J. Dairy Sci. 89:2649–2658

Effect of Essential Oil Active Compounds on Rumen Microbial Fermentation and Nutrient Flow in In Vitro Systems

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ABSTRACT

Two experiments were conducted to determine the effects of several essential oil active compounds on rumen microbial fermentation. In the first experiment, 4 doses (5, 50, 500, and 5,000 mg/L) of 5 essential oil compounds were evaluated using in vitro 24-h batch culture of rumen fluid with a 60:40 forage:concentrate diet (18% crude protein; 30% neutral detergent fiber). Treatments were control (CON), eugenol (EUG), guaiacol, limonene, thymol (THY), and vanillin. After 24 h, the pH was determined, and samples were collected to analyze ammonia N and volatile fatty acids (VFA). The highest dose of all compounds decreased total VFA concentration and increased the final pH. Eugenol at 5 mg/L tended to reduce the proportion of acetate and the acetate to propionate ratio, at 50 and 500 mg/L tended to reduce ammonia N concentration, and at 500 mg/L reduced the proportion of propionate and branched-chain VFA concentration, without affecting total VFA concentration. All other treatments had minor effects or changes occurred only after total VFA concentration decreased. In the second experiment, 8 dual-flow continuous culture fermenters (1,320 mL) were used in 3 replicated periods (6 d of adaptation and 3 d of sampling) to study the effects of THY and EUG on rumen microbial fermentation. Fermenters were fed 95 g/d of DM of a 60:40 forage:concentrate diet (18% crude protein; 30% neutral detergent fiber). Treatments were CON, 10 mg/L of monensin (positive control), and 5, 50, or 500 mg/L of THY and EUG, and were randomly assigned to fermenters within periods. During the last 3 d of each period, samples were taken at 0, 2, 4, and 6 h after the morning feeding and analyzed for peptides, amino acids, and ammonia N concentrations, and total and individual VFA concentrations. Monensin changed the VFA profile as expected, but inhibited nutrient digestion. Eugenol and THY decreased total VFA concentration and changed the VFA profile, and only 5 mg/L of THY tended to reduce the proportion of acetate, increased the proportion of butyrate, and increased the large peptides N concentration without decreasing total VFA concentration. Most of these essential oil compounds demonstrated their antimicrobial activity by decreasing total VFA concentration at high doses. However, EUG in batch fermentation and 5 mg/L of THY in continuous culture modified the VFA profile without decreasing total VFA concentration, and EUG in batch fermentation decreased ammonia N concentration.
Key words: essential oil, thymol, eugenol, rumen fermentation

INTRODUCTION

The use of antibiotics as growth promoters in animal feeds is facing reduced social acceptance due to the appearance of residues and resistant strains of bacteria (Gustafson and Bowen, 1997); antibiotic use has been prohibited in the European Union since January 2006 (Regulation 1831/2003/EC). Many herbs and plant extracts have antimicrobial activities against a wide range of bacteria, yeasts, and molds (Thompson, 1986; Voda et al., 2003). Essential oils are plant secondary metabolites responsible for the odor and color of plants and spices, and their antibacterial, antifungal, and antioxidant properties make them useful as natural additives in animal feeds. Most essential oils are classified as Generally Recognized as Safe (GRAS), and have been approved for food and beverage consumption by the US Food and Drug Administration (www.cfsan.fda.gov).

Essential oils are composed of more than 100 individual components (Guillén and Manzanos, 1998). Major components can constitute up to 95% of the essential oil, whereas other components are present only as traces (Davidson and Naidu, 2000). Phenolic components such as eugenol (present in clove bud), or thymol and carvacrol (present in oregano), are responsible for the antibacterial properties of many essential oils (Dorman and Deans, 2000), and appear to act as membrane permeabilizers (Helander et al., 1998). Although some essential oils and their active compounds have a wide spectrum of antimicrobial activity (Dorman and Deans, 2000), gram-positive bacterial are generally more sensitive to

Received October 3, 2005.
Accepted February 21, 2006.
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essential oils than are gram-negative bacteria (Smith-Palmer et al., 1998). However, few plant extracts have been tested for their effects on ruminal microbial fermentation (Cardozo et al., 2004; Castillejos et al., 2005; Busquet et al., 2006).

The objective of the present study was to evaluate the effects of different doses of 5 essential oil compounds on ruminal microbial fermentation in 2 in vitro studies.

MATERIALS AND METHODS

Experiment 1

Diet and Treatments. The effects of 5 essential oil compounds were evaluated in an in vitro batch fermentation (Tilley and Terry, 1963) trial with a 60:40 forage:concentrate diet (18% CP, 30% NDF, 22% ADF). The diet (DM basis) consisted of alfalfa hay (60.3%), ground corn grain (17.2%), ground barley grain (12.2%), soybean meal (9.7%), and a vitamin and mineral mixture (0.6%). The vitamin and mineral mixture contained, per kilogram of DM: 7 mg of Co; 167 mg of Cu; 33 mg of I; 2,660 mg of Mn; 27 mg of Se; 4,660 mg of Zn; 1,000 kIU of vitamin A; 200 kIU of vitamin D_3; 1,330 mg of vitamin E; 267 g of urea, 67 g of NaCl; 33 g of sulfur; and 300 g of MgO. The diet was formulated to meet NRC (2001) nutrient requirements for dairy cattle. Treatments were control (**CON**, no additive); thymol [**THY**, 5-methyl-2-(1-methylethyl)phenol; T-0501, Sigma-Aldrich Chemical, St. Louis, MO]; eugenol [**EUG**, 2-methoxy-4-(2-propenyl)-phenol; E-51791 Sigma-Aldrich Chemical]; limonene [1-methyl-4-(1-methylethenyl)cyclohexene; 183164, Sigma-Aldrich Chemical]; guaiacol (2-methoxyphenol; G-5502, Sigma-Aldrich Chemical); and vanillin (4-hydroxy-3-methoxybenzaldehyde; V-2375, Sigma-Aldrich Chemical). Four doses of each additive were used: 5, 50, 500, and 5,000 mg/L of the culture fluid.

Incubations were conducted using rumen fluid from 2 fistulated, lactating dairy cows fed a 60:40 forage:concentrate diet. Rumen fluid was strained through 2 layers of cheesecloth, and mixed in a 1:1 proportion with phosphate-bicarbonate buffer (McDougall, 1948). The incubation was conducted in a 90-mL tube containing 50 mL of diluted fluid and 0.5 g of the diet per tube. Each tube was gassed with CO_2 before sealing with rubber corks with a gas release valve. The 3 lowest doses of all compounds were dissolved in ethanol, and a total of 0.2 mL was added to the culture fluid. The 5,000 mg/L dose of all compounds (0.25 mL) was supplied directly to each tube plus 0.2 mL of ethanol. The CON treatment was also supplied with 0.2 mL of ethanol. Treatments were tested at an initial pH of 7.0. Incubations were conducted in a water bath at 39°C.

Sample Collection and Chemical Analyses. After 24 h, liquid samples were withdrawn from each tube to determine pH and analyze ammonia N and VFA concentrations.

Ammonia N was analyzed by colorimetry as described by Chaney and Marbach (1962), in which 4 mL of a 0.2 *N* HCl solution was added to 4 mL of filtered rumen fluid and frozen. Samples were centrifuged at 3,000 × *g* for 20 min, and the supernatant was used to determine ammonia N by spectrophotometry (Libra S21, Biochrom Technology, Cambridge, UK).

Samples for VFA analysis were prepared as described by Jouany (1982), and analyzed by gas chromatography: 1 mL of a solution made up of a 2 g/L solution of mercuric chloride, 2 g/L of 4-methylvaleric acid as an internal standard, and 20 g/L orthophosphoric acid, was added to 4 mL of filtered rumen fluid and frozen. Samples were centrifuged at 15,000 × *g* for 15 min, and the supernatant was analyzed by gas chromatography (model 6890, Hewlett Packard, Palo Alto, CA) using a polyethylene glycol nitroterephthalic acid-treated capillary column (BP21, SGE, Europe Ltd., Buckinghamshire, UK).

Statistical Analyses. Compounds were tested in duplicate, and fermentations were repeated on 2 separate days. Results of the batch fermentation experiment were analyzed as a randomized block design using the MIXED procedures of SAS (version 8.1, SAS Institute, 1989). Day was considered a random effect. Differences between means and CON within extract were tested using the Dunnett multiple comparison test and declared at $P < 0.05$.

Experiment 2

Fermenters, Diet, and Treatments. Eight 1,320-mL dual-flow continuous culture fermenters developed by Hoover et al. (1976) were used in 3 replicated periods. Each experimental period consisted of 6 d for adaptation and 3 d for sampling.

All fermenters were fed 95 g/d of DM of a diet formulated to meet or exceed current nutrient recommendations for lactating dairy cows (18.0% CP, 30.2% NDF, 21.7% ADF; NRC, 2001) and were fed in 3 equal portions at 0800, 1600, and 2400 h. The diet was the same as described in experiment 1.

Three different doses of THY and EUG were used in experiment 2. Treatments were a negative control without additive (CON), THY at 5 (**T5**), 50 (**T50**), and 500 mg/L (**T500**), and EUG at 5 (**E5**), 50 (**E50**), and 500 (**E500**) mg/L, and a positive control with monensin at 10 mg/L (**MON**; M5273, Sigma-Aldrich Chemical). Treatments were incorporated directly into the fermenter fluid 1 min before each feeding. The T5, T50,

E5, E50, and MON doses were dissolved in ethanol. The high dose was supplied directly to the fermenters plus the equivalent amount of ethanol (0.4 mL per feeding). Fermenters with the CON treatment were also supplied 0.4 mL of ethanol per feeding.

On d 1 of each period, fermenters were inoculated with undiluted ruminal fluid taken from a cow fed a 60:40 forage:concentrate diet. Fermentation conditions were maintained at a constant temperature of 39°C, and pH at 6.4 ± 0.05 by infusion of 3 *N* HCl or 5 *N* NaOH, and monitored and controlled by a computer and a Programmable Linear Controller (FieldPoint, National Instruments, Austin, TX). Anaerobic conditions were maintained by infusion of N_2 gas at a rate of 40 mL/min. Artificial saliva (Weller and Pilgrim, 1974) was continuously infused, and contained 0.4 g/L of urea to simulate recycled N. Infusion of saliva and flows of filtered liquid were set to maintain a liquid and solid dilution rates at 0.10 and 0.05 h^{-1}, respectively.

Sample Collection. During the final 3 d, 4 mL of filtered fermenter fluid was taken 2 h after the morning feeding to determine VFA concentration, and 36 mL was taken at 0, 2, 4, and 6 h after the morning feeding to determine tungstic acid-soluble N (**TA N**), TCA-soluble N (**TCA N**), and ammonia N concentrations. Results were used to calculate large peptides (**LPep N** = TCA N – TA N), small peptides plus amino acid (**SPep+AA N** = TA N – ammonia N), and ammonia N concentrations in fermenters.

During sampling days, collection vessels were maintained at 4°C to prevent microbial activity. Solid and liquid effluents were mixed and homogenized for 1 min at 24,000 rpm (Diax900, Heidolph, Nurnberg, Germany), and a 600-mL sample was removed by aspiration and frozen at –20°C. Upon completion of each period, effluents from the 3 sampling days were composited, mixed within fermenter, and homogenized for 1 min. Subsamples were taken for total N, ammonia N, and VFA analyses. The remainder of the sample was lyophilized. Dry samples were analyzed for DM, ash, NDF, ADF, and purine contents.

Bacterial cells were obtained from fermenter flasks on the last day of each experimental period. Solid- and liquid-associated bacteria were isolated using a combination of several detachment procedures (Whitehouse et al., 1994) selected to obtain the maximum detachment without affecting cell integrity. One hundred milliliters of a 2 g/L methylcellulose solution and small marbles (30 of 2 mm and 15 of 4 mm diameter) were added to each fermenter, and incubated in the same fermenter, mixed for 1 h at 39°C, to remove attached bacteria. After incubation, fermenter flasks were refrigerated for 24 h at 4°C. After this incubation, fermenter contents were agitated for 1 h to dislodge loosely attached bacteria. Finally, the fermenter contents were filtered through cheesecloth, and washed with saline solution (8.5 g/L NaCl). Bacterial cells were isolated within 4 h by differential centrifugation at 1,000 × *g* for 10 min to separate feed particles, and the supernatant was centrifuged at 20,000 × *g* for 20 min to isolate bacterial cells. Pellets were rinsed twice with saline solution and recentrifuged at 20,000 × *g* for 20 min. The final pellet was recovered with distilled water to prevent contamination of bacteria with ash. Bacterial cells were lyophilized and analyzed for DM, ash, N, and purine contents. Digestion of DM, OM, NDF, ADF, and CP, and flows of total, nonammonia, microbial, and dietary N were calculated as described by Stern and Hoover (1990).

Chemical Analyses. Effluent DM was determined by lyophilizing 200-mL aliquots in triplicate. The DM content of diets and bacterial samples was determined by drying samples for 24 h in a 103°C forced air oven (AOAC, 1990; method 950.01). Dry samples of diets, effluents, and bacteria were ashed overnight at 550°C in a muffle furnace (AOAC, 1990; method 942.05), and OM was determined by difference. Fiber components of diets and effluents were analyzed sequentially (Van Soest et al., 1991) using a thermostable α-amylase and sodium sulfite, and expressed without residual ash. Total N of diets, effluents, and bacterial samples was determined by the Kjeldahl method (AOAC, 1990; method 976.05). Sample CP was calculated as N × 6.25. Effluent total CP was determined in liquid samples.

Large peptide and SPep+AA N were determined as described by Winter et al. (1964). To determine TCA N, 4 mL of a 500 g/L TCA solution were added to 16 mL of filtered fermenter fluid. After 4 h at 5°C, tubes were centrifuged at 9,000 × *g* for 15 min. The supernatant was stored and frozen until analyzed for TCA N by the Kjeldahl procedure (AOAC, 1990; method 976.05). To determine TA N, 4 mL of a 100 g/L sodium tungstate solution and 4 mL of 1.07 *N* sulfuric acid were added to a 16-mL sample of filtered fermenter fluid. After 4 h at 5°C, tubes were centrifuged at 9,000 × *g* for 15 min. The supernatant was stored and frozen until it was analyzed for TA N by the Kjeldahl procedure (AOAC, 1990; method 976.05).

Ammonia N and VFA analysis were analyzed as described in the first experiment.

Samples of lyophilized effluent and bacterial cells were analyzed for purine content (adenine and guanine) by HPLC as described by Balcells et al. (1992), using allopurinol as the internal standard.

Statistical Analyses. All statistical analyses were conducted using SAS (version 8.1, SAS Institute, 1989). Results of VFA concentration and N fractions in fermenters were analyzed using PROC MIXED for re-

Table 1. Effect of limonene on pH, ammonia N, and total and individual VFA concentrations in in vitro fermentation

	Dose, mg/L					
	0	5	50	500	5,000	SEM
pH	6.46	6.49	6.50	6.50	6.60†	0.06
NH_3, mg/100 mL	21.9	20.8	20.6	18.7*	20.6	1.08
Total VFA, m*M*	140.4	136.9	134.1*	132.5*	126.2*	2.44
Acetate, mol/100 mol	64.9	64.3	63.9	64.6	63.7*	0.40
Propionate, mol/100 mol	20.6	20.8	20.9	20.9	21.0	0.25
Butyrate, mol/100 mol	10.5	10.8	11.0	10.4	10.7	0.19
BCVFA,[1] m*M*	3.00	3.05	2.96	2.80*	2.95	0.08
Acetate:propionate	3.55	3.40	3.40	3.40	3.36*	0.06

[1]BCVFA = Branched-chain VFA.

*Means within a column differ from control ($P < 0.05$); †means within a column differ from control ($P < 0.10$).

peated measures (Littell et al., 1998). The model accounted for the effects of treatments and days (for VFA during the sampling days) or hours (for the N fractions during the sampling days), and the interaction of treatment with days or hours. The period was considered a random effect. The VFA concentration and N fractions were analyzed using the compound symmetric covariance structure that yielded the largest Schwarz's Bayesian criterion. The significance of differences between means of treatments compared with CON were declared at $P < 0.05$ using the Dunnett option. Differences between each treatment and CON for N fractions at each hour, and between 0 h and 2, 4, and 6 h within treatments were tested using the Bonferroni option, and declared at $P < 0.05$.

The results of nutrient digestion and flows were analyzed as a completely randomized block design. Main effects and its interactions were determined with the ANOVA using the PROC MIXED of SAS (SAS Institute). Differences between each treatment and CON were tested using the Dunnett option and declared at $P < 0.05$.

RESULTS AND DISCUSSION

Experiment 1

The selection criteria to identify essential oil active compounds with positive effects on rumen microbial fermentation were an increase or no change in total VFA, a decrease in acetate proportion, an increase in propionate proportion, and a decrease in ammonia N concentration.

The effects of essential oil compounds on rumen microbial activity were different depending on the dose. The 5,000 mg/L dose of all compounds resulted in lower total VFA concentration and, consequently, higher rumen pH compared with CON (Tables 1 to 5). These results are consistent with their known antimicrobial activity, and agree with previous reports where essential oils have been supplied at similar doses and conditions (Evans and Martin, 2000; Busquet et al., 2005; Cardozo et al., 2005). The 5,000 mg/L dose was not selected because of its negative effect on rumen microbial fermentation, and will not be discussed further.

Table 2. Effect of guaiacol on pH, ammonia N, and total and individual VFA concentrations in in vitro fermentation

	Dose, mg/L					
	0	5	50	500	5,000	SEM
pH	6.14	6.28	6.26	6.19	6.59*	0.08
NH_3, mg/100 mL	31.2	21.4*	22.9*	26.0*	22.4*	4.05
Total VFA, m*M*	152.8	132.1*	131.4*	141.0	105.2*	7.64
Acetate, mol/100 mol	64.2	62.9†	62.8†	62.6*	62.8*	0.53
Propionate, mol/100 mol	20.3	22.1	22.0	21.3	19.4	0.86
Butyrate, mol/100 mol	11.1	10.9	10.9	11.6	13.6	0.32
BCVFA,[1] m*M*	3.77	2.87†	2.91†	3.35	2.27*	0.35
Acetate:propionate	3.31	2.89	2.90	3.03	3.51	0.22

[1]BCVFA = Branched-chain VFA.

*Means within a column differ from control ($P < 0.05$); †means within a column differ from control ($P < 0.10$).

Table 3. Effect of vanillin on pH, ammonia N, and total and individual VFA concentrations in in vitro fermentation

	Dose, mg/L					
	0	5	50	500	5,000	SEM
pH	6.14	6.15	6.13	6.11	6.40*	0.04
NH_3, mg/100 mL	31.2	32.3	32.8	32.6	26.3*	1.79
Total VFA, m*M*	152.8	156.9	153.6	148.3	115.4*	3.86
Acetate, mol/100 mol	64.2	64.0	63.5	62.9*	61.2*	0.47
Propionate, mol/100 mol	20.3	20.4	20.4	20.7	20.9*	0.33
Butyrate, mol/100 mol	11.1	11.1	11.5	11.6	13.0	0.22
BCVFA,[1] m*M*	3.77	4.00	4.12	3.99	2.93*	0.22
Acetate:propionate	3.31	3.28	3.27	3.20	3.12*	0.06

[1]BCVFA = Branched-chain VFA.

*Means within a column differ from control ($P < 0.05$).

Limonene is the most abundant monocyclic monoterpene in lemons (*Citrus limonum*), oranges (*Citrus aurantium*), grapefruit (*Citrus paradisi*), peppermint (*Mentha piperita*), spearmint (*Mentha spicata*), and other oils (Turner et al., 1999). Dorman and Deans (2000) demonstrated the antimicrobial activity of limonene, mainly against gram-negative bacteria. Limonene at 50 and 500 mg/L reduced total VFA concentration (–4.5 and –5.6%, respectively), suggesting that these doses were toxic to rumen bacteria. In addition, limonene at 500 mg/L reduced ammonia N (–14.6%) and branched-chain VFA (**BCVFA**, –6.6%) concentrations, suggesting that deamination of AA was inhibited (Allison et al., 1962; Table 1). In contrast, 5 mg/L of limonene did not modify rumen microbial fermentation. Oh et al. (1967) observed that limonene and other monoterpene hydrocarbons at 0.7 to 1.5 mL/L slightly promoted or had no effect on the activity of rumen microorganisms in sheep and deer. There are no other reports on the effects of limonene on rumen microbial fermentation. Therefore, there appears to be no benefit to using limonene as an additive to modify rumen microbial fermentation.

Guaiacol is a compound of the essential oil of peppermint (*Mentha piperita*), celery (*Apium graveolens*), birch (*Betula alba*), and juniper (*Juniperus communis*), among others (Magyar et al., 2004). Different doses of guaiacol had negative effects on rumen microbial fermentation (reduced VFA production), except at 500 mg/L. At this dose, guaiacol reduced the proportion of acetate (–2.5%) and ammonia N concentration (–16.7%) without reducing total VFA concentration (Table 2). It is not clear why the negative effects of guaiacol at 5 and 50 mg/L on total VFA concentration were not observed at 500 mg/L. There are no other reports in the literature on the effects on guaiacol on rumen microbial fermentation.

Vanillin is the major constituent of vanilla beans, the fruit of an orchid (*Vanilla planifola, Vanilla pompona,* or *Vanilla tahitensis;* Davidson and Naidu, 2000). Vanillin has strong antimicrobial properties against a number of bacteria, yeasts, and molds (Davidson and Naidu, 2000; Fitzgerald, et al., 2004). Recently, Fitzgerald et al. (2004) reported that the mode of action of vanillin on the inhibition of several food-related bacte-

Table 4. Effect of thymol on pH, ammonia N, and total and individual VFA concentrations in in vitro fermentation

	Dose, mg/L					
	0	5	50	500	5,000	SEM
pH	6.46	6.50	6.47	6.87*	7.38*	0.06
NH_3, mg/100 mL	21.9	21.8	21.5	14.9*	9.34*	1.17
Total VFA, m*M*	140.4	147.6	138.3	100.4*	64.9*	7.31
Acetate, mol/100 mol	64.9	64.8	64.1	66.7*	64.5	0.37
Propionate, mol/100 mol	20.6	20.7	20.9	16.8*	21.2*	0.17
Butyrate, mol/100 mol	10.5	10.6	10.9	13.2	10.6	0.21
BCVFA,[1] m*M*	3.02	3.10	2.98	1.76*	1.29*	0.14
Acetate:propionate	3.55	3.55	3.47	4.81*	3.38	0.31

[1]BCVFA = Branched-chain VFA.

*Means within a column differ from control ($P < 0.05$).

Table 5. Effect of eugenol on pH, ammonia N and total and individual VFA concentrations in in vitro fermentation

	Dose, mg/L					
	0	5	50	500	5,000	SEM
pH	6.46	6.42	6.43	6.56	7.35*	0.08
NH_3, mg/100 mL	21.9	19.9	17.1†	16.9†	10.4*	1.94
Total VFA, m*M*	140.4	134.8	137.0	133.4	66.1*	3.95
Acetate, mol/100 mol	64.9	63.8†	64.0	65.1	64.7	0.46
Propionate, mol/100 mol	20.6	21.0	20.9	19.8*	21.0	0.29
Butyrate, mol/100 mol	10.5	11.0	10.9	11.3	10.5	0.36
BCVFA,[1] m*M*	3.02	3.03	3.01	2.51*	1.31*	0.08
Acetate:propionate	3.55	3.36†	3.38	3.66	3.45	0.08

[1]BCVFA = Branched-chain VFA.

*Means within a column differ from control ($P < 0.05$); †means within a column differ from control ($P < 0.10$).

ria was related to its membrane disrupting activity. Vanillin did not modify rumen microbial fermentation, with the exception of the 500 mg/L dose that slightly reduced the proportion of acetate (–2.0%) without affecting total VFA concentration (Table 3). There are no other reports in the literature on the effects on vanillin on rumen microbial fermentation. Results indicate that vanillin seems not to be a good alternative to improve rumen microbial fermentation in 60:40 forage:concentrate diets.

Thymol is one of the major compounds of essential oil of thyme (*Thymus vulgaris*) and oregano (*Origanum vulgaris;* Dorman and Deans, 2000). Several studies demonstrated that THY has wide-spectrum antimicrobial activity, inhibiting gram-positive and gram-negative bacteria due to its capacity to act as membrane permeabilizer (Helander et al., 1998; Dorman and Deans, 2000; Walsh et al., 2003). Thymol at 500 mg/L reduced total VFA concentration (–28.5%), the proportion of propionate (–18.4%), ammonia N concentration (–31.9%), and BCVFA concentration (–41.7%), and increased the proportion of acetate (+1.8%), acetate to propionate ratio (+35.5%), and rumen pH (+6.3%; Table 4). The decrease in BCVFA and ammonia N concentration with T500 was consistent with the inhibition of the deamination process. In fact, McIntosh et al. (2003) observed that a blend of essential oil compounds containing THY reduced the rate of deamination of AA and inhibited the growth of *Clostridium sticklandii* and *Peptostreptococcus anaerobius,* 2 bacteria that belong to a specific group of hyper-producing ammonia species. Researchers suggested that this blend of essential oils reduced ammonia N concentration by inhibiting hyper-producing ammonia bacteria, a mechanism of action similar to that of MON (Russell et al., 1988). Borchers (1965), in the same batch cultures, observed that 1,000 mg/L of THY reduced ammonia N concentration, which agrees with the reduced ammonia N concentration observed with T500. However, Borchers (1965) did not study the effects of THY on VFA profile. The effects of THY on the VFA profile agree with the results of Evans and Martin (2000), in which 400 mg/L of THY resulted in a decrease in the acetate (–44.0%) and propionate (–78.2%) concentrations, and an increase in acetate to propionate ratio (+61.4%). Evans and Martin (2000) also observed an increase in the final pH, which agrees with the increased pH observed with T500. Moreover, Evans and Martin (2000) observed that at 90 and 180 mg/L, THY inhibited the growth of *Selenomonas ruminantium* and *Streptococcus bovis,* respectively; they suggested that THY disrupts membrane integrity and affects glucose transport. These results indicate that the THY has a strong antimicrobial activity and that the optimal dose may be difficult to define because there is a narrow range of doses between observing some effects (changes in the profile of VFA) and having negative effects (reduction in total VFA concentration).

Eugenol is one of the main compounds of clove (*Eugenia caryophyllata*) and cinnamon (*Cinnamomum zeylanicum*) oils (Davidson and Naidu, 2000), and has been shown to have antimicrobial activity against gram-positive and gram-negative bacteria (Dorman and Deans, 2000; Walsh et al., 2003). Treatments E5, E50, and E500 modified N metabolism and VFA proportions without affecting total VFA concentration, but changes were dose dependent (Table 5). The E5 treatment tended ($P < 0.10$) to reduce the proportion of acetate (–1.7%) and the acetate to propionate ratio (–5.4%); E50 tended ($P < 0.10$) to reduce and E500 reduced ammonia N concentration (–21.9 and –22.8%, respectively). Treatment E500 also reduced the proportion of propionate (–3.9%) and BCVFA concentration (–16.9%). The decrease in BCVFA and ammonia N concentration is consistent with the inhibition of the deamination of AA (Allison et al., 1962), and suggests that EUG may be an alternative for reducing ammonia N losses in the

Table 6. Effect of monensin, thymol, and eugenol on DM, OM, NDF, and ADF digestion in a dual-flow continuous culture

	Treatment[1]								
	CON	MON	T5	T50	T500	E5	E50	E500	SEM
True digestibility, %									
DM	51.0	43.8*	53.1	52.4	43.1*	49.4	53.9	51.4	1.97
OM	49.3	43.2*	49.7	49.9	39.2*	48.6	51.2	48.1	1.61
NDF digestibility, %	20.1	–0.48*	18.4	21.7	–1.96*	23.0	18.7	12.3	6.15
ADF digestibility, %	27.8	–0.94*	27.8	31.3	0.48*	31.3	30.4	20.1	5.86

[1]CON = control; MON = 10 mg/L of monensin; T5 = 5 mg/L of thymol; T50 = 50 mg/L of thymol; T500 = 500 mg/L of thymol; E5 = 5 mg/L of eugenol; E50 = 50 mg/L of eugenol; E500 = 500 mg/L of eugenol.

*Means within a row differ from control ($P < 0.05$).

rumen. Tamminga (1992) suggested that controlling microbial degradation of protein in the rumen could reduce N losses and improve the low efficiency of N retention in ruminants.

The effect of EUG in reducing the ammonia N concentration was the most interesting effect observed. Guaiacol at 500 mg/L also reduced ammonia N concentration and the proportion of acetate. Other treatments had small effects or reduced total VFA concentration.

Experiment 2

Monensin, THY, and EUG were selected for the second experiment. Monensin is widely used to improve the efficiency of feed use in cattle, decreasing ammonia production, and acetate to propionate ratio (Russell and Strobel, 1989), and was used as a positive control. The previous experiment showed that EUG was the most interesting essential oil compound in in vitro 24-h batch incubation. Thymol was also selected because it has received most attention from researchers (Borchers, 1965; Evans and Martin, 2000).

Monensin reduced the digestion of DM and OM, which is explained by the reduction of NDF and ADF digestion (Table 6). The negative effects of MON on diet digestion agree with some in vitro studies in which MON at 2 to 33 mg/L reduced DM, OM, and fiber digestion (Wallace et al., 1981; Russell and Strobel, 1988). However, in vivo studies with low doses (2 to 5 mg/L) of MON did not affect nutrient digestion, suggesting that a dose of 10 mg/L may be toxic to rumen bacteria. This effect of MON on NDF and ADF digestion is attributed to the inhibition of ruminal cellulolytic bacteria, such as cellulotytic ruminococci and *Butyrivibrio fibrisolvens,* which are particularly sensitive to ionophores (Russell and Strobel, 1989). Monensin reduced the proportion of acetate and butyrate, BCVFA concentration, and acetate to propionate ratio, and increased the proportion of propionate compared with CON, as expected, without affecting total VFA concentration (Table 7). Results of this experiment are in agreement with in vitro and in vivo studies that demonstrated that monensin supplementation maintained total VFA concentration, increased the proportion of propionate, and reduced the proportion of acetate and butyrate in ruminal fluid (Russell and Strobel, 1988; Yang and Russell, 1993; Busquet et al., 2005). Some studies also reported that the addition of monensin increased total VFA concentration (Richardson et al., 1976).

Thymol at 500 mg/L acted similarly to MON, reducing the digestion of DM, OM, NDF, and ADF digestion (Table 6). Thymol at 500 mg/L also reduced total VFA concentration, the proportion of acetate and valerate, BCVFA concentration, and acetate to propionate ratio, and increased the proportion of propionate and butyrate compared with CON. These negative effects of T500 on nutrient digestion agree with the results of experiment 1, in which THY at 500 and 5,000 mg/L reduced total VFA production and increased pH, and with results of Evans and Martin (2000), in which THY (400 mg/L) reduced total VFA production and inhibited the growth of gram-negative and positive bacteria. Thymol and MON caused the same negative effects on nutrient digestion, suggesting that THY might also act on cellulolytic species. Thymol at 50 mg/L had no effect on VFA profile. However, the lowest dose of THY (T5) tended to reduce ($P < 0.10$) the proportion of acetate, and increase the proportion of butyrate without affecting total VFA concentration and diet fermentation (Table 7). These results suggest that the range of doses between no effect and toxic effects in THY may be smaller compared with other active compounds (Cardozo et al., 2005), and that the selection of the optimal dose may require further studies with doses between 5 and 500 mg/L, to observe the same positive effects without affecting total VFA concentration.

Eugenol had no effect on DM, OM, NDF, and ADF digestion, which suggests that it did not modify overall diet fermentability (Table 6). Although EUG had no

Table 7. Effect of monensin, thymol, and eugenol on total VFA concentration and profile in a dual-flow continuous culture

	Treatment[1]								
	CON	MON	T5	T50	T500	E5	E50	E500	SEM
Total VFA, m*M*	105.5	103.7	102.5	103.9	52.8*	106.9	101.2	90.2*	4.32
VFA, mol/100 mol									
Acetate	69.9	48.9*	65.6†	67.5	51.6*	69.6	67.6	58.3*	1.50
Propionate	15.8	42.3*	16.9	16.7	20.0*	15.1	17.0	22.9*	1.24
Butyrate	9.01	4.69*	11.7*	10.0	25.6*	8.87	10.4	14.4*	0.88
Valerate	2.79	3.13	2.70	2.74	2.50*	2.84	2.78	3.73*	0.26
Isobutyrate	0.61	0.49	0.64	0.65	0.19*	0.67	0.53	0.34*	0.07
Isovalerate	2.16	0.44*	2.15	2.36	0.02*	2.95	1.63	0.28*	0.49
BCVFA,[2] m*M*	2.96	0.97*	2.86	3.10	0.11*	3.84	2.20	0.54*	0.57
Acetate:propionate	4.42	1.16*	3.92	4.05	2.58*	4.62	4.02	2.57*	0.31

[1]CON = control; MON = 10 mg/L of monensin; T5 = 5 mg/L of thymol; T50 = 50 mg/L of thymol; T500 = 500 mg/L of thymol; E5 = 5 mg/L of eugenol; E50 = 50 mg/L of eugenol; E500 = 500 mg/L of eugenol.

[2]BCVFA = Branched-chain VFA.

*Means within a column differ from control ($P < 0.05$); †means within a column differ from control ($P < 0.10$).

effect on nutrient digestion, E500 reduced total VFA concentration, the proportion of acetate, BCVFA concentration, and acetate to propionate ratio, and increased the proportion of propionate, butyrate, and valerate compared with CON (Table 7). In contrast, in experiment 1, E500 reduced the proportion of propionate and had no negative effect on total VFA concentration. The inconsistencies between studies may be attributed to the potential adaptation of microorganisms in the long-term fermentation studies. These results and other studies (Cardozo et al., 2004; Castillejos et al., 2005) suggest that effects from short-term fermentation studies should be interpreted with caution because a longer adaptation time may be required to observe real effects on rumen microbial fermentation.

The highest doses of THY, EUG, and MON reduced the proportion of acetate, BCVFA concentration, and acetate to propionate ratio, and increased the proportion of propionate. However, whereas T500 and E500 increased the proportion of butyrate, MON reduced it. The fermentation pattern observed in THY and EUG is different from that observed in MON, suggesting that the mechanism of action of THY and EUG may not be the same as that of MON. In fact, Helander et al. (1998) observed that monensin affects only some gram-positive bacteria, whereas essential oils inhibit gram-positive and gram-negative bacteria. Ruminal gram-positive bacteria are involved in fermentation processes that produce, among other products, acetate, butyrate, formate, lactate, hydrogen, and ammonia. On the other hand, ruminal gram-negative bacteria are involved in fermentation processes associated with the production of propionate and succinate (Russell and Strobel, 1989). Monensin reduced the proportion of butyrate because this additive inhibits the major butyrate producer, the ruminal gram-positive bacteria *Butyrivibrio fibrisolvens* (Russell and Strobel 1989). Moreover, T500 reduced the proportion of valerate and E500 increased it, suggesting that each essential oil compound may have a different mechanism of action. Essential oils comprise a large number of components and it is likely that their mode of action involves several targets in the bacterial cells.

Ammonia N concentration (9.91 ± 1.91 mg/100 mL), and flow of ammonia (0.31 ± 0.06 g/d), nonammonia (3.79 ± 0.14 g/d), bacterial (1.31 ± 0.17 g/d), and dietary N (2.47 ± 0.21 g/d), the degradation of CP (24.3 ± 6.5 %), and efficiency of microbial protein synthesis (28.4 ± 3.3 g bacterial N/kg of OM truly digested) were not affected by treatments (data not shown). However, the average concentration of LPep N was higher in T5, T500, and E500 (Table 8), suggesting that proteolysis was stimulated or peptidolysis was inhibited. Treatment T500 also increased the average concentration of SPep+AA N. The accumulation of LPep N and SPep+AA N suggest that proteolysis and peptidolysis were stimulated by T500. However, deamination was not affected because the average concentration of ammonia N was similar in all treatments (average of 12.1 mg/100 mL), and only MON unexpectedly tended to increase this fraction. In contrast, most in vitro studies reported that monensin decreased (Russell and Strobel, 1988) ruminal ammonia concentration, decreasing the deamination of AA. Russell et al. (1988) found a group of gram-positive bacteria that are monensin-sensitive, called hyper-ammonia-producing bacteria, which have a high specific activity for ammonia production. Although treatments T500, E500, and MON reduced BCVFA concentration, these treatments did not reduce ammonia N concentration as expected.

Table 8. Effect of monensin, thymol, and eugenol on N fraction concentrations

	Treatment[1]								
	CON	MON	T5	T50	T500	E5	E50	E500	SEM
N fractions,[2] mg/100 mL									
LPep N	5.14	6.49	7.49*	6.67	7.23*	6.53	6.42	7.25*	0.75
SPep+AA N	4.86	4.15	3.73	5.05	9.66*	4.46	4.07	4.97	0.61
Ammonia N	11.7	16.9†	13.4	14.0	10.7	13.7	9.45	11.6	1.97

[1]CON = control; MON = 10 mg/L of monensin; T5 = 5 mg/L of thymol; T50 = 50 mg/L of thymol; T500 = 500 mg/L of thymol; E5 = 5 mg/L of eugenol; E50 = 50 mg/L of eugenol; E500 = 500 mg/L of eugenol.

[2]Fractions: LPep N = N from large peptides; SPep+AA N = N from small peptides and AA.

*Means within a column differ from control ($P < 0.05$); †means within a column differ from control ($P < 0.10$).

CONCLUSIONS

Most essential oil compounds had important antimicrobial activity and decreased total VFA concentration, although at appropriate doses, these compounds also modified rumen microbial fermentation without decreasing total VFA concentration. Eugenol and guaiacol in batch fermentation reduced ammonia N concentration or changed VFA proportions. In continuous culture, MON also changed the proportions of VFA as expected but inhibited nutrient digestion. Eugenol and THY decreased total VFA concentration and changed VFA proportions, and only the lowest dose of THY modified the fermentation profile without decreasing total VFA concentration and diet fermentation. Further research is required to define the optimal doses and effects of these essential oils compounds on rumen microbial fermentation.

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