	Oxygenated compound (%)	Main components	%	Kovats retention[a] index
Citrus aurantifolia	34	16.44	α-Pinene	1.96	935
			β-Pinene	8.39	979
			p-Cymene	14.23	1027
			Limonene	51.37	1032
			γ-Terpinene	1.03	1060
			α-Terpineol	6.84	1198
			β-Bisabolene	1.31	1510
Eucalyptus citriodora	21	52.38	Citronellal	76.47	1088
			Isopulegol	1.11	1191
			Citronellol	3.18	1252
			Neoisopugenol	3.98	1319
			Citronnelyl acetate	3.13	1370
			γ-Elemene	1.18	1376
			Methy leugenol	2.62	1430
			Trans-paramenth-3,8 diol	1.37	1435
			Cis-paramenth-3,8 diol	1.06	1511
Laurus nobilis	22	59.06	Oct-1-en-3-ol	1.75	982
			Myrcene	29.09	991
			p-Cymene	1.26	1026
			Limonene	5.00	1031
			1,8-Cineole	1.87	1035
			Linalol	1.95	1101
			Chavicol	9.21	1255
			Eugenol	42.50	1357
Mentha piperita	25	92.51	Limonene	1.60	1031
			Menthone	28.49	1161
			Isomenthone	3.10	1168
			Neo-menthol	1.82	1173
			Menthol	45.53	1184
			Piperitone	6.59	1258
			Menthyl acetate	5.73	1292
Ocimum gratissimum	39	33.58	α-Thujene	5.80	928
			α-Pinene	1.69	935
			Myrcene	4.76	991
			α-Terpinene	1.25	1019
			p-Cymene	19.95	1028
			Limonene	1.17	1031
			γ-Terpinolene	17.52	1061
			Terpinen-4-ol	1.09	1183
			Thymol	27.56	1295
			α-Selinene	2.81	1495
			Caryophyllene oxide	1.73	1588
			α-Pinene	3.70	935
			Camphene	10.79	952
			β-Phellandrene	5.00	1033

Table 2. Contd.

Zingiber officinalis	35	12.59	1,8-Cineole	4.12	1035
			Terpinen-4-ol	1.31	1183
			Geranial	1.94	1269
			ar-Curcumene	11.64	1484
			α-Zingiberene	19.16	1498
			γ-Bulgarene	8.12	1505
			β-Bisabolene	7.17	1511
			β-Sesquiphellandrene	8.57	1527

current study are presented in Table 2. The EO *Citrus aurantifolia* mainly contained limonene (51.37%) and p-cymene (14.23%). The major compound in *Eucalyptus citriodora* was citronellal (76.47%). Those in *Z. officinalis* were camphene (10.79%), ar-curcumene (11.64%) and α-zingiberene (19.16%). *Laurus nobilis* was chiefly rich in chavicol (9.21%), myrcene (29.09%) and eugenol (42.50%). *Mentha piperita* mainly contained menthone (28.49%) and menthol (45. 53%). The abundant components in *O. gratissimum* were γ-terpinolene (17.52%), p-cymene (19.95%) and thymol (27.56%).

Effects of essential oils on *in vitro* rumen microbial fermentation

Incubation run 1

Off all the EO, *O. basilicum, E. citriodora, O. gratissimum and C. aurantifolia* significantly inhibited (Z' > 0 and relative decrease ≥ 15 %) methane production per g dry matter (DM) incubated at dosages of 300-1200 mg/L, whereas apparent DMD and gas production per g DM incubated were decreased (Z' > 0) mainly at 600 and 1200 mg/L relative to the control (Table 3). A substantial decrease (Z' > 0 and relative decrease ≥ 15%) in methane production per g DM incubated was apparent together with a reduction (Z' > 0) in apparent DMD and gas production per g DM incubated for *L. nobilis, C. citratus and M. piperita* mainly at dosages of 600-1200 mg/L. *Z. officinalis* and *L. multiflora* significantly inhibited (Z' > 0 and relative decrease ≥ 15 %) methane production per g DM incubated at dosage of 1200 mg/L together with a reduction (Z' > 0) in apparent DMD and gas production per g DM incubated at 1200 mg/L.

Incubation run 2

Most EO had globally negligible effects on methane production (Z' ≤ 0 and relative decrease < 15 %) at dosages of 25 to 150 mg/L (Table 4). At such dosages, EO treatments and control were overlapping (Z' ≤ 0) regarding apparent DMD and gas production per g DM incubated.

DISCUSSION

Chemical composition of essential oils

Results from the present study reveal the specificity in qualitative and quantitative composition of the EO from each plant species. Essential oil constituents of a given plant species may vary depending on harvesting seasons, geographical origin, analytical methodology and the part of the plant that they are extracted from (Burt, 2004). This may explain the divergence between the chemical composition of EO analyzed in the present study and that previously reported for their chemotypes (Benchaar et al., 2008; Cimanga et al., 2002; Marzouki et al., 2009). For example, a previous report (Marzouki et al., 2009) from Tunisia observed 1,8-cineole, α-terpinyl acetate and methyl eugenol as main components in the *L. nobilis* chemotype. Benchaar et al. (2008) reported camphene (14.1%), ar-curcumene (14.5%) and β-bisadolene (22.1%) as the major compounds in *Z. officinalis*. Similarly to this study, *O. gratissimum* from Democratic Republic of Congo was a thymol type, but it contained more thymol (53.2%) and less p-cymene (7.3%) (Cimanga et al., 2002). Cimanga et al. (2002) also identified eugenol and γ-terpinene as main components in *O. gratissimum* chemotype, whereas γ-terpinolene was absent. Iscan et al. (2002) identified menthol and menthone as major components in four chemotypes of *M. piperita*, but their concentrations were less than those found for *M. piperita* in the present study. A concentration of 53.53% was reported by Javari et al. (2011) for limonene in *C. aurantifolia* from Iran, which agrees with the results of the present study. Alpha terpineol and γ-terpinene are among other major components thought to be responsible for the antimicrobial properties of *C. aurantifolia*, but their concentrations were higher in the previous study than in the present study. The chemotype of *E. citriodora* from Democratic Republic of Congo was found to contain 72.7% citronellal (Cimanga et al., 2002),

Table 3. Effects of essential oils (150-1200 mg/L) on gas production, methane production and apparent dry matter disappearance after 48 of *in vitro* incubation.

Additive	Dosages (mg/L)	GP (mL/g DM)	Z' factor for GP	Methane (mL/g DM)	Z' factor for methane	Change in methane relative to CT (%)	Apparent DMD (g/kg)	Z' factor for apparent DMD
Control	0	160.4	-	44.4		-	456.8	-
Ocimum basilicum	150	154.9	Z' ≤ 0	40.0	Z' ≤ 0	-10.0	424.7	Z' ≤ 0
	300	133.7	Z' ≤ 0	36.2*	Z' > 0	-18.5	355.7	Z' ≤ 0
	600	104.2	Z' > 0	30.3*	Z' > 0	-31.8	244.1	Z' > 0
	1200	52.2	Z' > 0	22.8*	Z' > 0	-48.6	69.6	Z' > 0
Cymbopogon citratus	150	154.2	Z' ≤ 0	38.7	Z' ≤ 0	-12.8	428.2	Z' ≤ 0
	300	155.9	Z' ≤ 0	37.6	Z' ≤ 0	-15.4	429.0	Z' ≤ 0
	600	115.0	Z' > 0	33.7*	Z' > 0	-24.1	288.5	Z' > 0
	1200	82.0	Z' > 0	28.6*	Z' > 0	-35.5	175.3	Z' > 0
Eucalyptus citriodora	150	159.3	Z' ≤ 0	38.8	Z' ≤ 0	-12.6	441.7	Z' ≤ 0
	300	113.6	Z' > 0	33.9*	Z' > 0	-23.7	282.8	Z' > 0
	600	123.6	Z' > 0	33.0*	Z' > 0	-25.8	316.0	Z' > 0
	1200	60.2	Z' > 0	27.3*	Z' > 0	-38.4	63.7	Z' > 0
Ocimum gratissimum	150	153.3	Z' ≤ 0	38.5	Z' ≤ 0	-13.4	425.6	Z' ≤ 0
	300	136.4	Z' ≤ 0	35.4*	Z' > 0	-20.3	347.6	Z' ≤ 0
	600	80.9	Z' > 0	28.0*	Z' > 0	-37.0	170.3	Z' > 0
	1200	39.7	Z' > 0	23.6*	Z' > 0	-46.9	81.4	Z' > 0
Citrus aurantifolia	150	162.0	Z' ≤ 0	38.2	Z' ≤ 0	-14.1	439.5	Z' ≤ 0
	300	149.4	Z' ≤ 0	37.1*	Z' > 0	-16.5	384.8	Z' ≤ 0
	600	130.9	Z' > 0	32.3*	Z' > 0	-27.3	463.6	Z' > 0
	1200	60.8	Z' > 0	25.0*	Z' > 0	-43.8	167.5	Z' > 0
Lippia multiflora	150	160.1	Z' ≤ 0	37.5	Z' ≤ 0	-15.5	430.3	Z' ≤ 0
	300	163.7	Z' ≤ 0	39.4	Z' ≤ 0	-11.3	432.0	Z' ≤ 0
	600	152.8	Z' ≤ 0	37.7	Z' ≤ 0	-15.2	374.6	Z' ≤ 0
	1200	101.3	Z' > 0	31.9*	Z' > 0	-28.2	209.7	Z' > 0
Laurus nobilis	150	161.3	Z' ≤ 0	39.6	Z' ≤ 0	-10.8	430.9	Z' ≤ 0
	300	160.8	Z' ≤ 0	37.8	Z' ≤ 0	-15.0	399.8	Z' ≤ 0
	600	133.9	Z' > 0	35.2*	Z' > 0	-20.6	254.3	Z' > 0
	1200	61.8	Z' > 0	26.9*	Z' > 0	-39.5	62.7	Z' > 0
Zingiber officinalis	150	162.3	Z' ≤ 0	38.3	Z' ≤ 0	-13.8	431.1	Z' ≤ 0
	300	163.2	Z' ≤ 0	38.8	Z' ≤ 0	-12.5	399.9	Z' ≤ 0
	600	154.6	Z' ≤ 0	37.7	Z' ≤ 0	-15.1	328.4	Z' ≤ 0
	1200	139.9	Z' > 0	35.8*	Z' > 0	-19.4	274.2	Z' > 0
Mentha piperita	150	164.0	Z' ≤ 0	38.7	Z' ≤ 0	-12.9	455.4	Z' ≤ 0
	300	154.4	Z' ≤ 0	37.5	Z' ≤ 0	-15.6	475.5	Z' ≤ 0
	600	128.0	Z' ≤ 0	34.3*	Z' > 0	-22.8	316.7	Z' > 0
	1200	62.2	Z' > 0	26.5*	Z' > 0	-40.4	86.2	Z' > 0

CT: Control, DMD: dry matter disappearance, GP: cumulative gas production, *mean value for an additive differs significantly (relative decrease ≥15%) from the control within the column.

Table 4. Effects of essential oils (25-150 mg/L) on gas production, methane production and apparent dry matter disappearance after 48 h of *in vitro* incubation.

Additive	Dosages (mg/L)	GP (mL/g DM)	Z' factor for GP	Methane (mL/g DM)	Z' factor for methane	Change in methane relative to CT (%)	DMD (g/kg)	Z' factor for DMD
Control	0	205.5	-	22.8		-	463.9	-
Ocimum basilicum	25	184.9	Z' ≤ 0	20.1	Z' ≤ 0	-11.6	433.4	Z' ≤ 0
	50	189.9	Z' ≤ 0	21.1	Z' ≤ 0	-7.6	447.0	Z' ≤ 0
	100	177.6	Z' ≤ 0	19.9	Z' ≤ 0	-12.5	433.7	Z' ≤ 0
	150	148.2	Z' > 0	19.0	Z' ≤ 0	-16.4	429.6	Z' ≤ 0
Cymbopogon citratus	25	198.2	Z' ≤ 0	21.1	Z' ≤ 0	-7.2	451.3	Z' ≤ 0
	50	205.4	Z' ≤ 0	22.5	Z' ≤ 0	-1.3	447.5	Z' ≤ 0
	100	199.8	Z' ≤ 0	21.8	Z' ≤ 0	-4.1	454.1	Z' ≤ 0
	150	200.3	Z' ≤ 0	20.5	Z' ≤ 0	-10.2	449.2	Z' ≤ 0
Eucalyptus citriodora	25	201.0	Z' ≤ 0	20.5	Z' ≤ 0	-9.9	436.3	Z' ≤ 0
	50	203.0	Z' ≤ 0	23.1	Z' ≤ 0	1.5	451.5	Z' ≤ 0
	100	187.6	Z' ≤ 0	19.2	Z' ≤ 0	-15.9	432.9	Z' ≤ 0
	150	203.9	Z' ≤ 0	23.3	Z' ≤ 0	2.3	434.7	Z' ≤ 0
Ocimum gratissimum	25	209.6	Z' ≤ 0	22.4	Z' ≤ 0	-1.6	456.7	Z' ≤ 0
	50	205.6	Z' ≤ 0	21.9	Z' ≤ 0	-4.0	423.1	Z' ≤ 0
	100	197.4	Z' ≤ 0	19.4	Z' ≤ 0	-14.9	417.9	Z' ≤ 0
	150	199.3	Z' ≤ 0	20.6	Z' ≤ 0	-9.6	446.3	Z' ≤ 0
Citrus aurantifolia	25	204.1	Z' ≤ 0	21.0	Z' ≤ 0	-7.8	467.0	Z' ≤ 0
	50	207.2	Z' ≤ 0	22.1	Z' ≤ 0	-3.1	468.1	Z' ≤ 0
	100	197.2	Z' ≤ 0	18.6	Z' ≤ 0	-18.2	462.8	Z' ≤ 0
	150	207.7	Z' ≤ 0	20.8	Z' ≤ 0	-8.5	461.3	Z' ≤ 0
Lippia multiflora	25	204.6	Z' ≤ 0	21.0	Z' ≤ 0	-8.0	466.2	Z' ≤ 0
	50	208.2	Z' ≤ 0	23.4	Z' ≤ 0	2.7	477.7	Z' ≤ 0
	100	205.5	Z' ≤ 0	22.0	Z' ≤ 0	-3.6	450.2	Z' ≤ 0
	150	206.5	Z' ≤ 0	22.2	Z' ≤ 0	-2.7	471.8	Z' ≤ 0
Laurus nobilis	25	203.0	Z' ≤ 0	21.2	Z' ≤ 0	-6.7	459.1	Z' ≤ 0
	50	202.5	Z' ≤ 0	23.3	Z' ≤ 0	2.2	477.7	Z' ≤ 0
	100	198.5	Z' ≤ 0	21.7	Z' ≤ 0	-4.5	438.5	Z' ≤ 0
	150	193.2	Z' ≤ 0	22.5	Z' ≤ 0	-1.3	425.3	Z' ≤ 0
Zingiber officinalis	25	200.3	Z' ≤ 0	22.5	Z' ≤ 0	-1.3	473.5	Z' ≤ 0
	50	198.7	Z' ≤ 0	22.8	Z' ≤ 0	-0.1	472.8	Z' ≤ 0
	100	196.7	Z' ≤ 0	22.0	Z' ≤ 0	-3.3	460.9	Z' ≤ 0
	150	194.2	Z' ≤ 0	21.2	Z' ≤ 0	-7.1	455.7	Z' ≤ 0
Mentha piperita	25	194.5	Z' ≤ 0	20.3	Z' ≤ 0	-10.9	481.2	Z' ≤ 0
	50	195.8	Z' > 0	22.6	Z' ≤ 0	-0.8	477.7	Z' ≤ 0
	100	195.9	Z' ≤ 0	21.6	Z' ≤ 0	-5.1	457.0	Z' ≤ 0
	150	197.8	Z' ≤ 0	21.8	Z' ≤ 0	-4.3	467.9	Z' ≤ 0

CT: Control, DMD: dry matter disappearance, GP: cumulative gas production.

an observation which is in agreement with the present study.

Effects of essential oils on methane production and apparent DMD

From the results of this study, addition of most EO at dosages of 300-1200 or 600-1200 mg/L caused a significant decrease in methane production per g DM incubated except *Z. officinalis* and *L. multiflora* whose significant negative effects occurred at a more narrowdosage of 1200 mg/L. Consistent with these results regarding *O. gratissimum, M. piperita* and *L. nobilis*, Patra and Yu (2012) reported that *Thymus capitatus* (thymol type), *M piperita* (menthol type) and *Eugenia* spp. (eugenol type) inhibited methane production (per g DM) at 250-1000 mg/L. In this trial, *C. aurantifolia* reduced methane production (per g DM). This finding is in line with that of Kamalak et al. (2011) which showed that *Citrus sinensis* (limonene type), at 200-1200 mg/L, reduced methane production (per g DM). Results obtained with addition of *O. basilicum* are in agreement with Jahani-Azizabadi et al. (2011) which showed that *O. basilicum* (estragole type) reduced methane production (per g DM) at 1000 mg/L. Information relating to effects of *Z. officinalis* (α-zingiberene and ar-curcumene type), *L. multiflora* (p-cymene type), *C. citratus* (neral and geranial type) and *E. citriodora* (citronellal type) on methane production is scarce.

The decrease in apparent DMD together with gas production observed in this study suggests that EO may exert a general inhibition on rumen microbial fermentation. The extent of the observed effects of EO on apparent DMD and gas production depended on EO type and EO dosages. This is likely a reflection of the differences in EO composition within plant types (Benchaar et al., 2008; Burt, 2004). The reduction in apparent DMD with *Z. officinalis* and *L. multiflora* at 1200 mg/L and with the remaining EO at 600-1200 mg/L suggests that beneficial inhibition in methane production is likely offset by adverse effects on rumen microbial fermentation. Effects of *L. nobilis, O. gratissimum, O. basilicum, M. piperita* and *C. aurantifolia* on apparent DMD and gas production as observed in this work are consistent with previous cited studies (Kamalak et al., 2011; Jahani-Azizabadi et al., 2011; Patra and Yu, 2012) that worked on a range of dietary substrates. To the best knowledge of the authors, there is no study that examined the effects of *Z. officinalis* (α-zingiberene and ar-curcumene type), *L. multiflora* (p-cymene type), *C. citratus* (neral and geranial type) and *E. citriodora* (citronellal type) on apparent DMD and gas production.

In conclusion, reductions in enteric methane production with limited negative effects on apparent DMD seem to potentially occur *in vitro* with all EO (except *Z. officinalis* and *L. multiflora*) at dosages close to or less than 300 mg/L.

Conflict of Interests

The authors have not declared any conflict of interests.

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