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Effects of alfalfa extract, anise, capsicum, and a mixture of cinnamaldehyde and eugenol on ruminal fermentation and protein degradation in beef heifers fed a high-concentrate diet[1]

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ABSTRACT: Four Holstein heifers (360 ± 22 and 450 ± 28 kg of BW in Exp. 1 and 2, respectively) fitted with ruminal trocars were used in 4 × 4 Latin square designs to evaluate the effects on ruminal microbial fermentation of the following: Exp. 1, no additive, alfalfa extract (30 g/d, AEX), a mixture of cinnamaldehyde (0.18 g/d) and eugenol (0.09 g/d; CIE1), and AEX and CIE1 in combination; and Exp. 2, no additive, anise oil (2 g/d), capsicum oil (1 g/d), and a mixture of cinnamaldehyde (0.6 g/d) and eugenol (0.3 g/d). Heifers were fed a 90:10 concentrate:barley straw diet (16% CP; 25% NDF) for ad libitum intake. Each period consisted of 15 d for adaptation and 6 d for sampling. On d 16 to 18, DM and water intakes were measured. On d 19 to 21 ruminal contents were sampled at 0, 3, 6, 9, and 12 h after feeding to determine ruminal pH and the concentrations of VFA, L-lactate, large peptides, small peptides plus AA (SPep+AA), and ammonia N. On d 20 and 21, samples of ruminal fluid were collected at 0 and 3 h after feeding to determine protozoal counts. In Exp. 1, CIE1 and AEX decreased ($P < 0.05$) total DMI, concentrate DMI, and water intake. The increase ($P < 0.05$) in SPep+AA and the decrease ($P < 0.05$) in ammonia N when supplementing CIE1 suggest that deamination was inhibited. Treatment AEX increased ($P < 0.05$) the acetate to propionate ratio, which is less efficient for beef production. Treatment CIE1 increased ($P < 0.05$) counts of holotrichs. Effects of AEX and CIE1 were not additive for many of the measured metabolites. In Exp. 2, treatments had no effect on ruminal pH, total VFA concentration, and butyrate proportion. The capsicum oil treatment increased ($P < 0.05$) DMI, water intake, and SPep+AA N concentration and decreased ($P < 0.05$) acetate proportion, branched-chain VFA concentration, and large peptide N concentration. The cinnamaldehyde (0.6 g/d) and eugenol (0.3 g/d) treatment decreased ($P < 0.05$) water intake, acetate proportion, branched-chain VFA, L-lactate, and ammonia N concentrations and increased ($P < 0.05$) propionate proportion and SPep+AA N concentration. The anise oil treatment decreased ($P < 0.05$) acetate to propionate ratio, branched-chain VFA and ammonia N concentrations, and protozoal counts. The results indicate that at the doses used a mixture of cinnamaldehyde and eugenol, anise oil, and capsicum oil may be useful as modifiers of rumen fermentation in beef production systems.

Key words: plant extract, rumen fermentation

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INTRODUCTION

Diets high in cereal grains are energetically more efficient than high-fiber diets for beef cattle, but the resulting decrease in ruminal pH may increase the risk of acidosis (Nocek, 1997). Ionophores have been used in beef diets because of their ability to improve the efficiency of nutrient utilization and reduce the risk of ruminal acidosis and bloat (Chalupa et al., 1980; Bergen and Bates, 1984). However, the European Union legislation banned the use of antibiotics in animal feeds in January of 2006 (European Union, 2003). For this reason, industry is searching for alternative additives such as plant extracts that are generally recognized as safe for human and animal consumption. Previous in vitro studies in our laboratory (Cardozo et al., 2004, 2005; Busquet et al., 2005a,b, 2006) and others (Klita et al., 1996; Hristov et al., 1999) with different plant extracts and secondary plant metabolites showed the

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potential of some extracts including saponins, anise oil, capsicum extract, eugenol, and cinnamaldehyde to modify ruminal microbial fermentation. Calsamiglia et al. (2005) indicated that the combination of additives with different mechanisms of action may result in synergistic effects that may enhance ruminal fermentation.

The objective of this study was to evaluate the effects of alfalfa extract, anise, capsicum, and a combination of cinnamaldehyde and eugenol on ruminal microbial fermentation in beef heifers fed a high concentrate diet.

MATERIALS AND METHODS

Two trials were conducted with the same experimental design; the only differences were the initial BW of the animals and the additives used.

Animals and Housing

The research protocol was approved by the Campus Laboratory Animal Care Committee of the Universitat Autònoma of Barcelona (Spain). Four Holstein heifers (360 ± 22 and 450 ± 28 kg of BW in Exp. 1 and 2, respectively), each fitted with a 1-cm i.d. plastic ruminal trocar (Divasa Farmavic SA, Vic, Spain) were used in 4 × 4 Latin square designs. Heifers were individually housed in tie-stalls at the Unitat de Granges i Camps Experimentals of the Universitat Autònoma de Barcelona (Spain).

The diet consisted of barley straw and concentrate. The concentrate consisted of (DM basis) ground barley grain (30%), ground corn grain (21%), wheat bran (14%), soybean meal (15%), corn gluten feed (7%), sodium bicarbonate (0.5%), and a mineral and vitamin mixture (2.5%; 1 kg of DM of the vitamin and mineral mixture contained 1,562 kIU of vitamin A; 150 kIU of vitamin D; 2.5 g of vitamin E; 3.5 g of Zn; 2.0 g of Fe; 400 mg of Mn; 250 mg of Cu; 50 mg of Co; 38 mg of I; and 25 mg of Se). The diet (91% DM, 16% CP, 25% NDF, and 11% ADF; DM basis) was designed to meet or exceed nutrient recommendations of a 360 kg of BW Holstein heifer with an average daily gain of 1.15 kg/d (NRC, 1996).

Each experimental period consisted of 21 d (15 d for adaptation and 6 d for sample collection). Animals had ad libitum access to concentrate and barley straw offered once daily at 0900. Treatments were offered daily at 0900 and were mixed with 600 g of a mixture of soybean meal and mineral and vitamin mix of the diet to guarantee the consumption of the whole dose. Control animals received the same mixture but with no additive.

Sample Collection and Analyses

To avoid interference with ruminal sampling protocols, DM and water intake were measured on d 16, 17, and 18 of each period. Feed refusals were recorded before feeding. Refused concentrate and barley straw were manually separated with a sieve (0.5-cm screen) and weighed separately. To determine DMI, feed and refusal samples were collected daily and analyzed for DM. Water intake was also monitored using individual drinking cups equipped with individual flow meters (B98.32.50, Invensys model 510 C, Tashia SL, Artesa de Segre, Spain). Dry matter was determined by oven drying of the feed at 105°C for 24 h, and OM was determined by heating of the feed at 550°C for 4 h (AOAC, 1990). Nitrogen content was determined using the Kjeldahl procedure (AOAC, 1990), and ash-corrected NDF and ADF were determined sequentially using α-amylase and sodium sulfite (Van Soest et al., 1991).

On d 19, 20, and 21 of each period, samples of whole ruminal contents were collected at 0, 3, 6, 9, and 12 h after the morning feeding. The ruminal pH was measured immediately with a portable pH meter (model 507, Crison Instruments SA, Barcelona, Spain). Ruminal fluid was strained through 2 layers of cheesecloth, and 5 subsamples of the filtrate were taken for VFA, large peptides, small peptides plus AA, ammonia N, and lactate analyses.

Samples for VFA analysis were prepared as described by Jouany (1982) and analyzed by GLC (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 1.2 mL/min gas flow rate (29.9 cm/sec gas velocity). Ammonia N concentration was analyzed as described by Chaney and Marbach (1962) by spectrophotometry (Libra S21, Biochrom Analytical Instruments, Cambridge, UK). Tungstic acid-soluble N (**TAS N**) and trichloracetic acid-soluble N (**TCAS N**) were determined as described by Winter et al. (1964) and used to calculate (mg/100 mL) large-peptide N (**LPep N** = [TCAS N] – [TAS N]) and small-peptide plus AA N (**SPep+AA N** = [TAS N] – [ammonia N]). The L-lactate was analyzed according to Noll (1974) using an auto-analyzer (Model COBAS MIRA 89, Roche, Switzerland).

On d 20 and 21 of each period, at 0 and 3 h after the morning feeding, subsamples of filtrate were also collected for enumeration of protozoa by combining 8 mL of filtrate with 2 mL of methyl green:formaldehyde (38%, wt/wt) solution. Entodiniomorphs and holotrichs were identified (Dehority, 1993) and counted using a Neubauer Improved Bright-Line counting chamber (Hausser Scientific Partnership, Horsham, PA) as described by Veira et al. (1983). Protozoal counts were transformed to base 10 logarithms before statistical analysis.

Experimental Treatments

In Exp. 1, treatments were arranged in a 2 × 2 factorial, with the main factors being alfalfa extract (containing 10% malic acid and 1.5% saponins, DM basis) and a mixture of cinnamaldehyde and eugenol. Treat-

ments were: control (no additives), 30 g/d of alfalfa extract, **AEX**), a mixture of 0.18 g/d of cinnamaldehyde and 0.09 g/d of eugenol (**CIE1**), and the combination of the 2 treatments (**CIE1+AEX**).

In Exp. 2, treatments were: no additive (control), 2 g/d of anise extract (**ANI**; containing 10% anethole), 1 g/d of capsicum extract (**CAP**; containing 15% capsaicin), and a mixture of pure cinnamaldehyde (0.6 g/d) and eugenol (0.3 g/d; **CIE2**).

The extracts used in Exp. 1 and 2 were provided by Pancosma (Pancosma SA, 01200-Bellegarde-sur-Valserine Cedex, France).

For both experiments, the average ruminal concentration of the active components provided by the treatments was calculated assuming 1) an average ruminal volume of $BW^{0.57}$ (Owens and Goetsch, 1988), 2) a ruminal dilution rate of 10%/h, 3) no absorption of the active components through the ruminal wall, and 4) no degradation of the components within the rumen.

Statistical Analyses

Statistical analyses for both experiments were conducted using the PROC MIXED procedure of SAS (Version 8.2, SAS Inst. Inc., Cary, NC), and the differences were declared significant at $P < 0.05$. In Exp. 1, treatments were arranged as a 2×2 factorial and period, day, hour, AEX, CIE1, and their interactions were considered fixed effects, whereas animal was considered a random effect. Ruminal data collected at different times after feeding were analyzed for repeated measures (Littell et al., 1998). Repeated factors included days (for DM and water intakes), time after feeding (0, 3, 6, 9, and 12), and the day × time interaction (for pH, VFA, lactate, protein fractions, and protozoa). For each analyzed variable, heifer and period nested within treatment (the error term) were considered as a subject. The covariance structure that yielded the largest Schwarz' Bayesian criterion was considered to be the most desirable analysis.

In Exp. 2, statistical analyses were conducted as in Exp. 1 except that treatment was a single fixed effect. Significant differences between means of each treatment and control were tested using the Dunnett option.

RESULTS AND DISCUSSION

There is very limited information available on the effects of these plant extracts on rumen microbial fermentation, and most information is related to effects on rumen fermentation of dairy cattle (Broudiscou et al., 2002; Cardozo et al., 2004; Busquet et al., 2005a,b, 2006).

Experiment 1

Dry Matter and Water Intake. The CIE1 and AEX decreased ($P < 0.03$) total DM, concentrate, and water intakes compared with control (Table 1). There were no effects of extracts on barley straw DMI (average of 0.87 kg/d). The reductions in water intake in CIE1, AEX, and CIE1+AEX are likely associated with the reductions in DMI. There are no previous reports on the effects of a mixture of cinnamaldehyde and eugenol on DMI in growing heifers. However, Busquet et al. (2003) observed a 12% reduction in concentrate DMI in dairy cattle fed 0.6 g of cinnamaldehyde/kg of DM. Gurney et al. (1996) also observed that cinnamamide (the amide of cinnamaldehyde) decreased DMI by 17% in house mice. They noted that mice appeared to find the treated diet irritating to the mouth and paws. In contrast, the decreased DMI observed for AEX in the present trial is contrary to previous studies with malic acid or saponins. Feeding malic acid to dairy cows (Kung et al., 1982: level of inclusion 70 to 140 g/d of malate), dairy goats (Salama et al., 2002: level of inclusion 6.5 g/d of malate), or steers (Kung et al., 1982: level of inclusion 0.2 g of malate/kg of BW; Martin et al., 1999: level of inclusion 25 to 80 g/d of malate; Montaño et al., 1999: level of inclusion 80 g/d of malate) had no effect on DMI. Likewise, feeding saponins did not affect DMI in steers (Hussain and Cheeke, 1995; Hristov et al., 1999) or dairy cows (Wu et al., 1994). Therefore, the reduction in DMI cannot be explained by the presence of malic acid and saponins in AEX.

Rumen Fermentation Profile and Nitrogen Fractions. Because there were no interactions between treatment and time after feeding for any of the ruminal fermentation metabolites measured, only averages over time are presented (Table 2). There were no effects of AEX or CIE1 fed alone on ruminal pH (average of 5.91), total VFA (average of 136.8 m*M*), branched-chain VFA (average of 2.98 m*M*), and lactic acid (average of 0.06 m*M*) concentrations and proportions (mol/100 mol) of acetate (average of 56.5), propionate (average of 27.5), and butyrate (average of 12.1; Table 2), except for the increase ($P < 0.03$) in the acetate to propionate ratio for AEX. Previous research reported limited effects of saponins on total VFA concentration or profile in steers (Hussain and Cheeke, 1995; Hristov et al., 1999: level of inclusion 3.0 and 2.6 g/d of pure yucca saponins, respectively), sheep (Klita et al., 1996: level of inclusion 520 mg/d of pure alfalfa saponins), or dairy cows (Wu et al., 1994; Wang et al., 1998: level of inclusion 1.2 g/d and 22 mg/L of pure yucca saponins, respectively). Malate may increase or not change the total VFA concentration (Kung et al., 1982; Carro et al., 1999; Montaño et al., 1999), increase propionate proportion (Kung et al., 1982; Carro et al., 1999) and ruminal pH (Martin and Streeter, 1995; Martin et al., 1999; Montaño et al., 1999), and reduce lactate concentration (Carro et al., 1999). In the present trial, there were no effects of AEX on ammonia N concentration. Most of the reported research found inconsistent effects of saponins or malate on ammonia N concentration. Studies in sheep (Klita et al., 1996), dairy cows (Wu et al., 1994; Wang et al., 1998), and steers (Hussain and Cheeke, 1995) found

Table 1. Effect of natural plant extracts on DM and water intake in Holstein heifers (Exp. 1)

	Treatment[1,2]					*P*-value		
Item	Control	CIE1	AEX	CIE1+AEX	SEM	CIE1	AEX	CIE1 × AEX
Total DMI, kg/d	8.8	7.4	7.3	7.7	0.41	0.03	0.03	0.08
Concentrate DMI, kg/d	7.9	6.6	6.5	6.9	0.43	0.02	0.02	0.11
Barley straw DMI, kg/d	0.9	0.8	0.9	0.9	0.42	0.91	0.91	0.73
Water intake, L/d	44.3	38.0	36.8	39.4	2.06	0.01	0.01	0.04

[1]Data are means of 4 steers.
[2]CIE1 = mixture of cinnamaldehyde and eugenol; AEX = alfalfa extract; and CIE1+AEX = a combination of CIE1 and AEX.

no effect of saponins on ammonia N concentration. In contrast, other in vitro studies indicated that saponins reduce ammonia N concentration (Makkar et al., 1998: level of inclusion 1,200 mg/L of pure saponins). Hristov et al. (1999) also observed a lower ammonia N concentration 2 h after supplying 60 g/d of *Yucca schidigera* (containing 4.4% of saponins) to heifers. On the other hand, malate seems not to affect ammonia N concentration in ruminal fluid of sheep fed a 50:50 forage:concentrate ratio in a RUSITEC system (Carro et al., 1999: level of inclusion 750 mg/L of malate) and in dairy cows (Kung et al., 1982: level of inclusion 140 g/d of malate). If saponins and malate are the main active components in AEX, the lack of effect on ruminal pH and on propionate, lactate, and ammonia N concentrations could be attributed to the dose used. The estimated average ruminal concentration was of 6.3 mg/L for saponins and 41.7 mg/L for malate. The saponins and malate daily dose contained in AEX in the present trial was lower than other studies that found some effect of these additives on ruminal microbial fermentation (Makkar et al., 1998: level of inclusion 1,200 mg/L of pure saponins; Carro et al., 1999: level of inclusion 750 mg/L of malate).

The CIE1 affected N metabolism by increasing ($P < 0.05$) SPep+AA and decreasing ammonia N concentration, suggesting that deamination was inhibited. Cardozo et al. (2005) reported that cinnamaldehyde and eugenol increased total VFA concentration, and cinnamaldehyde also decreased the acetate to propionate ratio and ammonia N concentration in an in vitro batch culture fermentation study using ruminal fluid from heifers fed a 10:90 forage:concentrate diet and at a pH of 5.5. However, these effects were pH dependent, and at pH 7.0, cinnamaldehyde had the opposite effect, decreasing total VFA concentration and increasing the acetate to propionate ratio, and eugenol had no effects. The lack of an effect of CIE1 on VFA concentration and proportions in the present experiment could be attributed to the average high ruminal pH (5.95; Cardozo et al., 2005). The estimated average ruminal concentration was of 2.5 and 1.3 mg/L for cinnamaldehyde and eugenol, respectively. The cinnamaldehyde dose was within the range of the doses reported to have effects on ruminal microbial fermentation in vitro, and the concentration of eugenol was slightly below the concentration suggested to have effects (Cardozo et al., 2005).

The interaction between AEX and CIE1 was significant for total VFA, the proportions of acetate and propionate, and acetate:propionate ($P < 0.05$). These interac-

Table 2. Effect of natural plant extracts on average rumen pH, VFA and lactate concentrations, and N fractions in Holstein heifers (Exp. 1)

	Treatment[1,2]					*P*-value		
Item	Control	CIE1	AEX	CIE1+AEX	SEM	CIE1	AEX	CIE1 × AEX
Ruminal pH	5.95	5.95	5.94	5.80	0.06	0.25	0.19	0.06
Total VFA, m*M*	137.8	132.5	134.2	142.5	7.44	0.58	0.29	0.02
BCVFA,[3] m*M*	3.1	2.8	2.9	3.1	0.38	0.09	0.69	0.10
L-Lactate, m*M*	0.06	0.05	0.06	0.06	0.02	0.95	0.36	0.96
Individual VFA, mol/100 mol								
Acetate	55.8	56.8	58.6	54.8	1.01	0.31	0.19	0.01
Propionate	27.5	27.4	25.0	29.9	1.99	0.22	0.62	0.01
Butyrate	12.1	12.0	12.7	11.3	1.05	0.12	0.60	0.13
Acetate:propionate	2.03	2.08	2.35	1.83	0.10	0.83	0.03	0.02
Nitrogen fraction,[4] mg/100 mL								
LPep	10.1	9.0	10.1	10.1	1.01	0.49	0.47	0.49
SPep+AA	13.7	16.0	14.6	14.1	1.04	0.05	0.94	0.25
Ammonia	16.9	14.9	16.2	15.5	0.85	0.04	0.89	0.43

[1]Data are means of 4 steers.
[2]CIE1 = mixture of cinnamaldehyde and eugenol; AEX = alfalfa extract; and CIE1+AEX = a combination of CIE1 and AEX.
[3]BCVFA = Branched-chain VFA, including isobutyrate and isovalerate.
[4]LPep = large peptides; SPep+AA = small peptides plus AA.

Table 3. Effect of natural plant extracts on average protozoal population in Holstein heifers (Exp. 1)

Protozoa, $\log_{10}$ (counts/mL)	Treatment[1,2]					*P*-value		
	Control	CIE1	AEX	CIE1+AEX	SEM	CIE1	AEX	CIE1 × AEX
Entodiniomorphs	5.74	5.57	5.48	5.74	0.06	0.27	0.31	0.01
Holotrichs	4.37	4.70	4.33	4.64	0.05	0.01	0.18	0.74

[1]Data are means of 4 steers.
[2]CIE1 = mixture of cinnamaldehyde and eugenol; AEX = alfalfa extract; and CIE1+AEX = a combination of CIE1 and AEX.

tions suggest that the effects were not additive and that effects were different when fed together than when fed individually.

Protozoal Population. There were no time of sampling × treatment interactions for protozoal counts, and average treatment effects are reported (Table 3). The CIE1 increased ($P < 0.01$) holotrich counts, and although there is no clear explanation for that, it may be hypothesized that it is due to the numerical decrease in entodiniomorphs. There was a CIE1 × AEX interaction ($P < 0.01$) where the numerical decrease observed for CIE1 and AEX independently disappeared when both treatments were fed together. The effects of cinnamaldehyde or eugenol on protozoa have not been assessed previously. In contrast, the saponin content of AEX was expected to have an effect on protozoa, although the level of inclusion (estimated at 6.3 mg/L) was much lower than that reported in previous studies (ranging from 64 to 2,600 mg/L; Klita et al., 1996; Makkar et al., 1998; Hristov et al., 1999).

Experiment 2

Dry Matter and Water Intake. In this experiment, in addition to testing CAP and ANI, a dose of cinnamaldehyde and eugenol mix greater than in Exp. 1 was tested. The CAP increased ($P < 0.05$) total DMI, concentrate intake, and water intake; ANI tended ($P < 0.10$) to increase total DMI; and CIE2 decreased ($P < 0.05$) water intake compared with control (Table 4). However, barley straw DMI (average of 0.93 kg/d) was not affected by treatments. There are no previous reports on the effects of CAP on DM or water intake in growing heifers. However, there is evidence that capsaicin, the active component of capsicum oil, increases DM and water intake in rats (Zafra et al., 2003) and can stimulate appetite in humans (Calixto et al., 2000). The trend of ANI to increase DMI is in contrast to results of Nombekela et al. (1994), who tested the effects of anise as a flavoring additive in multiparous Holstein cows, but DMI was not affected. In contrast to results in Exp. 1, CIE2 had no effect on DMI in spite of the greater dose used. Busquet et al. (2003) reported that cinnamaldehyde decreased concentrate intake in dairy cattle, although the dose used (0.6 g/kg) was much greater than the one used in the present trial. The lack of agreement between Exp. 1 and 2 regarding the effect of the mix of cinnamaldehyde and eugenol on DMI is not clear.

Table 4. Effect of natural plant extracts on DM and water intake in Holstein heifers (Exp. 2)

Item	Treatment[1,2]				SEM
	Control	ANI	CAP	CIE2	
Total DMI, kg/d	7.6	8.0[a]	8.3[b]	7.8	0.13
Concentrate DMI, kg/d	6.7	7.0	7.3[b]	7.0	0.23
Barley straw DMI, kg/d	0.92	0.94	0.98	0.89	0.06
Water intake, L/d	36.3	37.4	45.7[b]	34.1[b]	0.43

[a]Within a row, means are different from control ($P < 0.10$).
[b]Within a row, means are different from control ($P < 0.05$).
[1]Data are means of 4 steers.
[2]ANI = anise oil; CAP = capsicum extract; and CIE2 = a mixture of cinnamaldehyde and eugenol.

Rumen Fermentation Profile. Because there were no interactions between treatment and time after feeding for any of the ruminal fermentation metabolites measured, only averages over time are presented (Table 5). Plant extracts had no effect on average ruminal pH (6.09) and average total VFA concentrations (154.5 m*M*, Table 5). Compared with control, molar proportion of acetate was lower ($P < 0.05$) in ANI, CAP, and CIE2, and that of propionate was greater ($P < 0.05$) in ANI and CIE2. The acetate to propionate ratio was lower ($P < 0.05$) in ANI; the concentration of branched-chain VFA was lower ($P < 0.05$) in ANI, CAP, and CIE2; and the concentration of L-lactate was lower ($P < 0.05$) in ANI and CIE2. These differences were most apparent 3 and 6 h after feeding (data not shown).

There has been very limited research on the effect of plant extracts on ruminal microbial fermentation. Cardozo et al. (2004) reported no effects of anise, cinnamon (containing 59% of cinnamaldehyde), and pepper (containing 12% of capsaicin) extracts on total and individual VFA concentrations. Busquet et al. (2006) also reported no effects of pure anethole (main component of anise), but cinnamaldehyde tended to decrease the acetate proportion, and clove bud oil (containing 81% eugenol) increased propionate proportion and decreased acetate proportion. However, whereas all these experiments were conducted in vitro under a dairy-type environment (high forage diets at pH 6.4), the effects of essential oils on ruminal microbial fermentation appear to be diet- and pH-dependent (Cardozo et al., 2005; Castillejos et al., 2005). When the effects were tested

Table 5. Effect of natural plant extracts on average of ruminal microbial fermentation profile and protein degradation in Holstein heifers (Exp. 2)

	Treatment[1,2]				
Item	Control	ANI	CAP	CIE2	SEM
Ruminal pH	6.10	6.07	6.14	6.05	0.07
Total VFA, m*M*	155.1	155.5	154.5	152.9	3.15
BCVFA,[3] m*M*	4.5	3.8[b]	4.0[b]	4.1[b]	0.16
L-Lactate, m*M*	0.33	0.27[a]	0.29	0.25[b]	0.04
Individual VFA, mol/100 mol					
Acetate	56.8	53.9[b]	53.9[b]	52.9[b]	0.61
Propionate	26.7	31.2[b]	29.8	30.5[b]	1.05
Butyrate	11.6	10.7	12.1	12.1	0.85
Acetate:propionate	2.3	1.8[b]	2.0	2.0	0.19
Nitrogen fraction,[4] mg/100 mL					
LPep	16.8	15.0	12.9[b]	13.6	1.17
SPep+AA	17.9	17.8	22.8[b]	21.0[b]	0.89
Ammonia	16.5	13.0[b]	15.3	13.9[b]	0.70

[a]Within a row, means are different from control ($P < 0.10$).
[b]Within a row, means are different from control ($P < 0.05$).
[1]Data are means of 4 steers.
[2]ANI = anise oil; CAP = capsicum extract; and CIE2 = a mixture of cinnamaldehyde and eugenol.
[3]BCVFA = Branched-chain VFA, including isobutyrate and isovalerate.
[4]LPep = Large peptides; SPep+AA = Small peptides plus AA.

using a 10:90 forage:concentrate diet and pH of 5.5, cinnamaldehyde, eugenol, and capsicum (doses from 0.3 to 30 mg/L) increased total VFA concentration, anise and capsicum (doses from 3 to 30 mg/L) decreased acetate and increased propionate proportions, and cinnamaldehyde (doses from 3 to 30 mg/L) decreased the acetate to propionate ratio, suggesting that the changes in the fermentation profile may be beneficial for beef-type production systems. In the present experiment, total VFA concentrations were not affected by treatments, but compared with control, the molar proportion of acetate decreased ($P < 0.05$) in ANI, CAP, and CIE, the molar proportion of propionate increased ($P < 0.05$) in ANI and CIE, and the acetate to propionate ratio decreased ($P < 0.05$) in ANI. Although these changes were in the same direction as those observed in vitro with a 10:90 forage:concentrate ratio diet (Cardozo et al., 2005), effects were smaller and can be attributed to the relatively high ruminal pH of heifers in the current experiment (average of 6.09) for the type of diet fed. It is likely that the management of heifers (fed individually with no animal-to-animal competition for feed) and the use of 0.5% bicarbonate in the diet may have prevented average pH from dropping below 5.8. The estimated average ruminal concentration of active compounds was of 24.4 mg/L for ANI, 12.0 mg/L of CAP, 7.3 mg/L of cinnamaldehyde, and 3.7 mg/L of eugenol. These doses were within the range of those reported to have effects on in vitro ruminal microbial fermentation (Cardozo et al., 2004; Cardozo et al., 2005; Busquet et al., 2006).

The addition of ANI, CAP, and CIE2 had small effects on the concentration of N fractions (Table 5). In the conditions of the present trial, CAP only decreased ($P < 0.05$) the average concentration of LPep N and increased ($P < 0.05$) SPep+AA N concentration without affecting ammonia N concentration. There is limited information on the effect of CAP on N fraction metabolism in the rumen. Cardozo et al. (2004) indicated that 0.22 mg/L of CAP had no effect on N fraction concentrations, and Busquet et al. (2005b) also reported that CAP (doses of 3 to 300 mg/L) had no effect on ammonia N concentration in a dairy-type ruminal environment. However, Cardozo et al. (2005) in an in vitro batch culture fermentation using ruminal fluid from heifers fed a 10:90 forage:concentrate diet found that doses from 0.3 to 30 mg/L of CAP decreased the ammonia N concentration. Results of the present experiment suggest that at the estimated concentration of 12.0 mg/L, CAP extract stimulated LPep N degradation, resulting in an accumulation of SPep+AA N in the rumen. Although the availability of SPep+AA N may enhance microbial protein synthesis (Hristov et al., 1999), the implication of these changes on microbial growth should be confirmed.

The accumulation ($P < 0.05$) of SPep+AA N and the lower ($P < 0.05$) branched-chain VFA and ammonia N concentrations in CIE2 in the present trial suggest that deamination activity was inhibited. In Exp. 1, CIE1 also increased SPep+AA N concentration and decreased ammonia N and branched-chain VFA concentration. Similar response was observed in vitro in a beef-type environment at low pH (Cardozo et al., 2005; doses from 0.3 to 30 mg/L of cinnamaldehyde and eugenol).

The addition of ANI decreased ($P < 0.05$) ammonia N concentration. This result is in contrast with a previous report (Cardozo et al., 2004), which indicated that ANI extract stimulated peptidolysis and deamination, resulting in an accumulation of ammonia N concentration. This inconsistency may be attributed to differences in diets and pH between the trials. Results from the

Table 6. Effect of natural plant extracts on average protozoal populations in Holstein heifers (Exp. 2)

Protozoa, log_{10} (counts/mL)	Treatment[1,2]				
	Control	ANI	CAP	CIE2	SEM
Entodiniomorphs	5.74	5.33[a]	5.64	5.59	0.07
Holotrichs	4.43	4.22[a]	4.40	4.29	0.08

[a]Within a row, means are different from control ($P < 0.05$).
[1]Data are means of 4 steers.
[2]ANI = anise oil; CAP = capsicum extract; CIE2 = mixture of cinnamaldehyde and eugenol.

present experiment suggest that ANI inhibited deamination of AA, although the lower concentration of ammonia N could be due to the reduction of protozoal counts.

Protozoal Populations. There were no time of sampling × treatment interactions for protozoal counts, and average treatment effects are reported (Table 6). Protozoal counts (entodiniomorphs and holotrichs) were only affected by ANI, which were lower ($P < 0.05$) compared with control. In contrast, CIE2 had no effect, which contradicts results observed in Exp. 1. There is very limited information on the effect of natural plant extracts and secondary compounds on ruminal protozoal counts. Rumen ciliate protozoa play diverse roles in ruminal metabolism, and in their absence the numbers of bacteria and starch degradation increase, and ammonia N concentration decreases (Van Nevel and Demeyer, 1988). Newbold et al. (2004) recently reported that a blend of essential oils containing thymol, limonene, and guaiacol had no effect on protozoal numbers. Although the mechanism of action of anise is not well understood, its lipophilic nature may allow them to cross the cell membrane of protozoa and yield antiprotozoal activity (Helander et al., 1998; Francis et al., 2002). There are no other reports available on the effect of ANI on ruminal protozoal counts.

IMPLICATIONS

Some plant extracts are able to modify intake and rumen microbial fermentation. However, the direction of these changes and their implications vary depending on the extract used. Whereas a mixture of cinnamaldehyde and eugenol appears to maintain or reduce dry matter intake, capsicum oil increased dry matter intake. Considering that reducing acetate and ammonia and increasing propionate concentrations is more efficient for beef production, capsicum oil, anise oil, or a mixture of cinnamaldehyde and eugenol may be useful as additives, but data on animal performance are required to confirm their benefits.

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