Effect of dietary fish oil supplements alone or in combination with sunflower and linseed oil on ruminal lipid metabolism and bacterial populations in lactating cows

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ABSTRACT

Fish oil (FO) alters ruminal biohydrogenation causing *trans* fatty acid (FA) intermediates to accumulate, but the effects of 18-carbon polyunsaturated FA supply on ruminal long-chain FA metabolism and microbial communities in cattle fed FO are not well established. Four cows fitted with rumen cannula were used in a 4 × 4 Latin square with 21-d experimental periods to evaluate the effects of FO alone or in combination with plant oils high in 18:2n-6 or 18:3n-3 on rumen microbial ecology and flow of FA at the omasum. Treatments comprised a basal grass silage-based diet containing no additional oil (control) or supplements of FO (200 g/d) or FO (200 g/d) plus 500 g/d of sunflower oil (SFO) or linseed oil (LFO). Flow of FA was determined using the omasal sampling technique. The relative abundance of key biohydrogenating bacteria was assessed by quantitative PCR on 16S rRNA genes in omasal digesta. Fish oil-supplemented treatments increased the amounts of *trans*-18:1, *trans*-18:2, and 20- to 22-carbon polyunsaturated FA escaping the rumen. Relative to the control, oil supplements had no effect on the amount of 18:0 leaving the rumen, but LFO decreased the flow of 18:0 at the omasum compared with SFO. Both SFO and LFO increased *trans*-18:1 relative to FO, whereas LFO resulted in the highest *trans*-18:2 and 20- to 22-carbon FA flow. Supplements of FO plus plant oils shifted biohydrogenation toward *trans*-10 18:1 formation. Compared with FO alone, the ruminal metabolism of 22:6n-3 in the rumen of lactating cows is more extensive on diets containing higher

amounts of 18-carbon polyunsaturated FA. However, the biohydrogenation of 22:5n-3 was less extensive in LFO than SFO, but showed no difference between FO and diets containing plant oils. Ruminal outflow of 20:5n-3 was not altered when plant oils were added to FO. Alterations in the amount of intermediates at the omasum or ruminal biohydrogenation pathways were not accompanied by major changes in analyzed bacterial populations. In conclusion, dietary supplements of FO alone or in combination with plant oils increase the amount of biohydrogenation intermediates containing 1 or more *trans* double bonds escaping the rumen, which may have implications for host metabolism and the nutritional quality of ruminant foods.

Key words: polyunsaturated fatty acid, plant oil, biohydrogenation, *Butyrivibrio*

INTRODUCTION

Due to the potential benefits for human health, there is interest in increasing *cis*-9,*trans*-11 CLA and long-chain n-3 fatty acids (FA) and lowering the concentration of medium-chain FA in ruminant milk (Lock and Bauman, 2004; Palmquist et al., 2005; Shingfield et al., 2013). Most of the *cis*-9,*trans*-11 CLA in milk originates from the desaturation of *trans*-11 18:1 in the mammary gland (Griinari et al., 2000). *Trans*-11 18:1 is the penultimate intermediate formed during the biohydrogenation of 18-carbon PUFA to 18:0 in the rumen (Harfoot and Hazlewood, 1988). Dietary supplements of plant oils high in 18:2n-6 (Shingfield et al., 2008a) and 18:3n-3 (Lor et al., 2004) or fish oil (FO; Shingfield et al., 2003) are known to increase *trans*-11 18:1 formation in the rumen in lactating cows. Feeding diets containing FO and sources of 18:2n-6 or 18:3n-3 increase *cis*-9,*trans*-11 CLA concentrations in milk from lactating cows (Whitlock et al., 2002; Palmquist and Griinari, 2006), but enrichment varies depending

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on the composition of the basal diet and the amount and source of lipid supplement. Furthermore, dietary supplements of oils containing 18:2n-6 or 18:3n-3, but not FO, lower the proportions of 12:0, 14:0, and 16:0 in milk fat (Shingfield et al., 2013).

In vitro studies have demonstrated that 18:2n-6 and 18:3n-3 decrease the biohydrogenation of 20:5n-3 and 22:6n-3 and cause *trans*-11 18:1 to accumulate (Chow et al., 2004; Wasowska et al., 2006). In growing cattle, dietary supplements of a mixture (1:1 wt/wt) of FO and linseed oil (**LO**) lowered ruminal biohydrogenation of 20:5n-3 and 22:6n-3 and increased *trans*-11 18:1 at the duodenum compared with the same amount of oil from FO alone (Shingfield et al., 2011). These findings suggest that dietary supplements of a mixture of plant oils and FO could be used to increase ruminal escape of *trans*-11 18:1, 18-carbon PUFA, and long-chain n-3 FA for incorporation into meat and milk.

Changes in ruminal biohydrogenation to dietary FO supplements are accompanied by alterations in the ruminal bacterial community capable of biohydrogenation (Kim et al., 2008; Huws et al., 2010; Shingfield et al., 2012). Populations of specific bacteria in the rumen were found to be altered by marine algae supplements in sheep fed diets containing sunflower oil (**SO**; Toral et al., 2012), but no reports exist on the effects of FO with plant oils on the abundance of biohydrogenating bacteria in cattle. Characterizing the effects on ruminal biohydrogenation is central to understanding the mechanisms underpinning physiological responses to lipid supplements in lactating ruminants and the effect on milk fat composition, which is a major source of SFA in the human diet (Eilander et al., 2015).

The present experiment tested the hypotheses that (1) plant-derived 18-carbon PUFA may lower ruminal biohydrogenation of long-chain n-3 FA in FO and increase *trans*-11 18:1, (2) that the resultant accumulation of intermediates is associated with changes in the key bacterial species known to be capable of biohydrogenation, and (3) that the effects may differ depending on whether 18:2n-6 or 18:3n-3 is the main source of PUFA. The experimental treatments were formulated to meet the objectives of providing new information on changes in ruminal biohydrogenation of long-chain polyenoic FA, metabolic pathways involved, and specific FA products and intermediates associated with these diets.

MATERIALS AND METHODS

Cows and Experimental Design

All experimental procedures were approved by the Animal Experiment Committee of MTT Agrifood

Research Finland in accordance with the guidelines established by the European Community Council Directive 86/609/EEC (European Council, 1986). Four multiparous Finnish Ayrshire cows (193 ± 16.2 d postpartum, 637 ± 22.3 kg of live weight, and producing 24.8 ± 4.73 kg of milk/d) fitted with rumen cannulas (i.d. 100 mm; Bar Diamond Inc., Parma, ID) were used in a 4×4 Latin square with 21-d experimental periods. Treatments comprised a basal diet containing no additional oil (control), or supplemented with 200 g of ultra refined herring and mackerel oil (EPAX 3000 TG Pronova Biocare AS, Aalesund, Norway; treatment **FO**), 200 g of FO and 500 g of SO (Raisioagro Ltd., Raisio, Finland; **SFO**) or 200 g of FO and 500 g of LO per day (Elix Oil Ltd., Somero, Finland; **LFO**; Supplemental Table S1; <https://doi.org/10.3168/jds.2017-13776>). Cows were housed in individual tiestalls within a dedicated metabolism unit with continuous access to water and salt block and were milked daily at 0700 and 1645.

Experimental Diets

Cows were offered grass silage and a cereal-based concentrate (forage:concentrate ratio 60:40, on a DM basis) as equal meals at 0600 and 1800 at 95% of ad libitum intake measured during 14-d before the start of the experiment. Experimental silage was prepared from primary growths of mixed timothy (*Phleum pratense*) and meadow fescue (*Festuca pratensis*). The concentrate supplement contained (g/kg): rolled barley (520), molassed sugar beet pulp (250), solvent extracted rapeseed meal (200) of low glucosinolate content (Raisioagro Ltd., Raisio, Finland), and a mineral and vitamin premix (30; Onni-Kivennäinen, Rehumelica, Vaasa, Finland). Oil supplements were fed in equal amounts at 0600 and 1800 by mixing with concentrate ingredients immediately before feeding.

Sampling and Chemical Analysis

Intake of each cow was measured daily. Representative samples of feed ingredients and feed refusals were collected daily over the last 5 d of each experimental period for analysis of chemical composition (Shingfield et al., 2002). Samples of ruminal fluid ($n = 8$) were collected from each cow at 1.5-h intervals from 0600 to 1500 h on d 20 (0600, 0730, 0900, 1030, 1200, 1330, and 1500 h, respectively). Samples (approximately 500 mL) were collected through rumen cannula using a vacuum pump and flexible tube and analyzed for pH, VFA and ammonia N concentrations (Shingfield et al., 2002). An additional 10-mL subsample of filtered rumen fluid was diluted with 30 mL of 0.9% (wt/vol) aqueous NaCl

containing 10% (vol/vol) of formaldehyde for visual assessment ($n = 6$) of protozoal numbers using a counting chamber (Fuchs-Rosenthal, Fortuna, Germany).

The flow of digesta at the omasal canal was determined using the omasal sampling technique (Ahvenjärvi et al., 2000) and a triple indigestible marker system based on Cr-EDTA, Yb-acetate (Dasico A/S, Birkerød, Denmark), and indigestible NDF as markers for liquid, small, and large particles, respectively (Shingfield et al., 2008b). The Cr-EDTA was prepared according to standard procedures (Binnerts et al., 1968); Cr-EDTA (978 g) and Yb-acetate (5.8 g) were dissolved in distilled water (6 kg) and infused separately into the rumen at a constant rate (4.2 mL/min) using a peristaltic pump (Watson-Marlow, High Wycombe, UK) starting at 1800 h on d 14 of each period. To facilitate rapid equilibration of marker concentrations in the rumen, cows also received priming doses of Cr-EDTA (1,500 g) and Yb-acetate (7.5 g) at the start of marker administration.

Spot samples (500 mL) of digesta entering the omasal canal were collected 3 times daily at 4-h intervals from d 17 to 20, to cover a 12-h period that was considered representative of the entire feeding cycle composited, and separated into large particle, small particle, and liquid phases (Ahvenjärvi et al., 2000). Each phase was freeze-dried and stored at -20°C , whereas subsamples of each fraction collected for FA and bacterial community analysis were stored at -80°C .

Marker concentrations and chemical composition of omasal digesta were determined using standard methods (Ahvenjärvi et al., 2000). Digesta flows entering the omasal canal were calculated after mathematical reconstitution for a triple marker method (Ahvenjärvi et al., 2000). Administration of markers was based on the excretion in feces. Thereafter, appropriate amounts of freeze-dried digesta previously stored at -80°C were weighted to yield two 10-g composite samples, one for FA analysis and the other for measurements of bacterial populations.

Whole-tract apparent digestibility coefficients were determined by total fecal collection over 120 h starting at 1800 h on d 17 of each experimental period. Urine was separated from feces by means of a light harness and flexible tubing attached to the vulva. Total feces excreted was weighed, thoroughly mixed, subsampled (5%, wt/vol), and stored at -20°C until analyzed for chemical composition using the same methods applied to feeds and marker concentrations using the same procedures applied to omasal digesta.

DNA Extraction from Omasal Digesta

Samples of freeze-dried, reconstituted omasal digesta (0.2 mL) were collected into lysis tubes from the Qbio-

gene FastDNA SPIN Kit (Qbiogene, Carlsbad, CA) for Soil before DNA extraction (Shingfield et al., 2012; Halmemies-Beauchet-Filleau et al., 2013). The integrity of the DNA was checked on 1% (wt/vol) agarose gels and the purity was assessed from the 260/280 nm ratio; purity of DNA was considered acceptable with ratios >1.8 .

Quantitative PCR of the Bacteria

For quantitative PCR reactions, the DNA was diluted 10 fold, except for the determination of total bacteria, when 100-fold dilutions were used. Quantitative PCR was performed using Sigma JumpStart PCR mixes with or without Sybr Green (Sigma-Aldrich, St. Louis, MO), as outlined earlier (Shingfield et al., 2012; Halmemies-Beauchet-Filleau et al., 2013). Primer sets and probes used in quantitative PCR are presented in Supplemental Table S2 (<https://doi.org/10.3168/jds.2017-13776>). The specificity of the primers and probes was validated on the same set of strains used in earlier reports (Paillard et al., 2007; Shingfield et al., 2012; Halmemies-Beauchet-Filleau et al., 2013). Calibration was carried out using DNA from a mixture of known target organisms. Responses were also tested using the same DNA added to DNA extracted from omasal digesta (Shingfield et al., 2012; Halmemies-Beauchet-Filleau et al., 2013). The specificity of different primer sets, 2 in combination with probes, to members of the *Butyrivibrio*/*Pseudobutyrvibrio* group is described in earlier reports (Shingfield et al., 2012; Halmemies-Beauchet-Filleau et al., 2013) and shown in Supplemental Figure S1 (<https://doi.org/10.3168/jds.2017-13776>).

FA Analysis

Fatty acid methyl esters of lipids in oil supplements and freeze-dried feed samples were prepared using standard methods (Shingfield et al., 2003). Lipids in 200 mg of freeze-dried samples of reconstituted omasal digesta were extracted in duplicate (Shingfield et al., 2003), organic extracts were combined and dried under oxygen-free N at 50°C , and lipids were converted to FAME (Kairenius et al., 2011). Fatty acid content was determined using the internal and external standards. Tridecanoic acid (0.1 wt/vol in hexane; T-135, Nu-Chek Prep Inc., Elysian, MN) and tridecanoic acid (0.1 wt/vol in absolute ethanol; N13-A, Nu-Chek Prep) were used as an internal standards for feed ingredients and omasal digesta, respectively. Trihexadecanoic acid (T-5888, Sigma-Aldrich) and hexadecanoic acid (S-4751, Sigma-Aldrich) were used to set up an external calibration curve for feed ingredients and omasal digesta, respectively.

Fatty acid methyl esters were quantified using a GC (6890; Agilent Technologies, Wilmington, DE) fitted with a 100-m fused silica capillary column (CP-Sil 88; Chrompack 7489, Chrompack International BV, Middelburg, the Netherlands) using a temperature gradient program and hydrogen as the carrier gas (Shingfield et al., 2003). Individual isomers of 18:1 and 18:2 were further resolved in a separate analysis under isothermal conditions at 170°C (Shingfield et al., 2003). Under these conditions, *trans*-10, *cis*-15 18:2 and *trans*-11, *cis*-15 18:2 eluted as a single peak. To resolve these isomers, analysis of FAME was repeated using a SLB-IL111 column (100 m × 0.25 mm i.d., 0.2 µm film thickness, Sigma-Aldrich) and helium as a carrier gas (Ventto et al., 2017). Peaks were initially identified based on retention time comparisons with authentic FAME standards (Nu-Chek Prep GLC #463; 21:0, #N-21-M; 23:0, #N-23-M; 24:0, #N-24-M; *trans*-11 20:1, #U-64-M; *cis*-12-21:1, #U-85-M; *cis*-14 23:1, #U-87-M, Larodan Fine Chemicals 18:4n-3, #10-1840; *cis*-9 20:1, #20-2001-1-4; 20:4n-3, #20-2024-1; 21:3n-3, #20-2103-1-4; 21:5n-3, #20-2105-1-4; *trans*-13 22:1, #20-2210-9; 22:5n-6, #20-2265-7; 23:5n-3, #20-2305-1-4; 24:5n-3, #20-2405-4; 25:0, #20-2500-7; 29:0, #20-2900-7; 9-O-18:0, #14-1800-5-4; 12-O-18:0, #14-1800-6-4; 13-O-18:0, #14-1800-7-4; 16-O-18:0, #14-1800-8-39 (Malmo, Sweden), and Sigma-Aldrich 26:0, #H-6389; 27:0, #H-6639; 28:0, #O-4129; 30:0 #T-1902; mixture of 18:2n-6 isomers, #L-8404; mixture of conjugated 18:2 isomers, #O-5632; mixture of 18:3n-3 isomers, #L-6031). Fatty acid methyl esters not available as commercial standards were identified in omasal digesta based on complementary silver-ion (Ag⁺) thin-layer chromatography and GC-MS analysis of FAME and 4,4-dimethyloxazoline derivatives prepared for each recovered FAME band (Kairenius et al., 2011; Halmemies-Beauchet-Filleau et al., 2013). The distribution of CLA isomers in omasal digesta was determined by Ag⁺-HPLC (Shingfield et al., 2003). Analysis was repeated using 2.0% (vol/vol) acetic acid in heptane to resolve *cis*-10, *trans*-12 CLA and *trans*-10, *cis*-12 CLA (Ventto et al., 2017).

Statistical Analysis

Dry matter intake recorded during the last 5 d of each experimental period were averaged before statistical analysis. Experimental data were analyzed by ANOVA for a 4 × 4 Latin square design with a model that included the random effects of cow and the fixed effects of period and experimental treatment using the mixed procedure of SAS (version 9.2, SAS Institute Inc., Cary, NC). Measurements of rumen pH and fermentation characteristics were analyzed by ANOVA for repeated measures with a model that included the

fixed effect of period, treatment, time, and treatment × time interactions and the random effect of cow using an autoregressive order one covariance structure. Denominator degrees of freedom were calculated using the Kenward-Roger method. Sums of squares were further separated into single degree of freedom orthogonal contrasts to test the effects due to FO (control vs. FO, SFO, and LFO), addition of plant oils in the diet (FO vs. SFO and LFO), and source of plant oil (SFO vs. LFO). Least squares means ± standard error of the means are reported and treatment effects were considered significant at $P \leq 0.05$. Treatment effects at $P > 0.05$ to 0.10 were considered close to significant.

RESULTS

Chemical Composition of Experimental Feeds

Experimental silage was of high quality, both in terms of nutritive value and fermentation characteristics (Supplemental Table S1; <https://doi.org/10.3168/jds.2017-13776>). Lipid in grass silage contained relatively high proportions of 16:0, 18:2n-6, and 18:3n-3, whereas 16:0, *cis*-9 18:1, and 18:2n-6 predominated in concentrates. Sunflower oil contained high proportions of 18:2n-6, 18:3n-3 was the major FA in LO, and FO was relatively rich in 20:5n-3, 22:5n-3, and 22:6n-3 (Supplemental Table S1).

Nutrient Intake, Digestibility, and Rumen Fermentation

Intakes of silage DM, total DM, OM, NDF, potentially digestible NDF, and CP were decreased ($P < 0.05$) by dietary oil supplements, and lowered further ($P < 0.05$) when FO was fed as a mixture with SO or LO (Table 1). Oil supplements increased ($P < 0.001$) the intakes of 16:2n-4, 16:3n-4, 16:4n-1, 18:4n-3, 20:3n-3, 20:4n-6, 20:4n-3, 20:5n-3, 21:5n-3, 22:3n-3, 22:5n-3, and 22:6n-3. By design, the intakes of *cis*-9 18:1 and 18:2n-6 were substantially higher ($P < 0.001$) for SFO than LFO, and 18:3n-3 intake was greater ($P < 0.001$) for LFO compared with SFO (Table 1). Relative to the control, oil supplements had no effect on nutrient digestibility, with the exception of a close to significant ($P = 0.09$) increase in whole-tract DM digestion (Supplemental Table S3; <https://doi.org/10.3168/jds.2017-13776>). Inclusion of plant oils with FO had no effect on nutrient digestion compared with FO alone (Supplemental Table S3).

Experimental treatments did not affect rumen pH, ammonia N, or total VFA concentrations, but altered molar VFA proportions (Table 2). Oil supplements lowered ($P < 0.05$) molar proportions of acetate and cap-

Table 1. Feed and nutrient intake of lactating cows fed a grass silage-based diet supplemented with fish oil or a mixture of fish oil with either sunflower oil or linseed oil

Item	Treatment ¹				SEM	P-value ²		
	Control	FO	SFO	LFO		1	2	3
Intake, kg/d								
Silage DM	10.9	10.1	7.93	8.43	0.67	<0.01	0.01	0.36
Total DM	18.2	17.4	14.9	13.9	1.18	<0.05	<0.05	0.33
OM	16.6	15.9	13.7	12.7	1.08	<0.05	<0.05	0.32
NDF	6.83	6.39	5.21	5.11	0.423	<0.01	0.01	0.80
pdNDF ³	5.61	5.25	4.26	4.21	0.345	<0.01	0.01	0.86
CP	2.85	2.70	2.26	2.05	0.189	0.01	0.01	0.28
Fatty acids, g/d								
14:0	1.41	15.3	15.4	15.1	0.11	<0.001	0.95	0.03
16:0	62.1	86.4	106	92.0	4.23	<0.001	0.01	0.01
16:1 <i>cis</i> -9	1.80	16.6	16.3	16.3	0.14	<0.001	0.07	0.77
16:1 <i>cis</i> -11	0.18	0.54	0.52	0.48	0.017	<0.001	0.07	0.14
16:1 <i>trans</i> -3	3.96	3.70	2.93	3.07	0.225	<0.01	<0.01	0.40
16:2 <i>cis</i> -6, <i>cis</i> -9 ⁴	0.00	0.39	0.39	0.39	0.003	<0.001	1.00	1.00
16:2 <i>cis</i> -9, <i>cis</i> -12 (16:2n-4)	0.00	2.11	2.11	2.11	0.006	<0.001	1.00	1.00
16:2 <i>cis</i> -11, <i>cis</i> -15 ⁵	0.00	2.12	2.12	2.12	0.004	<0.001	1.00	1.00
16:3 <i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12 (16:3n-4) ⁶	0.00	2.62	2.62	2.62	0.006	<0.001	1.00	1.00
16:4 <i>cis</i> -4, <i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13 (16:4n-3)	0.00	0.23	0.23	0.23	0.005	<0.001	1.00	1.00
16:4 <i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15 (16:4n-1) ⁷	0.00	3.93	3.93	3.93	0.006	<0.001	1.00	1.00
18:0	6.25	11.8	27.8	23.4	0.46	<0.001	<0.001	<0.001
18:1 <i>cis</i> -9	74.9	88.6	209	147	6.5	<0.001	<0.001	<0.001
18:1 <i>cis</i> -11	10.6	15.8	17.2	15.6	0.96	<0.01	0.56	0.24
18:2 <i>cis</i> -9, <i>cis</i> -12 (18:2n-6)	131	129	400	170	9.7	<0.001	<0.001	<0.001
18:3 <i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12 (18:3n-6)	0.00	0.43	0.43	0.43	0.000	<0.001	1.00	1.00
18:3 <i>cis</i> -9, <i>cis</i> -11, <i>cis</i> -14 (18:3n-4)	0.00	0.23	0.23	0.23	0.001	<0.001	1.00	1.00
18:3 <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15 (18:3n-3)	132	127	104	379	7.4	<0.001	<0.001	<0.001
18:4 <i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15 (18:4n-3)	0.00	6.03	6.30	6.03	0.010	<0.001	<0.001	<0.001
18:4 <i>cis</i> -9, <i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17	0.00	0.49	0.49	0.49	0.002	<0.001	1.00	1.00
20:0	3.18	3.60	4.33	3.54	0.177	<0.01	<0.05	<0.01
20:1 <i>cis</i> -11	0.00	3.15	3.96	4.06	0.015	<0.001	<0.001	<0.01
20:1 <i>cis</i> -13	0.00	0.44	0.44	0.44	0.002	<0.001	1.00	1.00
20:1 <i>cis</i> -15	0.00	0.08	0.08	0.08	0.003	<0.001	1.00	1.00
20:2 <i>cis</i> -11, <i>cis</i> -14 (20:2n-6)	0.00	0.34	0.34	0.57	0.002	<0.001	<0.001	<0.001
20:2 <i>cis</i> -8, <i>cis</i> -11	0.00	0.24	0.24	0.24	0.004	<0.001	1.00	1.00
20:3 <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14	0.00	0.67	0.67	0.79	0.011	<0.001	<0.01	<0.001
20:3 <i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17 (20:3n-3) ⁸	1.52	2.10	1.83	2.69	0.089	<0.001	<0.05	<0.001
20:4 <i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14 (20:4n-6) ⁹	0.00	1.84	1.84	1.84	0.005	<0.001	1.00	1.00
20:4 <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17 (20:4n-3)	0.00	1.76	1.76	1.76	0.006	<0.001	1.00	1.00
20:5 <i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17 (20:5n-3)	0.00	31.3	31.3	31.3	0.079	<0.001	1.00	1.00
21:0	1.33	1.28	1.10	1.07	0.080	<0.01	<0.01	0.54
21:5 <i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15, <i>cis</i> -18 (21:5n-3)	0.00	1.32	1.32	1.32	0.003	<0.001	1.00	1.00
22:0	3.80	3.79	6.42	3.66	0.228	0.001	<0.001	<0.001
22:1 <i>cis</i> -11	0.00	2.14	2.14	2.20	0.006	<0.001	<0.01	<0.001
22:2 <i>cis</i> -13, <i>cis</i> -16 (22:2n-6)	0.00	0.13	0.13	0.13	0.001	<0.001	1.00	1.00
22:3 <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19 (22:3n-3) ¹⁰	0.52	1.32	1.35	1.33	0.047	<0.001	0.58	0.77
22:4 <i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16 (22:4n-6)	0.00	0.14	0.14	0.14	0.001	<0.001	1.00	1.00
22:4 <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19 (22:4n-3)	0.00	0.16	0.16	0.16	0.003	<0.001	1.00	1.00
22:5 <i>cis</i> -4, <i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16	0.00	0.55	0.55	0.55	0.005	<0.001	1.00	1.00
22:5 <i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19 (22:5n-3)	0.00	3.60	3.60	3.60	0.009	<0.001	1.00	1.00
22:6 <i>cis</i> -4, <i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19 (22:6n-3)	0.00	20.0	20.0	20.0	0.066	<0.001	1.00	1.00
23:5 <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19, <i>cis</i> -20	0.00	0.03	0.03	0.03	0.001	<0.001	1.00	1.00
24:0	2.91	2.91	3.57	2.73	0.176	0.21	0.08	<0.001
24:5 <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15, <i>cis</i> -18, <i>cis</i> -21	0.00	0.30	0.30	0.30	0.002	<0.001	1.00	1.00
24:6 <i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15, <i>cis</i> -18, <i>cis</i> -21	0.00	0.32	0.32	0.32	0.003	<0.001	1.00	1.00
Σ SFA	95.1	146	183	160	5.97	<0.001	0.001	<0.01
Σ MUFA	94.3	138	260	197	7.73	<0.001	<0.001	<0.001
Σ PUFA	266	341	592	635	16.2	<0.001	<0.001	<0.05
Σ Total fatty acids	474	644	1052	1011	30.8	<0.001	<0.001	0.21

¹Refers to grass silage-based diet (forage:concentrate ratio 60:40, on a DM basis) supplemented with no additional oil (control), 200 g of fish oil (FO), 200 g of FO and 500 g of sunflower oil (SFO), or 200 g of FO and 500 g of linseed oil (LFO), respectively.

²Significance of single degree of freedom orthogonal contrasts comparing the control versus lipid supplemented diets (1), FO versus SFO and LFO (2), and SFO versus LFO (3).

³Potentially digestible NDF.

⁴Co-elutes with *trans*-9 17:1.

⁵Co-elutes with *trans*-10 17:1.

⁶Co-elutes with *trans*-9 18:1.

⁷Co-elutes with *trans*-9, *cis*-12 18:2.

⁸Co-elutes with *cis*-13 22:1.

⁹Co-elutes with *cis*-15 22:1.

¹⁰Co-elutes with *cis*-15 24:1 in FO supplemented treatments. Contains only *cis*-15 24:1 in a control treatment.

Table 2. Rumen fermentation characteristics and rumen microbial populations of lactating cows fed a grass silage-based diet supplemented with fish oil or a mixture of fish oil with either sunflower oil or linseed oil

Item	Treatment ¹				SEM	P-value ²		
	Control	FO	SFO	LFO		1	2	3
pH	6.67	6.63	6.70	6.67	0.081	0.98	0.58	0.79
Ammonia-N, mmol/L	4.61	4.70	3.60	4.03	0.771	0.53	0.31	0.65
Total VFA, mmol/L	106	104	95.5	96.3	3.98	0.15	0.12	0.89
Molar proportions, mmol/mol								
Acetate	653	649	619	613	7.4	0.01	<0.01	0.55
Propionate	184	182	217	222	9.8	0.07	0.01	0.74
Butyrate	128	134	126	126	7.2	0.93	0.41	1.00
Isobutyrate	7.27	7.40	7.30	7.01	0.263	0.85	0.15	0.20
Valerate	12.8	12.2	13.3	12.6	0.36	0.85	0.17	0.22
Isovalerate	11.0	11.4	14.2	17.0	1.31	0.05	<0.05	0.13
Caproate	4.44	4.04	3.55	2.66	0.256	0.01	0.01	<0.05
Molar acetate:propionate ratio, mol/mol	3.57	3.59	2.90	2.78	0.185	0.05	0.01	0.66

¹Refers to grass silage-based diet (forage:concentrate ratio 60:40, on a DM basis) supplemented with no additional oil (control), 200 g of fish oil (FO), 200 g of FO and 500 g of sunflower oil (SFO), or 200 g of FO and 500 g of linseed oil (LFO), respectively.

²Significance of single degree of freedom orthogonal contrasts comparing the control versus lipid supplemented diets (1), FO versus SFO and LFO diets (2), and SFO versus LFO (3).

roate. Compared with FO, plant oils plus FO lowered ($P < 0.05$) acetate and caproate, but increased ($P < 0.05$) molar proportions of propionate and isovalerate, whereas LFO decreased ($P < 0.05$) caproate compared with SFO (Table 2).

Nutrient Flow at the Omasum

Oil supplements lowered ($P < 0.05$) the flow of DM, VFA, and N at the omasum (Table 3). Compared with FO alone, the addition of plant oils decreased ($P < 0.05$) VFA and N at the omasum, but the effects of SFO and LFO did not differ. Inclusion of oil in the diet increased ($P < 0.05$) the flow of 16:0, total 16:1, 18:1, 18:2, *trans* FA (**TFA**), MUFA, and PUFA (Table 3) at the omasum. Compared with the control, oil supplements had no effect on the amount of 18:0 and SFA leaving the rumen. The same was true when FO was compared with SFO and LFO, but the flows of 18:0 and SFA differed ($P < 0.05$) between SFO and LFO (Table 3). Plant oils increased ($P < 0.05$) the amount of *trans*-18:1, total 18:2, TFA, MUFA, and PUFA at the omasum compared with FO alone, with the amounts of total 18:2, TFA, and PUFA being higher ($P < 0.05$) for LFO than SFO (Table 3).

Oil supplements decreased ($P < 0.01$) 18:2n-6 and 18:3n-3 at the omasum, with the amount of 18:3n-3 being lower ($P < 0.01$) when plant oils were fed compared with FO alone (Table 3). Relative to the control, oil supplements had no effect on *cis*-9,*trans*-11,*cis*-15 18:3 at the omasum, whereas the flow was higher ($P < 0.05$) on LFO than SFO. Inclusion of oils in the diet resulted in relatively minor increases ($P \leq 0.05$) or no change in the flow of odd- and branched-chain FA (**OBCFA**) at

the omasum, but increased ($P < 0.05$) 10-O-18:0 and 15-O-18:0 at the omasum compared with the control (Supplemental Table S4; <https://doi.org/10.3168/jds.2017-13776>). Plant oils plus FO increased ($P \leq 0.05$) the flow of 10-O-18:0 and 15-O-18:0 compared with FO alone.

Compared with the control, oil supplements increased ($P < 0.01$) *cis* (Δ 8–12) 16:1, *trans* (Δ 5–12) 16:1, and *trans,trans* (Δ 9,14; 10,14; 11,15) 16:2 at the omasum (Table 4 and Supplemental Table S5; <https://doi.org/10.3168/jds.2017-13776>). Flow of several *cis* (Δ 9–12) and *trans* (Δ 6, 7, 11, 12) 16:1 isomers and *trans*-9,*trans*-14 16:2 were higher ($P < 0.05$) for FO alone compared with FO plus plant oils, whereas the reverse was true for *trans*-10 16:1 and *trans*-10,*trans*-14 16:2. Relative to SFO, LFO increased ($P < 0.05$) *cis* (Δ 9, 10) 16:1, *trans* (Δ 8–10) 16:1, and *trans*-10,*trans*-14 16:2 and decreased ($P < 0.05$) *cis*-11 16:1 at the omasum (Table 4 and Supplemental Table S5).

Oil supplements altered the abundance of 18:1 and 18:2 isomers at the omasum, changes characterized by an increase ($P < 0.05$) in *cis* (Δ 11, 13, 15, 16) 18:1, *trans* (Δ 6–8, 10–13, 15) 18:1, *cis*-9,*trans*-12 18:2, *trans,cis* (Δ 9,12; 10,15; 11,15; 12,15) 18:2, and *trans,trans* (Δ 9,14; 10,15; 11,14) 18:2 at the omasum (Table 4 and Supplemental Table S6; <https://doi.org/10.3168/jds.2017-13776>). Inclusion of plant oils with FO increased ($P < 0.05$) the amount of *cis* (Δ 15, 16) 18:1, *trans* (Δ 6–8, 10) 18:1, and 18:2 intermediates other than *cis*-11,*cis*-14 18:2 compared with FO. Furthermore, the source of plant oil had a major influence on the amounts of specific 18:1 and 18:2 intermediates leaving the rumen. Compared with SFO, flows of *cis* (Δ 11, 15) 18:1, *trans*-11 18:1, and 18:2 intermediates

except *cis*-11,*cis*-14 18:2 were higher ($P < 0.05$) on the LFO treatment (Table 4 and Supplemental Table S6).

Oil supplements had no effect on *cis*-9,*trans*-11 CLA or total CLA at the omasum, but altered the relative abundance of minor isomers of CLA leaving the rumen (Table 4 and Supplemental Table S6; <https://doi.org/10.3168/jds.2017-13776>), causing an increase ($P < 0.05$) in *trans*-7,*cis*-9 CLA, *trans,trans* ($\Delta 8,10$; 9,11; 10,12) CLA and a decrease ($P < 0.05$) in *trans*-11,*cis*-13 CLA

and *trans*-11,*trans*-13 CLA. Relative to FO, plant oils plus FO resulted in higher ($P < 0.05$) *trans*-10,*cis*-12 CLA and *trans,trans* ($\Delta 8,10$; 10,12) CLA and lower ($P < 0.05$) *trans*-7,*cis*-9 CLA at the omasum (Table 4 and Supplemental Table S6). Compared with SFO, flows of *trans*-11,*cis*-13 CLA, and *trans,trans* ($\Delta 12,14$; 13,15) CLA were higher ($P < 0.05$) and the amount of *trans*-10,*cis*-12 CLA was lower ($P < 0.05$) on the LFO treatment (Table 4 and Supplemental Table S6).

Table 3. Flow of DM and nutrients at the omasum in lactating cows fed a grass silage-based diet supplemented with fish oil or a mixture of fish oil with either sunflower oil or linseed oil

Item	Treatment ¹				SEM	P-value ²		
	Control	FO	SFO	LFO		1	2	3
Flow, kg/d								
DM	11.5	10.7	8.76	8.49	0.96	<0.05	0.06	0.81
OM ³	8.70	8.12	6.70	6.38	0.775	0.06	0.08	0.72
NDF	3.55	3.37	2.54	2.50	0.356	0.07	0.06	0.93
pdNDF ⁴	2.55	2.42	1.75	1.75	0.290	0.10	0.08	1.00
VFA	1.50	1.28	0.93	1.01	0.157	0.01	<0.05	0.58
Flow, g/d								
N	444	418	339	297	37.9	<0.05	<0.05	0.36
NAN	437	412	335	294	37.2	<0.05	<0.05	0.36
Fatty acids, g/d								
12:0	0.89	0.94	0.60	0.67	0.089	0.10	0.01	0.50
14:0	2.22	6.17	4.98	5.60	0.519	0.001	0.20	0.42
16:0	56.0	86.2	90.2	79.4	6.69	0.01	0.86	0.26
Σ 16:1 <i>cis</i>	7.74	15.2	5.50	5.59	0.909	0.14	<0.001	0.91
Σ 16:1 <i>trans</i>	0.85	4.33	3.20	4.24	0.297	<0.001	0.12	<0.05
Σ 16:2 <i>cis,cis</i>	0.19	0.28	0.21	0.28	0.051	0.23	0.56	0.29
Σ 16:2 <i>trans,trans</i>	0.03	0.33	0.29	0.44	0.046	<0.001	0.48	0.04
18:0	226	130	258	93.7	33.8	0.17	0.31	0.01
Σ 18:1 <i>cis</i>	25.7	31.1	34.0	45.7	3.31	0.01	<0.05	<0.05
Σ 18:1 <i>trans</i>	46.3	135	255	289	25.2	0.001	<0.01	0.34
18:2 <i>cis</i> -9, <i>cis</i> -12 (18:2n-6) ⁵	17.8	11.9	9.94	6.76	1.779	<0.01	0.09	0.17
Σ 18:2 <i>cis,cis</i>	18.3	12.2	10.4	7.02	1.81	<0.01	0.12	0.25
Σ 18:2 <i>cis,trans/trans,cis</i>	4.37	11.2	14.7	64.1	3.21	<0.001	<0.001	<0.001
Σ 18:2 <i>trans,trans</i>	1.46	4.34	4.26	21.3	1.33	<0.01	<0.01	<0.001
Σ CLA	4.67	4.76	4.85	3.89	0.783	0.79	0.56	0.24
18:3 <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15 (18:3n-3) ⁶	7.42	5.86	2.67	4.37	0.517	<0.001	<0.01	0.01
18:3 <i>cis</i> -9, <i>trans</i> -11, <i>cis</i> -15	1.15	1.20	0.79	3.60	0.754	0.44	0.32	<0.05
20:0	3.74	6.58	5.73	2.58	0.973	0.32	0.09	0.06
24:1 <i>cis</i> -15	0.69	1.49	1.01	1.24	0.186	<0.05	0.15	0.40
26:0	10.2	13.9	5.40	4.07	1.202	<0.05	<0.001	0.23
28:0	6.89	9.01	5.09	3.53	0.668	0.15	<0.001	0.08
30:0	4.70	5.50	4.57	3.06	0.592	0.66	0.06	0.12
Unidentified ⁷	3.65	13.0	9.60	11.3	1.06	0.001	0.09	0.31
Σ TFA ⁸	60.8	183	305	408	29.8	<0.001	<0.01	<0.05
Σ SFA	333	291	431	231	40.6	0.75	0.46	0.01
Σ MUFA	83.9	204	312	361	30.2	0.001	0.01	0.5
Σ PUFA	48.7	78.4	76.6	145	6.97	<0.001	<0.01	<0.001
Σ Total fatty acids	470	587	829	749	43.8	<0.01	0.01	0.25

¹Refers to grass silage-based diet (forage:concentrate ratio 60:40, on a DM basis) supplemented with no additional oil (control), 200 g of fish oil (FO), 200 g of FO and 500 g of sunflower oil (SFO), or 200 g of FO and 500 g of linseed oil (LFO), respectively.

²Significance of single degree of freedom orthogonal contrasts comparing the control versus lipid supplemented diets (1), FO versus SFO and LFO diets (2), and SFO versus LFO (3).

³Measurements do not include volatile fatty acids.

⁴Potentially digestible NDF.

⁵Co-elutes with *cis*-9,*cis*-15 18:2 and *cis*-9,*cis*-14 18:2.

⁶Co-elutes with 20:1 *cis*-12.

⁷Sum of 31 minor fatty acids of indeterminate structure.

⁸*Trans* fatty acid.

Table 4. Flow of selected 16- and 18-carbon fatty acids at the omasum in lactating cows fed a grass silage-based diet supplemented with fish oil or a mixture of fish oil with either sunflower oil or linseed oil

Flow, g/d	Treatment ¹				SEM	P-value ²		
	Control	FO	SFO	LFO		1	2	3
16:1 <i>cis</i> -9 ³	1.44	3.99	2.64	3.26	0.253	0.001	<0.01	<0.05
16:1 <i>cis</i> -11	6.23	9.96	2.41	1.39	0.612	0.001	<0.001	<0.05
16:1 <i>trans</i> -9	0.00	0.27	0.22	0.35	0.037	0.001	0.75	0.05
16:1 <i>trans</i> -10	0.00	0.54	0.70	1.30	0.098	<0.001	0.01	<0.01
16:1 <i>trans</i> -11 ⁴	0.00	1.95	1.32	1.23	0.112	<0.001	0.01	0.60
16:2 <i>trans</i> -10, <i>trans</i> -14	0.00	0.10	0.15	0.26	0.018	<0.001	<0.01	<0.01
18:1 <i>cis</i> -9	17.7	17.7	17.6	17.5	2.14	0.95	0.94	0.95
18:1 <i>cis</i> -11	3.47	6.11	6.42	9.06	0.721	<0.01	0.07	<0.05
18:1 <i>cis</i> -15 ⁵	1.19	3.10	4.63	13.3	0.72	<0.001	<0.001	<0.001
18:1 <i>trans</i> -6-7-8	2.14	6.57	11.5	10.3	1.18	<0.01	<0.05	0.50
18:1 <i>trans</i> -10	2.29	7.36	11.3	10.3	10.51	0.001	<0.001	0.46
18:1 <i>trans</i> -11	21.6	69.2	66.8	114	11.29	<0.01	0.16	<0.05
18:1 <i>trans</i> -13-14	7.02	19.6	23.2	26.1	3.21	<0.01	0.21	0.52
18:2 <i>trans</i> -10, <i>cis</i> -15	0.00	0.81	5.20	35.8	1.86	<0.001	<0.001	<0.001
18:2 <i>trans</i> -11, <i>cis</i> -15	3.72	8.56	6.74	21.5	1.51	<0.01	<0.05	<0.001
18:2 <i>trans</i> -9, <i>trans</i> -14 ⁶	0.96	3.16	2.66	10.2	0.73	<0.01	0.01	<0.001
18:2 <i>trans</i> -10, <i>trans</i> -15 ⁷	0.15	0.49	1.02	8.54	0.542	<0.01	0.001	<0.001
18:2 <i>trans</i> -11, <i>trans</i> -14 ⁸	0.36	0.87	0.84	3.70	0.262	<0.01	<0.01	<0.001
CLA <i>cis</i> -9, <i>trans</i> -11	3.24	3.76	3.74	2.56	0.644	0.84	0.30	0.11
CLA <i>trans</i> -10, <i>cis</i> -12	0.08	0.01	0.21	0.08	0.025	0.45	<0.01	0.01
CLA <i>trans</i> -11, <i>cis</i> -13	0.64	0.46	0.13	0.39	0.095	0.01	0.07	<0.05
CLA <i>trans</i> -11, <i>trans</i> -13	0.32	0.09	0.06	0.17	0.038	<0.01	0.53	0.07
CLA <i>trans</i> -12, <i>trans</i> -14	0.13	0.07	0.06	0.15	0.021	0.19	0.14	0.01

¹Refers to grass silage-based diet (forage:concentrate ratio 60:40, on a DM basis) supplemented with no additional oil (control), 200 g of fish oil (FO), 200 g of FO and 500 g of sunflower oil (SFO), or 200 g of FO and 500 g of linseed oil (LFO), respectively.

²Significance of single degree of freedom orthogonal contrasts comparing the control versus lipid supplemented diets (1), FO versus SFO and LFO diets (2), and SFO versus LFO (3).

³Co-elutes with 17:0 *anteiso*.

⁴Co-elutes with *cis*-6 16:1.

⁵Co-elutes with 19:0, *trans*-9,*trans*-13 18:2, and *trans*-10,*trans*-14 18:2.

⁶Co-elutes with *trans*-11,*trans*-15 18:2.

⁷Co-elutes with *trans*-9,*trans*-12 18:2.

⁸Co-elutes with *trans*-10,*trans*-13 18:2, *cis*-9,*trans*-13 18:2, and *cis*-10,*trans*-14 18:2.

Oil supplements increased ($P < 0.05$) the flow of ≥ 20 -carbon FA at the omasum, including those contained in the FO supplement, several all-*cis* PUFA not supplied from the diet (20:2n-3, 21:3n-3, 21:4n-3, and 22:3n-6), and numerous intermediates containing at least 1 *trans* double bond, including *trans*-13,*cis*-17 20:2, *trans*-7,*cis*-11,*cis*-14,*cis*-17 20:4, *trans*-12,*cis*-16,*cis*-19 22:3, *cis*-7,*trans*-13,*cis*-16,*cis*-19 22:4, and *trans*-8,*cis*-13,*cis*-16,*cis*-19 22:4 (Table 5 and Supplemental Table S7; <https://doi.org/10.3168/jds.2017-13776>). Inclusion of plant oils plus FO decreased ($P < 0.05$) several all-*cis* PUFA, including 20:4n-6, 21:5n-3, 22:4n-6, and 22:6n-3, and increased ($P < 0.05$) multiple 20- and 22-carbon intermediates at the omasum, including *trans*,*cis* and *trans*,*trans* (Δ 11,15; 13,17) 20:2, *trans*-11,*cis*-14,*trans*-17 20:3, and 22:4n-3 compared with FO. Furthermore, LFO resulted in higher ($P < 0.05$) flows of specific 20- to 22-carbon intermediates, including *trans*-11,*cis*-15 20:2, *trans*,*trans* (Δ 10,16;

11,15; 13,17) 20:2, *cis*-10,*trans*-14,*cis*-19 22:3, 22:4n-6, *trans*-8,*cis*-13,*cis*-16,*cis*-19 22:4, 22:4n-3, and 22:5n-3, but decreased ($P < 0.05$) the amounts of others, including *trans*-14,*cis*-17 20:2, *cis*-9,*trans*-14,*trans*-17 20:3, and 22:3n-3, compared with SFO (Table 5 and Supplemental Table S7).

On all diets, the amount of most PUFA at the omasum was lower than the respective intake, with the exception of 20:2n-6, 20:4n-3, 22:2n-6, and 22:4n-3 on the FO, SFO, and LFO treatments (Supplemental Table S8; <https://doi.org/10.3168/jds.2017-13776>). Compared with the control, oil supplements increased ($P < 0.01$) the extent of apparent *cis*-9 18:1, 18:2n-6, and 18:3n-3 biohydrogenation in the rumen (Table 6). Supplements of plant oils with FO resulted in more extensive ($P < 0.05$) metabolism of *cis*-9 18:1, 18:2n-6, 18:3n-3, and 22:6n-3 compared with FO, whereas the effect was close to significant with 20:5n-3 ($P = 0.09$). Biohydrogenation of 18:3n-3 was higher ($P < 0.05$) on

Table 5. Flow of selected 20-, 21-, and 22-carbon fatty acids at the omasum in lactating cows fed a grass silage-based diet supplemented with fish oil or a mixture of fish oil with either sunflower oil or linseed oil

Flow, g/d	Treatment ¹					P-value ²		
	Control	FO	SFO	LFO	SEM	1	2	3
20:1 <i>cis</i> -11	0.47	2.13	2.39	3.76	0.267	<0.001	0.01	0.01
20:1 <i>trans</i> -10-11	0.14	0.88	0.57	0.61	0.092	0.001	<0.05	0.73
20:1 <i>trans</i> -14	0.10	1.46	0.86	0.65	0.203	0.01	<0.05	0.47
20:1 <i>trans</i> -15	0.10	3.86	2.66	1.27	0.575	0.01	<0.05	0.12
20:2 <i>cis</i> -11, <i>cis</i> -14 (20:2n-6)	0.09	0.65	0.60	0.55	0.050	<0.001	0.26	0.43
20:2 <i>cis</i> -14, <i>cis</i> -17 (20:2n-3)	0.00	0.55	0.53	0.38	0.069	0.001	0.32	0.17
20:2 <i>trans</i> -11, <i>cis</i> -15	0.00	0.39	0.40	1.35	0.100	<0.001	<0.01	<0.001
20:2 <i>trans</i> -13, <i>cis</i> -17	0.96	2.62	3.52	3.15	0.121	<0.001	<0.01	0.08
20:2 <i>trans</i> -9, <i>trans</i> -15 ³	0.26	1.78	1.66	2.07	0.227	0.001	0.76	0.25
20:3 <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14	0.02	0.12	0.10	0.17	0.014	<0.001	0.17	0.01
20:3 <i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17 (20:3n-3)	0.03	0.99	1.15	1.20	0.145	0.001	0.32	0.82
20:3 <i>cis</i> -9, <i>trans</i> -14, <i>trans</i> -17 ⁴	2.99	3.89	6.86	2.66	0.804	0.16	0.41	0.01
20:3 <i>trans</i> -9, <i>cis</i> -14, <i>cis</i> -17	0.05	0.98	0.94	0.96	0.126	0.001	0.85	0.89
20:4 <i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14 (20:4n-6)	0.00	0.14	0.05	0.08	0.007	<0.001	<0.001	<0.05
20:4 <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17 (20:4n-3)	0.00	1.71	1.45	1.93	0.196	<0.001	0.93	0.13
20:4 <i>trans</i> -7, <i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17	0.00	0.35	0.32	0.25	0.067	0.01	0.44	0.48
20:5 <i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17 (20:5n-3) ⁵	2.80	5.43	5.03	3.68	0.445	0.01	0.09	0.08
Σ 20-carbon fatty acids	12.2	41.6	40.4	34.0	2.617	<0.001	0.21	0.13
21:4 <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15, <i>cis</i> -18 (21:4n-3)	0.03	0.35	0.43	0.34	0.074	0.01	0.71	0.43
21:5 <i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15, <i>cis</i> -18 (21:5n-3)	0.00	0.32	0.17	0.22	0.035	<0.001	0.01	0.24
Σ 21-carbon fatty acids	0.03	0.79	0.71	0.71	0.065	<0.001	0.36	0.99
22:1 <i>cis</i> -11 ⁶	0.12	2.37	2.41	2.66	0.264	<0.001	0.63	0.54
22:1 <i>cis</i> -13	0.20	0.76	0.59	0.60	0.082	<0.01	0.12	0.94
22:2 <i>cis</i> -13, <i>cis</i> -16 (22:2n-6)	1.59	1.80	2.14	1.59	0.281	0.47	0.86	0.21
22:2 <i>trans</i> -12, <i>trans</i> -17	0.43	0.68	0.63	0.68	0.121	0.14	0.90	0.79
22:3 <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16 (22:3n-6)	0.10	0.25	0.28	0.26	0.047	<0.05	0.77	0.78
22:3 <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19 (22:3n-3)	0.05	0.50	0.53	0.40	0.036	<0.001	0.48	<0.05
22:3 Δ ^{10,13,18}	0.00	0.64	0.67	1.20	0.099	<0.001	0.06	0.01
22:3 <i>cis</i> -10, <i>trans</i> -14, <i>cis</i> -19	0.26	0.41	0.32	0.69	0.086	0.07	0.40	<0.05
22:3 <i>trans</i> -12, <i>cis</i> -16, <i>cis</i> -19	0.01	1.12	1.15	1.15	0.161	0.001	0.89	0.98
22:4 <i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16 (22:4n-6)	0.00	0.05	0.01	0.04	0.005	0.001	0.01	<0.01
22:4 <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19 (22:4n-3) ⁷	0.13	2.44	2.65	4.29	0.359	<0.001	<0.05	0.01
22:4 <i>trans</i> -8, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19	0.85	1.39	1.26	1.62	0.121	<0.001	0.54	0.01
22:5 <i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19 (22:5n-3)	0.27	0.78	0.58	1.01	0.091	<0.01	0.86	<0.05
22:5 Δ ^{5,10,10,13,16,19}	0.00	1.55	0.48	0.81	0.578	0.19	0.23	0.68
22:6 <i>cis</i> -4, <i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19 (22:6n-3)	0.02	2.03	0.88	1.03	0.115	<0.001	<0.001	0.40
Σ 22-carbon fatty acids	4.09	17.2	14.9	18.4	1.421	<0.001	0.76	0.10

¹Refers to grass silage-based diet (forage:concentrate ratio 60:40, on a DM basis) supplemented with no additional oil (control), 200 g of fish oil (FO), 200 g of FO and 500 g of sunflower oil (SFO), or 200 g of FO and 500 g of linseed oil (LFO), respectively.

²Significance of single degree of freedom orthogonal contrasts comparing the control versus lipid supplemented diets (1), FO versus SFO and LFO diets (2), and SFO versus LFO (3).

³Co-elutes with 21:0.

⁴Co-elutes with 22:0.

⁵Co-elutes with 24:0.

⁶Contains *trans*-11,*cis*-14,*cis*-17 20:3 as a minor component.

⁷Contains 8-O-16:0 as a minor component.

LFO than SFO, whereas the reverse was true for 22:5n-3 (Table 6).

Rumen Microbial Ecology

Experimental treatments had no effect on ruminal protozoal numbers (Table 7). Of the species known to be capable of biohydrogenation, the group *Butyrivibrio proteoclasticus* was the most abundant, comprising up to 1.27% of total bacteria. The group encompassing *Butyrivibrio fibrisolvens* and known *Pseudobutyrvibrio*

spp. were the next most numerous, whereas *Butyrivibrio hungatei* represented only a small proportion (0.04–0.05) of total *Butyrivibrio* + *Pseudobutyrvibrio*. *Streptococcus bovis* was present in 0.005% (average) of the bacterial community, but the numbers of *Propionibacterium acnes* were several orders of magnitude lower. *Megasphaera elsdenii* and the atypical *B. fibrisolvens* (which historically is the type strain) were below the limit of detection (10^{-7} of total bacteria) in all samples. Oil supplements had no effect on analyzed ruminal bacterial populations, whereas the abundance

Table 6. Apparent ruminal biohydrogenation of dietary UFA in lactating cows fed a grass silage-based diet supplemented with fish oil or a mixture of fish oil with either sunflower oil or linseed oil

Biohydrogenation, ¹ %	Treatment ²					P-value ³		
	Control	FO	SFO	LFO	SEM	1	2	3
16:1 <i>cis</i> -9	18.9	75.9	86.2	80.0	3.58	<0.001	0.15	0.26
16:2 <i>cis</i> -9, <i>cis</i> -12 (16:2n-4)	—	91.5	93.5	91.7	0.95	—	0.33	0.20
16:2 <i>cis</i> -11, <i>cis</i> -15	—	67.5	78.3	63.1	5.71	—	0.61	0.07
18:1 <i>cis</i> -9	76.4	80.0	91.7	88.2	1.22	<0.001	<0.01	0.08
18:1 <i>cis</i> -11	67.4	61.3	63.5	41.2	0.04	<0.05	0.06	<0.05
18:2 <i>cis</i> -9, <i>cis</i> -12 (18:2n-6)	86.5	91.0	97.6	96.0	0.74	<0.001	<0.01	0.17
18:3 <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15 (18:3n-3) ⁴	94.4	95.4	97.5	98.8	0.31	<0.01	<0.01	<0.05
20:5 <i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17 (20:5n-3)	—	82.6	84.0	88.2	1.40	—	0.09	0.07
21:5 <i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15, <i>cis</i> -18 (21:5n-3)	—	75.6	87.3	83.5	2.63	—	<0.05	0.25
22:5 <i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19 (22:5n-3)	—	78.5	83.7	72.0	2.43	—	0.83	<0.01
22:6 <i>cis</i> -4, <i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19 (22:6n-3)	—	89.9	95.6	94.9	0.54	—	<0.001	0.40

¹Calculated as [(intake, g/d – flow at the omasum, g/d)/intake, g/d] × 100.

²Refers to grass silage-based diet (forage:concentrate ratio 60:40, on a DM basis) supplemented with no additional oil (control), 200 g of fish oil (FO), 200 g of FO and 500 g of sunflower oil (SFO), or 200 g of FO and 500 g of linseed oil (LFO), respectively.

³Significance of single degree of freedom orthogonal contrasts comparing the control versus lipid supplemented diets (1), FO versus SFO and LFO diets (2), and SFO versus LFO (3).

⁴Co-elutes with *cis*-12 22:1.

of *Oribacterium* sp. was decreased ($P < 0.05$) on LFO compared with SFO (Table 7).

DISCUSSION

Dietary oil supplements decreased silage DMI even though cows were fed at a restricted level of intake. It is well established that FO and FO plus plant oils cause dose-dependent decreases in intake in lactating cows (Keady et al., 2000; Palmquist and Grinari, 2006; Shingfield et al., 2012). In the present study, the decrease in DMI to oil supplements were not ac-

companied by adverse effects on ruminal or total-tract nutrient digestion, but seemed to increase whole-tract DM digestion; this may be explained by longer ruminal retention time caused by lower intake (Huhtanen and Kukkonen, 1995).

Inclusion of oils in the diet had no effect on rumen pH, ammonia N, or total VFA concentrations, but altered rumen fermentation, effects that were more pronounced for SFO and LFO compared with FO, with no evidence of differences due to the source of plant oil. In relatively low amounts, FO has rather small effects on rumen fermentation characteristics in lactating cows;

Table 7. Rumen microbial populations of lactating cows fed a grass silage-based diet supplemented with fish oil or a mixture of fish oil with either sunflower oil or linseed oil

Microbial population, % of total bacteria ¹	Treatment ²					P-value ³		
	Control	FO	SFO	LFO	SEM	1	2	3
18:0-producing bacteria ⁴	1.22	1.04	0.89	0.73	0.180	0.16	0.33	0.54
Total <i>Butyrivibrio</i> + <i>Pseudobutyrvibrio</i>	1.47	1.24	1.10	0.93	0.193	0.14	0.36	0.56
<i>Butyrivibrio proteoclasticus</i>	1.27	1.10	0.94	0.77	0.182	0.16	0.32	0.54
<i>Butyrivibrio fibrisolvens</i> + <i>Pseudobutyrvibrio</i>	0.19	0.15	0.16	0.16	0.027	0.27	0.76	0.99
<i>Butyrivibrio hungatei</i>	0.051	0.051	0.044	0.042	0.0095	0.61	0.52	0.89
<i>Streptococcus bovis</i>	0.005	0.002	0.008	0.004	0.0029	0.99	0.31	0.31
<i>Propionibacterium acnes</i> , no. × 10 ⁻⁴	1.30	1.20	1.00	0.20	0.650	0.55	0.49	0.41
<i>Oribacterium</i> sp.	0.025	0.022	0.024	0.013	0.0027	0.12	0.37	<0.05
Protozoa numbers, 10 ⁵ /mL of rumen fluid	1.79	1.91	2.08	2.65	0.406	0.38	0.38	0.35

¹Populations determined based on quantitative PCR using primers and probes designed to target different members of the *Butyrivibrio*/*Pseudobutyrvibrio* group, *S. bovis*, *P. acnes*, and *Oribacterium* sp.

²Refers to grass silage-based diet (forage:concentrate ratio 60:40, on a DM basis) supplemented with no additional oil (control), 200 g of fish oil (FO), 200 g of FO and 500 g of sunflower oil (SFO), or 200 g of FO and 500 g of linseed oil (LFO), respectively.

³Significance of single degree of freedom orthogonal contrasts comparing the control versus lipid supplemented diets (1), FO versus SFO and LFO diets (2), and SFO versus LFO (3).

⁴Bacteria that are known to convert 18:1 *trans*-11 to 18:0, predominantly members of the *B. proteoclasticus* clade with some *B. hungatei* (Shingfield et al., 2012).

however, higher levels of FO, or when fed with SO, have been shown to induce larger changes in the relative proportions of gluconeogenic and ketogenic fermentation end-products (Shingfield et al., 2003; Palmquist and Grinari, 2006; Shingfield et al., 2012).

Alterations in ruminal biohydrogenation and the flow of FA at the omasum in response to oil supplements can be separated into the effects due to the metabolism of the long-chain UFA contained in FO and the associated effects on unsaturated 16- and 18-carbon FA, as well as the influence of the amount and source of dietary 18-carbon PUFA on these processes. Recoveries of 20:5n-3, 22:5n-3, and 22:6n-3 at the omasum on the FO, SFO, and LFO treatments were marginal relative to the intake, in agreement with earlier studies reporting that long-chain n-3 PUFA are extensively biohydrogenated in the rumen of lactating cows (Shingfield et al., 2003; Loores et al., 2005; Shingfield et al., 2012).

During incubations with rumen contents, 18:2n-6 inhibited 20:5n-3 and 22:6n-3 biohydrogenation (Wasowska et al., 2006). Studies in growing cattle (Shingfield et al., 2011) and nonlactating sheep (Toral et al., 2010) fed diets containing plant oils and FO also suggested that the supply of 18-carbon PUFA may influence the extent of ruminal 20- and 22-carbon PUFA biohydrogenation. The present experiment provided the first direct evidence that metabolism of 22:6n-3 in the rumen of lactating cows is more extensive on diets containing higher amounts of 18-carbon PUFA, and the effect seemed to be similar with 20:5n-3. However, the biohydrogenation of 22:5n-3 was less extensive in LFO than SFO, but showed no difference between FO and diets containing plant oils. Such findings may help to explain, at least in part, the difference in the enrichment of 20:5n-3, 22:5n-3, and 22:6n-3 in muscle and milk from cattle fed dietary FO supplements under different study designs (Shingfield et al., 2013). More extensive metabolism of UFA in the rumen on the FO, LFO, and SFO treatments compared with the control was consistent with biohydrogenation representing a survival mechanism by removing the bacteriostatic effects of UFA below a certain threshold that would otherwise inhibit the growth of specific rumen bacteria.

Even though oil supplements increased 20-, 21-, and 22-carbon PUFA intake, the amounts of 20:0, 21:0, and 22:0 reaching the omasum were not substantially higher than the control, indicating that ruminal biohydrogenation of ≥ 20 -carbon PUFA to saturated end-products is incomplete. Some 20-, 21- and 22-carbon biohydrogenation intermediates detected in omasal digesta of cows fed FO but not on the control diet have been shown to be formed during metabolism of 20:5n-3, 22:5n-3, and 22:6n-3 in the rumen of lactating cows (Shingfield et al., 2003; Kairenius et al., 2011; Shingfield et al.,

2012). The differences in the ruminal biohydrogenation of 20:5n-3 and 22:5n-3 and the different flow of 20- to 22-carbon intermediates at the omasum on LFO versus SFO suggests that the source of 18-carbon PUFA influences the kinetics of ruminal 20:5n-3, 22:5n-3, and 22:6n-3 metabolism.

The pathways of all-*cis* 20- to 22-carbon PUFA metabolism in the rumen are not well described. It has been suggested that the biohydrogenation of these FA may proceed via the reduction of *cis* double bonds closest to the carboxyl group, or via a specific mechanism that involves the sequential reduction of the *cis*-6 and *cis*-9 double bond (Kairenius et al., 2011). Flows of several all-*cis* 20- and 22-carbon FA, including 20:2n-6, 20:2n-3, 20:4n-3, 22:2n-6, 22:3n-6, and 22:4n-3 at the omasum, exceeded intake, which would implicate these FA as intermediates of 20:5n-3, 22:5n-3 or 22:6n-3 metabolism. No conjugated 20- to 22-carbon FA were detected in omasal digesta, consistent with earlier investigations (Kairenius et al., 2011; Shingfield et al., 2012), suggesting that biohydrogenation of long-chain PUFA does not involve isomerization and formation of intermediates containing a conjugated double bond system.

In the present study, oil supplements increased *trans*-16:1, *trans*-18:1, and *trans*-18:2 at the omasum relative to the control. The biohydrogenation of *cis*-9 18:1, 18:2n-6, and 18:3n-3 in oil-supplemented diets was also higher than biohydrogenation of these FA originating from ingredients of a control diet, being even greater when plant oils were fed with FO. Despite of a similar intake of 18-carbon PUFA and similar flow of *trans*-18:1, the flow of 18:0 at the omasum was lower and accumulation of *trans*-18:2 intermediates greater for LFO than SFO. Such findings indicate that the inhibitory effects of FO on the reduction of 18-carbon FA to 18:0 is influenced by the relative amounts of 18:2n-6 and 18:3n-3 in the diet.

On the FO treatment, *trans*-11 18:1 and *trans*-11, *cis*-15 18:2 were among the major biohydrogenation products. Inclusion of LO, but not SO, caused *trans*-11 18:1 to accumulate. In earlier studies, supplements of sunflower seeds or linseeds in combination with FO have been shown to elevate the proportion of *trans*-11 18:1 in ruminal (AbuGhazaleh et al., 2003) and duodenal digesta (Shingfield et al., 2011) of cattle. Some of these apparent discrepancies can be explained by differences in the influence of lipid supplements on the major biohydrogenation pathways in the rumen due to variation in 18-carbon FA intake within earlier investigations and the present study. In the present experiment, supplementing FO with sources of 18:2n-6 or 18:3n-3 caused *trans*-10 18:1 to accumulate, and, on the SFO treatment, *trans*-10 18:1 was the major biohydrogena-

tion intermediate escaping the rumen. High amounts of FO (Loor et al., 2005; Shingfield et al., 2012), high-starch, low-fiber diets (Piperova et al., 2002; Zened et al., 2013), and high-concentrate diets containing plant oils (Loor et al., 2004; Ventto et al., 2017) have been shown to promote a shift in ruminal biohydrogenation pathways, promoting the formation of *trans*-10 18:1; however, the underlying causes are not known. It has been shown that *trans*-10 18:1 is formed from 18:2n-6 via reduction of Δ 10,12 CLA isomers (Kepler et al., 1966; McKain et al., 2010) and may also be formed by isomerization of *cis*-9 18:1 (Mosley et al., 2002).

In vitro studies have suggested that a decrease in pH is associated with the shift from *trans*-11 18:1 to *trans*-10 18:1 in dual-flow continuous cultures (Fuentes et al., 2009). However, increases in *trans*-10 18:1 formation on the SFO and LFO treatment were not associated with a decrease in mean daily rumen pH. Similarly, increases in *trans*-10 18:1 formation in cows fed high-starch, low-fiber diets (Piperova et al., 2002; Zened et al., 2013), high-concentrate diets containing plant oils (Loor et al., 2004; Ventto et al., 2017), or high amounts of FO (Shingfield et al., 2012) were not accompanied by decreases in rumen pH. Further investigations are required to provide an insight into the mechanisms involved and the associated changes in the rumen microbial community.

The major pathway of ruminal 18:3n-3 metabolism involves an initial isomerization to *cis*-9,*trans*-11,*cis*-15 18:3 and sequential reduction of double bonds to yield *trans*-11,*cis*-15 18:2 and *trans*-11 18:1 as intermediates (Harfoot and Hazlewood, 1988). Relative to SFO, the amounts of alternative novel *trans*-18:2 isomers, including *trans*-10,*cis*-15 18:2, *trans*-9,*trans*-14 18:2, *trans*-11,*trans*-15 18:2, *trans*-10,*trans*-15 18:2, and *trans*-9,*trans*-12 18:2, were increased on LFO, providing further evidence that multiple products are formed during the biohydrogenation of 18:3n-3 (Jouany et al., 2007; Alves and Bessa, 2014). Inclusion of LO with FO also altered the major pathways described for 18:3n-3, resulting in *trans*-10,*cis*-15 18:2 replacing *trans*-11,*cis*-15 18:2 as the major 18:2 intermediate escaping the rumen. Recent studies have confirmed that *trans*-10,*cis*-15 18:2 is formed from 18:3n-3 in vivo (Alves and Bessa, 2014) and in vitro (Honkanen et al., 2016).

Irrespective of diet, *cis*-9,*trans*-11 was the major isomer of CLA in omasal digesta, confirming previous reports in lactating cows fed diets based on grass silage (Shingfield et al., 2003, 2008a, 2012). Compared with the control, oil supplements increased several geometric isomers of Δ 9,11 and Δ 10,12 CLA at the omasum, consistent with earlier reports in ruminants fed diets containing FO alone or in a combination with plant oils rich in 18:2n-6 or 18:3n-3 (Toral et al., 2010; Shingfield

et al., 2011). Inclusion of plant oils with FO increased ruminal *trans*-10,*cis*-12 CLA outflow in amounts greater for SFO than LFO. Typically, FO has minimal influence on ruminal *trans*-10,*cis*-12 CLA formation (Shingfield et al., 2003, 2010), whereas increases in dietary 18:2n-6 content often promote *trans*-10,*cis*-12 CLA synthesis (Sackmann et al., 2003; Shingfield et al., 2008a). Similarly, *trans*-11,*cis*-13 CLA and *trans*-11,*trans*-13 CLA at the omasum were higher on LFO than SFO, consistent with studies in vitro indicating geometric Δ 11,13 CLA isomers are formed during incomplete biohydrogenation of 18:3n-3 (Jouany et al., 2007; Honkanen et al., 2016).

Metabolism of 16-carbon UFA in the rumen is not well described. Formation of *trans*-16:1 and *trans*-16:2 isomers are thought to originate from incomplete biohydrogenation of 16:2n-4, 16:2n-7, 16:3n-4, 16:4n-1, and 16:4n-3 contained in FO (Shingfield et al., 2010, 2011, 2012). Irrespective of dietary lipid content, *trans*-11 16:1 was the major biohydrogenation intermediate on all oil-supplemented treatments. Including plant oils with FO altered the biohydrogenation of 16-carbon UFA, promoting the formation of *trans*-10-containing products (*trans*-10 16:1, *trans*-10,*trans*-14 16:2) in the rumen, suggesting that the mechanisms involved are common to both 16- and 18-carbon UFA.

Oil supplements had relatively minor effects on ruminal escape of OBCFA that originate from membrane lipid of rumen bacteria (Vlaeminck et al., 2006). Earlier reports in lactating cows have demonstrated that dietary supplements of FO (Shingfield et al., 2012), SO (Shingfield et al., 2008a), or LO (Loor et al., 2004) have variable effects on ruminal outflow of specific OBCFA that have been suggested to represent a proxy of the microbial community in the rumen (Vlaeminck et al., 2006).

Bacteria, rather than protozoa, are thought to be responsible for ruminal biohydrogenation, but relatively few strains capable of biohydrogenation have been identified (Harfoot and Hazlewood, 1988; Lourenço et al., 2010). The identification of specific microbial species able to catalyze 1 or more reactions remains challenging, as biohydrogenation intermediates and products may also be exchanged between populations. Among cultivated bacteria, all members of the *Butyrivibrio* group form *cis*-9,*trans*-11 CLA from 18:2n-6 much more rapidly than do other species, but only *B. proteoclasticus* is capable of reducing *trans*-11 18:1 to 18:0 (Wallace et al., 2006; Paillard et al., 2007). In the present experiment, the *Butyrivibrio* + *Pseudobutyrvibrio* group comprised 0.93 to 1.47% of total bacterial DNA, numbers higher than earlier estimates in cattle (Huws et al., 2010; Shingfield et al., 2012). The numbers of *B. proteoclasticus* did not differ due to treatment, con-

sistent with earlier reports in lactating cows fed FO (Shingfield et al., 2012). Even though oil supplements altered the amounts of 18-carbon biohydrogenation intermediates and 18:0 at the omasum, no changes in other populations of rumen *Butyrivibrio* group were observed. The population sizes of *S. bovis* and *P. acnes*, ruminal species known to hydrate rather than hydrogenate *cis*-9 18:1 and 18:2n-6 (Hudson et al., 2000; Kim et al., 2008), were extremely small (approximately $\leq 0.001\%$ of total bacteria) in omasal digesta and not affected by treatment.

It has often been assumed that the main FA in FO are responsible for the inhibitory effects on biohydrogenation, but based on the earlier investigations in vivo (Shingfield et al., 2012) or in vitro (Wasowska et al., 2006) it seems that FO inhibits ruminal biohydrogenation through a mechanism that is not solely explained by effects on *B. fibrisolvens*. Other bacteria, such as *Ruminococcus albus*, *Eubacterium* spp., and *Treponema* spp., have been described to hydrogenate FA (Yokoyama and Davis, 1971; Kemp et al., 1975). Recently we isolated strains of *Oribacterium* that converted 18:2n-6 to *cis*-9, *trans*-11 CLA and *trans*-11 18:1 (S. Muetzel and R. J. Wallace, unpublished data). Including FO in the diet of growing cattle has been shown to induce changes in the number of other rumen bacterial strains, such as *Anaerovibrio lipolytica*, *Fibrobacter succinogenes*, and *Ruminococcus flavefaciens* (Huws et al., 2010), suggesting a broader influence on ruminal lipid metabolism (Kim et al., 2008; Huws et al., 2010). Furthermore, a negative relationship between DNA concentrations from *B. proteoclasticus* strains and 18:0 at the duodenum was reported in earlier studies (Kim et al., 2008; Huws et al., 2010). No such relationship was observed in our experiment; it is possible, therefore, that unknown microbial species, not quantified here, are involved in FA biohydrogenation. Alternatively, direct inhibition of the biohydrogenation pathway, which does not lead to changes in the biohydrogenating community but alters the biohydrogenation products, could be the principal mode of action of the plant PUFA (Maia et al., 2007; Lourenço et al., 2010).

In summary, dietary supplements of FO alone or in combination with plant oils increase the amount of biohydrogenation intermediates escaping the rumen, but the alterations in the amount of intermediates at the omasum or ruminal biohydrogenation pathways were not accompanied by major changes in analyzed bacterial populations. The present experiment provided the first direct evidence that metabolism of 22:6n-3 in the rumen of lactating cows is more extensive on diets containing higher amounts of 18-carbon PUFA, with indication that the effect is similar for 20:5n-3, whereas the effect on 22:5n-3 biohydrogenation depended on the

source of 18-carbon PUFA. The source of 18-carbon PUFA influenced the kinetics of ruminal metabolism of these very long-chain FA. Further studies using functional metagenomics approaches are needed to provide a greater insight into the microbiology of diet-induced changes in bacterial biohydrogenation within the rumen, knowledge that can be used to develop novel strategies to enhance the beneficial FA content of ruminant products such as meat and milk.

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