Does supplemental 18:0 alleviate fish oil-induced milk fat depression in dairy ewes?

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ABSTRACT

Supplementation of dairy ewe diet with marine lipids may be an effective strategy for modulating milk fatty acid composition but induces milk fat depression (MFD). This syndrome has been associated with a shortage of 18:0 for uptake and Δ^9 -desaturation that may impair the capacity of the mammary gland to achieve an adequate fluidity for milk fat secretion. On this basis, it was suggested that supplemental 18:0 may contribute to alleviate marine lipid-induced MFD in sheep. To test this hypothesis, 12 lactating ewes were allocated to 1 of 3 lots and used in a 3×3 Latin square design with 3 periods of 28 d each and 3 experimental treatments: a total mixed ration without lipid supplementation (control) or supplemented with 20 g/kg of DM of fish oil alone (FO) or in combination with 20 g/kg of DM of 18:0 (FOSA). Diets were offered *ad libitum*, and animal performance and rumen and milk fatty acid composition were studied at the end of each period. After completing the Latin square trial and following a change-over design, the *in vivo* digestibility of supplemental 18:0 was estimated using 6 lactating sheep. As expected, diet supplementation with fish oil increased the milk content of some potentially health-promoting fatty acids (e.g., *cis*-9, *trans*-11 18:2, *trans*-11 18:1, 20:5n-3, 22:5n-3, and 22:6n-3), but reduced milk fat concentration and yield (–20% in both FO and FOSA treatments). Thus, although reductions in milk 18:0 and *cis*-9 18:1 output caused by FO (–81 and –51%, respectively) were partially reversed with FOSA diet (–49 and –27%, respectively), the addition of 18:0 to the diet did not prove useful to alleviate MFD. This response, which could not be fully accounted for by the low digestibility coefficient of supplemental 18:0, may challenge the theory of a shortage of this fatty acid as a mechanism to explain fish oil-induced MFD in sheep. Effects of FO and FOSA on rumen and milk fatty acid composition would support that increases in

the concentration of some candidate milk fat inhibitors (e.g., *cis*-9 16:1 or 10-oxo-18:0) might play a relevant role in this type of MFD.

Key words: mammary gland, marine lipid, rumen biohydrogenation, sheep, stearic acid

INTRODUCTION

Nutrition is a major factor determining ruminant milk fatty acid (FA) composition (Lock and Bauman, 2004; Chilliard et al., 2007). Some studies in dairy ewes (Capper et al., 2007; Toral et al., 2010a; Tsiplakou and Zervas, 2013) have shown that the addition of marine lipids to the diet (≤ 30 g/kg of DM) may be an effective strategy for enhancing the milk content of potentially health-promoting FA, such as *cis*-9, *trans*-11 CLA, *trans*-11 18:1, and very long chain n-3 PUFA. However, the application of this feeding strategy under practical farm conditions is hindered by the appearance of milk fat depression (MFD; Toral et al., 2010b; Bichi et al., 2013).

The biohydrogenation (BH) theory represents a unifying concept to explain diet-induced MFD and attributes this syndrome to an inhibition of milk fat synthesis by specific FA produced when the ruminal environment, and consequently the BH process, are altered (Bauman and Griinari, 2001). However, reports in dairy ewes, cows, and goats provide evidence that direct inhibition by FA with confirmed or putative antilipogenic effects (e.g., *trans*-10, *cis*-12 or *trans*-9, *cis*-11 CLA) would not be a major contributor to MFD on diets containing marine lipids (Bichi et al., 2013; Kaarenus et al., 2015; Toral et al., 2015); therefore, other intermediates or mechanisms should be involved. In line with this, marine lipid-induced MFD has been related to potential alterations of the milk fat fluidity (Lor et al., 2005; Shingfield and Griinari, 2007; Gama et al., 2008). Very long-chain n-3 PUFA are known to inhibit the ruminal saturation of *trans* 18:1 to 18:0 (Lor et al., 2005; Shingfield and Griinari, 2007; Toral et al., 2010c), which results in a shortage of 18:0 [melting point (MP) = 69.7°C] for mammary uptake that would constrain the synthesis of *cis*-9 18:1 (MP = 16.0°C) by Δ^9 -desaturation. In addition, marine lipids

Received August 24, 2015.

Accepted September 28, 2015.

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increase the content of *trans* 18:1 isomers, which have higher MP (40–66°C) than their equivalent *cis* isomers. Altogether, these changes would challenge the ability of the mammary gland to maintain milk fat MP below body temperature (Timmen and Patton, 1988) and may impair the capacity to achieve an adequate fluidity for milk fat secretion, which might account for this type of MFD (Shingfield and Griinari, 2007).

On this basis, it was suggested that supplemental 18:0 could contribute to alleviate marine lipid-induced MFD in dairy sheep. To test this hypothesis, lactating ewes were fed a diet supplemented with fish oil alone or in combination with 18:0 to examine the response in terms of animal performance, and rumen and milk FA composition.

MATERIALS AND METHODS

Animals, Experimental Design, and Management

All procedures involving animals were approved and completed in accordance with the Spanish Royal Decree 53/2013 for the protection of animals used for experimental purposes. Twelve lactating Assaf ewes (71.3 ± 2.68 kg of BW and 31.6 ± 1.53 DIM at the beginning of the assay) were allocated to 1 of 3 lots ($n = 4$), balanced according to milk production and composition, BW, DIM, and parity, and used in a 3×3 Latin square design to test the effects of 3 dietary treatments during 3 experimental periods of 28 d each. Diets consisted of a TMR, based on alfalfa hay and a concentrate (40:60), without lipid supplementation (control) or supplemented with 20 g of fish oil (Afa-mpes 121 DHA; Afamsa, Mos, Spain) per kilogram of diet DM alone (FO) or in combination with 20 g of 18:0 (Edenor C18–98MY; Oleo Solutions, York, UK) per kilogram of diet DM (FOSA). The ingredients and chemical composition of the experimental diets, which were prepared weekly and included molasses to reduce selection of dietary components, are presented in Table 1. The experimental diets, prepared every week, were stored in the dark at room temperature (approximately 5–14°C). All ewes were fed the control diet during 3 wk of adaptation before the start of the study. The TMR was offered twice daily, at 0930 and 1830 h, to ensure ad libitum intakes (10–15% of orts). Ewes had continuous access to clean drinking water and were milked twice daily at approximately 0900 and 1800 h in a single-side milking parlor with 10 stalls.

After completing the Latin square trial, 3 ewes on FOSA and 3 on FO were housed for 7 d in individual metabolic cages to examine the *in vivo* digestibility of supplemental 18:0. Following a change-over design, diets received by each ewe were then switched and offered for

21 more days. The digestibility trial was repeated again on the last 7 d of this second period. Measurements and samples from sheep fed the FO treatment were used to determine the fecal concentration of 18:0 when this FA was not added to the diet. During the time the animals were kept in metabolic cages, the management was the same as described previously but portable equipment was used for milking.

Measurements and Sampling Procedures

Diets and Intake. Feed intake was recorded during the last week of each experimental period by weighing the amount of DM offered and refused by each lot over the week. Samples of the diets and orts were collected with the same frequency, stored at -30°C , and then freeze-dried before chemical analysis.

Milk. Milk yield was recorded on d 25, 26, and 27 of each experimental period. With the same frequency, individual milk samples were collected and composited according to morning and evening milk yield. One aliquot was preserved with bronopol (D&F Control Systems Inc., San Ramon, CA) and stored at 4°C until analyzed for fat, CP, lactose, and TS. Milk FA composition was determined in untreated samples from each experimental lot that were composited according to individual milk yield and stored at -30°C until analysis.

Rumen Fluid. On d 28 of each experimental period, ewes were milked and given free access to morning rations for 1 h. Thereafter, feeds were removed and 3 h later samples of rumen fluid were collected from each ewe using a stomach tube (Ramos-Morales et al., 2014). Immediately after collection, the fluid was strained through a nylon membrane (400 μm ; Fisher Scientific S.L., Madrid, Spain) and frozen at -80°C . Samples were freeze-dried and stored at -80°C until analyzed for FA composition.

Digestibility of Supplemental 18:0. After 2 d of adaptation to the metabolic cages, DMI was recorded and feces were weighed daily over 5 consecutive days. Individual samples of feeds offered and refused and of feces were collected and stored at -30°C until analyzed for DM. Lipid composition was determined in subsamples that were freeze-dried and stored at -30°C .

Chemical Analysis

Diets and Orts. Dry matter concentration in diets and orts was determined according to the ISO (1999a) standard. Diet samples were also analyzed for ash (ISO, 2002), CP (ISO, 2009), and ether extract (Ankom Technology Method 2; Ankom Technology Corp. Macedon, NY, <https://www.ankom.com/>). Diet NDF and ADF were determined using an Ankom

Table 1. Formulation and chemical composition of the TMR without lipid supplementation (control) or supplemented with 20 g/kg of DM of fish oil alone (FO) or in combination with 20 g/kg of DM of 18:0 (FOSA)

Item	TMR ¹		
	Control	FO	FOSA
Ingredients, g/kg of fresh matter			
Dehydrated alfalfa hay	400	393	386
Whole corn grain	180	177	174
Whole barley grain	130	128	126
Soybean meal solvent, 440 g of CP/kg	150	147	145
Sugar beet pulp, pellets	70	69	68
Molasses, liquid	50	49	48
Fish oil ²	0	17	17
Stearic acid ³	0	0	17
Mineral supplement ⁴	18	18	17
Vitamin supplement ⁵	2	2	2
Chemical composition, g/kg of DM			
OM	911	902	904
CP	185	186	181
NDF	246	238	243
ADF	147	147	149
14:0	0.2	0.8	0.8
16:0	3.7	7.3	7.4
<i>cis</i> -9 16:1	0.1	0.9	0.8
18:0	0.8	1.9	20.5
<i>cis</i> -9 18:1	2.7	5.7	5.6
<i>cis</i> -11 18:1	0.2	0.8	0.8
18:2n-6	7.8	8.0	7.8
18:3n-3	1.9	2.0	1.9
20:5n-3	0.0	1.1	1.1
22:5n-3	0.0	0.3	0.3
22:6n-3	0.0	4.1	4.0
Total fatty acids	18	37	57

¹n = 3.²Semirefined tuna and sardine oil (Afampes 121 DHA; Afamsa, Mos, Spain); contained (g/100 g of total fatty acids): 14:0 (3.3), 16:0 (19.4), *cis*-9 16:1 (4.2), 17:0 (0.9), 18:0 (5.7), *cis*-9 18:1 (16.3), *cis*-11 18:1 (3.1), 18:2n-6 (2.1), 18:3n-3 (0.8), *cis*-11 20:1 (1.6), 20:5n-3 (6.0), 22:5n-3 (1.5), and 22:6n-3 (21.4).³Edenor C18-98MY (Oleo Solutions, York, UK); contained (g/100 g of total fatty acids): 16:0 (0.6), 18:0 (98.1), and *cis*-9 18:1 (0.4).⁴Declared as containing (g/kg): CaCO₃ (556), Ca₂HPO₄ (222), and NaCl (222).⁵Vitafac Ovino 0.2% AC (DSM Nutritional Products S.A., Madrid, Spain). Declared as containing: vitamin A (4,000,000 IU/kg), vitamin D₃ (1,000,000 IU/kg), vitamin E (5 g/kg), iron (17.5 g/kg), manganese (20 g/kg), cobalt (50 mg/kg), iodine (250 mg/kg), zinc (15 g/kg), selenium (100 mg/kg), sepiolite (100 g/kg), calcium (26.2 g/kg), and magnesium (6.15 g/kg).

2000 fiber analyzer (Ankom Technology Methods 13 and 12, respectively; Ankom Technology Corp.); the former was assayed with sodium sulfite and α -amylase, and both were expressed with residual ash. Fatty acid methyl esters of lipid in diets and Orts were prepared in a 1-step extraction-transesterification procedure using chloroform and 20 mL/L of sulfuric acid in methanol (Shingfield et al., 2003), and *cis*-12 tridecenoate (Larodan, Solna, Sweden) as an internal standard. Methyl esters were separated and quantified using a gas chromatograph (Agilent 7890A GC System, Santa Clara, CA) equipped with a flame-ionization detector and a 100-m fused silica capillary column (0.25 mm i.d., 0.2- μ m film thickness; CP-SIL 88, CP7489, Varian Ibérica S.A., Madrid, Spain) and hydrogen as the carrier gas (207 kPa, 2.1 mL/min). Total FAME profile in a 2- μ L

sample volume at a split ratio of 1:50 was determined using the temperature gradient program described in Shingfield et al. (2003). Peaks were identified based on retention time comparisons with commercially available standard FAME mixtures (GLC463, Nu-Chek Prep, Elysian, MN; and 18919-1AMP Supelco, Sigma-Aldrich, Madrid, Spain).

Milk. Fat, CP, lactose, and TS concentrations were determined by infrared spectrophotometry (ISO, 1999b) using a MilkoScan FT6000 (Foss, Hillerød, Denmark). Lipid in 1 mL of milk was extracted 3 times by sequential addition of 2.5 mL of diethylether and 2 mL of hexane, according to reference procedures (IDF, 1987a,b). Extracts were combined and evaporated to dryness at 60°C under nitrogen for 1 h, dissolved in hexane and methyl acetate, and transesterified to FAME using

freshly prepared methanolic sodium methoxide as a catalyst (Shingfield et al., 2003). Total FAME profile was determined by GC using the same chromatograph and temperature gradient program applied for the analysis of feeds, but isomers of 18:1 were further resolved in a separate analysis under isothermal conditions at 170°C (Shingfield et al., 2003). Peaks were identified based on retention time comparisons with the same FAME mixtures used for the analysis of feeds, other commercially available standards (U-37-M, U-43-M, U-45-M and U-64-M, Nu-Chek Prep; L6031, L8404 and O5632, Sigma-Aldrich; and 11-1600-8, 20-2024-1, 20-2210-9, 20-2305-1-4, 21-1211-7, 21-1413-7, 21-1614-7, 21-1615-7 and BR mixtures 2 and 3, Larodan), cross referencing with chromatograms reported in the literature (e.g., Shingfield et al., 2003; Halmemies-Beauchet-Filleau et al., 2011), and comparison with reference samples for which the FA composition was determined based on GC analysis of FAME and GC-MS analysis of corresponding 4,4-dimethyloxazoline derivatives (Bichi et al., 2013).

Rumen Fluid and Feces. Lipid in 200 mg of samples was extracted using 4 mL of a mixture of hexane and isopropanol (3:2, vol/vol; Shingfield et al., 2003). Extractions were repeated and organic extracts were combined, dried at 45°C under nitrogen, and converted to FAME by sequential base-acid catalyzed transesterification (Toral et al., 2010c) using *cis*-12 tridecenoate (Larodan) as an internal standard. Methyl esters were separated and quantified using the same methodology applied for the analysis of milk fat.

Calculations and Statistical Analyses

The digestibility coefficient of supplemental 18:0 was calculated as follows: (grams of supplemental 18:0 intake – grams of 18:0 recovered in the feces and corrected for fecal 18:0 recovery in the FO treatment)/grams of supplemental 18:0 intake. This calculation was based on the assumption that additional 18:0 does not influence rumen BH. Apparent transfer rates of 20:5n-3, 22:5n-3 and 22:6n-3 from supplemented diets to milk (corrected for their milk concentration in the control) were calculated as: grams milk fat yield $\times$ (FA concentration in milk fat – FA concentration in control milk fat)/FA intake.

The mean milk fat MP was estimated as the sum of the MP of FA weighted by their respective molar proportions as outlined in Toral et al. (2013). Individual FA melting points used for the estimations are reported in Supplementary Table S1 (<http://dx.doi.org/10.3168/jds.2015-10304>).

All data were subjected to statistical analysis for a 3 $\times$ 3 Latin square design (Kaps and Lamberson, 2009)

using the MIXED procedure of the SAS software package (version 9.4, SAS Institute Inc., Cary, NC). The statistical model for animal performance and milk FA composition data included the fixed effects of experimental treatment, period, and sampling day (considered as a repeated measure and assuming a covariance structure fitted on the basis of Schwarz's Bayesian information model fit criterion), and the random effect of the lot. The model for rumen FA composition data included the fixed effects of experimental treatment and period, and the random effect of the animal nested within the lot. Differences were declared significant at $P < 0.05$ and considered a trend toward significance at $P < 0.10$. Means were separated using the default pairwise *t*-test in the pdiff option of the lsmeans statement of the MIXED procedure and least squares means are reported.

RESULTS

Animal Performance, Milk Composition, and Digestibility of Supplemental 18:0

Diet supplementation with fish oil alone (FO) tended to decrease DMI in comparison with the control ($P = 0.088$; Table 2), but none of the treatments affected milk yield ($P = 0.41$); fish oil addition (both individually and in combination with 18:0) caused MFD, lowering milk fat content and secretion in a similar proportion (–20% relative to the control; $P < 0.001$). Reductions in the molar yield of milk <C16 FA were comparable in the 2 supplemented diets (–22%; $P < 0.001$), whereas that of C16 FA declined to a greater extent in FOSA than in FO (–30 and –25%, respectively; $P < 0.001$) and the secretion of >C16 FA was significantly lower in FO (–15%; $P < 0.001$). Milk protein content was slightly decreased after the inclusion of lipids in the diet (on average, –7%; $P < 0.001$), but its daily yield was only affected by the FO treatment (–8%; $P = 0.008$). The digestibility coefficient of supplemental 18:0 showed a high animal variation and averaged 0.484 ± 0.0603 .

Milk Fatty Acid Composition and Output

As shown in Table 3, feeding fish oil increased the milk concentration of some saturated short-chain FA (6:0 and 8:0; $P < 0.01$) and decreased that of all even-chain saturates with 12 to 20 carbon atoms ($P < 0.001$), with the reduction in 12:0 and 16:0 being the greatest in ewes fed FOSA ($P < 0.001$). Compared with the control, the response in the 18:0 concentration was higher in FO (–76%) than in FOSA (–37%; $P < 0.001$). This latter treatment also increased the proportion of 4:0 and decreased that of 10:0 ($P < 0.001$).

Large increments in the concentration of oxygenated FA (8-oxo-16:0 and 10- and 13-oxo-18:0) were associated with fish oil addition, particularly in the FO diet ($P < 0.001$). On the contrary, FOSA induced greater reductions in total odd-chain FA content than FO (-25 and -16% compared with the control, respectively; $P < 0.001$). Although the sum of branched-chain FA was only slightly increased with FO ($P = 0.012$; Table 3), both supplemented diets affected the concentrations of most individual FA within this group (for additional information, please see Supplementary Table S2; <http://dx.doi.org/10.3168/jds.2015-10304>).

The negative effect of fish oil on milk *cis*-9 18:1 concentration was partially counteracted by supplemental 18:0 (with reductions of 39 and 10% in FO and FOSA, respectively, compared with the control, $P < 0.001$, Table 4). The abundance of some minor 18:1 isomers (*cis*-12 and -16 and *trans*-4, -6+7+8, and -16 18:1) was also decreased with lipid supplements ($P < 0.001$), whereas that of most 16:1 (e.g., *cis*-9 and -11 and *trans*-9 16:1), other *cis*-18:1 (*cis*-11 and -15 18:1), and

major *trans*-18:1 isomers were increased ($P < 0.05$). Regarding these latter, the concentration of *trans*-11 18:1 was substantially higher in ewes on the FO than on the FOSA treatment ($P < 0.001$), whereas the opposite was found for *trans*-10 18:1 ($P < 0.001$), which reached the highest milk content in FOSA (where it was approx. 1.6 fold more abundant than *trans*-11 18:1). Both supplemented diets increased the concentration of very long-chain MUFA in a similar manner (e.g., *cis*-11 20:1 and *cis*-13 22:1; $P < 0.001$; Table 3).

The addition of fish oil to the diet decreased the proportion of *cis* 18-carbon PUFA in milk (i.e., 18:2n-6, *cis*-9, *cis*-15 18:2, and 18:3n-3; $P < 0.001$; Table 4), but increased that of most *trans* 18:2, such as *trans*-9, *cis*-12, *trans*-9, *trans*-12, *trans*-11, *cis*-15, and *trans*-11, *trans*-15 18:2, as well as conjugated *cis*-9, *trans*-11, *trans*-9, *cis*-11, and *trans*-10, *cis*-12 18:2 ($P < 0.05$). Differences in the effects of FO and FOSA were quantitatively minor, except for the *cis*-9, *trans*-11 CLA (with 3- and 2-fold increases, respectively, compared with the control; $P < 0.001$).

Table 2. Intake and milk yield and composition in dairy ewes fed a TMR without lipid supplementation (control) or supplemented with 20 g/kg of DM of fish oil alone (FO) or in combination with 20 g/kg of DM of 18:0 (FOSA)

Item	Treatment			SED ¹	P-value ²
	Control	FO	FOSA		
Intake, g/d					
DM	3,369	2,974	3,249	105.8	0.088
14:0	0.72 ^b	2.49 ^a	2.72 ^a	0.083	<0.001
16:0	12.56 ^c	21.81 ^b	24.03 ^a	0.071	<0.001
<i>cis</i> -9 16:1	0.19 ^b	2.54 ^a	2.79 ^a	0.089	<0.001
18:0	2.75 ^c	5.59 ^b	67.54 ^a	1.025	<0.001
<i>cis</i> -9 18:1	9.00 ^b	16.99 ^a	18.70 ^a	0.556	<0.001
<i>cis</i> -11 18:1	0.60 ^b	2.27 ^a	2.49 ^a	0.077	<0.001
18:2n-6	26.12	23.80	25.49	0.844	0.141
18:3n-3	6.29	5.92	6.34	0.212	0.235
20:5n-3	0.00 ^b	3.39 ^a	3.73 ^a	0.121	<0.001
22:5n-3	0.00 ^b	0.85 ^a	0.94 ^a	0.030	<0.001
22:6n-3	0.00 ^b	12.08 ^a	13.30 ^a	0.429	<0.001
Total fatty acids	61.53 ^c	110.80 ^b	184.08 ^a	4.442	<0.001
Yield, g/d					
Milk	3,157	3,093	3,212	86.8	0.407
Fat	168 ^a	134 ^b	136 ^b	4.4	<0.001
Protein	171 ^a	157 ^b	163 ^{ab}	3.9	0.008
Lactose	159	158	162	5.2	0.732
TS	523 ^a	472 ^b	486 ^b	13.5	0.004
Yield, mmol/d					
<C16	386 ^a	305 ^b	294 ^b	13.8	<0.001
C16	179 ^a	134 ^b	125 ^c	3.2	<0.001
>C16	150 ^a	126 ^b	148 ^a	4.5	<0.001
Composition, g/kg					
Fat	53.5 ^a	43.2 ^b	42.2 ^b	0.09	<0.001
Protein	54.4 ^a	50.9 ^b	50.7 ^b	0.05	<0.001
Lactose	50.2	50.9	50.3	0.04	0.186
TS	16.6 ^a	15.2 ^b	15.1 ^b	0.13	<0.001

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

¹SED = standard error of the difference for treatment effects.

²Probability of significant effects due to experimental treatment.

As shown in Table 3 and Supplementary Table S2 (<http://dx.doi.org/10.3168/jds.2015-10304>), the concentration of all very long-chain PUFA in milk was improved with fish oil supplements ($P < 0.001$). On average, 20:5n-3 and 22:6n-3 contents reached up to 0.41 and 1.20 g/100 g of total FA (Table 3), with an apparent transfer rate from fish oil into milk of 0.133 and 0.111, respectively. In this regard, no differences were observed between feeding fish oil alone or in combination with 18:0. However, 22:5n-3 was increased to a greater extent by FO than by FOSA ($P < 0.001$), and its apparent transfer efficiency to milk averaged 0.493.

Results on milk FA output (Supplementary Table S3; <http://dx.doi.org/10.3168/jds.2015-10304>) reflected the effects reported for milk FA concentrations, and reductions in 18:0 and *cis*-9 18:1 secretion caused by FO (−81 and −51%, respectively) were partially re-

versed with FOSA diet (−49 and −27%, respectively, $P < 0.001$). However, a few exceptions were noted in the general agreement with milk FA composition, and no increases in the output of 4:0, 6:0, 8:0, and *cis*-9 16:1 were observed in supplemented diets ($P > 0.10$), whereas decreases in the secretion of other de novo-synthesized FA were quantitatively important (e.g., 10:0, 12:0, 14:0, and 16:0; $P < 0.001$). Relative to the control, estimated milk fat MP was significantly lower in the milk from ewes on the FO and FOSA diets (−2.4 and −2.9°C, respectively; $P < 0.001$; Table 3).

Rumen FA Composition

In general, the effects of fish oil supplementation on rumen FA composition (Tables 5 and 6, and Supplementary Table S4; <http://dx.doi.org/10.3168/>

Table 3. Milk fatty acid (FA) composition in dairy ewes fed a TMR without lipid supplementation (control) or supplemented with 20 g/kg of DM of fish oil alone (FO) or in combination with 20 g/kg of DM of 18:0 [FOSA; data on 18-carbon unsaturated FA and on additional FA are reported in Table 4 and Supplementary Table S2 (<http://dx.doi.org/10.3168/jds.2015-10304>), respectively]

FA, g/100 g of total FA (unless otherwise noted)	Treatment			SED ¹	P-value ²
	Control	FO	FOSA		
4:0	2.64 ^b	2.73 ^b	2.98 ^a	0.066	<0.001
6:0	2.57 ^b	2.97 ^a	2.97 ^a	0.089	<0.001
8:0	2.85 ^b	3.39 ^a	3.19 ^a	0.132	0.002
10:0	10.86 ^a	10.84 ^a	9.71 ^b	0.272	<0.001
12:0	7.10 ^a	6.33 ^b	5.59 ^c	0.105	<0.001
14:0	13.23 ^a	12.40 ^b	12.13 ^b	0.197	<0.001
15:0	1.24 ^a	0.95 ^b	0.85 ^c	0.042	<0.001
16:0	27.18 ^a	24.70 ^b	22.74 ^c	0.307	<0.001
8-oxo-16:0	<0.01 ^c	0.12 ^a	0.11 ^b	0.004	<0.001
<i>cis</i> -9 16:1	0.84 ^b	1.15 ^a	1.10 ^a	0.063	<0.001
<i>cis</i> -11 16:1	0.04 ^b	0.05 ^a	0.05 ^a	0.002	<0.001
<i>trans</i> -9 16:1	0.09 ^c	0.27 ^a	0.20 ^b	0.017	<0.001
Σ 16:1	1.60 ^b	2.29 ^a	2.17 ^a	0.077	<0.001
18:0	5.52 ^a	1.33 ^c	3.50 ^b	0.185	<0.001
10-oxo-18:0	0.01 ^c	0.51 ^a	0.43 ^b	0.010	<0.001
13-oxo-18:0	<0.01 ^b	0.03 ^a	0.02 ^a	0.004	<0.001
Σ <i>cis</i> 18:1	11.07 ^a	7.23 ^c	10.29 ^b	0.204	<0.001
Σ <i>trans</i> 18:1	2.73 ^c	9.10 ^a	8.73 ^b	0.138	<0.001
Σ nonconjugated 18:2	3.34	3.26	3.50	0.097	0.066
Σ CLA	0.62 ^c	1.79 ^a	1.15 ^b	0.082	<0.001
20:0	0.21 ^a	0.16 ^c	0.18 ^b	0.005	<0.001
<i>cis</i> -9 20:1	0.03 ^b	0.08 ^a	0.08 ^a	0.005	<0.001
<i>cis</i> -11 20:1	0.05 ^b	0.32 ^a	0.30 ^a	0.009	<0.001
20:4n-6	0.09 ^b	0.18 ^a	0.18 ^a	0.012	<0.001
20:5n-3	0.06 ^b	0.42 ^a	0.41 ^a	0.008	<0.001
Σ unsaturated C20	0.34 ^c	1.18 ^a	1.12 ^b	0.026	<0.001
22:0	0.06 ^b	0.09 ^a	0.09 ^a	0.004	<0.001
<i>cis</i> -13 22:1	0.01 ^b	0.08 ^a	0.08 ^a	0.005	<0.001
22:5n-3	0.09 ^c	0.44 ^a	0.38 ^b	0.010	<0.001
22:6n-3	0.16 ^b	1.18 ^a	1.21 ^a	0.052	<0.001
Σ unsaturated C22	0.38 ^b	2.08 ^a	2.03 ^a	0.053	<0.001
Σ odd-chain FA	2.71 ^a	2.28 ^b	2.03 ^c	0.090	<0.001
Σ branched-chain FA	2.10 ^b	2.19 ^a	2.08 ^b	0.032	0.012
Estimated milk fat melting point, °C	37.01 ^a	34.61 ^b	34.08 ^c	0.200	<0.001

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

¹SED = standard error of the difference for treatment effects.

²Probability of significant effects due to experimental treatment.

Table 4. Milk 18-carbon unsaturated fatty acid (FA) composition in dairy ewes fed a TMR without lipid supplementation (control) or supplemented with 20 g/kg of DM of fish oil alone (FO) or in combination with 20 g/kg of DM of 18:0 (FOSA)

FA, g/100 g of total FA	Treatment			SED ¹	P-value ²
	Control	FO	FOSA		
<i>cis</i> -9 18:1 ³	9.89 ^a	6.02 ^c	8.91 ^b	0.17	<0.001
<i>cis</i> -11 18:1	0.67 ^c	0.97 ^b	1.06 ^a	0.027	<0.001
<i>cis</i> -12 18:1	0.38 ^a	0.11 ^c	0.17 ^b	0.017	<0.001
<i>cis</i> -13 18:1	0.10 ^b	0.08 ^c	0.12 ^a	0.007	<0.001
<i>cis</i> -15 18:1	0.08 ^b	0.12 ^a	0.12 ^a	0.005	<0.001
<i>cis</i> -16 18:1	0.06 ^a	0.03 ^b	0.03 ^b	0.003	<0.001
<i>trans</i> -4 18:1	0.03 ^a	0.02 ^b	0.02 ^b	0.002	<0.001
<i>trans</i> -5 18:1	0.01	0.01	0.01	0.002	0.677
<i>trans</i> -6+7+8 18:1	0.16 ^a	0.10 ^b	0.09 ^b	0.008	<0.001
<i>trans</i> -9 18:1	0.18 ^b	0.23 ^a	0.21 ^{ab}	0.014	0.013
<i>trans</i> -10 18:1	0.69 ^c	3.97 ^b	4.77 ^a	0.217	<0.001
<i>trans</i> -11 18:1	1.02 ^c	4.04 ^a	3.02 ^b	0.244	<0.001
<i>trans</i> -12 18:1	0.31 ^c	0.58 ^a	0.46 ^b	0.023	<0.001
<i>trans</i> -15 18:1 ⁴	0.58 ^a	0.43 ^b	0.64 ^a	0.038	<0.001
<i>trans</i> -16 18:1 ⁵	0.32 ^a	0.15 ^b	0.16 ^b	0.021	<0.001
<i>cis</i> -9, <i>cis</i> -12 18:2	2.63 ^a	1.97 ^c	2.20 ^b	0.084	<0.001
<i>cis</i> -9, <i>trans</i> -12 18:2	0.06	0.07	0.06	0.003	0.055
<i>cis</i> -9, <i>trans</i> -13 18:2	0.21 ^a	0.18 ^{ab}	0.17 ^b	0.012	0.024
<i>cis</i> -9, <i>trans</i> -14 18:2	0.07 ^a	0.03 ^b	0.03 ^b	0.004	<0.001
<i>cis</i> -12, <i>cis</i> -15 18:2	0.02 ^a	0.01 ^b	0.01 ^b	0.001	<0.001
<i>trans</i> -9, <i>cis</i> -12 18:2	0.03 ^c	0.09 ^a	0.08 ^b	0.003	<0.001
<i>trans</i> -9, <i>trans</i> -12 18:2 ⁶	0.07 ^c	0.16 ^b	0.17 ^a	0.003	<0.001
<i>trans</i> -11, <i>cis</i> -15 18:2	0.07 ^b	0.45 ^a	0.47 ^a	0.012	<0.001
<i>trans</i> -11, <i>trans</i> -15 18:2	0.012 ^c	0.033 ^a	0.028 ^b	0.0019	<0.001
<i>cis</i> -9, <i>trans</i> -11 CLA ⁷	0.51 ^c	1.55 ^a	1.02 ^b	0.085	<0.001
<i>trans</i> -9, <i>cis</i> -11 CLA	0.04 ^b	0.15 ^a	0.15 ^a	0.009	<0.001
<i>trans</i> -10, <i>cis</i> -12 CLA	0.01 ^b	0.03 ^a	0.02 ^a	0.002	<0.001
Other <i>trans</i> , <i>trans</i> CLA ⁸	0.06	0.06	0.06	0.004	0.548
<i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15 18:3	0.56 ^a	0.40 ^b	0.43 ^b	0.021	<0.001

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

¹SED = standard error of the difference for treatment effects.

²Probability of significant effects due to experimental treatment.

³Contains *trans*-13+14 18:1 as minor components.

⁴Contains *cis*-10 18:1 as minor component.

⁵Coelutes with *cis*-14 18:1.

⁶Coelutes with 19:0.

⁷Contains *trans*-8,*cis*-10 and *trans*-7,*cis*-9 CLA as minor components.

⁸Sum of *trans*-8,*trans*-10, *trans*-9,*trans*-11, *trans*-10,*trans*-12, and *trans*-11,*trans*-13 CLA.

jds.2015-10304) followed the same trend as those indicated for milk FA profile, with significant increments in the concentration of most 16:1 and major 18:1 and 18:2 isomers (e.g., *trans*-10 and *trans*-11 18:1, *trans*-9,*trans*-12, *trans*-11,*cis*-15 18:2, and *trans*-9,*cis*-11 and *trans*-10,*cis*-12 CLA), as well as in those of very long-chain MUFA and PUFA (e.g., *cis*-11 20:1, *cis*-13 22:1, 20:5n-3, 22:5n-3, and 22:6n-3; $P < 0.05$). Furthermore, decreases to FO and FOSA were also detected in some minor 18:1 isomers, such as *cis*-12 18:1, *trans*-4 and -16 18:1, or some 18-carbon PUFA, such as 18:3n-3 ($P < 0.01$; Table 6).

However, in contrast to observations in milk, the concentration of 18:0 in the rumen fluid of ewes fed FOSA was similar to that found in the control ($P = 0.88$; Table 5), whereas those of 14:0, 16:0, *cis*-9, *trans*-11,

and *trans*-10 18:1 and total odd-chain FA were increased alike with both fish oil supplements ($P < 0.05$; Tables 5 and 6). In the rumen fluid, the concentration of *trans*-10 18:1 never exceeded that of *trans*-11 18:1 (Table 6).

DISCUSSION

The main aim of dairy ewe diet supplementation with marine lipids is to modulate milk FA composition toward a potentially healthier profile for consumers (Reynolds et al., 2006; Toral et al., 2010b; Tsiplakou and Zervas, 2013). Accordingly, the concentration of *cis*-9,*trans*-11 CLA and its precursor for Δ^9 -desaturation in body tissues, *trans*-11 18:1 (Palmquist et al., 2005; Frutos et al., 2014), increased several-fold in the milk from

ewes on the FO treatment. On the other hand, dietary supplemental 18:0 (FOSA) led to the largest increase in milk *trans*-10 18:1, the health implications of which are not clear yet (Shingfield et al., 2008; Gebauer et al., 2011). The concentrations of 20:5n-3, 22:5n-3, and 22:6n-3 were relatively high in both FO and FOSA (in total, approximately 2 g/100 g of total milk FA), and their mean transfer efficiency from diet to milk was comparable to previous estimations in ewes fed fish oil (Capper et al., 2007; Toral et al., 2010a).

Diet-induced changes in milk FA profile were not accompanied by variations in milk yield, in agreement with most findings in ewes consuming marine lipids (Capper et al., 2007; Toral et al., 2010a; Bichi et al., 2013). The slight decrease in milk protein content with fish oil supplementation would also be consistent with other studies examining the effect of unsaturated fats on milk composition in sheep, even though the reasons for this response are still uncertain (Pulina et al., 2006; Toral et al., 2010a,b). We are not aware of published

data on the effects of highly pure 18:0 sources on dairy ewe performance, but works of a similar nature in cows show small increases or no changes in milk yield and protein content (e.g., Loftén et al., 2014; Piantoni et al., 2015). Other effects on cow performance include an increase in feed intake with the same 18:0 product used in the FOSA diet (Piantoni et al., 2015), which might contribute to explain the results on DMI in the present trial.

As expected, feeding fish oil caused MFD, with a mean reduction in milk fat content (20%) within the range observed for marine lipid supplementation in lactating sheep (17–31%; Capper et al., 2007; Toral et al., 2010b; Bichi et al., 2013). However, the addition of 18:0 to the diet did not prove useful to alleviate this syndrome, challenging the theory of the shortage of 18:0 for mammary uptake as a major mechanism responsible for marine lipid-induced MFD (Shingfield and Griinari, 2007; Gama et al., 2008; Toral et al., 2010a). In this regard, greater milk 18:0 and *cis*-9 18:1

Table 5. Rumen fatty acid (FA) composition in dairy ewes fed a TMR without lipid supplementation (control) or supplemented with 20 g/kg of DM of fish oil alone (FO) or in combination with 20 g/kg of DM of 18:0 [FOSA; data on 18-carbon unsaturated FA and on additional FA are reported in Table 6 and Supplementary Table S4 (<http://dx.doi.org/10.3168/jds.2015-10304>), respectively]

FA, g/kg of DM	Treatment			SED ¹	P-value ²
	Control	FO	FOSA		
12:0	0.09	0.08	0.06	0.014	0.217
14:0	0.54 ^b	0.70 ^a	0.68 ^a	0.061	0.020
15:0	0.27	0.32	0.29	0.027	0.136
16:0	6.49 ^b	9.40 ^a	9.09 ^a	0.439	<0.001
8-oxo-16:0	<0.01 ^c	0.13 ^a	0.09 ^b	0.017	<0.001
<i>cis</i> -9 16:1	0.05 ^c	0.45 ^b	0.54 ^a	0.033	<0.001
<i>cis</i> -11 16:1	0.01 ^b	0.03 ^a	0.02 ^a	0.002	<0.001
<i>trans</i> -9 16:1	<0.01 ^b	0.06 ^a	0.06 ^a	0.006	<0.001
Σ 16:1	0.20 ^b	0.97 ^a	1.05 ^a	0.050	<0.001
18:0	15.08 ^a	2.10 ^b	15.37 ^a	1.990	<0.001
10-oxo-18:0	0.06 ^c	0.82 ^a	0.58 ^b	0.093	<0.001
13-oxo-18:0	0.06	0.06	0.05	0.007	0.264
Σ <i>cis</i> 18:1	3.64 ^b	5.56 ^a	5.93 ^a	0.543	<0.001
Σ <i>trans</i> 18:1	5.98 ^b	14.48 ^a	12.13 ^a	1.564	<0.001
Σ nonconjugated 18:2	4.25	3.71	4.15	0.731	0.704
Σ CLA	0.07 ^b	0.12 ^a	0.10 ^a	0.011	0.002
20:0	0.23 ^b	0.22 ^b	0.30 ^a	0.017	<0.001
<i>cis</i> -9 20:1	<0.01 ^b	0.03 ^a	0.03 ^a	0.005	<0.001
<i>cis</i> -11 20:1	0.13 ^b	0.44 ^a	0.45 ^a	0.027	<0.001
20:4n-6	0.02 ^b	0.17 ^a	0.16 ^a	0.015	<0.001
20:5n-3	<0.01 ^c	0.19 ^b	0.28 ^a	0.022	<0.001
Σ unsaturated C20	0.20 ^b	1.19 ^a	1.29 ^a	0.062	<0.001
22:0	0.24	0.25	0.24	0.019	0.604
<i>cis</i> -13 22:1	<0.01 ^c	0.12 ^b	0.16 ^a	0.010	<0.001
22:5n-3	<0.01 ^b	0.34 ^a	0.33 ^a	0.047	<0.001
22:6n-3	0.01 ^c	1.40 ^b	1.86 ^a	0.114	<0.001
Σ unsaturated C22	0.04 ^c	2.29 ^b	2.78 ^a	0.158	<0.001
Σ odd-chain FA	0.56 ^b	0.74 ^a	0.71 ^a	0.042	<0.001
Σ branched-chain FA	0.83 ^c	1.19 ^a	1.05 ^b	0.065	<0.001
Σ total FA	39.36 ^c	47.24 ^b	57.27 ^a	2.534	<0.001

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

¹SED = standard error of the difference for treatment effects.

²Probability of significant effects due to experimental treatment.

output in FOSA than in FO (4.44 vs. 1.66 g of 18:0 and 11.27 vs. 7.53 g of *cis*-9 18:1 per day, respectively) would support an increase in the availability of these FA for MP maintenance and subsequent milk fat secretion in the former treatment, even though control levels were not achieved (8.71 g of 18:0 and 15.47 g of *cis*-9 18:1 per day, respectively). Given that the fall in 18:0 concentrations in the rumen of ewes fed FO was reversed with the FOSA diet, the weaker response in milk was speculated to be due to a low transfer efficiency of 18:0 into milk, which could derive from a limited intestinal digestibility or a poor mammary uptake from circulating plasma lipids.

In relation to 18:0 digestibility, average values approach 0.75 (Andrews and Lewis, 1970; Glasser et al., 2008; Loften et al., 2014), although it is known that the absorption is severely impaired at high duodenal

flows (Glasser et al., 2008). Thus, the low digestibility coefficient of supplemental 18:0 might be speculated to be due, at least in part, to the external supply of high amounts of 18:0 coupled with additional lipids from fish oil. Moreover, this coefficient was calculated on the premise that the 18:0 product would not affect the BH process, but the rumen FA results do not fully support this assumption, as discussed below. Recently, Piantoni et al. (2015) found a low digestibility coefficient for 18-carbon FA (0.55) in dairy cows fed a similar purified 18:0 supplement. In addition, it is uncertain whether the presentation form of the 18:0 product might have resulted in low solubility, impairing its incorporation in micelles, which would consequently reduce absorption rates (Pantoja et al., 1995; Rico et al., 2014). Regardless of this potential limitation, approximately 31 g/d of additional 18:0 would have been absorbed in the

Table 6. Rumen 18-carbon unsaturated fatty acid (FA) composition in dairy ewes fed a TMR without lipid supplementation (control) or supplemented with 20 g/kg of DM of fish oil alone (FO) or in combination with 20 g/kg of DM of 18:0 (FOSA)

FA, g/kg of DM	Treatment			SED ¹	P-value ²
	Control	FO	FOSA		
<i>cis</i> -9 18:1 ³	2.99 ^b	4.46 ^a	4.75 ^a	0.465	0.001
<i>cis</i> -11 18:1	0.38 ^b	0.86 ^a	0.83 ^a	0.076	<0.001
<i>cis</i> -12 18:1	0.31 ^a	0.11 ^b	0.11 ^b	0.057	0.001
<i>cis</i> -13 18:1	0.06 ^b	0.08 ^a	0.08 ^a	0.006	0.005
<i>cis</i> -15 18:1	0.08	0.11	0.11	0.015	0.053
<i>cis</i> -16 18:1	0.07 ^a	0.04 ^b	0.03 ^b	0.009	<0.001
<i>trans</i> -4 18:1	0.04 ^a	0.02 ^b	0.03 ^b	0.005	<0.001
<i>trans</i> -5 18:1	0.03	0.05	0.04	0.007	0.109
<i>trans</i> -6+7+8 18:1	0.31	0.18	0.14	0.085	0.107
<i>trans</i> -9 18:1	0.18	0.27	0.20	0.049	0.124
<i>trans</i> -10 18:1	2.01 ^b	5.41 ^a	5.27 ^a	1.399	0.026
<i>trans</i> -11 18:1	2.01 ^b	7.15 ^a	5.38 ^a	1.145	<0.001
<i>trans</i> -12 18:1	0.39 ^b	0.71 ^a	0.54 ^b	0.077	<0.001
<i>trans</i> -15 18:1 ⁴	0.43	0.44	0.41	0.038	0.785
<i>trans</i> -16 18:1 ⁵	0.30 ^a	0.15 ^b	0.13 ^b	0.035	<0.001
<i>cis</i> -9, <i>cis</i> -12 18:2	3.79	2.57	2.93	0.659	0.142
<i>cis</i> -9, <i>trans</i> -12 18:2	0.01 ^b	0.02 ^a	0.03 ^a	0.003	<0.001
<i>cis</i> -12, <i>cis</i> -15 18:2	0.02 ^a	0.01 ^b	0.01 ^b	0.001	<0.001
<i>trans</i> -9, <i>cis</i> -12 18:2	0.03 ^b	0.08 ^a	0.07 ^a	0.008	<0.001
<i>trans</i> -9, <i>trans</i> -12 18:2 ⁶	0.04 ^b	0.14 ^a	0.13 ^a	0.021	<0.001
<i>trans</i> -11, <i>cis</i> -15 18:2	0.15 ^b	0.49 ^a	0.54 ^a	0.070	<0.001
<i>trans</i> -11, <i>trans</i> -15 18:2	0.04 ^b	0.13 ^a	0.13 ^a	0.009	<0.001
<i>cis</i> -9, <i>trans</i> -11 CLA ⁷	0.02 ^b	0.06 ^a	0.04 ^{ab}	0.009	0.004
<i>trans</i> -9, <i>cis</i> -11 CLA	0.009 ^b	0.013 ^a	0.014 ^a	0.0019	0.011
<i>trans</i> -10, <i>cis</i> -12 CLA	0.002 ^b	0.008 ^a	0.008 ^a	0.0009	<0.001
Other <i>trans</i> , <i>trans</i> CLA ⁸	0.04	0.04	0.04	0.007	0.927
<i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15 18:3	0.64 ^a	0.34 ^b	0.41 ^b	0.084	0.002

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

¹SED = standard error of the difference for treatment effects.

²Probability of significant effects due to experimental treatment.

³Contains *trans*-13+14 18:1 as minor components.

⁴Contains *cis*-10 18:1 as minor component.

⁵Coelutes with *cis*-14 18:1.

⁶Coelutes with 19:0.

⁷Contains *trans*-8,*cis*-10 and *trans*-7,*cis*-9 CLA as minor components.

⁸Sum of *trans*-8,*trans*-10, *trans*-9,*trans*-11, *trans*-10,*trans*-12, and *trans*-11,*trans*-13 CLA.

digestive tract of ewes fed FOSA, which represents 2.6 times the estimated decrease in 18:0 used for milk fat yield in the FO treatment (approximately -12 g/d). This latter value was estimated from reductions in milk 18:0 and *cis*-9 18:1 yield in FO, assuming that diet is a major determinant of the relative contribution of Δ^9 -desaturase to milk FA profile (Palmquist et al., 2005; Chilliard et al., 2007) and that, according to Frutos et al. (2014), 51% of milk *cis*-9 18:1 in ewes fed a similar TMR would derive from 18:0 Δ^9 -desaturation. As an explanation for the low response of milk FA profiles to dietary 18:0 in terms of a low mammary uptake appears unlikely (Enjalbert et al., 1998), the question arises whether other factors might hamper the effects of this supplementation.

As previously mentioned, the synthesis of *cis*-9 18:1 by mammary Δ^9 -desaturation is considered a key mechanism in the regulation of milk fat fluidity in ruminants (Timmen and Patton, 1988; Taylor and MacGibbon, 2011). However, decreases in estimated milk fat MP in FO and FOSA indicate that lower concentrations of *cis*-9 18:1 (MP = 16°C) and greater of *trans*-18:1, particularly *trans*-10 and *trans*-11 18:1 (MP: 53 and 44°C , respectively), were counteracted by other modifications in milk FA profile, such as decreases in 12:0, 14:0, 16:0, and 18:0 (MP = 44, 54, 63.5, and 69.7°C , respectively) and increases in 4:0, 6:0, 8:0, *cis*-9, *trans*-11 CLA, 20:5n-3, 22:5n-3, and 22:6n-3 (MP = -7.9 , -3.4 , 16, -4.5 , -54 , -47 , and -44°C , respectively). In this regard, although some reports have shown that marine lipids may increase the mean MP of milk FA (Gama et al. 2008; Toral et al., 2010b), some others point to the opposite direction, with no changes or even decreases in this value (Toral et al., 2013, 2015; Kairenius et al., 2015). Therefore, results would indicate that the mammary gland has a high adaptation ability to avoid increases in MP exceeding the upper limit for the maintenance of milk fat fluidity at body temperature (Taylor and MacGibbon, 2011; Toral et al., 2013). However, all these estimates assume that the MP of individual FA are additive, without considering the nonrandom esterification of fatty acids to glycerol and its effect on triacylglycerol packing density and hence on overall milk fat MP (Taylor and MacGibbon, 2011). Furthermore, milk FA composition is only determined in lipids that have been successfully secreted, whereas, as suggested by Gama et al. (2008), FA with high MP might be speculated to accumulate in mammary epithelial cells and inhibit lipogenesis. Further research, including postruminal 18:0 administration or the study of mammary FA arteriovenous differences and milk triacylglycerol stereospecific distribution, would then be warranted before excluding an involvement of milk fat fluidity regulation in MFD.

In any event, on the basis of these results, it appears reasonable to suspect that other mechanisms may be contributing to the low milk fat syndrome caused by marine lipid diet supplementation, probably through the formation of antilipogenic intermediates under conditions of altered ruminal BH (Bauman and Griinari, 2001; Shingfield and Griinari, 2007). Changes in the molar yield of milk FA suggest that *de novo* FA synthesis was affected more extensively than uptake from plasma, which is a common feature of MFD induced by inhibitory intermediates (e.g., *trans*-10, *cis*-12 CLA; Bauman and Griinari, 2001; Shingfield and Griinari, 2007). The potential contribution of BH intermediates to fish oil-induced MFD would be supported not only by the effects of FO and FOSA on rumen FA composition, but also by some differences suggesting a greater disturbance of the microbial BH in ewes fed supplemental 18:0 (and to which the effects of the higher amount of fat on rumen bacterial populations may be contributing too; Nagaraja et al., 1997). Thus, for instance, the *trans*-10 18:1-to-*trans*-11 18:1 ratio was significantly higher in response to FOSA than to FO, both in rumen and milk fat, which is linked to rumen environmental alterations and could be associated with changes in specific biohydrogenating bacteria (Huws et al., 2010; Castro-Carrera et al., 2014). Modifications of the rumen bacterial community due to treatments would also be supported by differences in the profile of the milk odd- and branched-chain FA, which have been proposed as microbial biomarkers (Vlaeminck et al., 2015). Nonetheless, inconsistent effects on odd- and branched-chain FA were observed in rumen and milk fat concentrations, probably attributable to the confounding effects of postruminal modifications in this FA group, including *de novo* synthesis, desaturation, and elongation, which challenges their use as rumen biomarkers (Vlaeminck et al., 2015).

In relation to the putative biological properties of *trans*-10 18:1, doubts remain as to whether this *trans* 18:1 isomer is directly involved in MFD (Bauman and Griinari, 2001; Harvatine et al., 2009), but its accumulation in the rumen may be an indicator of favorable conditions for this syndrome to occur. With regard to conjugated FA with known or tentative antilipogenic effects (namely *trans*-10, *cis*-12, *cis*-10, *trans*-12, and *trans*-9, *cis*-11 18:2; Shingfield and Griinari, 2007), no association has been proven between their enrichment in rumen digesta or milk and reductions in milk fat synthesis due to marine lipids (Lor et al., 2005; Toral et al., 2010a; Kairenius et al., 2015), with the implication that other metabolites could play a more relevant role.

Among the FA provided with the fish oil, *cis*-11 18:1 has been proposed as a candidate milk fat inhibitor based on both its negative effect on *de novo* lipogen-

esis in adipocyte cultures (Burns et al., 2012) and the increases in its milk fat concentration during marine lipid-induced MFD (Gama et al., 2008; Kairenus et al., 2015; Toral et al., 2015). Its precursor for tissue elongation, *cis*-9 16:1, is also present in fish oil and has been shown to decrease intramuscular lipid content in sheep (Duckett et al., 2014). However, although the proportion of both MUFA was enhanced in rumen and milk fat in ewes fed FO and FOSA treatments, no clear evidence exists on the biological activity of *cis*-9 16:1 in lactating ruminants, and the abomasal infusion of a mixture of 18:1 FA containing *cis*-11 18:1 had no negative effects on milk fat synthesis (Shingfield et al., 2007). Recently, it has been speculated that an intermediate of 18:3n-3 BH, *trans*-10,*cis*-15 18:2, might be another putative antilipogenic FA (Alves and Bessa, 2014; Kairenus et al., 2015). Under our chromatographic conditions, this metabolite would presumably co-elute with *trans*-11,*cis*-15 18:2 (Kairenus et al., 2015), for which the concentration was increased several-fold in milk and rumen by both supplemented diets. Therefore, it might be expected that this latter change had most likely been accompanied by increments in *trans*-10,*cis*-15 18:2. Finally, increases in some keto-FA, especially 10-oxo-18:0, have been consistently associated with marine-lipid induced MFD (Bichi et al., 2013; Kairenus et al., 2015; Toral et al., 2015). The presence of an oxo group located on carbon 10 relative to the carboxyl group results in a similar structure to BH intermediates with putative inhibitory activity, such as *trans*-10 18:1 or *trans*-10,*cis*-12 CLA (Shingfield and Griinari, 2007; Kairenus et al., 2015), but no specific information has been reported on the potential biological effects of 10-oxo-18:0 on milk fat synthesis. Further investigation would be required to evaluate the antilipogenic action of these candidate milk fat inhibitors (e.g., *cis*-9 16:1, *cis*-11 18:1, *trans*-10,*cis*-15 18:2, 10-oxo-18:0) in lactating ruminants and provide evidence of their involvement in marine lipid-induced MFD.

CONCLUSIONS

As expected, supplementation of dairy ewe diet with fish oil modulates milk FA composition but reduces milk fat concentration and yield (−20% in both FO and FOSA treatments). The addition of 18:0 to the diet does not prove useful to alleviate the associated MFD, which cannot be fully accounted for by the low digestibility coefficient of supplemental 18:0 and may challenge the theory of a shortage of this FA as a mechanism to explain fish oil-induced MFD in sheep. Effects of FO and FOSA on rumen and milk FA composition would support that increases in the concentration of some candi-

date milk fat inhibitors (e.g., *cis*-9 16:1 or 10-oxo-18:0) might play a relevant role in this type of MFD. Further research is required to establish the biological activity of these and other putative antilipogenic FA and their actual involvement in marine lipid-induced MFD.

ACKNOWLEDGMENTS

This work was supported by the Council of Castile and Leon (CSI023U13) and the Spanish Ministry of Economy and Competitiveness (MINECO; AGL2011-23700). P. G. Toral and D. Carreño benefit from a Juan de la Cierva research contract and an FPI predoctoral grant, respectively, from the MINECO. The authors thank J. Brymer (Christie NHS Foundation Trust, Manchester, UK) for kindly revising the manuscript.

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