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The effects of cinnamaldehyde, monensin and quebracho condensed tannin on rumen fermentation, biohydrogenation and bacteria in continuous culture system

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ABSTRACT

The objective of this experiment was to evaluate the effects of different feed additives (cinnamaldehyde, monensin, and quebracho condensed tannin extract) on fermentation, *trans* fatty acids (FA) formation and selected strains of rumen bacteria. Four continuous culture systems were used in 4 × 4 Latin square designs with 4 periods of 10 days each. Treatment diets were: control diet (44:56 forage to concentrate; CON), control plus cinnamaldehyde (CIN) at 400 mg/L, control plus monensin (MON) at 12 mg/L, and control with quebracho condensed tannin extract (QTAN) at 100 g/kg of diet (DM basis). Fermenters were fed treatment diets three times daily at 120 g/day and overflow (effluent) samples were collected from each fermenter on days 8, 9 and 10 of each period to estimate nutrients digestibility and FA composition. On day 10 of each period, three samples were collected from each fermenter at 3 and 6 h post morning feeding for volatile fatty acids (VFA), ammonia-N and bacterial analyses. Compared with the CON diet, feed additives had no effects ($P > 0.05$) on apparent dry matter (DM), neutral detergent fiber (NDF) and organic matter (OM) digestibility but apparent protein digestibility decreased ($P < 0.01$) with the QTAN and CIN diets. Compared with the CON diet, the concentration of acetate decreased ($P < 0.05$) with the MON and CIN diets. The concentration of propionate increased ($P < 0.05$) with the MON and QTAN diets and was greatest with the MON diet. Ammonia-N concentration decreased ($P < 0.01$) with all feed additives and was least with the QTAN diet. The concentration of C18:0 decreased ($P < 0.01$) with the three feed additives and was least with the MON diet. Concentration of *trans* C18:1 and vaccenic acid (VA) increased ($P < 0.05$) with the MON and CIN diets and was greatest with the MON diet. Compared with the CON diet, the concentration of *c*9*t*11CLA increased ($P < 0.05$) only with the QTAN diet. The DNA abundance of *Butyrivibrio proteoclasticum* decreased ($P < 0.05$) with the MON and CIN diets while the DNA abundance for *Butyrivibrio* VA increased ($P < 0.05$) with all feed additives compared with the CON diet. Feed additives had no effects ($P > 0.05$) on the DNA abundance of *Anaerovibrio lipolytica* and *Butyrivibrio* SA. In conclusion, results demonstrate that the feed additives used in this study affected the fermentation and biohydrogenation process.

Abbreviations: ADF, acid detergent fiber; BH, biohydrogenation; CIN, cinnamaldehyde; CLA, conjugated linoleic acids; CON, control; CP, crude protein; DM, dry matter; EO, essential oils; FA, fatty acids; MON, monensin; NDF, neutral detergent fiber; OM, organic matter; QTAN, quebracho condensed tannin; VA, vaccenic acid; VFA, volatile fatty acids.

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Addition of feed additives reduced the formation of C18:0 but only MON and CIN increased VA formation. MON and CIN effects on VA formation may in part be explained by their effects on *B. proteoclasticum*.

1. Introduction

In recent years, a considerable amount of research work has focused on the fatty acid (FA) composition of ruminant products for the effects of their consumption on human health. Especially, FA such as conjugated linoleic acids (CLA) are active in the prevention of cancer, obesity and atherosclerosis in humans (Crumb, 2011). The most common CLA isomer is the *c*9*t*11 CLA, which is formed in the rumen during biohydrogenation (BH) of dietary C18:2*n*6 or in body tissue by Δ^9-desaturase from VA, another intermediate in ruminal BH of C18 unsaturated FA (Griinari et al., 2000).

Different dietary strategies have been used to decrease the rate of ruminal BH such as feeding plant and marine oils (AbuGhazaleh and Jacobson, 2007; Gudla et al., 2012), or algae (AbuGhazaleh et al., 2009) and increasing forage to concentrate ratio (AbuGhazaleh and Jacobson, 2007). Several feed additives have been also proposed to increase the content of CLA in milk fat. Ionophores such as monensin are known to reduce methane production by inhibiting the growth of gram-positive bacteria that produce hydrogen and that may interfere with the process of ruminal BH. Ionophores have been shown to decrease the rate of ruminal BH of unsaturated FA *in vitro* (Fellner et al., 1997) and increase the content of CLA in milk fat (AlZahal et al., 2008). However, the use of ionophores as a feed antibiotic in livestock has been banned in certain counties (*e.g.* EU) and criticized by others because of the possible presence of residues in food and the emergence of resistant strains of bacteria. Therefore, plant secondary metabolites such as essential oils (EO), saponins and tannins have been suggested as a potential means to manipulate bacterial populations involved in ruminal BH to modify the FA composition of ruminant-derived food products such as milk and meat. Although several studies have examined plant secondary metabolites effects on rumen fermentation and the greenhouse gases production (Makkar et al., 1995; Sliwinski et al., 2002; Cardozo et al., 2004; Busquet et al., 2006; Chaves et al., 2008; Hassanat and Benchaar, 2012), only few studies evaluated the effects of these compounds on BH. Additionally, little information is currently available about the effects of these plant metabolites on rumen microbial ecology, particularly, bacterial species believed to be involved in the BH process. Therefore, the main objective of this study was to evaluate the effects of two secondary metabolites such as QTAN from quebracho trees (*Schinopsis balansae*) and cinnamaldehyde and monensin on rumen *trans* FA formation and selected strains of rumen bacteria using continuous culture systems.

2. Materials and methods

2.1. Experimental design

Four 1700 ± 12 mL continuous culture fermenters (Stern and Hoover, 1990) were used in 4 × 4 Latin square designs with 4 periods of 10 days each. The first 7 days were used for adaptation and last 3 days for samples collection. Treatment diets were fed at 120 g/day (DM basis) in three equal portions during the day at 0800, 1500 and 2200 h. The diets were: (1) 44:56 forage to concentrate (CON), (2) CON plus cinnamaldehyde at 400 mg/L (1200 mg/day) (CIN), (3) CON plus monensin at 12 mg/L (36 mg/day) (MON), and (4) CON with quebracho condensed tannin at 100 g/kg of diet DM (QTAN; Tables 1 and 2). The forage consisted of grass hay while the concentrate mix contained corn, soybean meal, soy hulls, corn oil and minerals. Quebracho condensed tannin (purity of 0.70; Tannin Corporation, Peabody, MA, USA) was added to the diet by partially replacing some of the hay, corn and soy hulls. Cinnamaldehyde (C_9H_8O, purity of 0.98, Sigma–Aldrich, St. Louis, MO, USA); and monensin (Acros Organics Company, NJ, USA) were dissolved in ethanol and added directly into each fermenter with each feeding.

2.2. Continuous culture

Ruminal fluid was collected from a fistulated lactating Holstein cow fed (55:45 forage to concentrate diet; DM basis). At 2–4 h after the morning feeding, ruminal contents were collected into a plastic bag under anaerobic conditions. The rumen contents brought to the laboratory; were strained through 2 layers of cheesecloth, and used within 15 min. Approximately 1300 mL of the ruminal fluid were added to each of the four fermenters, containing 400 mL of prewarmed buffer. Cultures were stirred continuously at 120 rpm *via* a magnetic impeller stirrer unit and fermenter pH was maintained above 6.2 by adjusting buffer pH level with 1 N NaOH or 1 N HCl. Fermenters pH was measured daily before feeding using a portable pH meter at 0800, 1500 and 2400. Buffer was delivered continuously at a flow rate of 1.16 mL/min (0.10 h^{-1} liquid dilution rate), using a precision pump. Anaerobic conditions in fermenters were maintained by purged with N_2 gas (80 mL/min) and fermenter temperature was maintained at 39 °C. Flow rate of each fermenter was recorded every day at 08:00.

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Table 1
Ingredient and chemical composition of treatment diets.

	Treatment	
	CON	QTAN
Ingredient (g/100 g DM)		
Grass hay	44.0	39.0
Soybean meal	16.0	16.0
Ground corn	28.0	24.0
Soy hulls	9.0	8.0
Quebracho condensed tannin	–	10.0
Corn oil	2.0	2.0
Minerals and vitamins	1.0	1.0
Chemical composition, g/100 g DM		
DM	92.0	92.7
CP	12.1	13.1
ADF	24.4	22.2
NDF	40.9	39.8
Ash	5.3	5.9
Condensed tannin	–	7.0

CON = control, QTAN = quebracho condensed tannin.

Table 2
Fatty acids composition of treatment diets (mg/g of DM).

Fatty acid	Treatment	
	CON	QTAN
C14:0	0.10	0.10
C16:0	7.92	7.92
C18:0	2.33	2.33
C18:1*n*9	12.7	12.7
C18:2*n*6	30.4	30.4
C18:3*n*3	2.95	2.95
Total fatty acids	65.3	63.9

CON = control, QTAN = quebracho condensed tannin.

2.3. Sample collection and analysis

Starting on days 8, 9 and 10 of each period, the overflow (effluent) were collected into 2 L plastic flasks approximately 3/4th immersed into ice as outlined by AbuGhazaleh and Buckles (2007). Collected effluents were homogenized by stirring and 0.25 (v/v) subsamples were pooled into one sample and stored at −20 °C. Effluent samples were freeze dried and then analyzed for FA and nutrients content (DM, OM, CP, and NDF) to estimate digestibility. Effluent samples were thawed in a 50 °C water bath and then centrifuged (Beckman J2-21, GMI, Inc. Ramsey, MN, USA) in 250 mL plastic bottles at 500 rpm for 5 min. The supernatant was discarded, and to the sediment, more samples were added and centrifuged again. Finally the remaining sediments were freeze dried for at least 48 h, ground through 1 mm screen Wiley mill. Dry matter was determined by drying at 105 °C for 48 h (AOAC, 1990; Method 930.15). Samples were analyzed for CP (Method 976.05), ether extract (Method 920.39), and ash (Method 942.05) according to AOAC (2000) methods. The neutral detergent fiber (aNDFom) was determined using the Van Soest et al. (1991) procedure. The heat stable amylase and sodium sulphite were used to determine NDF. The acid detergent fiber (ADF) content was determined according to AOAC (1990; Method 973.18). Apparent digestibility was calculated as the difference between the contents in the initial samples and the residues remaining after incubation in the effluent (Khiaosa-Ard et al., 2009).

Treatment diets and effluent samples were methylated using the sodium methoxide ($NaOCH_3$) and HCl two-step procedures as outlined by Kramer et al. (1997) and analyzed in duplicate for FA as described by Gas Liquid Chromatography (GLC) as described by AbuGhazaleh and Jacobson (2007). Apparent biohydrogenation was calculated from C18:1*n*9, C18:2*n*6 and C18:3*n*3 proportions in dietary and effluent C18 FA, assuming that the total C18 FA input equals the total C18 FA output (Lourenço et al., 2008).

Two samples were collected from each fermenter on day 10 of each period at 3 and 6 h post morning feeding and then composited into one sample per fermenter for volatile fatty acids (VFA), ammonia-N and bacterial analyses. Samples for VFA analyses were mixed with 1 mL of freshly prepared 25% meta-phosphoric acid, centrifuged (IEC Centra GP8R, Needham Heights, MA) at 20,000 × *g* at 4 °C for 20 min and supernatant fluid were collected and stored at −20 °C until further analysis. Volatile fatty acids were analyzed on gas chromatograph as described by Jenkins (1987), using 2-ethylbutyric acid as an internal standard. For ammonia-N analysis, collected samples were centrifuged at 20,000 × *g* (IEC Centra GP8R, Needham Heights, MA) at 4 °C for 10 min. The supernatant were acidified with 0.5 mL 0.1 N HCl then analyzed for ammonia-N by Teco

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Table 3
PCR primers and starting DNA concentration for detection of selected ruminal bacteria.

Target bacterium	Primer sequences (5′–3′)	Starting DNA concentration (ng)/25 μL	Annealing temp (°C)	Product size (bp)
A. lipolytica	F GGGTGTTAGAAATGGATTCC R CTCTCCTGCACTCAAGAATT	80.5	57	597
B. proteoclasticus	F CCTAGTGTAGCGGTGAAATG R TTAGCGACGGCACTGAATGCCTAT	14	62	188
Butyrivibrio SA	F TCCGGTGGTATGAGATGGGC R GTCGCTGCATCAGAGTTTCCT	18	60	126
Butyrivibrio VA	F GCCTCAGCGTCAGTAATCG R GGAGCGTAGGCGTTTTAC	18	60	124

Diagnostics kit (Anaheim, USA) using a spectrophotometer. Bacterial samples collected from fermenters were frozen immediately in liquid nitrogen and stored at −80 °C until bacterial analysis. Microbial samples were thawed at room temperature and centrifuged at 500 rpm for 15 min, to separate solid and liquid associated bacteria, and the supernatant was transferred to new tubes. The DNA from the supernatants was then extracted using Microbial DNA Isolation kit (MO BIO Laboratories, Inc., USA). Concentration of DNA was measured by BioPhotometer (Eppendorf Scientific, Inc., USA) at A260 = 50 ng dsDNA/μL using disposable Eppendorf UVette cuvets.

Purified cultures of *Anaerovibrio lipolytica* (DSMZ No. 3074), *Butyribirio* VA (DSMZ 10316) and *Butyrivibrio* SA (DSMZ 10302) were obtained from DSMZ (German resource center for biological material, Germany). *Butyrivibrio proteoclasticus* (ATCC 51982) was obtained from ATCC (The Global Bioresource center, USA). The purified bacteria were used to generate standard curves in our relative quantitation analyses. Polymerase Chain Reaction (PCR) primers used in the present study are shown in Table 3. The PCR conditions *A. lipolytica* was described by Potu et al. (2011). The primer pairs for bacteria (*A. lipolytica*, *B. proteoclasticus*, *Butyrivibrio* SA and *Butyrivibrio* VA) were based on the description of Gudla et al. (2012) and Potu et al. (2011). The specificity of primers was confirmed using the BLAST program in the GeneBank Database. Primer pair for *Butyrivibrio proteoclasticum* was found online (using IDT, Integrated DNA Technologies). These primers were also tested for the requirements imposed by real-time quantitative PCR. Pure cultures of the previously described bacteria were grown in our laboratory under anaerobic conditions in Hungate tubes as specified by ATCC and DSMZ. The four bacteria were used to generate standard curves for the relative quantitation analyses as described by Potu et al. (2011). Tubes were incubated for 3–5 days at 37 °C to allow bacterial growth. Cultures were transferred to fresh medium from incubated tubes for 2 or 3 times to avoid dead cells. Approximately 1 mL of the culture was used to extract DNA as described before.

Individual species-specific real time quantitative PCR (qPCR) were performed using Bio-Rad iCycler MyiQ single color real-time PCR detection system (Bio-Rad laboratories, Inc, Hercules, CA, USA), using fluorescence detection of SYBR green mix (Bio-Rad laboratories, Inc., Hercules, CA, USA) as described by Potu et al. (2011). In one word, 12.5 μL SBYR Green mix, 2 μL of each primer, 20 ng of sample DNA template and RNAase free water were added to make a total volume of 25 μL. For initial denaturation, amplification involved one cycle at 95 °C for 10 min and then 40 cycles of 95 °C for 30 s followed by annealing at the temperatures for 30 s and then at 72 °C for 1 min except for *B. proteoclasticum* for which amplification involved one cycle at 95 °C for 3 min for initial denaturation and then 40 cycles of 95 °C for 30 s followed by annealing at 62 °C for 15 s and then at 72 °C for 30 s (Reilly and Attwood, 1998). Detection of the fluorescent product was set at the last step of each cycle. The melting curve was obtained by slow heating with a 0.1 °C/s increment from 65 °C to 95 °C, with fluorescence collection at 0.1 °C intervals. Samples were performed in triplicate along with standards of known bacterial DNA concentrations which were prepared in 10 fold dilutions. Samples and known standards (10^1–10^6 ng) were assayed on the same plate to allow for the quantification of bacteria present in sample. Standard curves, DNA sample quantification and melting curve analyses were obtained using iQ5 Optical System Software (version 2.1, Bio-Rad Laboratories, Inc., Hercules, CA, USA). Samples were amplified in triplicate along with dilution standards of known bacterial DNA concentrations. Samples and standards were assayed on the same plate to allow for the relative quantification of bacterial DNA present in sample.

2.4. Statistical analysis

Data were analyzed as a 4 × 4 Latin square design using the PROC MIXED of SAS (SAS Institute, Inc., Cary, NC. Version 9.1, 2003). The statistical model was including: fermenters, diet, and period. Fixed effects were diet and period. Random effect was fermenter. Results were expressed as least square means with standard error of the means. Means were compared using the PDIFF option of SAS. The significance threshold was set at $P < 0.05$.

3. Results

The effect of treatment diets on nutrients digestibility are presented in Table 4. Feed additives had no effects ($P > 0.05$) on apparent DM, NDF and OM digestibility compared with the CON diet (Table 4). Compared with the CON diet, apparent protein digestibility decreased ($P < 0.01$) only with the QTAN and CIN diets.

The effect of treatment diets on fermentation is presented in Table 5. The concentration of acetate decreased ($P < 0.05$) with the MON and CIN diets while the concentration of propionate increased ($P < 0.05$) with the MON and QTAN diets compared

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Table 4
The effect of treatment diets on nutrients digestibility.

	Treatment				MSE	*P*
	CON	QTAN	MON	CIN		
Digestibility, %						
DM	0.63	0.59	0.58	0.62	0.031	0.65
True protein	0.59[a]	0.39[b]	0.57[a]	0.38[b]	0.019	0.01
NDF	0.53	0.58	0.52	0.51	0.060	0.20
OM	0.62	0.51	0.53	0.51	0.019	0.72

[a] Means within raw with different superscripts differ ($P \leq 0.05$)
[b] Means within raw with different superscripts differ ($P \leq 0.05$)
CON = control, QTAN = quebracho condensed tannin, MON = monensin, and CIN= cinnamaldehyde.

Table 5
The effect of treatment diets on volatile fatty acids (mM) and ammonia-N level (mg/dL).

	Treatment				MSE	*P*
	CON	QTAN	MON	CIN		
Acetate	36.9[a]	34.9[a]	27.8[b]	29.5[b]	1.831	0.007
Propionate	22.9[c]	26.8[b]	32.8[a]	24.3[bc]	1.549	0.003
Butyrate	14.8	10.5	14.0	15.6	1.696	0.110
*Iso*butyrate	1.22[b]	1.44[b]	1.93[a]	1.82[a]	0.107	0.052
Valerate	3.75	2.72	3.78	3.24	0.409	0.147
*Iso*valerate	2.17	2.4	1.88	3.00	0.331	0.108
Acetate: propionate	1.61[a]	1.33[b]	0.83[c]	1.12[b]	0.127	0.004
Total VFA (mM)	82.3	78.9	82.5	79.0	2.671	0.110
NH_3-N (mg/dL)	36.1[a]	5.01[d]	35.7[b]	31.1[c]	0.151	0.010
pH	6.46	6.51	6.31	6.46	0.104	0.324

[a] Means within raw with different superscripts differ ($P \leq 0.05$).
[b] Means within raw with different superscripts differ ($P \leq 0.05$).
[c] Means within raw with different superscripts differ ($P \leq 0.05$).
[d] Means within raw with different superscripts differ ($P \leq 0.05$).
VFA = volatile fatty acids, NH_3-N = ammonia N, CON = control, QTAN = quebracho condensed tannin, MON = monensin, and CIN = cinnamaldehyde.

with the CON diet. *Iso*butyrate concentration was greater ($P < 0.05$) with the MON and CIN diets than the QTAN and CON diets. Total VFA concentration was not affected ($P > 0.05$) by treatment diets. Ammonia-N concentration decreased ($P < 0.01$) with the inclusion of feed additives and was least with the QTAN diet. Fermenter pH was similar between treatment diets ($P > 0.05$) averaging 6.43.

The effect of treatments on BH and effluent FA are presented in Tables 6 and 7. Compared with the CON diet, the addition of condensed tannin and cinnamaldehyde had no effects ($P > 0.05$) on the BH of C18 unsaturated FA while the addition of monensin reduced ($P > 0.05$) the BH of C18 unsaturated FA. Addition of monensin decreased ($P < 0.05$) the concentrations of C14 *iso* and C15:0 compared to other diets. However, the concentrations of C14:0 and C15 *anteiso* tended to increase ($P < 0.06$) with the CIN diet compared with the CON diet. Feed additives had no effects ($P > 0.05$) on the concentrations of C16 *iso*, C16:0 and C17:0 *anteiso* compared with the CON diet. The concentration of C18:0 decreased ($P < 0.01$) with the three feed additives and was least with the MON diet. Compared with the CON diet, the concentrations of *trans* C18:1 and VA increased ($P < 0.05$) with the MON and CIN diets and was greatest ($P < 0.05$) with the MON diet. The concentrations of *t*15, *t*16 and C18:1*c*9 increased ($P < 0.05$) with the CIN diet. Feed Additives had no effects ($P > 0.05$) on the concentrations of *t*4/5, *t*6/8, *t*9, *t*10 and *t*12 C18:1 isomers. The concentrations of C18:2*n*6 and C18:3*n*3 increased ($P < 0.05$) with the MON and CIN diets and were greatest with the MON diet. Concentration of *c*9*t*11 CLA increased ($P < 0.05$) only with the QTAN diet while total FA concentration decreased ($P < 0.05$) with the QTAN and MON diets.

The effects of treatment diets on the DNA abundance of selected strains of ruminal bacteria are presented in Table 8. Feed additives had no effects ($P > 0.05$) on the DNA abundance of *Butyrivibrio* SA and *A. lipolytica*. The DNA abundance of

Table 6
The effect of treatment diet on biohydrogenation of C18 unsaturated fatty acids.

Fatty acid	Treatment				MSE	*P*
	CON	QTAN	MON	CIN		
C18:1*n*9	0.72[a]	0.72[a]	0.65[b]	0.72[a]	0.01	0.01
C18:2*n*6	0.91[a]	0.89[a]	0.82[b]	0.89[a]	0.01	0.01
C18:3*n*3	0.87[a]	0.87[a]	0.81[b]	0.88[a]	0.01	0.02

[a] Means within raw with different superscripts differ ($P \leq 0.05$).
[b] Means within raw with different superscripts differ ($P \leq 0.05$).
CON = control, QTAN = quebracho condensed tannin, MON = monensin, and CIN = cinnamaldehyde.

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Table 7
The effect of treatment diets on effluent fatty acids (mg/g DM).

Fatty acid	Treatment				MSE	*P*
	CON	QTAN	MON	CIN		
C14 *iso*	0.22[b]	0.34[a]	0.05[c]	0.15[b]	0.049	0.01
C14:0	0.62	0.63	0.56	1.04	0.155	0.06
C15 *anteiso*	0.63	0.86	0.64	1.16	0.173	0.06
C15:0	0.90[b]	0.54[b]	0.31[c]	1.61[a]	0.161	0.01
C16 *iso*	0.10	0.12	0.07	0.09	0.029	0.40
C16:0	12.7	12.4	11.5	17.2	3.171	0.36
C17:0 *anteiso*	0.23	0.26	0.24	0.21	0.041	0.71
C18:0	61.9[a]	36.4[b]	10.4[c]	40.0[b]	1.570	0.01
*t*4/5	0.13	0.08	0.08	0.09	0.029	0.37
*t*6/8	0.13	0.06	0.07	0.11	0.060	0.15
*t*9	0.78	0.62	0.65	0.88	0.163	0.11
*t*10	3.60	2.99	3.55	3.03	0.525	0.22
*t*11 (VA)	7.64[c]	6.53[c]	12.2[a]	9.30[b]	0.467	0.01
*t*12	0.99	0.56	0.93	0.95	0.225	0.21
*t*15	0.71[b]	0.66[b]	1.46[a]	1.74[a]	0.200	0.01
*t*16	0.81[ab]	0.65[b]	0.26[b]	1.28[a]	0.262	0.01
trans C18:1	14.8[b]	12.9[b]	19.4[a]	18.1[a]	1.026	0.01
C18:1 *c*9	8.43[b]	8.13[b]	9.20[b]	13.1[a]	0.997	0.01
C18:2*n*6	6.61[c]	7.83[bc]	13.47[a]	8.59[b]	0.839	0.01
C18:3*n*3	0.58[c]	0.82[b]	1.15[a]	0.77[b]	0.077	0.01
*c*9*t*11CLA	0.57[b]	1.98[a]	0.33[b]	0.62[b]	0.414	0.01
*t*10*c*12CLA	0.48	0.19	0.24	0.30	0.095	0.08
TFA	133.9[a]	97.9[b]	85.8[b]	135.5[a]	8.33	0.01

[a] Means within raw with different superscripts differ ($P \leq 0.05$).
[b] Means within raw with different superscripts differ ($P \leq 0.05$).
[c] Means within raw with different superscripts differ ($P \leq 0.05$).
TFA = Total fatty acids, CON = control, QTAN = quebracho condensed tannin, MON = monensin, and CIN = cinnamaldehyde.

Butyrivibrio VA increased ($P < 0.05$) with all feed additives while the DNA abundance of *B. proteoclasticum* decreased ($P < 0.01$) only with the MON and CIN diets.

4. Discussion

Consistent with the findings of others (Sliwinski et al., 2002; Busquet et al., 2006; Animut et al., 2008; Benchaar et al., 2008; Lourenço et al., 2008; Yang et al., 2010), the apparent digestibility of DM, OM and NDF and total VFA production were not affected by the inclusion of feed additives. However, others studies have reported reductions in DM and NDF digestibility with monensin (Poos et al., 1979; Anassori et al., 2012) and tannin (Bhatta et al., 2009; Grainger et al., 2009). This discrepancy among the different studies may be related to several factors such as diet composition, the period of adaptation to the product, the time when the samples were collected, and the type and concentrations of the feed additives (McGuffey et al., 2001; Bodas et al., 2012). Although these feed additives had no detrimental effect on the apparent digestibility of DM, OM and NDF and the total VFA production, they had variable impacts on apparent protein degradability, VFA profiles, and ammonia-N concentration in the present study suggesting that these additives have a 'milder' effects on the ruminal microbial activity and fermentation.

Previous reports suggested that cinnamaldehyde and tannin have the ability to modify the growth of rumen proteolytic bacteria either directly or indirectly by preventing access to the protein. The reductions in protein degradation seen iwith the CIN and QTAN diets in this study therefore may suggest that proteolytic and peptidolytic activities were reduced. In

Table 8
The effect of treatment diets on the DNA abundance (pg) of selected rumen bacteria at a given starting concentration.

Bacteria	Treatment				MSE	*P*
	CON	QTAN	MON	CIN		
Butyrivibrio SA, 18 ng[x]	31.63	28.28	29.71	28.13	2.856	0.61
Butyrivibrio VA, 18 ng[x]	0.02[c]	0.04[a]	0.05[a]	0.03[b]	0.006	0.05
B. proteoclasticum, 10 ng[x]	0.019[b]	0.024[a]	0.002[c]	0.002[c]	0.002	0.01
A. lipolytica, 60 ng[x]	0.15^{-3}	0.20^{-3}	0.17^{-3}	0.17^{-3}	0.00004	0.37

[a] Means within raw with different superscripts differ ($P \leq 0.05$).
[b] Means within raw with different superscripts differ ($P \leq 0.05$).
[c] Means within raw with different superscripts differ ($P \leq 0.05$).
[x] Starting DNA concentration.
CON = control, QTAN = quebracho condensed tannin, MON = monensin, and CIN = cinnamaldehyde.

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previous continuous culture studies, Busquet et al. (2005b) and Tager and Krause (2010) reported that protein digestibility and bacterial nitrogen flow was depressed with cinnamon oil. Cardozo et al. (2004) also reported that 7.5 mg/kg DM cinnamaldehyde addition reduced peptidolysis in a continuous culture study. The suppressing of protein fermentation might be also due to formation of complexes between tannin and protein (Makkar et al., 1995) or the inhibition of the activities of the protease enzyme by tannin. Previous continuous culture (Khiaosa-Ard et al., 2009; Hassanat and Benchaar, 2012) and *in vivo* (McSweeney et al., 2001) experiments have also reported similar reductions in protein digestibility with tannin. The high affinity of tannins to proteins may explain the pronounced effect of tannins on protein apparent degradation in this study. It is well documented that tannins have higher affinity to proteins than to polysaccharides (Patra and Saxena, 2009). A decrease in the rumen protein degradability in the rumen is beneficial for ruminants as they increase the supply of dietary nitrogen to the lower intestine for production.

The decreases in ammonia-N concentration with the CIN and QTAN diets could be partly due to decrease in deamination of amino acids or low availability of amino acids resulted from decrease in protein degradation. Although the degradability of protein was not affected by the addition of monensin, the reduced ammonia-N concentration seen with the MON diet suggests that the deamination process was inhibited. Previous *in vitro* studies with monensin addition had also showed that the decrease in ammonia-N concentration was associated with an accumulation of amino acids or peptide indicating that monensin inhibits deamination to a greater extent than proteolysis (Whetstone et al., 1981; Russell and Martin, 1984).

The concentration of propionate increased with the MON and QTAN diets, while the concentration of acetate decreased with the MON and CIN diets. The pattern of VFA observed in the present study with these additives was marked by a strong decrease in acetate to propionate ratio, suggesting that these additives inhibited acetate producing bacteria particularly with the MON diet. Numerous *in vitro* and *in vivo* studies demonstrated that the addition of these additives increased the proportion of propionate and reduced the proportion of acetate and butyrate. Fellner et al. (1997) reported that the addition of monensin (2.9 μM of fermenter contents) decreased the molar percentage of acetate and increased the molar percentage of propionate, decreasing the ratio of acetate to propionate from 3.0 to 1.7. Ipharraguerre and Clark (2003) reviewed the effects of feeding monensin to lactating dairy cows and reported that monensin usually increased the molar proportion of propionate and decreased acetate to propionate ratio. In an *in vitro* experiment, 31.2 and 312 mg/L doses of cinnamaldehyde decreased the molar proportion of acetate and increased the molar proportion of propionate (Busquet et al., 2005a). Busquet et al. (2006) used varying doses of cinnamaldehyde from 3 to 3000 mg/L, and observed no effect on the molar proportion of acetate and butyrate, but an increase of the molar proportion of propionate with the highest dose. Bhatta et al. (2009) reported an increase in *in vitro* propionate production when supplementing condensed tannin from either quebracho (13.3 g/kg and 36.7 g/kg tannin of DM) or mimosa (15.0 g/kg tannin of DM). The acetate to propionate ratio decreased when feeding *Acacia mearnsii* extract as sources of tannin at a rate of 78.9 g/kg DM in a rumen simulation technique system (Khiaosa-Ard et al., 2009). The changes in VFA portions may be nutritionally advantageous, because propionate is one of the main sources of metabolizable energy for ruminants and it is energetically more effective. In the rumen, gram-positive bacteria are generally acetate and butyrate producing bacteria, while gram-negative bacteria are generally propionate-producing bacteria (Stewart, 1991). Therefore, the results of this study suggest that the feed additives ae more inhibitory to the gram-positive bacteria than gram-negative bacteria. Monensin is known to depress or inhibit rumen microorganism growth, especially gram positive bacteria, and stimulate the development of bacteria that produce propionic acid (McGuffey et al., 2001; Ipharraguerre and Clark, 2003). In an *in vitro* study, Chen and Wolin (1979) reported that bacteria that produce lactic, acetic, butyric and formic acids and hydrogen as main end products are susceptible to ionophores whereas succinic-and propionic acid-producing bacteria are resistant. Previous *in vitro* studies (Nemoto et al., 1995; Nelson et al., 1997) reported that growth and cellulolytic activity of *Fibrobacter succinogenes* were more inhibited by condensed tannin levels than the growth and activity of *Prevotella ruminicola* and *Streptococcus bovis* (Nelson et al., 1997). Similarly, McSweeney et al. (2001) observed a decrease of *F. succinogenes* and *Ruminococcus* spp. in ewes supplemented with 60 g of condensed tannin/kg of DM.

The lower concentration of C18:0 along with the greater concentrations of C18:2*n*6 and C18:3*n*3 in fermenters fed the MON diet suggest reductions in the apparent BH of C18:2*n*6 and C18:3*n*3 in fermenters fed the MON diet consistent with the findings (Fellner et al., 1997; Benchaar et al., 2006; Odongo et al., 2007). The observed reduction in their BH may indicate less lipid hydrolysis and/or isomerization of the free C18:2*n*6 and C18:3*n*3 in the monensin-fed fermenters. However, because the significant reduction in the C18:0 formation with the monensin diet in this study and the monensin known effects on the growth of gram-positive bacteria that produce hydrogen (Chen and Wolin, 1979), it is most likely that monensin effect on BH resulted from reductions in the first isomerization step of C18:2*n*6 and C18:3*n*3. Additionally, the lack of monensin effect on the DNA abundance of *A. lipolytica* further supports that monensin had no effects on lipids hydrolysis. The lack of effect of condensed tannin and cinnamaldehyde on the BH of C18 unsaturated FA is consistent with the findings of others (Benchaar and Chouinard, 2009; Khiaosa-Ard et al., 2009) but in contrast to others (Cabiddu et al., 2009; Vasta et al., 2009b). This inconsistency is most likely related to differences in supplement source, dose level and diets composition. The reduction in the formation of C18:0 along with the accumulation of *trans* C18:1, VA in particular, is consisting with the findings of others (Fellner et al., 1997; Benchaar et al., 2006; AlZahal et al., 2008) and may suggest that monensin interrupted the complete BH of C18 *trans* FA leading to a decrease in the concentration of C18:0 and increase in *trans* C18:1, VA in particular. In a continuous culture study, Fellner et al. (1997) reported that the concentration of VA was greater with monensin (2.5 μM final concentration) compared with control. Other *in vivo* studies (Benchaar et al., 2006; AlZahal et al., 2008) also reported greater VA concentration with monensin diets (350 mg/day and 22 g/kg of DM respectively). Since the vast majority of *c*9*t*11

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CLA is produced in animal tissues *via* Δ^9 desaturase using VA as its substrate (Griinari et al., 2000), increasing the formation and flow of VA from the rumen is therefore desirable as it would aid in the process of developing healther ruminant-derived food products. Although the mode of action could not be determined, monensin may have reduced group B bacteria and/or inhibits their reductase activity. Kemp and Lander (1984) classified bacteria involved in BH into two groups based on the reaction and end products of BH. Accordingly, group A bacteria, *R. albus* and *Butyrivibrio* sp., are able to hydrogenate C18:2*n*6 and C18:3*n*3, with VA being their major end product. Group B bacteria, *Fusocillus babrahamensis*, *Fusocillus* sp. and an unnamed gram-negative rod (R8/5), are able to utilize VA as one of the main substrates to form C18:0 as the end product. Wallace et al. (2006) reported that *B. proteoclasticus* has the ability to produce C18:0 from C18:2*n*6 and *B. proteoclasticum* is phylogenetically related to *Fusocillus* spp. Boeckaert et al. (2008) identified both *B. proteoclasticum* as well as *B. hungatei* groups as group B bacteria. In this study, the reduction in the concentration of C18:0 with the monensin diet was associated with reductions in the DNA abundance of *B. proteoclasticum* suggesting that monensin effect on VA formation may in part be explained by its effects on *B. proteoclasticum.* To the best of our knowledge, this study is the first to report monensin effects on *B. proteoclasticum.* Addition of cinnamaldehyde in this study also reduced the formation of C18:0 and increased the formation of VA but to a lower extent than that of monensin supplement. Such increase in the accumulation of *trans* C18:1, VA in particular, also suggests that cinnamaldehyde reduced the terminal step of BH, the reduction of VA to C18:0, possibly by affecting the group B bacteria. As with the MON diet, the reduction in the concentration of C18:0 with the CIN diet in this study was also associated with reductions in the DNA abundance of *B. proteoclasticum* which further supports the important role of these bacteria in *trans* C18:1 reductions to C18:0.

The greater concentration of *c*9*t*11 CLA in effluent of the QTAN diet compared with the CON diet is consistent with the findings of others (Khiaosa-Ard et al., 2009; Vasta et al., 2009a). The inclusion of condensed tannin in the diet did not affect the accumulation of C18:2*n*6 but increased the accumulation of *c*9*t*11 CLA suggesting that condensed tannin had no effect on oil hydrolysis. The increased *c*9*t*11 CLA with the QTAN diet may have resulted from a delay in the *c*9*t*11 CLA reduction step. The decrease in C18:0 concentration with the QTAN diet is consistent with the findings of Khiaosa-Ard et al. (2009) and Vasta et al. (2009a) whom also reported reductions in the concentrations of C18:0 when incubating ruminal fluid with tannins. Although C18:0 concentration decreased with the QTAN diet, the concentration of *trans* C18:1 and VA did not increase suggesting that other unknown BH intermediates may have been produced. For example, Jenkins et al. (2006) showed of the total C18:1*c*9 that disappeared from rumen cultures, approximately 70% was transferred into *trans* C18:1 and C18:0 and 30% was transformed into hydroxystearic acid and ketostearic acid. Additionally, the lack of QTAN diet effect on the DNA abundance of *B. proteoclasticus* further supports that condensed tannin had no effects on the group B bacteria. However, Vasta et al. (2009b) reported that inclusion of tannin to a barley-based concentrate and lucerne hay diet for sheep increased the population of *B. fibrisolvens*, while the growth of *B. proteoclasticus* reduced.

Using 16S rDNA sequence data, Paillard et al. (2007a) constructed a phylogenetic tree that contains two main groups of rumen *Butyrivibrio* bacteria called vaccenic acid-producing (*Butyrivibrio* VA) and stearic acid-producing (*Butyrivibrio* SA). Although both groups formed VA from C18:2*n*6, only *Butyrivibrio* SA bacteria produced C18:0 (Paillard et al., 2007b). Dietary supplements in this study had no effect on the DNA abundance for *Butyrivibrio* SA and to the best of our knowledge, this study is the first to report these feed additives effects on *Butyrivibrio* SA. Fuentes et al. (2009) and Gudla et al. (2012) also reported no correlations between *Butyrivibrio* SA and C18:0 flow under low rumen pH conditions. Kim et al. (2008) and Huws et al. (2010) also reported that DNA abundance from the *Butyrivibrio* SA-producing group did not correlate with the C18:0 concentrations suggesting that *Butyrivibrio* SA-producing group may not indeed by the main C18:0 producer in the rumen. These results, therefore, would suggest that other, yet-uncultivated microbial species might be involved in C18:0 production and might fulfill a more important role in the final step of the BH process. Additionally, it is still also possible that supplements decreased the capacity of these bacteria to hydrogenate *trans* C18:1 rather than the bacteria as such. A recent study by Maia et al. (2010) reported that the inhibitory-growth effects of unsaturated FA on bacteria may be mediated *via* metabolic means rather than disruption of cell membrane. Additionally, Huws et al. (2011) suggested that *Butyrivibrio* SA may not play the dominant role in C18:0 formation and other yet-uncultivated bacteria, particularly those belonging to the genera *Prevotella* and *Anaerovoax*, and unclassified *Clostridiales* and *Ruminococcaceae* may play a role in biohydrogenation.

5. Conclusions

Results from the present study shows that none of the feed additives had an effect on apparent digestibility of DM and NDF, however, apparent digestibility of protein was lower in diets supplemented with condensed tannin and cinnamaldehyde. Acetate concentration decreased with the addition of monensin and cinnamaldehyde while the concentration of propionate increased with the addition of monensin and condensed tannin. The concentration of C18:0 decreased with the three feed additives, particularly with monensin. The concentration of VA increased only with the MON and CIN diets and this increase corresponded with a decrease in the DNA abundance of *B. proteoclasticum*. None of the feed additives had any effects on the DNA abundance of *Butyrivibrio* SA and *A. lipolytica*. Monensin and cinnamaldehyde effects on VA formation may be therefore explained in part by their effects on *B. proteoclasticum*. Cinnamaldehyde could be a safe alternative to antibiotic (monensin) to alter bacterial populations involved in ruminal BH. However, further research is still needed to confirm these feed additives effects under *in vivo* conditions.

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Conflict of interest

Authors don't have conflict of interest with this manuscript.

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