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The Effect of Lipid Supplements on Ruminal Bacteria in Continuous Culture Fermenters Varies with the Fatty Acid Composition

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A single flow continuous culture fermenter system was used in this study to investigate the influence of dietary lipid supplements varying in their fatty acid content on the DNA concentration of selected rumen bacteria. Four continuous culture fermenters were used in a 4×4 Latin square design with four periods of 10 d each. Treatment diets were fed at 45 g/d (DM basis) in three equal portions during the day. The diets were: 1) control (CON), 2) control with animal fat source (SAT), 3) control with soybean oil (SBO), and 4) control with fish oil (FO). Lipid supplements were added at 3% of diet DM. The concentrations of total volatile fatty acids and acetate were not affected ($P>0.05$) by lipid supplements. Concentrations of propionate, iso-butyrate, valerate and iso-valerate were highest ($P<0.05$) with the FO diet compared with the other treatment diets. The concentration of *t*11 C18:1 (vaccenic acid, VA) in effluents increased ($P<0.05$) with SBO and FO diets and was highest with the SBO diet. The concentrations of C18:0 in effluents were lowest ($P<0.05$) for the FO diet compared with the other treatment diets. Concentrations of DNA for *Anaerovibrio lipolytica*, and *Butyrivibrio proteoclasticus* in fermenters were similar ($P>0.05$) for all diets. The DNA concentrations of *Butyrivibrio fibrisolvens* and *Ruminococcus albus* in fermenters were lowest ($P<0.05$) with the FO diet but were similar ($P>0.05$) among the other treatment diets. *Selenomonas ruminantium* DNA concentration in fermenters was highest ($P<0.05$) with the FO diet. In conclusion, SBO had no effect on bacterial DNA concentrations tested in this study and the VA accumulation in the rumen observed on the FO diet may be due in part to FO influence on *B. fibrisolvens*, *R. albus*, and *S. ruminantium*.

***Keywords*:** lipid, fatty acids, bacteria, fermenters

Conjugated linoleic acid (CLA) is a mixture of positional and geometric isomers of linoleic acid with two double bonds in conjugated arrangement. The primary CLA isomer in dairy products, *cis*-9, *trans*-11 (*c*9*t*11), is synthesized either in the rumen as an intermediate during the biohydrogenation (BH) of linoleic acid or from the endogenous conversion of vaccenic acid (VA, *trans*-11 C18:1) by Δ-9 desaturase in the mammary gland (Griinari *et al*., 2000). Approximately 80% of *c*9*t*11 CLA appearing in milk fat is synthesized in the mammary gland via Δ-9 desaturase (Mosley *et al*., 2006). Following reports that *c*9*t*11 CLA inhibits the growth of a number of cancer cell lines and suppresses chemically induced tumors development in animal models (Parodi, 2003); there has been growing interest in developing dairy products enriched with *c*9*t*11 CLA.

Providing plant and marine oils (Loor *et al*., 2002, 2005; Varadyova *et al*., 2007; Lee *et al*., 2008), algae (Boeckaert *et al*., 2007a, 2007b), and increasing forage to concentrate ratio (AbuGhazaleh and Jacobson, 2007) all have been shown to increase ruminal production of VA and subsequently milk *c*9*t*11 CLA content. Surprisingly, little information is currently available about the effects of feeding such diets on rumen microbial ecology, particularly, bacterial species believed to be involved in the BH process. The difficulties in measuring bacterial populations using the traditional culture-based *in vitro* methods along with the low accuracy and sensitivity of such methods may have contributed to that. However, recent advances in molecular microbiology techniques (denaturing gradient gel electrophoresis and real time PCR) have made quantifying microorganism population more accurate and sensitive

The rumen ecosystem is populated by a highly diverse collection of anaerobic microbes with the majority (70-80% of the microbial matter in the rumen) attached to feed particles in the digesta (Craig *et al*., 1987; McAllister *et al*., 1994). Among the different microbes present in the rumen, bacteria are considered to be the most actively involved in the BH of dietary unsaturated FA (Nam and Garnsworthy, 2007; Or-Rashid and Alzahal, 2008). It is generally agreed that the predominant ruminal celluloytic species such as *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, *Ruminococcus albus*, and *Butyrivibrio fibrisolvens* play fundamental roles in the BH of dietary unsaturated fatty acids (FA) (Maczulak *et al*., 1981). More recent studies have identified *B. fibrisolvens*, *B. hungatei*, and *Clostridium proteoclasticum*, which was recently reclassified as *Butyrivibrio proteoclasticus* by Christina *et al*. (2008) and Boeckaert *et al*. (2008), as the principal rumen bacteria in the BH of C18 unsaturated FA (Wallace *et al*., 2006; Maia *et al*., 2007). Wasowska *et al*. (2006) showed that addition of C20:5n3 (eicosapentaenoic acid, EPA) and C22:6n3 (docosahexaenoic acid, DHA) at 50 mg/L to pure cultures signifi-

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cantly inhibited growth of *B. fibrisolvens*, while linoleic acid had no effect. Other bacterial species (*B. hungatei*, *Selenomonas ruminantium*, and *B. proteoclasticus*) were also shown to be sensitive to the presence of unsaturated FA (Maia *et al*., 2007). Therefore, the main objective of this study was to evaluate the influence of dietary lipid supplements varying in their FA content on the DNA concentration of selected rumen bacteria using continuous culture fermenters. The effects of lipid supplements on fermentation and effluents FA composition were also evaluated.

Materials and Methods

Experimental protocol

Four single flow continuous fermenters (800 ml), as described by Teather and Sauer (1988), were used in 4×4 Latin square design with 4 periods of 10 d each. Treatment diets (50% alfalfa pellets, 50% concentrate) were fed (45 g^{-1} DM basis) in three equal portions during the day at 0800, 1500, and 2200 h. The diets were: 1) control (CON), 2) control with animal fat (SAT), 3) control with soybean oil (SBO), and 4) control with fish oil (FO). Rumo-fat, animal fat source, (Robt Morgan Inc., USA) and Menhaden FO (Omega Protein Inc., USA) were used in this study. Lipid supplements were added at 3% of diet DM. Corn, soybean meal, limestone, vitamins, and minerals made up the concentrate mix (Table 1). The chemical and FA composition for treatment diets and lipid supplements are presented in Tables 1 and 2.

Continuous culture

Two ruminally fistulated Holstein cows fed a total mixed ration (55:45 forage to concentrate ratio) were used for collection of ruminal contents. Whole ruminal contents were collected 2-4 h after the morning feeding, transferred to the laboratory in sealed bags, mixed, and then strained using a double-layered cheese cloth. Approximately 700 ml of the ruminal fluid was added to each of the four fermenters, containing 100 ml of prewarmed buffer (Weller and Pilgrim, 1974). Anaerobic conditions in fermenters were maintained by infusing CO_2 at 40 ml/min. Cultures were stirred continuously at 45 rpm and fermenter pH was measured daily before addition of feed using a portable pH meter at 0800, 1500, and 2400 h. Fermenter pH was maintained between 6.2-6.9 by adjusting buffer pH level as needed with 1 N NaOH or 1 N HCl. Fermenter temperature was maintained at 39°C using a circulating water bath. Buffer was delivered continuously at a flow rate of 1.25 ml/min (11% h^{-1} liquid dilution rate), using a precision pump. Flow rate of each fermenter was recorded every day at 08:00.

Table 1. Ingredients, chemical composition, and fatty acid profile of treatment diets

Ingredients (g/kg DM)	Treatment[a]			
	CON	SAT	SBO	FO
Alfalfa hay	500	500	500	500
Corn, cracked	340	320	320	320
Soybean hulls	60	56	56	56
Soybean meal, 48% CP	86	80	80	80
Limestone	10	10	10	10
Dicalcium phosphate	4	4	4	4
Animal fat[b]	0	30	0	0
Soybean oil	0	0	30	0
Fish oil	0	0	0	30
Chemical composition (g/kg DM)				
Dry matter	967	970	971	965
Acid detergent fiber (ADF)	258	265	257	260
Neutral detergent fiber (NDF)	351	342	35.4	343
Crude protein (CP)	166	175	162	167
Ether Extract	19	43	43	47
Ash	70	83	72	76
Fatty acids composition, (mg/g of DM)				
C14:0	0.2	0.3	0.1	2.6
C16:0	3.1	7.7	6.3	9.3
C18:0	0.7	15.4	2.4	1.7
C18:1n9	3.5	3.2	9.1	4.9
C18:2n6	7.7	7.0	22.7	8.0
C18:3n3	0.9	0.8	2.8	1.1
C20:5n3 (EPA)[d]	ND[e]	ND	ND	3.1
C22:5n3	ND	ND	ND	0.5
C22:6n3 (DHA)[e]	ND	ND	ND	1.9

[a] CON, control diet with no added fat; SAT, control diet with 30 g/kg (diet DM) added animal fat; SBO, control diet with 30 g/kg (diet DM) added soybean oil; FO, control diet with 30 g/kg (diet DM) added fish oil.
[b] Animal fat: Rumo-fat (Robt Morgan Inc., USA).
[c] ND, not detected or detected at <0.01.
[d] EPA, eicosapentaenoic acid.
[e] DHA, docosahexaenoic acid.

Table 2. 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)
Butyrivibrio fibrisolvens	Forward	TAACATGAGTTTGATCCTGGCTC	45	62	136
	Reverse	CGTTACTCACCCGTCCGC			
Ruminococcus albus	Forward	GTTTTAGGATTGTAAACCTCTGTCTT	45	60	270
	Reverse	CCTAATATCTACGCATTTCACCGC			
Anaerovibrio lipolytica	Forward	TGGGTGTTAGAAATGGATTCC	45	57	597
	Reverse	CTCTCCTGCACTCAAGAATT			
Selenomonas ruminantium	Forward	TGCTAATACCGAATGTTG	20	54	513
	Reverse	TCCTGCACTCAAGAAAGA			
Butyrivibrio proteoclasticus	Forward	TCCTAGTGTAGCGGTGAAATG	20	62	188
	Reverse	TTAGCGACGGCACTGAATGCCTAT			

Sample collection and analysis

Starting on days 7, 8, and 9 of each period, the overflow (effluent) was collected into 2 L plastic flasks and processed as outlined by AbuGhazaleh and Buckles (2007). Bacterial cells from effluent were isolated for analysis of FA composition as described by Czerkawski (1976). Treatment diets, lipid supplements, effluent, and bacterial samples were methylated using the sodium methoxide ($NaOCH_3$) and HCl two step procedure as outlined by Kramer *et al.* (1997) and analyzed in duplicate for FA as described by AbuGhazaleh and Buckles (2007).

On day 10 of each period, three 10-ml samples were collected from each fermenter, using pipet with large open mouth, at 3 h post morning feeding for VFA, ammonia-N (NH_3-N) and bacterial analysis as described by AbuGhazaleh *et al.* (2011). Samples for VFA analyses were prepared and analyzed as described by Jenkins (1987), using 2-ethylbutyric acid as an internal standard. Ammonia-N samples were centrifuged at 15,000×g at 4°C for 10 min and the supernatant was acidified with 0.5 ml of 0.1 N HCl then analyzed for NH_3-N by Teco Diagnostics kit (Anaheim, USA) using a spectrophotometer (Thermo Spectronic Genesys 5 Spectrophotometer, Artisan Scientific, USA). Bacterial samples collected from fermenters were frozen immediately in liquid nitrogen and stored at -80°C until bacterial analysis. Samples were thawed and centrifuged at 500×g for 15 min, to remove liquid associated bacteria, and the supernatant was discarded. To remove any trapped liquid associated bacteria, approximately 4 ml of phosphate buffered saline was then added to sediment, centrifuged again at 500×g for 15 min, and the supernatant was discarded. The DNA from pellets, containing the solid associated bacteria, was then extracted using the MO BIO Ultraclean™ Microbial DNA Isolation kit (MO BIO Laboratories, Inc., USA). Concentration of DNA was measured by BioPhotometer (Eppendorf Scientific, Inc., USA) at A_{260}=50 ng dsDNA/μl using disposable Eppendorf UVette cuvets.

Purified cultures of *Butyrivibrio fibrisolvens* (DSM 3071), *R. albus* (DSM 20455), *A. lipolytica* (DSM 3074), and *S. ruminantium* (DSM 2150) 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 this study are shown in Table 2. Primer pairs for *A. lipolytica* and *S. ruminantium* were as described by Tajima *et al.* (2001). Primer pairs for *B. fibrisolvens* and *R. albus* were described by Yang *et al*. (2009). The primer pair for *B. proteoclasticus* was produced using PrimerQuest at Integrated DNA Technologies (IDT, USA). The specificity of primers was confirmed using the BLAST program in the GenBank Database. The PCR products for all tested bacteria were sent to a DNA sequencing laboratory (GENEWIZ, USA) for validation (>97% homology).

Table 3. Coefficients of determination (R^2) and slope of the PCR standard curves for each reaction

	R^2	Slope
Ruminococcus albus	0.99	-3.366
Butyrivibrio fibrisolvens	1.0	-3.302
Anaerovibrio lipolytica	1.0	-3.336
Selenomonas ruminantium	0.99	-3.568
Butyrivibrio proteoclasticus	0.99	-3.569

The five purchased bacteria were grown in our laboratory in sealed Hungate tubes as specified by DSMZ and ATCC. Tubes were incubated for 3-5 d 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 using the MO BIO Ultraclean™ Microbial DNA Isolation kit. DNA concentrations were determined as previously described. In order to construct standard curves, serial dilutions (1/10) of isolated genomic DNA from each pure bacterium were used. The slopes and coefficient of determination (R^2) for the standard curves are presented in Table 3.

Individual species-specific real time quantitative PCR (qPCR) was performed using Bio-Rad iCycler MyiQ single color real-time PCR detection system (Bio-Rad Laboratories, USA), using fluorescence detection of SYBR green mix (Bio-Rad Laboratories). Briefly 12.5 μl SYBR green mix, 2 μl of each primer, sample DNA (starting concentration; Table 2), and RNAse free water were added to make a total volume of 25 μl. Amplification involved one cycle at 95°C for 10 min for initial denaturation and then 40 cycles of 95°C for 30 sec followed by annealing at the temperatures shown in Table 2 for 30 sec and then at 72°C for 1 min except for *B. proteoclasticus* where a modified temperature program was used to specifically detect the *B. proteoclasticus* as outlined by Reilly and Attwood (1998). The PCR amplification involved one cycle at 95°C for 3 min for initial denaturation, followed by 6 cycles of 95°C for 30 sec, 62°C for 15 sec, and 72°C for 30 sec and 25 cycles of 95°C for 15 sec, 62°C for 5 sec, and 72°C for 30 sec, with a final cycle of 72°C for 3 min (Reilly and Attwood, 1998). Detection of the fluorescent product was set at the last step of each cycle. Standard curves, DNA sample quantification and melting curve analyses were obtained using iQ5 Optical

Table 4. Fatty acid profile of lipid supplements (g/100 g FA)

Fatty acid	Animal fat[a]	Soybean oil	Fish oil
C14:0	2.9	0.1	10.1
C16:0	25.3	10.4	23.1
C18:0	63.5	4.0	3.9
C18:1n9	0.4	20.9	6.4
C18:2n6	0.0	53.8	1.3
C18:3n3	ND[b]	7.3	1.1
C20:5n3 (EPA)[c]	ND	ND	12.5
C22:5n3	ND	ND	1.9
C22:6n3 (DHA)[d]	ND	ND	9.0
Others	8.0	3.6	30.8

[a] Animal fat: Rumo-fat (Robt Morgan Inc.).
[b] ND, not detected or detected at <0.01.
[c] EPA, eicosapentaenoic acid.
[d] DHA, docosahexaenoic acid.

System Software (version 2.1, Bio-Rad Laboratories). Melting curve analysis was performed after each amplification step to determine the specificity of PCR product. The melting curve was obtained by slow heating with a 0.1°C/sec increment from 65°C to 95°C, with fluorescence collection at 0.1°C intervals. 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.

Statistical analysis

Data were analyzed as a 4×4 Latin square design using the PROC MIXED of SAS (2003; SAS Inst. Inc., USA) using the following model:

$$Y_{ijk} = \mu + T_i + P_j + F_k + e_{ijk}$$

Where μ was the overall mean, T was the lipid source, P was the period effect, F was the fermenter effect and e was the residual error. The random effect was fermenter. Results are expressed as least square means with standard error of the means. The significance threshold was set at $P<0.05$.

Results

The ingredient, chemical, and FA composition of treatment diets are presented in Table 1. The three lipid supplements were chosen because of the differences in their FA composition (Table 4) and the measured FA profiles were typical of the lipid supplements. Rumo-fat showed high contents of C18:0. Linoleic acid was the main FA in SBO while EPA and DHA were the main polyunsaturated FA in FO. The intake of individual FA therefore varied according to diet (Table 1). Intake of saturated FA (C18:0) was highest with the SAT diet, while intake of C18 unsaturated FA was highest with the SBO diet (Table 1). The intake of highly polyunsaturated FA (EPA, DHA, and C22:5n3) were highest with the FO diet.

The effects of lipid supplements on fermentation are presented in Table 5. Fermenter pH was similar between treatment diets ($P>0.05$). Ammonia-N concentration was higher ($P<0.05$) with lipid supplement diets compared with the CON diet and there were no significant differences in NH_3-N concentration ($P>0.05$) between diets with added lipid. The concentration of total VFA was not different ($P>0.05$) among the treatment diets (Table 5). Relative to the CON diet, the concentration of acetate was not affected ($P>0.05$) by lipid supplements whereas the concentration of propionate increased ($P<0.05$) with the FO diet. Higher concentrations ($P<0.05$) for iso-butyrate, valerate and iso-valerate and lower acetate to propionate ratios were observed with the FO diet relative to the other treatment diets. The concentration of butyrate was higher ($P<0.05$) with the CON and SAT diets than the SBO and FO diets.

The effect of lipid supplements on effluent FA is shown in Table 6. Diets supplemented with lipids had higher ($P<0.05$) total effluent FA concentration than the CON diet. Effluents from the SBO and FO diets had higher total FA concentration than the SAT diet. With the exception for C12:0, all other FAs were affected by lipid supplements. The concentrations of C18:0, the end product of BH, increased ($P<0.05$) with the SAT and SBO diets, and decreased with the FO diet when compared with the CON diet. Compared with the CON diet, the concentrations of total *trans* C18:1, VA and *c*9*t*11 CLA in effluents increased only in diets supplemented with SBO and FO. The *c*9*c*11 CLA was detected only in effluents from the SBO diet, while EPA, DHA, and C22:5n3 were detected only in effluents from the FO diet.

The effect of lipid supplements on bacterial FA composition is presented in Table 7. As with effluent FA, the concen-

Table 5. Effect of lipid source on volatile fatty acids (*m*M) and ammonia-N

	Treatment[a]				SEM
	CON	SAT	SBO	FO	
Total VFA	63.1	62.4	53.9	66.0	6.16
Acetate	32.5	33.6	28.7	32.0	3.91
Propionate	15.7^{x}	14.6^{x}	13.4^{x}	20.3^{w}	1.45
Butyrate	12.8^{w}	12.0^{w}	9.9^{x}	10.4^{x}	0.78
Iso-butyrate	0.4^{x}	0.3^{x}	0.2^{x}	0.7^{w}	0.11
Valerate	1.1^{x}	1.1^{xy}	0.9^{y}	1.3^{w}	0.06
Iso-valerate	0.7^{x}	0.8^{x}	0.8^{x}	1.3^{w}	0.11
Acetate:propionate	2.1^{x}	2.3^{w}	2.1^{wx}	1.5^{y}	0.10
NH_3-N (mg/dL)	9.0^{x}	10.6^{w}	11.0^{w}	12.5^{w}	0.98
pH	6.63	6.61	6.64	6.58	0.04

[a] CON, control diet with no added fat; SAT, control diet with 30 g/kg (diet DM) added animal fat; SBO, control diet with 30 g/kg (diet DM) added soybean oil; FO, control diet with 30 g/kg (diet DM) added fish oil.
[w,x,y] Values within rows with different superscript letters are significantly different ($P<0.05$).

Table 6. Effect of lipid source on effluent fatty acids (mg/g of DM)

Fatty acid	Treatment[a]				SEM
	CON	SAT	SBO	FO	
C12:0	0.07	0.07	0.07	0.09	0.008
C14:0	0.66^{xy}	0.80^{x}	0.61^{y}	3.49^{w}	0.054
C16:0	6.84^{y}	11.06^{x}	10.18^{x}	17.01^{w}	0.399
C18:0	7.78^{y}	21.99^{w}	15.89^{x}	3.51^{z}	1.224
VA[b]	3.74^{y}	3.47^{y}	11.82^{w}	9.32^{x}	0.730
C18:1 total *trans*	4.05^{y}	3.87^{y}	12.72^{w}	9.70^{x}	0.772
C18:1n9	3.66^{y}	3.18^{y}	7.68^{w}	4.72^{x}	0.194
C18:2n6	4.94^{x}	3.94^{xy}	6.79^{w}	3.17^{y}	0.342
C18:3n3	0.75^{x}	0.70^{x}	0.96^{w}	0.65^{x}	0.050
CLA[c] *c9t11*	0.18^{x}	0.16^{x}	0.47^{w}	0.39^{w}	0.085
C20:5n3 (EPA)[d]	ND	ND	ND	2.14^{w}	0.153
C22:5n3	ND	ND	ND	0.60^{w}	0.058
C22:6n3 (DHA)[f]	0.02^{x}	ND	0.02^{x}	1.46^{w}	0.115

[a] CON, control diet with no added fat; SAT, control diet with 30 g/kg (diet DM) added animal fat; SBO, control diet with 30 g/kg (diet DM) added soybean oil; FO, control diet with 30 g/kg (diet DM) added fish oil.
[b] VA, vaccenic acid.
[c] CLA, conjugated linoleic acid.
[d] EPA, eicosapentaenoic acid.
[e] ND, not detected or detected at <0.01.
[f] DHA, docosahexaenoic acid.
[w,x,y,z] Values within rows with different superscript letters are significantly different (P<0.05).

trations of VA, total *trans* C18:1 and *c9t*11 CLA in ruminal bacteria were higher (P<0.05) with the SBO and FO diets than the CON and SAT diets. The concentrations of C18:0 were higher (P<0.05) with the SBO and SAT diets, and lower with the FO diet when compared to the CON. The EPA, DHA, and C22:5n3 were present only in bacteria from the FO diet.

The effects of lipid supplement on the DNA concentration of the selected ruminal bacteria are presented in Table 8. Lipid supplementation had no effect (P>0.05) on the DNA concentrations of *A. lipolytica* and *B. proteoclasticus*. Addition of FO to diet resulted in lower (P<0.05) DNA concentrations for *R. albus* and *B. fibrisolvens*, but higher (P<0.05) DNA concentration for *S. ruminantium* when compared with other diets. Supplementing diets with saturated animal fat (SAT) and SBO had no effect (P>0.05) on the DNA concentrations for *R. albus* and *B. fibrisolvens* compared with the CON diet, however, saturated animal fat supplementation resulted in lower (P<0.05) DNA concentration for *S. ruminantium* compared with the CON diet.

Table 7. Effect of lipid source on bacterial fatty acids (g/100 g FA)

Fatty acid	Treatment[a]				SEM
	CON	SAT	SBO	FO	
C12:0	0.50^{w}	0.36^{x}	0.22^{y}	0.54^{w}	0.034
C14:0	3.46^{x}	2.77^{y}	1.57^{z}	6.28^{w}	0.274
C16:0	21.86^{x}	22.67^{x}	16.92^{y}	27.12^{w}	0.958
C18:0	31.47^{x}	40.52^{w}	41.95^{w}	5.42^{y}	3.052
VA[b]	8.31^{x}	7.76^{x}	16.09^{w}	15.57^{w}	0.979
C18:1total *trans* FA	10.60^{x}	9.23^{x}	18.27^{w}	19.77^{w}	1.040
C18:1n9	5.50^{x}	4.26^{y}	6.52^{w}	4.89^{xy}	0.265
C18:2n6	2.73^{w}	1.72^{x}	1.89^{x}	2.21^{wx}	0.321
C18:3n3	0.61^{y}	0.41^{y}	0.95^{x}	1.12^{w}	0.121
CLA[c] *c9t11*	0.49^{w}	0.24^{x}	0.45^{wx}	0.61^{w}	0.088
C20:5n3 (EPA)[d]	ND[e]	ND	ND	3.80	0.088
C22:5n3	ND	ND	ND	1.66	0.033
C22:6n3 (DHA)[f]	ND	ND	ND	3.03	0.044

[a] CON, control diet with no added fat; SAT, control diet with 30 g/kg (diet DM) added animal fat; SBO, control diet with 30 g/kg (diet DM) added soybean oil; FO, control diet with 30 g/kg (diet DM) added fish oil.
[b] VA, vaccenic acid.
[c] CLA, conjugated linoleic acid.
[d] EPA, eicosapentaenoic acid.
[e] ND, not detected or detected at <0.01.
[f] DHA, docosahexaenoic acid.
[w,x,y,z] Values within rows with different superscript letters are significantly different (P<0.05).

Table 8. Effect of lipid source on DNA abundance (pg) of selected rumen bacteria at a given starting concentration

	Treatment[a]				SEM
	CON	SAT	SBO	FO	
Ruminococcus albus, 45 ng*	5.17[w]	5.42[w]	5.71[w]	1.96[x]	1.027
Butyrivibrio fibrislovens, 45 ng*	126.71[w]	124.73[w]	120.26[w]	59.26[x]	20.03
Anaerovibrio lipolytica, 45 ng*	5.45	5.69	4.91	9.81	3.270
Selenomonas ruminantium, 20 ng*	177.51[x]	54.94[y]	75.57[xy]	691.16[w]	49.002
Butyrivibrio proteoclasticus, 20 ng*	1.05	2.13	1.71	0.86	0.694

[a] CON, control diet with no added fat; SAT, control diet with 30 g/kg (diet DM) added animal fat; SBO, control diet with 30 g/kg (diet DM) added soybean oil; FO, control diet with 30 g/kg (diet DM) added fish oil.
[w,x,y] Values within rows with different superscript letters are significantly different ($P<0.05$).
* Starting DNA concentration

Discussion

Lipid supplements in this study had no effect on fermenters total VFA or acetate concentrations, in agreement with others studies (Jenkins *et al*., 2003; Karnati *et al*., 2009). The increase in propionate and the decrease in butyrate concentrations with the FO diet were also reported in other studies (Kim *et al*., 2008; Lee *et al*., 2008; Vlaeminck *et al*., 2008) with the use of FO or algae. These changes may be linked to the alterations in the bacterial community seen in this study with the FO diet (discussed below).

The inclusion of SBO in diet resulted in the highest increase in effluent VA and total *trans* C18:1 concentrations. Relative to the CON diet, effluent VA and total *trans* C18:1 increased by approximately 2.2 fold with SBO supplementation. Vaccenic acid and other *trans* C18:1 isomers are formed as intermediates during BH of C18 unsaturated FA (Harfoot and Hazlewood, 1997). Indeed, the SBO diet had the highest proportions of C18 unsaturated FA among treatment diets (Table 1) averaging approximately 70% of total FA. Other studies also reported similar increases in VA and total *trans* C18:1 concentrations in rumen (Loor *et al*., 2002; Varadyova *et al*., 2007), and duodenal (Kucuk *et al*., 2008) with SBO inclusion in ruminant animals diet. Increasing the VA formation and flow from the rumen is the most limiting factor in enhancing milk *c9t11* CLA content as the majority of *c9t11* CLA in milk is a result of endogenous synthesis by Δ-9 desaturase, with VA as the substrate (Griinari *et al*., 2000).

Although not as effective as SBO, FO supplementation also increased effluent VA and total *trans* C18:1 concentrations by 1.5 and 1.4 fold, respectively, relative to the CON diet. Previous *in vitro* (AbuGhazaleh and Jenkins, 2004; Wasowska *et al*., 2006; Boeckaert *et al*., 2007b) and *in vivo* (Loor *et al*., 2005; Lee *et al*., 2008) studies reported a dramatic increase of total *trans* C18:1 and VA in the rumen when FO or marine algae were included in the diet. The increase in VA and total *trans* C18:1 concentrations with FO supplements cannot be attributed to differences in C18 unsaturated FA intake as both the FO and the CON diets had relatively similar C18 unsaturated FA content (Table 1). Previously, AbuGhazaleh *et al*. (2002) suggested that increases in rumen VA and total *trans* C18:1 accumulation with FO feeding is due to its ability to interrupt the complete BH of VA and *trans* C18:1 to C18:0. Subsequent studies (AbuGhazaleh and Jenkins, 2004; Boeckaert *et al*., 2007a) demonstrated that the DHA, and to a lesser extent EPA, in FO or their derivatives are responsible for inhibiting the terminal hydrogenation of *trans* C18:1 to C18:0, causing the accumulation of *trans* C18:1 in the rumen. Although the mode of action could not be determined, they speculated that FO may inhibit the growth and/or the reductase enzyme for the microorganism responsible for terminal hydrogenation of *trans* C18:1 to C18:0. The fact that the C18:0 concentrations in effluent were least and the VA to C18:0 ratio were highest with the FO diet compared with the other diets further confirm our previous findings.

Among the five bacteria examined in this study, only three appear to be affected by lipid supplements, primarily the FO. The DNA concentration of *B. fibrisolvens* and *R. albus* were significantly reduced with FO supplementation compared to other treatment diets indicating that FO inhibited their growth. Soybean oil and saturated fat supplementation had no effects on the growth of *B. fibrisolvens* and *R. albus* as DNA concentrations were similar to the CON diet. Wasowska *et al*. (2006) showed that EPA and DHA were more toxic to *B. fibrisolvens* than linoleic acid when added to pure cultures and linoleic acid toxicity toward *B. fibrisolvens* was transient as bacterium emerged from a lag phase 12 h after incubation. Recently, Maia *et al*. (2010) examined the effects of linoleic acid, EPA and DHA added to medium at 50 μg/ml on the growth of *B. fibrosolvens* JW11. Their results showed that EPA and DHA were more toxic than linoleic acid as the lag phase for *B. fibrosolvens* JW11 increased from 10 h with linoleic acid to >72 h with EPA and DHA. The sensitivity of *B. fibrisolvens*, *R. albus* and other bacterial strains to lipid supplements has been found to be influenced by FA composition (Maczulak *et al*., 1981; Paillard *et al*., 2007a; Maia *et al*., 2010) and levels of incubation (Maia *et al*., 2007). Maia *et al*. (2007) showed that most rumen bacterial species were able to grow in the presence of 50 μg linoleic acid per ml of medium, but not at 200 μg. In this study, linoleic acid concentration was approximately 10 μg/ml of fermenter fluid which may explain the lack of effects seen with the SBO diet. The mechanism(s) by which FO inhibits bacterial growth remains unclear but it may involve disruption of the cell membrane integrity (Keweloh and Heipieper, 1996) and/or cell metabolic reactions (Maia *et al*., 2010). In this study, FO supplementation significantly altered the FA composition of ruminal bacteria (Table 7) which may have disrupted the lipid bilayer structure and fluidity and therefore affecting bacterial growth. Whether the decrease in these bacteria with the FO supplement is responsible for accumulation of VA is by no means certain as other ruminal bacteria or microbes may be also involved.

Interestingly, no significant differences in the DNA concentrations of *B. proteoclasticus* were observed among treatment diets. *Butyrivibrio proteoclasticus*, which is closely related to *Butyrivibrio* (Paillard *et al*., 2007a), was identified to be the only known ruminal species to convert *trans* C18:1 to C18:0 (van de Vossenberg and Joblin, 2003; Wallace *et al*., 2006) and is among the most sensitive ruminal species to the toxic effects of dietary polyunsaturated FA (Wallace *et al*., 2006; Paillard *et al*., 2007b). Wallace *et al*. (2006) reported the *B. proteoclasticus* has ability to produce C18:0 from linoleic acid and its growth was inhibited when high levels of linoleic acid were present in diet. However, Kim *et al*. (2008) found that the decreased duodenal C18:0 flow in steers fed FO was not associated with *B. proteoclasticus* 16S rRNA gene concentrations in strained rumen fluid. Similarly, Huws *et al*. (2008) reported that DNA concentrations from the *Butyrivibrio* C18:0-producing group did not correlate with the C18:0 concentrations from rumen planktonic and biofilm samples. This suggests 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's still also possible that FO and SBO decreased the capacity of these bacteria to hydrogenate *trans* C18:1 rather than the bacteria as such. Recently, Paillard *et al*. (2007a) showed that the C18:0 producing bacteria had stronger butyrate kinase activity (> 600 U/mg protein) than the VA-producing bacteria (<40 U). In this study, butyrate concentration was significantly reduced with FO and SBO supplementation indicating possible inhibition in enzymatic activities of these bacteria.

Selenomonas ruminantium, non-fibrolytic bacteria that ferment soluble carbohydrates in the rumen (Tajima *et al*., 2001), was reported to be the predominant bacterium present in continuous cultures when incubated along with other cellulolytic bacteria (Chen and Weimer, 2001). Maia *et al*. (2007) showed that the growth of *S. ruminantium* was not inhibited when incubated with 50 μg/ml of linoleic acid, linolenic acid, EPA and DHA, as these bacteria were insensitive to polyunsaturated FA. In our study, DNA concentration of *S. ruminantium* was higher with the FO diet compared to the other treatment diets. It's unlikely that FO directly stimulated the growth of these bacteria. A possible explanation for the increase is that FO may have inhibited the growth of certain bacteria that suppress the growth of *S. ruminantium.* Many interactions among ruminal bacteria have been characterized before (Kudo *et al*., 1987; Chen and Weimer, 2001). For example, Chen and Weimer (2001) showed that the growth of *R. flavefaciens* and *F. succinogenes* were suppressed by inhibitors produced by *R. albus.* Therefore, one may speculate that the increase in DNA concentration of *S. ruminantium* with the FO diet may have resulted from the reduction in the DNA concentration for *R. albus*.

Based on lipolytic activity of *A. lipolytica* in pure cultures, Prins *et al*. (1975) suggested it was one of the bacterium involved in lipolysis in the rumen. In this study, we did not observe any effects of lipid supplements on the DNA concentration of *A. lipolytica*. Similar results were reported by Tajima *et al*. (2001) where no significant change was observed with *A. lipolytica* DNA concentration during a shift from forage-based diet to a grain-based diet. Maia *et al.* (2007) also reported no change in the growth of *A. lipolytica* upon incubating with PUFA at 50 μg/ml; demonstrating the low sensitivity of *A. lipolytica* to high levels of oils.

In conclusion, dietary lipid supplements in this study had no effects on total VFA and acetate concentrations. Propionate concentration increased and butyrate concentration decreased with the FO supplementation. Soybean oil and FO supplementations increased VA and *c*9*t*11 CLA content in effluents relative to other treatment diets. Stearic acid concentrations in effluents were least with FO diet. Fish oil supplementation decreased the DNA concentrations of *B. fibrisolvens* and *R. albus* and increased the DNA concentration of *S. ruminantium* relative to other treatment diets. Soybean oil and saturated fat supplementations had no effect on bacterial DNA concentrations tested in this study. The inhibitory effects of FO on BH (reduction of VA to C18:0) may be due in part to its influence on *B. fibrisolvens*, *R. albu*s, and *S. ruminantium*. Linking microbial changes to differences in FA flow to the small intestine can be the key to identify microorganisms responsible for VA accumulation which could potentially allow the development of novel strategies to manipulate rumen BH process leading to more enhancement in milk *c*9*t*11 CLA.

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