animal

Soybean oil and linseed oil supplementation affect profiles of ruminal microorganisms in dairy cows

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The objective of this study was to evaluate changes in ruminal microorganisms and fermentation parameters due to dietary supplementation of soybean and linseed oil alone or in combination. Four dietary treatments were tested in a Latin square designed experiment using four primiparous rumen-cannulated dairy cows. Treatments were control (C, 60 : 40 forage to concentrate) or C with 4% soybean oil (S), 4% linseed oil (L) or 2% soybean oil plus 2% linseed oil (SL) in a 4×4 Latin square with four periods of 21 days. Forage and concentrate mixtures were fed at 0800 and 2000 h daily. Ruminal fluid was collected every 2 h over a 12-h period on day 19 of each experimental period and pH was measured immediately. Samples were prepared for analyses of concentrations of volatile fatty acids (VFA) by GLC and ammonia. Counts of total and individual bacterial groups (cellulolytic, proteolytic, amylolytic bacteria and total viable bacteria) were performed using the roll-tube technique, and protozoa counts were measured via microscopy in ruminal fluid collected at 0, 4 and 8 h after the morning feeding. Content of ruminal digesta was obtained via the rumen cannula before the morning feeding and used immediately for DNA extraction and quantity of specific bacterial species was obtained using real- time PCR. Ruminal pH did not differ but total VFA (110 v. 105 mmol/l) were lower ($P < 0.05$) with oil supplementation compared with C. Concentration of ruminal NH_3-N (4.4 v. 5.6 mmol/l) was greater ($P < 0.05$) due to oil compared with C. Compared with C, oil supplementation resulted in lower ($P < 0.05$) cellulolytic bacteria (3.25×10^8 v. 4.66×10^8 colony-forming units (CFU)/ml) and protozoa (9.04×10^4 v. 12.92×10^4 cell/ml) colony counts. Proteolytic bacteria (7.01×10^8 v. 6.08×10^8 CFU/ml) counts, however, were greater in response to oil compared with C ($P < 0.05$). Among oil treatments, the amount of Butyrivibrio fibrisolvens, Fibrobacter succinogenes and Ruminococcus flavefaciens in ruminal fluid was substantially lower ($P < 0.05$) when L was included. Compared to C, the amount of Ruminococcus albus decreased by an average of 40% regardless of oil level or type. Overall, the results indicate that some ruminal microorganisms, except proteolytic bacteria, are highly susceptible to dietary unsaturated fatty acids supplementation, particularly when linolenic acid rich oils were fed. Dietary oil effects on ruminal fermentation parameters seemed associated with the profile of ruminal microorganisms.

Keywords: soybean oil, linseed oil, rumen fermentation, microorganisms

Implications

Level of dietary oil supplementation has been shown to reduce ruminal fermentation parameters in dairy cows. However, relatively little data exists pertaining to the effects of unsaturated oils on species of ruminal microorganisms. Lactating cows were fed linseed oil or soybean oil alone (4% of dry matter (DM)) or in combination (2% of DM from each oil) to examine effects on microbial populations, bacterial species and fermentation parameters. Oils reduced cellulolytic bacteria and protozoal counts, along with total ruminal volatile fatty acids compared to controls. Furthermore, linseed oil resulted in the most pronounced decrease in amount of Butyrivibrio fibrisolvens, Fibrobacter succinogenes and Ruminococcus flavefaciens in ruminal fluid assessed by real-time reverse transcriptase-PCR. The negative effect of polyunsaturated fatty acids on ruminal fermentation parameters is explained at least in part by reductions in cellulolytic bacteria.

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Introduction

Supplemental oil in the diet of dairy cows changes the composition of milk fat and enhances the concentration of conjugated linoleic acid (CLA), which has been shown to have potential health benefits, including anticarcinogenic properties (Piperova *et al.*, 2000; Roche *et al.*, 2001; Loor *et al.*, 2005a). Consumption of n-3 fatty acids also has been associated with positive effects on human health. Addition of soybean oil (SBO; high linoleic acid content) to lactating dairy cow diets is expected to increase n-6 and CLA, whereas linseed oil (LSO; high linolenic acid content) would increase n-3 and CLA in milk fat (Demeyer and Doreau, 1999; Dhiman *et al.*, 2000; Chilliard *et al.*, 2001). Further, oils higher in linoleic acid than in linolenic acid were more effective in enhancing CLA in milk fat (Bu *et al.*, 2007).

In ruminants, feeding large amounts of vegetable oil has the potential to inhibit ruminal fermentation (Jenkins, 1993; Machmüller *et al.*, 1998). High dietary oil levels have been shown to decrease the counts of ruminal bacteria and protozoa (Matsumoto *et al.*, 1991; Dohme *et al.*, 2001). It was found that when feeding 2.5% fish oil to lactating dairy cows, ruminal protozoa counts were higher as compared to 5% LSO and 5% sunflower oil (Loor *et al.*, 2005b).

Negative effects on DM intake (DMI) and ruminal fermentation are often reported when unsaturated oils are fed, but some researchers have hypothesized that the negative effect of the unsaturated fatty acids on ruminal digestion would be minimized if the diet contained a higher proportion of forage due to the ability of forage to promote normal ruminal function for maximum biohydrogenation (Palmquist, 1988). As suggested previously, both increased biohydrogenation of polyunsaturated fatty acids (PUFA) as well as competition between bacteria and feed particles for adsorption of fatty acids can explain the lower toxicity of PUFA with high-forage diets. A recent study with lactating cows observed greater ruminal fiber digestibility but lower duodenal microbial-N flow when a high-forage (F : C = 65 : 35, DM basis) diet was supplemented with LSO at 3% of DM than a control (Ueda *et al.*, 2003).

Linoleic and linolenic acid are the most abundant PUFA in fresh forage or feedstuffs (e.g. cottonseeds, linseeds) typically fed to dairy cows. It is not clear whether the presence of two (linoleic acid) *v.* three (linolenic acid) double bonds affects the profiles of ruminal microorganisms to different extents. Maia *et al.* (2007) evaluated the effect of different fatty acids on growth of pure strains of ruminal bacteria. Linolenic acid was more toxic than linoleic acid, whereas eicosapentaenoic acid and docosahexaenoic acid were more toxic than linolenic acid. Although a few studies have examined the negative effect of unsaturated fatty acids on some ruminal microbiota (Ferlay *et al.*, 1993; Oldick and Firkins, 2000), none has evaluated relative changes in the profiles of ruminal microbiota in response to dietary PUFA. Thus, the main objective of this study was to determine the effect of SBO and LSO supplementation of a high-forage diet (60% forage) on profiles of ruminal microorganisms.

Material and methods

Animals and diets

Four primiparous Holstein cows equipped with a rumen cannula (# 1C; Bar Diamond, Inc., Boise, Idaho, USA) were used in a 4×4 Latin square design. The average body weight and days in milk at the beginning of the experiment were 521 ± 38 kg and 45 ± 10, respectively. Dietary treatments were: control diet (C), C with 4% supplemental (wt/wt basis) SBO (S), C with 4% LSO (L), and C with 2% SBO and 2% LSO (SL). The C diet contained 60% forage and 40% concentrate (Table 1). Each period was 21 days, including 14 days to allow the cows to adjust to the diet, followed by 7 days for sampling. Cows were housed in a tie-stall barn and fed individually. The total mixed ration (TMR) diets were mixed once a day and fed twice daily in equal portions at 0800 and 2000 h. The oils were stored in a cold room at 4°C and were mixed evenly with the concentrate each morning. Supplements were stored in the shade in a cool environment until fed. Orts were restricted

Table 1 *Ingredient and chemical composition of total mixed diets in cows fed a control diet or C supplemented with soybean oil, linseed oil and soybean oil plus linseed oil*

	C	S	L	SL
Ingredients[†]	g/kg diet DM			
Alfalfa hay	100	100	100	100
Chinese wildrye (hay, ripe)	280	280	280	280
Maize silage	220	220	220	220
Maize	157	117	117	117
Wheat bran	30	20	20	20
Soybean meal	135	145	145	145
Cotton seed meal	50	50	50	50
Calcium carbonate	4	4	4	4
Calcium phosphate (dibasic)	14	14	14	14
NaCl	5	5	5	5
Premix[‡]	5	5	5	5
Soybean oil	0	40	0	20
Linseed oil			40	20
Nutrient composition (DM basis)				
NE_L (MJ/kg DM)[§]	6.0	6.6	6.6	6.6
Residual OM (OM minus CP, NDF and EE)	390	360	360	360
CP	161	161	161	161
NDF	423	414	414	414
ADF	272	270	270	270
EE	26	65	65	65
Ca	9	9	9	9
Available P	6	6	6	6

DM = dry matter; OM = organic matter; EE = ether extract.
[†]Cows were fed a basal diet (C) or basal diet supplemented with either 4.0% soybean oil (S), 4.0% linseed oil (L) or 2.0% soybean oil + 2.0% linseed oil (SL).
[‡]Contained (per kilogram of DM) a minimum 1 000 000 IU of vitamin A; 65 000 IU of vitamin D; 5000 mg of vitamin E; Fe 2000 mg; Cu 1750 mg; Zn 5500 mg; Mn 2550 mg; Se 75 mg; I 70 mg; and Co 40 mg.
[§]The NE_L values (MJ/kg DM) used for alfalfa hay, Chinese wildrye, maize silage, maize, wheat bran, soybean meal, cottonseed meal, soybean oil and linseed oil were 4.5, 4.7, 5.0, 8.1, 6.9, 9.2, 8.2, 23.7 and 23.7, respectively (China NY/t34, 2004).

to 5% to 10% of intake on an as-fed basis. Procedures used for animal care and handling were approved and conducted under established standards of the Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing, China.

Sampling and analysis

Samples of TMR were collected daily, frozen and stored at −20°C. Orts were sampled twice a week from each cow, composited for each treatment, frozen and stored at −20°C until analyses were performed. Weekly representative samples of TMR and orts from each treatment were analyzed for DM content. The DM content of feed ingredients was determined by oven-drying at 60°C for 48 h. Dried feed samples from each week during the experimental period were ground in a Cyclotec1093 mill (Tecator, Hoganas, Sweden) and analyzed for concentrations of DM, CP, ether extract, calcium and phosphorus (AOAC, 1990) and of NDF and ADF (Van Soest *et al.*, 1991). The NDF content was analyzed by the neutral detergent solution method after pretreatment with alpha-amylase. Samples were further dried at 105°C for 8 h to determine absolute DM and all chemical analyses are expressed on DM basis.

Ruminal fluid samples for volatile fatty acids (VFA), NH_3-N and pH were collected through the cannula every 2 h over 12 h on day 19 of each experimental period beginning just before the morning feeding. Whole ruminal contents were hand-collected from the anterior, dorsal and mid-ventral regions of the rumen and were squeezed through four layers of cheesecloth. The first 100 ml of strained ruminal fluid was discarded. Residual rumen fluid was immediately used to measure pH (PB-10 Standardize). Ruminal fluid samples were analyzed for NH_3-N (Broderick and Kang, 1980). The portion of ruminal fluid (20 ml) obtained for measurement of VFA was acidified with 5 ml metaphosphoric acid (25%, w/v). Acidified ruminal fluid was centrifuged at 10 000 × **g** for 10 min at 4°C. The supernatant was stored at −20°C pending determination of VFA by GLC (Model Agilent6890N; Agilent Co., Wilmington, Delaware, USA). Total and individual bacterial groups (cellulolytic, proteolytic, amylolytic bacteria and total viable bacteria) numbers were assessed using the roll-tube technique (Hungate, 1969). Protozoa counts were assessed by microscope (Olympus DP70; Olympus, Tokyo, Japan) (Boyne *et al.*, 1957; Dehority, 1984; Lu and Xie, 1991) in ruminal fluid samples collected at 0, 4 and 8 h after the morning feeding. The ruminal samples for DNA extraction were obtained via the cannula before the morning feeding. Whole ruminal contents from the anterior, dorsal and mid-ventral regions of the rumen were squeezed by hand through four layers of cheesecloth. The strained ruminal fluid was centrifuged at 15 000 × **g** for 15 min at room temperature. The supernatant was discarded. The residue was immediately added to sterile normal saline and stored at −80°C. Samples for bacterial groups and DNA extraction were taken on the same day as the samples that were collected for VFA analysis.

DNA extraction

Total DNA from the ruminal fluid was extracted as described by Tajima *et al.* (2001), with some modifications, using a Mini Bead-Beater-1 (Biospec Products, Inc., Bartlesville Oklahoma, OK, USA) for cell lysis. Briefly, 0.5 ml ruminal fluid mixed with 900 μl cetyl trimethyl ammonium bromide (CTAB) solution (100 mM Tris-HCl, 100 mM EDTA, 100 mM Na_3PO_4, 1.5 M NaCl, 1% CTAB, pH 8.0) in a 2 ml tube containing 1 g glass beads (150 μm in diameter) was shaken for 1 min at 4200 r.p.m. in a Mini Bead Beater. Tubes were then centrifuged at 12 000 × **g** for 10 min. The supernatant was transferred to a fresh tube and extracted with buffered phenol, chloroform and isoamyl alcohol (volume ratio, 25 : 24 : 1). The resultant supernatant was precipitated with two volumes of cold ethanol for 1 to 2 h. After centrifugation at 12 000 × **g** for 10 min at room temperature, the eluted DNA was washed with 70% ethanol, dried, dissolved in sterile Tris-EDTA (TE) buffer (1 M Tris-HCl, 0.5 M EDTA, pH 8.0), and treated with DNase-free RNase A. Samples were re-extracted with buffered phenol, precipitated with ethanol and dissolved in TE buffer. DNA was purified with the DNA fragment purification kit (TaKaRa Biotechnology Co., Ltd, Dalian, China) according to the manufacturer's recommendations. Eluted DNA was dissolved in sterile TE buffer, and stored at −20°C. DNA concentrations were measured at 260 nm with a Beckman DU800 spectrophotometer (Beckman Co., Fullerton, CA, USA). On average, the DNA used for these experiments possessed an $A260/A280$ ratio of 1.8.

Design and synthesis of PCR primers

Primers designed to detect the target species are listed in Table 2. Although primers to detect the chosen bacteria are available (Asanuma *et al.*, 2001; Koike and Kobayashi, 2001), we elected to re-design primers to obtain products of 80 to 300 bp (base pairs) as typically recommended (http://www.takara.com.cn/newproducts/015.htm). The 16S rDNA sequences of interest were downloaded from GenBank. Sequence regions specific for a given species (with 97% similarity) were searched online against the GenBank database using the Basic Local Alignment Search Tool (BLAST) family of programs to ensure the specificity of primers. The primers satisfied requirements of species conservation within a given bacteria, and specificity from other clusters of species in the phylogenetic tree. Primer sequences were then tested against online nucleotide databases to ensure their specificity. These primers also were tested for the requirements imposed by real-time quantitative PCR (see further below). Primers were designed and sequences were edited using DNAStar 5.0 (DNASTAR Inc., Madison, WI, USA). Oligonucleotides were synthesized by Sangon (Sangon Biological Engineering Technology and Service, Co., Ltd, Shanghai, China).

Conventional PCR

PCR was performed with the Takara *Taq*™ PCR kit (TaKaRa Biotechnology Co., Ltd) using PE9700 thermal cycler (PerkinElmer, Waltham, MA, USA). Amplification conditions were: one cycle at 95°C for 3 min of denaturation, 35 cycles at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min. A total of 25 μl of PCR mixture contained:

Table 2 *PCR primers and template DNA for detection of ruminal bacteria*

Target bacterium (Strain)		Primer	Primer concentration (μM)	Purified template DNA (ng)	Product size (bp)
Fibrobacter succinogenes (ATCC 19169[T])	Forward	5′-GCGGGATTGAATGTACCTTGAGA-3′	10	30	204
	Reverse	5′-TCCGCCTGCCCCTGAACTATC-3′			
Ruminococcus flavefaciens	Forward	5′-GATGCCGCGTGGAGGAAGAAG-3′	7	20	286
	Reverse	5′-CATTTCACCGCTACACCAGGAA-3′			
Butyrivibrio fibrisolvens	Forward	5′-TAACATGAGAGTTTGATCCTGGCTC-3′	10	25	136
	Reverse	5′-CGTTACTCACCCGTCCGC-3′			
Ruminococcus albus	Forward	5′-GTTTTAGGATTGTAAACCTCTGTCTT-3′	7	25	270
	Reverse	5′-CCTAATATCTACGCATTTCACCGC-3′			

2.5 μl 10× PCR buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM MgCl$_2$), 10 μM each primer and 20 ng of purified template DNA concentration, 2 μl dNTP mixture (each deoxynucleoside triphosphate 2.5 mM), 2.5 U *Taq*^{TM} DNA polymerase. PCR products were separated by electrophoresis on agarose gels and stained with ethidium bromide.

Sequence of amplicons

The specificity of amplification was confirmed by sequencing (Sangon Biological Engineering Technology and Service Co., Ltd). After detection of species-specific PCR amplifications in total rumen DNA, the PCR products were cloned into the TA cloning kit (TaKaRa Biotechnology Co., Ltd), and the transformants were picked randomly. Recombinant plasmids were extracted using the TaKaRa MiniBEST Plasmid Purification Kit Version 2.0 (TaKaRa Biotechnology Co., Ltd). After PCR amplification with the DNA plasmid (amplification conditions were as described above), the species-specific amplification was confirmed by sequencing (Sangon Biological Engineering Technology and Service Co., Ltd). Dilutions of purified plasmid DNA from each strain were used to construct species-specific calibration curves. These calibration curves were used for calculation of the copy of targeted species in total rumen DNA. The value of the slope of the species-specific calibration curves ranged between −3.2 and −3.6.

Real-time PCR

Quantification of each bacterial species was performed with an Applied Biosystems 7500 System Real-Time PCR (Model 7500; Applied Biosystems, Foster city, CA, USA). Optimal amplification conditions for each primer pair were achieved. The reaction mixture in 25 μl of the final volume contained 12.5 μl of SYBR green PCR 2× Mastermix (OBT Instruments Co., Ltd, Beijing, China), primer and template DNA (described in Table 2). Amplification involved one cycle at 95°C for 10 min for initial denaturation, and then 40 cycles at 95°C for 15 s, followed by annealing at 60°C for 30 s, and then 1 min at 72°C. Detection of fluorescent product was set at the last step of each cycle. Specificity of amplification was performed by analysis of melting curves after each amplification. The melting curve was obtained by slow heating at 0.1°C/s increments from 65°C to 95°C, with
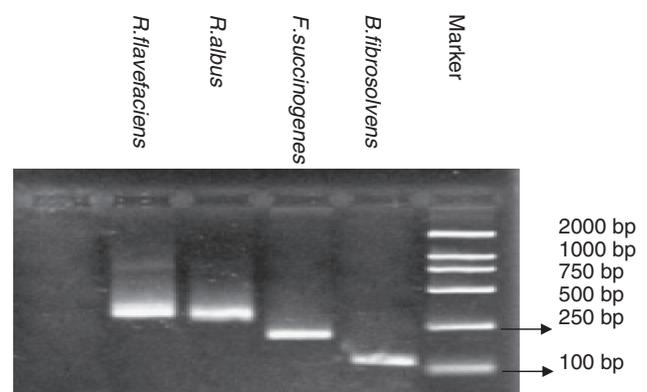
Figure 1 Amplification of the targeted species in total ruminal fluid DNA. DNA size marker (2000 bp) is in the far right lane.

fluorescence recording at 0.1°C intervals. Additional specificity analyses included product size verification by gel electrophoresis of samples after the PCR run, and melting point determination analysis (Figure 1).

Nucleotide sequence accession number

The 16S rDNA sequences described in this paper have been deposited in the GenBank database under accession no. AY169412 (*Butyrivibrio fibrisolvens*), X85098 (*Ruminococcus albus*), X85097 (*Ruminococcus flavefaciens*) and AJ496032 (*Fibrobacter succinogenes*).

Data analysis

All data were analyzed as a 4 × 4 Latin square using the MIXED procedure of SAS 8.2 (SAS Institute Inc., Cary, NC, USA). The statistical model included cow as random effect, and period and treatment as fixed effects. For the statistical analysis of ruminal fluid characteristics (pH, VFA, NH$_3$-N) and microorganism counts, sampling time and sampling time × treatment were added to the model and analyzed using repeated measures. Orthogonal contrasts included: C *v.* oil-supplemented diets to test the effect of oil supplementation; S *v.* L to test the effect of high S *v.* high L intake; and SL *v.* S + L to test the additive effect of S and L. The significance level was declared at $P < 0.05$ unless otherwise noted. Trends for significance were declared at $P = 0.05$ to 0.10.

Results

Chemical composition of the experimental diets

Ingredients and chemical composition of the diets are shown in Table 1. Each treatment contained 60% forage and 40% concentrate. Oils used for diets S, L and SL were substituted for corn grain on a weight basis to achieve the desired level of supplementation. Replacing corn grain with oil supplements increased the energy content of diets in the S, L and SL diets compared with the control (6.61 v. 5.98 MJ/kg of feed DM). Because the oil content of corn grain is typically <5% of dry matter (e.g. Dhiman et al., 1999; Loor et al., 2002), it is unlikely that it provided a quantitatively significant amount of PUFA for biohydrogenation. Milk yield and DMI during the study averaged 14.1 ± 0.3 (P = 0.75) and 12.6 ± 0.6 (P = 0.28) kg/day and no differences (P > 0.05) were observed between diets.

Ruminal fermentation parameters

Average ruminal pH and concentrations of total VFA and NH_3-N are shown in Table 3. Effect of time and time × treatment was not significant. Ruminal pH did not differ among treatments. Ruminal NH_3-N concentration was greater (4.4 v. 5.6 mmol/l), and the concentration of total VFA (110 v. 105 mmol/l) was lower due to oil supplementation. Molar proportion of acetate was not affected significantly, whereas that of propionate was significantly increased and butyrate significantly decreased for oil supplementation. Feeding the blend of S and L (SL) increased (P < 0.05) NH_3-N concentration to a greater extent than feeding them separately.

Microorganism counts

Cows fed diets with supplemental oil had lower (P < 0.05) cellulolytic bacteria (3.25×10^8 v. 4.66×10^8 colony-forming units (CFU)/ml) and protozoa counts (9.04×10^4 v. 12.92×10^4 cells/ml), but higher proteolytic bacteria (7.01×10^8 v. 6.08×10^8 CFU/ml) counts compared with cows fed the control diet (P < 0.05) (Table 4). Cows fed supplemental oil had numerically lower total viable bacteria than cows fed the control diet. Counts of total viable bacteria, proteolytic bacteria, cellulolytic bacteria and protozoa in cows fed S or L were similar.

Table 3 *Ruminal pH, VFA and ammonia-N concentration in cows fed a control diet or C supplemented with soybean oil, linseed oil or soybean oil plus linseed oil*

	Diet[†]					Contrast		
	C	S	L	SL		C v. oil[‡]	S v. L	SL v. S + L
	Mean				s.e.	P =		
pH	6.43	6.41	6.34	6.38	0.03	0.09	0.06	0.63
Total VFA (mmol/l)	110	104	105	106	4.26	*	0.81	0.61
Acetate (mol/100 mol)	69.4	70.2	69.7	69.6	0.69	0.07	0.57	0.73
Propionate (mol/100 mol)	19.5	19.6	20.4	20.2	0.28	***	***	0.15
Butyrate (mol/100 mol)	9.4	9.2	9.0	8.9	0.25	*	0.29	0.47
Valerate (mol/100 mol)	1.1	1.0	1.0	1.0	0.03	0.17	0.33	0.89
NH_3-N (mmol/l)	4.4	5.5	5.5	5.8	0.09	***	0.71	*

VFA = volatile fatty acids.
[†]Cows were fed a basal diet (C) or basal diet supplemented with either 4.0% soybean oil (S), 4.0% linseed oil (L) or 2.0% soybean oil + 2.0% linseed oil (SL).
[‡]C v. oil = C versus oil (S, L, SL).
*P < 0.05; ***P < 0.001.

Table 4 *Colony counts of ruminal microorganisms from cows fed a control diet and C supplemented with soybean oil, linseed oil, and soybean oil plus linseed oil*

	Diet[†]					Contrast		
	C	S	L	SL		C v. oil[‡]	S v. L	SL v. S + L
	Mean				s.e.	P =		
Amylolytic bacteria ($\times 10^7$ CFU/ml)	8.28	10.60	7.62	11.00	1.04	0.22	*	0.13
Total viable bacteria ($\times 10^9$ CFU/ml)	6.19	5.89	5.06	5.69	0.77	0.52	0.50	0.84
Proteolytic bacteria ($\times 10^8$ CFU/ml)	6.08	6.81	6.80	7.41	0.40	*	0.98	0.16
Cellulolytic bacteria ($\times 10^8$ CFU/ml)	4.66	3.30	2.82	3.63	0.30	***	0.27	0.14
Protozoa ($\times 10^4$ cells/ml)	12.92	9.92	8.17	9.04	1.11	**	0.23	0.99

CFU = colony-forming units.
[†]Cows were fed a basal diet (C) or basal diet supplemented with either 4.0% soybean oil (S), 4.0% linseed oil (L) or 2.0% soybean oil + 2.0% linseed oil (SL).
[‡]C v. oil = C versus oil (S, L, SL).
*P < 0.05; **P < 0.01; ***P < 0.001.

Table 5 *Quantification of ruminal bacteria by real time PCR in cows fed a control diet and C supplemented with soybean oil, linseed oil and soybean oil plus linseed oil*

Copies per template DNA (ng)	Diet[†]					Contrast		
	C	S	L	SL		C *v.* oil[‡]	S *v.* L	SL *v.* S + L
	Mean				s.e.	P =		
Butyrivibrio fibrisolvens $\times 10^8$	3.31	3.12	2.04	3.03	0.227	*	***	*
Ruminococcus albus $\times 10^7$	9.51	5.05	5.69	5.89	0.90	***	0.59	0.61
Fibrobacter succinogenes $\times 10^5$	7.95	4.83	0.48	2.64	2.06	*	0.13	0.99
Ruminococcus flavefaciens $\times 10^9$	2.08	1.99	1.50	1.87	0.16	0.11	*	0.50

[†]Cows were fed a basal diet (C) or basal diet supplemented with either 4.0% soybean oil (S), 4.0% linseed oil (L) or 2.0% soybean oil + 2.0% linseed oil (SL).
[‡]C *v.* oil = C versus oil (S, L, SL).
*$P < 0.05$; ***$P < 0.001$.

Quantification of ruminal bacteria

The resulting primer set (Table 2) produced PCR products of the expected size with test strains. All PCR products were of the expected size (Figure 1). The results of quantification are shown in Table 5. Overall, the quantity of *B. fibrisolvens*, *R. albus* and *F. succinogenes* DNA decreased ($P < 0.05$) by 18%, 42% and 67%, respectively, when cows were fed supplemental oil compared with control. Among oil treatments, DNA quantity of *B. fibrisolvens* and *R. flavefaciens* decreased ($P < 0.05$) by 35% and 25% in cows fed L *v.* S.

Discussion

Ruminal fermentation

Oil supplementation in our study led to lower total VFA concentration. It is suggested that unsaturated FA from oil could have interfered with ruminal fermentation resulting in greater gut fill. This is supported by the observed 3% reduction in 'residual organic matter' digestion (which could be mainly starch) (Table 1), which accounted for the reduction in VFA concentration (ca. 4% reduction). Additionally, the shift in carbohydrate sources (i.e. relatively more (hemi) cellulose due to less corn grain) because of the substitution of grain for oil in the diets coupled with the reduction in cell wall fermentation due to reduced bacterial activity, partly explained the reduction in total VFA concentrations.

Dietary oil supplementation effects on ruminal NH_3-N concentration have been variable. For example, in studies with sheep fed hay and concentrate, a decrease in ruminal NH_3-N was observed with LSO supplementation (Ikwuegbu and Sutton, 1982; Broudiscou *et al.*, 1994). In contrast, an increase in ruminal NH_3-N was observed in steers fed grass silage and concentrate (Scollan *et al.*, 2001) or fed supplemental SBO or LSO (Brokaw *et al.*, 2001; Ueda *et al.*, 2003). Our results were consistent with those found previously in bovine. In this study, protozoal counts decreased and proteolytic bacteria counts increased with supplemental oil, which likely accounted for the increase in ruminal NH_3-N concentration. Supplemental oil resulted in greater proteolytic bacteria counts, possibly due to a decrease in protozoa counts and predation. The greater content of proteolytic bacteria likely accelerated protein degradation,

causing ruminal NH_3-N concentration to increase. Furthermore, increased ruminal NH_3-N concentration could possibly be associated with a decrease in microbial protein synthesis by ruminal microbes.

Ruminal microorganisms

Over the years, it has been well recognized that supplemental oil affects certain aspects of microbial metabolic activity and/or modifies the microbial population in the rumen that are involved with cellulose digestion (White *et al.*, 1958; Ørskov *et al.*, 1978). Changes in the ruminal microbiota, as well as increased availability of free fatty acids in the rumen, are major factors affecting ruminal digestion. Some researchers have explained these effects based on (i) direct disruption of microbial cell membrane and cellular function by the unsaturated fatty acids, (ii) lipid coating of bacteria and feed particles, (iii) toxicity of cellulolytic bacteria by long-chain fatty acids and (iv) affecting bacterial population distribution due to antimicrobial effects of fatty acids (Jenkins, 1993). In the study of Ferlay *et al.* (1993), PUFA had a more negative effect on the metabolism of cellulolytic bacteria than saturated fatty acids. Few experiments, however, have evaluated changes in the profiles of ruminal microorganisms in response to dietary PUFA.

In the present study, cows fed supplemental oil had considerably lower numbers of cellulolytic bacteria, which likely accounted for the lower ruminal digestibility of NDF (56% *v.* 51%) and ADF (53% *v.* 50%) we observed (Hu *et al.*, 2007). Unsaturated fatty acids have been reported to have direct inhibitory effects on ruminal protozoa (Oldick and Firkins, 2000). Linoleic acid is toxic to ruminal protozoa and previous research has shown a consistent decrease in protozoal counts *in vivo* (Sutton *et al.*, 1983; Hristov *et al.*, 2004) and *in vitro* (Newbold and Chamberlain, 1988). Hristov *et al.* (2005) reported *in vitro* data showing 48%, 88% and 100% eradication of ruminal protozoa with inclusion of 0.25%, 0.5% and 1% linoleic acid, respectively, in the incubation media. Another study showed that oleic acid at 0.25%, 0.5% and 1% decreased protozoal counts by 26%, 45% and 78%, respectively (Hristov *et al.*, 2004). Further, Oldick and Firkins (2000) observed a linear decrease in ruminal protozoa with increasing degree of

unsaturation of dietary fats. In our study, a marked decrease in protozoa and cellulolytic bacteria numbers were observed when oils were supplemented. These effects were possibly associated with direct inhibition and/or coating action of the unsaturated fatty acids on microorganisms.

There is a paucity of data regarding the effects of supplemental oil on other bacterial (except for cellulolytic bacteria) populations in cattle. In the present study, total viable bacteria and amylolytic bacteria counts were not affected by oil. However, oil resulted in greater proteolytic bacteria counts possibly resulting from a decrease in protozoa as observed in cows fed L or S compared with fish oil (Loor *et al.*, 2005b).

The extreme complexity of the ruminal microbiota has been uncovered in numerous publications that employed isolation of pure cultures. However, culture-based approaches are very time- and labor-consuming, and the results, which are based on phenotypic characteristics, are not precise or conclusive. Modern molecular techniques based on sequence comparison of nucleic acids (DNA and RNA) offer certain advantages. These techniques can be used to provide molecular characterization and a classification scheme that predicts natural evolutionary relationships without cultivating the microorganisms (Mackie *et al.*, 2000), but also can be suitable for quantifying microorganisms (Reilly and Attwood, 1998). Advances in molecular microbial technique allowed us to quantify four ruminal bacteria that play key roles in aspects of ruminal biohydrogenation (e.g. lipid metabolism in ruminants). We specifically set out to design and validate PCR primers for detection of the chosen ruminal bacteria. These primers were designed to satisfy the stringent criteria required in real-time PCR quantification of a target in a microbial community DNA mixture. *Butyrivibrio fibrisolvens* is the most active bacterial species in the biohydrogenation of PUFA in the rumen (Jenkins, 1993). *Ruminococcus albus* also plays a role in the biohydrogenation of PUFA in the rumen. They remove unsaturated double bonds in order to detoxify the PUFA and enable growth of the bacterium (Devillard *et al.*, 2006).

In the present study, we observed that cows fed S or L or SL had lower DNA quantity of ruminal bacteria associated with biohydrogenation (*B. fibrisolvens* and *R. albus*) and also fibrolytic bacteria (*F. succinogenes* and *R. flavefaciens*). Kepler *et al.* (1966) reported that PUFA were particularly toxic, and other long-chain fatty acids were toxic to some of the cellulolytic bacteria also found in the rumen (Maczulak *et al.*, 1981). Furthermore, it appeared that L had a more potent inhibitory effect on ruminal microbiota. In the present study, cows fed L had lower DNA quantity of *B. fibrisolvens* and *R. flavefaciens* compared with cows fed S. Our results (except for *R. albus*) were consistent with those from a recent study by Maia *et al.* (2007), who reported that linolenic acid was more toxic than linoleic acid. In fact, the inhibitory effect of unsaturated oils on ruminal bacteria appears to be associated with the degree of unsaturation of the fatty acids, e.g. two unsaturated double bonds (linoleic acid) *v.* three double

bonds (linolenic acid). Oldick and Firkins (2000) observed a linear decrease in ruminal protozoa with an increasing degree of unsaturation of dietary fats when diets (48.5% maize silage and 51.5% concentrate) added 4.85% fat from prills of partially hydrogenated tallow, tallow or animal-vegetable fat.

Conclusions

Similar to previous studies, we observed that oil supplementation (4% of diet DM) to dairy cows decreases ruminal fermentation leading to lower VFA concentrations. Populations of ruminal microorganisms also were affected by LSO and SBO, with total protozoa and cellulolytic bacteria being reduced, and total proteolytic bacteria being increased by oils. Our data are some of the first to use molecular techniques to quantify detrimental effects of dietary PUFA on key ruminal bacteria with major roles in fiber digestion and biohydrogenation. In this context, our results clearly showed that, at the same level of supplementation linolenic acid was more potent than linoleic acid in reducing the amount of *B. fibrisolvens, F. succinogenes* and *R. flavefaciens.* Detrimental effects of oils on ruminal microorganisms could partly explain the observed effects on ruminal fermentation parameters.

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