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Quantitative analysis of ruminal bacterial populations involved in lipid metabolism in dairy cows fed different vegetable oils

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Vegetable oils are used to increase energy density of dairy cow diets, although they can provoke changes in rumen bacteria populations and have repercussions on the biohydrogenation process. The aim of this study was to evaluate the effect of two sources of dietary lipids: soybean oil (SO, an unsaturated source) and hydrogenated palm oil (HPO, a saturated source) on bacterial populations and the fatty acid profile of ruminal digesta. Three non-lactating Holstein cows fitted with ruminal cannulae were used in a 3 × 3 Latin square design with three periods consisting of 21 days. Dietary treatments consisted of a basal diet (Control, no fat supplement) and the basal diet supplemented with SO (2.7% of dry matter (DM)) or HPO (2.7% of DM). Ruminal digesta pH, NH_3–N and volatile fatty acids were not affected by dietary treatments. Compared with control and HPO, total bacteria measured as copies of 16S ribosomal DNA/ml by quantitative PCR was decreased (P < 0.05) by SO. Fibrobacter succinogenes, Butyrivibrio proteoclasticus *and* Anaerovibrio lipolytica *loads were not affected by dietary treatments. In contrast, compared with control, load of* Prevotella bryantii *was increased (*P < *0.05) with HPO diet. Compared with control and SO, HPO decreased (*P < *0.05) C18:2* cis *n-6 in ruminal digesta. Contents of C15:0 iso, C18:11* trans*-11 and C18:2* cis*-9,* trans*-11 were increased (*P < *0.05) in ruminal digesta by SO compared with control and HPO. In conclusion, supplementation of SO or HPO do not affect ruminal fermentation parameters, whereas HPO can increase load of ruminal* P. bryantii*. Also, results observed in our targeted bacteria may have depended on the saturation degree of dietary oils.*

Keywords: soybean oil, rumen fermentation, vegetable oil, palm oil

Implications

A better knowledge of the rumen microbiome may help us to understand, and eventually modulate, the effect of nutrition on milk fat production and quality. This work was conducted to evaluate the effect of two sources of dietary lipids: soybean oil (SO, an unsaturated source) and hydrogenated palm oil (HPO, a saturated source) on bacterial populations and the fatty acid (FA) profile of ruminal digesta. Contents of C15:0 iso, C18:11 *trans*-11 and C18:2 *cis*-9, *trans*-11 were increased by SO. Supplementation with SO or HPO (2.7% dry matter (DM)) did not affect ruminal fermentation parameters, whereas HPO can increase loads of ruminal *Prevotella bryantii*.

Introduction

Dietary polyunsaturated fatty acids (PUFA) have toxic effects on ruminal microorganisms, therefore, lipid supplementation often leads to changes in ruminal microbial populations and shifts in ruminal fermentation parameters (Zhang *et al*., 2008). Rumen microbes attempt to detoxify PUFA by biohydrogenation (Maia *et al*., 2010). Biohydrogenation pathways require an initial hydrolysis of ingested dietary glyceride by microbial lipases/esterases causing the release of FA (Prive *et al*., 2015) at this stage; *Anaerovibrio lipolytica* is recognized as one of the major species involved in lipid hydrolysis in ruminants (Prive *et al*., 2013). Wallace *et al*. (2006) proposed that *Butyrivibrio* genus contained the main bacterial species involved in the biohydrogenation process. However, Huws *et al*. (2011) demonstrated that as yet uncultured bacteria belonging to the genera *Prevotella* and *Anaerovoax*, and unclassified *Ruminococcaceae* and *Clostridiales* may play more important roles in ruminal biohydrogenation.

It is known that cellulolytic bacteria (*Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, *Ruminococcus albus* and *Butyrivibrio fibrisolvens*) are important in the biohydrogenation process of dietary sources of PUFA (Potu *et al*., 2011). Also, *Butyrivibrio proteoclasticus* has been reported to

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be the principal rumen bacteria involved in biohydrogenation of C18:1 FA (Boeckaert *et al*., 2008). On the other hand, *P. bryantii* has been described as a ruminal bacterium that is involved in oligosaccharolytic and xylanolytic activities (Tajima *et al*., 2001) and also *Prevotella* spp. has been reported as resistant to inhibitory effects of dietary PUFA (Huws *et al*., 2010).

Supplementing dairy cow diets with SO can increase milk bioactive FA such as C18:1 *trans*-11 (Allred *et al*., 2006; Vargas-Bello-Perez *et al*., 2015a). Also, SO has been shown to reduce cellulolytic bacteria, protozoal populations and total concentration of volatile fatty acids (VFA) (Yang *et al*., 2009). On the other hand, hydrogenated vegetable oils have been used to increase the energy content of dairy cow diets in housed (Kargar *et al*., 2012) and pasture systems (Schroeder *et al*., 2002) without effect on milk composition (Vargas-Bello-Perez *et al*., 2015b).

To our knowledge, no study on the effect of dietary HPO on ruminal bacterial populations in dairy cows has been published. Also, animal trials reporting use of oils and their effect on rumen microbiome have less risk of bias compared with *in vitro* studies. Therefore, the aim of this study was to make a quantitative analysis of bacterial populations involved in ruminal biohydrogenation (*F. succinogenes*, *B. proteoclasticus* and *A. lipolytica*) and *P. bryantii* (one of the most predominant ruminal bacteria) in dairy cows fed different vegetable oils (SO as an unsaturated source and HPO as a saturated source). The effect of fat supplements on the FA profile of ruminal digesta was another objective. Our hypothesis was that supplementation with saturated *v.* unsaturated oils would have different effects on bacterial populations that were or were not involved in biohydrogenation.

Table 1 *Ingredients and chemical compositions of control, soybean oil (SO) and hydrogenated palm oil (HPO) diets*

	Diet[1]		
	Control (% of DM)	SO (% of DM)	HPO (% of DM)
Ingredient composition			
Alfalfa hay	17	17	17
Corn silage	18	18	18
High-moisture corn	10	10	10
Soybean hulls	34	34	34
Wheat bran	19	19	19
Vitamin and mineral premix[2]	2	2	2
SO	0	2.7	0
HPO	0	0	2.7
Chemical composition (%)			
DM	53.6	53.6	53.6
CP	16.6	16.6	16.6
Ether extract	2.3	5.1	6.3
NDF	39.2	39.2	39.2
ADF	21.0	21.0	21.0
Lignin	3.6	3.6	3.6
Ash	6.0	6.0	6.0
FA composition (g/100 g of FA)			
C4:0	0.03	0.09	0.73
C6:0	0.05	0.04	0.01
C8:0	0.03	0.03	0.07
C10:0	1.63	0.15	0.10
C12:0	0.16	0.13	2.08
C14:0	0.26	0.15	1.70
C16:0	15.6	13.7	45.9
C18:0	18.7	18.8	36.3
C18:1 *cis*-9	0.42	1.78	0.04
C18:2 *cis* n-6	46.9	49.5	5.03
C18:3 *cis*-6,9,12	0.17	0.10	0.19
C18:3 *cis*-9,12,15	7.44	6.38	0.55

DM = dry matter; FA = fatty acids.
[1]Control = basal diet/no fat supplement; SO = basal diet + 170 g/day per cow of SO; HPO = basal diet + 170 g/day per cow of HPO.
[2]Contained per kg: 25 000 mg of P; 80 000 mg of Ca; 25 000 mg of Mg; 1612 mg of S; 300 000 IU of vitamin A; 50 000 IU of vitamin D_3 ; 1600 IU of vitamin E.

Material and methods

Animals and treatments

Three non-lactating Holstein cows (684.7 ± 84.7 kg BW) fitted with ruminal cannulae no. 3C (Bar Diamond Inc., Boise, ID, USA) were used in a 3 × 3 Latin square design with three periods consisting of 21 days. Cows were fed to satisfy the requirements of a dry cow in the last trimester of gestation consuming 10 kg DM daily (National Research Council, 2001). Dietary treatments (Table 1) were a basal diet (Control) containing 56% forage and 44% concentrate ratio with no fat supplement, and fat-supplemented diets containing SO (170 g/day per cow = 2.7% DM) and HPO (170 g/day per cow = 2.7% DM). The amounts of oil used were similar to those reported to alter rumen FA in previous studies (Yang *et al*., 2009; Vargas-Bello-Perez *et al*., 2015a). The most important FA in dietary oils were as follows: SO contained (g/100 g) 25 of C18:1 *cis*-9 and 51 of C18:2 *cis* n-6, whereas HPO contained 47 of C16:0 and 43 of C18:0. Oils were administrated separately and mixed manually into the daily total mixed ration (TMR) for each cow. Animals were housed in individual stalls (2.4 × 6 m) and had free access to fresh water. Animal care and procedures were carried out according to the guidelines of the Animal Care and Use Committee of the Pontificia Universidad Católica de Chile.

Samples

On the last day of each 21-day period, samples of whole ruminal digesta were collected from the anterior, dorsal and mid-ventral regions of the rumen at 0900 h (2 h post feeding) and were squeezed through three layers of cheesecloth. A quantity of 10 ml of residual ruminal fluid was immediately used to determine pH by using a pH meter (PP-201; GOnDO Electronic, Taipei, Taiwan), 10 ml were kept for NH_3–N analysis (Bal *et al*., 2000) and another 10 ml were preserved for VFA determination by adding 1 ml of 25% metaphosphoric acid. Samples were frozen (−20°C) for later analysis. The VFA

measurement were performed by gas chromatograph (GC-2010, Shimadzu Scientific Instruments, Columbia, MD, USA) equipped with a 30-m wall-coated open-tubular fused-silica capillary column (Stabilwax-DA; 30 m × 0.32 mm i.d., 0.25 μm film thickness; Restek, Bellefonte, PA, USA). Oven temperature was programmed for 145°C for 2 min and then increased from 145°C to 220°C at 4°C/min. The injector and flame ionization detector were maintained at 250°C and 300°C, respectively. Following pH determination, the strained ruminal fluid was centrifuged for 10 min at 3000 × **g** at room temperature. The supernatant was discarded and the residue was stored at −20°C until microbiology analysis.

DNA extraction

Samples from each cow and every period were weighed (240 ± 12 μg) and deposited in 1.5 ml Eppendorf tubes. Subsequently, 300 μl of phosphate-buffered saline solution were added and mixed to homogenize the sample. DNA was obtained by incubating the sample for 30 min at 37°C with lysozyme (1 μg/μl) and then for 30 min at 37°C with proteinase K (0.1 mg/ml). DNA extraction was performed using the PowerSoil DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's recommendations.

Quantitative PCR conditions

Primers (forward and reverse) used to target bacterial species of interest are described in Table 2. Primers for *A. lipolytica* (Tajima *et al*., 2001), *F. succinogenes* (Tajima *et al*., 2001), *B. proteoclasticus* (Huws *et al*., 2010) and *P. bryantii* (Tajima *et al*., 2001) were those reported in previous research. Once obtained, the primers were tested for specificity using the probe match function at the Ribosomal Database Project (RDP; https://rdp.cme.msu.edu/probematch/search.jsp), as described by Huws *et al.* (2007). The oligonucleotides from each target bacteria were synthetized from Integrated DNA Technologies (Coralville, IA, USA). These primers were also analyzed for the requirements necessary for real-time PCR.

Real-time PCR quantification (qPCR) of total ruminal bacteria and bacterial species of interest was performed on a Rotor Gene 6000 (Corbett Life Science, Brisbane, Australia). Quantification of total ruminal bacteria was accomplished by qPCR amplifying the V3–V4 region of the 16S ribosomal RNA (rRNA) gene using the conserved bacterial domain-specific primers 341f (5'-CCTACGGGAGGCAGCAG-3') and 788r (5'-GGACTACCAGGGTATCTAA-3'). PCR reactions were carried out in quadruplicate and in 10 μl final volume containing 1 μl of extracted DNA (1 : 1000 dilution), 25 pmol/μl of each primer, DNAse-free water and 2 × LightCycler® 480 DNA Master SYBR Green I (Roche Applied Science, Mannheim, Germany). PCR conditions started with an initial denaturation at 95°C for 5 min, followed by 50 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 10 s and extension at 72°C for 15 s. The reaction mixture for quantification of specific bacteria consisted of 1 μl of DNA template, 20 pmol/μl of each specific primer described in Table 2, DNAse-free water and 2 × LightCycler® 480 DNA Master SYBR Green I. The PCR program was similar to total bacterial quantification, except for annealing temperature. Annealing for *A. lipolytica* and *B. proteoclasticus* was performed at 62°C, and *F. succinogenes* and *P. bryantii*'s annealing was performed at 60°C. Specificity of qPCR reactions was confirmed by analyzing the temperature characteristics of melting curves – increase of temperature from 72°C to 95°C, holding 1 s on the first step and 5 s on next steps.

The number of copies of the target bacterial 16S ribosomal DNA (rDNA) were determined by the serial dilution of purified genomic DNA extracted from ruminal samples, with the objective to construct specific calibration curves, and thus calculate the concentration of total and target bacteria in samples. The bacterial concentrations were calculated considering the rRNA operon copy number of each bacterial genome described in GenBank as follows: *F. succinogenes*, three copies (Accession number CP001792.1); *P. bryantii*, four copies (Accession number NZ_AUKF00000000.1); *B. proteoclasticus*, six copies (Accession number NZ_JHWL00000000.1) and *A. lipolytica*, one copy (Accession number NZ_JHYA00000000.1). The qPCR efficiencies for bacterial species of interest were obtained using standard dilution curves in quadruplicate of *A. lipolytica*, *F. succinogenes*, *B. proteoclasticus* and *P. bryantii* 16S rDNA, respectively. The qPCR efficiencies were calculated according to the equation: $E = [(10(1/\text{slope})) - 1]$. Standard curves were generated using relative concentration *v.* the threshold cycle (*Ct*). The qPCR efficiencies (*E*) were calculated from the given slopes (*M*) in a Rotor Gene 6000 software. Based on the slopes of the standard curves, the qPCR efficiencies ranged from 80% to 97%. The transcripts studied showed high linearity: $R^2 > 0.99$.

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

Target bacteria		Primer[1]	Primer concentration (μM)	Purified template of DNA (ng)	Product size (bp)
Fibrobacter succinogenes	Forward	GGTATGGGATGAGCTTGC	20	30	500
	Reverse	GCCTGCCCCTGAACTATC			
Butyrivibrio proteoclasticus	Forward	TCCGGTGGTATGAGATGGGC	20	30	200
	Reverse	GTCGCTGCATCAGAGTTTCCT			
Prevotella bryantii	Forward	ACTGCAGCGCGAACTGTCAGA	20	26	550
	Reverse	ACCTTACGGTGGCAGTGTCTC			
Anaerovibrio lipolytica	Forward	TGGGTGTTAGAAATGGATTC	20	28	600
	Reverse	CTCTCCTGCACTCAAGAATT			

[1] *F. succinogenes*, *P. bryantii* and *A. lipolytica* primers were described by Tajima *et al.* (2001), whereas *B. proteoclasticus* primers were described by Huws *et al.* (2010).

In addition, to check the expected sizes of each PCR product, the amplicons were visualized by electrophoresis on a 1% (w/v) agarose gel which was stained using ethidium bromide and Lambda DNA/HindIII marker was used to compare the 16S rDNA amplification fragments.

Sequence analysis

To verify the correct amplification in the qPCR assays of specific bacteria, the PCR products were sequenced using the Macrogen USA (Rockville, MD, USA) sequencing service. The 16S rDNA sequences were compared with the available databases using the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and aligned with reference sequences using Sequence Match function at the RDP (https://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) to determine the approximate phylogenetic affiliations.

Fatty acid analysis

Lipids from oils, diets and ruminal digesta were extracted with chloroform/methanol (2 : 1, v/v) by the method of Bligh and Dyer (1959) and trans-esterified with sodium methoxide according to the method of Christie (1982) using a methylation reagent (1.75 ml methanol : 0.4 ml of 5.4 mol/l sodium methylate) and a termination reagent (1 g oxalic acid/30 ml diethyl ether) according to Chouinard *et al.* (1999). All chemicals and solvents used for this method were of analytical grade. A Gas Chromatography (GC) system (GC-2010) equipped with a 100-m column (Rt-2560 column 100 m × 0.32 mm × 0.20 μm column; Restek, Bellefonte, PA, USA) was used. The GC conditions were as follows: the oven temperature was initially set at 110°C for 4 min after injection, and then increased to 240°C (20°C/min) with equilibration time of 2 min. The inlet and flame ionization detector temperatures were 260°C, the split ratio was 15 : 1 and a 2 μl injection volume was used. The H_2 carrier gas flow to the detector was 40 ml/min, airflow was 400 ml/min and the flow of N makeup gas was 25 ml/min. FA peaks were identified by using a fatty acid methyl ester standard (FAME, Supelco 37 Component FAME mix; Supelco, Bellefonte, PA, USA).

Statistical analysis

Bacterial qPCR data were $\log_{10}$-transformed to attain normality. Data were analyzed as a 3 × 3 Latin square design using the GenStat (12th edition) statistical package (VSN International Ltd, Oxford, UK). Fixed effects were experimental periods and treatments, and the random effect was the cow. When significant treatment effects were detected, means were separated using Tukey's test. Probability of $P < 0.05$ was used to determine significant differences among means.

Results

Ruminal fermentation parameters and ruminal bacteria quantification

Cows consumed all their individual allocation of TMR (10 kg DM/cow per day) with no feed refusals. Rumen digesta pH, NH_3–N and total VFA were similar for the three dietary treatments and averaged 6.9, 13.3 mg/dl and 57.5 mmol/l, respectively. Molar proportions (mol/100 mol) of individual VFA were comparable across dietary treatments and averaged 63.8 for acetate, 22.7 for propionate, 11.2 for butyrate and 2.4 for valerate (Table 3).

Table 3 *Ruminal pH, NH_3–N and volatile fatty acids (VFA) from cows fed control, soybean oil (SO) and hydrogenated palm oil (HPO)*

	Diet[1]				
	Control	SO	HPO	SED	*P*-value
pH	6.90	6.88	6.89	0.10	0.78
NH_3–N (mg/dl)	13.6	11.9	14.3	1.22	0.32
Total VFA (mmol/l)	50.5	59.6	62.5	4.33	0.32
Molar proportion (mol/100 mol)					
Acetate	63.9	63.9	63.7	0.96	0.97
Propionate	22.5	22.8	22.8	1.30	0.99
Butyrate	11.2	11.2	11.3	0.30	0.97
Valerate	2.4	2.4	2.3	0.20	0.94

[1]Control = basal diet/no fat supplement; SO = basal diet + 170 g/day per cow of SO; HPO = basal diet + 170 g/day per cow of HPO.

In this study, ruminal bacterial populations involved in lipid metabolism were quantified by qPCR (Table 2). The obtained PCR products were checked by expected size and sequenced. All the PCR products corresponded to the expected size: *F. succinogenes* (500 bp), *B. proteoclasticus* (200 bp), *P. bryantii* (550 bp) and *A. lipolytica* (600 bp) (not shown) and the sequences corresponded to the target organism. Compared with control, total bacteria (copies of 16S rDNA/ml) was decreased ($P < 0.05$) by SO and increased ($P < 0.05$) by HPO. The load of target bacteria (bacteria/ml) was similar for all dietary treatments and averaged: 4.52 for *F. succinogenes*, 2.92 for *B. proteoclasticus* and 4.19 for *A. lipolytica* (Table 4). However, the load of *P. bryantii* was increased ($P < 0.05$) by HPO but not by SO.

Fatty acid composition of ruminal digesta

Data from the FA composition of ruminal digesta are shown in Table 5. The most abundant FA in ruminal digesta regardless of dietary treatment were (g/100 g) as follows: C14:0 (4.4), C15:0 (5.7), C16:0 (36.5), C18:0 (21.7), C18:1 *cis*-9 (5.4) and C18:3 *cis*-9,12,15 (4.3). Compared with control and SO, HPO decreased ($P < 0.05$) C18:2 *cis* n-6 (1.28 and 1.64 *v.* 0.75 g/100 g) and total contents of monounsaturated (15.61 and 17.9 *v.* 7.66 g/100 g) and unsaturated (22.54 and 25.79 *v.* 11.22 g/100 g) FA in ruminal digesta. Also, compared with control and HPO, SO increased ($P < 0.05$) contents (g/100 g) of C15:0 iso (1.92 and 1.04 *v.* 2.76), C18:1 *trans*-11 (0.96 and 0.23 *v.* 1.68) and C18:2 *cis*-9, *trans*-11 (1.42 and 0.42 *v.* 1.65). Dietary treatments did not affect contents (g/100 g) of the following FA: C10:0, C11:0, C12:0, C13:0, C14:0, C14:1, C15:0, C15:1 *cis*-10, C16:0, C16:0 iso, C16:1 *trans*-9 + C17:0 iso, C16:1 *cis*-9, C17:0, C17:1 *cis*-10, C18:0, C18:1 *cis*-9 and C18:3 *cis*-9,12,15.

Table 4 *Quantification of ruminal bacteria by PCR from cows fed control, soybean oil (SO) and hydrogenated palm oil (HPO)*

	Diet[1]				
	Control	SO	HPO	SED	*P*-value
Total bacteria (copies 16S rDNA/ml)[2]	11.84[b]	11.75[c]	12.06[a]	0.08	<0.01
Target bacterium (bacteria/ml)[2,3]					
Fibrobacter succinogenes	4.96	4.25	4.36	0.32	0.26
Butyrivibrio proteoclasticus	3.04	2.74	2.99	0.37	0.73
Prevotella bryantii	3.41[b]	3.51[b]	3.90[a]	0.08	0.04
Anaerovibrio lipolytica	4.10	4.20	4.28	0.23	0.76

rDNA = ribosomal DNA.
[a,b,c]Means in the same row with different superscripts are different ($P < 0.05$).
[1]Control = basal diet/no fat supplement; SO = basal diet + 170 g/day per cow of SO; HPO = basal diet + 170 g/day per cow of HPO.
[2]Log_{10}.
[3]Based on ribosomal operon copy number.

Table 5 *Fatty acid composition of ruminal digesta from cows fed control, soybean oil (SO) and hydrogenated palm oil (HPO)*

Fatty acids	Diet[1]				
(g/100 g of fatty acid)	Control	SO	HPO	SED	*P*-value
C10:0	2.35	1.77	1.63	0.90	0.71
C11:0	0.30	0.39	0.29	0.24	0.92
C12:0	0.57	0.63	0.21	0.17	0.23
C13:0	2.54	1.30	2.00	0.84	0.44
C14:0	4.10	5.47	3.69	1.53	0.53
C14:1	2.81	2.43	1.73	1.05	0.61
C15:0	7.10	6.17	3.94	2.21	0.42
C15:1 *cis*-10	3.41	2.21	1.13	0.79	0.10
C15:0 iso	1.92[b]	2.76[a]	1.04[c]	0.43	0.04
C16:0	35.06	32.84	41.62	3.89	0.17
C16:0 iso	2.40	1.35	0.28	2.23	0.52
C16:1 *trans*-9 + C17:0 iso	0.93	0.63	0.46	0.26	0.28
C16:1 *cis*-9	0.66	0.52	0.39	0.10	0.24
C17:0	1.59	1.74	0.79	0.48	0.21
C17:1 *cis*-10	0.61	0.78	0.69	0.30	0.85
C18:0	17.00	16.42	31.61	6.24	0.11
C18:1 *trans*-11	0.96[b]	1.68[a]	0.23[c]	0.06	<0.01
C18:1 *cis*-9	5.65	8.23	2.30	2.88	0.23
C18:2 *cis* n-6	1.28[a]	1.64[a]	0.75[b]	0.38	<0.01
C18:2 *cis*-9, *trans*-11	1.42[b]	1.65[a]	0.42[c]	0.06	0.03
C18:3 *cis*-9,12,15	4.81	5.41	2.53	1.50	0.24
∑Saturated fatty acids	70.44	66.54	85.64	7.03	0.07
∑Monounsaturated fatty acids	15.61[a]	17.90[a]	7.66[b]	3.26	0.04
∑Polyunsaturated fatty acids	6.92	7.88	3.56	1.87	0.16
∑Unsaturated fatty acids	22.54[a]	25.79[a]	11.22[b]	4.63	0.04

[a,b,c]Means in the same row with different superscripts are different ($P < 0.05$).
[1]Control = basal diet/no fat supplement; SO = basal diet + 170 g/day per cow of SO; HPO = basal diet +170 g/day per cow of HPO.

Discussion

In this study, ruminal pH and NH_3–N were not affected by dietary treatments, this partly agrees with the studies by Yang *et al.* (2009) who did not report ruminal pH changes when cows were fed SO and linseed oil, but did observe increases in ruminal NH_3–N concentration. Benchaar *et al.* (2012) reported no effect on pH, VFA and NH_3–N when dairy cows were supplemented with linseed oil at 2%, 3% and 4% DM. In the present study, lack of difference in ruminal fermentation parameters may be due to the amount (almost 3% of DM) of oil incorporated into the basal diet. Differences from other studies such as Yang *et al.* (2009), on the effect dietary oils on ruminal fermentation parameters in dairy cows may be explained by the amount of dietary oil and the

forage source used, for example, VFA patterns were not affected when cows were supplemented with linseed oil (3% DM) on a hay-based diet (Ueda *et al*., 2003), whereas on a corn silage-based diet they were changed (Doreau *et al*., 2009).

The chemical configuration of dietary lipids is associated with their effects on ruminal microorganisms. For example, PUFA are more toxic for biohydrogenating bacteria (e.g. *B. fibrisolvens*) than monoenoic FA (Lourenco *et al*., 2010). Consequently, SO, which is a rich source of C18:2 *cis* n-6, is expected to have strong negative effects on ruminal bacterial populations; this agrees in part with the reduction of total bacteria (copies of 16S rDNA/ml) caused by SO treatment observed in this study. During rumen byohydrogenation, C18:2 *cis* n-6 yields several intermediate compounds until reduction to C18:0 (Castagnino *et al*., 2015). In the present study, C18:1 *trans*-11 and C18:2 *cis*-9, *trans*-11 (biohydrogenation intermediate isomers) were increased in rumen contents with SO compared with control and HPO. This is important for milk production because those FA can escape from the rumen and be secreted in milk as shown by Bu *et al*. (2007) who observed increases in the C18:1 *trans*-11 and C18:2 *cis*-9, *trans*-11 concentrations of milk fat when dairy cows were supplemented with vegetable oils and oilseeds rich in C18:2 *cis* n-6.

Increases of C15:0 iso provoked by SO are particularly interesting, as branched-chain FA have been suggested to reflect rumen function (e.g. ruminal fermentation pattern) and also contribute to the formation of the main odd- and branched-chain FA in milk (Vlaeminck *et al*., 2006). The odd- (C15:0 and C17:0) and branched- (C13:0 iso, C14:0 iso, C15:0 iso, C16:0 iso, C17:0 iso, C18:0 iso, C13:0 anteiso, C15:0 anteiso, C17:0 anteiso) chain fatty acids (OBCFA) profile of the rumen bacteria appears to be largely determined by the FA synthase activity of the microorganism rather than by the precursor availability (Vlaeminck *et al*., 2006). Consequently, variation in the OBCFA profile leaving the rumen is expected to mirror changes in the relative abundance of specific bacterial populations in the rumen rather than an altered bacterial FA synthesis. In this study, supplementation with SO may have influenced the FA synthase activity of ruminal microorganisms, specifically from *Prevotella* spp. and *B. fibrisolvens* (Fievez *et al*., 2012). It has been suggested that higher proportions of iso-FA in solid-associated bacteria reflect their enrichment in cellulolytic bacteria (e.g. *B. fibrisolvens*), whereas higher proportions of anteiso-C15:0 in liquid-associated bacteria might indicate their enrichment in pectin- and sugar-fermenting bacteria (e.g. *Prevotella* spp.) (Bessa *et al*., 2009).

Normally in dairy cow diets, ruminal biohydrogenation of C18:2 *cis* n-6 varies between 70% and 95%, indicating that with the exception of diets containing marine lipids C18:0 is the major FA escaping from rumen (Shingfield *et al*., 2013). In the present study, this was corroborated by the FA profile of rumen digesta where C16:0 and C18:0 were the most predominant saturated FA (especially in HPO). Also, in the present study, HPO decreased ruminal C18:2 *cis* n-6, which may be explained by the levels of C18:2 *cis* n-6 in the HPO diet which was notably lower than control and SO.

It has been recognized that cellulolytic bacteria can be affected by dietary supplementation of lipids with high concentrations of PUFA (Paillard *et al*., 2007). This is explained by factors such as disruption of microbial cell membranes and cell function caused by PUFA and lipid coating of feed particles (especially fibrous components) and bacteria (Yang *et al*., 2009). The antimicrobial effect of lipids in the rumen is related to the cytotoxic effects of FA on membrane function of eukaryotic cells (Maia *et al*., 2010). Long-chain unsaturated FA appear to be more toxic to ruminal bacteria as they can attach to lipid bilayers in bacterial membranes (because of their hydrophobic and amphiphilic nature). The longer the chains, and the more double bonds, the easier it is for FA to attach and destroy membranes of bacteria (Zheng *et al*., 2005).

Although, *Prevotella* spp. has been reported to be resistant to dietary PUFA (Huws *et al*., 2010), in this study, *P. bryantii* load was increased by HPO (a saturated source), which agrees in part with the study by Choi *et al*. (2013) who reported that C16:0 and C18:0 have less antibacterial effect than PUFA (HPO diet contained 46 g of C16:0 and 36 g of C18:0 per 100 g of total FA). In concordance with that, it has been reported that consumption of animal fats (mainly saturated FA) has been associated with the presence of *Prevotella* and *Bacteroides* (Tremaroli and Bäckhed, 2012). Another possible explanation for increased *Prevotella* with the HPO diet may be the interaction of a saturated lipid source and a substrate (our basal diet comprised of 56% forage and 44% concentrate ratio). The *Prevotella* spp. are the dominant bacteria in the rumen (Stevenson and Weimer, 2007) and their ruminal populations vary according to different substrates, for example, on a hay diet, *Prevotella ruminicola* is predominant, whereas on a grain diet *P. bryantii* is predominant (Tajima *et al*., 2001). Our results are similar to those reported by Rico and Harvatine (2013), who fed dairy cows with a control diet composed of 60% forage and 40% concentrate and a low-fiber diet supplemented with 3 g/100 g of SO, later the authors Rico *et al*. (2015) studied the ruminal microbiome and found that the abundance of *P. bryantii* was lowered in the control diet.

This study used qPCR to analyze bacterial concentrations using their DNA and did not use isolation from pure cultures. Compared with culture-dependent studies, our results may be more precise because we avoided 'plate count anomaly' and the use of laborious protocols to isolate the target bacterial populations (Amann *et al*., 1995). The primers used in this study were previously validated (Tajima *et al*., 2001; Huws *et al*., 2010). Furthermore, the primers were checked using the probe match tool in the RDP (Huws *et al*., 2007; Cole *et al*., 2014). One interesting point is that the sum of the selected bacterial populations corresponded to half of the total bacterial, and this observation was independent of the diet used. Therefore, more studies should be performed to obtain a clear picture of the changes in ruminal bacterial populations; a metagenomic

approach could provide a deeper composition of ruminal populations.

Conclusions

In conclusion, supplementation with SO or HPO (2.7% DM) did not affect ruminal fermentation parameters, whereas HPO can increase loads of ruminal *P. bryantii*. Also, results observed in our target bacteria may have depended on the degree of saturation of dietary oils.

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