Effects of Cinnamaldehyde and Garlic Oil on Rumen Microbial Fermentation in a Dual Flow Continuous Culture

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ABSTRACT

Eight continuous culture fermentors inoculated with ruminal liquor from heifers fed a 50:50 alfalfa hay:concentrate diet (17.6% crude protein, 28.0% neutral detergent fiber) were used in 3 replicated periods to study the effects of cinnamaldehyde (CIN) and garlic oil (GAR) on rumen microbial fermentation. Treatments were no additive (negative control); 1.25 mg/L (MON) and 12.5 mg/L (MON10) of the ionophore antibiotic monensin (positive control); 31.2 mg/L CIN (CIN) and 312 mg/L (CIN10) of CIN; and 31.2 mg/L GAR (GAR) and 312 mg/L (GAR10) of GAR (*Allium sativa*). The MON10 caused expected changes in microbial fermentation patterns (a decrease in fiber digestion, ammonia N concentration, and proportions of acetate and butyrate; an increase in the proportion of propionate; and a trend to increase small peptide plus AA N concentration). The CIN decreased the proportion of acetate and branch-chained volatile fatty acids (VFA) and increased the proportion of propionate; CIN10 decreased the proportion of acetate and increased the proportion of butyrate compared with the control. The GAR10 increased the proportion of propionate and butyrate and decreased the proportion of acetate and branch-chained VFA compared with the control. The GAR10 also increased the small peptide plus amino acid N concentration, although no effects were observed on large peptides or ammonia N concentrations. The CIN and GAR10 resulted in similar effects as monensin, with the exception of the effects on the molar proportion of butyrate, which suggests that they might have a different mode of action in affecting in vitro microbial fermentation.

(Key words: rumen fermentation, garlic oil, cinnamaldehyde)

Abbreviation key: CIN = cinnamaldehyde, GAR = garlic oil, HMG-CoA = 3-hydroxy-3-methyl-glutaryl co-

enzyme A, LPep = large peptide, MON = monensin, SPep = small peptide, TA = tungstic acid.

INTRODUCTION

A goal of ruminant microbiologists and nutritionists is to manipulate the ruminal microbial ecosystem to improve the efficiency of converting feed to animal products consumable by humans. In dairy cattle, the use of antibiotics as feed additives, such as ionophore antibiotics, has proved to be a useful tool to reduce energy (in the form of methane) and nitrogen (in the form of ammonia) losses from the diet (McGuffey et al., 2001). However, the use of antibiotics as feed additives in dairy cows is banned in the European Union because of the fear of appearance of residues in milk (Russell and Houlihan, 2003). For this reason, scientists have recently become interested in evaluating other alternatives for manipulating gastrointestinal microflora in livestock. Plant extracts have been used for centuries for various purposes (traditional medicine, industrial applications, food preservatives) because of their antimicrobial properties (Davidson and Naidu, 2000) and because most of them are categorized under GRAS (Generally Recognized as Safe) for human consumption (FDA, 2004). The use of plant extracts appears as one of the most natural alternatives to the antibiotic use in animal nutrition.

In a preliminary study, the effects of different concentrations of various plant extracts were evaluated in a 24-h in vitro batch culture rumen microbial fermentation (Busquet et al., 2004). Cinnamaldehyde (CIN; main active compound of cinnamon oil) reduced the ammonia N concentration and increased the molar proportion of propionate compared with control. In the same in vitro trial, garlic oil (GAR; *Allium sativa*) increased the molar proportions of propionate and butyrate and reduced the molar proportion of acetate compared with the control. These results suggested that CIN and GAR may improve the efficiency of energy and N utilization in the rumen. However, results from 24-h in vitro batch culture rumen microbial fermentations should be interpreted with caution, because Cardozo et al. (2004), in

Received November 17, 2004.

Accepted March 8, 2005.

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a long-term continuous culture study, observed that the effects of some plant extracts on rumen microbial fermentation disappeared after 7 d of fermentation, suggesting that microorganisms may become adapted to the presence of these compounds. The aim of the present study was to evaluate the effects of 2 concentrations of CIN and GAR on rumen microbial fermentation in a long-term in vitro study. Two concentrations of the ionophore antibiotic monensin (**MON**) were also added as positive controls to compare its effects with those of the plant extracts in the same in vitro conditions.

MATERIALS AND METHODS

Apparatus and Experimental Design

Eight 1320-mL dual-flow continuous culture fermentors (Hoover et al., 1976) were used in 3 replicated periods of 9 d. On the first day of each period, all fermentors were inoculated with ruminal fluid obtained from 2 rumen-fistulated heifers (300 kg BW) fed a dehydrated alfalfa (30% of DM) and concentrate (70% of DM) diet. Fermentors were fed at 95 g DM/d in 3 equal portions/d (every 8 h); the diet contained 17.6% CP, 28.0% NDF, and 17.7% ADF. The diet (DM basis) consisted of alfalfa hay (49.4%), ground barley grain (24.0%), ground corn grain (15.4%), soybean meal (9.7%), white salt (NaCl, 0.3%), monobasic sodium phosphate ($\text{H}_2\text{NaPO}_4 \cdot 2\text{H}_2\text{O}$, 0.4%), and a vitamin and mineral mixture [0.4%, which contained (/kg): 7 mg Co, 167 mg Cu, 33 mg I, 2660 mg Mn, 27 mg Se, 660 mg Zn, 1000 kIU vitamin A, 200 kIU vitamin D₃, 1330 mg vitamin E, 2.67 g urea, 67 g NaCl, 33 g sulfur, and 300 g MgO]. The diet was designed to meet or exceed nutrient recommendations for a Holstein cow (650 kg BW) producing 30 kg of milk (NRC, 2001). Temperature (38.5°C), pH (6.4 ± 0.05), and liquid (10%/h) and solid (5%/h) dilution rates were maintained constant, and fermentation conditions were monitored with a personal computer and the LabView Software (FieldPoint; National Instruments, Austin, TX). Anaerobic conditions were maintained by infusion of N₂ at a rate of 40 mL/min. Artificial saliva (Weller and Pilgrim, 1974) was continuously infused into flasks and contained 0.4 g/L urea to simulate recycled N. Treatments were 2 fermentors with no additive (control), 2 fermentors with an average of 1.25 and 12.5 mg/L of culture fluid of the ionophore antibiotic MON (MON and MON10, respectively; Sigma Chemical, St. Louis, MO), 2 fermentors with an average of 31.2 and 312 mg/L of culture fluid of CIN (CIN and CIN10, respectively; C₉H₈O, purity of 98%), and 2 fermentors with an average of 31.2 and 312 mg/L of culture fluid of GAR (GAR and GAR10, respectively; *Allium sativa*; standardized at 0.7% of allicin). Cinnamaldehyde and garlic oil were provided by

Pancosma S.A. (Bellegarde-sur-Valserine Cedex, France). Average concentrations of GAR and CIN were selected based on previous in vitro research (Busquet et al., 2004). Average concentration of the low concentration of MON was calculated based on the estimated concentration in dairy cattle (350 mg/d per cow and a daily fluid flow through the rumen of 240 to 320 L/d). The low concentrations of CIN and GAR were dissolved in ethanol at a 1:10 ratio, and the high concentrations were supplied directly to the fermentors. The 2 concentrations of MON were also dissolved in ethanol; the low concentration (MON) was dissolved at a 1:250 ratio, and the high concentration (MON10) was dissolved at a 1:25 ratio. All additives were stored at 5°C in a smoked glass flask. The daily dose of the additives was divided in 3 fractions and dosed into the fermentors 1 min before each feeding to achieve the expected average concentration. The control, CIN10, and GAR10 treatments were also dosed with the equivalent amounts of ethanol (0.33 mL per feeding).

Each experimental period consisted of 9 d (6 d for adaptation and 3 d for sample collection). During the 9 d of each period, 8 mL of filtered fermentor fluid were taken 2 h after the morning feeding to determine VFA and ammonia N concentration, to study the adaptation process of the microorganisms to the presence of the additives. During the last 3 d of each period, 40 mL of filtered fermentor fluid were taken at 0, 2, 4, and 6 h after the morning feeding to determine tungstic acid (**TA**) soluble N, TCA-soluble N, and ammonia N.

During sampling days, collection vessels were maintained at 4°C to impede microbial action. Solid and liquid effluents were mixed and homogenized for 1 min, and a 500-mL sample was removed via aspiration. Upon completion of each period, effluent from the 3 sampling d was composited, mixed within fermentor, and homogenized for 1 min. Subsamples were taken for the determination of total N, ammonia N, and VFA concentration, and the remaining samples were lyophilized. Lyophilized, dry samples were analyzed for DM, ash, NDF, ADF, and purine contents.

Bacteria were obtained from the 3 sampling d effluents previously filtered through cheesecloth and washed with saline solution. Bacterial cells were isolated by differential centrifugation at $1000 \times g$ for 15 min to separate feed particles, and the supernatant was centrifuged at $25,000 \times g$ for 20 min to isolate the bacterial cells. Pellets were rinsed twice with saline solution and recentrifuged at $25,000 \times g$ for 25 min. The last rinse was performed 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 N, NAN, bacterial N, and dietary

N were calculated as described by Stern and Hoover (1990).

Chemical Analyses

Samples for VFA were prepared as described by Jouany (1982). One milliliter of a solution comprising 0.2% (wt/wt) of mercuric chloride, 0.2% (wt/wt) 4-methylvaleric acid (as an internal standard), and 2% (vol/vol) orthophosphoric acid was added to 4 mL of filtered rumen fluid and frozen. Samples were centrifuged at $3000 \times g$ for 30 min, and the supernatant fluid 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) at 275°C in the injector and at a gas flow rate of 29.9 mL/min.

For ammonia N determination, a 4-mL sample of filtered fermentor fluid was acidified with 4 mL of 0.2 N HCl and frozen. Samples were centrifuged at $25,000 \times g$ for 20 min, and the supernatant was analyzed by visible spectrophotometry (UV-120-01; Shimadzu, Kyoto, Japan) for ammonia N (Chaney and Marbach, 1962).

The TCA- and TA-soluble N were determined as described by Winter et al. (1964). A 16-mL sample of filtered fluid was added to 4 mL of 10% (wt/vol) sodium tungstate and 4 mL of 1.07 N sulfuric acid. After allowing the tubes to stand at 5°C for 4 h, they were centrifuged at $9000 \times g$ for 15 min. The supernatant was frozen until analyzed for TA soluble N by the Kjeldahl procedure (AOAC, 1990). To determine TCA soluble N, 4 mL of 50% (wt/vol) TCA solution were added to 16 mL of filtered fermentor fluid. After 4 h at 5°C, tubes were centrifuged at $9000 \times g$ for 15 min. The supernatant was frozen until analyzed for TCA soluble N by the Kjeldahl procedure. Based on the statements indicated by Licitra et al. (1996), results were used to calculate the following (mg/100 mL): 1) large peptide (**LPep**) = TCA soluble N – TA soluble N, and 2) small peptide (**SPep**) plus AA N = TA soluble N – ammonia N.

Effluents DM were calculated by lyophilizing 200-mL aliquots in triplicate with subsequent drying at 103°C in a forced-air oven for 24 h. Dry matter content of the diet and bacterial samples were determined by drying samples for 24 h in a 103°C forced-air oven. Dry samples of diet, effluents, and bacteria were ashed overnight at 550°C in a muffle furnace. Diet, effluents, and bacterial OM were determined by difference.

Total N of diet, effluents, and bacterial samples was determined by the Kjeldahl method (AOAC, 1990). Samples CP were calculated as $N \times 6.25$. Effluent N was determined in liquid samples.

The NDF and ADF of diet and effluents were analyzed by the detergent system, using the sequential procedure of Van Soest et al. (1991), with sodium sulfite and thermostable amylase and were corrected for ash. 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 an internal standard.

Statistical Analyses

All statistical analyses were conducted using SAS (version 8.1; SAS Inst., Inc., Cary, NC). Results for the determination of VFA and ammonia N concentrations (during the adaptation days) and N fractions (peptide, amino acid, and ammonia N; during the sampling days) were analyzed using PROC MIXED for repeated measures (Littell et al., 1998). The model accounted for the effects of treatments and days (for VFA and ammonia N concentration) or hours (for the protein fractions in d 7, 8, and 9) of sampling, and the interaction of treatment by days or treatment by hours. Period was considered a random effect. The statistical analyses of results of VFA and ammonia N concentrations and N fractions were subjected to 3 covariance structures: compound symmetric, autoregressive order one, and unstructured covariance. The covariance structure that yielded the largest Schwarz's Bayesian criterion was considered to be the most desirable analysis, and least square means for treatments are reported. Differences in average between treatments were declared at $P < 0.05$ using Tukey's multiple comparison test. For comparisons in N fractions between treatments at each hour, and between 0 h and 2, 4, and 6 h within treatments, the Bonferroni comparison test was used, and differences were declared at $P < 0.05$.

The results of DM, OM, NDF, ADF, and CP digestibilities; VFA concentrations (from effluent samples); and flows of total N, ammonia N, NAN, bacterial N, and dietary N were analyzed as a randomized block design. Main effects and their interactions were determined with ANOVA using the PROC MIXED procedure of SAS (version 8.1; SAS Inst., Inc.). Differences between treatments were declared at $P < 0.05$ using Tukey's multiple comparison test, and least squares means for treatments are shown.

RESULTS

Total VFA, individual VFA, and ammonia N concentrations at 2 h after feeding decreased in the first 2 d of fermentation in all treatments and remained constant thereafter (data not shown), indicating that 2 d were sufficient for the adaptation of rumen microflora to the

Table 1. Effect of different doses of monensin (MON), cinnamaldehyde (CIN), and garlic oil (GAR) on VFA concentrations.

Item	Treatment ¹							SEM
	Control	MON	MON10	CIN	CIN10	GAR	GAR10	
Total VFA, mM	87.4 ^b	89.7 ^b	104.4 ^a	85.7 ^b	88.0 ^b	93.8 ^{ab}	94.3 ^{ab}	3.28
(mol/100 mol)								
Individual VFA								
Acetate	61.2 ^a	60.2 ^{ab}	45.9 ^d	55.8 ^c	57.0 ^{bc}	58.5 ^{abc}	46.8 ^d	0.94
Propionate	20.5 ^d	22.4 ^{cd}	45.1 ^a	24.2 ^{bc}	21.6 ^{cd}	22.6 ^{cd}	27.4 ^b	1.24
Butyrate	10.8 ^c	10.3 ^c	4.3 ^d	13.1 ^{bc}	14.3 ^b	11.3 ^{bc}	19.4 ^a	1.47
Isobutyrate	1.0 ^a	0.9 ^{bc}	0.6 ^d	0.9 ^a	1.1 ^a	1.0 ^a	0.7 ^{cd}	0.03
Isovalerate	1.8 ^a	1.6 ^{ab}	0.6 ^d	1.4 ^b	1.4 ^{ab}	1.6 ^{ab}	0.9 ^c	0.10
Valerate	4.5 ^a	4.4 ^a	3.3 ^b	4.5 ^a	4.3 ^a	4.8 ^a	4.9 ^a	0.18
BCVFA ²	2.8 ^a	2.5 ^{ab}	1.2 ^c	2.3 ^b	2.5 ^{ab}	2.7 ^{ab}	1.6 ^c	0.14
C2:C3 ³	3.0 ^a	2.7 ^{ab}	1.0 ^d	2.3 ^b	2.6 ^{ab}	2.6 ^{ab}	1.7 ^c	0.14

^{a,b,c,d}Means within a row with different superscripts differ ($P < 0.05$).

¹MON = 1.25 mg/L MON, MON10 = 12.5 mg/L MON, CIN = 31.2 mg/L CIN, CIN10 = 312.0 mg/L CIN, GAR = 31.2 mg/L GAR, and GAR10 = 312.0 mg/L GAR.

²BCVFA = Branched-chain VFA, includes isobutyrate and isovalerate.

³Acetate:propionate.

fermentation conditions and to the presence of feed additives.

During sampling days, total VFA concentration was higher in MON10 compared with the control (Table 1). The MON10, CIN, and GAR10 treatments had lower molar proportions of acetate and branched-chain VFA and higher molar proportions of propionate compared with the control (Table 1). The CIN10 also decreased the molar proportion of acetate and increased the molar proportion of butyrate compared with the control. The acetate to propionate ratio was lower in MON10, CIN, and GAR10 treatments compared with the control. The high concentration of monensin (MON10) had lower molar proportions of butyrate and valerate, and GAR10 had a higher molar proportion of butyrate compared with the control.

The LPep N concentration (mg/100 mL) in the control treatment remained constant during the time between feedings, and there were no treatment effects on the average LPep N concentration (Table 2). The SPep + AA N concentration in the control increased from 0 to 4.2 mg/100 mL on the first 2 h after feeding and returned to pre-feeding levels at 4 h (Table 2). The average SPep + AA N concentration throughout all hours between feedings was higher in GAR10 and tended to be higher ($P = 0.07$) in MON10 compared with the control. The ammonia N concentration (mg/100 mL) in the control remained constant between feedings (Table 2). The ammonia N concentration at 2 and 4 h after feeding and the average between feedings was lower in MON10 compared with the control.

True DM and OM digestibility was similar in all treatments, although in MON10, true OM digestibility tended to be lower ($P = 0.07$) compared with the control

(Table 3). The NDF and ADF digestibilities were lower in MON10 compared with the control (Table 3).

There were no treatment effects on effluent ammonia N concentration, dietary or bacterial N flows, protein degradation, or efficiency of microbial protein synthesis compared with the control (Table 4).

DISCUSSION

In a previous in vitro study in the same dual-flow continuous culture system, Cardozo et al. (2004) observed that some of the effects of plant extracts on rumen microbial fermentation during the adaptation period (d 1 to 7) disappeared after d 7 of incubation, suggesting that rumen microbes were adapted to the additives within 7 d. In contrast, in the present trial, the changes in fermentation were observed within 48 h and remained unchanged until the end of the period. The lack of an adaptation process in the present trial may be attributed to the higher concentration of plant extracts used (30 and 300 mg/L) compared with the lower concentration (0.22 mg/L) used by Cardozo et al. (2004). Cinnamaldehyde and GAR had no effect on DM, OM, NDF, and ADF digestibilities or on total VFA concentration, which suggests that these additives did not modify overall diet fermentability. The MON10 reduced NDF and ADF digestibility and tended to reduce OM digestibility, but total VFA concentration was higher compared with the control. In general, in vitro studies show that ionophores decrease DM and OM digestion (Fuller and Johnson, 1981; Chalupa, 1988), although total VFA concentration may increase (Richardson et al., 1976), decrease (Richardson et al., 1976; Fuller and Johnson, 1981), or remain unchanged (Martin and

Table 2. Effect of different doses of monensin (MON), cinnamaldehyde (CIN), and garlic oil (GAR) N fraction concentrations (mg/100 mL) during the 6-h post-feeding cycle.

	Treatment ¹							
Time post-feeding, h	Control	MON	MON10	CIN	CIN10	GAR	GAR10	SEM
LPep ² soluble N								
0	6.8	3.9	4.7	7.8	8.9	8.3	6.3	1.83
2	7.6	7.3	6.0	7.4	6.7	7.6	6.0	1.83
4	7.6	6.4	5.9	4.6	4.9	7.6	5.2	1.83
6	5.2	5.0	5.1	5.6	5.5	6.3	4.3	1.83
Average	6.8	5.7	5.4	6.4	6.5	7.5	5.5	1.41
SPep ³ + AA soluble N								
0	0.0	0.2	1.5	0.3	2.2	2.0	3.1	2.33
2	4.2 ^z	3.6	6.6 ^z	4.4 ^z	3.4	6.2 ^z	5.9	2.33
4	1.7	2.1	5.6	3.1	3.7	2.4	5.1	2.33
6	1.8	2.0	4.0	2.2	2.0	2.9	4.4	2.33
Average	1.9 ^b	2.0 ^{ab}	4.4 ^{ab}	2.5 ^{ab}	2.8 ^{ab}	3.4 ^{ab}	4.6 ^a	2.12
Ammonia N								
0	19.8	18.7	15.2	15.9	17.6	17.8	18.7	2.48
2	20.6 ^a	19.6 ^{ab}	12.7 ^b	18.0 ^{ab}	19.2 ^{ab}	19.3 ^{ab}	17.9 ^{ab}	2.48
4	18.1 ^a	16.2 ^a	11.2 ^b	15.3 ^{ab}	15.8 ^{ab}	15.6 ^{ab}	15.9 ^{ab}	2.48
6	18.4	16.3	12.8	14.7	15.3	15.6	15.7	2.48
Average	19.2 ^a	17.7 ^{ab}	13.0 ^b	16.0 ^{ab}	17.0 ^{ab}	17.1 ^{ab}	17.0 ^{ab}	2.38

^{a,b}Means within a row with different superscripts differ ($P < 0.05$).

^zMeans within a column significantly differ from 0 h ($P < 0.05$).

¹MON = 1.25 mg/L MON, MON10 = 12.5 mg/L MON, CIN = 31.2 mg/L CIN, CIN10 = 312.0 mg/L CIN, GAR = 31.2 mg/L GAR, and GAR10 = 312.0 mg/L GAR.

²LPep = Large peptide.

³SPep = Small peptide.

Macy, 1985). The observed reduction in OM digestibility is thought to be due to a decrease in fiber degradation. The negative effect of monensin on NDF and ADF degradation is attributed to the higher sensitivity of cellulolytic ruminococci and other cellulolytic strains to the antibiotic (Schelling, 1984), although other cellulolytic species, such as *Fibrobacter succinogenes*, are resistant to monensin (Chen and Wolin, 1979). In contrast with in vitro observations, ruminal degradation of OM and cellulose in vivo is not normally decreased by MON (Schelling, 1984). In vivo, where a longer adaptation time is allowed, MON-resistant cellulolytic bacteria, such as *F. succinogenes*, may eventually replace rumi-

nococci and avoid the decrease in fiber degradation (Schelling, 1984).

The MON10 increased the molar proportion of propionate and decreased the molar proportions of acetate and butyrate, as expected. Numerous in vitro (Wallace et al., 1981; Chalupa, 1988) and in vivo (Richardson et al., 1976; Sauer et al., 1998) studies demonstrated that the addition of ionophores increased the proportion of propionate and reduced the proportion of acetate and butyrate. Gram-negative bacteria in the rumen, which mainly produce propionate and succinate, have a complex outer membrane that makes them impermeable to many large molecules such as ionophores. Therefore,

Table 3. Effect of different doses of monensin (MON), cinnamaldehyde (CIN), and garlic oil (GAR) on DM, OM, NDF, and ADF digestibilities.

	Treatment ¹							SEM
	Control	MON	MON10	CIN	CIN10	GAR	GAR10	
True digestibility								
DM, %	62.0	59.4	53.2	60.0	61.4	64.1	58.9	2.37
OM, %	59.6 ^{ab}	58.0 ^{ab}	53.0 ^b	58.7 ^{ab}	60.5 ^{ab}	62.3 ^a	57.6 ^{ab}	1.78
Fiber digestibility								
NDF, %	38.8 ^a	32.0 ^a	18.8 ^b	33.3 ^a	33.5 ^a	39.4 ^a	30.8 ^a	3.49
ADF, %	36.7 ^a	28.4 ^a	5.8 ^b	30.8 ^a	29.9 ^a	36.0 ^a	25.0 ^a	4.01

^{a,b}Means within a row with different superscript differ ($P < 0.05$).

¹MON = 1.25 mg/L MON, MON10 = 12.5 mg/L MON, CIN = 31.2 mg/L CIN, CIN10 = 312.0 mg/L CIN, GAR = 31.2 mg/L GAR, and GAR10 = 312.0 mg/L GAR.

Table 4. Effect of different doses of monensin (MON), cinnamaldehyde (CIN), and garlic oil (GAR) on N metabolism.

Item	Treatment ¹							SEM
	Control	MON	MON10	CIN	CIN10	GAR	GAR10	
N NH ₃ , mg/100 mL	21.5	19.8	16.0	18.5	20.6	20.1	19.0	1.67
N flow, g/d								
Total	3.5	3.4	3.5	3.4	3.5	3.5	3.5	0.04
Ammonia	0.6	0.6	0.5	0.5	0.6	0.6	0.6	0.05
Nonammonia	2.8	2.8	3.0	2.9	2.8	2.9	2.9	0.05
Dietary	1.3 ^{ab}	1.3 ^{ab}	1.6 ^a	1.1 ^{ab}	0.9 ^b	1.1 ^{ab}	1.4 ^{ab}	0.13
Bacterial	1.5	1.4	1.4	1.7	1.9	1.7	1.5	0.13
CP degradation, %	49.6 ^{ab}	47.9 ^{ab}	36.9 ^b	54.4 ^{ab}	65.7 ^a	56.1 ^{ab}	45.9 ^{ab}	5.33
EMPS, ² g/kg TOMD ³	27.6	26.6	27.7	30.6	34.5	29.7	28.5	2.04

^{a,b}Means within a row with different superscript differ ($P < 0.05$).

¹MON = 1.25 mg/L MON, MON10 = 12.5 mg/L MON, CIN = 31.2 mg/L CIN, CIN10 = 312.0 mg/L CIN, GAR = 31.2 mg/L GAR, and GAR10 = 312.0 mg/L GAR.

²EMPS = Efficiency of microbial protein synthesis.

³TOMD = True OM digestibility.

these gram-negative organisms are usually resistant to the action of MON (Bergen and Bates, 1984). In contrast, gram-positive bacteria, which mainly produce acetate, butyrate, and hydrogen, lack an outer membrane and are generally sensitive to ionophores (Bergen and Bates, 1984). This hypothesis was confirmed by the increase in the number of *Bacteroidetes* population (gram-negative bacteria) in MON10 compared with the control when samples of fermentor effluents from the present trial were analyzed by PCR (Ferre et al., 2004). The change in the VFA proportions as a result of the use of MON is often associated with a decline in methane production (Schelling, 1984). In contrast to other methane inhibitors, monensin inhibits methanogenesis indirectly by lowering the availability of hydrogen and formate (the primary substrates for methanogens), and methanogenic archaea, in general, are resistant to the antibiotic (Chen and Wolin, 1979). The low concentration of MON (1.25 mg/L) resulted in similar effects to those observed with MON10, but differences were smaller and nonsignificant compared with the control. The effects of similar concentrations of MON in continuous culture studies have been variable. Fellner et al. (1997), using 2 mg/L of MON, observed a decrease in acetate and butyrate proportions and an increase in propionate proportion compared with control, but others (Fuller and Johnson, 1981) found no effects on acetate and propionate proportions when using 0.8 and 1.07 mg/L of MON.

The GAR10 resulted in similar effects as MON10, except for the proportion of butyrate, which decreased with MON10 and increased with GAR10 compared with the control. These results agree with those observed in a previous in vitro batch culture fermentation study with different concentrations of GAR (Busquet et al., 2004). The decrease in butyrate production with the

use of MON is known to be due to the inhibition of the gram-positive bacteria *Butyrivibrio fibrisolvens* (Chen and Wolin, 1979), the major butyrate producer in the rumen. This difference in the butyrate concentration suggests that MON and GAR may have a different mode of action on rumen microbial fermentation. Results from the PCR analyses of the effluent samples (Ferre et al., 2004) indicated that GAR10 resulted in no stimulatory effects on gram-negative bacteria compared with the control, although MON10 resulted in a 2-fold increase, suggesting that both additives had different effects on gram-negative organisms. In fact, the effects observed with GAR10 were similar to those found in methane inhibitors such as amicaloral (Chalupa et al., 1980) or 2-bromoethanosulfonic acid (Martin and Macy, 1985), which, in contrast with MON, directly inhibit methanogenic archaea or the metabolic pathways of methane synthesis. Disposal of excess hydrogen produced by a direct inhibition of methane production results in increased concentrations of other hydrogen sinks such as propionate and butyrate (Demeyer and Van Nevel, 1975). Conversely, the production of acetate in the rumen results in large quantities of hydrogen and depends on the availability of reducing equivalents such as NAD⁺. It has been observed that high partial pressures of H₂ and high NADH/NAD⁺ ratios in the rumen derived from the inhibition of methanogenesis results in a decrease in acetate production (Miller, 1995). The fermentation pattern observed in GAR is consistent with the hypothesis that its mode of action is as a methane inhibitor. We hypothesize that this activity is mediated through the inhibition of the methane-producing microorganisms. The antimicrobial activity in garlic has been mainly attributed to its organosulfur compounds, particularly to allicin (Feldberg et al., 1988). Several mechanisms of action have been sug-

gested to explain the GAR antimicrobial effects, including the inhibition of RNA, DNA, and protein synthesis (Feldberg et al., 1988), among others. However, it appears that the main antimicrobial effect of the organosulfur compounds of GAR is due to their interaction with sulfidryl groups of proteins and other biological active molecules (Reuter et al., 1996). This interaction has been demonstrated by the loss of activity of some thiol-containing enzymes (i.e., papain, alcohol dehydrogenases) and by the reaction between different organosulfur compounds and cysteine to form other substances by a thiol-disulfide exchange reaction (Reuter et al., 1996). Although the mechanism of action by which GAR could decrease methane production is not known, it could be related to the capacity of its organosulfur compounds to inhibit the enzyme 3-hydroxy-3-methyl-glutaryl coenzyme A (**HMG-CoA**) reductase by a thiol-disulfide exchange reaction (Gebhardt and Beck, 1996). Methanogens and other *Archaea* have unique membrane lipids that contain glycerol joined by ether linkages to long-chain isoprenoid alcohols (De Rosa et al., 1986). The synthesis of the isoprenoid units in methanogenic archaea is catalyzed by the HMG-CoA reductase in a similar way as for cholesterol synthesis in humans. Numerous studies have demonstrated the inhibitory effects of garlic-derived organosulfur compounds on cholesterol biosynthesis in hepatocytes by inhibition of the HMG-CoA reductase (Gebhardt and Beck, 1996; Cho and Xu, 2000). Miller and Wolin (2001) demonstrated that products, such as lovastatin and mevastatin, that decrease cholesterol production in humans by inhibiting HMG-CoA reductase, have the potential to specifically inhibit rumen methanogenic archaea without affecting rumen fermentative bacteria (*Eubacteria*) because of their different membrane lipid composition.

The CIN and CIN10 treatments decreased the molar proportion of acetate compared with the control. This reduction was compensated with an increase in the proportion of propionate in CIN and an increase in the proportion of butyrate in CIN10. In a previous dual flow continuous culture study (Busquet et al., accepted), a concentration of 2.2 mg/L of CIN numerically ($P = 0.11$) decreased the molar proportion of acetate and numerically ($P = 0.11$) increased the molar proportion of butyrate compared with control, suggesting that low concentrations of CIN may be active as modulators of rumen microbial activity, agreeing with results observed in the present trial, although no effects on propionate were observed. In contrast, in a 24-h in vitro batch culture fermentation trial with a 50:50 forage:concentrate diet and different concentrations (3, 30, 300, and 3000 mg/L culture fluid) of CIN (Busquet et al., 2004), no effects on the proportion of acetate were observed, but at 300 mg/L, the proportion of butyrate

increased, and at 3000 mg/L, the proportion of propionate increased and the proportion of butyrate decreased compared with the control. The variability on the results observed between the different in vitro studies could have been due to different in vitro system (batch vs. continuous culture) or the length of incubation period (24 h in batch fermentations and 9 d in continuous culture). Cinnamaldehyde is the main active compound of cinnamon oil (Davidson and Naidu, 2000). Several studies have shown the antimicrobial effects of CIN over several targeted microorganisms (Helander et al., 1998). In general, gram-positive bacteria are more sensitive to the inhibition by CIN than the gram-negative (Ottara et al., 1997), although some studies have demonstrated that purified CIN was also highly effective against several gram-negative organisms (Helander et al., 1998). Helander et al. (1998) observed that, in contrast to other secondary plant metabolites (i.e., thymol or carvacrol), CIN exhibited neither outer membrane disintegrating activity nor depletion of intracellular ATP, suggesting that CIN could gain access to the periplasm and to the deeper parts of the cell, probably through the outer membrane traversing porin proteins (Nikaido, 1994). The mechanism of action of CIN as antimicrobial remains poorly understood, although some researchers suggested that the carbonyl group might be the active site (Wendakoon and Sakaguchi, 1995; Helander et al., 1998). In the PCR analysis of the effluent samples (Ferre et al., 2004), CIN resulted in a decrease in the number of gram-negative bacteria, and CIN10 resulted in an increase. These results are consistent with the ability of CIN to inhibit both gram-positive (most acetate- and butyrate-producing bacteria) and gram-negative bacteria (normally propionate-producing bacteria), probably in a dose-dependent manner, but they contradict the effects of CIN and CIN10 treatments on individual VFA proportions. The variable, and sometimes contradictory, effects of CIN make it difficult to establish a possible mechanism of action on rumen microbial fermentation.

There were no effects of the additives on the average LPep N concentration between feedings. The MON10 treatment tended to increase the average SPep + AA N concentration and reduced the ammonia N concentration, which suggests that the deamination process was inhibited. Most in vitro studies with MON resulted in a decrease in ammonia N concentration generally associated with an accumulation of AA or peptide N, which indicates that MON inhibits deamination to a greater extent than proteolysis (Wallace et al., 1981; Whetstone et al., 1981; Russell and Martin, 1984). In general, this reduction on ammonia N concentration in vitro is accompanied by a reduction in protein degradation and microbial protein synthesis, although the efficiency of

rumen bacterial protein synthesis is generally unchanged (Wallace et al., 1981; Whetstone et al., 1981). In the present trial, although this effect was not statistically significant, MON10 decreased protein degradation by 25% compared with the control, whereas bacterial and dietary N flows and efficiency of microbial protein synthesis were unaffected.

The GAR10 treatment significantly increased the SPep + AA soluble N concentration compared with the control, although it did not affect the average LPep or ammonia N concentrations, nor did it affect other rumen microbial N metabolism parameters. The mechanism by which GAR10 increased the SPep + AA soluble N concentration in culture fluid it is not known, but it is consistent with the decrease in the branch-chained VFA proportion. Branched-chain VFA result from the deamination of reduced AA (mainly branched-chain AA), which depends on the disposal of reducing equivalents. Methane inhibitors reduce the dehydrogenase activity in the rumen, which may partly be responsible for the inhibition of deamination of branched-chain AA and the lower production of branched-chain VFA (Hino and Russell, 1985).

In the present trial, CIN did not affect rumen microbial N metabolism, although it significantly decreased the molar proportion of branched-chain VFA compared with the control. In contrast, in the previous *in vitro* batch culture fermentation study (Busquet et al., 2004), the reduction in the proportion of the branched-chain VFA observed at high concentrations of CIN (300 and 3000 mg/L) was accompanied by a decrease in the ammonia N concentration.

CONCLUSIONS

The addition of MON to the continuous culture resulted in expected changes in rumen microbial fermentation. Changes in VFA and N metabolism indicate that CIN and GAR affected the fermentation profile and may be used as modulators of rumen microbial fermentation. However, their mechanisms of action may be different from that of MON. The decrease in the molar proportion of acetate and the increase in the molar proportion of propionate and butyrate in GAR are consistent with the effects observed with methane inhibitors. Further research is required to determine the mechanism of action and effects of CIN and to confirm the mechanism of action of GAR on rumen microbial fermentation and its effects on methane production.

ACKNOWLEDGMENTS

The authors thank Axiss France SAS for their financial support and technical assistance.

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