

Screening for the effects of natural plant extracts at different pH on in vitro rumen microbial fermentation of a high-concentrate diet for beef cattle¹

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ABSTRACT: Six natural plant extracts and three secondary plant metabolites were tested at five doses (0, 0.3, 3, 30, and 300 mg/L) and two different pH (7.0 and 5.5) in a duplicate $9 \times 5 \times 2$ factorial arrangement of treatments to determine their effects on in vitro microbial fermentation using ruminal fluid from heifers fed a high-concentrate finishing diet. Treatments were extracts of garlic (GAR), cinnamon (CIN), yucca (YUC), anise (ANI), oregano (ORE), and capsicum (CAP) and pure cinnamaldehyde (CDH), anethole (ATL), and eugenol (EUG). Each treatment was tested in triplicate and in two periods. Fifty milliliters of a 1:1 ruminal fluid-to-buffer solution were introduced into polypropylene tubes supplied with 0.5 g of DM of a 10:90 forage:concentrate diet (15.4% CP, 16.0% NDF; DM basis) and incubated for 24 h at 39°C. Samples were collected for ammonia N and VFA concentrations. The decrease in pH from 7.0 to 5.5 resulted in lower ($P < 0.05$) total VFA, ammonia N, branched-chain VFA concentration, acetate proportion, and acetate:propionate, and in a higher ($P < 0.05$) propionate proportion. The interaction between pH and doses was significant for all measurements, except for ATL and CDH for butyrate, ATL and EUG for acetate:propionate ratio, and ORE for ammonia N concentration. The high dose of all plant extracts

decreased ($P < 0.05$) total VFA concentrations. When pH was 7.0, ATL, GAR, CAP, and CDH decreased ($P < 0.05$) total VFA concentration, and ANI, ORE, CIN, CAP, and CDH increased ($P < 0.05$) the acetate:propionate. The CIN, GAR, CAP, CDH, ORE, and YUC decreased ($P < 0.05$), and EUG, ANI, and ATL increased ($P < 0.05$) ammonia N concentration. The effects of plant extracts on the fermentation profile when pH was 7.0 were not favorable for beef production. In contrast, when pH was 5.5, total VFA concentration did not change (ATL, ANI, ORE, and CIN) or increased ($P < 0.05$) (EUG, GAR, CAP, CDH, and YUC), and the acetate:propionate (ORE, GAR, CAP, CDH, and YUC) decreased ($P < 0.05$), which would be favorable for beef production. Ammonia N (ATL, ANI, CIN, GAR, CAP, and CDH) and branched-chain VFA (ATL, EUG, ANI, ORE, CAP, and CDH) concentrations also were decreased ($P < 0.05$), suggesting that deamination was inhibited. Results indicate that the effects of plant extracts on ruminal fermentation in beef cattle diets may differ depending on ruminal pH. When pH was 5.5, GAR, CAP, YUC, and CDH altered ruminal microbial fermentation in favor of propionate, which is more energetically efficient.

Key Words: Fermentation, Plant Extracts, Ruminal pH

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Introduction

Intensive beef production systems rely on feeding large quantities of cereal grains; however, highly fermentable diets may result in ruminal acidosis, bloat, and digestive and metabolic upsets (Nocek, 1997). Ionophores have been used successfully to improve the efficiency of beef production and to prevent or decrease

the incidence of digestive upsets (Chalupa et al., 1980; Bergen and Bates, 1984), but the use of ionophores in animal feeds will be restricted in the European Union in 2006 (OJEU, 2003). It has been estimated that the elimination of antibiotics from ruminant feeds will result in an increase in production costs of 3.5 to 5.0% (Carro and Ranilla, 2000). Therefore, it is necessary to identify potential feeding strategies and/or additives that will allow producers to maintain the current level of production without increasing the cost or the incidence of digestive upsets.

Natural plant extracts contain secondary metabolites that have shown antimicrobial activity (Cowan, 1999). Some of them have already been tested for their effects on ruminal microbial fermentation, including sarsapon-

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ins (Ryan et al., 1997), phenolic compounds (Evans and Martin, 2000), and essential oils (Cardozo et al., 2004; Busquet et al., 2005a). Most of these studies have been conducted using ruminal fluid from dairy cows fed high-forage diets. Microbial populations and ruminal fermentation conditions in cattle fed high-concentrate diets may be very different because of the type of substrate being fermented or the resulting pH. Therefore, the search for additives that will help to replace ionophores in beef diets needs to be tested in a high-concentrate, low-pH environment.

The objective of this trial was to test the effects of dose of six natural plant extracts and three purified secondary metabolites at two different pH (7.0 and 5.5) on the ruminal microbial fermentation profile in high-concentrate diets in an in vitro batch culture fermentation system.

Materials and Methods

Apparatus and Experimental Design

Thirty 100-mL polypropylene tubes with gas-release rubber stoppers described by Tilley and Terry (1963) were used for each additive. Ruminal fluid was obtained from two ruminally fistulated heifers (200 kg of BW) fed a 10:90 forage:concentrate diet. Ruminal fluid was strained through four layers of cheesecloth and transported to the laboratory in a prewarmed thermos. The ruminal fluid was mixed 1:1 with a buffer solution (Tilley and Terry, 1963). The mixed ruminal fluid was separated into two equal portions. The first was adjusted to pH 7.0, and the second was adjusted to pH 5.5 with 5 N NaOH or 3 N HCl, respectively. Both fluids were maintained at 39°C and flushed with CO₂-saturated gas for 15 min to maintain anaerobic conditions. Fifty milliliters of fluid were introduced into each polypropylene tube and quickly sealed with a gas-release rubber stopper. Each tube contained 0.5 g of the same 10:90 forage:concentrate diet fed to donor heifers, except that it was previously ground to pass a 1.5-mm screen (Hammer Mill Type O; P. Prat SA, Sabadell, Spain). The diet contained (DM basis) straw (10%), corn grain (30%), barley grain (25%), soybean meal (19%), tapioca (14%), sodium bicarbonate (0.5%), white salt (0.4%), calcium carbonate (0.4%), dicalcium phosphate (0.4%), and a vitamin and mineral mixture (0.3%; each kilogram of DM of the vitamin and mineral mixture contained 1,000,000 IU of vitamin A; 200,000 IU of vitamin D; 1,333 mg of vitamin E; 300 g of magnesium oxide; 67 g of sodium chloride; 33 g of sulfur; 2 g of zinc sulfate; 33 mg of iodine; 27 mg of selenium; 7 mg of cobalt sulfate; 167 mg of copper sulfate; 267 g of urea; 2.7 g of manganese sulfate; and 2.7 g of zinc methionate). The diet (15.4% CP, 16.0% NDF, and 8.2% ADF; DM basis) was formulated to meet NRC (1996) requirements for 180 kg heifers and growing at 1.2 kg/d. Treatments were no extract (control) and extracts of garlic (*Allium sativa*; **GAR**; 0.7% allicin), cinnamon (*Cinna-*

monum cassia; **CIN**; 59% cinnamaldehyde), yucca (*Yucca schidigera*; **YUC**; 8% sarsaponin), anise (*Pimpinella anisum*; **ANI**; 86% anethole), oregano (*Origanum vulgare*; **ORE**; 64% carvacrol and 16% thymol), capsicum (*Capsicum annuum*; **CAP**; 12% capsaicin), cinnamaldehyde (**CDH**; >99% cinnamaldehyde), anethole (**ATL**; >99% anethole), and eugenol (**EUG**; >99% eugenol). Plant extracts and main active components were provided by Pancosma SA (Bellegarde-sur-Valserine Cedex, France). Each treatment was tested at 0.3, 3, 30, and 300 mg/L in triplicate and in two replicated periods. All treatments and doses were dissolved in ethanol, and the control also was dosed with the equivalent amount of ethanol (0.015 mL). Treatments were dosed into the polypropylene tubes and incubated into a constant-temperature (39°C) horizontal shaking water bath for 24 h.

Chemical Analyses

The final pH of the batch after 24 h of fermentation was measured with a pH meter (Model 507; Crison Instruments, Alella, Barcelona, Spain), and samples were collected for determination of VFA and ammonia N concentrations.

Total and individual VFA were analyzed as described by Jouany (1982): 1 mL of a solution consisting of a 0.2% (wt/wt) solution of mercuric chloride, 0.2% (wt/wt) of 4-methylvaleric acid as an internal standard, and 2% (vol/vol) orthophosphoric acid was added to 4 mL of ruminal fluid and frozen at -20°C. Samples were centrifuged at 3,000 × g for 30 min, and the supernatant fraction 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 a gas flow rate of 29.9 mL/min. Branched-chained VFA were calculated as isobutyric + isovaleric acids.

Ammonia N concentration was analyzed as described by Chaney and Marbach (1962). A 4-mL sample of ruminal fluid was acidified with 4 mL of 0.2 N HCl and frozen at -20°C. Samples were centrifuged at 25,000 × g for 20 min, and the supernatant fraction was analyzed by spectrophotometry (Libra S21; Biochrom Technology; Cambridge, UK).

Calculations and Statistical Analyses

Data were analyzed by extract or active compound using the MIXED procedure of SAS (Version 8.2, SAS Inst., Inc., Cary, NC) for a randomized complete block design. Terms in the model contained effects of treatment, level of inclusion of extracts, two levels of pH, and their interactions. The day of the run was considered a random effect. Significant differences between means were declared at $P < 0.05$ using a multiple comparison test (Tukey, 1953).

Table 1. Effects of natural plant extracts at doses of 0, 0.3, 3, 30, and 300 mg/L and pH of 7.0 and 5.5 on total VFA concentration (mM)

Item ^a	pH 7.0					pH 5.5					SEM	<i>P</i> <		
	0	0.3	3	30	300	0	0.3	3	30	300		pH	Dose	pH × Dose
ATL	179 ^z	166 ^{zy}	158 ^{zy}	150 ^y	134 ^x	131 ^t	150 ^t	149 ^t	116 ^u	95 ^v	6.1	0.01	0.01	0.01
EUG	179 ^z	172 ^z	166 ^z	156 ^z	97 ^y	131 ^u	162 ^t	159 ^t	158 ^t	111 ^v	6.5	0.01	0.01	0.01
ANI	192 ^z	196 ^z	194 ^z	196 ^z	150 ^y	158 ^t	138 ^t	142 ^t	137 ^t	131 ^u	7.2	0.01	0.01	0.01
ORE	192 ^z	194 ^z	194 ^z	179 ^z	84 ^y	158 ^t	142 ^t	140 ^u	141 ^u	93 ^v	7.3	0.01	0.01	0.01
CIN	221 ^y	279 ^z	260 ^z	233 ^{zy}	166 ^x	161 ^t	162 ^t	167 ^t	170 ^t	115 ^u	4.1	0.01	0.01	0.01
GAR	221 ^y	204 ^{zy}	189 ^y	179 ^{yx}	172 ^x	161 ^u	168 ^u	192 ^t	206 ^t	95 ^v	9.4	0.01	0.01	0.01
CAP	178 ^z	162 ^y	160 ^y	158 ^y	97 ^x	127 ^u	153 ^t	155 ^t	155 ^t	101 ^v	9.2	0.01	0.01	0.01
CDH	178 ^z	169 ^{zy}	165 ^y	164 ^y	153 ^x	127 ^u	174 ^t	176 ^t	180 ^t	108 ^v	8.3	0.01	0.01	0.01
YUC	175 ^{zy}	182 ^z	166 ^y	159 ^y	135 ^x	131 ^u	173 ^t	166 ^t	109 ^{uv}	98 ^v	9.3	0.01	0.01	0.01

^aTreatments: ATL = anethole, EUG = eugenol, ANI = anise, ORE = oregano, CIN = cinnamon, GAR = garlic, CAP = capsicum, CDH = cinnamaldehyde, and YUC = yucca.

^{t,u,v}Means within a row at pH 5.5 followed by different superscripts differ, *P* < 0.05.

^{x,y,z}Means within a row at pH 7.0 followed by different superscripts differ, *P* < 0.05.

Results

The decrease in pH from 7.0 to 5.5 resulted in lower (*P* < 0.05) total VFA concentration (Table 1) and acetate proportion (Table 2), greater (*P* < 0.05) propionate proportion (Table 3), and lower (*P* < 0.05) butyrate proportion (Table 4), total branched-chain VFA concentration (Table 5), acetate:propionate (Table 6), and ammonia N concentration (Table 7). The final pH of mixed ruminal fluid was not affected by increasing the doses of treatments (average of 6.89 ± 0.27 for high pH and average of 5.48 ± 0.34 for low pH; data not shown).

The interaction between pH and extracts was significant (*P* < 0.05) for all treatments for the concentration of total VFA, branched-chain VFA, and ammonia N (Tables 1, 5, and 7) for the proportion of acetate, propionate, and butyrate (Tables 2 to 4) and for acetate:propionate (Table 6), with some exceptions: ORE on ammonia N concentration (Table 7), ATL and CDH on butyrate proportion (Table 4), and ATL and EUG on acetate:propionate (Table 6). These results indicate that the effects

of plant extracts on ruminal microbial fermentation were pH-dependent; therefore, these effects will be presented and discussed separately by pH.

Effects of Natural Plant Extract on Ammonia N and Total and Individual VFA Concentrations at pH 7.0

When the pH of the incubation media was 7.0, high doses (300 mg/L) of all extracts resulted in a strong inhibition (*P* < 0.05) of total VFA concentration ranging from 14% in CDH to 56% in ORE (Table 1). Because this effect was negative for ruminal microbial fermentation, data for individual VFA proportions of all extracts at 300 mg/L were removed from Tables 2 through 6, to improve readability and interpretation of results. Lower doses of ATL (30 mg/L), GAR (0.3, 3, and 30 mg/L), CDH (3, 3, and 30 mg/L), and CAP (0.3, 3, and 30 mg/L) decreased (*P* < 0.05) total VFA concentration compared with the control. In contrast, total VFA concentration was greater (*P* < 0.05) in CIN (0.3 and 3 mg/L) and was not affected by EUG, ANI, ORE, or YUC extracts compared with the control (Table 1).

Table 2. Effects of natural plant extracts at doses of 0, 0.3, 3, and 30 mg/L and pH of 7.0 and 5.5 on acetate proportions (mol/100 mol)

Item ^a	pH 7.0				pH 5.5				SEM	<i>P</i> <		
	0	0.3	3	30	0	0.3	3	30		pH	Dose	pH × Dose
ATL	53.9	55.7	56.6	55.5	49.6	50.7	50.2	48.5	1.73	0.01	0.01	0.04
EUG	53.9	55.0	53.9	57.2	49.6	51.4	51.2	51.2	1.82	0.01	0.01	0.04
ANI	57.9 ^y	62.8 ^z	62.6 ^z	61.6 ^z	51.6 ^t	48.7 ^t	45.2 ^u	44.9 ^u	1.41	0.01	0.01	0.01
ORE	57.9 ^y	62.3 ^z	62.6 ^z	63.2 ^z	51.6 ^t	48.7 ^{tu}	47.5 ^u	45.5 ^u	1.16	0.01	0.01	0.01
CIN	50.4 ^y	54.8 ^z	52.5 ^z	51.7 ^{zy}	47.3	48.2	48.2	48.0	0.52	0.01	0.01	0.01
GAR	50.4 ^z	50.6 ^z	49.9 ^z	47.1 ^y	47.3 ^t	46.5 ^t	45.9 ^{tu}	44.1 ^u	0.57	0.01	0.01	0.01
CAP	51.3 ^x	52.7 ^y	53.4 ^y	55.4 ^z	48.5 ^t	45.2 ^u	38.6 ^v	37.6 ^v	0.36	0.01	0.01	0.01
CDH	51.3	51.6	53.5	52.7	48.5 ^t	43.7 ^u	42.3 ^v	39.2 ^v	1.61	0.01	0.01	0.01
YUC	53.3	49.3	52.8	50.3	46.9 ^t	36.7 ^u	35.4 ^u	34.1 ^u	1.01	0.01	0.01	0.01

^aTreatments: ATL = anethole, EUG = eugenol, ANI = anise, ORE = oregano, CIN = cinnamon, GAR = garlic, CAP = capsicum, CDH = cinnamaldehyde, and YUC = yucca.

^{t,u,v}Means within a row at pH 5.5 followed by different superscripts differ, *P* < 0.05.

^{x,y,z}Means within a row at pH 7.0 followed by different superscripts differ, *P* < 0.05.

Table 3. Effects of natural plant extracts at doses of 0, 0.3, 3, and 30 mg/L and pH of 7.0 and 5.5 on propionate proportions (mol/100 mol)

Item ^a	pH 7.0				pH 5.5				SEM	<i>P</i> <		
	0	0.3	3	30	0	0.3	3	30		pH	Dose	pH × Dose
ATL	27.1	24.4	26.5	27.4	35.5 ^t	28.4 ^u	28.7 ^u	31.1 ^u	1.73	0.01	0.01	0.02
EUG	27.1	23.8	24.4	22.6	35.5 ^t	28.5 ^u	28.7 ^u	28.4 ^u	1.88	0.01	0.01	0.04
ANI	25.1 ^z	20.7 ^y	20.3 ^y	20.3 ^y	34.6 ^u	34.7 ^u	37.9 ^t	38.3 ^t	1.23	0.01	0.01	0.01
ORE	25.1 ^z	20.3 ^y	19.9 ^y	22.6 ^y	34.6 ^u	33.9 ^u	37.1 ^t	38.0 ^t	1.16	0.01	0.01	0.01
CIN	36.6 ^z	30.6 ^y	24.7 ^y	33.5 ^y	38.8	36.7	36.9	36.3	0.98	0.01	0.01	0.01
GAR	36.6	34.7	34.9	37.0	38.8 ^u	41.7 ^{tu}	42.1 ^{tu}	43.6 ^t	0.88	0.01	0.01	0.01
CAP	33.8 ^z	28.4 ^y	28.2 ^y	24.8 ^x	36.2 ^u	36.8 ^u	44.5 ^t	46.0 ^t	0.56	0.01	0.01	0.01
CDH	33.8 ^z	31.7 ^{zy}	27.6 ^y	28.9 ^y	36.2 ^u	39.6 ^{tu}	41.7 ^t	44.7 ^t	1.92	0.01	0.01	0.01
YUC	33.6 ^{zy}	35.8 ^z	29.9 ^y	34.8 ^{zy}	37.3 ^u	48.8 ^t	49.4 ^t	48.2 ^t	1.29	0.01	0.01	0.01

^aTreatments: ATL = anethole, EUG = eugenol, ANI = anise, ORE = oregano, CIN = cinnamon, GAR = garlic, CAP = capsicum, CDH = cinnamaldehyde, and YUC = yucca.

^{t,u,v}Means within a row at pH 5.5 followed by different superscripts differ, *P* < 0.05.

^{x,y,z}Means within a row at pH 7.0 followed by different superscripts differ, *P* < 0.05.

Table 4. Effects of natural plant extracts at doses of 0, 0.3, 3, and 30 mg/L and pH of 7.0 and 5.5 on butyrate proportions (mol/100 mol)

Item ^a	pH 7.0				pH 5.5				SEM	<i>P</i> <		
	0	0.3	3	30	0	0.3	3	30		pH	Dose	pH × Dose
ATL	10.4	8.4	9.2	9.7	8.1	9.8	9.9	9.1	2.78	0.10	0.24	0.06
EUG	10.4 ^z	8.4 ^y	8.5 ^y	8.1 ^y	8.1 ^v	9.5 ^u	9.5 ^u	9.5 ^u	0.26	0.02	0.04	0.01
ANI	7.9	8.5	8.6	8.5	7.2 ^v	9.5 ^u	9.5 ^u	9.7 ^u	0.33	0.01	0.03	0.01
ORE	7.9 ^y	8.4 ^{zy}	8.4 ^{zy}	8.8 ^z	7.2 ^v	9.7 ^u	10.1 ^u	9.6 ^u	0.28	0.01	0.03	0.01
CIN	7.8 ^z	7.3 ^y	7.1 ^y	7.1 ^y	7.3	7.5	7.5	7.5	0.11	0.44	0.85	0.01
GAR	7.8	8.0	8.1	8.1	7.3 ^v	7.3 ^v	7.4 ^{uv}	7.9 ^u	0.17	0.01	0.01	0.01
CAP	7.4 ^y	8.3 ^z	8.2 ^z	8.3 ^z	6.8 ^v	9.1 ^u	9.2 ^u	9.2 ^u	0.24	0.01	0.01	0.01
CDH	7.4 ^y	8.2 ^z	8.2 ^z	8.2 ^z	6.8	7.1	7.1	7.4	0.39	0.01	0.01	0.96
YUC	7.3	7.2	7.1	7.3	7.1 ^v	8.0 ^{uv}	7.4 ^v	8.0 ^u	0.22	0.05	0.04	0.01

^aTreatments: ATL = anethole, EUG = eugenol, ANI = anise, ORE = oregano, CIN = cinnamon, GAR = garlic, CAP = capsicum, CDH = cinnamaldehyde, and YUC = yucca.

^{u,v}Means within a row at pH 5.5 followed by different superscripts differ, *P* < 0.05.

^{y,z}Means within a row at pH 7.0 followed by different superscripts differ, *P* < 0.05.

Table 5. Effects of natural plant extracts at doses of 0, 0.3, 3, and 30 mg/L and pH of 7.0 and 5.5 on branched-chain VFA concentrations (mM)^a

Item ^b	pH 7.0				pH 5.5				SEM	<i>P</i> <		
	0	0.3	3	30	0	0.3	3	30		pH	Dose	pH × Dose
ATL	9.4 ^{zy}	10.9 ^z	8.2 ^y	7.2 ^y	7.4 ^u	5.7 ^u	5.8 ^u	3.2 ^v	1.19	0.01	0.01	0.01
EUG	9.4	11.2	11.1	9.3	7.4 ^u	5.7 ^v	5.7 ^v	5.8 ^v	0.08	0.01	0.01	0.03
ANI	10.5 ^y	12.5 ^z	13.5 ^z	12.6 ^z	8.2 ^u	4.8 ^v	4.7 ^v	4.4 ^v	0.76	0.01	0.01	0.01
ORE	10.5 ^y	12.6 ^z	13.4 ^z	9.9 ^y	8.2 ^u	4.6 ^v	3.5 ^v	4.5 ^v	0.84	0.01	0.01	0.01
CIN	5.8 ^y	6.6 ^z	7.1 ^z	6.6 ^z	4.3 ^u	3.9 ^v	4.0 ^v	4.1 ^v	0.12	0.01	0.01	0.01
GAR	5.8 ^y	7.5 ^z	6.6 ^{zy}	5.8 ^y	4.3	4.0	4.6	4.4	0.37	0.01	0.01	0.01
CAP	6.8 ^y	11.8 ^z	12.2 ^z	12.1 ^z	5.0 ^u	3.8 ^v	3.8 ^v	3.8 ^v	0.25	0.01	0.01	0.01
CDH	6.8 ^x	9.1 ^y	12.3 ^z	11.7 ^z	5.0 ^u	2.9 ^v	3.1 ^v	2.3 ^v	0.56	0.01	0.01	0.01
YUC	4.1 ^x	5.4 ^{yx}	8.3 ^z	6.6 ^{zy}	3.3	3.7	3.5	3.8	0.37	0.01	0.01	0.01

^aBranched-chain VFA = isobutyric + isovaleric acids.

^bTreatments: ATL = anethole, EUG = eugenol, ANI = anise, ORE = oregano, CIN = cinnamon, GAR = garlic, CAP = capsicum, CDH = cinnamaldehyde, and YUC = yucca.

^{u,v}Means within a row at pH 5.5 followed by different superscripts differ, *P* < 0.05.

^{x,y,z}Means within a row at pH 7.0 followed by different superscripts differ, *P* < 0.05.

Table 6. Effects of natural plant extracts at doses of 0, 0.3, 3, and 30 mg/L and pH of 7.0 and 5.5 on acetate-to-propionate ratio

Item ^a	pH 7.0				pH 5.5				SEM	<i>P</i> <		
	0	0.3	3	30	0	0.3	3	30		pH	Dose	pH × Dose
ATL	2.0	2.3	2.0	2.0	1.4	1.8	1.8	1.6	0.75	0.01	0.01	0.31
EUG	2.0	2.4	2.4	2.5	1.4	1.8	1.8	1.9	0.78	0.01	0.01	0.96
ANI	2.4 ^y	3.2 ^z	3.3 ^z	3.2 ^z	1.6	1.5	1.2	1.2	0.51	0.01	0.01	0.01
ORE	2.4 ^y	3.0 ^z	3.3 ^z	2.1 ^y	1.6 ^u	1.4 ^{uv}	1.4 ^{uv}	1.2 ^v	0.17	0.01	0.01	0.01
CIN	1.4 ^y	1.7 ^z	1.6 ^z	1.6 ^z	1.2	1.2	1.2	1.3	0.05	0.01	0.01	0.01
GAR	1.4 ^{zy}	1.5 ^z	1.4 ^{zy}	1.3 ^y	1.2 ^u	1.2 ^u	1.2 ^u	1.1 ^v	0.05	0.01	0.01	0.01
CAP	1.5 ^x	1.9 ^y	1.9 ^y	2.3 ^z	1.3 ^u	1.2 ^{uv}	0.9 ^v	0.8 ^v	0.04	0.01	0.01	0.01
CDH	1.5 ^y	1.7 ^{zy}	1.9 ^z	1.8 ^z	1.3 ^u	1.1 ^{uv}	1.0 ^{vw}	0.9 ^w	0.07	0.01	0.01	0.01
YUC	1.6 ^{zy}	1.4 ^y	1.8 ^z	1.5 ^{zy}	1.3 ^u	0.8 ^v	0.7 ^v	0.7 ^v	0.06	0.01	0.01	0.01

^aTreatments: ATL = anethole, EUG = eugenol, ANI = anise, ORE = oregano, CIN = cinnamon, GAR = garlic, CAP = capsicum, CDH = cinnamaldehyde, and YUC = yucca.

^{u,v,w}Means within a row at pH 5.5 followed by different superscripts differ, *P* < 0.05.

^{x,y,z}Means within a row at pH 7.0 followed by different superscripts differ, *P* < 0.05.

The GAR (30 mg/L) decreased (*P* < 0.05), and ANI, ORE, and CAP (0.3, 3, and 30 mg/L) and CIN (0.3 and 3 mg/L) increased (*P* < 0.05) the acetate proportion compared with the control (Table 2). Propionate proportion was less (*P* < 0.05) in ANI, ORE, CIN, and CAP (0.3, 3, and 30 mg/L) and CDH (3 and 30 mg/L) compared with the control (Table 3). The resulting acetate:propionate was greater (*P* < 0.05) in ANI, CIN, and CAP (0.3, 3, and 30 mg/L), ORE (0.3 and 3 mg/L), and CDH (3 and 30 mg/L) compared with the control (Table 6). The proportion of butyrate was greater (*P* < 0.05) in CAP and CDH (0.3, 3, and 30 mg/L) and in ORE (30 mg/L) and was less for EUG and CIN (0.3, 3, and 30 mg/L) compared with the control (Table 4). The ANI, CIN, CAP, and CDH (0.3, 3, and 30 mg/L), ORE (0.3 and 3 mg/L), GAR (0.3 mg/L), and YUC (3 and 30 mg/L) increased (*P* < 0.05) branched-chain VFA concentrations compared with the control (Table 5).

At pH 7.0, high doses (300 mg/L) of all treatments also decreased (*P* < 0.05) ammonia N concentration between 33% in CAP to 66% in CDH compared with the control

(Table 7). Lower doses of CIN, GAR, CAP, and CDH (0.3, 3, and 30 mg/L), YUC (3 and 30 mg/L), and ORE (30 mg/L) also decreased (*P* < 0.05) ammonia N concentration compared with the control. In contrast, low doses of ATL and ANI (0.3, 3, and 30 mg/L) and EUG (0.3 and 3 mg/L) increased (*P* < 0.05) ammonia N concentration compared with the control.

Effects of Natural Plant Extract on Ammonia N, and Total and Individual VFA Concentrations at pH 5.5

As with the pH 7.0 data, when pH of the incubation media was 5.5, high doses (300 mg/L) of all treatments resulted in an inhibition (*P* < 0.05) of total VFA concentration compared with the control, ranging from 20 to 41% in CAP and ORE, respectively (Table 1). Because this inhibition was negative for ruminal microbial fermentation, data of individual VFA proportions of all extracts at 300 mg/L were removed from Tables 2 through 6 to improve readability and interpretation of results. Supplementation of ATL, ORE, ANI, and CIN

Table 7. Effects of natural plant extracts at doses of 0, 0.3, 3, 30, and 300 mg/L and pH of 7.0 and 5.5 on ammonia N concentration (mg/100 mL)

Item ^a	pH 7.0					pH 5.5					SEM	<i>P</i> <		
	0	0.3	3	30	300	0	0.3	3	30	300		pH	Dose	pH × Dose
ATL	25.5 ^y	28.0 ^z	29.7 ^z	28.7 ^z	11.8 ^x	17.8 ^u	16.2 ^{ut}	13.9 ^s	13.7 ^s	14.4 ^{ts}	0.5	0.01	0.01	0.01
EUG	25.5 ^y	28.3 ^z	28.5 ^z	27.4 ^y	8.8 ^x	17.8 ^t	23.4 ^u	24.0 ^u	22.4 ^u	15.2 ^s	0.6	0.01	0.01	0.01
ANI	23.4 ^x	28.5 ^y	33.8 ^z	33.8 ^z	10.7 ^w	16.5 ^u	12.8 ^{ts}	11.7 ^s	14.1 ^t	15.6 ^{ut}	0.8	0.01	0.01	0.01
ORE	23.4 ^z	20.9 ^{zy}	19.9 ^{zy}	17.1 ^y	8.3 ^x	16.5 ^u	17.6 ^u	15.8 ^u	14.9 ^u	6.9 ^t	1.5	0.01	0.01	0.41
CIN	27.8 ^z	23.9 ^y	22.8 ^y	19.0 ^x	10.6 ^w	19.2 ^u	18.1 ^u	15.6 ^t	14.2 ^t	10.1 ^s	0.5	0.01	0.01	0.01
GAR	27.8 ^z	23.7 ^y	22.4 ^y	18.7 ^x	12.4 ^w	19.2 ^u	14.6 ^{ts}	13.8 ^s	15.7 ^t	18.1 ^u	0.5	0.01	0.01	0.01
CAP	29.2 ^z	26.1 ^y	23.9 ^y	22.1 ^y	19.3 ^x	18.6 ^u	15.2 ^t	12.6 ^s	13.0 ^s	17.5 ^u	0.5	0.01	0.01	0.01
CDH	29.2 ^z	26.9 ^y	22.8 ^x	11.8 ^w	9.6 ^v	18.6 ^u	15.4 ^t	15.6 ^t	14.0 ^t	10.1 ^s	1.2	0.01	0.01	0.01
YUC	27.2 ^z	23.4 ^z	20.1 ^y	13.7 ^x	15.5 ^x	17.0 ^{ts}	16.9 ^{ts}	19.9 ^u	16.6 ^{ts}	15.7 ^s	0.6	0.01	0.01	0.01

^aTreatments: ATL = anethole, EUG = eugenol, ANI = anise, ORE = oregano, CIN = cinnamon, GAR = garlic, CAP = capsicum, CDH = cinnamaldehyde, and YUC = yucca.

^{s,t,u}Means within a row at pH 5.5 followed by different superscripts differ, *P* < 0.05.

^{v,w,x,y,z}Means within a row at pH 7.0 followed by different superscripts differ, *P* < 0.05.

had no effect and EUG, CAP, and CDH (0.3, 3, and 30 mg/L), GAR (3 and 30 mg/L), and YUC (0.3 and 3 mg/L) increased ($P < 0.05$) total VFA concentration compared with the control.

The CAP, CDH, and YUC (0.3, 3, and 30 mg/L), ANI and ORE (3 and 30 mg/L), and GAR (30 mg/L) decreased ($P < 0.05$) the acetate proportion (Table 2). The ANI, ORE, CAP, and CDH (3 and 30 mg/L), GAR (30 mg/L), and YUC (0.3, 3, and 30 mg/L) increased ($P < 0.05$) and ATL and EUG (0.3, 3, and 30 mg/L) decreased ($P < 0.05$) the propionate proportion (Table 3). The resulting acetate:propionate was less ($P < 0.05$) for CAP and CDH (3 and 30 mg/L), YUC (0.3, 3, and 30 mg/L), ORE (30 mg/L), and GAR (30 mg/L) than for the control (Table 6). The EUG, ANI, ORE, and CAP (0.3, 3, and 30 mg/L), GAR (30 mg/L), and YUC (30 mg/L) increased ($P < 0.05$) butyrate proportion compared with the control (Table 4). The ATL (30 mg/L) and EUG, ANI, ORE, CAP, and CDH (0.3, 3, and 30 mg/L) decreased ($P < 0.05$) branched-chain VFA concentrations (Table 5). At pH 5.5, high doses (300 mg/L) of all treatments also resulted in a decrease in ammonia N concentration compared with the control at pH 5.5, ranging from 5 to 61% in ANI and ORE, respectively (Table 7). Moderate doses of ANI, GAR, CAP, and CDH (0.3, 3, and 30 mg/L) and ATL and CIN (3 and 30 mg/L) also resulted in moderate decreases ($P < 0.05$) in ammonia N concentration compared with the control at pH 5.5. In contrast, EUG (0.3, 3, and 30 mg/L) and YUC (3 mg/L) increased ($P < 0.05$) ammonia N concentration compared with the control.

Discussion

Effects of pH

The increase in propionate proportion and the decrease in total VFA and branched-chain VFA concentrations, acetate and butyrate proportions, acetate:propionate, and ammonia N concentration when pH was decreased from 7.0 to 5.5 were expected and agree with the results of previous studies (Shriver et al., 1986; Lana et al., 1998; Calsamiglia et al., 2002). Most fibrolytic ruminal bacteria, which are generally acetate and butyrate producers, are sensitive to low ruminal pH (Russell and Dombrowki, 1980; Hoover, 1986). In contrast, the amylolytic bacteria are acid-tolerant and are responsible for most of the production of propionate in the rumen (Wolin and Miller, 1988; Brockman, 1993).

Effects of Natural Plant Extracts

Natural plant extracts represent one of the alternatives to the use of antibiotic growth promoters in animal feeds (Kamel, 2001). Until recently, there has been very limited research on the effect of these extracts on ruminal microbial fermentation, and most research has been designed to examine their effects in dairy cattle-type environments (high-forage diets at pH > 6.2; Wu et al., 1994; Wilson et al., 1997; Cardozo et al., 2004). The

intent of the present screening was to identify natural plant extracts that would improve ruminal microbial fermentation in beef cattle diets. Total VFA concentration is the result of diet fermentation, and lower acetate:propionate reflects a shift in ruminal fermentation that is more efficient for beef production systems (Wolin and Miller, 1988; Brockman, 1993). Therefore, the main criteria for selecting the extracts were the increase or no change in total VFA concentration and the decrease in acetate:propionate. The benefits of modifying ammonia N concentration in beef cattle diets are more difficult to define. Whereas many diets result in high ammonia N concentrations in the rumen and losses of N in the urine (Tamminga, 1992), in beef cattle fed high-concentrate diets, ammonia N concentration is frequently, but not always, below the minimum concentration thought to be required to optimize microbial growth (Devant et al., 2000). Therefore, the increase or decrease in ammonia N concentration may be considered of interest depending on the diet being fed.

High doses of all extracts resulted in lower VFA concentration, which confirms their antimicrobial effect (Cowan, 1999). Similar results were obtained by Busquet et al. (2004) when supplementing high doses (3,000 mg/L) of plant extracts in an *in vitro* fermentation system fed a 50:50 forage:concentrate diet and pH 7.0. Evans and Martin (2000) also reported that high doses of thymol (400 mg/L; main active component of ORE) inhibited VFA production in mixed ruminal microbial fermentation. Furthermore, low and moderate doses of ATL, GAR, CAP, and CDH at pH 7.0 also decreased total VFA concentration, which would preclude its potential use in beef cattle diets; however, low and moderate doses of EUG, ANI, ORE, and YUC at pH 7.0 had no negative effects on total VFA concentrations, suggesting that these doses were not toxic to ruminal microbes. Busquet et al. (2004) observed similar responses with EUG, ANI, ORE, and YUC (doses from 3 to 30 mg/L) in a dairy cow-type environment. The supplementation with ANI, ORE, CIN, CAP, and CDH, however, resulted in a greater acetate:propionate, which is energetically less efficient because of the loss of a carbon as methane (Wolin and Miller, 1988). Therefore, there seems to be no potential benefit in using plant extracts for improving ruminal fermentation profile in beef production systems when ruminal pH is high. The average ammonia N concentration of the control at pH 7.0 (26.6 ± 3.38 mg/100 mL) was above the minimum concentration required for adequate ruminal microbial activity (Satter and Slyter, 1974). Compared with the control at pH 7.0, CIN, GAR, CAP, CDH, ORE, and YUC resulted in lower ammonia N concentration and agree with results reported by Busquet et al. (2004) with similar doses of ORE and CDH, by Grobner et al. (1982) with 1.45 mg/L of YUC, and by Ryan et al. (1997) with 100 mg/L of YUC. In contrast, EUG, ANI, and ATL increased ammonia N concentration, which also is consistent with previous reports (Cardozo et al., 2004; Bus-

quet et al., 2005a) and may be beneficial for beef cattle fed high-concentrate diets (Devant et al., 2000).

The effects of plant extracts on ruminal microbial fermentation at pH 5.5 were completely different than at pH 7.0. Most research on the effect of plant extracts on ruminal microbial fermentation has been conducted in fermentation conditions where pH was >6.0 (Wu et al., 1994; Cardozo et al., 2004; Busquet et al., 2005a, b) and data available on their effects at lower pH are limited. When ruminal pH was 5.5, total VFA concentrations were similar (ATL, ANI, ORE, and CIN) or greater (EUG, GAR, CAP, CDH, and YUC) than the control. The lower acetate; the greater propionate proportions in ANI, ORE, GAR, CAP, CDH, and YUC; and the resulting lower acetate:propionate in ORE, GAR, CAP, CDH, and YUC suggest a shift in the rumen microbial population that may result in a fermentation profile that will benefit beef production. In addition, the decrease in the acetate:propionate together with the increase in butyrate proportion observed in ANI, ORE, CAP, GAR, and YUC is consistent with the fermentation profile commonly found in methane inhibitors (Chalupa et al., 1980; Martin and Macy, 1985), suggesting a potential mechanism of action. In fact, Busquet et al. (2005b) already suggested that some of these extracts might act as methane inhibitors. The decrease in ammonia N concentration with ATL, ANI, CIN, GAR, CAP, and CDH suggests that these additives decreased deamination activity or that bacteria used peptides and AA as a N source, decreasing ammonia N concentration. In contrast, the accumulation of ammonia N concentrations in EUG and YUC suggests that these additives stimulated deamination activity, which could be desirable if ammonia N concentration limits microbial protein synthesis in feedlot cattle fed high percentages of concentrate (Devant et al., 2000).

The mechanism of action of most essential oils is related to their ability to disrupt cell membranes (Griffin et al., 1999; Dorman and Deans, 2000; Ultee et al., 2002). This results in a decreased proton-motive force across the cell membrane and decreased ATP synthesis, slowing microbial growth and eventually causing cell death (Ultee et al., 2002). Although essential oils are effective against gram-positive and gram-negative bacteria, the outer membrane of gram-negative bacteria seems to provide some degree of protection (Davison and Naidu, 2000), making them less sensitive to the presence of essential oils. However, some essential oils and their main active components, as CIN and CDH (Helander et al., 1998) or GAR (Busquet et al., 2005b), may have other mechanisms of action, acting in the cytoplasm or inner organelles of microbial cells. The overall effects of different essential oils on rumen microbial fermentation may be the result of different sensitivities of specific microbial populations to these compounds. The different response of natural plant extracts depending on pH was unexpected, but it may be related to the dissociated (hydrophilic) or undissociated (hydrophobic) status of the active molecules. Only the un-

dissociated, hydrophobic form of the molecule is able to interact with the bilayer cell membrane. As pH decreases, acids tend to become undissociated and more hydrophobic, therefore interacting more easily with cell membranes and exerting their antimicrobial effect. In fact, Juven et al. (1994) reported that the antimicrobial effect of the essential oil of thyme, CIN, and clove bud increased as pH decreased from 6.5 to 5.5. Furthermore, bacteria seem to be more susceptible to the effects of essential oils at low pH (Skandamis and Nychas, 2000). Therefore, it could be hypothesized that there is a close relationship between the ruminal pH and the antimicrobial effect of plant extracts caused by the higher proportion of undissociated, hydrophobic form of the active molecules and the higher susceptibility of specific microbial populations, resulting in changes in the ruminal fermentation profile. Results indicate that there seems to be a potential benefit of using some extracts to improve the ruminal fermentation profile in beef production systems when ruminal pH is low; however, the effects of daily fluctuation of pH generally observed in vivo require that further studies be conducted in vivo.

Implications

The effects of plant extracts on ruminal microbial fermentation are pH-dependent. At high pH, all extracts except cinnamon maintained or decreased total volatile fatty acids and maintained or increased the acetate to propionate ratio, suggesting that none of them seem beneficial for beef production. In contrast, when pH was 5.5, total volatile fatty acid concentration was greater with eugenol, garlic, capsicum, cinnamaldehyde, and yucca, and the acetate to propionate ratio was less with oregano, garlic, capsicum, cinnamaldehyde, and yucca. The effects of plant extracts on ammonia N concentrations were pH-dependent, but at pH 5.5, most of them decreased ammonia N concentrations. At the low ruminal pH expected in high-concentrate diets, oregano, garlic, capsicum, and yucca extracts and cinnamaldehyde are potentially useful in beef diets. Further studies are necessary to determine the effectiveness of these extracts on in vivo rumen microbial fermentation and animal performance.

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