Effects of essential oils and their components on in vitro rumen microbial fermentation

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Benchaar, C., Chaves, A. V., Fraser, G. R., Wang, Y., Beauchemin, K. A. and McAllister, T. A. 2007. **Effects of essential oils and their components on in vitro rumen microbial fermentation.** Can J. Anim. Sci. **87**: 413–419. The objective of this study was to investigate the effects of essential oils (EO) and essential oil compounds (EOC) on in vitro rumen microbial fermentation. Treatments were: control (no additive), cinnamon leaf oil (400 mg L^{-1}), clove leaf oil (200 mg L^{-1}), sweet orange oil (200 mg L^{-1}), oregano oil (200 mg L^{-1}), thyme oil (200 mg L^{-1}), carvacrol (400 mg L^{-1}), cinnamaldehyde (400 mg L^{-1}), eugenol (800 mg L^{-1}), and thymol (400 mg L^{-1}). Treatments were evaluated using in vitro 24-h batch culture of rumen fluid with a 51:49 forage:concentrate dairy ration [16.7% crude protein (CP), 34.4% neutral detergent fibre (NDF)]. Incubations were conducted in triplicate with gas production (GP) measured at 0, 2, 6, 8, 12, and 24 h, while pH, ammonia (NH_3), volatile fatty acid (VFA), in vitro dry matter (IVDMD) and neutral detergent fibre (IVNDFD) digestibilities were determined after 24 h of incubation. Among the EO and EOC evaluated, only the phenolic compounds, carvacrol, thymol, and eugenol affected ruminal fermentation, relative to the control. Carvacrol (400 mg L^{-1}) and eugenol (800 mg L^{-1}) increased pH and molar proportion of butyrate, and decreased molar proportion of propionate, IVDMD, IVNDFD, and GP. At the concentration of 200 mg L^{-1}, thymol increased final pH, and reduced molar proportion of propionate, IVNDFD, and GP. None of the EO or EOC affected NH_3 concentration, suggesting that at the doses tested in this study, protein degradation was not affected by EO and EOC. This study showed that among the EO and EOC investigated only phenolics exhibited antimicrobial activity as exemplified by reduced diet fermentability and a shift in VFA profile from less propionate towards more butyrate. These changes in fermentation may not be nutritionally beneficial to dairy cattle. The present study suggests that the types and concentrations of EO and EOC employed to alter ruminal fermentation must be carefully defined before their widespread use in ruminant nutrition can be recommended.

Key words: Essential oil, ruminal fermentation, in vitro batch culture, dairy cattle

Benchaar C., Chaves A. V., Fraser, G. R., Wang, Y., Beauchemin, K. A. et McAllister, T. A. 2007. **Effets des huiles essentielles et de leurs composés sur les fermentations ruminales microbiennes in vitro**. Can. J. Anim. Sci. **87**: 413–419. L'objectif de cette étude était d'examiner les effets des huiles essentielles (HE) et de leurs composés (CHE) sur les fermentations ruminales microbiennes in vitro. Les traitements ont été: contrôle (sans additif), HE de la feuille de cannelle (400 mg L^{-1}), HE de la feuille clou de girofle (200 mg L^{-1}), HE d'orange douce (200 mg L^{-1}), HE d'origan (200 mg L^{-1}), HE de thym (200 mg L^{-1}), carvacrol (400 mg L^{-1}), aldéhyde cinnamique (400 mg L^{-1}), eugenol (800 mg L^{-1}), et thymol (400 mg L^{-1}). Les traitements ont été évalués en utilisant la technique des cultures batch, par incubation dans du liquide ruminal pendant 24-h d'un substrat constitué d'une ration complète pour vaches laitières [51:49, fourrage:concentré; 15% protéines brutes (PB), 34.4% fibre au détergent neutre (NDF)]. Les incubations ont été effectuées en triplicata avec mesure de la quantité de gaz produit (GP) à 0, 2, 6, 8, 12 et 24 h. Le pH, les concentrations d'ammoniac (NH_3) et d'acides gras volatils (AGV), la digestibilité in vitro de la matière sèche (DIVMS) et celle du NDF (DIVNDF) on été déterminés après 24 h d'incubation. Parmi les HE et le CHE évalués, seuls les composés phénoliques carvacrol, thymol, et eugenol ont affecté les fermentations ruminales, comparativement au contrôle. Le carvacrol (400 mg L^{-1}), et l'eugenol (800 mg L^{-1}) ont augmenté le pH et la proportion molaire du butyrate, et ont réduit la proportion molaire du propionate, DIVMS, DIVNDF et GP. À la concentration de 200 mg L^{-1}, le thymol a augmenté le pH et a réduit la proportion molaire du propionate, DIVNDF, et GP. La concentration de NH_3 n'a pas été affectée par aucun des traitements évalués, suggérant qu'aux concentrations de HE et CHE évaluées dans cette étude, la dégradation des protéines n'a pas été modifiée. Cette étude a montré que parmi les HE et CHE évaluées, seuls les composés phénoliques ont exercé une activité antimicrobienne telle qu'illustrée par une réduction dans la fementation du substrat et une orientation du faciès fermentaire vers moins de propionate et plus de butyrate. Ce genre de changement dans les fermentations n'est pas nutritionnellement bénéfique pour la vache laitière. Cette étude suggère que le type et les concentrations de HE et CHE utilisées pour modifier les fermentations ruminales doivent être définis avec attention avant qu'ils ne soient recommandés pour utilisation à plus grande échelle dans l'alimentation des ruminants.

Mots clés: Huile essentielle, fermentations ruminales, culture batch in vitro, vache laitière

Abbreviations: **AFR**, average fermentation rate; **BEO**, blend of essential oil compounds; **DM**, dry matter; **CP**, crude protein; **EO**, essential oil; **EOC**, essential oil compound; **GP**, gas production; **IVDMD,** in vitro dry matter digestibility; **IVNDFD**, in vitro NDF digestibility; **NDF**, neutral detergent fibre; **VFA**, volatile fatty acid

In livestock production, antibiotics at non-therapeutic levels are commonly included in the diet to increase feed efficiency and prevent disease. The use of antibiotics in this manner has, however, been criticized because of the emergence of multi-drug resistant bacteria that may pose a risk to human health. Consequently, in recent years, considerable effort has been devoted towards developing alternatives to feed antibiotics. Among these alternatives, essential oils (EO) have received much attention (Benchaar et al. 2007; Fraser et al. 2007). Essential oils are the volatile and aromatic compounds that can be extracted from plants by distillation methods, in particular steam distillation (Greathead 2003). Contrary to their name, EO are not true oils (i.e., lipids) and are most commonly associated with the fragrance, the *Quinta essentia* of plants. Chemically, EO are secondary metabolites composed primarily of isoprenes or terpenes ($C_{10}H_{16}$) and may contain mixtures of diterpenes (C_{20}), triterpenes (C_{30}), tetraterpenes (C_{40}), hemiterpenes (C_5), and sesquiterpenes (C_{15}). When isoprenes are associated with additional elements, usually oxygen, they are termed terpenoids (Cowan 1999). Essential oils have been shown to have antimicrobial properties against different types of microorganisms including bacteria, protozoa, and fungi (Greathead 2003). A number of in vitro studies have been recently published on the effects of EO and their components (EOC) on ruminal fermentation and metabolism (McIntosh et al. 2003; Busquet et al. 2006). Results from those studies revealed variable effects of EO and their derivatives on rumen bacteria and ruminal fermentation (Busquet et al. 2006; Castillejos et al. 2006). Discrepancies between studies were attributed to different types and doses of EO (Busquet et al. 2006), but also to the in vitro technique (i.e., batch versus continuous culture) used (Fraser et al. 2007). The objective of this study was to examine in vitro the effects of five EO (cinnamon leaf, clove leaf, sweet orange, oregano, and thyme oils) and four EOC (carvacrol, cinnamaldehyde, eugenol, and thymol) on rumen microbial fermentation

MATERIAL AND METHODS

Ruminal Inoculum

Rumen inoculum was obtained from four ruminally fistulated, lactating Holstein dairy cows (617 ± 8.9 kg body weight; 45 ± 13 d in milk) fed a total mixed ration (18% CP, 33% NDF) consisting of whole crop barley silage (46.6%), alfalfa hay (4.5%), dry ground corn (6.8%), steam rolled barley (17.6%), pelleted dairy supplement (23.3%), and canola oil (1.2%). The pellets contained [dry matter (DM) basis] ground barley grain (14.1%), ground corn grain (0.05%), heat-processed canola meal (20.8%), beet pulp (11.9%), heat-treated soybean meal (20.6%), corn gluten meal (17.0%), dry molasses (6.5%), limestone (1.7%), dicalcium phosphate (2.7%), sodium bicarbonate (1.6%), and salt, minerals, and vitamins mixture (2.7%). The diet was formulated to meet nutrient requirements of the cows (NRC 2001) and was provided twice daily (0900 and 1600) ad libitum. Cows were cared for in accordance with the guidelines of the Canadian Council on Animal Care (CCAC 1993). Ruminal contents were collected from the anterior dorsal, anterior ventral, medium ventral, posterior dorsal, and posterior ventral locations within the rumen 2 h after the morning feeding. Pooled ruminal fluid was strained through four layers of cheesecloth into an insulated thermos and transported immediately to the laboratory. The strained ruminal fluid was continuously purged under free-oxygen CO_2 and kept at 39°C in a water bath prior to use in in vitro incubations.

Experimental Treatments

The experiment, repeated on 2 separate days, was conducted as a complete randomized block design with treatment as the main effect. Experimental treatments were: control (no additive), cinnamon leaf oil (400 mg L^{-1}; *Cinnamomum zeylanicum*, standardised at 76% of eugenol), clove leaf oil (200 mg L^{-1}; *Eugenia caryophyllata*, standardised at 85% of eugenol), sweet orange oil (200 mg L^{-1}; *Citrus sinensis*, standardised at 95% of limonene), oregano oil (200 mg L^{-1}; *Origanum vulgaris*, standardised at 64% of carvacrol), thyme oil (200 mg L^{-1}; *Thymus zygis*, standardised at 45% of thymol), eugenol (800 mg L^{-1}; purity > 99%), carvacrol (400 mg L^{-1}; purity > 98%), cinnamaldehyde (200 mg L^{-1}; purity > 99%), and thymol (200 mg L^{-1}; purity > 99%). Essential oils and EOC were provided by Pancosma S.A. (Bellegrade-sur-Valserine Cedex, France). Experimental treatments were evaluated in two separate incubation runs with clove leaf oil, cinnamon leaf oil, carvacrol, thymol, and eugenol included in a first run, and oregano oil, thyme oil, sweet orange oil, and cinnamaldehyde included in a second run.

Concentrations of EO and EOC employed in this study were selected based on preliminary screening of the selected compounds from 0 to 1000 mg L^{-1}. The lower concentration of each EO that resulted in any noticeable change in gas production was selected for further study in the present experiment.

Diet Substrate

A representative sample of the total mixed ration fed to cows was freeze-dried and ground through a 1-mm screen Wiley mill (standard model 4; Arthur M. Thomas, Philadelphia, PA) for later use in in vitro incubations.

In Vitro Batch Culture Incubations

In vitro incubations were conducted in 50-mL serum bottles containing 4.8 mL of strained ruminal fluid, 14.5 mL of phosphate-bicarbonate buffer (Menke et al. 1979), and 0.5 g of diet substrate. Triplicate bottles were prepared for each treatment and each incubation time (0, 2, 4, 6, 8, 12, and 24 h). The temperature of the bottles was raised to 39°C prior to inoculation. Stock solutions were prepared by dissolving EO and EOC in 99.5% ethanol (wt/vol). The additives were added to each culture bottle to achieve the final concentration needed. Equal volumes of ethanol were also added to control bottles. In all cases, the final concentration of ethanol in culture fluid was less than 2% (vol/vol). The bottles were purged with oxygen-free CO_2, sealed with a butyl rubber stopper, placed on a rotary shaker (Lab-Line Instruments Inc, Melrose Park, IL) in an incubator (Forma Scientific, model 39419-1, Marietta, OH) at 39°C.

For each incubation time, the bottles were removed from the incubator and gas production (GP) was measured using a water displacement technique (Fedorak and Hrudey 1983). After 24 h of incubation, the bottles were withdrawn from the incubator, uncapped and pH of the culture fluid was measured (Orion Model 260A, Fisher Scientific, Toronto, ON). The bottles were then placed into a crushed ice bath and immediately sampled for subsequent analysis of ammonia (NH_3). These subsamples (1.6 mL) were transferred into 2-mL microcentrifuge tubes containing 200 μL 65% trichloroacetic acid (TCA; wt/vol) and centrifuged at 14 000 × *g* for 10 min (Spectrafuse 16M, National Labnet Co., Edison, NJ) to precipitate particulate matter. The supernatants were transferred into 2-mL microcentrifuge tubes (Fisher Scientific, Ottawa, ON), frozen at –20°C until analyzed for NH_3 by the phenyl-hypochlorite reaction (Weatherburn 1967). An additional 1.5-mL sample was removed from each bottle for determination of volatile fatty acid (VFA) concentrations. These samples were acidified with 300 μL of 25% metaphosphoric acid (wt/vol), centrifuged, and analyzed by gas chromatography (Hewlett Packard model 5890; Brockville, ON) using a ZB-FFAP silica capillary column (30 m × 32 mm × 1 μm; Phenomenex, Torrance, CA).

In vitro DM digestibility (IVDMD) was determined at 24-h of incubation. The content of each bottle was transferred into pre-weighed 50 mL centrifuge tubes (Cole-Palmer, Montreal, QC), rinsed, and centrifuged at 400 × *g* for 10 minutes at + 4°C. Supernatants were discarded, and precipitates were dried at 55°C for 48 h and weighed to determine DM and NDF (Van Soest et al. 1991) concentrations for the estimation of IVDMD and in vitro NDF digestibility (IVNDFD), respectively.

Calculations and Statistical Analyses

Gas production data (corrected for substrate-free blanks) were fitted to a non-linear model (model no. 1) proposed by López et al. (1999):

$$GP = B \times [1 - e^{-k(t - L)}]$$

where B is the asymptotic gas production (mL g^{-1} DM incubated), k is the fractional fermentation rate (h^{-1}), L is the lag time (h), and t in the incubation time (h). An iterative least squares procedure using NLIN (SAS Institute, Inc. 2006) was used to estimate B, k, and L. Average fermentation rate (AFR, mL gas h^{-1}) was defined as:

$$(B \times k) / [(2 \times \ln 2) + (k \times L)]$$ (Hervás et al. 2005).

Results for pH, NH_3, VFA, IVDMD, and IVNDFD were analyzed using the PROC MIXED procedure of SAS (SAS Institute, Inc. 2007) for a randomized block design. Results for GP were analysed as repeated measures using PROC MIXED. The variance-covariance error structure was compound symmetry because it gave the lowest AIC values. Treatments were compared with control using the Dunnett test. Results are reported as least squares means and significance was declared at $P < 0.05$ unless otherwise stated.

RESULTS

Results on the effects of EO and EOC on fermentation characteristics, IVDMD, IVNDFD, and GP are presented in Tables 1 and 2.

Among the EO and EOC investigated, only cinnamon oil, carvacrol, thymol, and eugenol resulted in an increase ($P < 0.05$) in pH compared with the control. Total VFA concentration was not changed ($P > 0.05$) by any of the EO or EOC evaluated. Only carvacrol, thymol, and eugenol affected ($P < 0.05$) molar proportions of VFA compared with the control. The addition of carvacrol did not change ($P > 0.05$) the molar proportion of acetate but strongly decreased ($P < 0.05$) the molar proportion of propionate and increased ($P < 0.05$) that of butyrate. Relative to the control, the addition of thymol at 200 mg L^{-1} had no effect ($P > 0.05$) on the molar proportions of acetate and butyrate but reduced ($P < 0.05$) the molar proportion of propionate. Eugenol increased ($P < 0.05$) the molar proportion of butyrate and decreased ($P < 0.05$) the molar proportions of acetate and propionate, compared with the control. Of the EO and EOC evaluated, only carvacrol, thymol, and eugenol resulted in a decrease ($P < 0.05$) in GP compared with the control, which was consistent with a reduction in IVDMD. Kinetics of GP showed that relative to the control, only carvacrol decreased ($P < 0.05$) the fractional fermentation rate (k) and the AFR. With the exception of cinnamaldehyde, all EO and EOC decreased ($P < 0.05$) IVNDFD. None of the EO and EOC examined affected ($P > 0.05$) NH_3 concentration.

DISCUSSION

Results from this study revealed that among the EO and EOC examined only the phenolics (i.e., carvacrol, thymol, and eugenol) exhibited antimicrobial activities in vitro. Phenolic compounds such as thymol, carvacrol, and eugenol have been shown to possess high antimicrobial activity due to the presence of a hydroxyl group in the phenolic structure (Dorman and Dean 2000; Ultee et al. 2002; Burt 2004). Compounds with phenolic structures have a broad spectrum of activity against a variety of both Gram-positive and Gram-negative bacteria (Kim et al. 1995; Helander et al. 1998; Dorman and Deans 2000; Lambert et al. 2001). The mechanism of action by which phenolic compounds are thought to exert their antimicrobial activity is through the disturbance of the cytoplasmic membrane, disrupting the proton motive force, electron flow active transport, and coagulation of cell contents (Burt 2004). Helander et al. (1998) showed that thymol and carvacrol decreased the intracellular ATP pool and increased the extracellular ATP concentration of *E. coli*, an observation indicative of disruption of the cytoplasmic membrane.

The addition of carvacrol (400 mg L^{-1}) and eugenol (800 mg L^{-1}) resulted in an increase in pH. Recently, Busquet et al. (2006) reported effects of the phenolic compounds eugenol and carvacrol at concentrations of 3, 30, 300, and 3000 mg L^{-1} in 24-h in vitro batch culture incubations. In their study, final pH was not affected when these two phenolic compounds were supplied at the concentrations of 3 and 30 mg L^{-1}. However, the addition of these two compounds at high concentrations (i.e., 300 and 3000 mg L^{-1} for

Table 1. Effects of clove leaf oil, cinnamon leaf oil, carvacrol, thymol, and eugenol on in vitro batch culture rumen microbial fermentation

	Treatments[z]						
	Control	Clove	Cinnamon	Carvacrol	Thymol	Eugenol	SEM
pH	5.58	5.64	5.69*	6.01*	5.71*	5.92*	0.029
Ammonia (mM)	11.7	13.3	14.5	10.1	12.0	8.4	2.61
Total VFA (mM)	89.8	104.4	102.5	78.0	94.1	76.0	5.31
VFA (mol 100 mol^{-1})							
Acetate (A)	55.4	56.4	56.4	51.9	54.3	48.4	1.43
Propionate (P)	24.6	24.0	22.8	6.0	21.8	12.5	0.68
Butyrate	16.3	15.9	17.0	37.8	19.7	33.1	1.26
Others	3.7	3.7	3.8	4.3	4.2	6.0	0.19
A:P	2.3	2.4	2.5	8.7	2.5	3.9*	0.28
IVDMD (g kg^{-1} DM)	0.317	0.320	0.301	0.268*	0.305	0.245*	0.0107
IVNDFD (g kg^{-1} NDF)	0.277	0.198*	0.208*	0.122*	0.212*	0.072*	0.0135
GP[y] (mL g^{-1} DM)	171	170	170	145*	164*	152*	4.1
GP kinetic parameters[x]							
B (mL g^{-1} DM)	171	174	175	151	167	156	5.0
k (h^{-1})	0.140	0.137	0.138	0.110*	0.133	0.135	0.007
L (h)	0.9	0.7	0.8	1.1	1.0	1.0	0.12
AFR (mL gas h^{-1})	15.8	16.0	16.1	10.7*	14.6	13.7*	0.8

[z] Treatments were control (0 mg L^{-1}), clove leaf oil (200 mg L^{-1}), cinnamon leaf oil (400 mg L^{-1}), carvacrol (400 mg L^{-1}), thymol (200 mg L^{-1}), and eugenol (800 mg L^{-1}).
* Within a row, means followed by an asterisk differ significantly from the control ($P < 0.05$).
[y] Cumulative gas production.
[x] B, asymptotic gas production (mL g^{-1} DM); k, fractional fermentation rate (h^{-1}); L, lag (h); AFR, average fermentation rate (mL gas h^{-1}) calculated as $(B \times k)/[(2 \times \ln 2) + (k \times L)]$.

Table 2. Effects of oregano oil, thyme oil, sweet orange oil, and cinnamaldehyde on in vitro batch culture rumen microbial fermentation

	Treatments[z]					
	Control	Oregano	Thyme	Sweet orange	Cinnamal-dehyde	SEM
pH	5.52	5.6	5.57	5.56	5.54	0.065
Ammonia (mM)	11.8	13.2	12.4	10.9	11.2	1.68
Total VFA (mM)	94.0	101.3	104.9	105.9	70.7	9.57
VFA (mol 100 mol^{-1})						
Acetate (A)	56.9	57.6	57.4	56.0	56.6	1.72
Propionate (P)	23.5	22.9	23.0	23.6	24.2	2.73
Butyrate	15.9	15.6	15.7	16.3	15.0	7.36
Others	3.7	3.9	3.9	4.1	4.2	0.48
A:P	2.4	2.5	2.5	2.4	2.3	0.17
IVDMD (g kg^{-1} DM)	0.320	0.312	0.314	0.307	0.319	0.0575
IVNDFD (g kg^{-1} NDF)	0.235	0.169*	0.186*	0.148*	0.211	0.0101
GP[y] (mL g^{-1} DM)	168	166	161	169	165	4.3
GP kinetic parameters[x]						
B (mL g^{-1} DM)	177	168	170	178	172	5.2
k (h^{-1})	0.136	0.137	0.14	0.139	0.127	0.006
L (h)	0.6	0.9	0.6	0.5	1.0	0.22
AFR (mL gas h^{-1})	16	15	16	17	14	1.4

[z] Treatments were control (0 mg L^{-1}), oregano oil (200 mg L^{-1}), thyme oil (200 mg L^{-1}), sweet orange oil (200 mg L^{-1}), and cinnamaldehyde (200 mg L^{-1}).
*Within a row, means followed by an asterisk differ significantly from the control ($P < 0.05$).
[y] Cumulative gas production.
[x] B, asymptotic gas production (mL g DM^{-1}); k, fractional fermentation rate (h^{-1}); L, lag (h); AFR, average fermentation rate (mL gas h^{-1}) calculated as $(B \times k)/[(2 \times \ln 2) + (k \times L)]$.

carvacrol and 3000 mg L^{-1} for eugenol) increased pH. Evans and Martin (2000) and Castillejos et al. (2006) reported, respectively, that 400 and 500 mg L^{-1} of thymol increased final pH in 24-h in vitro batch culture incubations. A similar effect on pH was also observed in the current study with thymol but at a lower concentration (i.e., 200 mg L^{-1}) than the concentrations used by authors previously cited. In some of the studies cited previously (Busquet et al. 2006; Castillejos et al. 2006), the increased pH was associated with a reduction in total VFA concentration, reflecting a decrease in diet fermentability, which is consistent with the antimicrobial activity of phenolic compounds (Acamovic and Brooker 2005; Fraser et al. 2007). Such results indicate that phenolic compounds are likely to be detrimental for rumen microbial fermentation when administered at high doses. As the production of VFA represents the principal source of energy for ruminants, decreasing VFA production could yield adverse nutritional conse-

quences if this same effect was expressed in vivo.

In this study, among the EO and EOC examined, only carvacrol, thymol, and eugenol affected molar proportions of VFA compared with the control. The addition of 400 mg L^{-1} of carvacrol strongly decreased propionate molar proportion and increased that of butyrate. These results agree with those of Busquet et al. (2006), who reported that 300 mg L^{-1} of carvacrol decreased the proportion of propionate and increased that of butyrate. Using a dual-flow continuous culture fermenter maintained at constant pH, Cardozo et al. (2004) also observed that the addition (0.22 mg L^{-1}) of oregano oil (containing 64% of carvacol and 16% of thymol) increased the molar proportion of acetate and reduced that of propionate during the first 6 d of incubation. However, these effects disappeared after 7 d of fermentation, suggesting that ruminal microbes were able to adapt to oregano oil.

At the dose evaluated in the present study, eugenol (800 mg L^{-1}) increased the proportion of butyrate and decreased the proportions of acetate and propionate. Castillejos et al. (2006) observed that 500 mg L^{-1} of eugenol had no effect on the molar proportions of acetate and butyrate, but resulted in a decrease of the molar proportion of propionate. Busquet et al. (2006) observed that when used at the concentration of 300 mg L^{-1}, eugenol did not change molar proportions of acetate and propionate, but increased the proportion of butyrate.

The pattern of VFA observed in the present study with carvacrol and eugenol was marked by a strong decrease in the proportion of propionate and an increase in the proportion of butyrate, suggesting that at the concentrations evaluated, carvacrol (400 mg L^{-1}) and eugenol (800 mg L^{-1}) inhibited propionate-producing bacteria and favoured butyrate-producing bacteria. Similar VFA patterns were also observed when high concentration (i.e., 500 mg L^{-1}) of cinnamon leaf oil (containing 76% of eugenol) was added in continuous culture systems (Fraser et al. 2007). In the rumen, Gram-positive bacteria are generally acetate- and butyrate-producing bacteria, while Gram-negative bacteria are generally propionate-producing bacteria (Stewart 1991). These results suggest, therefore, that at the concentrations evaluated in our study, Gram-negative bacteria were sensitive to carvacrol (400 mg L^{-1}) and eugenol (800 mg L^{-1}), while Gram-positive bacteria were not. Burt (2004) suggested that Gram-positive bacteria appear to be more susceptible to the antibacterial properties of plant EO than Gram-negative bacteria. This may be expected as Gram-negative bacteria have an outer layer surrounding their cell wall that acts as a permeability barrier, limiting the access of hydrophobic compounds. However, Helander et al. (1998) reported that two phenolic compounds, thymol and caravacrol, also inhibited the growth of Gram-negative bacteria by disrupting the outer cell membrane. Puupponen-Pimiä et al. (2001) observed that phenolic compounds from berry extracts inhibited the growth of Gram-negative bacteria, but had no effect on Gram-positive bacteria. It appears that the small molecular weight of EO compounds allows them to penetrate the inner membrane of Gram-negative bacteria (Nikaido 1994; Dorman and Deans 2000).

In this study, the addition of thymol at 200 mg L^{-1} reduced the molar proportion of propionate compared with the control. Castillejos et al. (2006) reported that the addition of 500 mg L^{-1} of thymol in 24-h in vitro batch cultures resulted in lower proportion of propionate compared with the control. Evans and Martin (2000) observed that at the concentration of 180 mg L^{-1} (i.e., close to the concentration used in the current study), thymol completely inhibited the growth of *Streptococcus bovis*, one of the principal amylolytic bacteria species in the rumen. Therefore, the reduction in the molar proportion of propionate observed in this study may be accounted for by the inhibition of the growth of amylolytic bacteria.

Results of GP measurement revealed that only carvacrol, thymol, and eugenol resulted in a decrease in GP compared with the control, which was consistent with a reduction in IVDMD. The effect on DM digestibility was particularly marked with eugenol. Fraser et al. (2007) also observed a decrease in DM digestibility when cinnamon leaf oil (containing 76% of eugenol) was supplied at 500 mg L^{-1} in a RUSITEC. Using a dual-flow continuous culture fermenter maintained at constant pH, Castillejos et al. (2006) reported that at 500 mg L^{-1}, thymol reduced DM digestibility compared to the control, while no effect was observed on diet digestibility when eugenol was administered at the same concentration. However, a reduction in diet digestibility would have an adverse effect on animal productivity. These results suggest that at high concentrations phenolics exhibit broad-spectrum antimicrobial activity causing an inhibition of the overall fermentation process. Defining those types of EO and concentrations that specifically inhibit subpopulations in the rumen (e.g., hyper-ammonia producing bacteria) under a variety of dietary conditions could prove to be a significant challenge.

With the exception of cinnamaldehyde, all EO and EOC decreased IVNDFD. A decrease in NDF digestibility has often been observed in vitro when EO rich in phenolic compounds (Fraser et al. 2007) or pure phenolic compounds (Castillejos et al. 2006) were used. The fact that IVNDFD was reduced by almost all of the EO examined would suggest that fibrolytic bacteria may be particularly sensitive to these compounds.

Much of the current efforts in research on EO have been directed towards improving nitrogen (N) utilization in ruminants. Wallace et al. (2002) suggested that the anti-microbial properties of EO can be exploited to modulate activities of rumen microbial populations by reducing dietary protein degradation, thereby enhancing rumen N escape. In the current study, none of the EO and EOC compounds examined affected NH_3 concentration, suggesting that at the concentrations evaluated; these plant secondary metabolites had no dramatic impact on the deaminase activity of ruminal microorganisms.

McIntosh et al. (2003) used an in vitro 48-h incubation batch culture to determine the effects of a mixture of EO on proteolytic, peptidolytic, and deaminative activities in ruminal fluid collected from cows fed a silage-based diet supplemented with 1 g d^{-1} of a commercial blend of EO

compounds (BEO; Crina® ruminants; Akzo Surface Chemistry Ltd., Herfordshire, UK). The Crina® product contains between 10 and 30% of phenolic compounds including cresol, resorcinol, thymol, guaiacol, and eugenol (Rossi 1994). The BEO did not affect the degradation of any of the protein or peptide sources tested. However, the rate of NH_3 production from amino acids in casein acid hydrolysate was reduced by 9%, indicating that the effect of BEO on ruminal protein catabolism was at the final stage of the degradation process, namely, deamination. Newbold et al. (2004) also reported a 24% reduction in the deamination of amino acids when casein acid hydrolysate was incubated for 24-h in ruminal fluid obtained from sheep fed 100 mg d^{-1} of BEO. More recently, Castillejos et al. (2005) observed that when added at the concentration of 1.5 mg L^{-1} in a dual-flow continuous culture fermenter maintained at constant pH, BEO increased total VFA concentration but had no effect on protein metabolism (i.e., NH_3 concentration, bacterial and dietary N flows, degradation of CP, and microbial protein synthesis efficiency). The lack of effect of BEO on N metabolism in the study of Castillejos et al. (2005) was attributed to the fact that the dose of 1.5 mg L^{-1} may have been too low to inhibit the activity of ruminal bacteria. However, when Castillejos et al. (2007) used the same mixture at a higher concentration (i.e., 5 mg L^{-1}), there was still no effect of BEO on protein metabolism. McIntosh et al. (2003) and Newbold et al. (2006) suggested that BEO exerted its effects on ruminal bacteria only at concentrations in the range of 35 to 360 mg L^{-1}, higher than that likely to be achieved in vivo. Indeed, Benchaar et al. (2006, 2007) observed no changes in ruminal fermentation characteristics, including NH_3 concentration, when dairy cows were supplemented with 750 or 2000 mg d^{-1} of BEO. Assuming a rumen volume of 100 L and an outflow rate of 10% h^{-1} for an adult cow, ruminal concentration would be 3.1 and 8.3 mg L^{-1} for each of the doses, respectively. These concentrations are indeed much lower than the range of concentrations (i.e., 35 to 360 mg L^{-1}) required to alter N metabolism in ruminal bacteria as suggested by McIntosh et al. (2003). Therefore, despite their well-demonstrated antibacterial activity against a wide range of pathogenic bacteria, EO and their compounds seem to be active against ruminal bacteria only at high doses (i.e., 3000 mg L^{-1}) as showed recently by Busquet et al. (2006) in 24-h in vitro batch cultures.

CONCLUSION

This study showed that of the EO and EOC evaluated, only the phenolic compounds carvacrol, thymol, and eugenol affected ruminal fermentation. At the concentration evaluated carvacrol (400 mg L^{-1}) and eugenol (800 mg L^{-1}) increased pH and molar proportion of butyrate, and decreased molar proportion of propionate, IVDMD, IVNDFD, and gas production. At the concentration of 200 mg L^{-1}, thymol increased final pH, and reduced the molar proportion of propionate, IVNDFD, and GP. None of the EO and the compounds examined affected NH_3 concentration, suggesting that deaminative activity of ruminal bacteria was not affected by EO and EOC. At the doses evaluated in the current study, EO and EO compounds showed no beneficial effects on rumen microbial fermentation. Phenolic compounds exhibited antimicrobial activities mainly by reducing diet fermentability and shifting VFA pattern towards less propionate and more butyrate. If the same effects were expressed in vivo, phenolic compounds may not be beneficial for use in dairy cow nutrition to improve efficiency of feed utilization. Further research is required to asses in vivo the effects of EO and their constituents on rumen microbial fermentation.

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