Essential oils effect on rumen fermentation and biohydrogenation under *in vitro* conditions

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ABSTRACT: The effects of adding essential oils (EO) at different levels (125, 250, 500 mg/l) on rumen fermentation and biohydrogenation were examined in a rumen batch culture study. Treatments were: control without EO (CON), control with anise oil (ANO), cedar wood oil (CWO), cinnamon oil (CNO), eucalyptus oil (EUO), and tea tree oil (TEO). Essential oils, each dissolved in 1 ml of ethanol, were added to the culture flask containing 40 ml of buffer solution, 2 ml of reduction solution, 10 ml of rumen fluid, 25 mg of soybean oil, and 0.5 g of the diet. After 24 h of incubation in a water batch at 39°C, three samples were collected from each flask and analyzed for ammonia-N, volatile fatty acids (VFA), and fatty acids (FA). Expect for CNO, the proportions of acetate, propionate, and acetate to propionate ratios were not affected ($P > 0.05$) by EO addition. Addition of CWO, CNO, and TEO reduced total VFA concentrations ($P < 0.05$) regardless of dose level. The ammonia-N concentration was greater in cultures incubated with EO regardless of dose level. Compared with the CON, the concentrations of C18:0 and *trans* C18:1 were reduced ($P < 0.05$) with EO addition regardless of dose level. Compared with the CON, the concentration of linoleic acid was greater ($P < 0.05$) when EO were added at 500 mg/l. EO tested in this study had no effects on VFA profile but significantly reduced the formation of biohydrogenation products (C18:0 and *trans* C18:1).

Keywords: batch culture; *trans* fatty acids; rumen

INTRODUCTION

Milk fatty acids (FA) composition of ruminant animals is influenced by the consumed diet. Following ingestion, the rumen microorganisms transform the ingested unsaturated FA into mostly saturated FA plus some *trans* FA through the process of biohydrogenation. Among the *trans* FA produced, conjugated linoleic acids (CLA) have received the most attention. CLA are a mixture of geometric and positional isomers of linoleic acid (C18:2n6) in which two double bonds have a conjugated arrangement instead of methylene interruption. The major CLA isomer in ruminants' milk, *c*9*t*11, has been reported to have anti-carcinogenic properties in animal models and cell cultures (Crumb 2011). The *c*9*t*11 CLA is produced either in the rumen during the biohydrogenation of C18:2n6 or in the animal tissues by Δ^9-desaturase from vaccenic acid (VA; C18:1 *t*11), an intermediate in ruminal biohydrogenation of C18 unsaturated FA (Harfoot and Hazlewood 1997; Shingfield et al. 2010). Bichi et al. (2012) estimated that more than 74% of *c*9*t*11 CLA in cow's milk fat is made by the activity of the Δ^9-desaturase enzyme and therefore, increasing VA accumulation in the rumen and flow to the lower digestive track would be necessary to increase *c*9*t*11 CLA content in ruminants' milk.

Modulating rumen fermentation and the biohydrogenation process to increase the ruminal flow of VA through dietary manipulations and supplementations has received considerable attention in recent years. For example, using fish oil (AbuGhazaleh and Jenkins 2004; AbuGhazaleh and Ishlak 2014), algae (AbuGhazaleh et al. 2007; Toral et al. 2012), tannins (Khiaosa-Ard et al.

2009), and ionophores (Fellner et al. 1997) has been shown to alter rumen biohydrogenation and increase VA formation in the rumen by inhibiting the final step in the biohydrogenation of VA to stearic acid. However, due to rising cost of fish oil and the banned antibiotic use in animal feeds in many countries, the search for natural supplements that can alter rumen biohydrogenation and increase the formation of VA has been renewed (Calsamiglia et al. 2007).

EO are potentially promising natural alternatives to antibiotics and ionosphores for manipulating ruminal fermentation due to their ability to modify cell permeability in microbes and their toxicity to some strains of rumen microorganism, particularly the Gram-negative (Conner 1993; Helander et al. 1998; McIntosh et al. 2003; Calsamiglia et al. 2007). Although several studies have examined EO effects on rumen fermentation (McIntosh et al. 2003; Busquet et al. 2005a; Castillejos et al. 2007), little is still known about their effects on rumen biohydrogenation process. Because some of rumen bacteria involved in biohydrogenation are Gram-negative (Harfoot and Hazlewood 1997), it could be hypothesized that EO could be used as a feed supplement to alter rumen biohydrogenation and increase the flow of VA to duodenum. Therefore, the main objective of this study was to evaluate the effects of five EO on rumen biohydrogenation using batch cultures.

MATERIAL AND METHODS

Essential oils. The five EO used in this study were anise oil (*Illicium verum*), cedar wood oil (*Juniperus virginiana*), cinnamon oil (*Cinnamomum cassia*), eucalyptus oil (*Eucalyptus globulus*), and tea tree oil (*Melaleuca alternifolia*). The EO were purchased from NOW Foods essential oil company (Bloomingdale, USA). All EO were extracted by steam distillation.

Diets and treatments. The treatments were: control without EO (CON), control with anise oil (ANO), cedar wood oil (CWO), cinnamon oil (CNO), eucalyptus oil (EUO), and tea tree oil (TEO). Three doses of each EO were evaluated (125, 250, and 500 mg/l of the culture fluid). All EO were dissolved in ethanol and a total of 1.0 ml was added to the culture fluid. Controls were also dosed with the same amount of ethanol. Soybean oil, dissolved in ethanol, was added to the all treatment cultures at 25 mg per flask.

A ruminally fistulated Holstein cow fed a total mixed ration composed of 35% concentrate mix, 20% corn silage, and 45% alfalfa hay (dry matter (DM) basis) was used to collect ruminal contents. The rumen contents were collected 4 h after the morning feeding, transported to the laboratory under anaerobic conditions, and then strained through 2 layers of cheesecloth. Ten ml of strained ruminal fluid were added to erlenmeyer flasks containing 0.5 g of finely grounded diet, 40 ml of media, and 2 ml of reducing solution according to Goering and VanSoest (1970). The diet was composed (on a DM basis) of 55% alfalfa hay, 30% ground corn, 10% soy hulls, and 5% soybean meal. Each flask was gassed with CO_2, sealed with rubber corks with a gas release valve, and then incubated in a water batch at 39°C for 24 h. Each treatment was run in triplicate.

Sample collection and chemical analysis. After 24 h of incubation, three 5-ml samples were withdrawn from each culture flask (125 ml) for FA, volatile fatty acids (VFA), and ammonia-N determination. Collected samples were placed immediately in an ice bath and then stored at –20°C until analyses. The pH was measured immediately after samples were collected from each flask using a portable pH meter.

Samples for FA analysis were freeze dried and then methylated according to Kramer et al. (1997) and analyzed by gas chromatography as described by AbuGhazaleh and Jacobson (2007). For ammonia-N, samples were centrifuged at 20 000 *g* at 4°C for 10 min and then the supernatant from each sample was acidified with 0.5 ml 0.1 N HCl and stored at –20°C until analysis as outlined by Cotta and Russell (1982). Samples for VFA analysis were mixed with 1 ml of 25% meta-phosphoric acid, centrifuged at 20 000 *g* at 4°C for 20 min, and supernatant fluid was then collected and stored at –20°C until further analysis. Samples for VFA were prepared and analyzed as described by Jenkins (1987).

Statistical analysis. Data were analyzed using the Mixed Models procedure of SAS (Statistical Analysis System, Version 9.1, 2003). The fixed effect was treatment while replicate was the random effect. Differences between means of treatments were tested using the PDIFF and significance was declared at $P < 0.05$.

RESULTS

The effects of EO on cultures VFA and ammonia-N levels are presented in Tables 1–5. The addition of EO to rumen cultures had no effects ($P > 0.05$)

Table 1. Effect of anise oil (ANO) level on cultures volatile fatty acids (mole/100 mole) and ammonia level (mg/dl)

	CON	Treatment (mg/l)			MSE	*P*-value CON vs ANO
		125	250	500		
Acetate	42.51	42.83	41.72	43.24	1.009	0.91
Propionate	19.85	19.75	19.50	19.34	0.204	0.08
Butyrate	20.40	20.29	20.63	20.10	0.644	0.92
Isobutyrate	4.11[a]	3.75[b]	3.95[ab]	4.14[ab]	0.124	0.14
Valerate	6.02	6.01	6.40	5.87	0.272	0.72
Isovalerate	7.12	7.29	7.80	7.31	0.352	0.25
Acetate : propionate	2.14	2.16	2.14	2.24	0.052	0.39
VFA (mM)	76.97	66.10	71.53	71.40	4.227	0.32
NH_3-N (mg/dl)	31.83[b]	37.08[a]	38.62[a]	38.87[a]	2.199	0.07

VFA = volatile fatty acids, MSE = mean standard error, CON = control
[a,b]means within row with different superscripts differ ($P < 0.05$)

Table 2. Effect of cedar wood oil (CWO) level on cultures volatile fatty acids (mole/100 mole) and ammonia level (mg/dl)

	CON	Treatment (mg/l)			MSE	*P*-value CON vs CWO
		125	250	500		
Acetate	42.51	43.17	42.50	42.44	0.733	0.75
Propionate	19.85	19.55	19.58	19.98	0.470	0.71
Butyrate	20.40	19.95	20.22	20.46	0.315	0.51
Isobutyrate	4.11	3.96	4.27	3.88	0.233	0.73
Valerate	6.02	5.90	6.00	5.92	0.171	0.61
Isovalerate	7.12	7.47	7.43	7.32	0.273	0.25
Acetate : propionate	2.14	2.21	2.17	2.12	0.069	0.65
VFA (mM)	76.97[a]	55.78[b]	54.55[b]	63.75[b]	4.011	0.03
NH_3-N (mg/dl)	31.83[b]	42.70[a]	41.85[a]	38.85[a]	2.949	0.01

VFA = volatile fatty acids, MSE = mean standard error, CON = control
[a,b]means within row with different superscripts differ ($P < 0.05$)

Table 3. Effect of cinnamon oil (CNO) level on cultures volatile fatty acids (mole/100 mole) and ammonia level (mg/dl)

	CON	Treatment (mg/l)			MSE	*P*-value CON vs CNO
		125	250	500		
Acetate	42.51[c]	43.42[b]	46.69[a]	42.75[bc]	0.345	0.01
Propionate	19.85[b]	19.13[b]	18.98[b]	21.14[a]	0.515	0.81
Butyrate	20.40[a]	19.51[ab]	18.84[b]	18.37[b]	0.515	0.01
Isobutyrate	4.11	3.64	4.32	4.45	0.281	0.90
Valerate	6.02	5.93	5.53	6.17	0.165	0.33
Isovalerate	7.12	7.10	6.81	7.12	0.240	0.59
Acetate: propionate	2.14	2.34	2.40	2.03	0.106	0.24
VFA (mM)	76.97[a]	55.79[b]	59.48[ab]	51.33[b]	6.544	0.03
NH_3-N (mg/dl)	31.83[b]	39.25[a]	44.57[a]	38.15[a]	3.111	0.01

VFA = volatile fatty acids, MSE = mean standard error, CON = control
[a–c]means within row with different superscripts differ ($P < 0.05$)

Table 4. Effect of eucalyptus oil (EUO) level on cultures volatile fatty acids (mole/100 mole) and ammonia level (mg/dl)

	CON	Treatment (mg/l)			MSE	*P*-value CON vs ANO
		125	250	500		
Acetate	42.51	42.42	42.47	43.48	0.673	0.62
Propionate	19.85	19.78	19.87	19.59	0.238	0.60
Butyrate	20.40	20.46	20.17	19.76	0.365	0.40
Isobutyrate	4.11	3.89	3.89	3.72	0.207	0.16
Valerate	6.02	6.04	6.09	6.08	0.251	0.80
Isovalerate	7.12	7.42	7.51	7.37	0.283	0.22
Acetate : propionate	2.14	2.14	2.14	2.22	0.046	0.52
VFA (mM)	79.97	72.64	63.14	75.86	5.149	0.22
NH_3-N (mg/dl)	31.83[b]	44.82[a]	41.79[a]	38.52[a]	3.163	0.09

VFA = volatile fatty acids, MSE = mean standard error, CON = control
[a,b]means within row with different superscripts differ ($P < 0.05$)

Table 5. Effect of tea tree oil (TEO) level on cultures volatile fatty acids (mole/100 mole) and ammonia level (mg/dl)

	CON	Treatment (mg/l)			MSE	*P*-value CON vs TEO
		125	250	500		
Acetate	42.51	44.21	42.80	42.32	1.164	0.51
Propionate	19.85[a]	18.42[ab]	17.39[b]	18.42[ab]	0.715	0.02
Butyrate	20.40	20.40	20.69	21.97	0.394	0.10
Isobutyrate	4.11[a]	3.53[b]	4.11[a]	3.36[b]	0.124	0.01
Valerate	6.02	5.60	6.93	6.33	0.361	0.39
Isovalerate	7.12	6.88	8.09	7.60	0.290	0.14
Acetate : propionate	2.14	2.46	2.46	2.31	0.137	0.08
VFA (mM)	76.97[a]	49.46[b]	56.13[b]	56.27[b]	4.840	0.04
NH_3-N (mg/dl)	31.83	37.36	30.83	27.34	4.477	0.79

VFA = volatile fatty acids, MSE = mean standard error, CON = control
[a,b]means within row with different superscripts differ ($P < 0.05$)

Table 6. Effect of anise oil (ANO) level on cultures fatty acids (mg/flask)

	CON	Treatment (mg/l)			MSE	*P*-value CON vs ANO
		125	250	500		
C16:0	7.85[c]	7.74[c]	8.18[b]	8.74[a]	0.211	0.12
C18:0	19.80[a]	15.94[b]	15.26[b]	15.49[b]	1.001	0.03
C18:1 *trans*	4.26[a]	2.20[c]	2.54[c]	3.33[b]	0.155	0.01
C18:1 *trans*-11	3.16[a]	1.18[c]	1.55[c]	2.18[b]	0.151	0.01
C18:1 *cis*-9	2.99[b]	2.27[c]	3.14[b]	3.64[a]	0.115	0.80
C18:2n6	2.27[c]	2.04[d]	3.21[b]	3.59[a]	0.093	0.01
C18:3n3	0.54[b]	0.43[c]	0.62[ab]	0.55[b]	0.029	0.25
CLA*c*9*t*11	0.14	0.15	0.14	0.17	0.019	0.53
CLA*t*10*c*12	0.06[b]	0.09[a]	0.09[a]	0.09[a]	0.008	0.01
TFA	62.94[b]	75.17[a]	66.11[b]	75.16[a]	1.710	0.01

CLA = conjugated linoleic acid, TFA = total fatty acids, CON = control, MSE = mean standard error
[a–d]means within row with different superscripts differ ($P < 0.05$)

Table 7. Effect of cedar wood oil (CWO) level on cultures fatty acids (mg/flask)

	CON	Treatment (mg/l)			MSE	*P*-value CON vs CWO
		125	250	500		
C16:0	7.87^{a}	8.04^{a}	7.75^{a}	6.74^{b}	0.262	0.22
C18:0	19.80^{a}	18.17^{ab}	16.74^{bc}	15.04^{c}	0.827	0.02
C18:1 *trans*	4.29^{a}	2.60^{b}	2.19^{b}	2.21^{b}	0.170	0.01
C18:1 *trans*-11	3.15^{a}	1.53^{b}	1.21^{b}	1.25^{b}	0.182	0.01
C18:1 *cis*-9	2.94^{a}	2.55^{b}	3.03^{a}	2.98^{a}	0.083	0.35
C18:2n6	2.25^{c}	2.12^{c}	2.76^{b}	3.28^{a}	0.089	0.01
C18:3n3	0.52	0.48	0.48	0.40	0.052	0.22
CLA*c*9*t*11	0.14^{b}	0.12^{b}	0.16^{ab}	0.20^{a}	0.018	0.09
CLA*t*10*c*12	0.06	0.06	0.07	0.06	0.010	0.73
TFA	63.12^{ab}	64.62^{a}	61.24^{b}	57.83^{c}	0.850	0.09

CLA = conjugated linoleic acid, TFA = total fatty acids, CON = control, MSE = mean standard error
$^{a–c}$means within row with different superscripts differ ($P < 0.05$)

Table 8. Effect of cinnamon oil (CNO) level on cultures fatty acids (mg/flask)

	CON	Treatment (mg/l)			MSE	*P*-value CON vs CNO
		125	250	500		
C16:0	7.73^{a}	7.64^{ab}	6.63^{c}	7.18^{b}	0.181	0.02
C18:0	19.77^{a}	16.79^{ab}	15.38^{b}	14.69^{b}	1.088	0.02
C18:1 *trans*	4.27^{a}	2.93^{b}	2.37^{c}	2.58^{bc}	0.181	0.01
C18:1 *trans*-11	3.16^{a}	1.87^{b}	1.33^{c}	1.63^{bc}	0.152	0.01
C18:1 *cis*-9	2.94^{b}	2.85^{b}	2.41^{c}	3.44^{a}	0.119	0.74
C18:2n6	2.27^{b}	2.42^{b}	1.81^{c}	2.93^{a}	0.095	0.11
C18:3n3	0.54^{b}	0.53^{b}	0.69^{a}	0.48^{b}	0.031	0.30
CLA*c*9*t*11	0.14^{c}	0.20^{b}	0.27^{a}	0.18^{b}	0.018	0.01
CLA*t*10*c*12	0.06^{c}	0.06^{c}	0.12^{a}	0.09^{b}	0.009	0.01
TFA	62.94	64.41	66.11	74.29	4.190	0.21

CLA = conjugated linoleic acid, TFA = total fatty acids, CON = control, MSE = mean standard error
$^{a–c}$means within row with different superscripts differ ($P < 0.05$)

Table 9. Effect of eucalyptus oil (EUO) level on cultures fatty acids (mg/flask)

	CON	Treatment (mg/l)			MSE	*P*-value CON vs EUO
		125	250	500		
C16:0	7.95^{a}	7.70^{a}	8.40^{a}	6.88^{b}	0.342	0.38
C18:0	19.77^{a}	15.80^{b}	16.14^{b}	14.77^{b}	1.196	0.02
C18:1 *trans*	4.20^{a}	2.39^{b}	2.69^{b}	2.24^{b}	0.177	0.01
C18:1 *trans*-11	3.15^{a}	1.34^{b}	1.66^{b}	1.47^{b}	0.176	0.01
C18:1 *cis*-9	2.98^{a}	2.39^{b}	3.01^{a}	2.46^{b}	0.028	0.01
C18:2n6	2.27^{b}	2.09^{b}	2.26^{b}	2.72^{a}	0.146	0.25
C18:3n3	0.54	0.50	0.59	0.47	0.078	0.77
CLA*c*9*t*11	0.14	0.10	0.12	0.14	0.036	0.52
CLA*t*10*c*12	0.06	0.07	0.06	0.07	0.013	0.70
TFA	64.44	65.42	65.07	62.91	4.430	1.00

CLA = conjugated linoleic acid, TFA = total fatty acids, CON = control, MSE = mean standard error
^{a,b}means within row with different superscripts differ ($P < 0.05$)

Table 10. Effect of tea tree oil (TEO) level on cultures fatty acids (mg/flask)

	CON	Treatment (mg/l)			MSE	P-value CON vs TEO
		125	250	500		
C16:0	7.87[a]	7.48[b]	7.89[a]	7.77[a]	0.111	0.18
C18:0	19.77[a]	17.79[ab]	18.10[ab]	17.12[b]	0.895	0.05
C18:1 *trans*	4.27[a]	2.81[b]	3.05[b]	3.14[b]	0.169	0.01
C18:1 *trans*-11	3.17[a]	1.89[b]	1.86[b]	2.02[b]	0.090	0.01
C18:1 *cis*-9	2.94[ab]	2.51[b]	2.90[ab]	3.45[a]	0.228	0.34
C18:2n6	2.30[b]	2.03[b]	2.37[b]	3.51[a]	0.145	0.06
C18:3n3	0.54[b]	0.54[b]	0.53[b]	0.57[a]	0.007	0.61
CLA*c*9*t*11	0.14[a]	0.11[b]	0.12[ab]	0.10[b]	0.010	0.02
CLA*t*10*c*12	0.06	0.06	0.08	0.06	0.009	0.06
TFA	62.94[a]	57.57[c]	61.46[ab]	59.86[bc]	1.109	0.03

CLA = conjugated linoleic acid, TFA = total fatty acids, CON = control, MSE = mean standard error
[a–c]means within row with different superscripts differ (P < 0.05)

on the proportions of acetate, propionate, or acetate to propionate ratios in comparison with the CON. Compared with the CON, the proportions of butyrate were also not affected (P > 0.05) by EO except for CNO cultures where butyrate was lower (P < 0.05) with the 250 and 500 mg/l cultures. The proportions of isobutyrate, valerate, and isovalerate were also not affected (P > 0.05) by EO addition. Compared with the CON, total VFA concentrations in cultures were reduced (P < 0.05) only with the addition of CWO, CNO, and TEO and the reduction was not affected by the dose level. Addition of EO to cultures had more pronounce effects of ammonia-N concentration. Except for TEO, ammonia-N concentrations were increased (P < 0.05) by the addition of all other EO regardless of dose level. The pH in cultures averaging 6.67 ± 0.22 were not affected (P > 0.05) by EO addition.

The effects of EO on cultures FA concentrations (mg/culture) are presented in Tables 6–10. Compared with the CON, the concentrations of C18:0 were lower (P < 0.05) in cultures incubated with EO. The EO dose level, however, had no effects on C18:0 concentrations except for CWO where the reductions were more pronounced with the 500 mg/l. Similarly, the concentrations of *trans* C18:1 and VA were also lower (P < 0.05) in the EO cultures in comparison with the CON. The concentrations of *c*9*t*11 CLA and *t*10*c*12 CLA were affected by the addition of EO, particularly at the 250 and 500 mg/l dose levels. In comparison with the CON, the concentration of *c*9*t*11 CLA was greater in cultures incubated with CWO and CNO but lower in cultures incubated with TEO. In comparison with the CON, the concentration of *t*10*c*12 CLA was greater only in cultures incubated with ANO and CNO. Compared with the CON, the concentration of C18:2n6 was greater (P < 0.05) in cultures incubated with EO at the 500 mg/l dose level. Oleic acid (C18:1 *c*-9) concentration was also greater (P < 0.05) in cultures incubated with ANO and CNO. Linolenic acid (C18:3n3) concentration in cultures, however, was not affected by EO addition.

DISCUSSION

In this study, the EO that resulted in (a) an increase in the biohydrogenation intermediate products (e.g. VA or CLA), (b) a decrease in the biohydrogenation end product (C18:0), (c) a decrease in ammonia-N concentration, and/or (d) an increase in the proportion of propionate is considered of beneficial effects. Davidson and Naidu (2000) reported that anethol, the major compund of ANO (up 80–90%), has strong antimicrobial activity against both Gram-positive and Gram-negative bacteria. Addition of ANO to cultures did not affect the total VFA suggesting that ANO had no inhibitory effects on microbial fermentation. Using an *in vitro* batch fermentation, Busquet et al. (2006) reported no effects on VFA concentration when ANO was added at up to 300 mg/l but reported a decrease in VFA concentrations at 3000 mg/l of ANO. However, Cardozo et al. (2006) reported that feeding 2 g of anise oil per day to beef heifers fed

high concentrate diets decreased acetate to propionate ratio and branched-chain VFA. All doses of ANO increased ammonia-N concentration in our study. Cardozo et al. (2004) also reported an increase in ammonia-N concentration in continuous cultures fed ANO. However, Busquet et al. (2006) reported no change in ammonia-N concentration when ANO was used at up to 3000 mg/l. The higher concentrations of ammonia-N seen with ANO may have resulted from an increase in the deamination of amino acids by hyper-ammonia producing (HAP) bacteria (Castillejos et al. 2005; Benchaar et al. 2006). McIntosh et al. (2003) demonstrated that EO inhibited growth of some HAP bacteria (i.e. *Clostridium sticklandii* and *Peptostreptococcus anaerobius*), but other HAP bacteria (e.g. *Clostridium aminophilum*) were less sensitive. HAP bacteria are present in low numbers in the rumen (1% of the rumen bacterial population), but they possess a very high deamination activity. Wallace (2004) reported that the number of HAP bacteria was reduced by 77% in sheep receiving a low protein diet supplemented with EO at 100 mg daily, but that EO had no effect on HAP bacteria when sheep were fed a high-protein diet.

The active compounds in CWO are cedrol, thujopsene, α-cedrene, bornyl acetate, and 3-carene (Davidson and Naidu 2000; Dorman and Deans 2000). Although the components of VFA were not affected by CWO, addition of CWO decreased total VFA concentration suggesting some inhibitory effects of CWO on microbial fermentation. Addition of CWO to rumen cultures increased ammonia-N concentration. In contrast to our results, Hristov et al. (2008) reported no effects of CWO on microbial fermentation when CWO was added to rumen cultures at 10 and 100 mg/l. The higher concentrations of ammonia-N seen with CWO (also EUO and CNO) may have resulted from either an increase in the deamination of amino acids by HAP bacteria (Castillejos et al. 2005; Benchaar et al. 2006) and/or an increase in bacterial lysis. According to Wallace et al. (2002) and McIntosh et al. (2003), the growth of *Ruminobacter amylophilus* (amylolytic bacterium) was not affected when a blend of EO was used but its lysis in the stationary growth phase greatly increased. The higher ammonia-N concentration seen with EO cultures may indicate that rumen efficiency was decreased with EO addition as more nitrogen would be escaping as ammonia instead of passing out of the rumen as amino acids for absorption.

The two main compounds in CNO are cinnamaldehyde (up to 75%) and eugenol (up to 10%) (Davidson and Naidu 2000). Addition of CNO to cultures reduced total VFA concentration in agreement with Busquet et al. (2006) who reported significant reductions in total VFA concentration when CNO was added to cultures at 300 mg/l. Lourenco et al. (2008) also reported a decrease in total VFA concentration when 500 mg/l of cinnamaldehyde was added to dual-flow continuous culture fermenters. Although acetate to propionate ratio was not affected, the highest dose of CNO increased propionate proportion. All doses of CNO resulted in significant increases in ammonia-N concentrations in cultures. In contrast, Cardozo et al. (2006) reported that a mixture of cinnamaldehyde (0.6 g per day) and eugenol (0.3 g per day) to beef heifers decreased ammonia N concentrations. However, cinnamaldehyde and CNO effects on N metabolism have not been consistent – some studies reported no effects (Busquet et al. 2005b; Chaves et al. 2008a) while others reported some changes in N metabolism (Cardozo et al. 2004; Busquet et al. 2005a). Using *in vitro* batch culture, Busquet et al. (2006) observed that high doses of CNO (3000 mg/l) and cinnamaldehyde (300 and 3000 mg/l) reduced ammonia-N concentration, however, the effects were not seen when low doses (3 and 30 mg/l) were used. Chaves et al. (2008a) reported that 250 mg/l of cinnamon leaf essential oil (containing 76% of eugenol) had no effects on deaminative activity of rumen bacteria and ammonia-N concentration *in vitro*. The addition of 500 mg/l of cinnamon leaf essential oil in the Rusitec fermenter decreased ammonia-N concentration, whereas no effects were observed in the dual flow system (Fraser et al. 2007). Cinnamaldehyde and CNO effects on N-metabolism may depend on rumen pH and diet composition. For example, Cardozo et al. (2005) reported higher acetate to propionate ratio and lower total VFA concentration when cinnamaldehyde and CNO were added to rumen cultures incubated at pH of 7.0. However, the cultures pH was reduced to 5.5, they observed an increase in total VFA and ammonia-N concentration and a decrease in the acetate to propionate ratio with cinnamaldehyde and CNO.

The active compounds in EUO are 1,8-cineole (65–85%), piperitone, α-phellandrene, β-eudesmol, and pinene (Davidson and Naidu 2000; Dorman and Deans 2000). Addition of EUO to cultures

did not affect rumen fermentation or total VFA concentration. Similarly, Hristov et al. (2008) reported no effects of EUO on microbial fermentation when EUO was added to rumen cultures at 10 and 100 mg/l. In contrast to these results, the VFA concentrations were reduced by the inclusion of EUO at 0.66, 1.0, 1.33, and 1.66 µl/ml (Kumar et al. 2009). Also, Thao et al. (2014) reported that 2 ml of EUO per day administered to swamp buffaloes reduced the proportions of acetate and acetate to propionate ratio but increased propionate proportion. Like the previous EO, EUO also increased the concentrations of ammonia-N in our cultures. In contrast, Sallam et al. (2009) reported a decrease in ammonia-N concentration when EUO was added to rumen cultures at 25, 20, 100, 150 µl/75 ml.

The terpinen-4-ol, γ-terpinene, α-pinene, cymene, α-terpineol, and terpinolene are the main active components in TEO (Cox et al. 2000; Hammer et al. 2003). Total VFA concentration decreased with TEO regardless of dose level. Hristov et al. (2008) also reported significant reductions in total VFA concentration when TEO was added to cultures at 100 mg/l. Although the acetate to propionate ratio and butyrate proportions were not affected, TEO tended to decrease propionate proportion. However, Castillejos et al. (2008) reported that TEO at 500 mg/l reduced the proportions of acetate and butyrate and acetate to propionate ratio but increased propionate proportion. The different outcomes of our study and that of Castillejos et al. (2008) might be explained by the differences in the basal diet. Castillejos et al. (2008) used 10 : 90 barley straw : concentrate diet compared to a 55 : 45 alfalfa hay : concentrate in this study. Unlike the other EO in this study, TEO did not affect ammonia-N concentration in agreement with Castillejos et al. (2008) who also reported no effects when TEO was added at up to 500 mg/l. Also, Malecky et al. (2009) observed no change in ruminal ammonia-N concentration, when dairy goats were supplemented with monoterpene blend at doses of 0.043 or 0.43 g/kg.

Although C18:0 concentration decreased with the addition of EO to cultures, the concentration of *trans* C18:1 did not increase suggesting that other unknown biohydrogenation intermediates may have been produced. The higher concentration of C18:2n6 along with the lower formation of C18:0 with the addition of 500 mg/l of EO to cultures suggest a lower biohydrogenation activity in these cultures. Decreased disappearance of C18:2n6 may suggest that EO either reduced oils hydrolysis or C18:2n6 isomerization in culture. The greater concentrations of CLA in CNO, ANO, and CWO cultures may suggest that these EO had an effect on the reductase step of biohydrogenation. Studies that examined EO effects on biohydrogenation are scarce. Lourenco et al. (2008) reported that in dual-flow continuous culture fermenters a 500 mg/l of cinnamaldehyde resulted in an increase of the ruminal accumulation of *t*10*c*12 CLA. On the other hand, milk FA composition of cows supplemented daily with 1 g of cinnamaldehyde (i.e. 43 mg/g of DM) was not affected (Benchaar and Chouinard 2008). Addionally, feeding cinnamaldehyde to growing lambs at 200 mg/kg of DM had also no effects on their back fat or liver *trans* FA composition (Chaves et al. 2008b).

CONCLUSION

Results from this study showed that except for effects on ammonia-N, EO tested in this study had moderate effects on rumen fermentation. The reduction in the formation of *trans* FA and C18:0 with some EO may indicate shifts in the biohydrogenation pathways toward the formation of other unidentified intermediate FA. It is unlikely that these moderate *in vitro* effects would correspond to any substantive impact on ruminal fermentation *in vivo*.

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