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Fatty acid composition and bacterial community changes in the rumen fluid of lactating sheep fed sunflower oil plus incremental levels of marine algae

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ABSTRACT

Supplementation of ruminant diets with plant oils and marine lipids is an effective strategy for lowering saturated fatty acid (FA) content and increasing the concentration of *cis*-9,*trans*-11 conjugated linoleic acid and long-chain n-3 FA in ruminant milk. However, changes in populations of ruminal microorganisms associated with altered biohydrogenation of dietary unsaturated FA are not well characterized. Twenty-five lactating Assaf ewes were allocated at random to 1 of 5 treatments composed of dehydrated alfalfa hay and concentrates containing no additional lipid (control), or supplemented with 25 g of sunflower oil and 0 (SO), 8 ($SOMA_1$), 16 ($SOMA_2$), or 24 ($SOMA_3$) g of marine algae/kg of diet dry matter. On d 28 on diet, samples of rumen fluid were collected for lipid analysis and microbial DNA extraction. Appearance and identification of biohydrogenation intermediates was determined based on complementary gas chromatography and Ag+-HPLC analysis of FA methyl esters. Total bacteria and the *Butyrivibrio* group were studied in microbial DNA by terminal RFLP analysis, and real-time PCR was used to quantify the known *Butyrivibrio* bacteria that produce *trans*-11 18:1 or 18:0. Dietary supplements of sunflower oil alone or in combination with marine algae altered the FA profile of rumen fluid, which was associated with changes in populations of specific bacteria. Inclusion of marine algae in diets containing sunflower oil resulted in the accumulation of *trans* 18:1 and 10-O-18:0 and a marked decrease in 18:0 concentrations in rumen fluid. At the highest levels of supplementation ($SOMA_2$ and $SOMA_3$), marine algae also promoted a shift in ruminal biohydrogenation pathways toward the formation of *trans*-10 18:1 at the expense of *trans*-11 18:1. Changes in the concentration of biohydrogenation intermediates were not accompanied by significant variations in the abundance of known cultivated ruminal bacteria capable of hydrogenating unsaturated FA. However, certain bacterial groups detected by terminal RFLP (such as potentially uncultured *Lachnospiraceae* strains or *Quinella*-related bacteria) exhibited variations in their relative frequency consistent with a potential role in one or more metabolic pathways of biohydrogenation in the rumen.

Key words: lactating ewe, marine algae, ruminal bacteria, biohydrogenation intermediate

INTRODUCTION

The inclusion of plant oils rich in 18:2n-6 and fish oil or marine algae (**MA**) in the diet is an effective nutritional strategy to increase concentrations of *cis*-9,*trans*-11 conjugated linoleic acid (**CLA**) and 22:6n-3 in bovine (AbuGhazaleh et al., 2002; Shingfield et al., 2006; Invernizzi et al., 2010) and ovine milk (Reynolds et al., 2006; Toral et al., 2010a,b). However, the increases in milk fat CLA content in response to a combination of plant oils and marine lipids have often been accompanied by alterations in ruminal biohydrogenation (**BH**) pathways, leading to a shift toward the formation of *trans*-10 18:1 at the expense of *trans*-11 18:1 and a decrease in milk fat synthesis in cattle (Shingfield et al., 2006; Invernizzi et al., 2010) and sheep (Toral et al., 2010a,b). Nevertheless, relatively few studies have characterized the effect of plant oils and marine lipids in the diet on the abundance of specific FA in ruminal digesta (AbuGhazaleh et al., 2002; Toral et al., 2010c).

Traditional culture-based methods for studying the effects of diet composition on ruminal bacteria and protozoa are less sensitive and accurate compared with molecular microbial methods based on 16S/18S rRNA genes. Use of culture-independent techniques have shown that alterations in the formation of specific BH intermediates to fish oil (Kim et al., 2008; Huws et al., 2010; Liu et al., in press) or marine algae (Boeckaert et al., 2007, 2008) in the diet may involve changes in specific populations of ruminal bacteria and protozoa in cattle. However, investigations of the effect of lipid supplements on the abundance of microorganisms involved in ruminal BH in sheep are limited (Boeckaert et al., 2009; Belenguer et al., 2010).

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Previous investigations indicated that changes in the FA composition of rumen fluid in nonlactating sheep fed diets containing fish oil and sunflower oil (**SO**, *Helianthus annus*; Toral et al., 2010c) did not provide a complete explanation for the effects of these lipid supplements on milk production and milk fat composition in lactating sheep (Toral et al., 2010a). Analysis of ruminal microbial communities using terminal RFLP (**T-RFLP**) also revealed that fish oil and SO induced comparable changes in certain groups of bacteria belonging to the family *Lachnospiraceae* or to the clostridial cluster IX in lactating and nonlactating sheep, whereas the effect on other groups possibly involved in ruminal BH differed (Belenguer et al., 2010).

The objective of this study was therefore to examine and characterize the changes in the bacterial ecology and appearance of biohydrogenation intermediates in the rumen of sheep fed diets that alter milk FA composition and inhibit mammary lipogenesis.

MATERIALS AND METHODS

Animals, Diets, and Experimental Design

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. Twenty-five lactating Assaf ewes of mean parity 3.7 ± 0.10, BW 85 ± 1.7 kg, and 97 ± 1.0 DIM producing 2.27 ± 0.101 kg of milk/d were used. Ewes were allocated at random to 5 experimental treatments (5 animals/treatment): containing no additional lipid supplements (control diet) or supplemented with 25 g of SO and 0, 8, 16, or 24 g of MA/kg of diet DM (SO, **SOMA$_1$**, **SOMA$_2$**, and **SOMA$_3$** diets, respectively). Experimental diets were composed of dehydrated alfalfa hay (*Medicago sativa*) and concentrates (forage:concentrate ratio 485:515) and fed as TMR to minimizing the sorting of dietary components. Sunflower oil (Carrefour, S.A., Madrid, Spain) and MA (DHA Gold Animal Feed Ingredient, Martek Biosciences Corp., Columbia, MD) replaced other dietary ingredients on a proportional basis. Dietary ingredients and chemical composition of experimental diets are shown in Table 1.

The experiment lasted 4 wk and rations were prepared weekly and offered ad libitum twice daily at 0900 and 1900 h. Ewes were housed in tie stalls and had continuous access to fresh water. Effects of treatments on intake, milk production, and milk FA composition have been reported elsewhere (Toral et al., 2010b).

Table 1. Ingredients and chemical composition (g/kg of DM) of the experimental diets[1]

Item	Control	SO	SOMA$_1$	SOMA$_2$	SOMA$_3$
Ingredients (g/kg of fresh matter)					
Dehydrated alfalfa hay	484	474	470	466	462
Whole corn grain	136	133	131	130	129
Whole barley grain	175	170	169	168	167
Soybean meal	97	95	94	93	92
Beet pulp	49	47	47	47	46
Molasses	37	36	36	36	36
Feed supplement[2]	22	21	21	21	21
Sunflower oil[3]	0	24	24	24	24
Marine algae[4]	0	0	8	15	23
Chemical composition (g/kg of DM)					
OM	896	900	897	893	899
CP	161	159	158	159	158
NDF	308	304	296	300	293
ADF	198	195	190	191	187
Ether extract	26	50	54	57	63

[1]Refers to TMR based on dehydrated alfalfa hay and concentrates containing no additional lipid (control), or supplemented with 25 g of sunflower oil and 0 (SO), 8 (SOMA$_1$), 16 (SOMA$_2$), or 24 (SOMA$_3$) g of marine algae/kg of diet DM.

[2]Contained (g/kg) $NaHCO_3$ (333), $CaCO_3$ (311), Ca_2HPO_4 (133), mine salt (111), and mineral and vitamins (111; INA OV1, Evialis, Madrid, Spain).

[3]Contained (g/kg) 16:0 (52.7), 18:0 (42.1), *cis*-9 18:1 (347), *cis*-11 18:1 (7.7), 18:2n-6 (479), 20:0 (2.7), *cis*-11 20:1 (1.6), 22:0 (7.1), 24:0 (2.2), other (11), and total fatty acids (953).

[4]DHA Gold Animal Feed Ingredient (Martek Biosciences Corp., Columbia, MD), contained (g/kg of DM) OM (913), CP (103), and ether extract (403). Fatty acid composition (g/kg of lipid): 12:0 (2.9), 14:0 (99.3), 14:2n-3 (1.5) 15:0 (4.2), 16:0 (245), *cis*-9 16:1 (1.6), 16:2n-3 (1.2), 16:3n-3 (1.5), 18:0 (5.8), *cis*-11 18:1 (1.2), 18:3n-6 (2.1), 18:4n-3 (3.0), 20:0 (1.5), 20:3n-6 (3.7), 20:4n-3 (8.1), 20:4n-5 (1.6), 20:4n-6 (4.7), 20:4n-7 (12.5), 20:5n-3 (14.0), 22:4n-9 (2.8), 22:5n-3 (3.6), 22:5n-6 (147), 22:6n-3 (369), 26:0 (3.9), other (18.3), unidentified (5.6), and total fatty acids (955).

Rumen Sample Collection

On d 29 of the experiment, ewes were given free access to morning rations for 3 h. Thereafter, feeds were removed and 3 h later samples of rumen fluid were collected from each ewe using a stomach tube. Immediately after collection, subsamples (approximately 50 mL) of rumen fluid were frozen at –80°C, freeze-dried, and stored at –80°C until analysis.

FA Analysis

Fatty acid methyl esters (**FAME**) of lipid in SO and MA were prepared in a 1-step extraction-transesterification procedure (Shingfield et al., 2003). Lipid in 200 mg of freeze-dried rumen fluid was extracted and converted to FAME by base-acid catalyzed transesterification (Toral et al., 2010c). Methyl esters were quantified by GC using a temperature gradient program (Shingfield et al., 2003) and isomers of 18:1 and 18:2 were further resolved in a separate analysis under isothermal conditions (Shingfield et al., 2003). Peaks were routinely identified using a mixture of authentic standards and retention time comparisons with samples of rumen digesta collected from sheep fed fish oil and SO (Toral et al., 2010c) and omasal digesta from cows fed fish oil (Kairenius et al., 2011), for which the FA composition was determined based on GC analysis of FAME and GC-MS analysis of corresponding 4,4-dimethyloxazoline derivatives. The distribution of CLA isomers in rumen fluid was determined by Ag+-HPLC (Shingfield et al., 2003).

Microbial Community Analysis

After thorough mixing, DNA was extracted from samples of freeze-dried rumen fluid (Belenguer et al., 2010). Duplicates were combined and used as templates for quantitative real-time PCR (**qPCR**) amplification and T-RFLP analysis. Sample DNA concentration was determined by spectrophotometry (NanoDrop ND-1000 Spectrophotometer, Nanodrop Technologies, Wilmington, DE).

The qPCR analysis was carried out using the Applied Biosystems StepOne Plus Real Time PCR system (Applied Biosystems, Foster City, CA) to investigate the abundance of *Butyrivibrio* capable of producing *trans*-11 18:1 and 18:0. The 16S rRNA gene-targeted primer sets included those for total bacteria (Maeda et al., 2003) and *Butyrivibrio* spp. that produce *trans*-11 18:1 (R. J. Wallace, Rowett Institute of Nutrition and Health, Aberdeen, UK, personal communication) and 18:0 (molecular beacon approach; Paillard et al., 2007). All procedures were performed as outlined previously (Belenguer et al., 2010).

Two different T-RFLP analyses were performed. The first used a universal bacteria-specific primer pair set (Hongoh et al., 2003) and 3 restriction enzymes (*Hha*I, *Msp*I, and *Hae*III) for total bacteria analysis. The second analysis was based on *Butyrivibrio* group-specific primers (Boeckaert et al., 2008) and 1 restriction enzyme (*Hha*I; Belenguer et al., 2010). The lengths of the fluorescently labeled terminal restriction fragments (**T-RF**) were determined by using the size standard ET-550-R (GE Healthcare Life Sciences, Buckinghamshire, UK) using the GeneMarker Analysis software (SoftGenetics, State College, PA).

Sample data (size, bp, and peak area for each T-RF) were analyzed as outlined by Castillo et al. (2007) and used to determine the number of T-RF (richness) and the Shannon-Wiener and Shannon evenness indices (Hill et al., 2003). In silico restriction for the major rumen bacteria with the primers and enzymes used in the analysis, obtained from the Ribosomal Database Project II Web site (http://rdp.cme.msu.edu/index.jsp; Cole et al., 2009), was used to infer the potential bacterial composition of rumen fluid.

Statistical Analysis

Data from FA composition of rumen fluid and qPCR and the relative frequencies of the T-RF were analyzed by 1-way ANOVA, using the MIXED procedure of the SAS software package, version 9.1 (SAS Inst. Inc., Cary, NC). Because some qPCR results did not satisfy the assumptions of data normality, data were transformed to $\log_{10}$ before ANOVA. The statistical model included the fixed effect of treatment and random effect of animal. Means were separated using the "pdiff" option of the "lsmeans" statement of the MIXED procedure. In addition, linear and quadratic components of the response to incremental amounts of MA in the diet were evaluated using orthogonal polynomial contrasts. Least squares means are reported and treatment effects were declared significant at $P < 0.05$ and considered a trend toward significance at $P < 0.10$.

The matrix with the T-RFLP data obtained by the 3 single-enzyme digestions for the total bacteria and by the *Hha*I digestion for the *Butyrivibrio* group was analyzed using hierarchical clustering with the Ward's method based on Jaccard distances (1 – Jaccard coefficient) with the Community Analysis Package 4 software (Pisces Conservation Ltd., Lymington, UK).

RESULTS

FA Composition of Rumen Fluid

Dietary supplements of SO did not affect the proportion of total saturated FA in rumen fluid, whereas the

inclusion of MA resulted in a substantial decrease (63.7 vs. 32.7% of total FA for Control and SOMA, respectively ($P < 0.001$); data not reported). Compared with the control, the concentration of 18:0 was marginally increased (+16%) on the SO treatment but was, on average, 88% lower in ewes fed diets containing MA ($P < 0.001$; Table 2). Dietary SO alone or in combination with MA lowered the proportions of 16:0 and 2 minor saturated FA, 20:0 and 28:0, in the rumen ($P < 0.05$) and increased that of 22:0 ($P < 0.05$). Inclusion of MA in the diet also linearly increased 14:0 concentrations ($P < 0.001$).

Concentrations of oxygenated 18-carbon FA (9-, 10-, 13-, and 15-O-18:0) were low in the digesta of ewes fed control and SO treatments, whereas the inclusion of MA resulted in a quadratic increase in ruminal 10-O-18:0 concentrations ($P < 0.001$; Table 2). In contrast, the abundance of total odd- and branched-chain FA in the rumen of ewes fed diets containing lipid supplements was decreased (–34%, $P < 0.001$; data not presented), because of lower concentrations of most 15- to 19-carbon odd- and branched-chain FA ($P < 0.05$).

Experimental treatments resulted in isomer-dependent changes in the relative abundance of 16-carbon FA in rumen fluid (Table 3). All lipid supplements decreased ruminal *cis*-9 16:1 concentrations, whereas MA quadratically increased *trans* 16:1 concentrations ($P < 0.001$).

For all treatments, *trans*-11 was the most abundant 18:1 isomer (Table 3). With the exception of *trans*-10 and *trans*-11 18:1, concentrations of *trans*-4 to *trans*-15 18:1 were greater for $SOMA_1$ compared with other treatments ($P < 0.001$). In contrast, ruminal *trans*-10 18:1 concentrations were greater for $SOMA_2$ compared with the control, SO, or $SOMA_1$ treatments, whereas the abundance for $SOMA_3$ was intermediate relative to $SOMA_1$ and $SOMA_2$ ($P < 0.01$). Supplementing the diet with SO increased ruminal *cis*-12 18:1 concentrations ($P < 0.001$), whereas MA had divergent effects on *cis* 18:1 isomer concentrations (Table 3).

Sunflower oil in the diet had no effect on 18:2 isomers in rumen fluid, other than a decrease in *cis*-9,*cis*-12 18:2 and an increase in *cis*-11,*cis*-14 18:2 concentrations ($P < 0.001$; Table 4). However, inclusion of SOMA in the diet increased ($P < 0.001$) the relative abundance of most 18:2 nonconjugated isomers in rumen fluid (Table 4). Concentrations of the major CLA isomer in rumen fluid, *cis*-9,*trans*-11, were increased on $SOMA_1$ ($P < 0.01$), whereas treatments had relatively minor effects on the abundance of other CLA isomers.

As expected, SO in the diet had no substantial effect on the abundance of 20- and 22-carbon unsaturated FA in rumen fluid (Table 5). Because MA contained small amounts of 20-carbon FA, the changes in their relative proportion in the rumen fluid to SOMA treatments were marginal compared with the control and SO diets. Furthermore, little variation in the low concentration of 22:1 intermediates was observed in response to MA in the diet. However, incremental amounts of MA resulted in dose-dependent increases in most 22-carbon PUFA, changes that were highly significant due to the low abundance of these FA in rumen fluid of ewes fed the control and SO treatments. Ruminal concentrations of 22:5n-6 and 22:6n-3 increased linearly ($P < 0.001$) in response to graded amounts of MA in the diet, changes that were also accompanied by the appearance of unique 22-carbon intermediates in rumen fluid including Δ10,13,17 22:3, *cis*-7,*trans*-13,*cis*-16,*cis*-19 22:4, and *trans*-5,*cis*-10,*cis*-13,*cis*-16,*cis*-19 22:5. No 22-carbon FA containing a conjugated double bond or unique 22:6 isomers were detected in rumen fluid of sheep fed SO and MA.

Bacterial Community Analysis by T-RFLP

Rumen bacterial T-RFLP analysis generated on average 32.9 ± 1.10, 54.7 ± 1.57, and 67.6 ± 4.58 fragments with the enzymes *Hha*I, *Msp*I, and *Hae*III, respectively, with substantial variation between animals. Hierarchical clustering analysis grouped the bacterial community structures according to dietary treatments (Figure 1a), resulting in 2 major clusters, with most samples obtained from animals fed the control or SO diet being grouped together and separate from those collected from ewes fed diets containing MA. Samples of the SO treatment formed a subgroup within the first cluster, and most samples from ewes on $SOMA_1$ grouped together and segregated from samples of $SOMA_2$ and $SOMA_3$ (Figure 1a).

The diversity indices were similar for all treatments after *Hha*I and *Hae*III digestions (Table 6). However, according to the Shannon-Wiener index, the *Msp*I digestion indicated greater diversity of ruminal bacteria for SO and $SOMA_1$ compared with $SOMA_2$ and $SOMA_3$.

Several different T-RF exhibited variations in their relative frequency out of the total peak area due to treatments. The relative frequency of T-RF compatible with *Quinella*-related bacteria increased in a linear (150 plus 268 bp with *Msp*I, and 244 plus 288 bp with *Hae*III) or quadratic (98 bp with *Hha*I) manner with incremental amounts of MA in the diet (Table 7), although these fragments exhibited considerable variation between animals. Certain T-RF that may correspond to uncultured bacteria belonging to the subphylum *Clostridia* (380, 217, and 309 bp with *Hha*I, *Msp*I, and *Hae*III, respectively) were detected in lower relative proportions ($P < 0.05$) in samples from ewes fed MA than on the SO or control diet. In contrast,

Table 2. Effect of diet supplementation with sunflower oil alone or in combination with incremental amounts of marine algae on FA composition of rumen fluid in lactating ewes

FA (g/100 g of FA)	Treatment[1]					SED[2]	*P*-value	Contrast[3]
	Control	SO	$SOMA_1$	$SOMA_2$	$SOMA_3$			
12:0	0.14	0.11	0.19	0.14	0.20	0.071	0.65	
13:0	0.03	0.02	0.03	0.04	0.05	0.010	0.36	
13:0 *iso*	0.03	0.02	0.03	0.04	0.03	0.009	0.27	
13:0 *anteiso*	<0.01	<0.01	0.01	<0.01	<0.01	0.002	0.33	
14:0	0.53^{c}	0.41^{c}	0.94^{b}	1.03^{b}	1.38^{a}	0.138	<0.001	L
14:0 *iso*	0.19	0.12	0.14	0.11	0.15	0.031	0.12	
15:0	0.90^{a}	0.60^{b}	0.65^{b}	0.55^{b}	0.62^{b}	0.075	<0.01	
15:0 *iso*	0.46^{a}	0.22^{c}	0.26^{bc}	0.33^{b}	0.29^{bc}	0.038	<0.001	
15:0 *anteiso*	1.11^{a}	0.60^{c}	0.62^{c}	0.79^{b}	0.76^{bc}	0.079	<0.001	
Σ 15:1 *trans*	0.02^{d}	0.01^{d}	0.08^{c}	0.14^{b}	0.18^{a}	0.019	<0.001	L
16:0	25.74^{a}	18.58^{b}	18.82^{b}	20.85^{b}	21.14^{b}	1.360	<0.001	
16:0 *iso*	0.65^{a}	0.43^{b}	0.31^{b}	0.35^{b}	0.42^{b}	0.077	<0.01	
10-O-16:0	0.01^{cd}	<0.01^{d}	0.02^{c}	0.09^{b}	0.11^{a}	0.007	<0.001	LQ
17:0	0.52^{a}	0.29^{b}	0.31^{b}	0.28^{b}	0.27^{b}	0.038	<0.001	
17:0 *iso*[4]	0.41^{a}	0.20^{c}	0.28^{b}	0.27^{b}	0.26^{bc}	0.032	<0.001	
7-methyl-hexadec-7-enoate	0.006^{bc}	0.005^{c}	0.009^{b}	0.009^{b}	0.014^{a}	0.0013	<0.001	L
cis-10 17:1	0.11^{a}	0.08^{b}	0.05^{c}	0.05^{c}	0.05^{c}	0.010	<0.001	LQ
11-cyclohexyl 11:0	0.15^{a}	0.09^{b}	0.06^{bc}	0.04^{c}	0.04^{c}	0.015	<0.001	L
18:0	31.26^{b}	36.43^{a}	5.44^{c}	2.76^{c}	2.60^{c}	1.693	<0.001	LQ
18:0 *iso*	0.04^{a}	0.02^{b}	0.02^{b}	0.02^{b}	0.02^{b}	0.007	0.02	
9-O-18:0	0.01^{c}	0.01^{c}	0.02^{b}	0.02^{ab}	0.03^{a}	0.003	<0.001	LQ
10-O-18:0	0.03^{b}	0.07^{b}	2.96^{a}	3.04^{a}	3.17^{a}	0.488	<0.001	LQ
13-O-18:0	0.04	0.05	0.06	0.05	0.06	0.012	0.37	
15-O-18:0	0.03	0.01	0.02	0.01	0.02	0.014	0.65	
Σ 18:1 *cis*	8.42^{c}	8.71^{c}	9.16^{bc}	11.47^{a}	10.67^{ab}	0.845	<0.01	L
Σ 18:1 *trans*	11.38^{d}	21.41^{c}	46.79^{a}	40.74^{b}	40.45^{b}	2.007	<0.001	LQ
Σ 18:2 nonconjugated	11.04^{a}	6.67^{b}	4.44^{c}	6.65^{b}	5.35^{bc}	0.759	<0.001	
Σ CLA[5]	0.48	0.50	0.70	0.34	0.36	0.134	0.09	L
cis-9,*cis*-12,*cis*-15 18:3	1.58^{a}	0.75^{b}	0.48^{c}	0.71^{bc}	0.53^{bc}	0.119	<0.001	
cis-9,*trans*-11,*cis*-15 18:3	0.05^{ab}	0.02^{c}	0.06^{a}	0.02^{bc}	0.02^{bc}	0.014	0.04	Q
cis-8,*cis*-11,*cis*-14,*cis*-17 18:4	<0.01^{b}	<0.01^{b}	<0.01^{b}	0.02^{a}	0.02^{a}	0.003	<0.001	L
3,7,11,15-tetramethyl 16:0	0.15^{a}	0.19^{a}	0.21^{a}	0.07^{b}	0.13^{ab}	0.036	0.01	L
20:0	0.48^{a}	0.37^{b}	0.33^{bc}	0.31^{c}	0.30^{c}	0.026	<0.001	L
Σ 20:1	0.17^{cd}	0.13^{d}	0.23^{bc}	0.30^{ab}	0.32^{a}	0.040	<0.001	L
21:0	0.04	0.03	0.04	0.04	0.05	0.008	0.31	LQ
cis-9,*cis*-12,*cis*-15,*cis*-18 21:4[6]	0.02^{b}	0.01^{b}	0.02^{b}	0.04^{a}	0.04^{a}	0.005	<0.001	
cis-6,*cis*-9,*cis*-12,*cis*-15,*cis*-18 21:5	—	—	0.04^{b}	0.18^{a}	0.18^{a}	0.016	<0.001	Q
22:0[7]	0.34^{c}	0.47^{b}	0.54^{a}	0.45^{b}	0.44^{b}	0.030	<0.001	
Σ 22:1	0.04^{d}	0.03^{e}	0.07^{c}	0.10^{b}	0.14^{a}	0.006	<0.001	L
23:0	0.14^{b}	0.09^{c}	0.18^{a}	0.11^{c}	0.11^{c}	0.016	<0.001	Q
cis-14 23:1	0.01^{c}	<0.01^{c}	0.04^{b}	0.02^{b}	0.02^{b}	0.004	<0.001	Q
cis-15 24:1	0.04^{b}	0.03^{b}	0.06^{a}	0.07^{a}	0.07^{a}	0.006	<0.001	LQ
cis-16 25:1	—	—	0.01^{c}	0.02^{b}	0.04^{a}	0.006	<0.001	
26:0	0.22^{b}	0.19^{b}	0.30^{a}	0.20^{b}	0.22^{b}	0.032	0.02	
cis-17 26:1	0.01^{bc}	<0.01^{c}	0.01^{bc}	0.03^{b}	0.09^{a}	0.013	<0.001	LQ
27:0	0.009^{bc}	0.004^{c}	0.015^{ab}	0.017^{ab}	0.021^{a}	0.005	0.03	
28:0	0.13^{a}	0.09^{b}	0.13^{a}	0.10^{b}	0.09^{b}	0.015	0.01	Q
30:0	0.29^{b}	0.29^{b}	0.46^{a}	0.24^{b}	0.22^{b}	0.063	0.01	

[a–e]Within a row, different superscripts indicate significant differences ($P < 0.05$).

[1]Refers to TMR based on dehydrated alfalfa hay and concentrates containing no additional lipid (control), or supplemented with 25 g of sunflower oil and 0 (SO), 8 ($SOMA_1$), 16 ($SOMA_2$), or 24 ($SOMA_3$) g of marine algae (MA)/kg of diet DM.

[2]SED = standard error of the difference.

[3]Indicates significant ($P < 0.05$) linear (L) or quadratic (Q) components of the response to incremental MA supplementation of a TMR containing sunflower oil estimated by orthogonal polynomial contrasts using data for SO, $SOMA_1$, $SOMA_2$, and $SOMA_3$ treatments.

[4]Coelutes with *trans*-9 16:1.

[5]CLA = conjugated linoleic acid.

[6]Coelutes with 24:1.

[7]Coelutes with *trans*-10,*trans*-14,*trans*-17 20:3.

Table 3. Effect of diet supplementation with sunflower oil alone or in combination with incremental amounts of marine algae on 16:1 and 18:1 isomer concentrations of rumen fluid in lactating ewes

Isomer (g/100 g of FA)	Treatment[1]					SED[2]	*P*-value	Contrast[3]
	Control	SO	$SOMA_1$	$SOMA_2$	$SOMA_3$			
cis-9 16:1[4]	0.85[a]	0.51[b]	0.56[b]	0.56[b]	0.62[b]	0.053	<0.001	
cis-10 16:1	<0.01[b]	<0.01[b]	0.01[a]	0.02[a]	0.02[a]	0.002	<0.001	LQ
cis-11 16:1	0.03[a]	0.01[b]	0.01[b]	<0.01[b]	0.01[b]	0.002	<0.001	
cis-13 16:1	0.006[b]	0.005[b]	0.013[a]	0.006[b]	0.007[b]	0.0013	<0.001	Q
trans-5 16:1	—	—	0.009[a]	0.007[b]	0.008[ab]	0.0010	<0.001	LQ
trans-6–7 16:1	0.03[c]	0.02[b]	0.10[a]	0.10[a]	0.09[a]	0.013	<0.001	LQ
trans-10 16:1	<0.01[c]	<0.01[c]	0.02[a]	0.01[b]	0.01[b]	0.001	<0.001	LQ
cis-9 18:1[5]	6.83[c]	6.61[c]	7.31[bc]	9.90[a]	8.97[ab]	0.912	<0.01	L
cis-11 18:1	0.63[b]	0.56[b]	1.10[a]	0.98[a]	1.07[a]	0.125	<0.001	LQ
cis-12 18:1	0.54[b]	1.14[a]	0.25[c]	0.18[c]	0.23[c]	0.062	<0.001	LQ
cis-13 18:1	0.12[bc]	0.08[c]	0.15[ab]	0.12[bc]	0.18[a]	0.023	<0.01	L
cis-15 18:1[6]	0.17	0.16	0.21	0.20	0.17	0.024	0.12	
cis-16 18:1	0.13[a]	0.15[a]	0.14[a]	0.07[b]	0.05[b]	0.014	<0.001	L
trans-4 18:1	0.14[b]	0.19[b]	0.37[a]	0.14[b]	0.18[b]	0.033	<0.001	LQ
trans-5 18:1	0.09[d]	0.11[cd]	0.39[a]	0.17[bc]	0.20[b]	0.027	<0.001	Q
trans-6, -7, -8 18:1	0.47[c]	1.11[b]	2.06[a]	0.67[bc]	0.62[c]	0.212	<0.001	LQ
trans-9 18:1	0.30[c]	0.67[b]	1.47[a]	0.88[b]	0.83[b]	0.132	<0.001	Q
trans-10 18:1	0.60[c]	1.30[c]	4.67[bc]	17.30[a]	11.58[ab]	3.875	<0.01	L
trans-11 18:1	6.24[d]	12.98[cd]	30.54[a]	17.40[bc]	23.14[ab]	4.195	<0.001	
trans-12 18:1	0.73[d]	1.25[c]	2.32[a]	1.34[b]	1.29[b]	0.192	<0.001	Q
trans-13 18:1	1.38[c]	2.01[b]	3.28[a]	1.78[bc]	1.65[bc]	0.293	<0.001	LQ
trans-15 18:1[7]	0.70[b]	0.94[a]	1.06[a]	0.73[b]	0.67[b]	0.085	<0.001	L
trans-16 18:1[8]	0.73[a]	0.85[a]	0.62[a]	0.34[b]	0.29[b]	0.074	<0.001	L

[a–d]Within a row, different superscripts indicate significant differences ($P < 0.05$).

[1]Refers to TMR based on dehydrated alfalfa hay and concentrates containing no additional lipid (control), or supplemented with 25 g of sunflower oil and 0 (SO), 8 ($SOMA_1$), 16 ($SOMA_2$), or 24 ($SOMA_3$) g of marine algae (MA)/kg of diet DM.

[2]SED = standard error of the difference.

[3]Indicates significant ($P < 0.05$) linear (L) or quadratic (Q) components of the response to incremental MA supplementation of a TMR containing sunflower oil estimated by orthogonal polynomial contrasts using data for SO, $SOMA_1$, $SOMA_2$, and $SOMA_3$ treatments.

[4]Coelutes with 17:0 *anteiso*.

[5]Contains *trans*-14 18:1 as a minor component.

[6]Coelutes with 19:0.

[7]Contains *cis*-10 18:1 as a minor component.

[8]Contains *cis*-14 18:1 as a minor component.

the relative abundance of some others compatible with uncultivated bacteria of the order *Clostridiales* (390 bp with *Hha*I, 227 with *Msp*I, and 276 with *Hae*III) was greater ($P < 0.05$) in samples from the SO or $SOMA_1$ treatments compared with the control. In addition to fragments matching with the 3 enzymes listed in Table 7, treatments also altered other T-RF. For example, a 65-bp T-RF obtained with *Hha*I, compatible with bacteria of the phylum *Firmicutes*, showed a lower relative proportion in samples on $SOMA_3$ (data not shown).

Butyrivibrio Population Analysis by T-RFLP

Even though diversity indices did not change in response to treatments, possibly as a result of substantial between-animal variation (Table 6), most of the T-RFLP profiles of the *Butyrivibrio* population in samples from the control and SO were grouped together and separated from those from MA-containing diets (Figure 1b).

The relative frequency of the 300-bp fragment was higher in samples on $SOMA_2$ and $SOMA_3$ treatments compared with the control ($P < 0.05$; Table 7), whereas the relative proportion of the 164-bp T-RF was greater when diets contained SO alone ($P < 0.01$), but decreased in a quadratic manner ($P < 0.05$) in response to incremental amounts of MA in the diet.

Microbial Population Analysis by qPCR

Quantification of the *Butyrivibrio trans*-11 18:1-producing bacteria showed no significant changes in response to treatments either in their relative proportion of the total bacteria (mean 0.23 ± 0.033%) or when expressed on the basis of DNA concentration (mean 0.16 ± 0.038 pg/ng of total DNA; Table 8). Inclusion of SO or MA or both in the diet had no effect on the DNA concentration of the 18:0-producing *Butyrivibrio* (*Butyrivibrio proteoclasticus* group) bacteria, whose

Table 4. Effect of diet supplementation with sunflower oil alone or in combination with incremental amounts of marine algae on 18:2 isomer concentrations of rumen fluid in lactating ewes

Isomer (g/100 g of FA)	Treatment[1]					SED[2]	*P*-value	Contrast[3]
	Control	SO	$SOMA_1$	$SOMA_2$	$SOMA_3$			
cis-9,*cis*-12 18:2	10.65[a]	6.30[b]	3.50[d]	5.49[bc]	4.05[cd]	0.755	<0.001	L
cis-11,*cis*-14 18:2	0.03[b]	0.07[a]	<0.01[c]	<0.01[c]	<0.01[c]	0.005	<0.001	LQ
cis-12,*cis*-15 18:2	0.013[a]	0.012[ab]	0.009[bc]	0.011[abc]	0.008[c]	0.0019	0.04	
cis-9,*trans*-12 18:2	0.03[b]	0.03[b]	0.05[a]	0.04[a]	0.05[a]	0.005	<0.001	LQ
cis-11,*trans*-15 18:2[4]	0.01[c]	0.01[c]	0.05[b]	0.11[a]	0.09[a]	0.015	<0.001	LQ
trans-9,*cis*-12 18:2	0.03[b]	0.06[b]	0.19[a]	0.20[a]	0.18[a]	0.028	<0.001	LQ
trans-11,*cis*-15 18:2	0.19[c]	0.09[c]	0.46[b]	0.56[b]	0.72[a]	0.074	<0.001	L
trans-11,*trans*-14 18:2[5]	0.03[b]	0.03[b]	0.04[b]	0.08[a]	0.11[a]	0.010	<0.001	LQ
trans-11,*trans*-15 18:2	0.02[b]	0.03[b]	0.08[a]	0.08[a]	0.11[a]	0.017	<0.001	L
trans-12,*cis*-15 18:2	<0.01	<0.01	<0.01	<0.01	0.01	0.002	0.30	
cis-9,*trans*-11 CLA[6]	0.29[b]	0.33[b]	0.54[a]	0.18[b]	0.20[b]	0.092	<0.01	L
cis-12,*trans*-14 CLA	<0.01	<0.01	<0.01	<0.01	<0.01	0.001	0.35	
trans-7,*cis*-9 CLA	<0.01	<0.01	<0.01	<0.01	<0.01	0.002	0.24	
trans-8,*cis*-10 CLA	<0.01	<0.01	<0.01	<0.01	<0.01	0.001	0.42	
trans-9,*cis*-11 CLA	0.01	<0.01	<0.01	<0.01	<0.01	0.002	0.33	
trans-10,*cis*-12 CLA	0.04	0.03	0.02	0.04	0.03	0.014	0.77	
trans-11,*cis*-13 CLA	<0.01	0.01	0.01	<0.01	0.01	0.004	0.65	
trans-8,*trans*-10 CLA	0.01	<0.01	<0.01	<0.01	<0.01	0.003	0.51	LQ
trans-9,*trans*-11 CLA	0.03[b]	0.03[b]	0.05[a]	0.03[b]	0.05[a]	0.006	<0.01	L
trans-10,*trans*-12 CLA	0.02	0.04	0.02	0.03	0.02	0.008	0.13	L
trans-11,*trans*-13 CLA	0.03	0.03	0.02	0.02	0.02	0.010	0.69	
trans-12,*trans*-14 CLA	<0.01	<0.01	<0.01	<0.01	<0.01	0.001	0.58	

[a–d]Within a row, different superscripts indicate significant differences ($P < 0.05$).

[1]Refers to TMR based on dehydrated alfalfa hay and concentrates containing no additional lipid (control), or supplemented with 25 g of sunflower oil and 0 (SO), 8 ($SOMA_1$), 16 ($SOMA_2$), or 24 ($SOMA_3$) g of marine algae (MA)/kg of diet DM.

[2]SED = standard error of the difference.

[3]Indicates significant ($P < 0.05$) linear (L) or quadratic (Q) components of the response to incremental MA supplementation of a TMR containing sunflower oil estimated by orthogonal polynomial contrasts using data for SO, $SOMA_1$, $SOMA_2$, and $SOMA_3$ treatments.

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

[5]Contains *trans*-10,*trans*-13 18:2 as a minor component.

[6]CLA = conjugated linoleic acid.

abundance was always low (mean 0.45 ± 0.071 pg/ng of total DNA) and varied substantially between animals.

DISCUSSION

Recent studies have investigated the effect of fish oil (Kim et al., 2008) or MA (Boeckaert et al., 2008) in the diet on ruminal lipid metabolism and microbial ecology in cattle, but no reports exist for lactating sheep. Because ruminal cannulation is not feasible in lactating ewes, rumen fluid was collected via stomach tube after 28 d on diet, a period long enough to allow the effects of SO and MA in the diet on milk fat composition and animal performance to be determined (Toral et al., 2010b).

Changes in the relative abundance of FA in rumen fluid to SO and SOMA are consistent with the effects of 18:2n-6 rich plant oils (Atkinson et al., 2006) and fish oil (Chikunya et al., 2004; Sinclair et al., 2005) on the flow of FA at the duodenum in sheep.

Decreases in 18:0 concentrations as percentage of total long-chain FA (≥18-carbon FA) on the SOMA treatments were 3-fold greater in rumen fluid compared with milk fat in the same ewes (Toral et al., 2010b), despite the desaturation of 18:0 to *cis*-9 18:1 in the mammary gland. These findings could be interpreted as evidence of compensatory mechanisms operating in the mammary gland in response to decreases in 18:0 availability that may be important in the overall regulation of milk fat fluidity and lipogenesis in ruminants (Shingfield et al., 2010a).

Relative abundance of a T-RF compatible with uncultured bacteria of the family *Lachnospiraceae* (Boeckaert et al., 2009), which clustered within the 18:0-producing branch, increased with SO and was decreased in samples of rumen fluid of ewes fed MA-containing diets. However, rumen liquid-associated bacteria are thought to have only a minor role in 18:0 formation (Boeckaert et al., 2009). Moreover, the *B. proteoclasticus* group, the only culturable ruminal species known to convert *trans*-11 18:1 or *trans*-10 18:1 to 18:0 (Lourenço et al., 2010; McKain et al., 2010) was neither abundant nor altered by SO or SOMA treatment relative to the control, consistent with the hypothesis that these bacteria

Table 5. Effect of diet supplementation with sunflower oil alone or in combination with incremental amounts of marine algae on 20- and 22-carbon unsaturated FA concentrations of rumen fluid in lactating ewes

Isomer (g/100 g of FA)	Treatment[1]					SED[2]	*P*-value	Contrast[3]
	Control	SO	$SOMA_1$	$SOMA_2$	$SOMA_3$			
cis-5 20:1	0.008	0.007	0.015	0.016	0.024	0.0078	0.24	
cis-8 20:1	—	—	0.001^{b}	0.026^{ab}	0.058^{a}	0.0208	0.04	
cis-11 20:1	0.094^{c}	0.088^{c}	0.164^{b}	0.193^{a}	0.173^{b}	0.0094	<0.001	LQ
cis-13 20:1	0.004^{bc}	0.003^{c}	0.010^{a}	0.007^{b}	0.010^{a}	0.0014	<0.001	L
cis-14 20:1	0.003	0.002	<0.001	0.001	0.003	0.0013	0.16	
trans-6, -7, -8 20:1	0.027^{a}	0.012^{c}	0.012^{c}	0.017^{b}	0.016^{bc}	0.0023	<0.001	L
trans-12 20:1	0.008	0.006	0.010	0.007	0.007	0.0017	0.21	
trans-13 20:1	0.006	0.004	0.006	0.006	0.005	0.0017	0.59	
trans-14 20:1[4]	0.015	0.010	0.014	0.024	0.027	0.0064	0.06	
cis-11,*cis*-14 20:2	0.019^{c}	0.013^{d}	0.025^{b}	0.029^{ab}	0.030^{a}	0.0021	<0.001	LQ
cis-14,*cis*-17 20:2	0.009^{b}	0.005^{b}	0.009^{b}	0.020^{a}	0.021^{a}	0.0040	<0.01	
trans-14,*cis*-17 20:2	0.005^{ab}	0.004^{bc}	0.003^{bc}	0.006^{a}	0.003^{c}	0.0010	0.05	
Δ10,14,17 20:3	—	—	0.004^{ab}	0.004^{a}	0.006^{a}	0.0013	<0.01	
Δ11,14,17 20:3	—	—	0.003^{b}	0.004^{b}	0.006^{a}	0.0009	<0.001	
Δ11,14,18 20:3	0.017^{a}	0.010^{bc}	0.009^{c}	0.014^{ab}	0.015^{a}	0.0022	<0.001	
cis-8,*cis*-11,*cis*-14 20:3	0.002^{d}	0.001^{d}	0.013^{c}	0.020^{b}	0.033^{a}	0.0022	<0.001	L
cis-11,*cis*-14,*cis*-17 20:3	0.005^{bc}	0.002^{d}	0.004^{cd}	0.006^{b}	0.008^{a}	0.0009	<0.001	L
trans-10,*trans*-14,*cis*-17 20:3	—	—	0.015^{a}	0.004^{c}	0.008^{b}	0.0014	<0.001	LQ
trans-9,*trans*-14,*trans*-17 20:3	—	—	0.004^{b}	0.011^{a}	0.009^{a}	0.0014	<0.001	
cis-5,*cis*-8,*cis*-11,*cis*-14 20:4[5]	0.005^{d}	0.003^{d}	0.015^{c}	0.023^{b}	0.036^{a}	0.0020	<0.001	L
cis-8,*cis*-11,*cis*-14,*cis*-17 20:4	0.304^{a}	0.175^{b}	0.242^{ab}	0.247^{a}	0.248^{a}	0.0338	0.02	LQ
trans-7,*cis*-11,*cis*-14,*cis*-17 20:4	—	—	0.007^{a}	0.008^{a}	0.006^{a}	0.0016	<0.001	
cis-5,*cis*-8,*cis*-11,*cis*-14,*cis*-17 20:5 + 24:0	0.376	0.313	0.362	0.336	0.336	0.0233	0.10	
cis-13 22:1	0.022^{d}	0.017^{d}	0.040^{c}	0.072^{b}	0.103^{a}	0.0040	<0.001	L
cis-15 22:1	0.002^{d}	0.002^{d}	0.006^{c}	0.011^{b}	0.020^{a}	0.0016	<0.001	L
cis-13,*cis*-16 22:2	0.025^{a}	0.011^{b}	0.009^{b}	0.008^{b}	0.007^{b}	0.0028	<0.001	
Δ10,13,17 22:3	—	—	0.043^{a}	0.006^{b}	0.007^{b}	0.0049	<0.001	Q
cis-10,*cis*-13,*cis*-16 22:3	0.028^{b}	0.019^{b}	0.027^{b}	0.168^{a}	0.082^{b}	0.0370	<0.01	Q
cis-13,*cis*-16,*cis*-19 22:3	0.006^{b}	0.006^{b}	0.151^{a}	0.152^{a}	0.159^{a}	0.0249	<0.001	LQ
cis-10,*trans*-14,*cis*-19 22:3	0.036^{a}	0.026^{b}	0.014^{c}	0.030^{ab}	0.017^{c}	0.0038	<0.001	
trans-12,*cis*-16,*cis*-19 22:3	—	—	0.118^{a}	0.051^{b}	0.050^{b}	0.0038	<0.001	LQ
Δ8,13,16,19 22:4[6]	—	—	0.054^{b}	0.292^{a}	0.306^{a}	0.0390	<0.001	LQ
cis-7,*cis*-10,*cis*-13,*cis*-16 22:4[7]	0.062^{c}	0.038^{c}	0.196^{b}	0.331^{a}	0.346^{a}	0.0268	<0.001	LQ
cis-10,*cis*-13,*cis*-16,*cis*-19 22:4	0.023^{d}	0.016^{d}	0.125^{c}	0.311^{b}	0.445^{a}	0.0254	<0.001	L
cis-7,*trans*-13,*cis*-16,*cis*-19 22:4[6]	—	—	0.005^{b}	0.093^{a}	0.073^{a}	0.0178	<0.001	L
cis-4,*cis*-7,*cis*-10,*cis*-13,*cis*-16 22:5	0.025^{d}	0.019^{d}	0.378^{c}	0.965^{b}	1.426^{a}	0.0656	<0.001	L
cis-7,*cis*-10,*cis*-13,*cis*-16,*cis*-19 22:5	—	—	0.074^{a}	0.079^{a}	0.099^{a}	0.0148	<0.001	LQ
trans-5,*cis*-10,*cis*-13,*cis*-16,*cis*-19 22:5	—	—	0.115^{a}	0.112^{a}	0.131^{a}	0.0265	<0.001	
cis-4,*cis*-7,*cis*-10,*cis*-13,*cis*-16,*cis*-19 22:6	0.008^{d}	0.004^{d}	0.413^{c}	0.965^{b}	1.607^{a}	0.1201	<0.001	L

[a–e]Within a row, different superscripts indicate significant differences ($P < 0.05$).

[1]Refers to TMR based on dehydrated alfalfa hay and concentrates containing no additional lipid (control), or supplemented with 25 g of sunflower oil and 0 (SO), 8 ($SOMA_1$), 16 ($SOMA_2$), or 24 ($SOMA_3$) g of marine algae (MA)/kg of diet DM.

[2]SED = standard error of the difference.

[3]Indicates significant ($P < 0.05$) linear (L) or quadratic (Q) components of the response to incremental MA supplementation of a TMR containing sunflower oil estimated by orthogonal polynomial contrasts using data for SO, $SOMA_1$, $SOMA_2$, and $SOMA_3$ treatments.

[4]Contains *cis*-9 20:1 as a minor component.

[5]Contains *cis*-16 22:1 as a minor component.

[6]Eluted before *cis*-10,*cis*-13,*cis*-16,*cis*-19 22:4 during GC analysis.

[7]Coelutes with 25:0.

may not play a major role in 18:0 formation in the rumen (Boeckaert et al., 2009; Belenguer et al., 2010; Huws et al., 2010, 2011).

Despite a relatively high abundance of 16:0 in MA (25.6% of total FA), decreases in ruminal 16:0 concentrations were comparable for SO and SOMA treatments, which can be explained, at least in part, by the relatively high variance between animals. However, current data are based on measurements of the relative proportions of FA, and therefore such changes may simply be a reflection of the increase in the total lipid content in the rumen.

The decrease in odd- and branched-chain FA abundance in rumen fluid to SO alone or in combination with MA, relative to the control, was in agreement with the changes reported in samples collected from

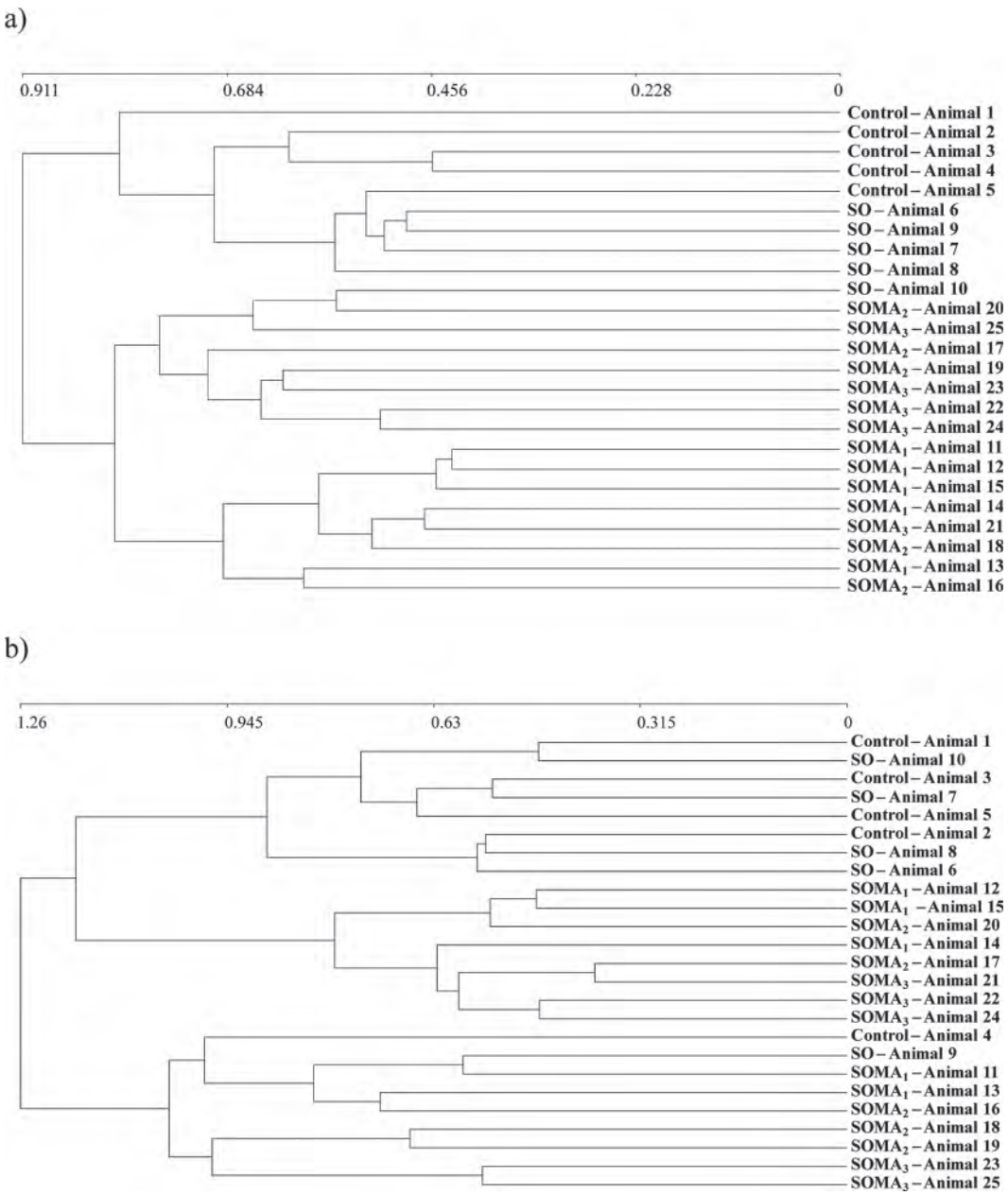

Figure 1. Terminal RFLP-derived Ward's method with Jaccard distance dendrograms showing the effect on total bacteria (a) and *Butyrivibrio* population (b) in DNA extracted from the rumen fluid of lactating ewes fed diets containing no additional lipid (control), sunflower oil alone (SO) or in combination with 8, 16, or 24 g of marine algae/kg of diet DM ($SOMA_1$, $SOMA_2$, and $SOMA_3$, respectively).

nonlactating sheep fed diets containing SO and fish oil (Toral et al., 2010c). Lipids in the diet are known to inhibit bacterial lipid synthesis de novo (Schmidely et al., 2008). In cattle, MA in the diet has been reported to decrease (Boeckaert et al., 2007), have no effect (Or-Rashid et al., 2008), or increase (Boeckaert et al., 2008) concentrations of ruminal odd- and branched-chain FA.

An increase of 10-O-18:0 in rumen fluid of ewes fed MA is consistent with the effects reported for fish oil in combination with SO (Toral et al., 2010c) in sheep, whereas earlier studies reported that fish oil in the diet promoted the accumulation of 10-OH-18:0 (Kitessa et al., 2001). Evidence exists that ruminal bacteria, such as *Selenomonas ruminantium* (Hudson et al., 1995), are capable of hydrating *cis*-9 18:1 or *trans*-10 18:1 to 10-OH-18:0, and others, including *Propionibacterium acnes* (McKain et al., 2010), can further oxidize 10-OH-18:0 to 10-O-18:0.

Table 6. Effect of diet supplementation with sunflower oil alone or in combination with incremental amounts of marine algae on diversity indices (richness, R; Shannon-Wiener, H; and Shannon evenness, E) calculated from the total bacteria and the *Butyrivibrio* group-specific terminal RFLP profiles of rumen fluid in lactating ewes

Group	Diversity index	Treatment[1]					SED[2]	*P*-value	Contrast[3]
		Control	SO	$SOMA_1$	$SOMA_2$	$SOMA_3$			
Total bacteria									
*Hha*I	R	32.8	37.2	34.4	29.4	30.6	3.27	0.17	
	H	2.32	2.71	2.49	2.06	2.18	0.258	0.14	L
	E	0.66	0.75	0.70	0.61	0.64	0.062	0.21	L
*Msp*I	R	53.8^{bc}	57.4^{ab}	64.2^{a}	48.2^{c}	50.0^{bc}	3.63	<0.01	L
	H	3.09^{ab}	3.37^{a}	3.31^{a}	2.81^{b}	2.91^{b}	0.184	0.02	L
	E	0.78	0.83	0.80	0.73	0.74	0.041	0.12	L
*Hae*III	R	61.2	62.6	66.6	79.8	67.6	15.17	0.76	
	H	3.03	3.34	3.33	3.29	3.13	0.254	0.68	
	E	0.74	0.81	0.80	0.77	0.74	0.035	0.23	
Butyrivibrio									
*Hha*I	R	29.6	25.6	22.4	26.4	21.8	4.55	0.44	
	H	2.35	2.30	2.25	2.17	2.07	0.177	0.54	
	E	0.70	0.72	0.74	0.67	0.68	0.031	0.18	

[a–c]Within a row, different superscripts indicate significant differences ($P < 0.05$).

[1]Refers to TMR based on dehydrated alfalfa hay and concentrates containing no additional lipid (control), or supplemented with 25 g of sunflower oil and 0 (SO), 8 ($SOMA_1$), 16 ($SOMA_2$), or 24 ($SOMA_3$) g of marine algae (MA)/kg of diet DM.

[2]SED = standard error of the difference.

[3]Indicates significant ($P < 0.05$) linear (L) components of the response to incremental MA supplementation of a TMR containing sunflower oil estimated by orthogonal polynomial contrasts using data for SO, $SOMA_1$, $SOMA_2$, and $SOMA_3$ treatments.

Rumen fluid of sheep fed diets supplemented with MA had a greater relative abundance of T-RF compatible with bacteria identified in the rumen (Yang et al., 2010) that are phylogenetically related to *Quinella ovalis*, and may have a metabolism similar to that of *S. ruminantium* (Krumholz et al., 1993). Recent studies have also reported an increase in comparable T-RF in sheep fed diets containing SO and fish oil (Belenguer et al., 2010).

On certain diets, the addition of fish oil (Loor et al., 2005) or MA (Boeckaert et al., 2008; Or-Rashid et al., 2008) has been shown to promote a shift in ruminal BH toward increased formation of *trans*-10 18:1 at the expense of *trans*-11 18:1. The results from a recent study suggest that, in high amounts, *trans*-10 18:1 may inhibit mammary lipogenesis in the bovine (Shingfield et al., 2010a). Therefore, identifying the mechanisms underlying the shift in ruminal BH promoting *trans*-10 18:1 formation in the rumen is an important component in developing a more complete understanding of the role of diet and lipid supplements on milk fat synthesis. Inclusion of SO and fish oil in the diet did not stimulate a major change in the ratio of *trans*-10 18:1 to *trans*-11 18:1 in rumen fluid of nonlactating sheep after 11 d on diet (Toral et al., 2010c), whereas a combination of SO and fish oil or MA over a 28-d period in lactating sheep resulted in a substantial increase in the relative proportion of *trans*-10 18:1 in milk (Toral et al., 2010a,b). In the present study, the ratio of *trans*-10 18:1 to *trans*-11 18:1 in rumen fluid was increased on the $SOMA_2$ and $SOMA_3$ diets, which may be attributable to greater amounts of 22-carbon PUFA from MA supplements in the rumen inhibiting the growth of certain *trans*-11 18:1-producing bacteria. However, qPCR quantification did not support a major effect of dietary treatments on known *Butyrivibrio trans*-11 18:1-producing bacteria, consistent with earlier investigations in sheep (Belenguer et al., 2010).

The ruminal bacteria involved in *trans*-10 18:1 formation during isomerization of *cis*-9 18:1 or as an intermediate of 18-carbon PUFA biohydrogenation remain unclear (Jenkins et al., 2008; McKain et al., 2010). To date, only a few rumen bacterial strains have been shown capable of metabolizing 18:2n-6 to *trans*-10,*cis*-12 CLA, including *P. acnes* (Lourenço et al., 2010). The conversion of *trans*-10,*cis*-12 CLA to *trans*-10 18:1 may, however, be catalyzed by *Butyrivibrio*-related species (McKain et al., 2010). In this study, a T-RF compatible with the latter type of microorganisms, such as yet uncultured *Lachnospiraceae* strains, increased with $SOMA_2$ and $SOMA_3$ compared with the control, which is consistent with the changes reported in rumen fluid of sheep fed diets containing SO and fish oil (Belenguer et al., 2010). It is tempting to speculate that these bacteria are able to rapidly convert *trans*-10,*cis*-12 CLA to 18:1 isomers, mostly *trans*-10 18:1 (McKain et al.,

Table 7. Effect of diet supplementation with sunflower oil alone or in combination with incremental amounts of marine algae on the relative frequencies over the total peak area (%) of some terminal restriction fragments (T-RF) identified by terminal RFLP and potential compatible bacteria in rumen fluid of lactating ewes

T-RF (bp)	Compatible bacteria[1]	Treatment[2]					SED[3]	*P*-value	Contrast[4]
		Control	SO	$SOMA_1$	$SOMA_2$	$SOMA_3$			
Total bacteria									
98 (*Hha*I)	Unclassified	8.62^{bc}	3.96^{c}	16.82^{ab}	21.12^{a}	14.99^{ab}	5.364	0.03	LQ
150 + 268 (*Msp*I)	*Veillonellaceae*	6.45	3.78	13.30	10.25	16.56	4.633	0.08	L
244 + 288 (*Hae*III)	(*Quinella*-related)	1.52^{b}	2.55^{b}	3.06^{ab}	6.43^{a}	7.37^{a}	2.103	0.04	L
380 (*Hha*I)	Unclassified	3.73^{ab}	9.43^{a}	1.36^{b}	0.22^{b}	0.00^{b}	2.791	0.02	L
217 (*Msp*I)	*Clostridia*	4.44^{a}	2.75^{ab}	1.64^{b}	1.19^{b}	2.26^{b}	0.989	0.03	
309 (*Hae*III)		6.34^{a}	4.06^{b}	2.37^{bc}	1.64^{c}	3.67^{b}	0.853	<0.001	L
390 (*Hha*I)	Unclassified	1.94^{b}	4.97^{a}	4.28^{a}	1.35^{b}	2.07^{b}	1.043	<0.01	L
227 (*Msp*I)	*Clostridiales*	0.98^{c}	4.10^{ab}	5.20^{a}	1.69^{bc}	2.28^{bc}	1.311	0.02	
276 (*Hae*III)		8.00^{b}	17.11^{a}	10.21^{ab}	6.14^{b}	7.67^{b}	3.580	0.04	Q
Butyrivibrio (*Hha*I)									
149	*Lachnospiraceae*	23.20	29.21	26.41	28.64	26.06	6.162	0.87	
162		15.10	17.67	11.33	14.56	17.34	4.189	0.57	
164		1.36^{b}	3.50^{a}	1.00^{b}	0.70^{b}	0.27^{b}	0.676	<0.01	LQ
300		8.77^{c}	11.38^{bc}	16.62^{abc}	23.25^{a}	20.30^{ab}	4.798	0.04	

[a–c]Within a row, different superscripts indicate significant differences ($P < 0.05$).

[1]Potentially compatible bacteria for each T-RF length pair and for the *Butyrivibrio* T-RF.

[2]Refers to TMR based on dehydrated alfalfa hay and concentrates containing no additional lipid (control), or supplemented with 25 g of sunflower oil and 0 (SO), 8 ($SOMA_1$), 16 ($SOMA_2$), or 24 ($SOMA_3$) g of marine algae (MA)/kg of diet DM.

[3]SED = standard error of the difference.

[4]Indicates significant ($P < 0.05$) linear (L) or quadratic (Q) components of the response to incremental MA supplementation of a TMR containing sunflower oil estimated by orthogonal polynomial contrasts using data for SO, $SOMA_1$, $SOMA_2$, and $SOMA_3$ treatments.

2010), based on the lack of differences in the former between treatments. A low concentration of this CLA isomer in rumen fluid is in line with the low concentration in milk fat and provides further support that increases in ruminal outflow of *trans*-10,*cis*-12 CLA are not a major component of the decreases in mammary lipogenesis in lactating ewes fed SO and MA (Toral et al., 2010b).

The concentrations of 18:2n-6 and 18:3n-3 in the rumen fluid were lower on diets containing lipid supplements compared with the control treatment, which is in agreement with earlier measurements of rumen digesta

Table 8. Effect of diet supplementation with sunflower oil alone or in combination with incremental amounts of marine algae on the quantity of bacterial DNA[1] of *trans*-11 18:1- and 18:0-producing *Butyrivibrio* determined by real-time PCR in rumen fluid of lactating ewes

Item	Treatment[2]					SED[3]	*P*-value	Contrast[4]
	Control	SO	$SOMA_1$	$SOMA_2$	$SOMA_3$			
trans-11 18:1-producing bacteria								
Percentage	–0.74 (0.20)	–0.60 (0.28)	–0.69 (0.23)	–0.77 (0.18)	–0.78 (0.26)	0.172	0.84	
DNA concentration	–0.94 (0.15)	–0.89 (0.14)	–0.93 (0.13)	–0.95 (0.13)	–0.92 (0.27)	0.226	0.99	
18:0-producing bacteria								
Percentage	–0.56 (0.37)	–0.39 (0.49)	–0.66 (0.23)	–0.76 (0.19)	–0.81 (0.17)	0.172	0.14	L
DNA concentration	–0.38 (0.60)	–0.27 (0.62)	–0.47 (0.34)	–0.54 (0.35)	–0.54 (0.36)	0.188	0.58	

[1]Data are expressed as a $\log_{10}$ of the percentage (nontransformed values in parentheses) of the total genomic bacterial DNA and of the specific DNA concentration (pg/ng of total DNA; nontransformed values in parentheses) in total DNA.

[2]Refers to TMR based on dehydrated alfalfa hay and concentrates containing no additional lipid (control), or supplemented with 25 g of sunflower oil and 0 (SO), 8 ($SOMA_1$), 16 ($SOMA_2$), or 24 ($SOMA_3$) g of marine algae (MA)/kg of diet DM.

[3]SED = standard error of the difference.

[4]Indicates significant ($P < 0.05$) linear (L) or quadratic (Q) components of the response to incremental MA supplementation of a TMR containing sunflower oil estimated by orthogonal polynomial contrasts using data for SO, $SOMA_1$, $SOMA_2$, and $SOMA_3$ treatments.

FA composition in nonlactating sheep fed fish oil alone (Kitessa et al., 2001) or in combination with SO (Toral et al., 2010c). This may be the result of 2 effects: simple dilution due to SO and MA increasing total FA concentrations in the rumen or more extensive hydrogenation of dietary 18-carbon PUFA in the rumen in direct response to FA in the MA supplement, an effect that has been reported to occur in sheep fed diets containing fish oil (Chikunya et al., 2004; Sinclair et al., 2005).

In sheep, 22:6n-3 is hydrogenated to a lesser extent in the rumen when supplied as MA rather than fish oil in the diet (Sinclair et al., 2005). In cattle, ruminal BH of this PUFA has been reported to increase in direct relation to the amount of fish oil in the diet (Kim et al., 2008; Lee et al., 2008; Shingfield et al., 2010b). Inclusion of MA in the diet was associated with the appearance of Δ10,13,17 22:3, Δ8,13,16,19 22:4, *cis*-7,*trans*-13,*cis*-16,*cis*-19 22:4, and *trans*-5,*cis*-10,*cis*-13,*cis*-16,*cis*-19 22:5, which supports earlier findings on the metabolic fate of 22-carbon PUFA in the rumen in sheep (Toral et al., 2010c) and lactating cows (Kairenius et al., 2011).

CONCLUSIONS

Supplementing the diet with SO alone or in combination with MA altered the FA composition of rumen fluid, an effect that was also associated with changes in populations of specific bacteria in lactating ewes. Inclusion of MA in diets containing SO resulted in the accumulation of *trans* 18:1 and 10-O-18:0 and in a marked decrease in 18:0 concentrations in rumen fluid. At the highest levels of supplementation (16 and 24 g/kg of DM), MA also promoted a shift in ruminal BH pathways toward the formation of *trans*-10 18:1 at the expense of *trans*-11 18:1. Changes in the concentration of BH intermediates were not accompanied by significant variations in the abundance of known cultivated ruminal bacteria capable of hydrogenation of unsaturated FA. However, certain bacterial groups detected by T-RFLP (such as possibly uncultured *Lachnospiraceae* strains or *Quinella*-related bacteria) exhibited variations in their relative frequency consistent with a potential role in ruminal BH.

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