Evaluating the effects of six essential oils on fermentation and biohydrogenation in *in vitro* rumen batch cultures

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ABSTRACT: The effects of six essential oils (EO) on rumen fermentation and biohydrogenation were evaluated under *in vitro* conditions. Three doses (125, 250, and 500 mg/l) of EO were evaluated using *in vitro* 24 h batch culture of rumen fluid with a 55 : 45 forage : concentrate diet. Treatments were control (CON), control with Siberian fir needle oil (FNO), citronella oil (CTO), rosemary oil (RMO), sage oil (SAO), white thyme oil (WTO), and clove oil (CLO). Treatments were incubated in triplicate in 125 ml flasks containing 500 mg of finely ground total mixed ration (TMR), 25 mg of soybean oil, 10 ml of the strained ruminal fluid, 40 ml of media, and 2 ml of reducing solution. After 24 h, the pH was determined and samples were collected to analyze ammonia N, volatile fatty acids (VFA), and fatty acids (FA). Cultures pH was not affected by EO averaging 6.6 ± 0.2. In general, high EO doses reduced the total VFA concentration except for SAO and RMO. Relative to CON, all EO decreased ($P < 0.05$) ammonia N concentrations except for the highest dose of WTO. Except for SAO, EO did not modify acetate to propionate ratio. Relative to CON, the addition of CTO and FNO increased ($P < 0.05$) the proportions of isobutyrate and decreased ($P < 0.05$) the proportions of valerate and isovalerate. The concentrations (mg/culture) of C18:0 and C18:1 *trans* FA decreased ($P < 0.05$) with CTO, FNO, RMO, and SAO relative to CON. Most tested EO in this study had little to no effects on conjugated linoleic acids (CLA), and linoleic and linolenic acids concentrations. In 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.

Keywords: plant extracts; volantile fatty acids; *trans* fatty acids; *in vitro*

Abbreviations: CLA = conjugated linoleic acids; CLO = clove oil; CON = control; CTO = citronella oil; EO = essential oils; FA = fatty acids; FNO = Siberian fir needle oil; RMO = rosemary oil; SAO = sage oil; TMR = total mixed ration; VFA = volatile fatty acids; WTO = white thyme oil

Conjugated linoleic acids (CLA) have recently attracted significant attention because of their health benefits in a variety of models of metabolic and chronic inflammatory diseases. Among the many CLA isomers, *c*9*t*11 CLA has received the most attention because of its recognized health benefits as a cancer chemopreventive (Kennedy et al., 2010; Crumb 2011). The *c*9*t*11CLA is synthesized either in the rumen as an intermediate during the biohydrogenation of linoleic and linolenic acids (Harfoot and Hazlewood, 1987; Lee and Jenkins, 2011) or in tissues by Δ-9 desaturase from vaccenic acid (*t*11 C18:1; VA), another important intermediate in ruminal

biohydrogenation of C18 unsaturated fatty acids (FA) (Grinari and Bauman, 1999). Piperova et al. (2002) and Kay et al. (2004) have estimated that more than 90% of *c*9*t*11CLA in milk fat is made by the activity of the Δ-9 desaturase enzyme and therefore, increasing VA flow from the rumen to the lower digestive tract would be necessary to increase milk *c*9*t*11 CLA content.

Antibiotic ionophores have been successfully used in reducing energy and protein losses in the rumen (Van Nevel and Demeyer, 1988). However, the use of antibiotics in animal feeds has been queried due to the potential of appearance of residues in animal products (Russell and Houlihan, 2003). For this reason, there is an increasing interest in evaluating the potential use of plants and plant extracts to modify rumen microbial fermentation (Calsamiglia et al., 2006). Essential oils (EO) are naturally occurring volatile components that can be extracted from plants by distillation methods, in particular steam distillation (Greathead, 2003). The antimicrobial properties of EO have been demonstrated against a wide range of microorganisms including bacteria, protozoa, and fungi (Dean and Ritchie, 1987; Sivropoulou et al., 1996; Chao et al., 2000).

Essential oils benefit ruminal nitrogen metabolism by inhibiting selectively bacteria that ferment amino acids (McIntosh et al., 2003). As to the effect of EO on ruminal biohydrogenation, Benchaar et al. (2006, 2007) reported no effects of EO on milk FA profiles when supplementing dairy cows. However, Lourenco et al. (2009) reported that EO rich in the monoterpenes such as limonene and carvone resulted in the ruminal accumulation of *c*9*t*11 CLA, suggesting some effects of the latter on the extent of ruminal biohydrogenation *in vitro*. Additionally, Durmic et al. (2008) reported the ability of selected Australian plants extracts and EO to inhibit the growth and/or activity of important ruminal biohydrogenating bacteria such as *Butyrivibrio fibrisolvens* and *Butyrivibrio proteoclasticus.*

It is impractical and technically impossible to screen *in vivo* the vast number of EO available (more than 3000) (Van de Braak and Leijten, 1999). Therefore, researchers have heavily built on *in vitro* models to investigate effects of EO and their main constituents on rumen fermentation and consequently predict *in vivo* effects. It is important to study the level of supplementation in order to avoid rumen fermentative depression at excessive high dosages. Therefore, the objective of this experiment was to evaluate effects of different doses of six EO on *in vitro* rumen microbial fermentation *trans* FA formation.

MATERIAL AND METHODS

Essential oils

The effects of six EO on microbial fermentation and biohydrogenation were evaluated using an *in vitro* batch fermentation system. The six oils were Siberian fir needle oil (*Abies sibirica*), citronella oil (*Cymbopogon winterianus*), rosemary oil (*Rosmarinus officinalis*), sage oil (*Salvia officinalis*), white thyme oil (*Thymus vulgaris*), and clove oil (*Eugenia caryophyllus*). Essential oils were purchased from NOW Foods essential oil company (Bloomingdale, USA). All EO were extracted by steam distillation.

Diets and treatments

Treatments were: control without EO (CON), control with Siberian fir needle oil (FNO), citronella oil (CTO), rosemary oil (RMO), sage oil (SAO), white thyme oil (WTO), and clove oil (CLO). 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 0.2 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 all treatment cultures at 25 mg/flask.

Ruminal contents were collected 4 h after morning feeding from a ruminally fistulated Holstein cow fed a total mixed ration (TMR) composed of 35% concentrate mix, 20% corn silage, and 45% alfalfa hay (DM basis). The rumen contents were brought to the laboratory in a plastic bag under anaerobic conditions, strained through 2 layers of cheesecloth, and used within 15 min. Cultures were maintained in 125 ml erlenmeyer flasks containing 500 mg of finely ground TMR, 10 ml of strained ruminal fluid, 40 ml of media, and 2 ml of reducing solution according to Goering and Van Soest (1970). The TMR 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 before sealing with rubber corks with a gas release valve. Incubations were conducted in triplicate in a water batch at 39°C for 24 h.

Sample collection and chemical analysis

Three 5 ml samples were withdrawn from each culture flask at 24 h while being stirred with a magnetic bar under a stream of CO_2 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 collecting 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 Liquid Chromatography (GLC) as described by AbuGhazaleh and Jacobson (2007). Samples for VFA analysis were mixed with 1 ml of freshly prepared 25% meta-phosphoric acid, centrifuged (IEC Centra GP8R, Needham Heights, USA) 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 analyses were prepared as described by Jenkins (1987) using 2-ethylbutyric acid as an internal standard. A gas chromatograph system Shimadzu GLC-2010 (Shimadzu Scientific Instruments, Inc., Columbia, USA) equipped with a flame-ionization detector and 30 m SP-2560 fused silica capillary column (Restek Stabil WAX DA column, Bellefonte, USA) were used for the analysis.The helium carrier gas was maintained at a linear velocity of 23 cm/s. The oven temperature was programmed to 65°C for 3 min, increased at 12°C/min to a final temperature of 225°C which was held for 9 min. The column temperature was maintained at 65°C and flame ionization detector temperature at 225°C. For ammonia N, the 5 ml collected sample was centrifuged at 20 000 *g* (IEC Centra GP8R, Needham Heights, USA) at 4°C for 10 min. The supernatant was then acidified with 0.5 ml 0.1 *N* HCl and stored at –20°C until analysis. Acidified samples were thawed and analyzed for ammonia N as outlined by Cotta and Russell (1982).

Statistical analysis

Data were analyzed using the Mixed Models procedure of SAS (Statistical Analysis System, Version 9.3, 2011) using treatment as the fixed effect and replicate as the random effect. Differences between means of treatments were tested using the PDIFF option. All results were expressed as Least Squares Means (LSM) and significance was declared at $P < 0.05$.

RESULTS

The effects of EO on cultures VFA and ammonia N levels are presented in Table 1. Relative to CON, total VFA concentrations in cultures were reduced ($P < 0.05$) with the addition of FNO, CTO, CLO, and WTO and the decrease was not affected by the dose level except for CLO where it was the lowest with the 500 mg/l dose. Except for FNO, the addition of other EO had no effects ($P > 0.05$) on the proportions of propionate and butyrate relative to CON. The proportions of propionate and butyrate and the acetate to propionate ratios were significantly lower with FNO, particularly at 250 and 500 mg/l doses, relative to CON. The proportion of acetate was significantly altered with EO addition; however, the effect was variable among EO. Relative to CON, the proportion of acetate was greater ($P < 0.05$) in cultures supplemented with FNO, RMO, and CLO and lower ($P < 0.05$) when CTO and WTO were added at 125 and 250 mg/l, respectively. Relative to CON, the addition of CTO and FNO increased ($P < 0.05$) the proportion of isobutyrate and decreased ($P < 0.05$) the proportions of valerate and isovalerate. Addition of EO to cultures had more pronounced effects of ammonia N concentration. Ammonia N concentrations decreased ($P < 0.05$) by all the doses of all EO except for the highest dose of WTO. None of the added EO had any effect on cultures pH. Cultures pH averaged 6.6 ± 0.2.

The effects of EO on cultures FA concentrations are presented in Table 2. Relative to CON, the concentrations (mg/culture) of C18:0 decreased ($P < 0.05$) with FNO, CTO, RMO, and SAO. Except for CLO, the concentrations of *trans* C18:1 and *t*11 C18:1 were also lower in EO cultures. Relative to CON, the concentrations of *c*9*t*11 CLA and *t*10*c*12 CLA were reduced ($P < 0.05$) with FNO. The concentration of *t*10*c*12 CLA was also lower ($P < 0.05$) in SAO cultures relative to CON. Relative to CON, the concentration of linoleic acid was not affected by EO except for FNO where linoleic acid concentration was lower, particularly at the 125 mg/l dose of FNO. The concentration of linolenic acid was lower in cultures supplemented with CLO, SAO, and WTO.

DISCUSSION

The aim of the experiment was to examine the effects of six EO on microbial fermentation and

Table 1. Effect of Siberian fir needle oil, citronella oil, rosemary oil, clove oil, sage oil, and white thyme oil levels on volatile fatty acids (mole/100 mole) and ammonia N level of cultures

	Treatment (mg/l)				SEM
	CON	125	250	500	
Siberian fir needle oil					
Acetate	37.66^{b}	39.89^{a}	39.49^{a}	40.56^{a}	0.564
Propionate	22.42^{a}	22.07^{ab}	21.95^{b}	21.89^{b}	0.186
Butyrate	23.57^{a}	22.47^{ab}	21.99^{b}	21.50^{b}	0.586
Isobutyrate	2.86^{b}	3.63^{a}	3.55^{a}	3.64^{a}	0.144
Valerate	6.59^{a}	5.88^{b}	5.94^{b}	5.96^{b}	0.288
Isovalerate	6.90^{a}	6.52^{ab}	6.61^{ab}	6.45^{b}	0.168
Acetate : propionate	1.68^{b}	1.81^{a}	1.80^{a}	1.85^{a}	0.028
Total VFA (mM)	79.87^{a}	72.37^{b}	62.87^{c}	82.30^{a}	1.218
NH_3-N (mg/dl)	29.54^{a}	20.01^{b}	19.16^{b}	17.02^{c}	0.802
pH	6.77	6.75	6.67	6.59	0.123
Citronella oil					
Acetate	37.66^{b}	40.59^{a}	38.68^{ab}	38.12^{b}	0.805
Propionate	22.42^{b}	22.19^{b}	22.40^{b}	22.76^{a}	0.148
Butyrate	23.57^{ab}	21.81^{b}	24.39^{a}	24.88^{a}	0.954
Isobutyrate	2.76^{c}	3.51^{a}	3.10b^{b}	2.98^{b}	0.095
Valerate	6.59^{a}	5.74^{b}	5.01^{b}	5.20^{b}	0.351
Isovalerate	6.90^{a}	6.16^{b}	6.00^{b}	6.06^{b}	0.104
Acetate : propionate	1.68^{b}	1.83^{a}	1.75^{ab}	1.67^{b}	0.088
Total VFA (mM)	79.87^{a}	62.44^{b}	62.46^{b}	63.48^{b}	3.262
NH3-N (mg/dl)	29.54^{a}	21.05^{b}	15.68^{c}	15.74^{c}	1.061
pH	6.72	6.62	6.59	6.63	0.101
Rosemary oil					
Acetate	37.66^{b}	40.03^{a}	38.90^{ab}	39.92^{a}	0.525
Propionate	22.42^{b}	22.45^{b}	23.12^{a}	22.64^{ab}	0.235
Butyrate	23.57	21.72	22.99	22.56	0.830
Isobutyrate	2.86	3.04	2.79	3.01	0.128
Valerate	6.59	6.15	5.90	6.54	0.371
Isovalerate	6.90	6.61	6.31	6.78	0.312
Acetate: propionate	1.68^{b}	1.78^{a}	1.68^{b}	1.70^{ab}	0.042
Total VFA (mM)	79.87	76.98	72.77	84.54	4.215
NH3-N (mg/dl)	29.81^{a}	18.57^{c}	16.43^{c}	22.81^{b}	1.351
pH	6.66	6.71	6.58	6.61	0.099
Clove oil					
Acetate	37.66^{b}	38.49^{a}	39.11^{a}	38.40^{a}	0.319
Propionate	22.42^{ab}	22.67^{a}	21.87^{b}	22.27^{ab}	0.253
Butyrate	23.57^{a}	22.65^{b}	22.99^{ab}	23.12^{ab}	0.360
Isobutyrate	2.86a	2.80^{b}	2.86^{a}	2.85^{a}	0.020
Valerate	6.59	6.61	6.45	6.61	0.151
Isovalerate	6.92	6.78	6.75	6.75	0.093
Acetate : propionate	1.68^{b}	1.70^{b}	1.79^{a}	1.72^{b}	0.026
Total VFA (mM)	79.87^{a}	63.23^{b}	65.32^{b}	54.96^{c}	2.319
NH_3-N (mg/dl)	29.54^{a}	25.09^{b}	22.22^{b}	25.07^{b}	1.632
pH	6.69	6.62	6.71	6.60	0.101

Table 1 to be continued

	Treatment (mg/l)				SEM
	CON	125	250	500	
Sage oil					
Acetate	37.66^{ab}	38.55^{a}	38.30^{a}	37.11^{b}	0.494
Propionate	22.42^{b}	22.00^{c}	23.45^{a}	22.65^{bc}	0.167
Butyrate	23.57^{a}	22.90^{ab}	22.74^{b}	23.51^{ab}	0.336
Isobutyrate	2.86^{ab}	2.90^{ab}	2.80^{b}	2.95a^{a}	0.060
Valerate	6.59	6.11	6.50	6.44	0.334
Isovalerate	6.90	6.54	6.53	6.87	0.189
Acetate : propionate	1.68^{a}	1.68^{a}	1.63^{ab}	1.61^{b}	0.020
Total VFA (mM)	79.87	73.22	81.68	83.34	8.015
NH3-N (mg/dl)	29.54^{a}	25.68^{ab}	22.96^{bc}	20.12^{c}	1.978
pH	6.71	6.65	6.73	6.59	0.121
White thyme oil					
Acetate	37.66^{c}	37.84^{c}	39.16^{a}	38.21^{bc}	0.149
Propionate	22.42^{a}	23.09^{a}	20.00^{b}	22.79a^{a}	0.303
Butyrate	23.57^{ab}	23.06^{b}	24.94^{a}	22.79^{b}	0.683
Isobutyrate	2.86	2.86	2.93	2.84	0.067
Valerate	6.59^{ab}	6.33^{b}	7.26^{a}	6.80^{ab}	0.292
Isovalerate	6.92	6.56	7.14	6.77	0.283
Acetate : propionate	1.68^{b}	1.65^{b}	1.89^{a}	1.66^{b}	0.042
Total VFA (mM)	79.87^{a}	67.22^{b}	67.75^{b}	66.41^{b}	4.417
NH3-N (mg/dl)	29.54^{a}	25.89^{ab}	24.75^{b}	28.18^{a}	1.407
pH	6.59	6.63	6.67	6.60	0.089

CON = control, VFA = volatile fatty acids, NH3-N = ammonia N

^{a,b,c}means within row with different superscripts differ ($P \leq 0.05$)

biohydrogenation. Effects of EO on microbial fermentation were considered positive when (*i*) total VFA concentration and propionate proportion increased, (*ii*) the acetate proportion or acetate to propionate ratio decreased, and/or (*iii*) ammonia N concentration decreased. Similarly, effects of EO on microbial biohydrogenation were considered positive when biohydrogenation intermediate product (*t*11 C18:1 and CLA) or linoleic acid increased and the biohydrogenation end product (C18:0) decreased.

The active compounds in FNO (*Abies sibirica*) are α-pinene, β-pinene, limonene, bornyl acetate, camphene, borneol, and carene (Roshchin et al., 1998). Addition of FNO to rumen cultures decreased total VFA and ammonia N concentrations suggesting some inhibitory effects of FNO on microbial fermentation. In contrast to these results, Soliva et al. (2008) reported that in a rumen simulation technique (Rusitec) a 57 mg/l of pine oil (*Pinus mugo*), which contains α-pinene, β-pinene, limonene, camphene, carene, myrcene as active compounds (Krauze-Baranowska et al., 2002), had no effects on rumen microbial fermentation, however, it must be noted that they used a lower dose than that in our experiment. FNO decreased the total FA concentrations of the cultures except for the highest dose. It is possible that reduction of the total VFA or ammonia N concentrations in cultures might reduce the microbial *de novo* FA synthesis (Sauvant and Bas, 2001). Although all doses of FNO decreased the biohydrogenation end product, biohydrogenation intermediate products did not increase possibly suggesting that other unkown biohydrogenation intermediate FA were produced. However, Lourenco et al. (2009) reported that EO mixture of limonene and carvone resulted in increase of the ruminal accumulation of *c*9*t*11 CLA.

CTO (*Cymbopogon winterianus*) active compounds are citronelal, citronelol, geraniole, geranial, and *d*-limonene (Burdock, 2002). All doses

Table 2. Effect of Siberian fir needle oil, citronella oil, rosemary oil, clove oil, sage oil, and white thyme oil levels on fatty acids (mg/flask) of cultures

	Treatment (mg/l)				SEM
	CON	125	250	500	
Siberian fir needle oil					
C16:0	9.24^{a}	8.65^{c}	8.91^{b}	8.65^{c}	0.065
C18:0	20.33^{a}	19.06^{ab}	17.78^{ab}	16.81^{bc}	0.063
C18:1 *trans*	4.77^{a}	3.17^{bc}	3.26^{bc}	2.86^{bc}	0.207
C18:1 *t*11	3.47^{a}	1.99^{b}	2.07^{b}	1.76^{b}	0.122
C18:1 *c*9	3.94^{a}	2.73^{b}	3.27^{b}	2.93^{b}	0.154
C18:2n6	3.15^{a}	2.20^{c}	2.66^{b}	2.40^{bc}	0.099
C18:3n3	0.72	0.52	0.58	0.58	0.059
CLA*c*9*t*11	0.23^{a}	0.16^{b}	0.19^{b}	0.20^{b}	0.017
CLA*t*10*c*12	0.15^{a}	0.08^{b}	0.08^{b}	0.10^{b}	0.010
TFA	68.92^{a}	63.90^{ab}	64.45^{ab}	60.98^{b}	1.425
Citronella oil					
C16:0	9.33	8.50	8.65	9.09	0.240
C18:0	20.50^{a}	17.08^{b}	17.42^{b}	17.11^{b}	0.149
C18:1 *trans*	4.77^{a}	2.92^{b}	3.19^{b}	3.42^{b}	0.128
C18:1 *t*11	3.47^{a}	1.78^{b}	2.04^{b}	2.22^{b}	0.110
C18:1 *c*9	3.90^{a}	3.02^{c}	3.86^{b}	4.48^{a}	0.104
C18:2n6	3.12	3.27	3.51	4.93	0.464
C18:3n3	0.72	0.58	0.58	0.59	0.056
CLA*c*9*t*11	0.23	0.17	0.22	0.21	0.021
CLA*t*10*c*12	0.14	0.08	0.11	0.12	0.013
TFA	68.92	60.65	65.61	65.93	2.217
Rosemary oil					
C16:0	9.25	8.66	8.78	8.73	0.333
C18:0	20.33^{a}	17.76^{ab}	16.48^{b}	17.52^{b}	0.954
C18:1 *trans*	4.77^{a}	2.84^{b}	2.87^{b}	3.42^{b}	0.258
C18:1 *t*11	3.47^{a}	1.75^{b}	1.80^{b}	2.22^{b}	0.217
C18:1 *c*9	3.90^{a}	2.60^{b}	3.46^{a}	3.20^{ab}	0.234
C18:2n6	3.12^{a}	2.20^{b}	3.13^{a}	2.69^{ab}	0.234
C18:3n3	0.72	0.55	0.66	0.70	0.112
CLA*c*9*t*11	0.23^{a}	0.15^{b}	0.23^{a}	0.19^{ab}	0.022
CLA*t*10*c*12	0.14^{a}	0.06^{b}	0.13^{a}	0.10^{ab}	0.022
TFA	68.92^{a}	57.36^{b}	61.85^{b}	61.64^{ab}	2.527
Clove oil					
C16:0	9.25	8.74	9.09	8.89	0.242
C18:0	20.33	20.84	20.87	21.23	1.186
C18:1 *trans*	4.77^{ab}	4.53^{b}	5.35^{a}	5.25^{a}	0.252
C18:1 *t*11	3.47^{ab}	3.33^{b}	3.96^{a}	3.96^{a}	0.237
C18:1 *c*9	3.90^{b}	3.94^{b}	4.87^{a}	4.43^{a}	0.186
C18:2n6	3.12^{b}	3.10^{b}	4.00^{a}	3.43^{ab}	0.306
C18:3n3	0.72^{ab}	0.54^{c}	0.74^{a}	0.61^{bc}	0.048
CLA*c*9*t*11	0.23v	0.24^{b}	0.34^{a}	0.26^{b}	0.015
CLA*t*10*c*12	0.14^{b}	0.11^{c}	0.18^{a}	0.16^{b}	0.005
TFA	68.92	66.93	70.41	68.92	2.844

Table 2 to be continued

	Treatment (mg/l)				SEM
	CON	125	250	500	
Sage oil					
C16:0	9.25	9.81	8.84	8.70	0.883
C18:0	20.33^{a}	18.69^{ab}	18.38^{ab}	17.70^{b}	1.036
C18:1 *trans*	4.77^{a}	3.26^{b}	3.52^{b}	3.35^{b}	0.251
C18:1 *t*11	3.47^{a}	2.01^{b}	2.34^{b}	2.17^{b}	0.194
C18:1 *c*9	3.90	3.79	3.75	3.51	0.220
C18:2n6	3.12^{ab}	2.75^{b}	3.43^{a}	3.07^{ab}	0.205
C18:3n3	0.72^{a}	0.55^{b}	0.57^{b}	0.58^{b}	0.040
CLA*c*9*t*11	0.23	0.22	0.17	0.17	0.035
CLA*t*10*c*12	0.14^{a}	0.10^{b}	0.08^{b}	0.08^{b}	0.014
TFA	68.92	65.19	64.11	61.70	3.699
White thyme oil					
C16:0	9.25	9.04	8.90	8.03	0.266
C18:0	20.33	17.99	19.59	18.72	1.009
C18:1 *trans*	4.77^{a}	3.36^{b}	4.88^{a}	3.66^{b}	0.123
C18:1 *t*11	3.47^{a}	2.20^{c}	3.60^{a}	2.47^{b}	0.142
C18:1 *c*9	3.89^{b}	3.47^{c}	5.20^{a}	3.67^{c}	0.076
C18:2n6	3.12	2.90	3.71	3.12	0.214
C18:3n3	0.72^{a}	0.59^{ab}	0.61^{ab}	0.53^{b}	0.063
CLA*c*9*t*11	0.23	0.21	0.21	0.18	0.028
CLA*t*10*c*12	0.14^{a}	0.08^{b}	0.13^{ab}	0.11^{ab}	0.014
TFA	68.92^{ab}	61.30^{c}	71.04^{a}	62.24^{bc}	2.289

CON = control, CLA = conjugated linoleic acid, TFA = total fatty acids

^{a,b,c}means within row with different superscripts differ ($P \leq 0.05$)

of CTO also reduced the total VFA, suggesting that some changes in the microbial population or activity had occurred. CTO addition increased the proportions of isobutyrate, acetate, and the acetate to propionate ratio and decreased isovalerate proportion and ammonia N concentrations in rumen cultures. The reduction in the concentrations of both C18:0 and *trans* C18:1 seen in CTO cultures and the lack of effects on linoleic acid concentration further suggest that other biohydrogenation intermediate FA were produced in these cultures. For example, Jenkins et al. (2006) showed of the total oleic acid that it disappeared from rumen cultures, approximately 70% was transferred into *trans* C18:1 and C18:0 and 30% was transformed into hydroxystearic acid and ketostearic acid.

SAO (*Salvia officinalis*) contains α- and β-thujene, camphor, 1,8-cineol, borneol, pinene, and cariophyllene (Marino et al., 2001; Burt, 2004) and RMO (*Rosmarinus officinalis*) contains 1,8-cineol, borneol, camphor, bornyl acetate, pinene, limonene, camphene, terpineol, and verbenone as their main active compounds (Baratta et al.,1998; Burt, 2004). In general, the 250 and 500 mg/l doses of these EO decreased the acetate to propionate ratio without affecting total VFA concentration. All doses of these EO also reduced ammonia N concentration. In contrast to our results, Castillejos et al. (2008) reported that 50 and 500 mg/l of SAO and RMO had no effects on *in vitro* batch fermentation. Like the previous EO, these two EO also reduced the concentrations of C18:0 and *trans* C18:1 in cultures without affecting linoleic acid concentration. The reduction in total FA concentration seen with the RMO cultures may be due to the reduction of ammonia N concentration and therefore *de novo* FA synthesis (Sauvant and Bas, 2001).

The main active compound of CLO (*Eugenia caryophyllus*) is eugenol (up to 95%) which is a phenolic compound with strong antimicrobial

activity against gram-positive and negative bacteria (Davidson and Naidu, 2000; Dorman and Deans, 2000). Addition of CLO to cultures reduced total VFA concentration in agreement with Busquet et al. (2006) who also reported significant reductions in total VFA concentration when CLO was added to cultures at 300 mg/l. In contrast, Castillejos et al. (2008) reported that 50 and 500 mg/l of CLO increased total VFA concentration in batch cultures. CLO increased the proportion of acetate and the acetate to propionate ratio but did not modify propionate proportion in cultures. In contrast, Castillejos et al. (2006) reported that 500 mg/l of eugenol reduced propionate proportion. All doses of CLO reduced ammonia N concentration. Previous studies also reported that 300 and 500 mg/l of eugenol resulted in decreases in ammonia N concentrations (Busquet et al., 2006; Castillejos et al., 2006). However, Castillejos et al. (2008) reported that 50 mg/l of CLO increased ammonia N concentrations and 500 mg/l of CLO had no effect on ammonia N concentrations. The higher concentrations of linoleic acid in the 250 and 500 mg/l CLO cultures in this study suggested a lower biohydrogenation activity by microbes in these cultures. Decreased disappearance of linoleic acid may suggest that CLO either reduced oils hydrolysis or linoleic acid isomerization in cultures. The accumulations of C18:1 *trans* and C18:1 *c*9 in cultures with the 250 and 500 mg/l CLO may suggest some effects on the reductase step of biohydrogenation. Moreover, the concentrations of *c*9*t*11 CLA and *t*10*c*12 CLA in these cultures were higher compared to CON which supports the CLO possible effects on the reductase step of biohydrogenation.

The two main active phenolic compounds in WTO (*Thymus vulgaris*) are thymol and carvacrol and both account for up to 60% of the total identified compounds in WTO (Lawrence and Reynolds, 1984). All doses of WTO resulted in significant reductions in total VFA concentration in cultures. In contrast to our study, Castillejos et al. (2008) reported that 50 and 500 mg/l of WTO increased total VFA concentration. Castillejos et al. (2006) also reported in *in vitro* batch fermentation and continuous culture that 50 mg/l of thymol, one of the main active compounds of WTO, had no effects on rumen microbial fermentation but 500 mg/l of thymol reduced VFA concentration. WTO increased the proportion of acetate but did not modify propionate and the acetate to propionate ratio in rumen cultures. In contrast, Evans and Martin (2000) observed in pure microbial cultures that 400 mg/l of thymol reduced propionate production. The little effects of WTO on ammonia N concentration may be due to the interaction between the phenolic compounds (thymol and carvacrol) and proteins. Previous studies reported significant reductions in ammonia N concentration with the addition of thymol to culture at 500–1000 mg/l (Brochers, 1965; Castillejos et al., 2006).

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.

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