Assessment of the Effects of Cinnamon Leaf Oil on Rumen Microbial Fermentation Using Two Continuous Culture Systems¹

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ABSTRACT

Two continuous culture (CC) systems, the rumen simulation technique (Rusitec) and a dual-flow (DF) fermenter, were used to evaluate effects of the essential oil from cinnamon leaf (CIN) on rumen microbial fermentation. Incubations (d 1 through 8 for adaptation and d 9 through 16 for sampling) were conducted concurrently in the 2 systems, with CIN added at 0 (control) and 500 mg/L of rumen fluid culture. Eight Rusitec (920 mL; dilution rate = 2.9%/h) and 6 DF (1,300 mL; dilution rate = 6.3%/h) fermenters were randomly assigned to treatment. Inoculum was prepared from 4 ruminally cannulated lactating Holstein cows fed a total mixed ration consisting of 51% forage and 49% concentrate (dry matter basis). Ruminal pH, total volatile fatty acid (VFA) concentration, and diet digestibility were reduced by CIN addition in the Rusitec but were not affected by CIN administration in the DF. The addition of CIN in the Rusitec decreased apparent N disappearance, $\text{NH}_3\text{-N}$ concentration, and molar proportions of branched-chain VFA. In contrast, in the DF no effect of CIN was observed on apparent N degradation, $\text{NH}_3\text{-N}$ concentration, and molar proportion of branched-chain VFA. In the Rusitec, the molar proportion of acetate was similar between treatments on d 9 and 13, but was lower from d 10 to 12 and higher on d 14 to 16 with CIN than with control (interaction of treatment $\times$ sampling day). The molar proportion of acetate remained unaffected by CIN addition in the DF. In both CC systems, the molar proportion of propionate was decreased whereas that of butyrate was increased by CIN addition. In the DF, CIN decreased microbial N flow and efficiency of microbial protein synthesis. Protozoa numbers were lower with CIN than with control in both CC fermenters. In the

Rusitec, CIN increased ^{15}N enrichment in total bacterial fractions, but no effect was observed on the production of microbial N. This study showed that CIN exhibited antimicrobial activity in both CC systems, but the effects were more pronounced in the Rusitec than in the DF system. These differences are likely a reflection of the higher dilution rate in the DF resulting in a lower effective concentration of CIN than in Rusitec. Based on these changes in rumen microbial fermentation, supplementation of CIN at the concentration evaluated in this study may not be nutritionally beneficial to ruminants.

Key words: cinnamon leaf oil, rumen microbial fermentation, rumen simulation technique, dual-flow fermenter

INTRODUCTION

With increasing public concern that the routine use of antibiotics and growth promoters in livestock feeds may heighten bacterial resistance to antibiotics, interest in evaluating plant extracts, and more recently essential oils (EO), as natural alternatives to antibiotics has arisen (Greathead, 2003). Contrary to what their name might suggest, essential oils are not oils (i.e., lipids) because they consist solely of volatile, aromatic compounds that can be extracted from plants mainly by steam distillation. Structurally, they are variable mixtures of principally terpenoids—mainly monoterpenes (C_{10}) and sesquiterpenes (C_{15}), although diterpenes (C_{20}) may also be present—and a variety of low molecular weight aliphatic hydrocarbons, acids, alcohols, aldehydes, acyclic esters or lactones, and sometimes N- and S-containing compounds, coumarins, and homologues of phenylpropanoids (Dorman and Deans, 2000). Essential oils have been shown to have antimicrobial activity against a variety of microorganisms, including gram-positive and gram-negative bacteria, protozoa, and fungi (Helander et al., 1998; Greathead, 2003). The mechanism by which EO are thought to exert their antimicrobial activity is by disrupting the cell wall structures, affecting electron transport, ion gradients, protein translocation, phosphorylation steps, and other enzyme-dependent reactions (Ultee et al., 1999; Dorman and Deans, 2000).

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A number of recent *in vitro* studies using batch or continuous cultures have evaluated effects of various EO and their compounds on ruminal microorganisms and ruminal metabolism and have reported varied results (Busquet et al., 2006; Castillejos et al., 2006). Inconsistencies among studies may be attributed to factors such as the chemical composition of the EO, the concentration used, and interactions among the bioactive agents in EO (Dorman and Deans, 2000). The *in vitro* technique used may also influence results. For instance, Busquet et al. (2005a) showed that garlic oil and 2 of its compounds, diallyl disulfide and allyl mercaptan (300 mg/L of rumen fluid), reduced total VFA concentration in a 24-h batch culture. When evaluated at the same concentration in a continuous culture (CC) system under controlled pH conditions, allyl mercaptan reduced total VFA concentration but no effect was observed for garlic oil and diallyl disulfide.

In vitro batch culture studies must be interpreted with caution because they report effects over a set incubation time (24 or 48 h) and do not account for the possible adaptation of rumen microbes to EO compounds. Conversely, CC systems are advantageous in that they allow for longer term effects, but there is no standardization among systems. Discrepancies among studies using different CC systems may be attributed to the type of system used, dilution rate, and ruminal bacterial activity (Vázquez-Añón et al., 2001).

Accordingly, the objective of this study was to use 2 CC systems: the rumen simulation technique (**Rusitec**; Czerkawski and Breckenridge, 1977) and a dual-flow (**DF**) fermenter (Hoover et al., 1976), to examine the effects of cinnamon leaf oil (**CIN**) on rumen microbial fermentation.

MATERIALS AND METHODS

Experimental Design and Treatments

The experiment was designed as a complete randomized block with 2 dietary treatments and replication within each treatment (4 replications in the Rusitec system and 3 replications in the DF system). The experimental period consisted of 16 d, whereby the first 8 d (d 1 to 8) served as an adaptation period to allow for equilibration of microbial populations, followed by 8 d of sampling (d 9 to 16). The 2 experimental treatments were 1) control (**CON**, no additive), and 2) addition of CIN (*Cinnamomum zeylanicum*; standardized at 76 g/kg of eugenol; Pancosma S. A., Bellegarde-sur-Valserine, France) at a concentration of 500 mg/L of culture ruminal fluid. The concentration of 500 mg/L was selected based on a previous batch-culture screening study conducted in our laboratory (unpublished data), which revealed that 500 mg/L of CIN was the threshold beyond

Table 1. Chemical composition of the barley silage and the concentrate feed administered to the continuous culture fermenters

Item	Barley silage	Concentrate
DM, %	32.5	93.6
OM, % of DM	92.7	89.9
CP, % of DM	12.0	23.9
NDF, % of DM	49.2	24.5
ADF, % of DM	30.7	8.71
Starch, % of DM	35.0	64.2
Ether extract, % of DM	2.20	2.70
Gross energy, Mcal/kg of DM	4,042.3	4,112.1

which changes in the fermentation pattern were observed.

Source of Inoculum

Rumen inoculum was obtained 2 h after the morning feeding from 4 ruminally fistulated, lactating Holstein dairy cows fed a TMR (16.7% CP, 34.4% 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 (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 a salt, mineral, and vitamin mixture (2.7%). Pooled rumen fluid inoculum for use in the Rusitec was squeezed through 4 layers of cheesecloth into an insulated thermos. A small amount (approximately 160 g) of solid rumen content was also collected for initial inoculation of the fermenters. Mixed ruminal contents for use in the DF were pooled and homogenized for 1 min using a Waring commercial blender (Waring Products Division, New Hartford, CT) under a stream of CO₂ to detach feed particle-associated (**FPA**) bacteria. The homogenized material was then squeezed through 2 layers of cheesecloth into an insulated thermos. All procedures with the cows were performed in accordance with the guidelines of the Canadian Council on Animal Care (CCAC, 1993).

Diet Substrate

Whole-crop barley silage and a concentrate mix fed to dairy cows were used as dietary substrates (Table 1). The barley silage was sampled at various locations within a bunker silo and samples were immediately stored at -20°C. Just prior to the beginning of the experiment, feedstuffs were freeze-dried and ground through a 4.5-mm screen (Arthur H. Thomas Co., Philadelphia, PA).

In the Rusitec system, equal quantities of barley silage and concentrate were incubated in separate polyester bags (80 × 120 mm; pore size of 50 µm; B. & S.H. Thompson, Ville Mont-Royal, QC, Canada). In the DF system, equal quantities of barley silage and concentrate (50:50, DM basis) were hand-mixed to obtain a TMR and incubated as free-floating particles in the fermenters.

Experimental Apparatuses and Operation

Rusitec. A Rusitec unit (Czerkawski and Breckenridge, 1977) equipped with 8 fermenters each of 920-mL nominal capacity was used in this study. To begin the experiment, each fermentation vessel ($n = 8$) was filled with 200 mL of warmed McDougall's buffer (pH 8.2; McDougall, 1948) modified to contain 1.0 g/L of $(\text{NH}_4)_2\text{SO}_4$, and 700 mL of filtered rumen fluid. In addition, solid rumen digesta (20 g), barley silage (5 g, DM basis), and concentrate (5 g, DM basis) contained in 3 separate polyester bags were placed within each fermentation vessel. After 24 h, the solid rumen digesta bag was replaced with 2 polyester bags containing either 5 g of barley silage or concentrate. Thereafter, 2 nylon bags (one of concentrate and the other of barley silage) were replaced daily, allowing each bag to remain in the fermenter for 48 h. Artificial saliva was continuously infused into the fermenters at a dilution rate of 2.9%/h. During nylon bag exchange, each fermentation vessel was flushed with O_2 -free CO_2 to maintain anaerobic conditions. Effluent accumulation was determined daily during feed-bag exchange and collected in a 2.0-L container immersed in an ice water bath to impede microbial growth. Beginning on d 9, CIN (500 mg/L of culture ruminal fluid) was administered once daily to each of the 4 treatment fermenters. The daily dose of CIN was deposited directly into the fermentation fluid at the time of morning feeding.

DF Fermenter. A 6-unit, dual-effluent system similar to that described by Hoover et al. (1976) was used concurrently with the Rusitec in this study. Each fermentation unit had a 1.3-L nominal capacity. To begin, 275 mL of warmed McDougall's buffer (pH 8.2; McDougall, 1948) modified to contain 1.0 g/L of $(\text{NH}_4)_2\text{SO}_4$, 1,000 mL of strained rumen fluid, and 15 g of TMR (DM basis) were added to each fermenter. Thereafter, the fermenters were manually given 30 g (DM basis) of diet per day, divided into 2 equal feedings. Fermenters were maintained at a constant temperature of 39°C and were infused with a constant stream of N_2 at a rate of 20 mL/min to maintain anaerobic conditions. Infusion of artificial saliva and flow of filtered liquid were set to maintain solid and liquid dilution rates of approximately 2.0 and 4.3%/h, respectively. Daily effluent was collected in a 4.0-L container immersed in an ice water bath to inhibit

microbial growth. Beginning on d 9, CIN (500 mg/L of culture ruminal fluid) was administered once daily to each of the 3 treatment fermenters. The daily dose of CIN was deposited directly into the fermentation fluid at the time of morning feeding.

Sample Collection: Rusitec Apparatus

DM Disappearance. In situ DM disappearance at 48 h was determined daily from d 9 to 16. Feed bags were removed from each fermenter, washed in cold, running tap water until the water running off was clear, and dried at 55°C for 48 h. To ensure there was sufficient sample for analysis, silage and concentrate bag residues were pooled over 2 and 3 d, respectively, ground through a 1-mm screen in a Wiley mill (standard model 4; Arthur H. Thomas) and subsequently analyzed for NDF, ADF, and total N.

Fermentation Gas and End-Products. Fermentation gas was collected into reusable 2,000-mL, vinyl urine collection bags (Bard Inc., Mississauga, ON, Canada) attached to each fermenter. Just prior to feed-bag exchange, daily total gas production from each fermenter was determined using a wet-type gas flow meter (Alexander-Wright, London, UK). From d 9 to 16, just prior to determination of total gas, gas samples were taken from the septum of collection bags using a 26-gauge needle (Becton Dickinson, Franklin Lakes, NJ). Twenty-milliliter samples were transferred to evacuated 6.8-mL exetainers (Labco Ltd., Wycombe, Bucks, UK) for immediate analysis of methane (CH_4) concentration. Fermenter pH was recorded (Orion model 260A, Fisher Scientific, Toronto, ON, Canada) daily at the time of feed-bag exchange. To determine VFA concentration, 4.0-mL subsamples of fermenter liquid taken directly from the fermentation vessels at the time of feed-bag exchange were placed in screw-capped vials preserved with 400 µL of 25% (wt/wt) metaphosphoric acid and immediately frozen at -20°C until analysis. At the same time, 4.0-mL subsamples of fermenter fluid were also collected, placed in screw-capped vials, preserved with 400 µL of TCA, and immediately frozen at -20°C until analyzed for NH_3 -N concentration.

Protozoa Enumeration. Protozoa counts were determined daily on pooled fluid samples collected from both the 48-h barley silage and concentrate feed bags from each fermenter. Bags were gently pressed to expel fermentation fluid and a 5.0-mL subsample of rumen fluid was obtained and preserved using 5.0 mL of methyl green formalin-saline solution (Ogimoto and Imai, 1981). Protozoa samples were stored in darkness at room temperature until counting. Protozoa were enumerated microscopically in a Levy-Hausser counting chamber (Hausser Scientific, Horsham, PA). Each sample was

counted twice and if the average of the duplicates differed by more than 10%, counts were repeated.

Microbial Protein Synthesis. Bacteria in the fermenters were labeled using ^{15}N . On d 8, McDougall's buffer (McDougall, 1948) was modified by replacing the $(\text{NH}_4)_2\text{SO}_4$ with 1.0 g/L of ^{15}N -enriched $(\text{NH}_4)_2\text{SO}_4$ (Sigma Chemical Co., St. Louis, MO; minimum ^{15}N enrichment 10.01 atom%) until the end of the experiment. On d 15 and 16, effluent samples (250 mL) preserved on ice were collected from the effluent container to obtain a sample of liquid fraction bacteria. Both the 48-h bag residues and 24-h bag residues (on 16 d) were processed, with the barley silage and concentrate processed separately, to obtain the FPA and the feed particle-bound (FPB) bacterial fractions. Bag residues were removed from the fermentation vessels and gently squeezed, and then were placed in a plastic bag with 20 mL of McDougall's buffer and processed for 60 s in a Stomacher 400 laboratory blender (Seward Medical Ltd., London, UK). The processed liquid was gently squeezed out, poured off, and retained. The feed residues were washed 2 additional times using 10 mL of buffer in each wash. All wash buffer was retained and pooled with the initially expressed fluid to obtain the FPA bacterial fraction, and the total volume was recorded. The washed, solid feed residues were representative of the FPB bacterial fraction.

To determine ^{15}N concentration, effluent liquid samples were centrifuged ($20,000 \times g$, 30 min, 4°C) and the resulting pellets were washed using deionized water and centrifuged 3 times ($20,000 \times g$, 30 min, 4°C). The pellet was then resuspended in distilled water and frozen at -20°C until it was lyophilized. The FPA bacterial samples collected from the stomaching process were centrifuged ($500 \times g$, 10 min, 4°C). The supernatant was poured off, centrifuged again ($20,000 \times g$, 30 min, 4°C), and the resulting pellet was washed 3 times as previously described. The pellet was then resuspended in distilled water and frozen at -20°C until it was lyophilized. Washed feed residues (FPB fraction) were dried at 55°C for 48 h, weighed for DM determination, ground, and frozen at -40°C until analyzed for total N and ^{15}N concentrations.

Sample Collection: DF Fermenter

Diet Digestibility. From d 9 to 16, daily effluent was homogenized and a 250-mL subsample was taken, centrifuged ($20,000 \times g$, 30 min, 4°C), and the solid fraction was dried at 55°C for 48 h for the determination of DM (indigestible portion plus microbial fraction). The daily undigested residues were subsequently analyzed for OM, NDF, ADF, and total N.

Fermentation Gases and End-Products. Fermenter pH was recorded at 2-h intervals daily using a pH meter (model 4505, Chemtrix, Inc., Hillsboro, OR). On d 9 to 16, just prior to the morning feeding, gas samples were taken from the septum located on the top of the fermenter using a 26-gauge needle (Becton Dickinson) and 20-mL samples were transferred to evacuated 6.8-mL exetainers (Labco Ltd.) for immediate analysis of CH_4 . At the same time, 4.0-mL subsamples of fermenter liquid were taken directly from the fermenters and preserved as previously described for the Rusitec for analysis of $\text{NH}_3\text{-N}$ and VFA concentrations. Samples were stored at -20°C until analyzed.

Protozoa Enumeration. During the sampling period (d 9 to 16), 5.0-mL subsamples of fermentation media were taken directly from the fermenters just prior to the morning feeding and processed as described previously with Rusitec samples for subsequent enumeration of protozoa.

Microbial Protein Synthesis. Microbial protein synthesis was estimated using ^{15}N as a microbial marker. On d 8, McDougall's buffer solution was modified by replacing the $(\text{NH}_4)_2\text{SO}_4$ with ^{15}N -enriched $(\text{NH}_4)_2\text{SO}_4$ (1.0 g/L; Sigma Chemical Co., minimum ^{15}N enrichment 10.01 atom %). On the last 2 d of the sampling period (d 15 to 16), the effluent was homogenized and filtered through 2 layers of preweighed cheesecloth. A 500-mL subsample was taken from the filtered liquid and centrifuged as described previously for the Rusitec samples to isolate liquid-associated bacteria. The solid fraction of the effluent retained in the cheesecloth was lyophilized, weighed for DM determination, and subsequently ground for measurement of total N and ^{15}N .

Chemical Analyses

Analytical DM content of barley silage, concentrate, effluent feed residue in the DF, and the undigested feed residues of both barley silage and concentrate contained in the polyester bags in the Rusitec were determined by oven drying at 105°C for 48 h in a forced-air oven (AOAC, 1990, method 930.15). Ash content of barley silage, concentrate, and the effluent feed residue in the DF was determined by combustion at 550°C overnight, and OM was subsequently calculated as 100 minus the percentage ash (AOAC, 1990, method 942.05). Total N content of barley silage, concentrate, effluent feed residue in the DF, and the undigested feed residues of both barley silage and concentrate contained in the polyester bags in the Rusitec was determined by combustion assay (model NA 1500, Carlo Erba Instruments, Rodano, Italy). Crude protein was calculated as $\text{N} \times 6.25$. The NDF (Van Soest et al., 1991) and ADF (AOAC, 1990, method 973.18) contents from barley silage, concentrate, the effluent

feed residue in the DF, and the undigested feed residues of both silage and concentrate in the Rusitec were determined using an Ankom²⁰⁰ fiber analyzer (Ankom Technology Corp., Fairport, NJ). Heat-stable α -amylase and sodium sulfite were used during the NDF procedure. Volatile fatty acid concentration was determined by GLC (Hewlett-Packard model 5890, Agilent Technologies, Mississauga, ON, Canada) equipped with a 30-m (0.32-mm i.d.) Zebron FFAP column (Phenomenex, Florence, CA). The concentration of $\text{NH}_3\text{-N}$ in fermentation fluid samples was determined using the phenol-hypochlorite procedure adapted for a Technicon Autoanalyzer II (Broderick and Kang, 1980). Methane concentration in the gas sample was determined using a Varian 3600 gas chromatograph equipped with 180-cm Poropak QS (Alltech and Associates Inc., Deerfield, IL). Nitrogen gas was used as the carrier gas at a flow rate of 30 mL/min. Total N and ^{15}N content of the bacterial pellets were determined by mass spectrometry using a model NA 1500 N analyzer (Carlo Erba Instruments).

Calculations

For the Rusitec, incorporation of ^{15}N into microbial N in the effluent, FPA, and FPB fractions was calculated based on sample size (weight and volume) and ^{15}N enrichment in the fractions produced daily. Total microbial ^{15}N incorporation was calculated as the sum of incorporations in the effluent, 24-h, and 48-h feed bags (Wang et al., 2001).

Microbial N (MN) yield in both CC systems (in milligrams) was estimated using the following equation:

$$\text{MN} = \frac{\text{APE in RN}}{\text{APE in MN}} \times \text{RN}$$

where APE in RN is the percent excess of ^{15}N in the solid residue, APE in MN is the percent excess of ^{15}N in the microbial fraction of the effluent, and RN is the total N in the residue (in milligrams; Wang et al., 2000).

Statistical Analyses

For each CC system, data were analyzed as repeated measures using the MIXED procedure of SAS (SAS Institute, 2000). The MIXED model accounted for the repeated measures (sampling day), the fixed effects of treatment, and the interaction between treatment and sampling day. Results are reported as least squares means ($\pm$ SEM). Significance was declared at $P \leq 0.05$ and a trend at $0.05 < P \leq 0.15$ unless otherwise stated. Protozoal counts yielded a nonnormal distribution and normality was unable to be induced via log transformation; therefore, only mean protozoal counts ($\pm$ SEM) are reported.

RESULTS

Rusitec

There was no interaction ($P > 0.05$) between treatment and sampling day for ruminal pH and total VFA concentration, which increased ($P < 0.01$) and decreased ($P < 0.01$), respectively, with CIN addition (Table 2). However, there were interactions between treatment and sampling day for $\text{NH}_3\text{-N}$ concentration ($P = 0.04$) and molar proportions of acetate ($P < 0.01$), propionate ($P < 0.01$), butyrate ($P < 0.01$), isobutyrate ($P = 0.02$; data not shown), and valerate ($P < 0.01$; data not shown). The concentration of $\text{NH}_3\text{-N}$ was similar between treatments on d 9 (i.e., first day of CIN administration) but was lower for CIN than for CON on d 10 to 16. The magnitude of decrease in $\text{NH}_3\text{-N}$ concentration increased from d 10 to 12 and remained unchanged from d 12 until the end of the experimental period (Figure 1). The molar proportion of acetate was similar between treatments on d 9 and 13, but was lower from d 10 to 12 and higher on d 14 to 16 with CIN than with CON (Figure 2a). Addition of CIN decreased the molar proportion of propionate and the magnitude of this decrease increased over sampling days (Figure 2b). The butyrate molar proportion was similar between treatments on d 9, and increased on d 10 to 16 with CIN addition. The magnitude of this change increased from d 10 to 11 and remained constant from d 11 to the end of the sampling period (Figure 2c). The molar proportion of valerate was similar between treatments on d 9, but was increased with CIN addition on d 10 to 13 and did not differ between treatments from d 14 to the end of the experiment (data not shown). The isobutyrate molar proportion was similar between treatments on d 9 to 10, but was lower for CIN than for CON on d 11 to 16 (data not shown). There was no treatment $\times$ sampling day interaction for the molar proportion of isovalerate, which was lower for CIN than for CON ($P < 0.01$). The acetate:propionate ratio was similar between treatments from d 9 to 11, but was higher for CIN than for CON from d 12 to 16 (data not shown), resulting in a treatment $\times$ sampling day interaction ($P = 0.01$).

There was no interaction ($P = 0.13$) between treatment and sampling day for total gas production, which was lower ($P = 0.01$) for CIN than for CON. However, CH_4 concentration was similar between treatments on d 9 to 10 and d 15 to 16 but was lower for CIN than for CON on d 11 to 14 (data not shown), resulting in a treatment $\times$ sampling day interaction ($P < 0.01$).

Effects of CIN addition on nutrient disappearance measured in the Rusitec are presented in Table 3. There was an interaction ($P = 0.05$) between treatment and sampling day for apparent DM disappearance of barley silage at 48 h, which was lower for CIN than for CON.

Table 2. Effects of the essential oil from cinnamon leaf (CIN) on the fermentation characteristics measured using the rumen simulation technique (Rusitec)

Item	Treatment		SEM	Effect ¹ (<i>P</i> =)		
	Control	CIN		Trt	Sampling day	Trt × sampling day
pH	6.88	6.94	0.008	<0.01	<0.01	0.76
NH ₃ -N, mg/L	21.3	15.1	0.49	<0.01	<0.01	0.04
Total VFA, mM	43.3	25.5	1.70	<0.01	0.72	0.28
VFA, mol/100 mol						
Acetate (A)	52.9	53.8	1.05	0.55	<0.01	<0.01
Propionate (P)	26.1	13.0	0.62	<0.01	<0.01	<0.01
Butyrate	14.2	25.8	0.81	<0.01	<0.01	<0.01
Isobutyrate	1.02	0.71	0.043	<0.01	<0.01	0.02
Valerate	4.27	5.70	0.389	0.04	<0.01	<0.01
Isovalerate	1.49	1.00	0.101	<0.01	<0.01	0.28
A:P	2.04	7.95	0.685	<0.01	<0.01	0.01
Gas production, mL/24 h	1,338	1,071	52	0.01	0.10	0.13
CH ₄ , % in gas	2.21	1.84	0.184	0.19	0.01	<0.01

¹Probabilities associated with *F*-tests for treatment (Trt), sampling day, and the interaction between treatment and sampling day.

The magnitude of this decrease increased over sampling days (data not shown). There was no interaction ($P > 0.05$) between treatment and sampling day for the disappearance of any other measured nutrients. Addition of CIN reduced ($P < 0.01$) apparent DM and N disappearance of concentrate at 48 h but did not affect ($P = 0.81$) apparent N disappearance of barley silage at 48 h. The 48-h disappearance of NDF from barley silage tended ($P = 0.11$) to decrease and that of the concentrate was reduced ($P < 0.01$) by CIN. Supplementation with CIN also decreased ADF disappearance of both the barley silage ($P < 0.01$) and the concentrate ($P < 0.01$). The addition of CIN reduced protozoa numbers (46.9 ± 166.3 vs. $2,314.7 \pm 352.5$ protozoa/mL) by as much as 2 logs.

Figure 1. Effect of cinnamon leaf essential oil (CIN, ■) on NH₃-N concentration (mg/L) in the Rusitec over sampling days compared with the control (▲). *Means differ from the control; $P < 0.05$.

Effects of CIN addition on microbial protein synthesis measured in the Rusitec are presented in Table 4. Total microbial incorporation of ¹⁵N (i.e., total of all bacterial fractions: effluent, FPA, and FPB) was increased ($P = 0.03$) by the addition of CIN. In the effluent fraction, enrichment of ¹⁵N into microbial N was increased ($P = 0.02$) in the treatment fermenters. In the FPA bacterial fraction, the addition of CIN tended to reduce ($P = 0.07$) and reduced ($P = 0.04$) ¹⁵N incorporation into microbial N in barley silage at 24 and 48 h, respectively. For the concentrate, CIN reduced ($P = 0.03$) the incorporation of ¹⁵N into the FPA bacterial fraction at 24 h, whereas no effect was observed at 48 h. In the FPB fraction, CIN increased ¹⁵N incorporation into microbial N from the barley silage and concentrate feeds at both 24 h ($P = 0.05$) and 48 h ($P = 0.04$). Estimated total production of microbial N (i.e., from all bacterial fractions) tended ($P = 0.12$) to be lower with the CIN treatment than with CON (Table 4). Estimated production of microbial N in the effluent bacterial fraction was not changed ($P = 0.38$) by CIN addition. In the FPA bacterial fraction, CIN reduced the production of microbial N from both the barley silage ($P < 0.01$) and concentrate ($P < 0.01$) at both 24 and 48 h. In the FPB fraction, the production of microbial N from barley silage was unaffected by the CIN treatment at 24 ($P = 0.41$) and 48 h ($P = 0.88$), whereas it was increased ($P < 0.01$) by CIN from the concentrate at both incubation times.

DF Fermenter

No treatment × sampling day interactions ($P > 0.05$) were observed for the various ruminal fermentation characteristics measured in the DF (Table 5). Addition

Figure 2. Effect of cinnamon leaf essential oil (CIN, ■) on molar proportions (mol/100 mol) of a) acetate, b) propionate, and c) butyrate in the Rusitec over sampling days compared with the control (▲). *Means differ from the control; $P < 0.05$.

of CIN had no effect on fermenter pH ($P = 0.68$), $\text{NH}_3\text{-N}$ ($P = 0.17$), total VFA concentration ($P = 0.60$), or acetate molar proportion ($P = 0.75$). The interaction between sampling day and treatment tended ($P = 0.06$) to be significant for the molar proportion of propionate, which was not changed by CIN addition on d 9, but decreased on d 10 to 16 (Figure 3). The magnitude of this decrease increased from d 10 to 13 and remained constant from d 14 until the end of the sampling period. In contrast, adding CIN increased ($P = 0.02$) the butyrate molar proportion, and no change was observed for isobutyrate ($P = 0.78$), valerate ($P = 0.50$), or isovalerate ($P = 0.93$) molar proportions. A treatment $\times$ sampling day interaction was observed ($P = 0.04$) for the acetate:propionate ratio, which was similar between treatments on d 9 but was increased by CIN from d 10 to 16. The magnitude of this increase remained constant from d 13 to 16 (data not shown). There was a trend ($P = 0.07$) for an interaction between treatment and sampling day for CH_4 concentration, expressed as a percentage of total fermentation gas. On d 9 to 10 and d 14 to 16, CH_4 concentration was similar between treatments but was increased by CIN on d 11 to 13 (data not shown).

Effects of CIN addition on nutrient digestibility measured in the DF are presented in Table 6. There were interactions between treatment and sampling day for DM ($P = 0.03$) and OM ($P = 0.05$) digestibilities. For both variables (data not shown), DM and OM digestibilities were similar ($P > 0.05$) between treatments on d 9 to 10 and d 13 to 14, but were decreased ($P < 0.05$) by CIN addition on d 11 to 12. On d 15, DM and OM digestibilities increased with addition of CIN; however, on d 16 they were similar between treatments. Conversely, no treatment $\times$ sampling day interaction ($P > 0.05$) was observed for the digestibility of NDF and ADF, which were reduced ($P = 0.03$ and < 0.01 , respectively) with CIN addition. Protozoa numbers were reduced ($P < 0.05$) by the addition of CIN in DF (390.0 ± 959.7 vs. $3,939.1 \pm 987.2$ protozoa/mL).

Flow of total N ($P = 0.48$), $\text{NH}_3\text{-N}$ ($P = 0.21$), NAN ($P = 0.31$), and dietary N ($P = 0.64$) were unaffected by CIN addition (Table 7). However, adding CIN decreased ($P = 0.01$) microbial N flow and efficiency of microbial protein synthesis ($P = 0.01$). Degradation of CP tended ($P = 0.06$) to decrease with CIN supplementation.

DISCUSSION

Ruminal Fermentation Characteristics and Nutrient Digestion

In the present study, 2 CC systems were used to evaluate EO from cinnamon leaf for its potential as a ruminal modifier. To our knowledge, this is the first time that

Table 3. Effects of the essential oil from cinnamon leaf (CIN) on apparent disappearances of DM, NDF, and ADF measured using the rumen simulation technique (Rusitec)

Item	Treatment		SEM	Effect ¹ (<i>P</i> =)		
	Control	CIN		Trt	Sampling day	Trt × sampling day
DM disappearance at 48 h, %						
Barley silage	59.8	48.8	0.64	<0.01	0.39	0.05
Concentrate	82.1	68.0	1.31	<0.01	0.32	0.60
N disappearance at 48 h, %						
Barley silage	57.6	57.2	1.11	0.81	0.63	0.23
Concentrate	71.3	55.4	2.76	<0.01	0.67	0.99
NDF disappearance at 48 h, %						
Barley silage	29.9	29.1	0.30	0.11	0.16	0.60
Concentrate	21.0	14.0	1.21	<0.01	<0.01	0.49
ADF disappearance at 48 h, %						
Barley silage	6.41	5.06	0.161	<0.01	0.25	0.55
Concentrate	7.04	4.52	0.457	<0.01	0.39	0.36

¹Probabilities associated with *F*-tests for treatment (Trt), sampling day, and the interaction between treatment and sampling day.

these 2 types of CC systems were used simultaneously in the same experiment.

In the Rusitec, CIN addition increased fermenter pH and decreased total VFA concentration, indicating that the CIN reduced diet fermentability, an observation consistent with the antimicrobial activity of EO compounds

(Acamovic and Brooker, 2005). Results presented here are consistent with results on the effects of EO compounds at high doses, as reported in previous in vitro studies (Evans and Martin, 2000; Castillejos et al., 2006). Using 24-h batch cultures, Castillejos et al. (2006) reported that at a high dose (5,000 mg/L), eugenol, guaia-

Table 4. Effect of the essential oil from cinnamon leaf (CIN) on incorporation of ¹⁵N into microbial N in effluent, feed particle-associated (FPA), and feed particle-bound (FPB) fractions during 24- and 48-h incubations of barley silage and concentrate in the rumen simulation technique (Rusitec)

Fraction	Treatment		SEM	Effect ¹ (<i>P</i> =)
	Control	CIN		
Microbial incorporation of ¹⁵ N				
Total, ² µg/24 h	1,905.5	2,244.7	84.05	0.02
Effluent, µg/24 h	1,226.6	1,468.8	51.44	0.02
FPA				
Silage, µg/24 h	133.0	91.5	13.12	0.07
Silage, µg/48 h	99.8	71.3	7.79	0.04
Concentrate, µg/24 h	122.9	90.6	7.71	0.03
Concentrate, µg/48 h	118.6	99.8	11.42	0.29
FPB				
Silage, µg/24 h	55.1	80.8	7.54	0.05
Silage, µg/48 h	86.5	118.5	8.88	0.04
Concentrate, µg/24 h	30.3	105.4	13.11	<0.01
Concentrate, µg/48 h	32.9	168.04	5.80	<0.01
Production of microbial N				
Total, mg/24 h	58.8	53.2	2.20	0.12
Effluent, mg/24 h	37.5	35.1	1.78	0.38
FPA				
Silage, mg/24 h	3.67	2.07	0.189	<0.01
Silage, mg/48 h	2.92	1.55	0.138	<0.01
Concentrate, mg/24 h	3.43	1.75	0.231	<0.01
Concentrate, mg/48 h	4.90	2.41	0.462	<0.01
FPB				
Silage, mg/24 h	1.80	1.69	0.086	0.41
Silage, mg/48 h	2.53	2.56	0.178	0.88
Concentrate, mg/24 h	0.89	2.38	0.202	<0.01
Concentrate, mg/48 h	1.18	3.66	0.139	<0.01

¹Probabilities associated with *F*-tests for effect of treatment.

²Total incorporation is the sum of all incorporations in the effluent, FPA and FPB fractions obtained from both the silage and concentrate feed bags.

Table 5. Effects of the essential oil from cinnamon leaf (CIN) on rumen fermentation characteristics measured in the dual-flow continuous culture system

Item	Treatment		SEM	Effect ¹ (<i>P</i> =)		
	Control	CIN		Trt	Sampling day	Trt × sampling day
pH	7.00	6.96	0.079	0.68	0.11	0.59
NH ₃ -N, mg/L	18.6	19.9	0.60	0.17	0.10	0.22
Total VFA, mM	75.6	69.4	8.01	0.60	0.53	0.57
VFA, mol/100 mol						
Acetate (A)	69.7	68.2	1.62	0.51	0.75	0.49
Propionate (P)	15.1	9.4	0.54	<0.01	0.02	0.06
Butyrate	10.3	17.1	1.72	0.02	0.52	0.28
Isobutyrate	1.07	1.03	0.096	0.78	0.89	0.94
Valerate	2.11	2.37	0.267	0.50	0.47	0.99
Isovalerate	1.80	1.83	0.234	0.93	0.57	0.75
A:P	4.69	7.65	0.556	0.01	0.04	0.04
CH ₄ , % in gas	3.36	5.58	1.042	0.20	<0.01	0.07

¹Probabilities associated with *F*-tests for treatment (Trt), sampling day, and the interaction between treatment and sampling day.

col, limonene, thymol, and vanillin reduced total VFA concentration and consequently increased pH compared with the control. However, in the same study, at a dose of 500 mg/L, effects on ruminal pH and VFA concentration were apparent with limonene but not with the other EO examined. Using 24-h in vitro batch cultures of mixed ruminal bacteria, Evans and Martin (2000) reported that thymol at a dose of 400 mg/L increased pH in the fermentation cultures, whereas at doses of 50, 100, and 200 mg/L, no effects were observed. Because the production of VFA serves as the principal source of energy for the ruminant animal, decreasing VFA production could yield adverse nutritional consequences if this same effect were expressed in vivo.

Figure 3. Effect of cinnamon leaf essential oil (CIN, ■) on the molar proportion (mol/100 mol) of propionate in the dual-flow fermenter over sampling days compared with control (▲). *Means significantly differ from the control; *P* < 0.05.

The negative effect of CIN on nutrient digestion in the Rusitec corroborates the reduction in total VFA concentration and the concomitant increase in fermenter pH. In the Rusitec, DM disappearance from both barley silage and concentrate was reduced; however, the magnitude of the reduction in DM disappearance was greater for concentrate than for barley silage. This may imply that microbial populations involved in concentrate digestion were more affected by CIN than those involved in barley silage digestion. Percentage of NDF disappearance from concentrate was decreased with CIN addition; however, that from barley silage was only slightly reduced, suggesting that the reduction in DM disappearance from the barley silage could be attributed to a decline in the soluble carbohydrate fraction of silage. The manner in which diet disappearance was altered might suggest that digestibility of the soluble carbohydrate fraction of the feedstuffs was more negatively affected than structural carbohydrate digestion.

In contrast to the effects observed with the Rusitec, CIN addition in the DF fermenter did not change ruminal pH or total VFA concentration. The lack of effect of CIN on total VFA concentration indicated that diet fermentability was not changed by CIN addition in the DF system as shown by apparent disappearances of DM and OM (Table 6). This discrepancy in the effects of CIN between the 2 CC systems may be due to functional differences between the 2 types of fermenters, in particular the dilution rate, which was twice as high in the DF than in the Rusitec (6.3 vs. 2.9%/h), resulting in a lower effective concentration of CIN in the DF than in the Rusitec.

The antiprotozoal effect of the CIN may also contribute to the decrease in fiber digestibility observed in both CC systems, because it is estimated that protozoa con-

Table 6. Effects of the essential oil from cinnamon leaf (CIN) on apparent disappearances of DM, OM, NDF, and ADF in the dual-flow continuous culture system

Item	Treatment		SEM	Effect ¹ (<i>P</i> =)		
	Control	CIN		Trt	Sampling day	Trt × sampling day
Apparent disappearance, %						
DM	54.2	53.2	0.39	0.12	0.06	0.03
OM	52.6	51.5	0.43	0.10	0.02	0.05
NDF	39.3	35.3	0.91	0.03	0.04	0.22
ADF	13.8	11.2	0.15	<0.01	0.79	0.61

¹Probabilities associated with *F*-tests for treatment (Trt), sampling day, and the interaction between treatment and sampling day.

tribute upward of 25 to 30% of total ruminal fiber digestion (Lee et al., 2000). Contrary to the antiprotozoal effect of CIN in the present study (2 log reductions), Newbold et al. (2004) and Benchaar et al. (2006, 2007) observed no change in protozoa numbers when sheep and dairy cows were fed a commercial mixture of EO compounds (Crina ruminants, Crina S. A., Switzerland) at doses of 100 mg/d and 2 g/d, respectively. The antiprotozoal effect observed in the current study could be explained by the high dose of CIN used compared with doses examined in vivo (Benchaar et al., 2006, 2007).

Despite the reduction in numbers of protozoa in the treatment fermenters, the contribution of CH₄ gas to the total gas production in the Rusitec remained unchanged on d 9 to 10 and d 15 to 16 relative to the control, but was decreased by the CIN from d 11 to 14. Ruminal protozoa play a significant role in CH₄ production (Moss et al., 2000), because a direct relationship has been identified between rumen protozoa numbers and methanogenesis (Klieve and Hegarty, 1999). Rumen protozoa are estimated to provide a habitat for up to 20% of ruminal methanogens, and methanogens living on and within protozoa are thought to be responsible

for an estimated 37% of CH₄ emissions from ruminant animals (Klieve and Hegarty, 1999). Our results indicate that although a synergism between protozoa and methanogens exists, methanogens can still actively produce significant quantities of CH₄ in the absence of a fully functional population of protozoa.

In the current study, a treatment × sampling day interaction was observed for the molar proportions of individual VFA (except for isovalerate) when CIN was administered in the Rusitec. In contrast, CIN addition in the DF did not result in a treatment × sampling day interaction for the molar proportions of individual VFA, with the exception of propionate (*P* = 0.06). The absence of an interaction effect in DF may be attributed to the lack of effect of CIN on rumen microbes due to the higher dilution rate and to the lower effective concentration of CIN in DF than in Rusitec.

Other in vitro studies using a DF fermenter under controlled pH conditions (Cardozo et al., 2004; Busquet et al., 2005a) also reported effects of EO and their compounds on molar proportions of individual VFA over days. Cardozo et al. (2004) noted that garlic, cinnamon, anise, oregano, and pepper extracts (0.22 mg/L of culture

Table 7. Effects of the essential oil from cinnamon leaf (CIN) on N degradation and efficiency of microbial protein synthesis in the dual-flow continuous culture system

Item	Treatment		SEM	Effect ¹ (<i>P</i> =)
	Control	CIN		
Dietary N input, g/d	5.02	5.02	—	—
N flow, mg/d				
Total	954.7	966.4	11.9	0.48
Ammonia-N	388.7	464.5	39.9	0.21
NAN	565.9	501.9	42.3	0.31
Microbial	324.4	286.4	9.10	0.01
Dietary	241.6	215.5	37.04	0.64
EMPS ²	21.8	19.2	0.63	0.01
True CP degradation, % of N intake ³	87.4	86.5	0.34	0.06

¹Probabilities associated with *F*-tests for treatment (Trt), sampling day, and the interaction between treatment and sampling day.

²EMPS = efficiency of microbial protein synthesis (g of microbial N/kg of OM fermented).

³Corrected for microbial N.

fluid) altered the molar proportions and total VFA concentration during the adaptation period (d 1 to 7). After d 7 of incubation, however, these effects disappeared, suggesting that the ruminal microbes were able to adapt to the presence of EO administered at this concentration. In contrast, Busquet et al. (2005a) observed no change in total VFA concentration and diet digestibility when cinnamaldehyde and garlic oil were administered at 31 and 312 mg/mL of culture fluid in a DF system under controlled pH conditions. However, changes in the VFA pattern (i.e., molar proportions of individual VFA) were apparent within 48 h after these EO compounds were added to the fermenter and remained unchanged until the end of the experimental period. The results of Busquet et al. (2005a) suggest that the adaptation of rumen microbes to EO (i.e., replacement of sensitive bacteria by more resistant bacteria) or lack thereof may be explained by the different dosages used. Rumen microbes are able to adapt to EO when these compounds are administered at low doses, but at higher doses, the effect of EO appears to be sustained over time (i.e., 9 d of continuous culture fermentation). Based on these observations, Cardozo et al. (2004) and Busquet et al. (2005c) warned that data from short-term *in vitro* studies may lead to erroneous conclusions and should be interpreted with caution. Therefore, there is a need for long-term *in vivo* studies to assess the efficacy of EO under *in vivo* conditions where rumen microbes are exposed for a longer period (i.e., weeks) compared with *in vitro* conditions (i.e., 9 d in continuous culture systems). It is interesting to note that in this study, similar to the study of Busquet et al. (2005a), some of the effects of CIN on ruminal fermentation were apparent within 2 to 3 d after the administration of this EO in both CC systems. The effects stabilized 3 d after CIN addition in the fermenters and remained unchanged until the end of the sampling period.

The chemical composition of EO may also have implications on the results observed at given dosages. Castillejos et al. (2006) observed variation in the effects of 5 different EO compounds (i.e., eugenol, guaiacol, limonene, thymol, and vanillin) at increasing dosage levels (5, 50, 500, and 5,000 mg/L of fermentation culture fluid) on fermentation products in 24-h batch culture of rumen fluid. At a dose of 5,000 mg/L, all compounds decreased the concentration of total VFA. The monoterpene limonene reduced total VFA concentration at doses of 50 and 500 mg/L, but did not alter molar proportions of individual VFA. The phenolic guaiacol reduced total VFA concentration at doses of 5 and 50 mg/L and altered the molar proportion of acetate, but for unknown reasons was not effective at altering total VFA concentration at 500 mg/L. The aldehyde vanillin and the phenolic eugenol were ineffective at altering total VFA concentration

at doses of 5, 50, or 500 mg/L, and the phenolic thymol reduced total VFA concentration at a dose of 500 mg/L. These results illustrate that effects of EO on rumen microbial activities vary with the chemical structure and the dose of the EO compound. This variability in the effect of EO on rumen fermentation as a result of differences in activity and composition suggests that it may be challenging to develop an EO extract that consistently results in a favorable change in the profile of VFA.

The pattern of VFA in both CC systems was marked by a reduction in the proportion of propionate and an increase in that of butyrate, a result that implies that CIN favored populations of bacteria that produce butyrate and may have inhibited those that produce propionate. In the rumen, many predominant gram-positive bacteria are involved in the fermentation processes that produce, among other end-products, acetate, butyrate, formate, lactate, and hydrogen (Stewart, 1991). Rumen amylolytic bacteria generally ferment soluble dietary carbohydrates (starches and sugars) to produce propionate and succinate as major end-products, and these bacteria are generally gram-negative (Van Soest, 1982; Stewart, 1991). A reduction in the digestibility of soluble carbohydrates in the Rusitec and changes in patterns of VFA in both systems suggest that CIN inhibited gram-negative bacteria to a greater extent than gram-positive bacteria. Because fiber disappearance was reduced by CIN in both systems, this hypothesis does not imply that gram-positive, cellulolytic bacteria were unaffected by CIN addition, but that the inhibitory effects were less pronounced in this subpopulation. Eugenol is the primary constituent of the EO extract from cinnamon leaf. Unlike the ruminal modifier monensin, which targets primarily gram-positive bacteria (Russell and Strobel, 1989), phenolic compounds have been shown to have a broad spectrum of activity against both gram-positive and gram-negative bacteria (Kim et al., 1995; Helander et al., 1998; Dorman and Deans, 2000; Lambert et al., 2001). The antibacterial activity of phenolic compounds such as eugenol has been associated with their ability to disrupt the outer cell membrane (Dorman and Deans, 2000; Walsh et al., 2003). Consequently, eugenol as the primary phenolic in CIN may have resulted in a general inhibition of both gram-positive and gram-negative bacteria, resulting in an overall decline in digestion and total VFA production.

Recent *in vitro* studies both in short-term batch culture and long-term CC have reported similar effects of high doses of phenolic EO compounds (e.g., eugenol, thymol, carvacrol) on rumen microbial fermentation (Evans and Martin, 2000; Busquet et al., 2005a, 2006; Castillejos et al., 2006). Evans and Martin (2000) observed a decrease in acetate and propionate concentrations and an increase in the acetate to propionate ratio when thy-

mol (400 mg/L) was added in batch cultures of mixed ruminal bacteria. Using a DF fermenter maintained under controlled pH conditions, Castillejos et al. (2006) observed that thymol (500 mg/L) reduced digestibility of DM, OM, NDF, and ADF, and reduced total VFA concentration and the molar proportion of acetate, whereas the molar proportions of propionate and butyrate were increased. Increases in butyrate in the study by Castillejos et al. (2006) were significant; as in our study, the molar proportion of butyrate exceeded that of propionate. In the same study, eugenol (500 mg/L) decreased total VFA concentration and the molar proportion of acetate, but increased the molar proportions of propionate and butyrate without altering feed digestibility relative to the control. Although eugenol is the main component of CIN, results of the present study using the DF differ from those of Castillejos et al. (2006). Discrepancies in the results between the 2 studies are probably due to interactions between the primary compound eugenol and the secondary compounds within CIN, but also to different experimental conditions. In the study of Castillejos et al. (2006), each experimental period consisted of 9 d (6 d for adaptation and 3 d for sampling) compared with 16 d (8 d for adaptation and 8 d for sampling) in our study. Fermentation conditions were maintained at a constant pH (6.4 ± 0.05) in the experiment of Castillejos et al. (2006), whereas fermentation pH was not controlled in our study. Busquet et al. (2006) reported effects of the plant secondary metabolites eugenol and clove bud oil (85% of eugenol) at doses of 3, 30, 300, and 3,000 mg/L in 24-h in vitro batch cultures. Despite the high eugenol content of clove bud oil, this EO and its main component exerted different effects. At 3,000 mg/L, the molar proportion of butyrate was not changed by clove bud oil, but it was decreased with the addition of eugenol. At 300 mg/L, clove bud oil decreased acetate molar proportion, whereas eugenol exerted no effect. These results suggest that minor compounds within the clove bud EO acted synergistically with eugenol to produce the observed effects. It is equally possible that the fermentation responses observed in the present study were due to secondary components within CIN.

Nitrogen Metabolism and Microbial Protein Synthesis

In the Rusitec, addition of CIN decreased $\text{NH}_3\text{-N}$ concentration and proportions of isobutyrate and isovalerate, which arise from AA metabolism. These reductions may have been caused by a decrease in the disappearance of N from the concentrate, as N disappearance of barley silage was not changed. Therefore, changes in $\text{NH}_3\text{-N}$ and branched-chain VFA (BCVFA) concentra-

tions may suggest that both proteolysis and deamination processes were inhibited by the addition of CIN.

In the DF, CIN slightly reduced N degradation ($P = 0.06$); however, no changes were observed for $\text{NH}_3\text{-N}$ concentration or molar proportions of BCVFA, suggesting that CIN did not affect the proteolysis or deamination processes to the same extent as in the Rusitec. Consistent with the present study, recent studies have reported varied effects of EO containing phenolics on $\text{NH}_3\text{-N}$ concentration. Castillejos et al. (2006) observed no effect of thymol and eugenol on $\text{NH}_3\text{-N}$ concentration when administered at doses of 5, 50, and 500 mg/L in a DF fermenter under controlled pH conditions. In 24-h batch cultures, Busquet et al. (2006) reported variable effects of various phenolic EO compounds on $\text{NH}_3\text{-N}$ concentration. At 300 mg/L, eugenol tended to decrease $\text{NH}_3\text{-N}$ concentration and carvacrol exerted no effect. Oregano oil (69% carvacrol) decreased $\text{NH}_3\text{-N}$ concentration and clove bud oil (85% eugenol) exerted no effect. At a dose of 3,000 mg/L, all these EO compounds decreased $\text{NH}_3\text{-N}$ concentration. Again, responses to EO vary with the EO and the dose used; however, the reduction in $\text{NH}_3\text{-N}$ observed at high doses may in part be due to the reduction in overall diet fermentability. The fact that both DM and protein digestion were reduced in the present study suggests that the antimicrobial activity of EO are of a broad-spectrum nature.

In the Rusitec, CIN increased the total microbial incorporation of ^{15}N without affecting the total production of microbial N, a result that may reflect a shift of the bacterial populations toward those that utilize predominantly ammonia as a N source. The bacterium *Butyrivibrio fibrisolvens* utilizes ammonia and is considered a major producer of butyrate in the rumen (Miller and Jenesel, 1979). A predominance of this species may account for the increased proportion of butyrate observed with CIN treatment in the Rusitec.

The reduction in incorporation of ^{15}N into the FPA bacterial fraction from silage at 24 h (tendency at 48 h) and concentrate at 24 h suggests that the CIN may have inhibited the growth of secondary colonizing bacteria to a greater extent than primary colonizing bacteria. Secondary colonizers subsist on soluble substrates produced by primary colonizing or fibrolytic bacteria and may be more susceptible to antimicrobials within the biofilm community (McAllister et al., 1994). In the FPB fraction, microbial N production in the barley silage at both incubation times remained unaffected by treatment, further suggesting that the CIN did not inhibit primary colonizing cellulolytic bacteria as much as secondary colonizers.

The apparent increase in ^{15}N incorporation into bacterial fractions may also result from the antiprotozoal effect of the CIN. Ruminal protozoa ingest large numbers

of bacteria, decreasing net microbial production and bacterial protein flow from the rumen to the duodenum (Ivan et al., 2000). Consequently, bacterial predation by protozoa yields an inverse relationship between population numbers of these 2 groups of microorganisms (Russell, 2002). A reduction in protozoa as a result of inclusion of CIN could therefore lead to an increase in bacterial numbers due to a reduction in predatory activity.

In the DF, the lack of effect of CIN on the flow of nondigested feed N and CP degradation is consistent with the lack of effect of the CIN on $\text{NH}_3\text{-N}$ concentration and the molar proportions of BCVFA. Addition of CIN reduced the flow of microbial N, which is consistent with the antimicrobial activity of EO and the tendency observed for reduced DM ($P = 0.12$) and OM ($P = 0.10$) disappearances. Microbial efficiency (expressed as milligrams of N produced per kilogram of ruminally fermented OM) was significantly reduced by CIN and can be accredited to the tendency for the reduction in OM digestion and the significant reduction in microbial N flow with CIN addition. Because production of microbial protein serves as a major source of high-quality protein for the animal and can account for between 50 to 90% of the protein entering the intestine (Beauchemin, 2002), inhibition of microbial protein synthesis could have negative effects on animal productivity.

CONCLUSIONS

In the present study, 2 CC systems, the Rusitec and the DF fermenter, were used to evaluate CIN for its potential as a ruminal modifier. In the Rusitec, the EO from CIN exhibited antimicrobial activity through a reduction in diet digestibility and total VFA concentration. Conversely, there were no effects of CIN on concentration of total VFA and diet fermentability in the DF. The addition of CIN altered N metabolism in the Rusitec, decreasing N disappearance, $\text{NH}_3\text{-N}$ concentration, and the molar proportions of BCVFA. Contrarily, no effect of CIN in the DF was observed on N degradation, $\text{NH}_3\text{-N}$ concentration, and the molar proportions of BCVFA. In the Rusitec, CIN increased ^{15}N enrichment in bacterial fractions, but had no effect on the production of microbial N, whereas in the DF, microbial N flow and efficiency of microbial protein synthesis were decreased by CIN addition. Discrepancies in the effects of CIN between the 2 CC systems may be due to the higher dilution rate and consequently the lower effective CIN concentration in the DF than in the Rusitec.

Results from this study show that CIN exhibited antimicrobial activities in both CC systems, but the effects were more pronounced with the Rusitec than with the DF. However, because reductions in total VFA concentration, diet fermentability, and efficiency of microbial

protein synthesis are nutritionally unfavorable consequences, supplementation with CIN at the dosage evaluated may have adverse effects on the metabolism and productivity of ruminants.

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