CSIRO PUBLISHING
Animal Production Science
https://doi.org/10.1071/AN17469

Essential oils from *Lippia turbinata* and *Tagetes minuta* persistently reduce *in vitro* ruminal methane production in a continuous-culture system

F. Garcia[A,B,F], *P. E. Vercoe*[C], *M. J. Martínez*[D], *Z. Durmic*[C], *M. A. Brunetti*[D], *M. V. Moreno*[D], *D. Colombatto*[A,E], *E. Lucini*[B] *and J. Martínez Ferrer*[D]

[A]Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Godoy Cruz 2290, Buenos Aires, C1425FQB, Argentina.
[B]Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, Félix Aldo Marrone 746, Córdoba, 5000, Argentina.
[C]School of Agriculture and Environment, University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia.
[D]Instituto Nacional de Tecnología Agropecuaria, Ruta Nacional 9, Km 636, Manfredi, 5988, Argentina.
[E]Facultad de Agronomía, Universidad de Buenos Aires, Avenida San Martín 4453, Buenos Aires, C1417DSQ, Argentina.
[F]Corresponding author. Email: fgarcia@agro.unc.edu.ar

Abstract. The aim of the present study was to evaluate the impact of essential oils (EO) from *Lippia turbinata* (LT) and *Tagetes minuta* (TM) as well as the rotation of both EO on fermentation parameters *in vitro*. Daily addition of LT, TM, or a 3-day rotation between them (TM/LT), as well as a control (without EO), was evaluated using the rumen simulation technique (Rusitec). The experiment lasted 19 days, with a 7-day adaptation period, followed by 12 days of treatment (Days 0–12). The EO were dissolved in ethanol (70% vol/vol) to be added daily to fermenters (300 μL/L) from Day 0. Daily measurements included methane concentration, total gas production, apparent DM disappearance and pH, which started 2 days before the addition of treatments. On Days 0, 4, 8 and 12 apparent crude protein disappearance and neutral detergent fibre disappearance, ammonia and volatile fatty acid concentration and composition were determined. Methane production was significantly inhibited shortly after addition of both EO added individually, and persisted over time with no apparent adaptation to EO addition. The TM/LT treatment showed a similar effect on methane production, suggesting that rotating the EO did not bring further improvements in reduction or persistency compared with the inclusion of the EO individually. Gas production, total volatile fatty acid concentration and composition and apparent crude protein disappearance were not affected by EO addition. Compared with the control, a 5% reduction of apparent DM disappearance and a 15% reduction of neutral detergent fibre disappearance were observed with the addition of EO. Only TM and TM/LT reduced ammonia concentration. Given the significant and persistent antimethanogenic activity of both EO, and the potential of *T. minuta* to modify nitrogen metabolism, EO from these plant species are of interest for developing new feed additives with potential application in ruminant nutrition that are also likely to be acceptable to consumers.

Additional keywords: adaptation, greenhouse gases, supplements, ruminants, Rusitec, semi-continuous cultures.

Received 13 July 2017, accepted 11 February 2018, published online 24 May 2018

Introduction

Climate change is one of the most serious challenges faced by mankind, with the livestock sector emerging as a significant contributor to total anthropogenic emissions of greenhouse gases (GHG; Steinfeld *et al*. 2006). However, ruminants play an important role in providing high quality protein essential for human diets, and the global demand for livestock products will continue to increase (Opio *et al*. 2013). The largest source of GHG emissions in ruminant production is methane, which accounts for ~47% of the sector's emissions, and more than 90% of total methane emissions (Opio *et al*. 2013). Methane has a warming potential 28 times greater than that of carbon dioxide (IPCC 2013), and it is a loss of productive energy for the animal, since a mole of methane contains the same gross energy as acetate (i.e. 0.88 MJ/mol; Bodas *et al*. 2012). Therefore, reducing enteric methane emissions from ruminants is beneficial from both an environmental (i.e. reduced contribution of the agricultural sector to total GHG emissions) and a nutritional (i.e. improved feed efficiency and animal productivity) perspective (Steinfeld *et al*. 2006).

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The use of dietary additives as ruminal modifiers is one of the most effective mitigation strategies (Veneman *et al*. 2016). In the past five decades, in-feed antibiotics have been used to mitigate ruminal methane, but this practise is facing scrutiny due to consumer concerns over antibiotic resistance (Robinson *et al*. 2017). As a consequence, there are some countries and/or markets where the use of antibiotics as ruminal modifiers has been, or will be, restricted. Natural products with antimicrobial activity, particularly plant secondary metabolites, have been shown to be effective in manipulating ruminal methane and methanogens (Banik *et al*. 2016). Among the plant compounds evaluated, essential oils (EO) have shown potential to be used as additives in ruminant nutrition (Acamovic and Brooker 2005; Benchaar and Greathead 2011; Khiaosa-ard and Zebeli 2013). However, there are also reports that benefits associated with EO can be lost or diminish over time due to shifts in microbial populations or adaptation of individual microbial species to EO (McIntosh *et al*. 2003; Cardozo *et al*. 2004; Busquet *et al*. 2005*a*). This can potentially be overcome by making use of the fact that EO are very complex natural mixtures of compounds that can contain ~20–60 components with different chemical functional groups and at quite different concentrations (Dorman and Deans 2000; Dewick 2002; Bakkali *et al*. 2008).

Preliminary *in vitro* batch-culture studies from our group demonstrated that selected EO from Argentinean native species might have positive effects on rumen microbial fermentation, by inhibiting methane production, and potentially biohydrogenation, which is another hydrogen-utilising pathway, without affecting overall ruminal fermentation. Moreover, altering biohydrogenation in the rumen may improve the fatty acid composition of meat and milk, with potential positive effects on human health (Lock and Bauman 2004). In the present paper, we focussed on methane, considering that EO from *Lippia turbinata* and *Tagetes minuta* when added to batch culture with a mix of ruminal microbes had the potential to inhibit methane production by 60% and 90% compared with a control without EO (Martínez Ferrer *et al*. 2014; Garcia *et al*. 2015, 2016). We chose EO from *L. turbinata* and *T. mintuta* for further evaluation here because their long-term persistency and possible microbial adaptation have not been examined.

The genus *Lippia* is composed of aromatic shrubs that accumulate high amounts of EO (Juliani *et al*. 2004), mainly found in South and Central America (Terblanché and Kornelius 1996). The EO from *L. turbinata* exhibit bactericidal, fungicidal and virucidal activity (Dellacasa *et al*. 2003; García *et al*. 2003; Pérez-Zamora *et al*. 2016). *Tagetes minuta* is also an aromatic plant native from South America, that has a variety of uses, and it is known for its biocidic properties, acting against bacteria, fungi and insects (Héthélyi *et al*. 1986; Senatore *et al*. 2004; Garcia *et al*. 2012). The composition of its oil is a distinctive characteristic of this group of plants, as it has components that are only present in this genus (e.g. dihydrotagetone, tagetone and tagetenone; Chamorro *et al*. 2008). The composition of these two EO is very different; the EO from *L. turbinata* has a great predominance of hydrocarbons, while the EO from *T. minuta* is mainly represented by oxygenated terpene compounds. Therefore, it is possible that the mechanism of their bioactivity is also different and alternating their use may increase the potential to overcome microbial adaptation.

The aim of the present study was to evaluate the impact of *L. turbinata* and *T. minuta* EO, and their rotational use, on methane production over a period of time. We hypothesised that methanogenesis can be reduced by the addition of these two EO; and that the potential adaptation of rumen microorganisms to EO can be overcome by sequentially alternating the use of these two different EO.

Materials and methods

Experimental design

Essential oils from *L. turbinata* (LT) and *T. minuta* (TM) were used as methane-mitigation additives. Daily supply of LT, TM or their sequential rotational use every 3 days (TM/LT), as well as controls (basal substrate), were evaluated using the rumen simulation technique (Rusitec) as described by Czerkawski and Breckenridge (1977). A small amount of sunflower oil (1 g/100 g DM) was added to the base substrate to ensure that there was enough oil present to enable the evaluation of any effect on biohydrogenation of long-chain fatty acids (results are not presented in the present paper). Controls included basal substrate enriched with sunflower oil (control) and a basal substrate without sunflower oil (negative control), to evaluate any effect of the addition of sunflower oil on methane production. The treatments were randomly allocated to the experimental units (fermentation vessels), with three replicates per treatment. The 15 fermentation vessels were set in two Rusitec units (eight in one, and seven in the other) and the two were run simultaneously.

The experiment lasted 19 days, including a 7-day adaptation period to allow equilibration of microbial populations (Martínez *et al*. 2011), followed by 12 days of treatment addition (Days 0–12) defined as treatment period.

Plant material

Lippia turbinata and *T. minuta* were collected in Córdoba, Argentina (31°28′77″S, 64°12′32″W) between February and March 2014. Aerial parts of plants were harvested at full flowering (strongest antimicrobial activity; Burt 2004), air-dried at room temperature, and plant destillate was obtained by steam distillation using an all-glass Clevenger-type apparatus. The EO were dried over anhydrous sodium sulfate and stored at −20°C until further analyses. The EO composition was analysed by gas chromatography coupled with mass spectrometry, using a Perkin Elmer Clarus 600 GC-MS (Waltham, Massachusetts, United States) instrument equipped with two columns: Supelcowax 10 (30 m × 0.25 μm) and DB-5 fused silica column (30 m × 0.25 μm). The carrier gas was helium at an initial temperature of 60°C for 3 min, and then increased 4°C/min up to 240°C. Compounds were identified by comparing their retention time and mass spectra with published data (Adams 1995) and National Institute of Standards and Technology libraries (NIST version 3.0). The proportion of each compound was calculated by dividing its peak area by the total area of all the compounds detected in EO.

Rusitec fermentation

Fermentation was performed using two units of Artificial Rumen Rusitec S (Sanshin Industrial Co. Ltd, Takada-higashi, Kohoku-

ku, Yokohama, Japan). Each Rusitec unit was equipped with a water bath in which the vessels (1-L effective volume) were allocated to maintain fermentation temperature (39°C) throughout the whole experiment. Rumen content was obtained from three ruminally fistulated adult sheep accustomed to a diet of 1 kg of oaten chaff and 0.2 kg of lupin grain (per sheep/day) for 2 weeks before rumen-fluid collection. Animals were managed according to protocols approved by the University of Western Australia Animal Care and Use Committee.

Ruminal contents were collected 2 h after the morning feeding by using a vacuum pump via a rumen cannula and immediately transferred to the laboratory in a pre-warmed thermos. Rumen contents were strained to separate fluid from large feed particles and rumen fluid and digesta were pooled separately. Vessels were filled with 360 mL of warmed McDougall's buffer of pH 8.2 (McDougall 1948) modified to contain 1.0 g/L of $(NH_4)_2SO_4$, as suggested by Fraser *et al*. (2007), and 240 mL of filtered rumen fluid (initial working volume: 600 mL). On the first day, a bag (7 × 17 cm, 100-μm pore size) with whole rumen digesta (60 g wet weight) and a bag with substrate (15 g DM basis) were placed within each fermentation unit. Vessels were flushed with nearly 2 L of N_2 each, so as to maintain anaerobic conditions.

After 24 h, the bag with solid rumen digesta was replaced with a bag containing 15 g DM of substrate. Thereafter, one bag was replaced daily, which meant that each bag remained in the fermenter for 48 h. Substrate was a feedlot-type diet for lambs (EasyOne, Milne Feeds, Perth, WA, Australia), ground through a 4-mm screen. The nutrient composition of the basal diet was as follows: organic matter, 930 g/kg DM; crude protein (CP), 149 g/kg DM; neutral detergent fibre (NDF), 509 g/kg DM; and *in vitro* DM digestibility, 707 g/kg DM. During the adaptation period, 12 fermenters were supplied with basal substrate plus 200 μL of sunflower oil added straight into feed bags immediately before they were exchanged, while negative control vessels (n = 3) received only the 15 g DM of basal substrate.

Stock solutions of each EO were dissolved in ethanol (70% vol/vol) to achieve a final dose of 12 μL/g DM incubated. The dose (300 μL/L) was selected on the basis of previous *in vitro* batch-culture screening studies (J. Martínez Ferrer, unpubl. data). A volume of 1 mL of this solution was added to fermenters once daily at feeding time, from Day 0 up to the end of the experiment. Control vessels received an equivalent amount (1 mL) of ethanol (70% vol/vol).

Artificial saliva was continuously infused into the fermenters at a dilution rate of 4%/h, by using a peristaltic pump (Thermo Scientific FH100M Series, Thermo Scientific Inc., Waltham, Massachusetts, United States). The pH of the fermentation medium was monitored daily to maintain it at ~6.5–6.9. Effluent from each fermentation vessel was collected in 1-L bottles fitted with Alltech® Economy Mobile Phase Caps 2-Hole Screw Cap (Columbia, Maryland, United States) placed in a refrigerator to stop microbial growth and effluent accumulation was determined daily after feed-bag exchange.

Measurements, sample collection and analyses

Gas and methane production

Gas was collected into reusable 4000-mL silver wine-bladder bags attached to each effluent bottle and replaced daily just after feeding. Total gas production from each fermenter was determined by emptying the bag with a 200-mL syringe. Just before determination of total gas, methane concentration was measured directly from the bag by gas chromatography using a Varian CP-4900 Quad Micro-GC (Varian Inc., Palo Alto, California, United States). The injector temperature was 50°C, and the PoraPLOT Q column (10 m × 0.25 mm) was held constantly at 60°C during analysis, while the thermal conductivity detector temperature was 60°C. The carrier gas was helium and its flow was adjusted to 24 mL/min. Methane content was calculated by external-standard regression curve created by three levels of methane (20%, 10% and 5%) generated from a standard gas mixture.

DM, NDF and CP disappearance

Feed bags removed from fermenters were immediately placed in ice and washed under cold tap water until the water running off was clear, and then dried at 60°C for 48 h. The apparent DM disappearance was calculated from the loss in weight of the bag. Substrate and residues of Days 2, 6, 10 and 14 (corresponding to bags that were fed on Treatment days 0, 4, 8 and 12) were analysed for CP and NDF, so as to be able to estimate apparent CP disappearance and NDF disappearance respectively. Analysis of CP was performed by the Kjeldahl method (954.01; AOCS 1998) and NDF analysis was performed according to Van Soest *et al*. (1991) by using an Ankom 220 Fibre Analyser unit (Ankom Technology Corporation, Fairport, New York, United States). It was assayed with a heat-stable amylase and expressed inclusive of residual ash.

Ammonia, volatile fatty acids and pH

On Days 0, 4, 8 and 12, samples of the liquid fraction were collected 3 h post-feeding and stored immediately at −20°C until analysis of ammonia (1 mL with 200 μL 2 M HCl), and volatile fatty acids (VFA; 1 mL with 200 μL 1 M NaOH). Samples were taken directly from a port on the top of the fermentation vessels. The ammonia concentration in the fermentation fluid was determined by spectrometry with a Boehringer Mannheim Test kit 1112732 (R-Biopharm AG, Darmstadt, Hesse, Germany) on Cobas Mira S autoanalyser (Roche Diagnostics, F Hoffmann-La Roche Ltd, Basel, Switzerland), using principles of enzymatic assay described by Bergmeyer and Beutler (1985). Samples for VFA analysis were centrifuged and the supernatant was analysed by gas chromatography using Agilent GC equipment (6890 Series, Agilent Technologies Inc., Santa Clara, California, United States). Injector temperature was 260°C and the column (capillary column HP-FFAP, 30 m × 0.53 mm × 1.0 m) temperature was held constant at 240°C, while the flame ionisation-detector temperature was 265°C. Carrier gas was hydrogen and its flow was adjusted to 6.6 mL/min. Branched-chained VFA were calculated as the sum of iso-butyric acid and iso-valeric acid. The pH of the fluid was measured daily, using a pH meter (Waterproof EcoTestr Model WD-35423-10, Oakton Instruments, Vernon Hills, Illinois, United States), immediately before feeding and 3 h after the exchange of substrate bags.

Statistical analyses

Data were analysed using a mixed linear model with repeated-measures, using R Platform (DCOM) under InfoStat software (Di Rienzo *et al*. 2016). The mixed model accounted for treatment, day and their interaction as fixed effects, and Rusitec units (n = 2) and fermentation vessels within Rusitec units (eight vessels in one unit, and seven in the other) as random effects. Different covariance structures were evaluated for each variable and the one that yielded the lowest Akaike and Bayesian information criteria was considered to be the most desirable for analysis. Differences among means were tested using Di Rienzo, Guzman and Casanoves (DGC, Di Rienzo *et al*. 2002) multiple-comparison test and were declared statistically significant when $P \leq 0.05$. Differences among means with $0.05 < P \leq 0.10$ were accepted as representing tendencies to differences.

Results

There were no significant differences among fermentation vessels for any of the variables evaluated on Day 0. Our main focus was on effects of EO added individually or combined in time (their rotational use) on methane production. Therefore, although long-chain fatty acid profile was determined, these results are not presented in the paper. The negative control differed from the control in methane production when expressed per gram of NDF degraded ($P < 0.001$; 26.0 ± 1.51 and 21.3 ± 1.55 mL/g degraded NDF respectively) and in pH ($P < 0.001$; 6.81 ± 0.02 and 6.74 ± 0.02 respectively), whereas NDF disappearance tended (P = 0.090) to be lower (0.242 ± 0.011 and 0.268 ± 0.011 g/g respectively). The rest of the variables were similar among controls (data not shown).

Essential oil yield and composition

The yield of EO from *L. turbinata* was 2.8 mL/100 g of plant distillate, and *T. minuta* yield was 1.7 mL/100 g of plant distillate. Chemical composition of both EO is shown in Table 1. Of the 11 compounds identified for *L. turbinata*, two were sesquiterpenes (i.e. caryophyllene oxide and β caryophyllene) and the rest were monoterpenes. The main chemical functional group in this EO was hydrocarbons, with four compounds accounting for 71% of the total composition. The other functional groups present were alcohols (6%), ethers (8%), epoxides (6%), ketones (6%) and ketone-alcohols (3%). The major compound was limonene (62%; Fig. 1*a*), followed by bornyl acetate (8%; Fig. 1*b*) and carvone (6%; Fig. 1*c*). The oxygenated terpene compounds represented ~30% of the composition.

Nine compounds were identified for *T. minuta*, with only one sesquiterpene (i.e. spathulenol), while the rest were monoterpenes. Of the four chemical functional groups present, ketones were the most abundant (81%), followed by hydrocarbons (11%), alcohols (6%) and epoxides (2%). Main compounds were verbenone (42%; Fig. 1*d*), *cis* tagetone (28%; Fig. 1*e*), limonene (6%; Fig. 1*a*) and *trans* tagetone (6%; Fig. 1*f*). Oxygenated terpene compounds represented almost 90% of the composition.

Methane, gas and substrate disappearance

Effects of EO treatments on methane, total gas and substrate disappearance are presented in Table 2. There was a significant interaction of treatment by day for methane concentration (mL/100 mL), and daily methane production (mL/g DM; Fig. 2*a*), either when expressed on an incubated or degraded DM basis. The methane concentration was 60% lower for LT than for the control, and over 75% lower for TM and TM/LT than for the control on the first day of treatment addition. Methane concentration was reduced further by 10% on Day 2, and continued to diminish, reaching over a 90% reduction from Day 6 onward for the three EO treatments. When expressed on a degraded NDF basis, methane production was also lower by above 90%, but there was no interaction of treatment by day for this variable.

There was a significant treatment per day interaction for gas production ($P = 0.014$; Fig. 2*b*). The LT produced 24% and 10% less gas than did the control on Days 1 and 10 respectively, while TM also produced between 10% and 30% less gas than did the control on Days 1–5, and on Day 10 respectively. The TM/LT produced between 10% and 40% less gas than did the control on Days 1, 2, 4 and 12.

Compared with the control, apparent DM disappearance was reduced ~5% with the addition of EO (P = 0.015), and was affected by incubation day (P < 0.001), but no interaction between treatment and day was detected for this variable (P = 0.21; Fig. 2*c*). The NDF disappearance was affected by day (P = 0.004), being ~10% higher on Day 8 (data not shown), and tended ($P = 0.090$) to be lower with EO treatments

Table 1. Chemical composition of essential oils from *Lippia turbinata* and *Tagetes minuta*

n.d., not detected

Component	Chemical formula	Proportion of component (%) *Lippia turbinata*	*Tagetes minuta*
	Alcohols		
cis-Carveol	$C_{10}H_{18}O$	2.1	n.d.
Linalool	$C_{10}H_{18}O$	n.d.	1.4
Spathulenol	$C_{15}H_{24}O$	n.d.	4.6
α-Terpineol	$C_{10}H_{18}O$	3.8	n.d.
	Ethers		
Bornyl acetate	$C_{12}H_{20}O_2$	8.4	n.d.
	Epoxide		
Caryophyllene oxide	$C_{15}H_{24}O$	2.7	n.d.
α-Pinene oxide	$C_{10}H_{16}O$	n.d.	2.3
Piperitone oxide	$C_{10}H_{16}O_2$	3.2	n.d.
	Hydrocarbons		
Camphene	$C_{10}H_{16}$	3.3	n.d.
β-Caryophyllene	$C_{15}H_{24}$	3.0	n.d.
Limonene	$C_{10}H_{16}$	62.1	6.0
β-*trans*-Ocimene	$C_{10}H_{16}$	n.d.	5.1
Sabinene	$C_{10}H_{16}$	2.2	n.d.
	Ketones		
Carvone	$C_{10}H_{14}O$	6.0	n.d.
Piperitenone	$C_{10}H_{14}O$	n.d.	4.6
cis-Tagetone	$C_{10}H_{16}O$	n.d.	27.9
trans-Tagetone	$C_{10}H_{16}O$	n.d.	5.9
Verbenone	$C_{10}H_{14}O$	n.d.	42.2
	Ketone alcohols		
Cinerolone	$C_{10}H_{14}O_2$	3.2	n.d.

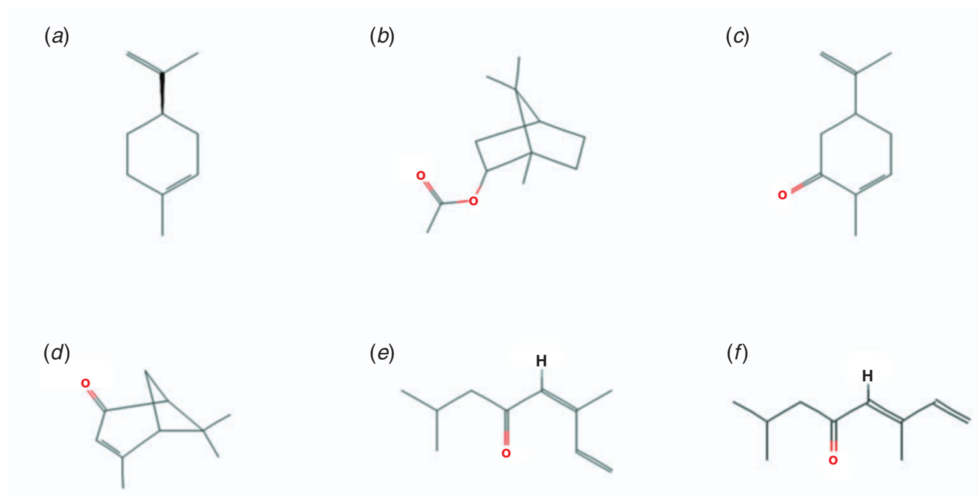

Fig. 1. Chemical structures of the main compounds present in *Lippia turbinata*: (*a*) limonene, (*b*) bornyl acetate and (*c*) carvone; and in *Tagetes minuta*: (*d*) verbenone, (*e*) *cis* tagetone, (*a*) limonene and (*f*) *trans* tagetone.

Table 2. Effect of essential oils of *Lippia turbinata* (LT), *Tagetes minuta* (TM) or their alternate use (TM/LT) on methane, total gas and substrate disappearance of the substrate fermented in the Rusitec

Dose of EO is 300 µL/L. Values are means ± s.e.m. Pretreatment values are averages across all fermenters (n = 15) on Day 0 (before treatment addition). Treatment period: Days 4, 8 and 12 for neutral detergent fibre and crude protein disappearance and Days 1 to 12 for the rest of the variables. Probabilities (F-test) for treatment (T), sampling day (D), and their interaction (T × D) for the treatment period are shown. Means within each row during the treatment period followed by different letters (a, b) are statistically significantly different (DGC test; $P \leq 0.05$) or have a tendency (x, y) to be different (DGC test; $0.05 < P \leq 0.10$) between the treatments

Variable	Pretreatment	Treatment period				*P*-value		
		Control	LT	TM	TM/LT	T	D	T × D
				Gas production				
Methane (mL/100 mL total gas)	3.36 ± 0.153	5.25a ± 0.103	0.61b ± 0.039	0.49b ± 0.039	0.55b ± 0.039	<0.0001	<0.0001	<0.0001
Methane (mL/day)	25.42 ± 1.746	44.97a ± 1.614	4.90b ± 0.344	3.64c ± 0.344	4.11c ± 0.344	<0.0001	<0.0001	<0.0001
Methane (mL/g DM)	1.69 ± 0.115	3.01a ± 0.109	0.33b ± 0.024	0.24c ± 0.024	0.27c ± 0.024	<0.0001	<0.0001	<0.0001
Methane (mL/g DM) degraded	3.13 ± 0.217	5.42a ± 0.246	0.61b ± 0.050	0.47b ± 0.050	0.52b ± 0.050	<0.0001	<0.0001	<0.0001
Methane (mL/g NDF degraded)	14.29 ± 0.860	21.27a ± 1.545	2.16b ± 0.237	2.04b ± 0.237	2.44b ± 0.266	<0.0001	0.5253	0.5058
Total gas production (mL/day)	481.9 ± 22.98	580.0a ± 34.09	560.4a ± 34.09	493.6a ± 34.15	513.8a ± 34.09	0.3818	<0.0001	0.0141
				Substrate apparent disappearance (g/g)				
Dry matter	0.535 ± 0.0065	0.554a ± 0.0058	0.534b ± 0.0058	0.525b ± 0.0058	0.532b ± 0.0060	0.0152	<0.0001	0.2131
Neutral detergent fibre	0.238 ± 0.0090	0.268x ± 0.0107	0.233y ± 0.0107	0.223y ± 0.0107	0.226y ± 0.0116	0.0903	0.0036	0.6077
Crude protein	0.722 ± 0.0102	0.711a ± 0.0177	0.724a ± 0.0177	0.725a ± 0.0185	0.718a ± 0.0177	0.2515	0.1389	0.8741

than in the control (~15%). There were no treatment, day or interaction treatment by day effects detected for apparent CP disappearance.

Fermentation parameters

The effects of EO addition on ammonia, VFA and pH are shown on Table 3. Effects of day and treatment were observed for ammonia concentration, but not for their interaction. Overall, ammonia concentration declined over the measurement period (Fig. 3*a*). In comparison to Day 0, there was a decrease from Day 0 to Day 12 in ammonia concentration (from 446.2 to 344.6 mg/L; Fig. 3*a*). When compared with the control, TM and TM/LT reduced (P = 0.002) ammonia concentration by 21% and 14% respectively.

Total VFA concentration was not affected by treatment, day or their interaction, neither the molar proportions of butyrate, iso-butyrate, iso-valerate, valerate and branched-chain VFA were affected. Molar proportion of acetate tended (P = 0.054) to decrease from 52.8 to 49.6 mol/100 mol from Day 4 to Day 12, whereas molar proportion of propionate decreased (P = 0.004) from 18.7 to 15.3 mol/100 mol; thus, these concurrent reductions meant that the acetate to propionate ratio was maintained. In contrast, molar proportion of caproate increased (P = 0.002) from 4.0 to 7.3 mol/100 mol between Day 4 and Day 12, without an effect of treatment or the interaction of treatment by day.

The interaction was significant for pre- and post-feeding pH, which was higher with EO addition for almost all treatment days (Fig. 3*b*, *c*).

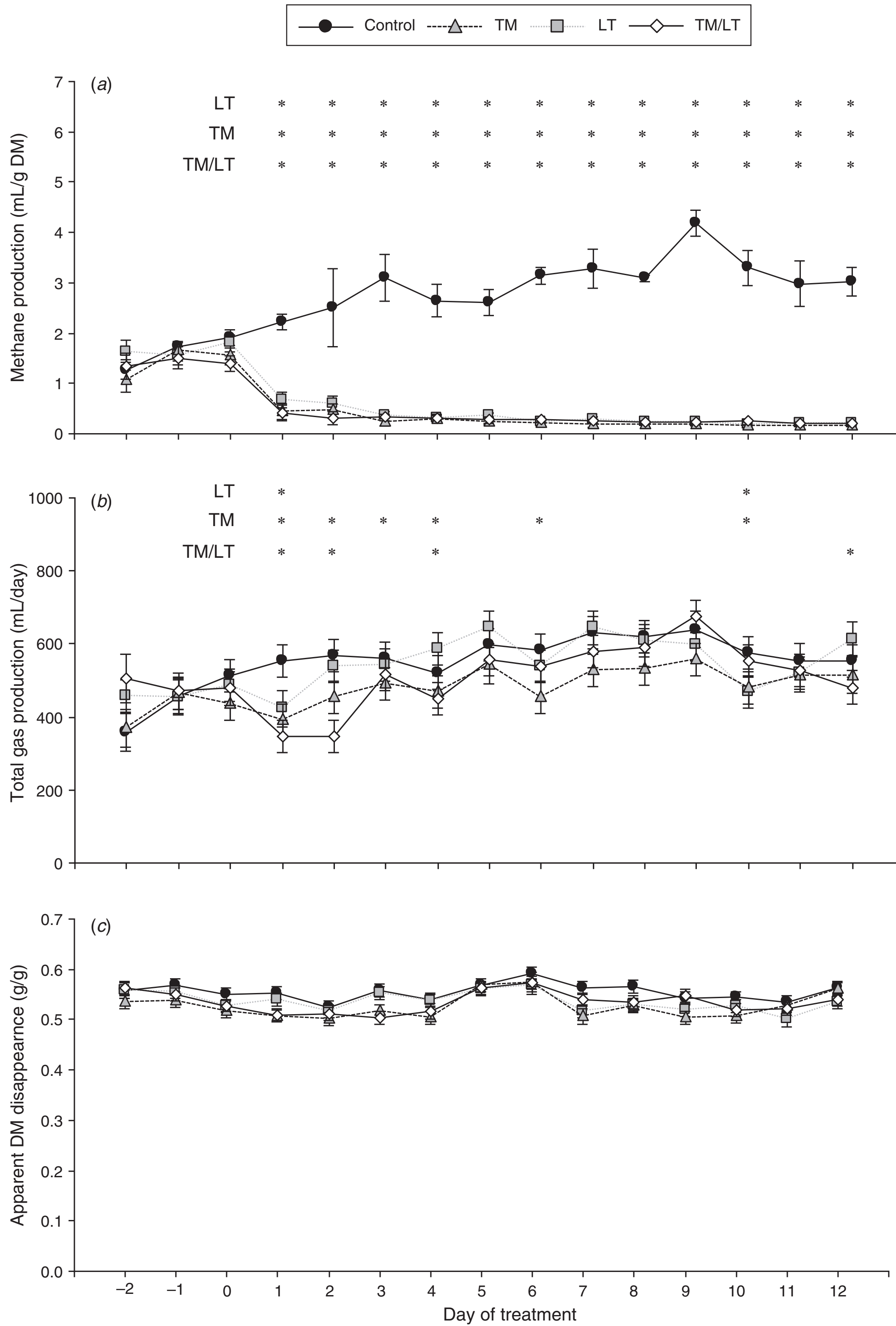

Fig. 2. Effect of essential oils (EO) of *Lippia turbinata* (LT), *Tagetes minuta* (TM) and their alternate use (TM/LT) on (*a*) methane production over 24 h, (*b*) total gas production and (*c*) apparent DM disappearance over 48 h incubation. Days –2, –1 and 0 are from the adaptation period; EO were added daily from the end of Day 0. Bars represent the standard errors of the mean. In *a* and *b*, asterisks indicate significant statistical differences compared with control (DGC test; $P < 0.05$).

Table 3. Effect of essential oils of *Lippia turbinata* (LT), *Tagetes minuta* (TM) or their alternate use (TM/LT) on average daily fermentation parameters evaluated in the Rusitec

Dose of EO is 300 µL/L. Values are means ± s.e.m. Pretreatment values are averages across all fermenters (n = 15) on Day 0 (before treatment addition). Treatment period: Days 1 to 12 for pH; Days 4, 8 and 12 for the rest of the variables. A : P, acetate : propionate ratio. VFA, volatile fatty acids. Probabilities (F-test) for treatment (T), sampling day (D), and their interaction (T × D) for the treatment period are shown. Means within each row during the treatment period followed by different letters (a, b) are statistically significantly different (DGC test; $P \leq 0.05$) between the treatments

Variable	Pretreatment	Treatment period				*P*-value		
		Control	LT	TM	TM/LT	T	D	T × D
Ammonia (mg/L)	446.2 ± 7.99	392.9a ± 11.19	375.0a ± 11.19	312.0b ± 11.19	336.0b ± 11.19	0.0019	0.0001	0.6558
Total VFA (mmol/L)	121.9 ± 3.42	119.6 ± 5.08	126.0 ± 5.08	118.4 ± 5.08	119.3 ± 5.08	0.7815	0.3473	0.3792
			Individual VFA (mol/100 mol)					
Acetate	54.0 ± 1.05	51.3 ± 1.48	52.7 ± 1.48	49.4 ± 1.48	50.5 ± 1.48	0.4085	0.0544	0.1495
Propionate	20.5 ± 0.72	15.5 ± 0.98	16.1 ± 0.98	16.3 ± 0.98	17.9 ± 1.05	0.5282	0.0035	0.9689
Butyrate	13.9 ± 0.46	13.6 ± 0.67	12.9 ± 0.67	13.8 ± 0.67	14.0 ± 0.70	0.8214	0.4605	0.3527
Iso-butyrate	0.47 ± 0.011	0.42 ± 0.025	0.41 ± 0.025	0.43 ± 0.025	0.41 ± 0.023	0.9599	0.1171	0.6814
Iso-valerate	3.77 ± 0.222	7.10 ± 1.558	5.42 ± 1.558	8.49 ± 1.558	7.34 ± 1.558	0.6781	0.1585	0.2649
Valerate	3.21 ± 0.474	5.56 ± 2.266	7.54 ± 2.563	4.14 ± 1.897	4.70 ± 1.355	0.8192	0.5956	0.7191
Caproate	2.93 ± 0.440	6.48 ± 1.425	4.59 ± 1.010	7.34 ± 1.589	6.11 ± 1.194	0.4781	0.0022	0.2580
Branched-chain VFA	4.23 ± 0.221	7.53 ± 1.549	5.80 ± 1.549	8.92 ± 1.549	7.76 ± 1.549	0.6586	0.1589	0.2609
A : P	2.52 ± 0.192	3.52 ± 0.332	3.33 ± 0.257	3.12 ± 0.221	3.01 ± 0.198	0.7016	0.1858	0.8824
			pH					
Pre-feeding	6.72 ± 0.015	6.54a ± 0.015	6.69b ± 0.015	6.71b ± 0.015	6.68b ± 0.015	0.0001	<0.0001	0.0005
3 h post-feeding	6.89 ± 0.016	6.74a ± 0.017	6.82b ± 0.017	6.82b ± 0.017	6.79b ± 0.017	0.0009	<0.0001	0.0011

Discussion

The addition of EO obtained from *L. turbinata* (LT) and *T. minuta* (TM) to a Rusitec system containing rumen fluid from sheep resulted in a 90% inhibition of methane production over the course of the experiment, with only minor reductions in total gas production, apparent DM disappearance and fibre disappearance. The two EO had distinct chemical profiles that differed from one another and are novel in comparison to other compounds that have been shown to have bioactivity towards ruminal microorganisms. Our results supported the hypothesis that EO from these two native plant species from Argentina have the potential to modify ruminal microbial activity to selectively reduce methanogenesis. However, the hypothesis that alternating the two EO sequentially would extend the duration of treatment effectiveness could not be confirmed, because no adaptation was observed with the EO individually over the course of the experiment.

The inhibition of methane production started shortly after the addition of EO from *L. turbinata* and *T. minuta*, and it persisted throughout the experimental period. It has been reported that ruminal microbes might be able to build up tolerance to additives (McIntosh *et al*. 2003; Cardozo *et al*. 2004), which was why the rotational use of both EO treatments in time was proposed in the present experiment. No adaptation (loss of effectiveness) was observed over time with any of the EO treatments, and the combined treatment of TM/LT did not bring further improvements compared with the inclusion of the EO individually. The lack of adaptation in the present study may be attributed to the duration of the experiment and/or to the dose used. The experimental period may not have been long enough to evaluate the ability of the ruminal microbiota to adapt to EO. The doses used in the present experiment were higher (300 µL/L, ~270 mg/L) than what was previously reported (0.22 mg/L) by Cardozo *et al*. (2004) when evaluating plant oils and extracts. In a later study, the same group (Busquet *et al*. 2005*b*) also observed no adaptation at 31.2 and 312 mg/L for *A. sativa* oil and the pure compound cinnamaldehyde. At high doses, the effect of EO appears to be sustained over time in continuous cultures (Fraser *et al*. 2007). It seems that when the dose is adequate, the decrease in methane production can be obtained with small effects on overall fermentation, and that the effect can be maintained. It should be noted that an increase in methane production and concentration was observed up to 3 days beyond the adaptation period in the control. This may be an indication that the system was not entirely stabilised when treatments were initiated. However, this is unlikely to have influenced the results of the study because the effects of the treatments were rapid and severe. Indeed, it is possible that the effects of EO addition may have been of greater magnitude if their administration had started earlier.

Substrate enrichment with sunflower oil (1% of DM), to facilitate studying the effects of these EO on fatty acid biohydrogenation (results not presented or discussed here), reduced methane production 18% relative to NDF apparent disappearance. Sunflower oil lipids have been shown to be effective in reducing methane emissions. In an *in vitro* system, crushed sunflower seed reduced methane production by 23% and 40% when added to an oil inclusion level of 3% and 6% of DM respectively (Machmüller *et al*. 1998), which was associated with a decrease in protozoal counts. Also, growing steers receiving sunflower oil at 5% of dietary DM (McGinn *et al*. 2004) produce 22% less methane. In both cases, sunflower oil efficiency as a methane-mitigation agent is reduced when depression in fermentable organic matter, mainly through its fibre, is taken into account. In the present study, addition of

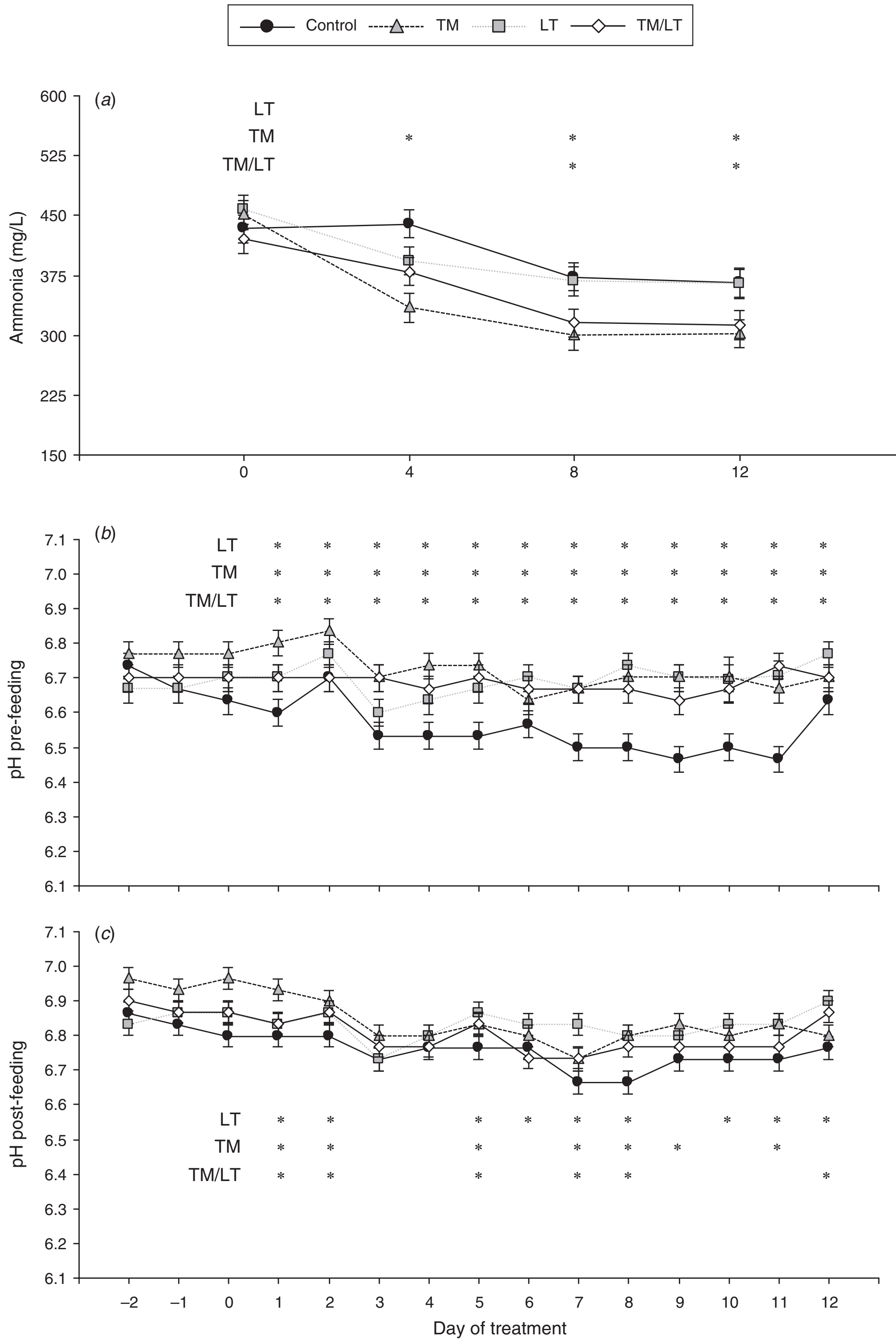

Fig. 3. Effect of essential oils (EO) of *Lippia turbinata* (LT), *Tagetes minuta* (TM) and their alternate use (TM/LT) on (*a*) ammonia concentration and (*b*) pre- and (*c*) post-feeding pH in the fermentation fluid. Days –2, –1 and 0 are from the adaptation period; EO were added daily from the end of Day 0. Bars represent the standard error of the mean. Asterisk indicates significant statistical differences compared with control (DGC test; $P < 0.05$).

sunflower oil to the basal substrate was not aimed to act as a methane-mitigation agent. Even though it reduced methane production (control vs negative control), this reduction was of a magnitude almost 10 times lower than when comparing EO effects against the control.

Microbial diversity has been reported to be lost over time in continuous-culture fermenters (Johnson *et al*. 2009), and it could be argued that a less diverse microbial (methanogenic) community may also be less resilient to alterations. However, as microbial populations were not evaluated in the present study, we cannot make a definitive conclusion about the mechanism behind the decrease in methane production. Further *in vivo* experiments would be needed, as true rumen microbial community may react in a different way and allow adaptation to methane inhibitors.

The inhibition of methane was accompanied by a decrease in daily gas production only on the first days of EO addition, and then returned to be similar to the control. This probably is an indication that other microbial populations may have, in fact, been adapting to the presence of EO, while methanogens may not have been adapting (as the reduction in methane persisted).

A 5% reduction in apparent DM disappearance was observed, while the magnitude of the effect was greater for NDF disappearance (~15%). A possible explanation for this could be that cellulolytic bacteria appear to be more sensitive to EO and its components than are other rumen microbes (Patra and Yu 2012; Cobellis *et al*. 2016). The reduction of fibre disappearance may also explain a part, but not all, of the inhibition of methane production, as the magnitude of the reduction was six times greater for methane than for fibre (90% and 15% respectively).

In the current study, only TM and TM/LT reduced ammonia concentration. Given that LT had no effect on ammonia, it is probable that the effect on ammonia concentration observed in TM/LT treatment was due to TM, rather than to the combination of both EO. Despite the reduction in ammonia concentration caused by TM and TM/LT, there was no effect on apparent CP disappearance. However, protein breakdown, amino acid fermentation, as well as microbial protein synthesis would need to be taken into account to understand the full significance of these findings. McIntosh *et al*. (2003) proposed that the main effect of EO on protein catabolism is on the deamination of amino acids. In their study with a blend of EO, they found a significant decrease in the rate of ammonia production from free amino acids, but no effect on the breakdown of proteins. In pure cultures, they observed that a group of 'hyper-ammonia producing' species was severely inhibited by the same blend of EO. Similar findings have been reported *in vivo* where addition of EO decreased both the number and diversity of hyper-ammonia producing bacteria in the rumen (McEwan *et al*. 2002), which was associated with a decrease in the rate of ammonia production from amino acids. Furthermore, a 24% inhibition of deaminase activity was also reported *in vivo* when this blend of EO was supplemented to sheep (Newbold *et al*. 2004).

Molar proportions of VFA (except for valerate) were affected by time of incubation; however, there were no effects of EO observed on the total VFA concentration and composition. Although propionate is expected to increase when methanogenesis is inhibited, Ungerfeld (2015) hypothesised that some propionate producers may not adapt well to continuous culture and, therefore, they do not benefit from favourable thermodynamic conditions for propionate production occurring when methanogenesis is inhibited (Janssen 2010).

The effects on pH may be explained by toxicity of EO to amylolytic species, which may slow the breakdown of starch (McIntosh *et al*. 2003). Although that would suggest potential benefits of these EO on pH control, results from this *in vitro* experiment, where pH is controlled by buffer addition, should be interpreted with caution.

Essential oils are complex mixtures of numerous molecules, and it is this complexity that might enable them to interact with multiple molecular targets. What determines the antimicrobial activity of compounds within EO is unclear, but the presence of oxygen in the chemical structure would appear to be important (Benchaar and Greathead 2011). This could be true for *T. minuta*, being composed of mainly oxygenated compounds, but not for *L. turbinata*. In general, compounds with phenolic structures (i.e. carvacrol, eugenol or thymol) are considered to have stronger antimicrobial activity than do other non-phenolic secondary plant metabolites (Busquet *et al*. 2005*a*, 2006; Cobellis *et al*. 2016). In the present study, neither of the EO evaluated had phenolic compounds, yet they exhibited great capacity to modulate rumen fermentation towards decreasing methane production, supporting the suggestion by Lin *et al*. (2013) that other chemical groups besides phenols could also be considered as highly active to modulate microbial activity. In addition, the combination of variable chemical structures is what may enhance the effect of EO, as evidenced by a more potent effect of the whole EO than that of single compounds (Busquet *et al*. 2006; Macheboeuf *et al*. 2008; Cobellis *et al*. 2016).

Limonene was the main compound found in *L. turbinata* (62%), and was also present in *T. minuta*, albeit at much lower concentrations (6%), being the only compound present in both EO. Similar to the present study, *Citrus aurantifolia* (51% limonene) significantly inhibited methane production at 300 mg/L (Kouazounde *et al*. 2016). Further, Castillejos *et al*. (2006) reported a reduction in ammonia concentration only at high doses of limonene (500 mg/L) and it may explain why the LT treatment had no effect on ammonia. The effect observed in the TM/LT combination treatment is likely to be associated with other compounds within *T. minuta*. The EO treatments in the present study had no effect on the total VFA concentration, which is similar to that observed by other authors using limonene or other EO at these concentrations (Castillejos 2005; Joch *et al*. 2016). Conversely, at concentrations similar to the dose we evaluated, limonene reduced NDF and true DM degradability, total gas and methane production, total VFA production and composition as well as ammonia concentration (Cattani *et al*. 2016). The discrepancy in the toxicity of limonene to rumen microbes in all these studies could be due to differences in the substrate used (Klevenhusen *et al*. 2012), the type of experiment (open vs closed *in vitro* systems) and the type of inoculum used (animal donor and its diet).

Bornyl acetate was the second major compound in *L. turbinata* (8%). Increasing doses of bornyl acetate (500, 1000 and 2000 μL/L) decreased methane production and did

not affect VFA production compared with the control (Joch *et al*. 2016). Although these doses were significantly higher than those used in the present study (25 and 12.5 µL/L in LT and TM/LT respectively), bornyl acetate might have contributed in the bioactivity of EO from *L. turbinata*. The antimicrobial activity of this compound may be related to the presence of an acetate moiety in the structure (Dorman and Deans 2000).

Carvone was the third major compound in *L. turbinata* (6%). *Anethum graveolens* (standardised at 47% carvone) at 3000 mg/L did not affect total VFA concentration, but increased the molar proportion of acetate and butyrate, while propionate was decreased (Busquet *et al*. 2005*a*). The antimicrobial activity of *A. graveolens* was attributed to the presence of carvone as the main compound. *Anethum graveolens* also reduced deamination of amino acids *in vitro* (Macheboeuf *et al*. 2008), and the effect was achieved at ~2.5 mM of carvone (equivalent to 376 mg/L). This is ~20 times the dose we used for this compound, which may explain the lack of effect on ammonia concentration by LT treatment.

The antibacterial activity of other individual compounds present in *L. turbinata* was also reported for α terpineol, linalool, α pinene, sabinene, β caryophyllene and β ocimene; however, we could not find any literature of specific uses in ruminant evaluation.

Compounds present in *L. turbinata* are ones commonly reported in other EO, but the composition of *T. minuta* EO is very distinctive and a feature for that genus (Chamorro *et al*. 2008). The antimicrobial activity of *T. minuta* oil has previously been ascribed to ketone fractions (Héthélyi *et al*. 1986; Senatore *et al*. 2004), in which the presence of an oxygen function in the framework has been reported to increase the antimicrobial properties of terpenoids (Dorman and Deans 2000). The concentrations of verbenone, *cis* and *trans* tagetones and piperitenone found are likely to be sufficient to account for the observed antimicrobial activity. Further progress in determining the active components of this EO may be achieved by fractionating and determining the antimicrobial activity of individual components as, to the authors' knowledge, none of the individual compounds has been previously described to modify rumen microbial activity.

Although present in small amounts, some chemical groups or compounds could still be of interest. Compounds with the epoxide group might have bioactivity (i.e. caryophyllene oxide, α pinene oxide and piperitone oxide). An epoxide is a cyclic ether with a three-atom ring. This ring approximates an equilateral triangle, which makes it strained, and, hence, highly reactive, more so than other ethers. Other compounds of interest could be caryophyllene and sabinene. The first is a sesquiterpene notable for having a cyclobutane ring, as well as a *trans*-double bond in an eight-membered ring, which makes it larger than monoterpenes, and may contribute with its antimicrobial activity (Dewick 2002). Sabinene thujane-type monoterpenes are unusual monoterpenes with a cyclopropane ring in a bicyclo skeleton (Dewick 2002). It is a double bond isomer of thujone, a compound which is highly toxic for microorganisms (Chizzola *et al*. 2004).

Essential oils are complex and given the level of interest in them as potential rumen modifiers, particularly in relation to the decrease in methanogenesis and improving energy efficiency in ruminants, it is important to characterise their chemical profiles thoroughly. It is also important to characterise and understand the variation in the composition of EO in each plant, which can be affected by growth stage, region and environment in which they grow. Without this characterisation, it is difficult to determine the mechanism of action related to their bioactivity and enhance the persistency of their effects. In addition, it will also be important to look at specific effects on ruminal microbial species to enable a link to be established to the chemical profiles and doses, to better understand those mechanisms.

Conclusions

The addition of essential oils from *Lippia turbinata* and *Tagetes minuta* decreased *in vitro* methane production almost 10-fold, with a slight reduction in substrate disappearance. The essential oil from *T. minuta* also appears to modify nitrogen metabolism in the rumen, reflected by the reduction in ammonia concentration, but this needs to be studied in more detail than was possible in the present study. In conclusion, given the significant and persistent antimethanogenic activity of both essential oils and the potential of *T. minuta* to modify nitrogen metabolism, these plant species can be considered as candidates of interest in the development of new feed additives to manipulate microbial activity, with potential application in ruminant nutrition. Further *in vivo* evaluation is critically important to confirm the persistency of effects of these natural products for their potential applicability as methane-mitigation additives.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

The authors thank Bidhyut Banik and Xixi Li for their guidance in the use of the Rusitec system; Joy Vadhanabhuti for the predisposition and guidance in all the work carried out in the laboratory; and Charlotte Ruiz, Eve Dauriac and Mélanie Heudelot for helping preparing the material needed for the experiment. Florencia Garcia gratefully acknowledges the National Scientific and Technical Research Council (CONICET, Argentina) for the provision of a doctoral grant; and the Department of Education of the Australian Government for the provision of an Endeavour Research Fellowship (ID ERF_RDDH_113649_2013). We also acknowledge the financial contribution of the National Institute of Agricultural Technologies.

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