Research Article

Received: 21 January 2014 Revised: 4 June 2014 Accepted article published: 13 June 2014 Published online in Wiley Online Library: 15 July 2014

(wileyonlinelibrary.com) DOI 10.1002/jsfa.6785

Effects of essential oils from medicinal plants acclimated to Benin on *in vitro* ruminal fermentation of *Andropogon gayanus* grass

Jacques B Kouazounde,[a] Long Jin,[a] Fidele M Assogba,[b] Marc A Ayedoun,[c] Yuxi Wang,[a] Karen A Beauchemin,[a] Tim A McAllister[a*] and Joachim D Gbenou[b]

Abstract

BACKGROUND: Plants from West Africa commonly used in both human and veterinary medicine contain various secondary metabolites. However, their potential in mitigating ruminal methane production has not been explored. This study examined the effects of seven essential oils (EOs) from plants acclimated to Benin at four dosages (100, 200, 300 and 400 mg L^{-1}), on *in vitro* rumen microbial fermentation and methane production using *Andropogon gayanus* grass as a substrate.

RESULTS: Compared to control, *Laurus nobilis* (300–400 mg L^{-1}), *Citrus aurantifolia* (300–400 mg L^{-1}) and *Ocimum gratissimum* (200–400 mg L^{-1}) decreased ($P < 0.05$) methane production (mL g^{-1} DM) by 8.1–11.8%, 11.9–17.8% and 7.9–30.6%, respectively. Relative to the control, reductions in methane (mL g^{-1} DM) of 11.4%, 13.5% and 14.2% were only observed at 400 mg L^{-1} for *Eucalyptus citriodora*, *Ocimum basilicum* and *Cymbopogon citratus*, respectively. These EOs lowered methane without reducing concentrations of total volatile fatty acids or causing a shift from acetate to propionate production. All EOs (except *M. piperita*) reduced ($P < 0.05$) apparent dry matter (DM) disappearance of *A. gayanus*.

CONCLUSIONS: The current study demonstrated that EOs from plants grown in Benin inhibited *in vitro* methane production mainly through a reduction in apparent DM digestibility.
© 2014 Society of Chemical Industry

Keywords: essential oil; *in vitro*; rumen; volatile fatty acids; digestibility; methane

INTRODUCTION

Ruminant livestock emit methane as an unavoidable by-product of the rumen microbial fermentation of carbohydrates and proteins by anaerobic microbes.[1,2] Enteric methane is of fundamental importance as an electron sink that maintains the intrinsic oxidation–reduction reactions that are pivotal to rumen function.[2] However, enteric methane losses have a negative impact on both the host and environment as it lowers the efficiency of feed utilization and is a potent greenhouse gas.[2] It is thus desirable to develop strategies that mitigate enteric methane production.

Natural plant secondary metabolites such as essential oils (EOs), tannins and saponins show promise as a means of reducing enteric methane from ruminants.[3] However, the potential of extracts from West African plants to modify ruminal fermentation and to reduce enteric methane production has not been explored, despite a long history of use of these secondary metabolites in both human and veterinary medicine.[4] Therefore, the purpose of this study was to investigate the potential of EOs from medicinal plants acclimated to Benin (West Africa) to modify *in vitro* rumen microbial fermentation and reduce methane production from *Andropogon gayanus* grass.

MATERIALS AND METHODS

Essential oils

Six EOs were prepared by processing various parts (leaves, rhizomes or fruit peel) of six medicinal plants (Table 1) collected by the departments of Oueme (Porto Novo, Sèmè, Djérègbé), Plateau (Kétou) and Zou (Setto) in Benin. The plants were botanically identified by the National Herbarium of the University of Abomey Calavi (UAC) where voucher specimens are deposited. Appropriate plant fractions to obtain the desired volume of EO were air dried

* *Correspondence to: Tim A McAllister, Agriculture and Agri-Food Canada, Lethbridge Research Centre P.O. Box 3000, Lethbridge, AB, Canada T1J 4B1. E-mail: tim.mcallister@agr.gc.ca*

a *Agriculture and Agri-Food Canada, Lethbridge Research Centre, P.O. Box 3000, Lethbridge, AB, Canada T1J 4B1*

b *Laboratoire de Pharmacognosie et des Huiles Essentielles, Faculté des Sciences et Techniques, Faculté des Sciences de la Santé, Université d'Abomey-Calavi, ISBA Champ de Foire, 01 BP 918, Cotonou, Benin*

c *Laboratoire de Phytochimie, Faculté des Sciences et Techniques de Natitingou, Faculté de Médecine de Parakou, Université de Parakou, BP 123, Parakou, Benin*

Table 1. List of medicinal plants and plant part from which essential oils were extracted and main volatile components (≥100 g kg^{-1}) in plant extracts as determined by GC-MS

Scientific name of plant	Registration number	Plant part	Main component	Concentration (g kg^{-1})	Kovats retention[a] index
Citrus aurantifolia	AP 2086	Fruit peel	*p*-Cymene	142.3	1027
(Christm). Swing	HNB		Limonene	513.7	1032
Eucalyptus citriodora Hook	AAC 181 HNB	Leaves	Citronellal	764.7	1088
Laurus nobilis L.	AP 2065	Leaves	Myrcene	290.9	991
	HNB		Eugenol	425.0	1357
Mentha piperita L.	AAC177	Leaves	Menthone	284.9	1161
	HNB		Menthol	455.3	1184
Ocimum gratissimum L.	AAC 176	Leaves	*p*-Cymene	199.5	1028
	HNB		γ-Terpinolene	175.2	1061
			Thymol	275.6	1295
Zingiber officinalis Rosc.	AP 2095	Rhizomes	Camphene	107.9	952
	HNB		ar-Curcumene	116.4	1484
			α-Zingiberene	191.6	1498

[a] Retention time of a volatile component relative to the retention times of adjacently eluting normal alkanes on a capillary column.

for 72 h and subjected to steam distillation for 180–240 min using a Clevenger-type apparatus.[5] The recovered EOs were dried using a small amount of anhydrous magnesium sulfate and stored at 4 °C until use. The EOs were analyzed by gas chromatography (GC) and GC coupled to mass spectrometry (GC-MS) for chemical composition (Fig. 1 and Table 1). 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; Chrompack, Middelburg, Netherlands; 25 m × 0.25 mm, 0.25 µm film thickness) was used. The oven was kept at 50 °C for 5 min and programmed to increase to 220 °C (2 °C min^{-1}). Nitrogen was used as the carrier gas at a flow of 1 mL min^{-1} with

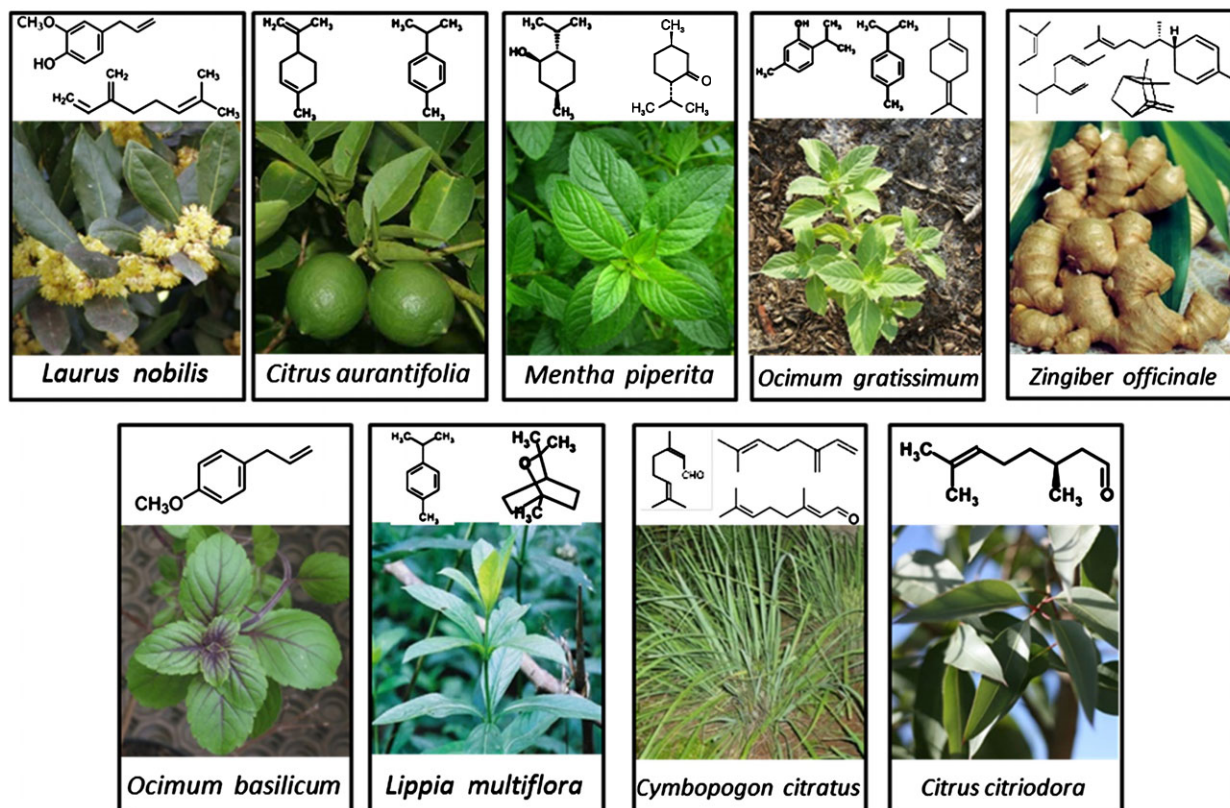

Figure 1. Selected medicinal plants from Benin and associated principal essential oils (≥100 g kg^{-1}; volatile components) as identified by GC-MS analysis and assessed in rumen fluid batch cultures.

 © 2014 Society of Chemical Industry

injector and detector temperatures of 240 and 250 °C, respectively. GC-MS was performed (model 5970; Hewlett–Packard, Palo Alto, CA, USA) using a DB-5 non-polar capillary column (25 m × 0.25 mm) with ionization energy of 70 eV. The column was kept at 50 °C for 5 min and programmed to subsequently increase to 300 °C (5 °C min^{-1}). Helium (0.9 mL min^{-1}) was used as the carrier gas and EO constituents were identified on the basis of their Kovats retention indices and mass spectral fragmentation, using standards, literature data[6] and an established laboratory data bank (Laboratory of Pharmacognosy and Essential Oils at the University of Abomey-Calavi, UAC, Benin).

An additional three EOs, *Cymbopogon citratus* (DC.) Stapf, *Lippia multiflora* Moldenke and *Ocimum basilicum* L. were obtained from the Laboratory of Pharmacognosy and Essential Oils, UAC, Benin. The main components in these EOs (Fig. 1) as extracted by hydrodistillation and analyzed by GC-MS were (in g kg^{-1}): estragole 849.8 for *O. basilicum*; geranial 71.7, thymol 84.6, eucalyptol 100.9, and *p*-cymene 218.9 for *L. multiflora*; and myrcene 107.8, neral 307.5, and geranial 394.2 for *C. citratus*.[7]

In vitro experimental design and treatment

Preliminary *in vitro* batch culture incubations were conducted in a single run to screen the effects of all nine EOs at four dosages (150, 300, 600 and 1200 mg L^{-1} inoculum) on methane production, gas production and dry matter disappearance (DMD) using *A. gayanus* grass as a substrate. This dosage range was chosen as per a previous study,[8] which observed that most EOs inhibited methane production in *in vitro* incubations at dosages above 300 mg L^{-1}. The results revealed that most EOs decreased DMD and gas production at dosages between 300 and 1200 mg L^{-1}. Therefore, all EOs were evaluated in a second preliminary screening at lower dosages of 25, 50, 100 and 150 mg L^{-1} in a single run in an effort to limit their adverse effect on DMD. From preliminary screenings, most EOs caused a drastic reduction in DMD and gas production at 600 and 1200 mg L^{-1} with negligible effects on methane production at 25 to 150 mg L^{-1}.

Based on the results from screening assays, a main study was carried out through a series of *in vitro* batch culture to evaluate EOs at four dosages (100, 200, 300 and 400 mg L^{-1}) where methane production was most likely to be reduced with no or a limited decline in DMD. *Lippia multiflora* and *Zingiber officinalis* were excluded from the main study as they exhibited negligible effects on *in vitro* methane production at dosages under 1200 mg L^{-1}. The main study was repeated twice in two runs.

Incubation sets containing only inoculum and substrate served as controls and those containing only inoculum served as blanks within screening and main runs. The blanks served to correct for fermentation residues, gas and methane production resulting from the inoculum.

Substrate, ruminal inoculum and *in vitro* batch incubations

A single lot of dried *A. gayanus* grass was used as the substrate for all *in vitro* batch incubations. *A. gauanus* is the principal low-quality forage fed to ruminants in West Africa and is representative of the low-quality tropical grasses which are often associated with high enteric methane emissions. The aerial part of the forage was collected by cutting plants 10 cm above the soil surface at the flowering stage between 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 for 3 days followed by oven drying at 60 °C for 48 h. Grass was then ground through a 1 mm screen with all collected samples combined.

Two ruminally fistulated non-lactating cows fed barley grain and barley silage in a 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).[9] Rumen fluid was collected 2 h after the morning feeding as previously described[10] and mixed 1:2 with a mineral buffer[11] to obtain inoculum.

In vitro batch incubations were conducted as described by Wang *et al.*[12] In each run, incubations were performed in 125-mL serum vials in triplicate for all treatments and for control and blank samples. Vials pre-loaded with 500 mg of substrate were warmed to 39 °C, and inoculum (40 mL) was dispensed under a stream of O_2-free CO_2 and an appropriate amount of each EO was added using a pipette to obtain the final desired concentration. Vials were immediately sealed with a foldable rubber stopper and affixed to a rotary shaking incubator (120 rpm) at 39 °C for 48 h.

Sampling and analysis

Gas production from each culture vial was measured at 6, 12, 24 and 48 h of incubation using a water displacement technique.[13] Prior to measuring gas production at each time point, 10 mL of headspace gas was sampled for methane analysis.[14,15]

After 48 h of incubation, each vial was removed from the incubator and contents were transferred into a pre-weighed 50-mL centrifuge tube and centrifuged at 500 × *g* (4 °C, 10 min) to separate a liquid and solid fraction which contained residual feed particles and associated microbial mass.[16] The fermentation residue from each vial was washed with dH_2O and centrifuged (500 × *g*, 4 °C, 10 min) two times, dried at 50 °C and weighed to determine apparent dry matter disappearance (DMD).[16] The supernatant was processed for VFA and ammonia analysis.[17] The substrate was analyzed for DM and organic matter (OM) content,[18] neutral detergent fiber (NDF) and acid detergent fiber (ADF) as described by Van Soest.[19] Heat-stable α-amylase and sodium sulfite were used in NDF procedure and expressed inclusive of residual ash. Combustion analysis (NA2100; Carlo Erba Instruments, Rodano, Milan, Italy) was used to determine nitrogen. The substrate used in this study had nutrient composition (g kg^{-1} in DM) of OM 920.3, NDF 678.8, ADF 346.5 and CP 104.7.

Calculations and statistical analysis

Total VFA and gas production per g DM incubated; cumulative methane (per g of DM incubated and DMD) as well as ammonia-N in the incubation fluid were estimated after 48 h of incubation. Apparent dry matter disappearance (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.[16]

Net gas production data collected over time was fitted using the SAS MODEL[20] procedure to the equation[21] $GP = b(1 - e^{-ct})$ separately for each EO treatment, dosage, run and replication, where GP (in mL g^{-1} DM incubated) is the cumulative gas production, *b* (in mL g^{-1} DM incubated) is the potential gas production, *c* (in h^{-1}) is the gas production rate, and *t* (in h) is the gas measurement time. The estimated coefficients (*b* and *c*) were output to a file for further analysis as described below. PROC UNIVARIATE[20] was used to test the residuals for outliers and normality, and obvious outliers were removed before undertaking further analyses.

 © 2014 Society of Chemical Industry

As only one control was used for all treatment combinations, treatments did not have a factorial structure. Therefore, in order to compare all treatment combinations with the control, an analysis was performed using the seven EOs and four dosages combined with the control to form one treatment variable (EO_DOSAGE) with 29 levels ($7 \times 4 +$ Control). PROC MIXED[20] was then used to analyze the estimated coefficients (*b* and *c*) and other dependent variables measured at the end of the experiments with EO_DOSAGE in the model as a fixed effect and RUN as a random effect. Dunnett's test was used to compare all treatment levels to the control. A second analysis was performed, after removing the control treatment, to test for EO by dosage interactions using PROC MIXED with EO, dosage and their interaction considered as fixed effects, and RUN as a random effect in the model. Orthogonal polynomials were fitted to test for linear and quadratic effects of EO dosage.

RESULTS

Screening assays served to identify the dosage to be used in the main study, thus the ensuing results were neither reported nor discussed. In the main study, all interactions between EOs and DOSAGE were significant ($P < 0.05$). Cumulative gas production (GP) exhibited a quadratic response to all EOs ($P < 0.05$; Table 2). The rate of gas production was linearly decreased ($P < 0.05$) by *O. basilicum* and responded quadratically ($P < 0.05$) with *O. gratissimum*. With the exception of *M. piperita*, all EO either linearly decreased or quadratically ($P < 0.05$) impacted methane production per g DM. A quadratic effect on methane production per g DM was apparent for *O. basilicum*, *C. citratus*, *E. citriodora* and *O. gratissimum,* whereas a linear response was observed for *C. aurantifolia* and *L. nobilis*. *O. basilicum*, *E. Citriodora* and *C. aurantifolia* linearly increased ($P < 0.05$) methane production per g DMD whereas *O. gratissimum* and *L. nobilis* altered it in a quadratic manner ($P < 0.05$).

Concentrations of total VFA (TVFA) per g of DM incubated was linearly decreased ($P < 0.05$) by *E. citriodora* and *O. gratissimum* while it responded quadratically to *C. citratus* [($P < 0.05$); Table 3]. Acetate was quadratically ($P < 0.05$) affected by *C. citratus*, *E. citriodora*, *O. gratissimum* and *L. nobilis* while it was linearly increased ($P < 0.05$) by the remaining EO ($P < 0.05$). Propionate was quadratically affected ($P < 0.05$) by *E. citriodora*, *O. gratissimum* and *C. aurantifolia*. The acetate to propionate ratio (acetate:propionate) was quadratically ($P < 0.05$) affected by *C. citratus*, *E. citriodora* and *O. gratissimum* whereas it was linearly increased ($P < 0.05$) by other EO. Of all EOs, *C. citratus*, *O. gratissimum*, *C. aurantifolia* and *O. basilicum* affected the molar proportion of butyrate quadratically ($P < 0.05$) while it was linearly decreased by *L. nobilis* ($P < 0.05$). Addition of *E. citriodora* and *O. gratissimum* linearly decreased ($P < 0.05$) ammonia-N concentration while *L. nobilis* and *M. piperita* resulted in a quadratic relationship ($P < 0.05$; Table 3).With the exception of *M. piperita*, all EO linearly decreased or quadratically impacted DMD ($P < 0.05$; Table 3). The quadratic effect occurred with *C. citratus*, *E. citriodora* and *L. nobilis*.

DISCUSSION

Effects of essential oils on methane production

In the current study, all EOs with the exception of *M. piperita* inhibited methane production per unit of DM incubated. However, the observed negligible effects on methane production when expressed on a DMD basis suggest the decrease in methane was mainly due to a reduction in digestibility. Furthermore, the observed decrease in propionate production and increase in acetate:propionate is contrary to a direct inhibitory effect of EO on methane production.[1] Together, these results indicate a lack of specificity in the influence of these EOs on methanogenesis. There were significant interactions between EO and dosage on methane production suggesting dependence of our findings on EO type and dosage. This may be due to differences in the biological activity of EOs against methanogens or hydrogen producing bacteria owing to differences in the types and amount of phenolics isolated in extracts.[22]

The selection of *A. gayanus* as a substrate for *in vitro* studies could have also influenced the efficacy of EOs at reducing methanogenesis as previous work has suggested that EO extracts are more effective at lowering methane production from concentrate as opposed to forage diets.[8] One of the goals of this work was to apply EOs to feed from the same region from which they were derived. As *A. gayanus* is one of the most common native grasses in the tropical and sub-tropical savannas of Africa, it was a logical choice as a substrate for the present study.

Of all assayed EOs, *O. gratissimum* appeared to possess the most potent anti-methanogen activity. Effects exhibited by *O. gratissimum* on methane production (DM incubated basis) are in agreement with Patra and Yu[23] who observed that inclusion of *Thymus capitatus* (thymol type) at 250–1000 mg L^{-1} decreased methane production (per g DM) and increased acetate:propionate, but this EO also reduced methane production per g of DMD. Our results may be explained by the presence of thymol in *O. gratissimum*. In a recent review, Benchaar and Greathead[8] reported that thymol had the potential to depress methane production with a minimum active dosage of 300 mg L^{-1} with *p*-cymene (20 mg L^{-1}) reducing methane production by 30% *in vitro*.

The potency of limonene and *p*-cymene (main components in *C. aurantifolia*) to inhibit methane production has been reported in previous studies.[3,8,24] Similar to the current experiment, Kamalak *et al.*[25] noted that *Citrus sinensis* (limonene type), at 200–1200 mg L^{-1}, reduced methane production (per g DM) with a concomitant increase in acetate:propionate. Sallam and Abdelgaleil[24] reported that methane production (per g DM) was depressed by *Citrus reticulata* (limonene type) at 667–1000 μL L^{-1}.

Effects exhibited by *L. nobilis* on methane production (per g DM) are similar to those reported for other eugenol type EO such as *Cinnamomum zeylanicum* and *Eugenia* spp.[23,25,26] In contrast, Patra and Yu[23] observed that *Eugenia* spp. decreased methane production per g DMD. Consistent with effects of *L. nobilis*, Benchaar and Greathead[8] reported that eugenol decreased methane production at 400 and 500 mg L^{-1}.

Jahani-Azizabadi *et al.*[27] observed that *O. basilicum* (estragole type) inhibited methane production per g DM, in agreement with the current study. In contrast, this author also observed that *O. basilicum* decreased methane production per g DMD. Our results with *M. piperita* contradict Patra and Yu[23] who observed a decrease in methane production (per g DM and per g DMD) at dosages of 250–1000 mg L^{-1}. Agarwal *et al.*[28] also observed that, at 0.33–2 mL L^{-1}, *M. piperita* inhibited methane production per g DMD, which disagrees with our results at 100–400 mg L^{-1}. The divergence between our results and these previous studies may be due to variations in diet type, minor components and/or concentration of menthol in chemoypes of *M. piperita*. There is a dearth of information on the effects of *C. citratus* and *E. citriodora* on methane production.

 © 2014 Society of Chemical Industry

Table 2. Effect of essential oils on *in vitro* gas and methane production from *Andropogon gayanus*

Additive	Dosage ($mg\ L^{-1}$)	Gas production kinetics *b* ($mL\ g^{-1}$ DMI)	*c* (h^{-1})	GP-48 h ($mL\ g^{-1}$ DMI)	Methane ($mL\ g^{-1}$ DMI)	Methane ($mL\ g^{-1}$ DMD)
Control	0	268.1	0.018	152.7	25.8	58.2
	SE	10.56	0.001	2.04	0.405	1.352
Ocimum basilicum	100	250.6	0.016	149.0	25.7	60.3
	200	245.1	0.018	144.1*	24.7	64.6
	300	226.1*	0.019	136.2*	25.6	68.2*
	400	203.1*	0.020	127.0*	22.3*	72.0*
	Contrast	L	L	Q	Q	L
Cymbopogon citratus	100	260.1	0.019	153.0	25.7	58.3
	200	259.0	0.019	152.6	25.8	57.9
	300	260.9	0.017	145.4*	25.5	58.4
	400	207.4*	0.020	125.2*	22.2*	59.9
	Contrast	Q	–	Q	Q	–
Eucalyptus citriodora	100	246.1	0.021	153.3	26.0	57.9
	200	247.8	0.020	151.1	26.7	59.5
	300	220.9*	0.020	138.3*	24.5	59.0
	400	196.1*	0.022*	128.5*	22.9*	61.7
	Contrast	Q	–	Q	Q	L
Ocimum gratissimum	100	233.4*	0.021	148.1	25.4	57.9
	200	218.8*	0.021	137.4*	23.8*	60.7
	300	202.2*	0.019	128.6*	23.3*	62.4
	400	151.2*	0.025*	106.3*	17.9*	58.8
	Contrast	Q	Q	Q	Q	Q
Citrus aurantifolia	100	253.2	0.019	150.2	25.6	56.4
	200	250.2	0.019	147.5	25.3	53.7
	300	232.7*	0.019	136.1*	22.7*	60.9
	400	210.6*	0.019	125.8*	21.2*	60.0
	Contrast	L	–	Q	L	L
Laurus nobilis	100	240.9	0.021	150.5	24.9	59.1
	200	235.2*	0.021	147.9	24.7	55.3
	300	228.0*	0.022*	143.5*	23.7*	59.8
	400	201.8*	0.023*	133.2*	22.8*	62.3
	Contrast	L	–	Q	L	Q
Mentha piperita	100	235.2*	0.022*	150.6	25.1	56.6
	200	228.7*	0.022*	149.1	25.0	56.6
	300	215.7*	0.023*	142.8*	24.2	55.4
	400	209.8*	0.023*	138.4*	24.4	58.2
	Contrast	L	–	Q	–	–
Statistics						
Degrees of freedom, *df*						
	EO × dosage	135	138	136	136	130
SEM	EO	10.56	0.001	2.04	0.41	1.35
	Dosage	10.10	0.001	2.00	0.41	1.36
P-value	EO	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
	Dosage	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
	EO × dosage	0.013	0.023	< 0.001	< 0.001	0.005

*Mean value for an additive differs significantly ($P < 0.05$) from the control within the column.
GP, cumulative gas production; DMI, dry matter incubated; L, linear contrast ($P < 0.05$); Q, quadratic contrast ($P < 0.05$).
EO, essential oil.

Effects of essential oils on volatile fatty acid production

Compared to the control, most EO caused no change in TVFA concentration, suggesting that even the highest dose of EO was too low to depress VFA production. This is not consistent with the adverse effects of these EO on DMD and GP and may be due to a higher sensitivity and/or resistance of some specific types of cellulolytic bacteria to EO.[16] Alternatively, a reduction in methane could have contributed to reduced GP and biomass feed particle adherent bacteria may have resulted in an underestimation of substrate DMD. However, given the inhibitory effects of EO on microbial populations, this underestimation is likely to be less than for residue in treatment than control vials.

 © 2014 Society of Chemical Industry

Table 3. Effect of essential oils on *in vitro* ruminal fermentation of *Andropogon gayanus*

Additive	Dosage (mg L^{-1})	TVFA (mmol g^{-1} DMI)	Volatile fatty acids Acetate	Propionate	Butyrate	Valerate	BCVFA	A:P	DMD (g kg^{-1})	Ammonia-N (mmol L^{-1})
Control	0	7.1	65.5	22.7	6.8	1.4	3.6	2.9	440.3	19.1
	SE	0.819	1.178	0.271	0.449	0.123	0.597	0.090	8.61	3.376
Ocimum basilicum	100	7.1	65.9	22.4	6.7	1.4	3.4	2.9	435.9	17.8
	200	6.0	67.5*	20.6*	6.4	1.3*	3.5	3.3*	405.9	18.7
	300	6.6	69.6*	19.5*	6.3	1.3*	3.2	3.6*	377.2*	17.2
	400	7.0	71.8*	18.0*	6.9	1.3*	2.3*	4.0*	312.4*	16.0
	Contrast		L	L	Q	L	Q	L	L	–
Cymbopogon citratus	100	8.4	65.8	22.1	7.1	1.4	3.6	3.0	440.5	16.7
	200	9.8*	66.6	21.0*	7.4	1.3*	3.5	3.2*	445.9	15.0
	300	8.4	68.1*	20.3*	6.8	1.3*	3.4	3.4*	436.6	16.4
	400	7.2	72.2*	18.4*	6.1	1.2*	2.5*	3.9*	372.5*	13.9
	Contrast	Q	Q	L	Q	L	Q	Q	Q	–
Eucalyptus citriodora	100	8.7	65.7	21.7	7.6	1.3*	3.5	3.0	449.4	18.0
	200	7.7	66.0	21.5	7.5	1.3*	3.6	3.1	448.1	17.5
	300	7.9	68.6*	18.9*	7.9*	1.2*	3.4	3.7*	413.9	14.0
	400	7.0	70.3*	17.4*	7.9*	1.2*	3.1*	4.1*	371.6*	12.5*
	Contrast	L	Q	Q	–	L	L	Q	Q	*L*
Ocimum gratissimum	100	8.2	66.3	21.7	7.0	1.3*	3.5	3.1	438.9	16.6
	200	7.4	68.8*	20.8*	6.2	1.3*	2.9*	3.3*	393.0*	14.6
	300	6.3	70.4*	19.8*	5.9*	1.2*	2.6*	3.6*	372.6*	13.9
	400	5.2	74.3*	15.8*	6.5	1.3*	2.0*	4.7*	306.7*	11.9*
	Contrast	L	Q	Q	Q	Q	L	Q	L	L
Citrus aurantifolia	100	7.2	66.2	22.4	6.4	1.3*	3.5	3.0	455.1	16.7
	200	6.5	67.2*	22.2	5.9*	1.3*	3.5	3.0	419.2	19.4
	300	6.3	69.7*	21.2*	5.4*	1.2*	2.5*	3.3*	363.4*	14.1
	400	6.2	70.8*	19.7*	5.9*	1.2*	2.2*	3.6*	354.5*	15.5
	Contrast	–	L	Q	Q	L	L	L	L	–
Laurus nobilis	100	7.2	65.6	22.5	6.9	1.4	3.6	2.9	422.5	20.0
	200	7.2	65.8	22.2	6.9	1.4	3.4	3.0	447.1	16.2
	300	6.5	66.5	22.0	6.6	1.4	3.4	3.0	397.3*	14.9
	400	6.2	68.3*	20.8*	6.4	1.3*	3.2	3.3*	366.5*	19.2
	Contrast	–	Q	L	L	L	L	L	Q	Q
Mentha piperita	100	7.0	65.0	22.8	6.9	1.4	3.8	2.9	443.1	18.3
	200	6.9	65.1	22.5	6.9	1.4	3.9	2.9	442.7	15.5
	300	6.7	66.1	21.6	6.8	1.4	4.0	3.1	437.2	15.5
	400	6.6	66.9*	21.1*	6.6	1.3*	3.6	3.2*	419.4	18.6
	Contrast	–	L	L	–	L	Q	L	–	Q
Statistics										
Degrees of freedom, *df*										
	EO × dosage	137	132	123	137	138	134	133	136	134
SEM	EO	0.82	1.18	0.27	0.45	0.12	0.60	0.09	0.86	3.38
	Dosage	0.82	1.21	0.27	0.46	0.12	0.60	0.09	0.92	3.43
P-value	EO	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.003
	Dosage	< 0.001	< 0.001	< 0.001	0.005	0.005	< 0.001	< 0.001	< 0.001	< 0.001
	EO × dosage	0.108	< 0.001	< 0.001	< 0.001	0.009	< 0.001	< 0.001	< 0.001	0.050

Results for volatile fatty acids (VFAs) are given as mmol L^{-1} VFA per 100 mmol L^{-1} total volatile fatty acids.
*Mean value for an additive differs significantly ($P < 0.05$) from the control within the column.
BCVFA, branched-chain VFA; A:P: acetate: propionate ratio; DMI, dry matter incubated; L, linear contrast ($P < 0.05$); Q, quadratic contrast ($P < 0.05$).
EO, essential oil.

 © 2014 Society of Chemical Industry

On the whole, assayed EO shifted VFA profiles towards less propionate and butyrate and more acetate, with significant interactions between EO and dosage effects. In a number of studies,[23,29,30] thymol, *Thymus zygis* (thymol 62.1%, *p*-cymene 17.03%) and *T. capitatus* exerted effects similar to *O. gratissimum* on VFA profile, but decreased TVFA concentration. Benchar *et al.*[31] reported that at 200 mg L^{-1}, thymol reduced propionate production with no change in acetate or TVFA concentration.

Regarding *L. nobilis*, apart from the trend in butyrate production, some similarities were observed between our study and others[30,32,33] when eugenol and eugenol based EO were included up to 800 mg L^{-1}. Busquet *et al.*[33] noted that up to 300 mg L^{-1}, *Syzygium aromaticum* (85% of eugenol) had no impact on TVFA concentration, supporting our results. However, *S. aromaticum* has also been observed to decrease acetate production without altering propionate.[33] Similar to the present study, Patra and Yu[23] noted that *Eugenia* spp. decreased propionate production without any change in acetate production. In contrast, TVFA concentration decreased and butyrate production increased with *Eugenia* spp.

Effects exhibited by *M. piperita* on VFA in the current study agree with Patra and Yu[23] who observed no change in TVFA concentration with *M. piperita* (menthol type) at 250–1000 mg L^{-1}, whereas acetate and acetate:propionate increased and propionate decreased at 1000 mg L^{-1}, but these two studies diverge regarding the impact on butyrate production. The alteration in VFA profile with *M. piperita* (100–400 mg L^{-1}) agrees with Agarwal *et al.*[28] whom reported that *M. piperita* had no effect on VFA at low concentrations but decreased TVFA at high concentrations.

A number of reports[24,25,30] observed that limonene (50–5000 mg L^{-1} and 400–800 μL L^{-1}), *C. reticulata* (333–1000 μL L^{-1}) and *C. sinensis* (100–1200 mg L^{-1}) decreased TVFA concentration, contradicting results obtained with *C. aurantifolia* (100–400 mg L^{-1}) in the present study. These differences may arise due to differences in the concentration of limonene, the principal EO in this plant. Kamalak *et al.*[25] noted that *C. sinensis*, at 200–1200 mg L^{-1}, shifted the VFA pattern towards more acetate and less propionate.

Effects of essential oils on *in vitro* digestibility

Essential oils have been shown to have the potential to modulate rumen microbial fermentation by altering rumen microbial activity. In the current study, all EOs with the exception of *M. piperita* consistently decreased DMD as reflected by a decline in GP, suggesting that they broadly inhibit rumen microorganisms. This may be governed by EO type (chemical composition) and assayed dosages as suggested by the significant interactions between EOs and dosage effects on DMD.

Of all EOs assayed, *O. gratissimum* appeared to be the most detrimental to rumen microbial activity with clear inhibition at dosages of 200–400 mg L^{-1}. Consistent with this study, Martinez *et al.*[29] observed a decrease in DMD of an alfalfa hay:barley grain mixture (30:70) using extracts from *T. zygis* at 1.35 mL L^{-1}. Inclusion of *T. capitatus* at dosages of 250–1000 mg L^{-1} also decreased DMD and GP (DM incubated basis) from an alfalfa hay:concentrate mixture (50:50).[23] Our results may be explained by the presence of thymol in *O. gratissimum* as phenonolic monoterpes have been shown to have activities against both Gram-positive and Gram-negative bacteria due to their hydrophobicity, low molecular weight and hydroxyl functional group.[22]

Laurus nobilis is rich in eugenol, and consistent with our work, Patra and Yu[23] observed that *Eugenia* spp. decreased DMD along with GP (DM incubated basis) from ground alfalfa hay:concentrate (50:50) at dosages of 250–1000 mg L^{-1}. *Cinnamomum zeylanicum* (76% of eugenol) also decreased DMD and GP from barley silage and dairy concentrate (50:50) at 500 mg L^{-1} in a Rusitec system.[26] Eugenol is a phenolic monoterpene with antimicrobial activity against a broad spectrum of microorganisms.[22,34] A previous study[31] reported a decrease in DMD and GP (DM incubated basis) from a 51:49 forage:concentrate diet when eugenol was added at 800 mg L^{-1}.

Similar to the current experiment, it was observed that *O. basilicum* decreased DMD along with GP (DM incubated basis) of a 80:20 alfalfa hay:concentrate diet at 1000 mg L^{-1}.[27] A previous report[35] indicated that *O. basilicum* exhibited antimicrobial activity against a wide range of Gram-positive and Gram-negative bacteria due its high content of estragole, corroborating the reduction in digestibility observed in the present experiment.

Information related to effects of *C. citratus* (citral type) and *E. citriodora* (citronellal type) on rumen microbial fermentation *in vitro* is scarce. The adverse effect of *E. citriodora* on DMD may be due to citronellal as it has been shown to strongly inhibits rumen microbial activity and exhibit activity against both Gram-positive and Gram-negative bacteria.[34,36]

M. piperita is rich in menthol and menthone, and in agreement with our results, Patra and Yu[23] observed negligible effects on DMD while GP (DM incubated basis) decreased from ground alfalfa hay:dairy concentrate (50:50) when it was included at 250 mg L^{-1}. In contrast, DMD of ground alfalfa hay: concentrate at (50:50) and (80:20) were adversely affected by *M. piperita* (menthol type) when it was included at dosages of 500–1000 mg L^{-1} and 1 mL L^{-1}, respectively.[23,27]

Consistent with effects exhibited by *C. aurantifolia* in the present study, others reported that *Citrus limon* (1000 mg L^{-1}), *C. reticulata* (333–1000 μL L^{-1}) and *C. sinensis* (200–1200 mg L^{-1}) contain chiefly limonene and decreased DMD and GP (DM incubated basis) from a range of dietary substrates.[24,25,27] In an *in vitro* study,[24] limonene was found to be responsible for the effects of *C. reticulata* on rumen microbial activity.

CONCLUSION

In summary, most of the EOs evaluated in this *in vitro* study modified rumen microbial fermentation and reduced ruminal production of methane from low quality grass forage. However, at the dosages used, the reduction in methane was achieved mainly through a reduction in digestibility. Further research is needed to determine an effective dose rate and whether these EO could potentially benefit ruminal fermentation and lower methane production when used with other feed types.

ACKNOWLEDGEMENTS

The authors have benefited from technical support from Krysty Munns, Darrel Vedres, John Baah, Smart Wendy, Zhongjun Xu and Toby Entz. This project was financially supported by the Canadian Beef Cattle Research Council as part of a program to assess and identify plant extracts that may be used as alternatives to antibiotics.

REFERENCES

1 Moss AR, Jouany J-P and Newbold J, Methane production by ruminants: Its contribution to global warming. *Ann Zootechnol* **49**:231–253 (2000).

© 2014 Society of Chemical Industry

2 Bodas R, Prieto N, García-González R, Andrés S, Giráldez FJ and López S, Manipulation of rumen fermentation and methane production with plant secondary metabolites. *Anim Feed Sci Technol* **176**:78–93 (2012).
3 Patra AK and Saxena J, A new perspective on the use of plant secondary metabolites to inhibit methanogenesis in the rumen. *Phytochemistry* **71**:1198–1222 (2010).
4 Stark TD, Mtui DJ and Balemba OB, Ethnopharmacological survey of plants used in the traditional treatment of gastrointestinal pain, inflammation and diarrhea in Africa: Future perspectives for integration into modern medicine. *Animals* **3**:158–227 (2013).
5 Clevenger JF, Apparatus for the determination of volatile oil. *J Am Pharm Assoc* **17**:345–349 (1928).
6 Baba-Moussa F, Adjanohoun A, Attakpa ES, Kpavodé L, Gbénou JD, Akpagana K, *et al*, Antimicrobial activity of three essential oils from Benin on five oral germs: Test of mouthbaths. *Ann Biol Res* **3**:5192–5199 (2012).
7 Adams RP, *Identification of Essential Oils Components by Gas Chromatography/Quadrupoloe Mass Spectrometry*. Allured Publishing Corporation, Carol Stream (2001).
8 Benchaar C and Greathead H, Essential oils and opportunities to mitigate enteric methane emissions from ruminants. *Anim Feed Sci Technol* **166**:338–355 (2011).
9 Canadian Council of Animal Care (CCAC), *Guide to the Care and Use of Experimental Animals*, ed. by Olfert ED, Cross BM and McWilliam AA. CCAC, Ottawa, ON (1993).
10 Wang Y, Barbieri LR, Berg BP and McAllister TA, Effects of mixing sainfoin with alfalfa on ensiling, ruminal fermentation and total tract digestion of silage. *Anim Feed Sci Technol* **135**:296–314 (2007).
11 Menke KH, Raab L, Salewski A, Steingass H, Fritz D and Schneider W, The estimation of the digestibility and metabolisable energy content of ruminant feeding stuffs from the gas production when they are incubated with rumen liquor in vitro. *J Agric Sci* **93**:217–222 (1979).
12 Wang Y, McAllister TA, Yanke LJ, Xu ZJ, Cheeke PR and Cheng KJ, *In vitro* effects of steroidal saponins from *Yucca schidigera* extract on rumen microbial protein synthesis and ruminal fermentation. *J Sci Food Agric* **80**:2114–2122 (2000).
13 Fedorak PM and Hrudey SE, A simple apparatus for measuring gas production by methanogenic cultures in serum bottles. *Environ Technol Lett* **4**:425–432 (1983).
14 Wang Y, Xu Z, Bach SJ and McAllister TA, Effects of phlorotannins from *Ascophyllum nodosum* (brown seaweed) on *in vitro* ruminal digestion of mixed forage or barley grain. *Anim Feed Sci Technol* **145**:375–395 (2008).
15 Chaves AV, Thompson LC, Iwaasa AD, Scott SL, Olson ME, Benchaar C, *et al*, Effect of pasture type (alfalfa vs grass) on methane and carbon dioxide production by yearling beef heifers. *Can J Anim Sci* **86**:409–418 (2006).
16 Narvaez N, Wang Y, Xu ZJ and McAllister TA, Effects of California chaparral plants on *in vitro* ruminal fermentation of forage and concentrate diet. *J Sci Food Agric* **93**:550–559 (2013).
17 Wang Y, McAllister TA, Newbold CJ, Rode LM, Cheeke PR and Cheng K-J, Effects of *Yucca schidigera* extract on fermentation and degradation of steroidal saponins in the rumen simulation technique (RUSITEC). *Anim Feed Sci Technol* **74**:143–153 (1998).
18 Association of Official Analytical Chemists (AOAC), *Official Methods of Analysis*, 17th edition, 2nd revision. AOAC, Gaithersburg, MD (2003).
19 Van Soest PJ, Robertson JB and Lewis BA, Methods for dietary fiber, neutral detergent fiber, and non-starch polysaccharides in relation to animal nutrition. *J Dairy Sci* **74**:3583–3597 (1991).
20 SAS Institute Inc, SAS OnlineDoc® 9.3.1. SAS Institute Inc., Cary, NC (2012).
21 Blummel M and Ørskov ER, Comparison of *in vitro* gas production and nylon-bag degradability of roughage in predicting feed intake in cattle. *Anim Feed Sci Technol* **40**:109–119 (1993).
22 Dorman HJD and Deans SG, Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *J Appl Microbiol* **88**:308–316 (2000).
23 Patra AK and Yu Z, Effects of essential oils on methane production, fermentation, abundance and diversity of rumen microbial populations. *Appl Environ Microbiol* **78**:872–877 (2012).
24 Sallam SMA and Abdelgaleil SAM, Effect of different levels of citrus essential oil and its active component on rumen microbial fermentation and methane production *in vitro*. *Cuban J Agric Sci* **44**:367–372 (2010).
25 Kamalak A, Atalay AI, Ozkan CO, Tatliyer A and Kaya E, Effect of essential orange (*Citrus sinensis L.*) oil on rumen microbial fermentation using *in vitro* gas production technique. *J Anim Plant Sci* **21**:764–769(2011).
26 Fraser GR, Chaves AV, Wang Y, McAllister TA, Beauchemin KA and Benchaar C, Assessment of the effects of cinnamon leaf oil on rumen microbial fermentation using two continuous culture systems**.** *J Dairy Sci* **90**:2315–2328 (2007).
27 Jahani-Azizabadi H, Mesgaran MD, Vakili AR, Rezayazdi K and Hashemi M, Effect of various medicinal plant essential oils obtained from semi-arid climate on rumen fermentation characteristics of a high forage diet using *in vitro* batch culture. *Afr J Microbiol Res* **5**:4812–4819 (2011).
28 Agarwal N, Shekhar C, Kumar R, Chaudhary LC and Kamra DN, Animal effect of peppermint (*Mentha piperita*) oil on *in vitro* methanogenesis and fermentation of feed with buffalo rumen liquor. *Anim Feed Sci Technol* **148**:321–327 (2009).
29 Martinez S, Madrid J, Hernandez F, Megias MD, Sotomayor JA and Jordan MJ, Effect of thyme essential oils (*Thymus hyemalis* and *Thymus zygis*) and monensin on *in vitro* ruminal degradation and volatile fatty acid production. *J Agric Food Chem* **54**:6598–6602 (2006).
30 Castillejos L, Calsamiglia S and Ferret A, Effect of essential oil active compounds on rumen microbial fermentation and nutrient flow in *in vitro* systems. *J Dairy Sci* **89**:2649–2658 (2006).
31 Benchaar C, Chaves AV, Fraser GR, Wang Y, Beauchemin KA and McAllister TA, Effects of essential oils and their components on *in vitro* rumen microbial fermentation. *Can J Anim Sci* **87**:413–419 (2007).
32 Chaves AV, He ML, Yang WZ, Hristov AN, McAllister TA and Benchaar C, Effects of essential oils on proteolytic, deaminative and methanogenic activities of mixed ruminal bacteria. *Can J Anim Sci* **88**:117–122 (2008).
33 Busquet M, Calsamiglia S, Ferret A and Kamel C, Plant extracts affect *in vitro* rumen microbial fermentation. *J Dairy Sci* **89**:761–771 (2006).
34 Burt S, Essential oils: Their antibacterial properties and potential applications in foods – A review. *Int J Food Microbiol* **94**:223–253 (2004).
35 Wan J, Wilcock A and Coventry MJ, The effect of essential oils of basil on the growth of *Aeromonas hydrophila* and *Pseudomonas fluorescens*. *J Appl Microbiol* **84**:152–158 (1998).
36 Oh HK, Sakai T, Jones MB and Longhurst WM, Effect of various essential oils isolated from Douglas Fir needles upon sheep and deer rumen microbial activity. *Appl Microbiol* **15**:777–784 (1967).

 © 2014 Society of Chemical Industry