Effect of fish oil and sunflower oil on rumen fermentation characteristics and fatty acid composition of digesta in ewes fed a high concentrate diet

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ABSTRACT

Studies in ruminants have shown that supplementing the diet with a mixture of fish oil (FO) and sunflower oil (SO) enhances the concentration of *cis*-9, *trans*-11 conjugated linoleic acid (CLA), 20:5 n-3, and 22:6 n-3 in milk because of alterations in ruminal biohydrogenation, but the intermediates formed under these conditions are not well characterized. Five ewes fitted with rumen cannula and fed a high concentrate diet were used to examine the effect of a mixture (30 g/kg of DM) of FO and SO (1:2, wt/wt) on temporal changes in rumen fermentation characteristics and the relative abundance of biohydrogenation intermediates in ruminal digesta collected after 0, 3, and 10 d on diet. Appearance and identification of biohydrogenation intermediates was determined based on complementary gas-liquid chromatography and Ag+-HPLC analysis of fatty acid methyl esters and gas chromatography-mass spectrometry analysis of corresponding 4,4-dimethyloxazoline derivatives. Inclusion of FO and SO in the diet had no effect on rumen pH, volatile fatty acid concentrations, or nutrient digestion, but altered the fatty acid composition of ruminal digesta, changes that were characterized by time-dependent decreases in 18:0 and 18:2 n-6 and the accumulation of *trans* 16:1, *trans* 18:1, 10-O-18:0, and *trans* 18:2. Lipid supplements enhanced the proportion of 20:5 n-3 and 22:6 n-3 in digesta and resulted in numerical increases in *cis*-9, *trans*-11 conjugated linoleic acid concentrations, but decreased the relative abundance of *trans*-10, *cis*-12 conjugated linoleic acid. Furthermore, detailed analysis revealed the appearance of several unique 20:1, 20:2, 22:1, 22:3, and 22:4 products in ruminal digesta that accumulated over time, providing the first indications of 20 and 22 carbon fatty acid intermediates formed during the biohydrogenation of long-chain unsaturated fatty acids in sheep. In conclusion, FO and SO in a high concentrate diet caused a time-dependent inhibition of the complete

biohydrogenation of 16 and 18 carbon unsaturated fatty acids, and resulted in the accumulation of *trans* 16:1, *trans* 18:1, and *trans* 18:2, 20, and 22 carbon metabolites in ruminal digesta of sheep, with no evidence of a shift in ruminal biohydrogenation pathways toward *trans*-10 18:1 formation.

Key words: biohydrogenation, fish oil, sheep, *trans* fatty acid

INTRODUCTION

Studies in ruminants have shown that inclusion of fish oil (FO) in the diet alone or in combination with plant oils increases *cis*-9, *trans*-11 conjugated linoleic acid (CLA), 20:5 n-3, and 22:6 n-3 contents in milk (Shingfield et al., 2003, 2006; Toral et al., 2010a), with the level of enrichment being related to a large extent to the biohydrogenation of dietary unsaturated fatty acids (FA) in the rumen.

Plant oils rich in 18:2 n-6 are known to enhance the accumulation and ruminal outflow of *trans*-18:1 and *cis*-9, *trans*-11 CLA (Kucuk et al., 2004; Atkinson et al., 2006; Shingfield et al., 2008). Inclusion of FO containing 20:5 n-3 and 22:6 n-3 inhibits the complete biohydrogenation of 18 carbon unsaturated FA causing an increase in the supply of *trans*-18:1 available for incorporation in milk fat triacylglycerides and tissue lipids (Shingfield et al., 2003; Lee et al., 2008; Shingfield et al., 2010b). Even though 20:5 n-3 and 22:6 n-3 are extensively hydrogenated in the rumen, the products formed and the mechanisms involved are not known.

Measurements of temporal changes in milk FA composition have provided evidence that the inclusion of plant oils in high concentrate diets (Bauman et al., 2000; Roy et al., 2006) or FO and sunflower oil (SO) in TMR (Shingfield et al., 2006; Cruz-Hernandez et al., 2007) result in time-dependent changes in ruminal biohydrogenation. Recent studies in sheep have also shown that supplementing a high concentrate diet with 10 g of FO and 20 g of SO/kg of diet increases milk fat *cis*-9, *trans*-11 CLA, 20:5 n-3, and 22:6 n-3 concentrations. However, the enrichment of *cis*-9, *trans*-11 CLA in milk increased within 7 d on diet but declined thereafter

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(Toral et al., 2010a). Transient increases in milk *cis*-9, *trans*-11 CLA content were also associated with a progressive decrease in DMI and milk fat synthesis over time (Toral et al., 2010a), effects that may, at least in part, be related to the effect of lipid supplements on nutrient digestion in the rumen (Wachira et al., 2000; Fievez et al., 2003; Shingfield et al., 2003). Furthermore, the decreases in mammary lipogenesis were not explained by increases in milk fat concentrations of biohydrogenation intermediates known to inhibit milk fat synthesis in cows (Shingfield and Griinari, 2007), suggesting that other metabolites, or mechanisms, or both are involved. In the current experiment, the effect of FO and SO on temporal changes in rumen fermentation patterns and the accumulation of biohydrogenation intermediates in sheep fed high concentrate diets were examined based on the analysis of samples collected after 0, 3, and 10 d on diet. Fatty acid composition of ruminal digesta was analyzed using GLC and Ag+-HPLC analysis of FA methyl esters (**FAME**) and GC-MS analysis of corresponding 4,4-dimethyloxazoline (**DMOX**) derivatives to provide further insight into the metabolic fate of long-chain FA in the rumen.

MATERIALS AND METHODS

Animals and Diets

All experimental procedures were performed in accordance with the Spanish Royal Decree 1201/2005 for the protection of animals used for experimental and other scientific purposes. Five dry Merino ewes (approximately 3 yr old; mean BW 63.0 ± 5.99 kg) each fitted with a rumen cannula were individually housed in raised-floor pens (1.5×2 m). Diets comprised alfalfa hay (mean particle size >4 cm) and a concentrate supplement (35:65 forage:concentrate ratio, DM basis) containing 0 (control diet) or 30 g/kg of DM of a mixture (1:2 wt/wt) of FO and SO (**SFO**). Oils replaced other dietary ingredients on a proportionate basis. Ingredients and chemical composition of the experimental diets are shown in Table 1. Fatty acid composition of the control diet, FO, and SO is reported in Table 2. Diets were fed as a TMR to avoid selection of dietary components as 2 meals (60% at 900 h and 40% at 1800 h) at a rate of 41 g of DM/kg of BW^{0.75} equivalent to proportionately 0.8 of ad libitum intake measured over a 15-d period immediately before the start of the experiment. Ration mixes were prepared every 4 d and adjusted for changes in component DM content. Semirefined tuna and sardine oil (Afampes 121 DHA, Afamsa, Vigo, Spain) and SO (Carrefour S.A., Madrid, Spain) were stored in the dark at 4°C before incorporation into daily rations. Animals had continuous access to fresh clean water.

Table 1. Ingredient and chemical composition of experimental diets¹

Item	Control	SFO
Ingredient (g/kg of fresh matter)		
Dehydrated alfalfa hay	350	341
Whole corn grain	203	198
Soybean meal	163	158
Whole barley grain	122	119
Beet pulp	73	71
Molasses	53	51
Salt premix ²	33	32
Minerals and vitamins ³	4	4
Sunflower oil ⁴	0	18
Fish oil ⁵	0	9
Composition (g/kg of DM)		
OM	882	891
CP	172	163
NDF	269	279
ADF	156	172
Starch	274	253
Fatty acids	46	75

¹Control = no oil supplementation; SFO = supplemented with 20 g of sunflower oil plus 10 g of fish oil/kg of DM.

²Contained (g/kg): 375 NaHCO₃; 350 CaCO₃; 150 Ca₂HPO₄; and 125 NaCl.

³INA OV1 (Evalis, Madrid, Spain). Contained: 60 g/kg of S; 30 g/kg of Mg; 12 g/kg of Fe; 10 g/kg of Zn; 10 g/kg of Mn; 6 g/kg of choline; 200 mg/kg of Co; 200 mg/kg of I; 36 mg/kg of Se; 200 mg/kg of vitamin B₁; 200 mg/kg of vitamin B₂; 1,250 IU/g of vitamin A; 250 IU/g of vitamin D₃; and 3 IU/g of DL- α -tocopheryl acetate.

⁴Carrefour S.A. (Madrid, Spain).

⁵Semirefined tuna and sardine oil (Afampes 121 DHA, Afamsa, Vigo, Spain).

Experimental Design

The experiment was conducted over 2 consecutive 11-d periods. In the first period, the control diet was fed over an 11-d adaptation period to establish a baseline and thereafter all ewes were fed the SFO diet during the second 11-d period. In vivo, in situ, and in vitro determinations were conducted before the start of oil supplementation (d 0, control) and after 3 d (**SFO**₃) and 10 d (**SFO**₁₀) on the SFO diet. Even though treatment was confounded with experimental period, this approach was used rather than a more robust switch-back or change-over design to avoid possible residual carry-over effects of FO and SO on rumen fermentation and lipid metabolism (Cruz-Hernandez et al., 2006). The same experimental design has been used previously to examine the effects of FO on ruminal biohydrogenation and milk fat composition (Kitessa et al., 2001; Shingfield et al., 2003; Roy et al., 2006).

Experimental Procedures

In Vivo Studies. Dry matter intake was measured daily. Rumen fluid was collected from each sheep after 0, 3, and 10 d on the SFO diet (control, **SFO**₃, and **SFO**₁₀, respectively) at 0, 1.5, 3, 6, and 9 h after morn-

Table 2. Fatty acid composition of the control diet and the oils

Fatty acid (g/kg of total fatty acids)	Control diet	Sunflower oil ¹	Fish oil ²
12:0	0.55	<0.01	0.06
14:0	0.40	0.06	4.07
15:0	0.24	0.02	0.87
16:0	19.85	5.53	19.45
<i>cis</i> -9 16:1	0.26	0.09	4.97
<i>cis</i> -11 16:1	0.00	<0.01	0.24
<i>trans</i> -6 16:1	0.00	0.00	0.60
<i>cis</i> -9, <i>cis</i> -12 16:2	0.00	0.00	0.29
<i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12 16:3	0.00	0.00	0.23
<i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15 16:4	0.00	0.00	0.22
<i>cis</i> -4, <i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13 16:4	0.00	0.00	0.04
17:0	0.23	0.05	0.99
<i>cis</i> -9 17:1	0.06	0.03	0.56
18:0	2.92	4.42	5.11
<i>cis</i> -9 18:1	14.99	36.36	14.70
<i>cis</i> -11 18:1	0.88	0.81	2.68
<i>cis</i> -12 18:1	0.00	0.00	0.08
<i>cis</i> -13 18:1	0.00	0.00	0.17
<i>cis</i> -9, <i>cis</i> -12 18:2	42.15	50.28	2.23
<i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12 18:3	0.19	0.00	0.11
<i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15 18:3	10.16	0.06	0.65
<i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15 18:4	0.00	0.00	0.91
19:0	0.00	0.00	0.27
20:0	0.66	0.29	0.35
<i>cis</i> -9 20:1	0.05	0.02	0.24
<i>cis</i> -11 20:1	0.31	0.16	2.20
<i>cis</i> -13 20:1	0.00	<0.01	0.21
<i>cis</i> -11, <i>cis</i> -14 20:2	0.10	0.00	0.31
<i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14 20:3	0.00	0.00	0.12
<i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17 20:3	0.00	0.00	0.18
<i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14 20:4	0.00	0.00	1.47
<i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17 20:4	0.00	0.00	0.51
<i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17 20:5	0.00	0.00	6.05
<i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15, <i>cis</i> -18 21:5	0.00	0.00	0.21
22:0	0.78	0.74	0.21
<i>cis</i> -11 22:1	0.00	0.00	1.68
<i>cis</i> -13 22:1	0.00	0.00	0.34
<i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16 22:4	0.00	0.00	0.22
<i>cis</i> -4, <i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16 22:5	0.00	0.00	1.06
<i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19 22:5	0.00	0.00	1.31
<i>cis</i> -4, <i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19 22:6	0.00	0.00	17.26
24:0	2.49	0.23	0.00
<i>cis</i> -15 24:1	0.00	0.00	0.68
<i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19, <i>cis</i> -21 24:5	0.00	0.00	0.08
<i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19, <i>cis</i> -21 24:6	0.00	0.00	0.01
Other	2.73	0.85	4.98
Unidentified	0.00	0.00	0.83

¹Contained 953 g of total fatty acids/kg.²Contained 954 g of total fatty acids/kg.

ing feeding for the measurement of pH and ammonia, lactic acid, and VFA concentrations (Toral et al., 2009). In addition, samples of ruminal digesta were collected at 0, 3, 6, 12, 18, and 24 h postfeeding and stored at -30°C immediately after collection. Digesta was pooled to provide composite samples for each animal and sampling day, freeze dried, and stored at -80°C until submitted for FA determinations.

In Situ Studies. After 0, 3, and 10 d on the SFO diet (control, SFO₃, and SFO₁₀, respectively), in situ rumen degradation of alfalfa hay used as a reference

feed was estimated using nylon bags that were incubated in duplicate for 12 and 24 h. Concentrations of DM, CP, and NDF in incubation residues were assessed according to standard procedures (Toral et al., 2009).

In Vitro Studies. After 0 d (control) and 11 d on the SFO diet, in vitro ruminal fermentation was assessed by the gas production technique using rumen inocula collected from each of the 5 experimental ewes. Fifteen samples [5 inocula (replicates) $\times$ 3 flasks/inoculum] per substrate (alfalfa hay and control and SFO diets; 500 mg) were incubated in sealed serum

flasks at 39°C with 10 mL of rumen fluid and 40 mL of phosphate-bicarbonate buffer, prepared as outlined previously (Hervás et al., 2005). Accumulated head-space gas pressures were measured at 2, 4, 6, 8, 10, 15, 18, 21, 24, 29, 35, 48, and 72 h postinoculation. Pressure values, corrected for the amount of OM incubated and gas released from blanks (i.e., rumen fluid plus buffer medium, without substrate), were used to generate gas volume estimates using a predictive equation derived from earlier simultaneous measurements of pressure and volume (Hervás et al., 2005). In vitro DM disappearance after 72 h of incubation was estimated by filtering fermentation residues.

Chemical Analysis

Samples of diets were collected, stored at -30°C, and freeze dried. Diet samples were analyzed for DM (ISO, 1999), ash (ISO, 2002), CP (ISO, 2005), and starch (ISO, 2000). Concentrations of NDF and ADF were determined according to the instructions of the manufacturer (Ankom Technology Corp., Macedon, NY) that included residual ash. Determinations of NDF were made using sodium sulfite and α -amylase.

Fatty acid methyl esters of lipid in FO, SO, and freeze-dried samples of TMR were prepared in a 1-step extraction-transesterification procedure using chloroform (Sukhija and Palmquist, 1988) and 2% (vol/vol) sulfuric acid in methanol (Shingfield et al., 2003). Fatty acid content was determined using tritridecanoic (T-135; Nu-Chek Prep Inc., Elysian, MN) as an internal standard. Lipid in 200 mg of freeze-dried ruminal digesta was extracted in duplicate using 4 mL of a mixture (3:2, vol/vol) of hexane and isopropanol following the adjustment of digesta pH to 2.0 using 2 M hydrochloric acid (Shingfield et al., 2003). Organic extracts were combined and dried under nitrogen at 50°C. Lipid dissolved in 2 mL of hexane was converted to FAME using a base-acid catalyzed transesterification procedure with freshly prepared 0.5 M sodium methoxide in methanol for 5 min at 20°C followed by reaction with 1% (vol/vol) sulfuric acid in methanol at 50°C for 30 min.

Methyl esters were quantified by GLC using a gas chromatograph (model 6890; Hewlett-Packard, Wilmington, DE) equipped with a flame-ionization detector, selective quadrupole mass detector (model 5973N; Agilent Technologies Inc., Wilmington, DE) and 100-m fused silica capillary column (i.d. 0.25 mm) coated with a 0.2- μ m film of cyanopropyl polysiloxane (CP-Sil 88, Chrompack 7489; Chrompack, Middelburg, the Netherlands) using hydrogen as the carrier and fuel gas. Total FAME profile in a 2- μ L sample at a split ratio of 1:50 was determined using a temperature gradient program (Shingfield et al., 2003). Isomers of 18:1 and

18:2 methyl esters were further resolved in a separate analysis under isothermal conditions at 170°C (Shingfield et al., 2003). Peaks were routinely identified using a mixture of authentic FAME standards (GLC #463, N-21-M, N-23-M, N-24-M, U-37-M, U-39-M, U-43-M, U-54-M, U-64-M, U-85-M, and U-87-M: Nu-Chek Prep Inc.; 10-1840, 11-1600-8, 20-2001-1-4, 20-2024-1, 20-2103-1-4, 20-2105-1-4, 20-2210-9, 20-2265-7, 20-2305-1-4, 20-2405-4, 2000-2500-7, 20-2900-7, BR2, and BR3: Larodan Fine Chemicals, Malmö, Sweden; H-6389, H-6639, L-6031, O-4129, O-5632, and T-1902: Sigma-Aldrich, St. Louis, MO).

Fatty acids not available as commercial standards were identified based on GC-MS analysis of DMOX derivatives prepared from FAME according to reference procedures (Shingfield et al., 2006). The mass spectrometer was operated at 230°C in the electron impact ionization mode and mass spectra were recorded under an ionization energy of 70 eV using helium as the carrier gas and the same temperature gradient used for routine analysis of FAME (Shingfield et al., 2006). Electron impact ionization spectra of DMOX derivatives were used to locate double bonds based on atomic mass unit distances, with an interval of 12 atomic mass units between the most intense peaks of clusters of ions containing n and $n - 1$ carbon atoms being interpreted as cleavage of the double bond between carbon n and $n + 1$ in the FA moiety. Identification was verified based on comparisons with an online reference library of DMOX derivative electron impact ionization spectra (<http://lipidlibrary.aocs.org/ms/masspec.html>) and previous determinations of FA composition in omasal digesta of cows fed FO identified based on fractionation of FAME by argentation silver-ion thin-layer chromatography and GC-MS analysis of corresponding DMOX derivatives (P. Kairenius, V. Toivonen, and K. J. Shingfield, MTT Agrifood Research Finland; unpublished data). Analysis allowed for an unambiguous identification of monoenoic and dienoic FA in digesta, but double bond geometry of specific biohydrogenation intermediates could not be confirmed because of the inability of GC-MS analysis to discriminate between geometric isomers. Double bond geometry of Δ 9,11,15 18:3 was assigned according to published reports (Gómez-Cortés et al., 2009). Partial gas chromatograms indicating the separation of 20 and 22 carbon FAME are shown in Figures 1 and 2, respectively.

The distribution of CLA isomers in ruminal digesta was determined by HPLC using 4 silver-impregnated silica columns (ChromSpher 5 lipids, 250 $\times$ 4.6 mm, 5 μ m particle size; Varian Ltd., Walton-on-Thames, UK) coupled in series and 0.1% (vol/vol) acetonitrile in heptane as the mobile phase (Shingfield et al., 2003). Isomers were identified using an authentic CLA methyl

ester standard and chemically synthesized *trans*-9, *cis*-11 CLA (Shingfield et al., 2005) using *cis*-9, *trans*-11 CLA as a landmark isomer.

Calculations and Statistical Analysis

Gas production data were fitted to an exponential model [$G = A \times (1 - e^{-k \times \text{time}})$] to provide parameters describing gas release (G) in terms of cumulative gas production (A ; mL of gas/g of OM incubated) and fractional fermentation rate (k ; h^{-1}). Calculations were made using the NLIN procedure of SAS (version 9.1, SAS Institute Inc., Cary, NC). Effective degradability was estimated assuming a rumen particulate outflow (k_p ; h^{-1}) of 0.042 (Hervás et al., 2005).

All data were evaluated by a 1-way ANOVA using the MIXED procedure of SAS with a model that included the fixed effect of treatment [control, SFO₃, and SFO₁₀ for in vivo and in situ measurements and control vs. 11 d on the SFO diet (SFO₁₁) for in vitro gas production data]. Measurements of rumen fermentation parameters were analyzed by ANOVA for repeated measures using the MIXED procedure of SAS with a model that included the fixed effects of treatment, hours postfeeding, and their interaction. Differences between treatment means were evaluated using the pdiff option of the LS means statement in the MIXED procedure of SAS and declared significant at $P < 0.05$. Treatment effects at $P < 0.10$ were considered to reflect a trend toward significance.

RESULTS AND DISCUSSION

Intake and Rumen Fermentation Parameters

Ewes consumed all the feeds offered throughout the experiment, which was probably related to animals being fed at a restricted level rather than an indication of lipid supplements having no role in the regulation of intake. Previous studies have demonstrated that supplementing diets with FO often decreases DMI in sheep (Wachira et al., 2002; Fievez et al., 2003; Toral et al., 2010a).

Relative to the control, the SFO diet had no effect on mean rumen pH, ammonia, lactic acid, or VFA concentrations (Table 3). No significant changes in these parameters were observed when analyzed separately for each sampling time postfeeding (data not shown). Inclusion of SFO had no effect on molar VFA proportions (Table 3) other than a transient increase in molar butyrate proportions after 3 d, or on the molar acetate:propionate ratio in agreement with earlier observations (Chikunya et al., 2004). In contrast, several studies in cattle (Keady and Mayne, 1999; Shingfield et

Figure 1. Partial gas chromatogram indicating the separation of 20:1 isomers obtained using a temperature gradient for fatty acid methyl esters prepared from lipid in ruminal digesta of ewes fed a high concentrate diet supplemented with 20 g of sunflower oil plus 10 g of fish oil (SFO)/kg of DM, after 0 (dotted line) and 3 (solid line) d on diet. The y-axis represents arbitrary response units. Identification was verified based on argentation silver-ion thin-layer chromatography fractionation of fatty acid methyl esters and electron impact ionization spectra recorded during GC-MS analysis of methyl esters and corresponding 4,4-dimethyloxazoline derivatives. Peak identification: 1 = 20:0; 2 = Δ 7,9 17:2; 3 = *cis*-6, *cis*-9, *cis*-12 18:3; 4 = unresolved *trans*-6, -7, -8 20:1; 5 = unresolved *trans*-9, -10 20:1; 6 = *cis*-5 20:1; 7 = *trans*-11 20:1; 8 = *trans*-12 20:1; 9 = *trans*-13 20:1; 10 = *cis*-8 20:1; 11 = unresolved *trans*-14 20:1 and *cis*-9 20:1; 12 = *trans*-15 20:1; 13 = *cis*-11 20:1; 14 = unresolved *cis*-9, *cis*-12, *cis*-15 18:3 and *cis*-12 20:1; 15 = *cis*-13 20:1; 16 = *cis*-14 20:1; 17 = unresolved *trans*-7, *cis*-9 conjugated linoleic acid (CLA), *trans*-8, *cis*-10 CLA, and *cis*-9, *trans*-11 CLA.

al., 2003; Shingfield et al., 2010b) and sheep (Wachira et al., 2000; Fievez et al., 2003) have reported that FO alters rumen fermentation characteristics. The underlying reasons for the variable effects of FO or marine lipids on rumen VFA profiles both between and within ruminant species are not obvious but may be related to selective changes in rumen microbial populations (Boeckaert et al., 2008b; Belenguer et al., 2010).

Supplementation with SFO had no significant effect on in vitro ruminal fermentation characteristics, irrespective of the substrate incubated, with respective mean values for the reference alfalfa hay, control, and SFO diets of 206, 314, and 299 mL/g of OM for the asymptotic gas production; 0.08, 0.08, and 0.09 h^{-1} for the fractional gas production rate; and 371, 512, and 529 g/kg of DM for the effective degradability. In situ measurements indicated that the SFO diet was associated with a numerical increase in the ruminal DM, NDF, and CP degradation (Table 3), but the effects

on DM and NDF disappearance were not considered significant. Overall, current data suggest that inclusion of SO and FO in the diet has no negative effect on rumen function, consistent with earlier reports in cattle or sheep fed diets containing moderate amounts of FO or plant oils (Kucuk et al., 2004; Sinclair et al., 2005; Shingfield et al., 2010b).

FA Composition of Ruminal Digesta

Inclusion of FO and SO in the diet resulted in time-dependent alterations in the FA composition of ruminal digesta, changes that were characterized by decreases in 18:0 and 18:2 biohydrogenation intermediates and concomitant increases in the accumulation of 16:1, 10-O-18:0, 18:1, 20, and 22 carbon FA (Table 4).

Supplementation with SFO was associated with a time-dependent increase of 10-O-18:0 in ruminal digesta (Table 4). Early studies reported that FO promoted the formation of 10-OH-18:0 in the rumen of sheep (Kitessa et al., 2001), whereas more recent studies have indicated that 10-OH-18:0 formed during the dehydration of *cis*-9 18:1 and *trans*-11 18:1 in vitro is reduced to 10-O-18:0 (Jenkins et al., 2006; McKain et al., 2010). Ruminal digesta was also found to contain 13-O-18:0 and 15-O-18:0 (Table 4), suggesting that double bonds at other positions along the carbon chain may also be hydrated in the rumen (Hudson et al., 2000).

Relative concentrations of most odd- and branched-chain FA with 17 or fewer carbon atoms were decreased on the SFO diet (Table 4). Despite no change or substantial increase in the abundance of longer chain odd-numbered carbon FA, the total content of odd- and branched-chain FA was 35% lower in digesta in ewes fed the SFO diet compared with the control. Previous reports examining the effect of diets containing FO or marine algae and plant oils rich in 18:2 n-6 have reported similar changes in the concentration of these FA in ovine milk (Toral et al., 2010a,b), bovine milk (Shingfield et al., 2006; Cruz-Hernandez et al., 2007), and ruminal digesta of lactating cows (AbuGhazaleh et al., 2002). In contrast, supplementing diets with marine algae was reported to increase odd- and branched-chain FA concentrations in ruminal digesta and milk fat in cows (Boeckaert et al., 2008a,b).

Increases in the relative abundance in 16 carbon intermediates were isomer-dependent and attributable in the most part to the accumulation of *trans*-6, -7, -10, and -13 (Table 5). Few investigations have examined the appearance of 16 carbon FA in the rumen, but FO in the diet has been reported to enhance the flow of *trans* 16:1 isomers with double bonds from Δ 6–13 at the duodenum in growing cattle (Shingfield et al., 2010b). Increases in ruminal outflow of *trans* 16:1 has

Figure 2. Partial gas chromatogram indicating the separation of 22:1 isomers obtained using a temperature gradient for fatty acid methyl esters prepared from lipid in ruminal digesta of ewes fed a high concentrate diet supplemented with 20 g of sunflower oil plus 10 g of fish oil (SFO)/kg of DM, after 0 (dotted line) and 3 (solid line) d on diet. The y-axis represents arbitrary response units. Identification was verified based on argentation silver-ion thin-layer chromatography fractionation of fatty acid methyl esters and electron impact ionization spectra recorded during GC-MS analysis of methyl esters and corresponding 4,4-dimethyloxazoline derivatives. Peak identification: 1 = unresolved Δ 9,14,17 20:3 and 22:0; 2 = *cis*-8, *cis*-11, *cis*-14 20:3; 3 = unresolved *trans*-10, -11 22:1; 4 = *trans*-12 22:1; 5 = *trans*-13 22:1; 6 = unresolved *trans*-14 22:1 and *cis*-9 22:1; 7 = *trans*-15 22:1; 8 = unresolved *cis*-11 22:1 and *trans*-16 22:1; 9 = unresolved *trans*-17 22:1 and *cis*-12 22:1; 10 = *cis*-13 22:1; 11 = *cis*-14 22:1; 12 = *cis*-11, *cis*-14, *cis*-17 20:3; 13 = *cis*-15 22:1; 14 = unresolved *cis*-5, *cis*-8, *cis*-11, *cis*-14 20:4 and *cis*-16 22:1.

been attributed to incomplete biohydrogenation of 16:2 n-4, 16:3 n-4, 16:4 n-1, and 16:4 n-3 in the rumen (Shingfield et al., 2010b), but based on the transformations of *trans*-9 18:1 during incubations with rumen inocula (Proell et al., 2002) it is possible that one or more *trans* 16:1 isomers detected in rumen digesta in the current study originate from *trans*-6 16:1 contained in FO.

Supplementing the diet with FO and SO increased the accumulation of all *trans* 18:1 intermediates over time (Table 6). It is well established that FO (Kitessa et al., 2001; Wachira et al., 2002; Shingfield et al., 2003) and plant oils rich in 18:2 n-6 (Kucuk et al., 2004; Atkinson et al., 2006; Shingfield et al., 2008) in the diet increase the flow of *trans* 18:1 leaving the rumen in sheep and cattle, indicating that the collection of rumen digesta samples in this experiment represented a useful approach to examine the effect of SO and FO on biohydrogenation of unsaturated FA in the rumen. However, inferences drawn on the effect of oils on rumi-

Table 3. Mean rumen fermentation characteristics and disappearance of dry matter (DMD), crude protein (CPD), and NDF from alfalfa hay incubated for 24 h in the rumen of sheep fed a high concentrate diet supplemented with 20 g of sunflower oil plus 10 g of fish oil (SFO)/kg of DM, after 0 (control), 3 (SFO₃), and 10 (SFO₁₀) d on diet

Item	Treatment			SED ¹	P-value ²		
	Control	SFO ₃	SFO ₁₀		T	H	T × H
pH	6.26	6.22	6.30	0.071	0.501	<0.001	0.972
Ammonia (mg/L)	326	404	425	50.9	0.131	<0.001	0.999
Lactic acid (mmol/L)	0.66	0.76	0.81	0.091	0.256	<0.001	0.015
VFA (mmol/L)	175	189	167	14.4	0.294	<0.001	0.876
Molar proportion (mmol/mmol)							
Acetate	65.2	63.9	64.6	0.89	0.350	0.285	0.985
Propionate	16.5	16.4	17.0	0.73	0.641	<0.001	0.769
Butyrate	13.5 ^b	15.1 ^a	14.2 ^{ab}	0.59	0.038	<0.001	0.823
Others ³	4.78	4.59	4.17	0.320	0.160	<0.001	0.950
Molar ratio							
Acetate:propionate	4.09	3.99	4.01	0.228	0.894	<0.001	0.927
(Acetate + butyrate):propionate	4.97	4.93	4.91	0.284	0.980	<0.001	0.886
Disappearance (g/g incubated)							
DMD	0.579	0.616	0.610	0.0168	0.099	—	—
CPD	0.727 ^b	0.767 ^a	0.753 ^a	0.0108	0.009	—	—
NDFD	0.302	0.360	0.357	0.0278	0.108	—	—

^{a,b}Within a row, different superscripts indicate significant differences ($P < 0.05$).

¹SED = standard error of the difference.

²Probability of significant effects due to treatment (T), hours postfeeding (H), and their interaction (T × H).

³Calculated as the sum of isobutyrate, isovalerate, valerate, and caproate.

nal biohydrogenation are based on the measurement of relative proportions of FA in digesta, but it is possible that ruminal outflow of certain intermediates may not be altered on the SFO diet despite measured changes in their relative abundance.

Even though plant oils rich in 18:2 n-6 and FO cause *trans* 18:1 to accumulate in the rumen, the mode of action explaining these effects differs. Studies in vitro have shown that 20:5 n-3 and 22:6 n-3 in FO specifically inhibit the reduction of *trans* 18:1 to 18:0 (AbuGhazaleh and Jenkins, 2004; Wąsowska et al., 2006), whereas the higher ruminal outflow of *trans* 18:1 in ruminants fed sources of 18:2 n-6 is accompanied by an increase in ruminal outflow of 18:0 (Kucuk et al., 2004; Atkinson et al., 2006; Shingfield et al., 2008). Incubations with rumen inocula indicate that metabolism of both 18:2 n-6 and 22:6 n-3 causes *trans* 18:1 to accumulate in the absence of changes in 18:0 formation in vitro (AbuGhazaleh and Jenkins, 2004; Chow et al., 2004; Boeckaert et al., 2007). In this experiment, feeding SFO resulted in increased *trans* 18:1 concentrations and a corresponding decrease in 18:0 in digesta over time, suggesting that the long-chain FA in FO were responsible for the inhibition of complete hydrogenation of 18 carbon unsaturated FA in the rumen. Data from this and earlier studies (Wachira et al., 2000; Shingfield et al., 2006; Duckett and Gillis, 2010) highlight that inclusion of small amounts of FO in combination with moderate or relatively high quantities of plant oils represents an effective nutritional strategy for inhibiting the reduction of *trans* 18:1 to 18:0 in the rumen.

Isomers of *trans* 18:1 were found to accumulate over time on the SFO diet, but there was no evidence of a significant shift in ruminal biohydrogenation toward *trans*-10 18:1 at the expense of *trans*-11 18:1 (Table 6). Previous studies in cows have demonstrated that diets containing FO and SO result in the rapid enrichment of *trans*-11 18:1 in milk fat that declines over time, which is associated with concomitant increases in *trans*-10 18:1 concentrations (Shingfield et al., 2006; Cruz-Hernandez et al., 2007). Measurements of milk fat composition or FA flow at the duodenum also indicate that plant oils enhance ruminal formation of *trans*-10 18:1 in cows fed high concentrate diets (Griinari et al., 1998; Loores et al., 2004) in direct relation to time on diet (Roy et al., 2006). Indirect comparisons of milk FA composition between ruminants fed diets containing moderate amounts of 18:2 n-6 rich oils tend to suggest that the propensity for a shift in ruminal biohydrogenation pathways toward *trans*-10 18:1 is lower in sheep and goats compared with cows (Shingfield et al., 2010a). However, recent experiments demonstrated that concentrations of *trans*-10 18:1 were marginally higher than *trans*-11 18:1 in milk from ewes fed high concentrate diets supplemented with SO and marine lipids (Toral et al., 2010a,b). The reasons underlying differences in the accumulation of *trans* 18:1 metabolites in ruminal digesta in this experiment compared with changes in the *trans* 18:1 isomer profile in milk reported previously are difficult to reconcile. It is possible that changes in ruminal biohydrogenation to SO and FO occur over a longer period in dry compared with lactating ewes, although measurements of bovine

Table 4. Fatty acid composition of lipid in ruminal digesta of sheep fed a high concentrate diet supplemented with 20 g of sunflower oil plus 10 g of fish oil (SFO)/kg of DM, after 0 (control), 3 (SFO₃), and 10 (SFO₁₀) d on diet

Fatty acid (g/100 g of fatty acids)	Treatment			SED ¹	P-value
	Control	SFO ₃	SFO ₁₀		
12:0	<0.01	0.01	0.01	0.004	0.341
14:0	0.68	0.64	0.71	0.098	0.779
16:0	17.93 ^a	14.92 ^b	15.22 ^b	0.399	<0.001
18:0	43.66 ^a	29.05 ^b	10.39 ^c	4.691	<0.001
20:0	0.73 ^a	0.88 ^a	0.49 ^b	0.098	0.006
22:0 ²	0.48 ^b	0.77 ^a	0.59 ^b	0.060	0.001
26:0	0.27 ^a	0.23 ^b	0.23 ^b	0.018	0.043
28:0	0.16 ^a	0.12 ^b	0.12 ^b	0.009	0.001
30:0	0.34	0.40	0.44	0.066	0.370
Σ unsaturated 16	0.77 ^b	0.78 ^b	1.01 ^a	0.081	0.019
Σ 18:1 <i>cis</i>	6.12	7.94	9.71	1.370	0.066
Σ 18:1 <i>trans</i>	7.06 ^c	26.72 ^b	41.57 ^a	2.745	<0.001
Σ 18:1	13.18 ^c	34.66 ^b	51.28 ^a	3.291	<0.001
Σ 18:2 ³	11.48 ^a	5.98 ^b	6.04 ^b	1.500	0.004
Σ CLA ⁴	0.23	0.43	0.51	0.177	0.300
Σ unsaturated 20	1.20 ^b	2.15 ^a	2.20 ^a	0.077	<0.001
Σ unsaturated 22	0.25 ^b	2.20 ^a	2.26 ^a	0.120	<0.001
Σ unsaturated 24	0.10 ^c	0.17 ^b	0.21 ^a	0.010	<0.001
<i>cis</i> -17 26:1	<0.01 ^c	0.01 ^b	0.03 ^a	0.003	<0.001
11-cyclohexyl 11:0	0.06 ^a	0.03 ^b	0.04 ^{ab}	0.009	0.020
13:0	0.07 ^a	0.04 ^b	0.03 ^b	0.011	0.011
13:0 <i>iso</i>	0.09 ^a	0.03 ^b	0.03 ^b	0.013	0.001
13:0 <i>anteiso</i>	0.01	0.01	0.01	0.005	0.552
<i>cis</i> -12 13:1	0.33 ^a	0.14 ^b	0.12 ^b	0.051	0.002
14:0 <i>iso</i>	0.16 ^a	0.05 ^b	0.04 ^b	0.027	0.001
15:0	0.87 ^a	0.59 ^b	0.59 ^b	0.033	<0.001
15:0 <i>iso</i>	0.47 ^a	0.17 ^b	0.21 ^b	0.050	<0.001
15:0 <i>anteiso</i>	0.84 ^a	0.27 ^b	0.26 ^b	0.053	<0.001
Σ unsaturated 15	0.49 ^a	0.18 ^b	0.16 ^b	0.032	<0.001
16:0 <i>iso</i>	0.41 ^a	0.14 ^b	0.13 ^b	0.085	0.010
17:0	0.58	0.53	0.50	0.032	0.069
17:0 <i>iso</i> ⁵	0.32	0.28	0.38	0.039	0.061
7-methyl-hexadec-7-enoate	0.01 ^c	0.12 ^b	0.14 ^a	0.006	<0.001
<i>trans</i> -10 17:1	0.00 ^c	0.02 ^b	0.03 ^a	0.001	<0.001
18:0 <i>iso</i> ⁶	0.08 ^a	0.03 ^b	0.03 ^b	0.013	0.004
3,7,11,15-tetramethyl 16:0	0.07 ^a	0.18 ^b	0.18 ^b	0.035	0.009
21:0	0.06 ^b	0.11 ^a	0.10 ^a	0.008	<0.001
<i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15, <i>cis</i> -18 21:5	0.00 ^b	0.03 ^a	0.03 ^a	0.006	<0.001
23:0	0.19 ^c	0.30 ^a	0.24 ^b	0.024	0.003
<i>cis</i> -14 23:1	0.01 ^b	0.03 ^{ab}	0.04 ^a	0.009	0.007
25:0	0.08	0.07	0.07	0.015	0.915
27:0	0.02	0.01	0.02	0.006	0.535
29:0	0.05	0.03	0.04	0.018	0.519
10-O-18:0	0.04 ^b	0.74 ^b	3.11 ^a	0.471	<0.001
13-O-18:0	0.05	0.08	0.06	0.019	0.277
15-O-18:0	0.01	0.01	0.02	0.005	0.272
Summary					
Σ saturates	68.97 ^a	51.10 ^b	34.40 ^c	4.553	<0.001
Σ monounsaturates	15.37 ^c	38.29 ^b	54.61 ^a	3.306	<0.001
Σ polyunsaturates	15.40 ^a	9.79 ^b	10.45 ^b	2.020	0.033
Σ n-6 polyunsaturates	11.29 ^b	5.64 ^a	5.41 ^a	1.479	0.003
Σ n-3 polyunsaturates	2.83	2.05	2.33	0.392	0.174
Σ OBC ⁷	5.31 ^a	3.43 ^b	3.45 ^b	0.178	<0.001

^{a-c}Within a row, different superscripts indicate significant differences ($P < 0.05$).

¹SED = standard error of the difference.

²Contains Δ9,14,17 20:3 as a minor component.

³Sum of 18:2 fatty acids excluding isomers of conjugated linoleic acid.

⁴CLA = conjugated linoleic acid.

⁵Coelutes with *trans*-9 16:1.

⁶Coelutes with *trans*-11 17:1.

⁷OBC = odd- and branched-chain fatty acids containing 11 to 29 carbon atoms.

Table 5. Concentration of 15 and 16 carbon unsaturated fatty acids of lipid in ruminal digesta of sheep fed a high concentrate diet supplemented with 20 g of sunflower oil plus 10 g of fish oil (SFO)/kg of DM, after 0 (control), 3 (SFO₃), and 10 (SFO₁₀) d on diet

Fatty acid (g/100 g of fatty acids)	Treatment			SED ¹	P-value
	Control	SFO ₃	SFO ₁₀		
<i>trans</i> -5 15:1	0.01 ^b	0.01 ^b	0.03 ^a	0.005	0.005
<i>trans</i> -11 15:1	0.44 ^a	0.15 ^b	0.11 ^b	0.039	<0.001
<i>trans</i> -13 15:1	0.03 ^a	0.02 ^b	0.02 ^b	0.005	0.050
<i>cis</i> -9 16:1 ²	0.51	0.43	0.56	0.071	0.242
<i>cis</i> -11 16:1	0.08 ^a	0.05 ^b	0.03 ^b	0.014	0.018
<i>cis</i> -13 16:1	0.00 ^b	0.03 ^a	0.04 ^a	0.006	<0.001
<i>trans</i> -5 16:1	0.00 ^b	0.02 ^a	0.01 ^a	0.006	0.022
<i>trans</i> -6, -7 16:1	0.01 ^c	0.06 ^b	0.10 ^a	0.012	<0.001
<i>trans</i> -8 16:1	0.00 ^c	0.02 ^b	0.03 ^a	0.003	<0.001
<i>trans</i> -10 16:1	0.00 ^c	0.03 ^b	0.05 ^a	0.007	<0.001
<i>trans</i> -11 16:1	0.08	0.07	0.10	0.018	0.298
<i>trans</i> -12 16:1	0.08 ^a	0.04 ^b	0.04 ^b	0.012	0.002
<i>trans</i> -13 16:1	<0.01 ^c	0.02 ^b	0.03 ^a	0.005	<0.001
<i>cis</i> -9, <i>cis</i> -12 16:2	<0.01 ^b	0.01 ^a	0.01 ^a	0.002	0.002

^{a-c}Within a row, different superscripts indicate significant differences ($P < 0.05$).

¹SED = standard error of the difference.

²Coelutes with 17:0 *anteiso*.

milk FA composition (Roy et al., 2006; Shingfield et al., 2006) suggest that alterations in ruminal biohydrogenation pathways would be expected within 10 d on diet. However, measurements in cows have provided evidence to indicate that increases in *trans*-10 18:1 concentrations in ruminal digesta and milk fat in response to marine algae as a source of 22:6 n-3 may continue after 14 d on diet (Boeckaert et al., 2008a,b).

Relative to the control, the SFO diet altered the composition of 18:2 isomers in ruminal digesta, resulting in time-dependent decreases in the relative abundance of 18:2 n-6 and 18:3 n-3 and the accumulation of 18:2 biohydrogenation intermediates including *trans*-9, *cis*-12 18:2, *trans*-11, *cis*-15 18:2, and *trans*-11, *trans*-15 18:2 (Table 7). Studies in cattle have indicated that the flow of *trans*-9, *cis*-12 18:2 at the omasum is enhanced on

Table 6. Concentration of 18:1 isomers of lipid in ruminal digesta of sheep fed a high concentrate diet with 20 g of sunflower oil plus 10 g of fish oil (SFO)/kg of DM, after 0 (control), 3 (SFO₃), and 10 (SFO₁₀) d on diet

Fatty acid (g/100 g of fatty acids)	Treatment			SED ¹	P-value
	Control	SFO ₃	SFO ₁₀		
<i>cis</i> -9 18:1	5.02	6.30	7.82	1.322	0.148
<i>cis</i> -11 18:1	0.56 ^b	0.64 ^b	1.01 ^a	0.099	0.002
<i>cis</i> -12 18:1	0.29	0.35	0.29	0.047	0.394
<i>cis</i> -13 18:1	0.04 ^b	0.15 ^a	0.13 ^a	0.028	0.005
<i>cis</i> -15 18:1 ²	0.14 ^b	0.33 ^a	0.31 ^a	0.033	<0.001
<i>cis</i> -16 18:1	0.07 ^b	0.18 ^a	0.15 ^a	0.031	0.013
<i>trans</i> -4 18:1	0.09 ^b	0.25 ^a	0.24 ^a	0.040	0.003
<i>trans</i> -5 18:1	0.05 ^b	0.21 ^a	0.24 ^a	0.034	<0.001
<i>trans</i> -6, -7, -8 18:1	0.32 ^b	1.74 ^a	1.72 ^a	0.166	<0.001
<i>trans</i> -9 18:1	0.19 ^c	1.20 ^b	1.57 ^a	0.166	<0.001
<i>trans</i> -10 18:1	0.46 ^c	1.54 ^b	2.11 ^a	0.251	<0.001
<i>trans</i> -11 18:1	4.07 ^c	14.36 ^b	28.45 ^a	2.339	<0.001
<i>trans</i> -12 18:1	0.46 ^b	1.88 ^a	2.20 ^a	0.211	<0.001
<i>trans</i> -13, -14 18:1	0.63 ^b	3.05 ^a	3.01 ^a	0.413	<0.001
<i>trans</i> -15 18:1 ³	0.38 ^b	1.35 ^a	1.22 ^a	0.175	0.003
<i>trans</i> -16 18:1 ⁴	0.41 ^b	1.14 ^a	0.81 ^{ab}	0.185	0.007

^{a-c}Within a row, different superscripts indicate significant differences ($P < 0.05$).

¹SED = standard error of the difference.

²Coelutes with 19:0.

³Contains *cis*-10 18:1 as a minor component.

⁴Contains *cis*-14 18:1 as a minor component.

Table 7. Concentration of 18 carbon polyunsaturated fatty acids in lipid of ruminal digesta of sheep fed a high concentrate diet supplemented with 20 g of sunflower oil plus 10 g of fish oil (SFO)/kg of DM, after 0 (control), 3 (SFO₃), and 10 (SFO₁₀) d on diet

Fatty acid (g/100 g of fatty acids)	Treatment			SED ¹	P-value
	Control	SFO ₃	SFO ₁₀		
<i>cis</i> -9, <i>cis</i> -12 18:2	11.25 ^a	5.47 ^b	5.17 ^b	1.465	0.002
<i>cis</i> -11, <i>cis</i> -14 18:2	0.03	0.02	0.01	0.006	0.092
<i>cis</i> -12, <i>cis</i> -15 18:2	0.01	0.01	0.01	0.001	0.321
<i>cis</i> -9, <i>trans</i> -12 18:2	0.03 ^b	0.03 ^{ab}	0.05 ^a	0.008	0.035
<i>cis</i> -11, <i>trans</i> -15 18:2 ²	0.01 ^b	0.02 ^b	0.04 ^a	0.005	<0.001
<i>trans</i> -9, <i>cis</i> -12 18:2	0.03 ^c	0.14 ^b	0.19 ^a	0.022	<0.001
<i>trans</i> -11, <i>cis</i> -15 18:2	0.10 ^b	0.17 ^b	0.34 ^a	0.037	<0.001
<i>trans</i> -12, <i>cis</i> -15 18:2	0.00 ^c	0.02 ^b	0.05 ^a	0.007	<0.001
<i>trans</i> -11, <i>trans</i> -14 18:2 ³	0.02 ^b	0.03 ^b	0.05 ^a	0.007	0.002
<i>trans</i> -11, <i>trans</i> -15 18:2	0.02 ^c	0.06 ^b	0.12 ^a	0.013	<0.001
<i>cis</i> -9, <i>trans</i> -11 CLA ⁴	0.09	0.33	0.43	0.167	0.170
<i>cis</i> -12, <i>trans</i> -14 CLA	0.001 ^a	<0.001 ^b	<0.001 ^b	0.0001	0.010
<i>trans</i> -8, <i>cis</i> -10 CLA	<0.01	<0.01	<0.01	0.001	0.744
<i>trans</i> -9, <i>cis</i> -11 CLA	<0.01	<0.01	<0.01	0.001	0.714
<i>trans</i> -10, <i>cis</i> -12 CLA	0.04 ^a	0.02 ^b	0.01 ^c	0.004	<0.001
<i>trans</i> -11, <i>cis</i> -13 CLA	0.02	0.02	0.01	0.006	0.213
<i>trans</i> -8, <i>trans</i> -10 CLA	<0.01	<0.01	<0.01	0.002	0.889
<i>trans</i> -9, <i>trans</i> -11 CLA	0.01	0.02	0.03	0.007	0.128
<i>trans</i> -10, <i>trans</i> -12 CLA	0.02	0.01	<0.01	0.003	0.083
<i>trans</i> -11, <i>trans</i> -13 CLA	0.02 ^a	0.01 ^{ab}	<0.01 ^b	0.004	0.016
<i>trans</i> -12, <i>trans</i> -14 CLA	0.008 ^a	0.007 ^a	0.005 ^b	0.001	0.015
<i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12 18:3	0.01	0.01	0.01	0.001	0.413
<i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15 18:3 ⁵	2.38 ^a	0.87 ^b	0.69 ^b	0.278	<0.001
<i>cis</i> -9, <i>trans</i> -11, <i>cis</i> -15 18:3 ⁶	0.02	0.04	0.05	0.018	0.340

^{a-c}Within a row, different superscripts indicate significant differences ($P < 0.05$).

¹SED = standard error of the difference.

²Contains *trans*-9, *trans*-12 18:2 as a minor component.

³Contains *trans*-10, *trans*-13 18:2 as a minor component.

⁴CLA = conjugated linoleic acid.

⁵Contains *cis*-12 20:1 as a minor component.

⁶Double bond geometry confirmed based on published reports (Gómez-Cortés et al., 2009).

diets containing SO (Shingfield et al., 2008), whereas FO causes an increase in ruminal outflow of *trans*-11, *cis*-15 18:2 formed during 18:3 n-3 metabolism (Shingfield et al., 2003; Lee et al., 2008; Shingfield et al., 2010b). Observed reductions in the concentrations of 18:2 n-6 and 18:3 n-3 in digesta are most probably explained by increases in the percentages of other FA rather than reflecting an absolute decrease in the abundance in the rumen. Despite extensive biohydrogenation in the rumen, the flow of 18:2 n-6 and 18:3 n-3 at the duodenum can be expected to be higher in direct relation to the increases in dietary supply (Kucuk et al., 2004; Looor et al., 2004; Atkinson et al., 2006).

Inclusion of FO and SO in the diet had no effect on *cis*-9, *trans*-11 CLA or total CLA concentrations in ruminal digesta (Table 7), consistent with reports of FA flow at the duodenum in sheep fed high concentrate diets containing 18:2 n-6 rich plant oils or FO (Kucuk et al., 2004; Sinclair et al., 2005; Atkinson et al., 2006). Furthermore, concentrations of *cis*-9, *trans*-11 CLA are increased in milk from sheep fed the same amount of

SO and FO as evaluated in this experiment (Toral et al., 2010a). Current data indicate that the enrichment of *cis*-9, *trans*-11 CLA in ovine milk from ewes fed FO and SO can, for the most part, be explained by higher endogenous synthesis in the mammary gland using *trans*-11 18:1 as a substrate (Palmquist et al., 2005) rather than increased outflow of *cis*-9, *trans*-11 CLA from the rumen.

Relatively low concentrations of *trans*-10, *cis*-12 CLA in the rumen digesta on the control diet were found to decrease in response to the SFO treatment, whereas abundance of *trans*-9, *cis*-11 CLA was not affected. Ruminal digesta was devoid of *cis*-10, *trans*-12 CLA. Studies in cattle have shown that typically FO has no effect on the amount of *trans*-10, *cis*-12 CLA leaving the rumen (Shingfield et al., 2003; Lee et al., 2008; Shingfield et al., 2010b), but plant oils rich in 18:2 n-6 often enhance ruminal *trans*-10, *cis*-12 CLA outflow in sheep and cattle (Kucuk et al., 2004; Atkinson et al., 2006; Shingfield et al., 2008). Previous studies reported that the progressive decline in the milk fat content in

Table 8. Concentration of 20 carbon unsaturated fatty acids of lipid in ruminal digesta of sheep fed a high concentrate diet supplemented with 20 g of sunflower oil plus 10 g of fish oil (SFO)/kg of DM, after 0 (control), 3 (SFO₃), and 10 (SFO₁₀) d on diet

Fatty acid (g/100 g of fatty acids)	Treatment			SED ¹	P-value
	Control	SFO ₃	SFO ₁₀		
<i>cis</i> -5 20:1	0.02	0.03	0.02	0.009	0.411
<i>cis</i> -8 20:1	0.04	0.03	0.04	0.016	0.786
<i>cis</i> -11 20:1	0.11 ^c	0.24 ^b	0.49 ^a	0.061	<0.001
<i>cis</i> -13 20:1	0.02 ^c	0.04 ^b	0.05 ^a	0.003	<0.001
<i>cis</i> -14 20:1	<0.01 ^b	0.02 ^a	0.01 ^b	0.003	0.005
<i>trans</i> -6, -7, -8 20:1	0.03 ^a	0.02 ^b	0.02 ^b	0.003	0.007
<i>trans</i> -9, -10 20:1	0.00 ^c	0.04 ^a	0.03 ^b	0.004	<0.001
<i>trans</i> -11 20:1	<0.01 ^b	0.07 ^a	0.06 ^a	0.007	<0.001
<i>trans</i> -12 20:1	<0.01 ^b	0.07 ^a	0.06 ^a	0.009	<0.001
<i>trans</i> -13 20:1	<0.01 ^c	0.09 ^a	0.06 ^b	0.009	<0.001
<i>trans</i> -14 20:1 ²	0.03 ^c	0.12 ^a	0.09 ^b	0.010	<0.001
<i>trans</i> -15 20:1	<0.01 ^b	0.22 ^a	0.06 ^b	0.048	0.002
<i>cis</i> -11, <i>cis</i> -14 20:2	<0.01 ^c	0.04 ^b	0.06 ^a	0.007	<0.001
<i>cis</i> -10, <i>trans</i> -15 20:2 ³	0.00 ^b	0.03 ^a	0.03 ^a	0.005	<0.001
<i>trans</i> -11, <i>cis</i> -17 20:2 ³	<0.01 ^b	0.03 ^a	0.02 ^a	0.005	<0.001
<i>trans</i> -13, <i>cis</i> -17 20:2 ³	<0.01 ^c	0.03 ^a	0.02 ^b	0.006	<0.001
<i>trans</i> -14, <i>cis</i> -17 20:2 ³	<0.01 ^b	0.02 ^a	<0.01 ^b	0.003	0.020
<i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14 20:3	<0.01 ^c	0.01 ^b	0.03 ^a	0.003	<0.001
<i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17 20:3	<0.01 ^b	0.02 ^a	0.03 ^a	0.006	0.009
<i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14 20:4 ⁴	<0.01 ^b	0.06 ^a	0.07 ^a	0.010	<0.001
<i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17 20:4	0.38 ^a	0.33 ^b	0.27 ^c	0.020	<0.001
<i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17 20:5 + 24:0	0.48 ^b	0.57 ^a	0.64 ^a	0.036	0.003

^{a-c}Within a row, different superscripts indicate significant differences ($P < 0.05$).

¹SED = standard error of the difference.

²Coelutes with *cis*-9 20:1.

³Double bond geometry assigned based on retention times compared with *cis*-8, *cis*-11 20:2; *cis*-11, *cis*-14 20:2; and *cis*-14, *cis*-17 20:2 and the elution order of a mixture of 18:2 methyl esters of known composition.

⁴Contains *cis*-16 22:1 as a minor component.

lactating ewes fed high concentrate diets containing FO and SO (Toral et al., 2010a) were not accompanied by an increase in milk *trans*-10, *cis*-12 CLA content or substantial change in *trans*-9, *cis*-11 CLA concentrations. Overall available data are consistent with the view that other biohydrogenation intermediates, or mechanisms, or both are involved in milk fat depression on diets containing FO (Chilliard et al., 2007; Shingfield and Griinari, 2007; Shingfield et al., 2010a).

Inclusion of FO and SO in the diet resulted in time-dependent increases in concentration of 20 carbon FA in digesta (Table 8). Detailed analysis confirmed the occurrence of *cis*-14 20:1 and several *trans* 20:1 and 20:2 isomers not contained in FO, indicating that these FA must be formed during ruminal biohydrogenation of FO and SO. No 20 carbon FA containing a conjugated double bond or other unique 20:4 or 20:5 products were identified in digesta. Owing to the complex composition of FO, it is not possible to establish the origins of these biohydrogenation intermediates. Extensive investigations of the fate of 18 carbon unsaturated FA in the rumen have not yielded any substantive evidence that the carbon chain of unsaturated FA is elongated or shortened during biohydrogenation in the rumen (Jen-

kins et al., 2008). Relative abundance of 20 carbon FA in FO (Table 2) would tend to suggest that most of the 20 carbon intermediates in ruminal digesta may arise from the biohydrogenation of 20:5 n-3 in the rumen.

Supplementing the diet with SO and FO also caused an increase in the accumulation of *cis* and *trans* 22:1 isomers and several 22:3 and 22:4 products in digesta (Table 9). Because Δ 10,13,17 22:3, Δ 10,14,19 22:3, Δ 12,16,19 22:3, Δ 8,13,16,19 22:4, and Δ 7,13,16,19 22:4 were not supplied from the diet or formed in the rumen of sheep fed the control diet, their appearance in ruminal digesta on the SFO diet confirms that these FA represent products formed during the biohydrogenation of 22 carbon unsaturated FA. However, no 22:5 or 22:6 intermediates or 22 carbon FA containing a conjugated bond were detected.

Even though intermediates produced during the initial stages of biohydrogenation are known to be transient and rapidly hydrogenated (Palmquist et al., 2005; Jenkins et al., 2008), several geometric and positional isomers of CLA and *cis*-9, *trans*-11, *cis*-15 18:3 were identified in ruminal digesta (Table 7). An absence of conjugated 20 and 22 carbon FA could be considered as evidence that the biohydrogenation of long-chain FA

Table 9. Concentration of 22 and 24 carbon unsaturated fatty acids of lipid in ruminal digesta of sheep fed a high concentrate diet supplemented with 20 g of sunflower oil plus 10 g of fish oil (SFO)/kg of DM, after 0 (control), 3 (SFO₃), and 10 (SFO₁₀) d on diet

Fatty acid (g/100 g of fatty acids)	Treatment			SED ¹	P-value
	Control	SFO ₃	SFO ₁₀		
<i>cis</i> -11 22:1 ²	<0.01 ^b	0.28 ^a	0.29 ^a	0.005	<0.001
<i>cis</i> -13 22:1	0.02 ^c	0.05 ^b	0.10 ^a	0.013	<0.001
<i>cis</i> -14 22:1	0.00 ^b	0.09 ^a	<0.01 ^b	0.020	<0.001
<i>cis</i> -15 22:1	<0.01 ^b	0.03 ^a	0.03 ^a	0.003	<0.001
<i>trans</i> -10, -11 22:1	0.02 ^c	0.07 ^a	0.04 ^b	0.009	<0.001
<i>trans</i> -12 22:1	<0.01 ^b	0.05 ^a	0.01 ^b	0.008	<0.001
<i>trans</i> -13 22:1	0.00 ^b	0.09 ^a	0.02 ^b	0.013	<0.001
<i>trans</i> -14 22:1 ³	<0.01 ^b	0.10 ^a	0.01 ^b	0.016	<0.001
<i>trans</i> -15 22:1	<0.01 ^b	0.12 ^a	0.02 ^b	0.018	<0.001
<i>trans</i> -17 22:1 ⁴	<0.01 ^b	0.25 ^a	0.03 ^b	0.047	<0.001
<i>cis</i> -13, <i>cis</i> -16 22:2	0.03	0.04	0.04	0.006	0.184
Δ10,13,17 22:3 ⁵	0.00 ^c	0.04 ^b	0.07 ^a	0.011	<0.001
Δ10,14,19 22:3 ⁵	<0.01 ^b	0.06 ^a	0.05 ^a	0.007	<0.001
Δ12,16,19 22:3 ⁶	<0.01 ^b	0.04 ^a	0.06 ^a	0.011	<0.001
<i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16 22:3	0.00 ^b	<0.01 ^b	0.02 ^a	0.004	0.003
<i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19 22:3	<0.01 ^c	0.02 ^b	0.04 ^a	0.006	0.001
Δ7,13,16,19 22:4 ⁷	<0.01 ^b	0.02 ^a	0.02 ^a	0.002	<0.001
Δ8,13,16,19 22:4 ⁷	0.00 ^b	0.02 ^b	0.04 ^a	0.010	0.008
<i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19 22:4	0.03 ^b	0.13 ^{ab}	0.22 ^a	0.050	0.009
<i>cis</i> -4, <i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16 22:5	0.00 ^c	0.04 ^b	0.08 ^a	0.014	<0.001
<i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19 22:5	<0.01 ^c	0.09 ^b	0.18 ^a	0.018	<0.001
<i>cis</i> -4, <i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19 22:6	<0.01 ^b	0.55 ^a	0.88 ^a	0.158	<0.001
<i>cis</i> -11 24:1	0.02 ^a	<0.01 ^b	0.01 ^b	0.003	0.002
<i>cis</i> -15 24:1	0.07 ^c	0.15 ^b	0.18 ^a	0.010	<0.001
<i>cis</i> -16 24:1	<0.01	0.01	0.02	0.004	0.077

^{a-c}Within a row, different superscripts indicate significant differences ($P < 0.05$).

¹SED = standard error of the difference.

²Contains *trans*-16 22:1 as a minor component.

³Coelutes with *cis*-9 22:1.

⁴Contains *cis*-12 22:1 as a minor component.

⁵Isomers eluted before *cis*-10, *cis*-13, *cis*-16 22:3 during GC analysis.

⁶Eluted after *cis*-10, *cis*-13, *cis*-16 22:3 but before *cis*-13, *cis*-16, *cis*-19 22:3 during GC analysis.

⁷Isomers eluted before *cis*-10, *cis*-13, *cis*-16, *cis*-19 22:4 during GC analysis.

in the rumen does not proceed via the formation of intermediates containing a conjugated bond system.

In conclusion, supplementing a high concentrate diet with FO and SO had no effect on rumen fermentation characteristics in sheep but altered the FA composition of lipid in ruminal digesta consistent with a time-dependent inhibition of the complete biohydrogenation of 16, 18, 20, and 22 carbon unsaturated FA. Furthermore, temporal changes in the accumulation of *trans* 18:1 intermediates offered no support that FO and SO in the diet induced a shift in ruminal biohydrogenation pathways toward the formation of *trans*-10 18:1. Several unique 20:1, 20:2, 22:1, 22:3, and 22:4 intermediates were detected in digesta, indicating that the hydrogenation of 20:5 n-3, 22:5 n-3, and 22:6 n-3 in the rumen involves a complex series of reactions.

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