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EFFECT OF SOME AUSTRALIAN NATIVE SHRUBS ESSENTIAL OILS ON *IN VITRO* RUMEN MICROBIAL FERMENTATION OF A HIGH-CONCENTRATE DIET

H. Jahani-Azizabadi[2*], Z. Durmic[1], J. Vadhanabhuti[1] and P. E Vercoe[1]

[1]School of Animal Biology, The University of Western Australia M085, 35 Stirling Hwy, Crawley WA 6009 Australia
[2]Department of Animal Science, Faculty of Agricultures, University of Kurdistan, P O Box:416, Kurdistan, Iran

[*]Corresponding author E-mail address: ho.jahani@uok.ac.ir

ABSTRACT

The aim of the present study was to investigate the dose-response effect of selected Australian EOs on *in vitro* rumen fermentation characteristics in order to find a combination of EO and dose that may be effective against methane and ammonia-N, while maintaining rumen fermentation. Selected EO treatments significantly ($P<0.05$) reduced methane (between 25% and 97% reduction) and ammonia-N (between 19% to 70% reduction) concentrations when compared to control, and change occurred in a dose-dependent manner. However, other fermentation parameters were also reduced in all of these, but in *Santalum spicatum* EO treatment the effect on methane (50% reduction) and ammonia-N (59% reduction) were greater than the effect on overall fermentation (i.e. VFA reduced only up to 20%). Results of the present study demonstrated that at doses tested, it was not possible to dissociate the effects of EOs on *in vitro* fermentation, except in *S. spicatum* EO, where selected doses may reduce methane and ammonia N with less negative effect on overall fermentation by rumen microbes.

Keywords: essential oils, methane, ammonia-N, rumen fermentation, *in vitro.*

INTRODUCTION

Rumen fermentation of feedstuff consumed by the animal provides energy for the animal, but includes some disadvantages, such as methane emission and ammonia production and excretion. Methane represents 8% to 12% loss of gross energy intake in ruminants (Johnson and Johnson , 1995), and it is a potent greenhouse gas that has a global warming potential 21 times higher than that of CO_2 (Crutzen, 1995). Further, valuable protein consumed by ruminant is metabolized by rumen microbes into nitrogen (N) and over 80% of N consumed is excreted in feces and urine (Tamminga, 1992). Ruminant nutritionists have been interested in enhancing energy and protein efficiency, by decreasing methane and ammonia-N excretion. The use of feed additives such as ionophores that can modify rumen fermentation has been found useful in this respect and widely applied (Van Nevel, and Demeyer, 1988; McGuffey *et al.,* 2001). However, increased public concerns about the risk of the use of these synthetic, subtherapeutic levels of antimicrobials resulted in a ban on their use as animal feed additives in EU (European Commission, 2003). Plant essential oils (EOs) have been studied in recent years as a natural alternative for these in-feed antibiotics, in particular for a decrease of rumen methane production and ammonia nitrogen (N) excretion (Busquet *et al.,* 2005; Busquet *et al.,* 2006; Castillejos *et al.,* 2007; Jahani-Azizabadi *et al.,* 2011). Our recent preliminary findings have implied that selected EOs from Australian plants may also affect rumen fermentation including methane production *in vitro* (Durmic *et al.,* 2014) .The study focused on one relatively high dose of EOs that, while being very potent at reducing on methane, also had an effect on other fermentation parameters.

Several authors have found that it is possible to dilute out negative effects of EOs, while maintaining desirable bioactivity (Castillejos *et al.,* 2007; Castillejos *et al.,* 2008; Hungate, 1966). Therefore, the aim of the present study was to investigate the effect of selected Australian EOs on *in vitro* rumen fermentation characteristics at various doses to find out if there is an optimal dose that affects methane and ammonia N without affecting other fermentation parameters.

MATERIALS AND METHODS

The effects of eight commercial Australian EOs were examined in an *in vitro* batch fermentation system (Durmic *et al.,* 2014). This experiment was conducted from Jun to Agust 2012 on anaerobic lab of department of animal science of university of Western Australia. The experimental substrate used was a concentrate-based diet (Ellenbank pellet, Debenham Australia, Pty Ltd) which was ground to pass 1-mm screen. Concentrate-based diet was selected because the targeted animals for this feed manipulation are likely to be in intensive systems and fed this type of diet. The ingredients and chemical composition of experimental diet are shown in Table 1. Rumen fluid for incubations was obtained from three adults ruminally-fistulated sheep (49.5 ± 2.5 kg, body

weight), that were acclimatized for two weeks on a diet of 1kg of oaten chaff, 250 g of lupin grain and 25g mineral mix (per head/day). This fibrous diet for donor sheep was selected in order to promote methanogen population in their rumen (McDougall, 1948). The ruminal content was collected on experimental day, 2 h after the morning feeding to obtain an inoculum with the most active microbial population. It was immediately transferred to the laboratory in a pre-warmed thermos flask, then strained through one layer of cheesecloth to eliminate large feed particles and placed in an anaerobic chamber (Coy Vinyl Anaerobic Chamber; Coy Laboratory Products Inc., USA). Buffer (Durmic *et al.,* 2014) was added to strained rumen fluid until pH reached to 7.1, and then 10 ml of the mix was dispensed into a 30-ml anaerobic tube (Bellco tube, Bellco Glass Inc., Vineland, NJ, USA) containing 0.1 g dry matter (DM) of the experimental diet and the appropriate dose of EO. Treatments were control (substrate+ 0.20 ml of 70% ethanol), and EO from *Agonis fragrans*, *Eucalyptus plenissima, Leptospermum pettersoni, Melaleuca alternifolia, Melaleuca ericifolia, Melaleuca teretifolia, Santalum spicatum* and *Lavandula angustifolia,* obtained commercially from Paperbark Co., Harvey, Western Australia. Four different doses of EO were tested: 62.5, 125, 250 and 500 µl/g DM incubated (DMI), with three replicates for each treatment and control, run in a single batch. All EO were individually dissolved (v/v) in 70% ethanol prior to inclusion in the assay. Control included tubes containing substrate only and an equivalent amount of 70% ethanol. Three Bellco tubes that contained buffered rumen fluid only provided a blank to correct gas produced from nutrient content of initial rumen fluid. Each tube was sealed with a butyl rubber stopper and aluminum cap and placed in a shaking incubator (at 50 rpm) for 24 h at 39 ºC.

At the end of the incubation, the gas pressure in the tubes was recorded using pressure transducer (Greisinger electronic GmbH, Regenstauf, Germany) and a sample of the gas (5 ml) was collected into a 10 ml evacuated Exetainer vial (Labco, UK). The tubes were then transferred to an ice bath to stop fermentation. Samples for ammonia-N (0.2 ml of 2MHCl was added to 1 ml of culture liquid) and volatile fatty acid (VFA, 0.2 ml of 1M NaOH was added to 1ml of culture liquid) were collected and frozen for analysis. Finally, each bottle content was filtered (42 µm pore size), the residual was oven dried (60 ºC for 48 h) and used to calculate *in vitro* dry matter disappearance (IDMD).

Chemical analysis: Samples for VFA analysis were prepared as described by Durmic *et al.* (2010). Briefly, samples were centrifuged and the supernatant was analyzed by gas chromatography (Aligent, 6890 Series, Technologies Inc., Santa Clara, CA, USA) with HP 6890 injector, capillary column HP-FFAP, 30 m × 0.53 mm ×1.0 m, FID detector and HP Chemstation software. Temperatures were 240°C, 260°C and 265ºC in the oven, injector and detector, respectively. The carrier gas (hydrogen) flow was adjusted to 6.6 ml/ min. Total VFA were calculated as the sum of acetate, propionate, butyrate, isobutyrate, valerate and isovalerate, while individual main VFA were analyzed as the proportion in total VFA. Branched-chained VFA (BCVFAs) were calculated as the sum of isobutyrate and isovalerate. The ammonia N concentration in the fermentation fluid was determined by spectrometry with a Boehringer Mannheim Test kit 1112732 (R-Biopharm AG, D-64293 Darmstadt, Germany) on Cobas Mira S auto-analyzer (Roche Diagnostics, F Hoffman-La Roche Ltd., CH-4002 Basel, Switzerland), using principles of enzymatic assay described by Bergmeyer and Beutler (1985). Methane content of the produced gas was determined using gas chromatography (Micro gas Chromatograph, Varian 3600; Varian Inc., Palo Alto, CA, USA). The instrument had 60 m HP-1capillary column using helium as the carrier gas. The injector temperature was 50°C, and the column was held constantly at 60°C during analysis, while the flame ionization detector temperature was 60°C. Carrier gas flow was adjusted to 24 ml/min. Methane content was calculated by external standard regression curve which provided by standard gas mixture (contains methane, hydrogen and carbon dioxide; 20, 30 and 30 ml/100 ml, respectively).

Calculations and statistical analysis: Total gas production was reported as ml/24 h of incubation (cumulative gas) and methane production was reported as ml/24h and ml/mg IDMD. Substrate fermentation efficiency (SFE) was calculated as mg IDMD/ml cumulative gas [18]. *In vitro* DM disappearance (IDMD) was calculated using flowing formula:

IDMD (g/g) = (IDM- remained DM after 24h of incubation)/IDM

Gas pressure was converted into volume using a formula total volume of gas (TVM) = $[(\text{Gas pressure}_{kPa} + 101.3_{kPa}) \times 20_{mL}]/101.3_{kPa}$.

Data were statistically analyzer using GLM procedure of SAS (1999) with following statistically model; $y=\mu+T_i+ e_{ij}$, where y= depended variable, µ= overall mean, T_i= effect of EO and e_{ij}= residual error. The standard error of the mean (SEM) determined with MEAN procedure. Dunnett's test was used to compare the means with those of the control ($P< 0.05$). Linear and quadratic orthogonal contrasts were tested using the CONTRAST statement of SAS (1999).

RESULTS AND DISCUSSION

Microbial gas and methane production: Effects of EOs on gas production and methane are presented in Table 2. Relative to the control, the addition of *A. fragrans* and *E. plenissima* EOs (at 250 and 500 µl/g DMI), *M.*

alternifolia, M. ericifolia, M. teretifolia and *L. agustifolia* EOs (at 125, 250 and 500 µl/g DMI) and *L. pettersoni* EOs at all doses resulted in a decrease (P<0.05) in gas production. The gas production was affected linearly in *E. plenissima, M. ericifolia, M. teretifolia* and *L. angustifolia* Eos and quadratically in *A. fragrans*, *L. pettersoni* and *M. alternifolia* EOs. The addition of *S. spicatum* EO did not have a significant effect on total gas production at any of tested levels.

Methane production (ml/24h) was lower (P<0.05) in *A. fragrans* and *E. plenissima* (at 500 µl/g DMI), *M. ericifolia* and *M. teretifolia* (at 125, 250 and 500 µl/g DMI), *L. angustifolia* (at 250 and 500 µl/g DMI) and in *M. alternifolia* EOs treatments (at 125, 250 and 500 µl/g DMI, except at 125 µl/g DMI for ml/mg DMI), compared to control. When expressed as ml/mg IDMD, methane was reduced at all levels in *S. spicatum* and *L. pettersoni* EOs, except at 62.5µl/g DMI in *L. pettersoni* EO. With increasing dose, methane decreased linearly in all EOs treatments, except quadratically in *L. pettersoni.* The results from the current study confirmed that EOs from selected Australian plants may have a noticeable effect on rumen microbial fermentation and the effect occurs in a dose-dependent manner. When EO were supplied at high levels (250 µl/g DMI and above), the majority of treatments resulted in significant reduction of methane concentration, but also in other fermentation parameters (gas production, IDMD and total VFA).The inhibitory effect on microbial fermentation confirmed potent antimicrobial activity of EO (Reuter *et al.,* 1996; Davidson and Naidu, 2000) and agree with previous *in vitro* batch culture reports where high doses of EO have been tested (Busquet *et al.,* 2005; Busquet *et al.,* 2006; Castillejos *et al.,* 2007; Durmic *et al.,* 2014; Jahani-Azizabadi *et al.,* 2011).

The study provided further evidence on the bioactivity of these EOs, either aligning or contrasting other studies. For example, Castillejos *et al.* (2008) reported that addition of *L. angustifolia* could not modify rumen microbial fermentation, while we have found a significant effect of this EOs, in particular on methane production and VFA concentration. Conversely, a recent study found that methane was decreased and propionate increased *in vivo* when buffalo diet was supplemented with of eucalyptus leaves (Thao *et al.,* 2015) and our study had confirmed similar effects and further revealed that it is likely to be associated with extractable compounds (EOs) in this plant (Zrira *et al.,* 2004). These findings may reflect some differences or similarities in methodologies and approaches, but also imply the need to look into specific plant secondary compounds.

The most potent EOs in terms of reducing methane was *L. angustifolia* (reduction up 96%) and *Melaleuca* species were highly effective in this respect (reduction ranged in methane from 31 to 94 %) and these EOs were highly effective in reducing *in vitro* methane even at lower doses. The high antimicrobial potential of *Melaleuca* EOs is not surprising (Hammer *et al.,* 2003; Durmic *et al.,* 2014) and while species of *Melaluca* have been reported to have anti-methanogenic effect (Castillejos *et al.,* 2008; Durmic *et al.,* 2014) to our knowledge this is the first report that *L. angustifolia* may have that property. However, *S. spicatum* was the only EO causing a significant reduction in methane (up to 50%) at all doses that were coupled with relatively low effect on overall fermentation.

IDMD and VFA and ammonia-N concentrations: When compared to the control, the IDMD was significantly reduced (P<0.05) with as little as 125µl/g DMI in *L. pettersoni, M. alternifolia* and *M. teretifolia* treatments, 250 µl/g DMI in *A. fragrans, E. plenissima, M. ericifolia* and *L. angustifolia,* but this decrease for *S. spicatum* treatment was observed only at high inclusion levels (at 500 µl/g DMI). When compared to control, the SFE was higher (P<0.05) in *L. pettersoni, M. alternifolia, M. ericifolia* and *M. teretifolia* EO treatments at 500µl/g DMI; and in *L. angustifolia* EO treatment (at 250 and 500 µl/g DMI), while, *S. spicatum, A. fragrans* and *E. plenissima* EOs had no effect on SFE (Table 2). The addition of *A. fragrans* and *E. plenissima* EOs resulted in a linear decrease in IDMD and quadratic increase in SFE, whereas *L. pettersoni* (highest at 62.5 µl/g IDMD and lowest at 500 µl/g DMI), *M. alternifolia*, *M. ericifolia*, *M. teretifolia, S. spicatum* and *L. angustifolia* (highest at 62.5 µl/g DMI and lowest at 500 µl/g DMI) resulted in a quadratic effect. The SFE changed linearly in *M. ericifolia* and *M. teretifolia*. Effects of EOs on VFA concentration and proportion of individual VFA are reported in Table 3. The addition of all EO and at all levels tested resulted in a reduction (P<0.05) in total VFA concentrations, but the reduction was the least in *S. spicatum* (up to 20%). Total VFA concentrations relative to control were reduced linearly in *A. fragrans*, *E. plenissima* and *L. pettersoni* EOs, and quadratically in the others. When Australian EOs were tested at higher doses, there was a decrease in IDMD and total VFA concentrations, especially in genus *Melaluca*, and this agrees with our previous *in vitro* studies when these EO were used at high doses (Durmic *et al.,* 2014). This also confirmed findings of other researchers where EOs inhibited IDMD, gas production and VFA production (Kumara *et al.,* 2009b; Jahani-Azizabadi *et al.,* 2011, Jahani-Azizabadi *et al.,* 2014).

Relative to the control, the molar proportion of acetate was higher (P<0.05) in all EO excluding *S. spicatum*. This effect was quadratic in all EOs that caused the effect. The molar proportion of propionate generally decreased with increasing concentration in all treatments except *S. spicatum* EO. The quadratical effect was observed in *M. ericifolia* and *L. angustifolia* and linear in the other EO treatments. In contrast, the molar proportion

of propionate linearly increased (P<0.05) in *S. spicatum* EO compared with control. The molar proportion of butyrate increased (P<0.05) in all EO treatments, except *S. spicatum* where there was no effect. The molar proportion of BCVFAs decreased (P<0.05) with selected doses of EOs and with increasing dose, the effect was quadratic in all treatments. With the increased dose, the proportion of acetate to propionate (C2:C3) decreased quadratically in *S. spicatum* EO (highest at 62.5µl/g DMI and lowest at 125 µl/g DMI). In other treatments, the values increased quadratically. The VFA are a major source of metabolizable energy for ruminants (Van Soest, 1982) and it important that these are maintained when developing rumen manipulation strategies. We have now demonstrated that at lower doses, the bioactive effects on rumen fermentation in at least one EO may dissociate and became more specific, targeting only selected microbes and pathways. The most promising candidate appears to be *S. spicatum* EO, as it caused a significant reduction in methane and ammonia-N, with a lesser effect on VFA. Another interesting finding is that *S. spicatum* was the only EO that was actually promoting propionate (up to 22% compared to control). Propionate is considered as a valuable energy fuel for the animal, and hence this is a highly desirable effect and comparable to that of some in-feed antibiotics (Kobayashi, 2010). Propionate is often implied as an alternative hydrogen acceptor in methane mitigation, and it is likely that these two were coupled in *S. spicatum* treatments, but further studies are needed to confirm this. In contrast with ionophores or other feed additives such as tannins that decrease ruminal methane production by affecting overall microbial fermentation (Chen and Wolin, 1979; Newbold *et al.,* 1988; MOSS *et al.,* 2000) this particular EO appears to have a more specific antimicrobial nature and apparently targeting methane-producing microbes and/or pathways. Other EOs were also effective in reducing methane and ammonia-N, but in these, a significant reduction in VFA production occurred, usually greater than 20%. Interestingly, other fermentation parameters, such as microbial gas production or dry matter disappearance were not impaired with any of the low-dose treatments. While later is considered a nutritionally-favorable effect in ruminants, these findings also imply that we can explore the doses further and potentially find a dose that is also more gentle towards VFA-producing microbes.

The addition of all EO and at levels, except *M. ericifolia* at 62.5 µl/g DMI, resulted in a decrease (P<0.05) in ammonia N concentration compared with control (Table 3). This decrease effect with increasing dose was quadratical for all EO treatments. All EOs, except for one, significantly reduced ammonia N at the lowest doses tested. Microbes involved in N metabolism in the rumen seem to be very sensitive to Eos (McIntosh *et al.,* 2003), and Australian EOs seem not to be an exception in that respect. While this effect may have been the result of a desirable decrease in rumen degradation of substrate protein (Van Nevel and Demeyer, 1988) it is also possible that the bacteria involved in deamination process were altered (Allison *et al.,* 1962), as the effect was readily associated with a reduction in VFA. Nevertheless, results of the present study suggested that selected Australian EOs may have a potential to reduce ammonia-N losses in the rumen, but further studies are needed to confirm the exact mechanism of this effect.

Table 1. Chemical and ingredients composition of experimental diet.

	Amount
Ingredients (g kg^{-1} DM)	
Lucerne Hay	125
Wheat grain	406
Powdered Molasses	50
Minerals	30
Cold pressed Canola	388
Raspberry Flavour	1
Chemical composition (% of DM)	
Crude protein (CP)	23.0
Soluble protein (% CP)	44.0
Neutral detergent fiber	20.7
Acid detergent fiber	9.8
ME (Mcal/kg DM)	3.3

Table 2. Effect of essential oils from selected Australian shrubs on fermentation variables measured in gas and solid phase after 24h *in vitro* rumen microbial fermentation of a concentrate-based diet.

Treatment	**Dose (µl/g DMI)**						**Effects**	
	0	**62.5**	**125**	**250**	**500**	**SEM**	**Linear**	**Quadratic**
A. fragrans								
IDMD	0.77	0.79	0.79	0.68*	0.67*	0.010	*	NS
SFE	5.53	6.35	6.30	6.15	8.46	0.070	NS	*
Gas (ml/24h)	14.0	12.5	12.4	11.1*	8.0*	0.212	NS	*
Methane (ml/24h)	1.26	1.33	1.24	1.03	0.27*	0.027	*	NS
Methane(ml/mg IDMD)	0.016	0.017	0.016	0.015	0.004*	<0.001	*	NS
E. plenissima								
IDMD	0.77	0.80	0.76	0.68*	0.68*	0.010	*	NS

SFE	5.53	5.40	5.43	5.88	5.90	0.120	NS	*
Gas (ml/24h)	14.0	14.89	14.07	11.62*	7.62*	0.182	*	NS
Methane (ml/24h)	1.26	1.87*	1.78*	1.12	0.34*	0.029	*	NS
Methane (ml/mg IDMD)	0.016	0.023*	0.023*	0.016	0.005*	0.002	*	NS
L. pettersoni								
IDMD	0.77	0.70	0.67*	0.57*	0.52*	0.010	NS	*
SFE	5.53	6.07	6.91	6.54	10.50*	0.420	NS	NS
Gas (ml/24h)	14.0	11.58*	9.75*	9.63*	5.10*	0.769	NS	*
Methane (ml/24h)	1.26	0.95*	0.31*	0.31*	0.14*	0.036	*	NS
Methane (ml/mg IDMD)	0.016	0.014	0.005*	0.006*	0.003*	0.004	NS	*
M. alternifolia								
IDMD	0.77	0.78	0.68*	0.63*	0.62*	0.010	NS	*
SFE	5.53	5.80	6.31	7.87	9.05	0.240	NS	*
Gas (ml/24h)	14.0	13.50	10.96*	8.00*	6.91*	0.343	NS	*
Methane (ml/24h)	1.26	1.51*	0.87*	0.41*	0.34*	0.088	*	*
Methane (ml/mg IDMD)	0.016	0.019	0.013	0.007*	0.005*	0.007	*	*
M. ericifolia								
IDMD	0.77	0.70	0.72	0.67*	0.63*	0.010	NS	*
SFE	5.53	5.70	7.38	7.56	31.70*	1.120	NS	*
Gas (ml/24h)	14.0	12.25	9.83*	8.88*	2.05*	0.206	NS	*
Methane (ml/24h)	1.26	1.30	0.85*	0.59*	0.08*	0.022	*	*
Methane (ml/mg IDMD)	0.016	0.019	0.011*	0.009*	0.001*	<0.001	*	*
M. teretifolia								
IDMD	0.77	0.71	0.65*	0.61*	0.49*	0.010	NS	*
SFE	5.53	5.64	6.71	8.20	33.50*	0.490	NS	*
Gas (ml/24h)	14.0	12.62	9.77*	7.47*	1.47*	0.131	*	*
Methane (ml/24h)	1.26	1.41	0.32*	0.24*	0.05*	0.011	*	*
Methane (ml/mg IDMD)	0.016	0.020	0.005*	0.004*	0.001*	<0.001	*	*
S. spicatum								
IDMD	0.77	0.74	0.73	0.69	0.67*	0.010	NS	*
SFE	5.53	5.68	5.77	5.60	5.38	0.060	NS	NS
Gas (ml/24h)	14.0	13.05	12.68	12.41	12.44	0.223	NS	*
Methane (ml/24h)	1.26	0.86*	0.69*	0.66*	0.63*	0.028	*	*
Methane (ml/mg IDMD)	0.016	0.012*	0.009*	0.009*	0.009*	<0.001	*	*
L. angustifolia								
IDMD	0.77	0.73	0.63	0.58*	0.52*	0.020	NS	*
SFE	5.53	5.52	6.13	11.60*	35.90*	0.550	NS	*
Gas (ml/24h)	14.0	13.28	11.31*	4.52*	1.47*	0.295	*	*
Methane (ml/24h)	1.26	1.50*	1.09	0.14*	0.04*	0.010	*	*
Methane (ml/mg IDMD)	0.016	0.021*	0.015	0.003*	0.001*	<0.001	*	*

*Mean within a row with an asterisk differs from the control (P< 0.05).
IDMD= *in vitro* dry matter disappearance; SFE= substrate fermentation efficiency; DMI= dry matter incubated; NS= non significant

Table 3. Effect of some of Australian native shrub essential oils on ammonia N and total and individual volatile fatty acids (VFA) concentration compared with control after 24h *in vitro* rumen microbial fermentation of a concentrate-based fermentation substrate.

Treatment	Dose (µl/g DMI)						Effects	
	0	62.5	125	250	500	SEM	Linear	Quadratic
A. fragrans								
Ammonia N (mg/l)	173	105*	114*	105*	70.0*	2.40	NS	*
Total VFA (mmol/l)	110.2	92.6*	80.7*	68.2*	49.9*	2.50	*	*
Individual, mol/100 mol								
Acetate	55.2	54.3	53.4	64.2*	65.3*	0.93	*	*
Propionate	26.5	29.8*	24.7	18.0*	18.1*	0.71	*	*
Butyrate	12.3	10.4	17.1*	12.1	12.3	0.23	NS	*

BCVFA	2.63	2.27	1.77*	1.67*	1.93*	0.06	NS	*
C2:C3	2.08	1.82	2.16	3.14*	3.61*	0.06	*	*
E. plenissima								
Ammonia N (mg/l)	173	136*	102*	109*	60*	2.22	NS	*
Total VFA (mmol/l)	110.2	98.2*	90.5*	68.2*	47.2*	1.62	*	*
Individual, mol/100 mol								
Acetate	55.2	54.5	54.4	58.7	63.1*	0.67	*	*
Propionate	26.5	28.3	26.8	14.7*	18.8*	0.53	*	*
Butyrate	12.3	11.6	13.1	-	13.1	0.44	NS	*
BCVFA	2.63	2.50	2.27	1.77*	2.10*	0.04	NS	*
C2:C3	2.08	1.92	2.03	4.02*	3.35*	0.058	*	*
L. pettersoni								
Ammonia N (mg/l)	173.0	87.5*	61.0*	42.0*	50.3*	2.65	NS	*
Total VFA (mmol/l)	110.2	61.5*	49.7*	44.6*	40.6*	1.95	*	*
Individual, mol/100 mol								
Acetate	55.2	55.8	67.4*	66.1*	63.1*	1.66	NS	*
Propionate	26.5	14.5*	16.8*	18.2*	18.5*	0.33	*	*
Butyrate	12.3	25.4*	12.1	11.6	13.9	1.18	*	*
BCVFA	2.63	1.67*	1.70*	1.83*	2.30	0.22	*	NS
C2:C3	2.08	3.83*	4.03*	3.63*	3.43*	0.15	*	*
M. alternifolia								
Ammonia N (mg/l)	173	129*	134*	106*	81.0*	3.24	*	*
Total VFA (mmol/l)	110.2	84.6*	77.5*	53.8*	47.1*	1.95	NS	*
Individual, mol/100 mol								
Acetate	55.2	52.8	51.6	57.2	63.4*	0.612	*	*
Propionate	26.5	30.2*	27.8	21.1*	18.7*	0.464	*	*
Butyrate	12.3	11.2	15.4	16.9*	13.3	0.686	NS	*
BCVFA	2.63	2.50	2.03*	1.83*	2.00*	0.10	NS	*
C2:C3	2.08	1.75	1.86	2.72*	3.38*	0.06	*	*
M. ericifolia								
Ammonia N (mg/l)	173.0	162.0	110.0*	74.0*	70.3*	0.10	NS	*
Total VFA (mmol/l)	110.2	80.8*	82.4*	58.5*	42.7*	2.76	NS	*
Individual, mol/100 mol								
Acetate	55.2	55.2	54.7	70.2*	58.9	0.48	*	*
Propionate	26.5	26.9	22.9*	15.2*	20.6*	0.55	NS	*
Butyrate	12.3	11.9	17.8*	10.6	15.6	0.63	NS	*
BCVFA	2.63	2.63	1.77*	1.60*	2.30	0.63	NS	*
C2:C3	2.08	2.05	2.41	2.61*	2.86*	0.07	*	*
M. teretifolia								
Ammonia N (mg/l)	173.0	74.3*	50.3*	37.0*	55.7*	1.50	*	*
Total VFA (mmol/l)	110.2	76.1*	54.2*	49.2*	43.7*	1.05	NS	*
Individual, mol/100 mol								
Acetate	55.2	53.8	68.4*	66.2*	56.4	0.42	*	*
Propionate	26.5	16.9*	16.9*	18.4*	20.1*	0.30	*	*
Butyrate	12.3	25.4*	11.2	11.2	18.6*	0.53	*	*
BCVFA	2.63	1.40*	1.60*	1.73*	2.10*	0.07	*	*
C2:C3	2.08	3.20*	4.04*	3.60*	2.80*	0.06	*	*
S. spicatum								
Ammonia N (mg/l)	173.0	118.0*	94.7*	102.0*	71.0*	3.03	*	*
Total VFA (mmol/l)	110.2	87.4*	86.8*	92.6*	88.5*	1.51	NS	NS
Individual, mol/100 mol								
Acetate	55.2	52.0	51.9	52.4	52.4	0.19	*	*
Propionate	26.5	31.6*	32.8*	32.5*	32.6*	0.23	*	*
Butyrate	12.3	11.0	10.9	10.4	10.5	0.24	*	*
BCVFA	2.63	2.30	1.77*	1.73*	1.70*	0.64	NS	*
C2:C3	2.08	1.65*	1.58*	1.61*	1.60*	0.02	*	*

L. angustifolia								
Ammonia N (mg/l)	173.0	122.0*	105.0*	68.3*	71.3*	2.75	*	*
Total VFA (mmol/l)	110.2	90.7*	72.3*	48.2*	43.9*	1.78	NS	*
Individual, mol/100 mol								
Acetate	55.2	54.8	56.1	63.3*	55.1	0.59	NS	*
Propionate	26.5	27.2	18.7*	18.8*	19.8*	0.31	NS	*
Butyrate	12.3	12.8	20.7*	12.3	17.2*	0.57	*	*
BCVFA	2.63	2.30	1.47*	1.83*	2.20*	0.05	*	*
C2:C3	2.08	2.02	3.01*	3.37*	2.77*	0.08	NS	*

*Mean within a row with an asterisk differs from the control (P< 0.05).
DMI= dry matter incubated; NS= non significant

Conclusions: In conclusion, this study revealed an interesting candidate amongst Australian EOs, i.e. *S. spicatum* EO that may have the potential to improve rumen microbial fermentation characteristics of a high concentrate diet by reducing methane, ammonia-N and promoting propionate production. Future studies should focus on progressing these findings toward optimizing doses and confirming effect *in vivo*. It is also necessary to examine and identify specific plant secondary compounds in this and other EOs directly responsible for the effect, as well as the mechanisms by which they act. The findings from this and future studies may warrant further investigation of these Eos (especially *S. spicatum* EO) into possible applications in animal diets.

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