Fish oil-induced milk fat depression and associated downregulation of mammary lipogenic genes in dairy ewes

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ABSTRACT

Several studies in dairy cows have shown a relationship between milk fat depression (MFD) and alterations caused in lipogenic gene expression by dietary nutrients. However, information on small ruminants is not only scarce but also inconsistent. Therefore, this experiment was conducted in dairy ewes to study the effect of a diet known to induce MFD on milk fatty acid (FA) composition and mRNA abundance of key candidate genes involved in mammary lipogenesis. Twelve lactating Assaf ewes (on average 63 d in milk) were randomly assigned to 2 treatments consisting of a total mixed ration based on alfalfa hay and concentrates (50:50), supplemented with 0 (control) or 17 g of fish oil/kg of diet dry matter (FO). Profiles of milk FA and mRNA abundance of candidate genes in biopsied mammary tissue were examined before starting the treatments and after 1 and 4.5 wk on the diets. As expected, FO induced MFD and modified milk FA composition. Compared with the control, reductions in milk fat concentration and yield were not detected on d 7, but reached up to 25 and 22%, respectively, on d 30. However, increases in confirmed or putative antilipogenic FA (*trans*-10,*cis*-12 and *trans*-9,*cis*-11 18:2, *cis*-9 16:1, *cis*-11 18:1, and oxo-FA) were already established on the early stage of the treatment and lasted until the end of the feeding period. These changes were accompanied by decreases in the mRNA abundance of genes encoding lipogenic enzymes. The coordinated nature of the downregulation, which tended to affect most studied metabolic pathways, including FA activation (*ACSS1*), de novo synthesis (*ACACA* and *FASN*), uptake and transport (*LPL* and *FABP3*), desaturation (*SCD1*), and esterification (*AGPAT6*), supports the involvement of a central regulator of milk fat synthesis. In this regard, without ruling out the potential contribution of *PPARG*, our results suggest that *SREBF1*

would have a relevant role in the MFD syndrome in sheep fed FO. Among the other studied transcription factors, the tendency to a downregulation of *INSIG1* was associated with that of *SREBF1*, whereas no variation was detected for *SCAP* or *THRSP*. Fish oil had no significant effects on the transcript abundance of *CD36*, *GPAM*, *DGAT1*, *LPIN1*, and *XDH*. Overall, changes in potential antilipogenic FA and mRNA abundance of candidate lipogenic genes support a relationship between them and suggest that FO-induced MFD in dairy ewes would be mediated by transcriptional mechanisms. **Key words:** fatty acid, gene expression, marine lipid, nutrigenomics, sheep

INTRODUCTION

Without ruling out the potential involvement of other causative factors, it is widely accepted that the low-milk fat syndrome is mediated by alterations of ruminal fermentation resulting in the formation of specific bioactive fatty acids (FA) that will exert antilipogenic effects in the mammary gland (Bauman and Griinari, 2001; Shingfield et al., 2010). However, the molecular mechanisms by which these FA are able to decrease mammary synthesis of fat remain uncertain (Bauman et al., 2011). Based on studies in dairy cows, the low-milk fat syndrome, commonly referred to as milk fat depression (MFD), has been hypothesized to be caused by alterations in lipogenic gene expression by dietary nutrients (Harvatine and Bauman, 2006; Angulo et al., 2012; Bionaz et al., 2015). The downregulation of the mRNA abundance of key genes involved in milk fat synthesis by *trans*-10,*cis*-12 CLA is likely the best-known example of nutrigenomics in this area (Shingfield et al., 2010; Bauman et al., 2011; Hussein et al., 2013).

Nevertheless, information about nutrigenomics and lipid metabolism in small ruminants is not only scarce but also inconsistent (Shingfield et al., 2013). For example, Hussein et al. (2013) reported that *trans*-10,*cis*-12 CLA-induced MFD in lactating ewes involves the *SREBF* family and a coordinated downregulation of genes related to mammary lipid synthesis (e.g., *ACA*-

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CA, *FASN*, *SCD1*, *AGPAT6*, *SREBF1*, or *INSIG1*). In contrast, several studies in dairy sheep (Dervishi et al., 2012; Bichi et al., 2013a; Castro-Carrera et al., 2015) or dairy goats (Bernard et al., 2008; Tsiplakou et al., 2009; Toral et al., 2013) observed a weak relationship between changes in milk FA production and mammary mRNA abundance of key candidate genes and transcription factors involved in lipid metabolism. Yet, Bichi et al. (2013a), who studied that relationship after 54 d on marine lipids, suggested that transcriptional regulation in dairy ewes might have been established earlier during the feeding period. This speculation was based on the findings of Invernizzi et al. (2010) in mammary tissue from dairy cows fed saturated lipids or soybean oil and fish oil, who observed that transcriptional control mechanisms altering the expression of several lipogenic genes (e.g., *PPARG*, *LPIN1*, *SREBF1*, *ACSS1*, *ACA-CA*, *FASN*, or *LPL*) were affected by d 7 postfeeding, but variations between diets had disappeared on d 21.

This experiment was therefore conducted in dairy ewes to study the effect of dietary supplementation with marine lipids, which is known to induce MFD in this species (Capper et al., 2007; Bichi et al., 2013b; Toral et al., 2016b), on milk fat composition and mRNA abundance of key candidate genes involved in mammary lipogenesis. Based on previous reports (Toral et al., 2010; Hussein et al., 2013), we hypothesized that changes in FA composition and gene expression would be detected early during the feeding period, whereas the milk fat decrease would only be observed later on. To test this hypothesis, transcript and FA profiles were examined before starting the assay and after 1 and 4.5 wk on a control or a fish oil supplemented diet.

MATERIALS AND METHODS

Animals, Experimental Diets, and Management

All experimental procedures 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 (BW = 77.8 kg, SD = 7.81; DIM = 63, SD = 7.8; milk production = 2.5 kg/d, SD = 0.35) were housed in individual tie stalls and randomly allocated to 1 of 2 groups (n = 6). They were used to test the effects of 2 dietary treatments consisting of a TMR, based on alfalfa hay (particle size >4 cm) and concentrates (50:50), without lipid supplementation (control) or supplemented with 17 g of fish oil (Afampes 121 DHA; Afamsa, Mos, Spain)/kg of diet DM (**FO**). The ingredients and chemical composition of the experimental diets, which were prepared weekly and included molasses to reduce selection of components, are presented in

Table 1. Formulation and chemical composition of the TMR without lipid supplementation (control) or supplemented with 17 g of fish oil/kg of DM (**FO**)

Item	TMR	
	Control	FO
Ingredient, g/kg of fresh matter		
Dehydrated alfalfa hay	500	493
Whole corn grain	140	138
Whole barley grain	100	99
Soybean meal solvent 440, g of CP/kg	150	148
Sugar beet pulp, pellets	50	49
Molasses, liquid	40	39
Fish oil ¹	0	15
Mineral supplement ²	18	18
Vitamin supplement ³	2	2
Chemical composition, g/kg of DM		
OM	904	902
CP	187	181
NDF	251	240
ADF	155	145
14:0	0.60	1.16
16:0	5.49	8.78
<i>cis</i> -9 16:1	0.00	0.77
18:0	1.07	2.04
<i>cis</i> -9 18:1	4.17	7.22
<i>cis</i> -11 18:1	0.31	0.89
18:2n-6	12.53	12.74
18:3n-3	3.15	3.26
20:5n-3	0.00	1.08
22:5n-3	0.00	0.27
22:6n-3	0.00	3.86
Total fatty acids	28.1	43.7

¹Semi-refined tuna and sardine oil (Afampes 121 DHA; Afamsa, Mos, Spain); contained (g/100 g of total fatty acids): 14:0 (3.1), 16:0 (18.6), *cis*-9 16:1 (4.2), 17:0 (0.8), 18:0 (5.4), *cis*-9 18:1 (17.2), *cis*-11 18:1 (3.2), 18:2n-6 (2.3), 18:3n-3 (0.9), *cis*-11 20:1 (1.8), 20:5n-3 (5.9), 22:5n-3 (1.5), and 22:6n-3 (21.2).

²Declared as containing (g/kg): CaCO₃ (556), Ca₂HPO₄ (222), and NaCl (222).

³VITAFAC 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).

Table 1. All ewes were fed the control diet during 4 wk of adaptation before the start of the study. The TMR were offered twice daily, at 0930 and 1830 h, to ensure ad libitum intakes. Ewes had continuous access to clean drinking water and were milked at approximately 0900 and 1800 h in a dedicated 1 × 10-stall milking parlor (DeLaval, Madrid, Spain).

Measurements and Sampling Procedures

Diets. Representative samples of the experimental diets, collected weekly, and the fish oil were stored at −30°C until analysis. Feed intake was measured on d 0 (pretreatment), 7, and 30 by weighing the amount of DM offered and refused by each ewe.

Milk. Milk yield was recorded on d 0 (pretreatment), 7, and 30, and individual milk samples were collected and composited according to morning and evening milk yield. One aliquot of composite milk was stored at -30°C until milk FA composition analysis, whereas another aliquot was preserved with bronopol (D & F Control Systems Inc., San Ramon, CA) and stored at 4°C until analyzed for fat and CP.

Biopsies. Two weeks before starting the experiment (i.e., the administration of the 2 experimental diets; pretreatment) and on d 8 and 31 on the treatments, mammary tissue samples were biopsied from each ewe. Biopsies were performed with local anesthesia of the area by subcutaneous injection of lidocaine (Anesvet, Laboratorios Ovejero S.A., León, Spain). A 4-cm incision was made at the midpoint section of a half-udder, through the skin and underlying fascia until the mammary capsule was clearly visible. Tissue samples (≈ 25 mg) were then collected using disposable needles (12-gauge $\times$ 10 cm, MN1210, Bard Magnum, Bard Biopsy Systems, Tempe, AZ) for Magnum reusable core biopsy instrument (MG1522, Bard Biopsy Systems), immediately frozen in liquid N_2 , and stored at -80°C until RNA extraction. Briefly (see Toral et al., 2015b for details), the wound was treated with adrenaline, and the incision was closed with suture clips and sprayed with topical antibiotic (oxytetracycline) and a wound-healing product (micronized aluminum powder). Ewes also received injections of antibiotic (amoxicillin and colistin), anti-inflammatory (ketoprofen), and systemic hemostatic drugs (ethamsylate). No incidences of mastitis were detected for any ewe in the study.

Laboratory Analysis

Diets. Samples were analyzed for DM (ISO, 1999a), ash (ISO, 2002), and CP (ISO, 2009). The NDF and ADF were determined using an Ankom²⁰⁰⁰ fiber analyzer (Ankom Technology Methods 13 and 12, respectively; Ankom Technology Corp., Macedon, NY); the former was assayed with sodium sulfite and α -amylase, and both were expressed with residual ash. Fatty acid methyl esters of lipid in freeze-dried samples of TMR were prepared in a 1-step extraction-transesterification procedure using chloroform (Sukhija and Palmquist, 1988) and 2% (vol/vol) sulfuric acid in methanol (Shingfield et al., 2003), 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, Agilent Technologies Inc., Santa Clara, CA) equipped with a flame-ionization detector and a 100-m fused silica capillary column (0.25 mm i.d., 0.2- μm film thickness; CP-SIL 88, CP7489, Varian Ibérica S.A., Madrid, Spain) and hydrogen as

the carrier gas (207 kPa, 2.1 mL/min). The total FAME profile in a 2- μL sample volume at a split ratio of 1:50 was determined using a temperature gradient program (Shingfield et al., 2003). Peaks were identified based on retention time comparisons with commercially available standard FAME mixtures (Nu-Chek Prep, Elysian, MN; and Sigma-Aldrich, Madrid, Spain).

Milk. Fat and CP concentrations were determined by infrared spectrophotometry (ISO, 1999b) using a MilkoScan FT6000 (Foss, Hillerød, Denmark). Lipid in 1 mL of milk was extracted using diethylether and hexane (5:4, vol/vol) and transesterified to FAME using freshly prepared methanolic sodium methoxide as a catalyst (Shingfield et al., 2003). The total FAME profile was determined by gas chromatography 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 (Nu-Chek Prep, Sigma-Aldrich, and Larodan), cross referencing with chromatograms reported in the literature (Shingfield et al., 2003; Halmemies-Beauchet-Filleau et al., 2011), and comparison with reference samples for which the FA composition was determined based on gas chromatography analysis of FAME and GC-MS analysis of corresponding 4,4-dimethyloxazoline derivatives (Bichi et al., 2013b).

Mammary Tissue, RNA Isolation, and Quantitative PCR. Total RNA from the mammary secretory tissue was isolated and purified (PureLink RNA Mini kit, Invitrogen, Carlsbad, CA) as described in Bonnet et al. (2013), with the exception that the tissue was homogenized using a bead beater (Mini-bead Beater-8, BioSpec Products, Bartlesville, OK). The concentration and purity of RNA was determined by spectrophotometry using a Nano-Drop ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE), and RNA integrity by capillary electrophoresis (Agilent 2100 Bioanalyzer, Agilent Technologies Inc.). The average RNA integrity number of the samples was 8.1 ± 0.46 . For quantitative PCR (qPCR) analysis, cDNA was synthesized using the High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA) and the lack of DNA contamination was verified by a PCR amplification using *ACTB* primers flanking an intron (Supplemental Table S1; <http://dx.doi.org/10.3168/jds.2016-11019>). The qPCR was carried out (Bonnet et al., 2013) using a 7500 Real-Time PCR System (Applied Biosystems) and specific primers for the main candidate genes and transcription factors involved in mammary lipogenesis. These genes are de-

Table 2. Candidate genes studied in the mammary tissue of experimental ewes

Metabolic pathway	Gene symbol	Gene name
Fatty acid activation	<i>ACSS1</i>	Acyl-CoA synthetase short-chain family member 1
De novo fatty acid synthesis	<i>ACACA</i>	Acetyl-CoA carboxylase α
	<i>FASN</i>	Fatty acid synthase
Uptake and intracellular transport of fatty acids	<i>LPL</i>	Lipoprotein lipase
	<i>CD36</i>	CD36 molecule
	<i>FABP3</i>	Fatty acid-binding protein 3
Δ^9 -desaturation of fatty acids	<i>SCD1</i>	Stearoyl-CoA desaturase (delta-9-desaturase)
Esterification of fatty acids to glycerol	<i>GPAM</i>	Glycerol-3-phosphate acyltransferase, mitochondrial
	<i>DGAT1</i>	Diacylglycerol O-acyltransferase 1
	<i>AGPAT6</i>	1-Acylglycerol-3-phosphate O-acyltransferase 6
	<i>LPIN1</i>	Lipin 1
Lipid droplet formation	<i>XDH</i>	Xanthine dehydrogenase
Regulation of transcription	<i>SREBF1</i>	Sterol regulatory element binding transcription factor 1
	<i>INSIG1</i>	Insulin-induced gene 1
	<i>SCAP</i>	SREBF chaperone
	<i>THRSP</i>	Thyroid hormone responsive
	<i>PPARG</i>	Peroxisome proliferator activated receptor gamma
Reference genes	<i>PPIA</i>	Peptidylprolyl isomerase A
	<i>UXT</i>	Ubiquitously expressed prefoldin-like chaperone
	<i>EIF3K</i>	Eukaryotic translation initiation factor 3 subunit K

scribed in Table 2 and were selected on the basis of previous works on lipid metabolism in ruminants (e.g., Bernard et al., 2008; Bauman et al., 2011; Ma and Corl, 2012). To account for variations in RNA integrity and quantification and cDNA synthesis, mRNA abundance was normalized using the geometric mean of 3 reference genes (Table 2), which have been identified as suitable internal controls (Bionaz and Looor, 2007; Bonnet et al., 2013) and were confirmed as the most stable genes among those quantified using the geNorm procedure (Vandesompele et al., 2002). The primer sequences and qPCR performance are shown in Supplemental Tables S1 and S2, respectively (<http://dx.doi.org/10.3168/jds.2016-11019>). The abundance of gene transcripts was expressed as the $\log_2$ value of mRNA copy number relative to the geometric mean of the 3 reference genes.

Statistical Analysis

All statistical analyses were performed using the SAS software package (version 9.4, SAS Institute Inc., Cary, NC). After the second biopsies, 2 animals from each group were removed from the assay because they showed a slow recovery. Animal performance, milk FA composition, and mammary mRNA abundances of candidate genes ($\log_2$ -transformed values) were analyzed for repeated measures using PROC MIXED. For each variable, the covariance structure (e.g., compound symmetric, first-order autoregressive, heterogeneous first-order autoregressive, first-order antedependence, or unstructured) was selected on the basis of Schwarz's Bayesian information model fit criterion. The statistical model included the fixed effects of diet, sampling time,

their interaction, and the pretreatment record (i.e., d 0 for animal performance and milk FA composition and d –13 for gene mRNA abundances) as a covariate. When the P -value for the interaction of diet $\times$ sampling time was <0.10 , a previously defined orthogonal contrast (FO-d 30 vs. control-d 7 + control-d 30 + FO-d 7 for animal performance and milk FA composition data, and FO-d 31 vs. control-d 8 + control-d 31 + FO-d 8 for mammary mRNA abundance data) was used to test that the effect of FO supplementation was not detected early (d 7–8) but late (d 30–31) during the feeding period. Differences were declared significant at $P < 0.05$ and considered a trend toward significance at $P < 0.10$, and least squares means (adjusted for the covariance) are reported throughout.

RESULTS

Animal Performance and Milk Composition

Fish oil supplementation had no effect on DMI and milk yield, but significantly modified milk composition (Table 3). Compared with the control, reductions in milk fat concentration and yield were not detected on d 7, but reached up to 25 and 22%, respectively, on d 30. The milk protein content also tended to be decreased by FO, but to a lower extent (–6%) and on both sampling days. Time exerted a negative effect on milk yield.

Milk Fatty Acid Composition

Addition of fish oil to the diet had a marked influence on milk FA composition (Table 4 and Supplementary

Table S3; <http://dx.doi.org/10.3168/jds.2016-11019>). Variations in relative proportions of FA derived from mammary uptake were generally more relevant than those observed in de novo synthesized FA, and most changes were already detected on d 7 and comparable on both sampling days. For example, concentrations of the antilipogenic *trans*-10,*cis*-12 CLA and other *trans* 18:2 intermediate metabolites, such as *trans*-9,*cis*-12 and *trans*-11,*cis*-15 18:2, or *cis*-9,*trans*-11, *trans*-11,*cis*-13, and *trans,trans* CLA, were increased several times compared with the control. These increases were similar on d 7 and 30. On the contrary, the large increment in *trans*-9,*cis*-11 CLA was greater at the end of the trial, coincident with a rise in *trans*-10 18:1 and a less pronounced increase in *trans*-11 18:1.

Notable variations in milk long-chain FA also included reductions in the major C18 FA, namely 18:0 and *cis*-9 18:1, which averaged 69 and 41%, respectively, as well as an increase in oxo-FA, which tended to be greater on d 30, and in *cis*-11 18:1. Similarly, the proportions of very long-chain n-3 PUFA (i.e., 20:5n-3, 22:5n-3, and 22:6n-3) were augmented with this treatment, whereas the milk concentration of major PUFA in the control (namely, 18:2n-6 and 18:3n-3) was negatively affected by oil supplementation. The percentage of some short- and medium-chain FA in milk was also modified by the FO diet, with decreases in 4:0, *cis*-9 12:1, 14:0, and *cis*-9 14:1, and increases in 8:0, *cis*-9 16:1 and *trans*-9 16:1 that were comparable in both sampling days. However, the concentration of milk 10:0 was increased in the early stage of the treatment (d 7), and that of 6:0 tended to be higher at the end point (d 30).

On a molar basis, FA with less than 16 carbons accounted for 43% of the reduction in milk fat yield with FO on d 30, and those with 18 or more carbon atoms for 27%, whereas the other 30% was explained by C16 FA (data not shown in tables).

mRNA Abundance of Candidate Genes

As shown in Table 5, the mRNA abundance of most candidate genes was affected by either diet or time. The lipogenic genes *ACACA*, *ACSS1*, and *AGPAT6*, and the transcription factor *SREBF1* were downregulated in the mammary tissue of ewes fed FO. Also, the abundance of *FABP3*, *LPL*, *SCD1*, and *INSIG1* transcripts tended to be reduced by marine lipid supplementation. All these changes in gene expression were already identified on d 8 and remained stable afterward, whereas those of *FASN* were only detected on d 31. With regard to the effect of time on candidate gene expression, greater mRNA abundances of *PPARG1* and *THRSP* were found on d 31 compared with d 8, and the opposite behavior was observed for *ACSS1*, *DGAT1*, *GPAM*, *LPIN1*, *SCAP*, and *SREBF1*.

DISCUSSION

Dietary supplementation of dairy ewes with marine lipids is known to modulate milk FA composition toward a potentially healthier profile for consumers (Capper et al., 2007; Bichi et al., 2013b; Toral et al., 2016b). However, it induces MFD, which has a negative effect on ovine cheese making and hampers the implementation of this feeding strategy. Because the causative factors and mechanisms involved in this syndrome are still uncertain, as in dairy cows, MFD remains an active area of research (Hussein et al., 2013; Bionaz et al., 2015; Toral et al., 2015a).

In the present study, fish oil was used as a mean of experimentally decreasing milk fat concentration and yield in sheep, based on earlier reports indicating that this species is not prone to develop MFD with plant oils (Mele et al., 2006; Pulina et al., 2006; Castro-Carrera et al., 2015). This is probably the first but not the only

Table 3. Intake, and milk yield and composition in lactating ewes fed a TMR without lipid supplementation (control) or supplemented with 17 g of fish oil/kg of DM (FO)

Item	Pretreatment ¹		Day 7		Day 30		SED ²	P-value ³			
	Control	FO	Control	FO	Control	FO		D	T	D × T	Contrast ⁴
DMI, g/d	3,375	3,465	3,390	3,281	3,308	3,107	198.3	0.221	0.313	0.710	
Milk, g/d	2,535	2,552	2,564	2,701	2,089	2,172	199.2	0.362	0.002	0.834	
Fat, %	5.92	5.77	5.89	5.90	5.98	4.51	0.054	0.062	0.065	0.043	0.005
Fat, g/d	150.5	146.9	151.0	159.0	125.4	97.5	11.33	0.156	0.002	0.041	<0.001
Protein, %	5.26	5.21	5.26	5.00	5.39	5.08	0.158	0.065	0.110	0.614	
Protein, g/d	133.7	132.4	135.2	135.4	112.3	108.4	20.92	0.879	0.067	0.865	

¹Data collected on d 0.

²SED = standard error of the difference.

³Probability of significant effects due to diet (D), time (T), and their interaction (D × T).

⁴Probability of the orthogonal contrast: FO-d 30 vs. control-d 7 + control-d 30 + FO-d 7.

difference when comparing this syndrome in ovine and bovine. As expected, feeding FO caused MFD, with a reduction in milk fat content (25% on d 30) that was within the range observed in lactating sheep consuming marine lipids (17–31%; Capper et al., 2007; Toral et al., 2010; Bichi et al., 2013b).

The MFD syndrome has been defined in dairy cows as a reduction in milk fat synthesis with no change in the yield of milk and other components (Bauman et al., 2011). In dairy ewes, however, MFD is often accompanied by small decreases in milk protein concentration (approximately 5–7%; Toral et al., 2010, 2016b), which is consistent with our results. Mechanisms involved in milk protein depression might be speculated to be

independent from those underlying the inhibition in lipogenesis, because reductions in protein content also occur in the absence of changes in milk fat synthesis when sheep receive lipid supplements (e.g., Mele et al., 2006; Pulina et al., 2006). This is in agreement with our observations on d 7, when MFD had not been detected yet.

Another difference between dairy cows and sheep is that several nutritional assays in cows have shown a relationship between marine lipid-induced MFD and changes in mammary gene expression (e.g., Ahnadi et al., 2002; Harvatine and Bauman, 2006; Angulo et al., 2012), whereas this link is not so clear in ewes. For instance, Bichi et al. (2013a) could not detect any

Table 4. Milk fatty acid composition in lactating ewes fed a TMR without lipid supplementation (control) or supplemented with 17 g of fish oil/kg of DM (FO; data on additional fatty acids are reported in Supplemental Table S3; <http://dx.doi.org/10.3168/jds.2016-11019>)

Fatty acid, g/100 g of total fatty acids	Pretreatment ¹		Day 7		Day 30		SED ²	P-value ³			Contrast ⁴
	Control	FO	Control	FO	Control	FO		D	T	D × T	
4:0	3.00	2.90	2.80	2.50	2.85	2.72	0.111	0.034	0.046	0.175	
6:0	2.73	2.68	2.65	2.81	2.69	2.95	0.130	0.138	0.011	0.080	0.022
8:0	2.97	3.00	3.02	3.54	3.03	3.48	0.214	0.039	0.626	0.536	
10:0	10.58	11.22	10.96	12.37	10.89	11.56	0.601	0.108	0.010	0.020	0.714
<i>cis</i> -9 10:1	0.28	0.25	0.29	0.27	0.32	0.32	0.019	0.558	0.004	0.666	
12:0	6.65	7.25	7.21	7.34	6.84	6.72	0.400	0.996	0.002	0.247	
<i>cis</i> -9 12:1	0.11	0.11	0.13	0.11	0.14	0.10	0.012	0.039	0.677	0.638	
14:0	12.69	12.95	13.01	11.72	13.01	11.77	0.411	0.001	0.936	0.927	
<i>cis</i> -9 14:1	0.21	0.20	0.22	0.17	0.24	0.20	0.025	0.019	0.132	0.707	
16:0	27.09	25.72	26.72	24.66	25.78	25.16	1.274	0.182	0.760	0.338	
<i>cis</i> -9 16:1	0.70	0.61	0.74	0.90	0.79	1.06	0.097	0.028	0.056	0.233	
<i>trans</i> -9 16:1	0.08	0.07	0.08	0.37	0.08	0.26	0.082	0.005	0.298	0.247	
18:0	6.43	6.93	6.00	2.12	6.05	1.65	0.327	<0.001	0.443	0.242	
<i>cis</i> -9 18:1 ⁵	12.32	11.54	11.18	7.39	12.07	6.32	0.677	<0.001	0.839	0.058	<0.001
<i>cis</i> -11 18:1	0.79	0.76	0.73	0.98	0.78	1.06	0.085	0.001	0.265	0.745	
<i>trans</i> -10 18:1	0.19	0.24	0.36	0.62	0.38	2.78	0.675	0.031	0.004	0.005	<0.001
<i>trans</i> -11 18:1	1.08	0.97	1.13	5.65	1.19	4.40	0.510	<0.001	0.053	0.037	<0.001
10- + 13-oxo-18:0	<0.01	<0.01	<0.01	0.23	<0.01	0.37	0.505	<0.001	0.057	0.073	<0.001
<i>cis</i> -9, <i>cis</i> -12 18:2	2.46	2.50	2.39	2.02	2.41	1.92	0.137	0.004	0.512	0.340	
<i>cis</i> -9, <i>trans</i> -12 18:2	0.07	0.06	0.07	0.08	0.07	0.09	0.009	0.079	0.308	0.073	0.026
<i>cis</i> -9, <i>trans</i> -13 18:2	0.17	0.17	0.17	0.24	0.18	0.26	0.025	0.002	0.348	0.768	
<i>cis</i> -9, <i>trans</i> -14 18:2	0.07	0.08	0.07	0.05	0.07	0.04	0.008	0.010	0.232	0.663	
<i>trans</i> -9, <i>cis</i> -12 18:2	0.02	0.02	0.03	0.07	0.03	0.08	0.009	<0.001	0.418	0.304	
<i>trans</i> -11, <i>cis</i> -15 18:2	0.07	0.07	0.08	0.36	0.07	0.38	0.054	<0.001	0.731	0.615	
<i>cis</i> -9, <i>trans</i> -11 CLA ⁶	0.54	0.44	0.51	2.28	0.53	2.28	0.307	<0.001	0.938	0.920	
<i>trans</i> -9, <i>cis</i> -11 CLA	0.03	0.03	0.03	0.12	0.04	0.16	0.021	0.001	0.008	0.033	<0.001
<i>trans</i> -10, <i>cis</i> -12 CLA	<0.01	<0.01	<0.01	0.02	<0.01	0.02	0.004	<0.001	0.688	0.647	
<i>trans</i> -11, <i>cis</i> -13 CLA	0.01	0.02	0.01	0.04	0.01	0.03	0.007	0.001	0.347	0.266	
Other <i>trans</i> , <i>trans</i> CLA ⁷	0.03	0.03	0.03	0.04	0.03	0.04	0.007	0.013	0.803	0.719	
18:3n-3	0.62	0.64	0.60	0.53	0.58	0.46	0.047	0.056	0.027	0.238	
20:5n-3	0.06	0.05	0.07	0.28	0.07	0.35	0.041	<0.001	0.203	0.239	
Σ unsaturated C20	0.33	0.34	0.33	0.97	0.40	0.99	0.099	<0.001	0.562	0.729	
22:5n-3	0.10	0.10	0.10	0.35	0.10	0.35	0.041	<0.001	0.863	0.956	
22:6n-3	0.05	0.05	0.04	0.78	0.03	0.99	0.107	<0.001	0.213	0.188	
Σ unsaturated C22	0.21	0.21	0.18	1.39	0.21	1.60	0.165	<0.001	0.320	0.478	

¹Data collected on d 0.

²SED = standard error of the difference.

³Probability of significant effects due to diet (D), time (T), and their interaction (D × T).

⁴Probability of the orthogonal contrast: FO-d 30 vs. control-d 7 + control-d 30 + FO-d 7.

⁵Contains *trans*-13, -14, and -15 18:1 and *cis*-10 18:1 as minor components.

⁶Contains *trans*-8,*cis*-10 and *trans*-7,*cis*-9 CLA as minor components.

⁷Sum of *trans*-8,*trans*-10, *trans*-9,*trans*-11, *trans*-10,*trans*-12, and *trans*-11,*trans*-13 CLA.

significant relationship between the MFD observed in dairy sheep after 54 d of diet supplementation with microalgae and the mRNA abundance of the studied lipogenic genes. However, concurring with our results and based on findings by Invernizzi et al. (2010), the authors suggested that transcriptional regulation mechanisms could be established early during the feeding period.

In line with this, we observed higher contents of *trans*-10,*cis*-12 and *trans*-9,*cis*-11 18:2, 2 CLA isomers with confirmed or putative, respectively, antilipogenic properties after only 7 d on FO. Yet, these increments were lower than those reported to affect milk fat synthesis when administered separately (Perfield et al., 2007; Shingfield et al., 2010; Hussein et al., 2013). This contributes to its actual involvement in FO-induced MFD remaining uncertain and supports that other less well-known inhibitor FA might play a relevant role in the low- milk fat syndrome. In this regard, our results agree with recent reports that suggest a potential influence of *cis*-9 16:1, *cis*-11 18:1, and oxo-FA (Kairenius et al., 2015; Toral et al., 2015a, 2016b). Another FA being putatively involved in MFD (Alves and Bessa, 2014; Kairenius et al., 2015) is *trans*-10,*cis*-15 18:2. Under our chromatographic conditions, this intermediate probably coelutes with *trans*-11,*cis*-15 18:2 (Kairenius et al., 2015), the unresolved peak showing noticeable increases with FO.

Increments in these potentially antilipogenic FA were observed on d 7 and lasted until the end of the experiment. However, the higher concentration of *trans*-10 18:1 (an intermediate FA with yet-equivocal effects on lipogenesis; Kadegowda et al., 2009; Shingfield et al., 2010) only on d 30 is an exception to the general trend in the temporal pattern of milk FA responses. All these changes were inversely related to variations in the mRNA abundance of genes encoding key lipogenic enzymes, which supports a relationship between them (Gervais et al., 2009; Kadegowda et al., 2009; Hussein et al., 2013) and suggests that FO-induced MFD in dairy ewes would be mediated by downregulation of lipogenic genes. This mechanism, previously observed in other dairy ruminants (Shingfield et al., 2010; Bauman et al., 2011; Bionaz et al., 2015), would have tended to affect most studied metabolic pathways, including FA activation (*ACSS1*), de novo synthesis (*ACACA* and *FASN*), uptake and transport (*LPL* and *FABP3*), desaturation (*SCD1*), and esterification (*AGPAT6*).

The enzyme encoded by *ACACA* has been traditionally considered as responsible for the rate-limiting step in de novo lipogenesis (Bauman and Davis, 1974), but simultaneous decreases in the transcript abundance of this gene and *FASN* are common features of MFD (e.g., Ahnadi et al., 2002; Peterson et al., 2003; Hussein et al., 2013). Despite both genes would share common regulatory mechanisms (Bernard et al., 2008), reduc-

Table 5. mRNA relative abundance (log₂ transformed data) of candidate genes in mammary tissue of lactating ewes fed a TMR without lipid supplementation (control) or supplemented with 17 g of fish oil/kg of DM (FO)

	Pretreatment ¹		Day 8		Day 31			P-value ³			
Item	Control	FO	Control	FO	Control	FO	SED ²	D	T	D × T	Contrast ⁴
Lipogenic gene											
<i>ACACA</i>	7.09	7.22	7.26	6.61	6.95	6.25	0.385	0.030	0.175	0.910	
<i>ACSS1</i>	7.72	7.80	7.77	7.30	7.61	6.54	0.228	0.015	0.031	0.101	
<i>AGPAT6</i>	6.91	7.52	7.50	6.96	7.53	6.54	0.266	0.004	0.274	0.211	
<i>CD36</i>	7.41	7.45	7.19	7.34	7.13	7.55	0.289	0.171	0.712	0.471	
<i>DGAT1</i>	6.91	6.82	6.81	6.82	6.56	6.34	0.198	0.502	0.011	0.293	
<i>FABP3</i>	6.88	7.38	6.67	6.45	7.10	5.88	0.729	0.066	0.885	0.295	
<i>FASN</i>	7.93	8.34	8.58	8.26	9.32	7.83	0.442	0.060	0.230	0.004	0.002
<i>GPAM</i>	6.88	7.08	6.64	6.87	6.18	6.50	0.310	0.106	0.068	0.829	
<i>LPIN1</i>	6.11	7.05	7.32	6.56	6.73	6.13	0.588	0.178	0.095	0.776	
<i>LPL</i>	6.92	7.10	7.31	6.81	7.08	6.51	0.304	0.054	0.116	0.803	
<i>SCD1</i>	7.17	7.02	7.40	6.97	7.53	6.69	0.326	0.054	0.549	0.138	
<i>XDH</i>	7.42	7.36	7.14	7.20	6.99	7.48	0.310	0.254	0.718	0.257	
Transcription factor											
<i>INSIG1</i>	7.10	7.35	7.40	6.43	6.86	6.19	0.488	0.057	0.158	0.556	
<i>PPARG</i>	6.91	7.07	6.39	6.46	6.82	6.78	0.204	0.927	0.015	0.663	
<i>SCAP</i>	6.93	7.10	6.85	6.92	6.41	6.53	0.246	0.540	0.026	0.877	
<i>SREBF1</i>	6.68	7.47	7.20	6.10	6.76	5.63	0.367	0.004	0.069	0.941	
<i>THRSP</i>	5.28	5.50	5.71	5.58	7.10	6.96	0.831	0.793	0.030	0.991	

¹Data collected on d -13.

²SED = standard error of the difference.

³Probability of significant effect due to diet (D), time (T), and their interaction (D × T).

⁴Probability of the orthogonal contrast: FO-d 31 vs. control-d 8 + control-d 31 + FO-d 8.

tions in *FASN* mRNA abundance were not observed on the early stage of the treatment.

De novo FA synthesis might have also been limited by the lower mRNA abundance of *ACSS1*, indirectly involved in this metabolic pathway through acetate activation. Although the available information on this gene in lactating ruminants is scant and inconsistent (Mach et al., 2011; Hussein et al., 2013), our results suggest its implication in fish oil-induced MFD in dairy ewes.

Among the candidate genes codifying for enzymes responsible for FA uptake, *LPL* has received a great deal of attention. Available data suggest that, under feeding conditions increasing milk long-chain FA yield (e.g., the addition of plant oils to the diet of small ruminants; Bernard et al., 2008; Castro-Carrera et al., 2015), *LPL* transcript abundance would not be limiting. However, downregulation of this lipogenic gene has been consistently associated with decreases in milk fat synthesis (Harvatine and Bauman, 2006; Angulo et al., 2012; Hussein et al., 2013), which agrees with our results and supports a role in ovine MFD.

Little and erratic information exists about other genes potentially involved in mammary FA uptake and transport in ruminants, such as *CD36* or *FABP3*. The stability of *CD36* transcript abundance observed in the FO treatment as well as in a variety of other feeding conditions (Invernizzi et al., 2010; Hussein et al., 2013; Castro-Carrera et al., 2015) might reflect a low relevance in the transcriptional mechanisms mediating diet-induced changes in lipogenesis. On the other hand, and despite the co-expression of *CD36* and *FABP3* in the bovine mammary gland (Spitsberg et al., 1995), we detected a tendency for lower mRNA abundance of *FABP3* with FO, which may indicate a possible involvement in marine lipid-induced MFD in ewes. This contrasts with the increase reported by Invernizzi et al. (2010) in cows suffering MFD caused by the consumption of FO and soybean oil, and also with the lack of variation in ovine CLA-induced MFD (Hussein et al., 2013).

Following the pathways of milk fat synthesis in the mammary epithelial cell, before the synthesis of milk triacylglycerols, some FA can be subjected to the action of Δ^9 -desaturase, an enzyme that deserves particular attention due to its key role in milk fat composition (Bernard et al., 2013; Toral et al., 2015b). The nutritional regulation of *SCD1* gene expression involves complex interactions between dietary factors and regulatory events that have not been well characterized yet (Bernard et al., 2013). Still, consistent with our results, several studies have shown a downregulation of *SCD1* during MFD induced by marine and plant lipid supple-

mentation (Ahnadi et al., 2002; Peterson et al., 2003; Angulo et al., 2012) or CLA administration (Baumgard et al., 2002; Gervais et al., 2009), the latter not only in cows but also in dairy ewes (Hussein et al., 2013). Increases in 20:5n-3 and *trans*-10,*cis*-12 CLA, which were observed from the early stage of FO treatment, might have been involved in this inhibition (Kadegowda et al., 2009; Bernard et al., 2013).

Due to the diet-induced differences in the availability of *cis*-9-containing FA and substrates for mammary Δ^9 -desaturation, it was not possible to associate the tendency to a downregulation of *SCD1* with systematic reductions in its product milk concentrations. However, the inhibition might have contributed to a further decline in the already low *cis*-9 18:1 milk content derived from the shortage of 18:0 of ruminal origin (Shingfield et al., 2003; Toral et al., 2016b). Decreases in these FA have been linked to altered milk fat fluidity and tentatively to the negative effect of marine lipids on mammary lipogenesis (Gama et al., 2008; Shingfield et al., 2010; Bichi et al., 2013b), although some recent results in dairy ewes (Toral et al., 2016b) question its relevance. In any event, it is probably worth mentioning that Bionaz and Looor (2008) speculated that *FABP3* would provide 18:0 to the SCD, which would worsen the situation in our study.

The last steps of milk fat synthesis include esterification of FA to glycerol and secretion of milk fat globules. Among the studied candidate genes (i.e., *GPAM*, *AGPAT*, *DGAT1*, *LPIN1*, and *XDH*), the lack of variation in *DGAT1* transcript abundance during MFD (Invernizzi et al., 2010; Bichi et al., 2013a; Hussein et al., 2013) agrees with our findings and is probably explained by its known posttranscriptional regulation (Bernard et al., 2008). Conversely, the reduction in *AGPAT6* mRNA abundance with FO, in line with previous reports (Baumgard et al., 2002; Peterson et al., 2003; Hussein et al., 2013), would suggest a greater relevance of transcriptional regulating mechanisms. Based on our results and on inconsistent changes detected in response to diet-induced MFD (Peterson et al., 2003; Invernizzi et al., 2010; Hussein et al., 2013), this link appears less clear for *GPAM*, *LPIN1*, and *XDH*. Reasons for the specific downregulation of *AGPAT6* in ewes fed FO are not clear, but its greater affinity for short- and medium-chain SFA (Bernard et al., 2008) leaves room for speculation about a relationship with the inhibition of de novo lipogenesis.

Overall, the coordinated nature in all these lipogenic gene changes support the involvement of a central regulator of milk fat synthesis (Bernard et al., 2008; Shingfield et al., 2010; Bauman et al., 2011). The *SREBF1* and *PPARG* transcription factors seem to play a promi-

nent nutrigenomic role through different mechanisms (Bionaz et al., 2015). Thus, *PPARG*-mediated effects in gene expression do not necessarily involve variations in its mRNA abundance (Kadegowda et al., 2009; Shi et al., 2013), whereas downregulation of *SREBF1* is frequently detected during MFD induced by CLA (Gervais et al., 2009; Hussein et al., 2013) or marine lipid supplementation (Harvatine and Bauman, 2006; Invernizzi et al., 2010; Angulo et al., 2012), as observed in our study. Furthermore, there may be complex interactions between them: *PPARG* could partly control the expression of *SREBF1*, which in turn could affect the activity of *PPARG* by increasing the production of its natural agonists (Bionaz et al., 2015).

Other transcription factors, such as *INSIG1* and *SCAP*, are known to be involved in *SREBF1* activation to its mature form (the nuclear SREBP1; Sakai et al., 1998; Yang et al., 2002). So far, no clear association between lipid supplementation and changes in *SCAP* transcript abundance has been demonstrated, in contrast to diet-induced changes in both *INSIG* and *SREBF1* mRNA abundances during MFD (Harvatine and Bauman, 2006; Mach et al., 2011; Hussein et al., 2013), which concurs with our observations. Reduced *SREBF1* transcript abundance in ewes fed FO may be indicative of an inhibition of SREBP1 signaling pathway and of its target genes (Bernard et al., 2008; Ma and Corl, 2012), which could contribute to explain the negative effects of FO on the expression of a large number of our candidate genes, including *ACACA*, *FASN*, *LPL*, *FABP3*, and *SCD1*.

Concerning *THRSP*, although there is evidence indicating that this gene acts as a relevant transcriptional regulator in the mammary responses to antilipogenic FA in bovine (Bauman et al., 2011), neither our results nor the little available information in dairy ewes (Bichi et al., 2013a; Hussein et al., 2013) allow confirmation of a similar role in this species. Further research is necessary to elucidate whether observed differences are attributable to interspecies features in the transcriptional regulation of mammary lipogenic genes or in the ruminal metabolism of PUFA (Shingfield et al., 2010; Toral et al., 2016a).

Finally, it is probably worth mentioning that our results do not allow us to conclude a causative relationship between downregulation and lower milk fat synthesis. In fact, some of the enzymes encoded by the candidate genes that we studied might be no longer necessary during MFD, or not as much, and therefore their inhibition might be a consequence of rather than a cause for the low-milk fat syndrome. This would also merit further investigation.

CONCLUSIONS

Reductions in milk fat concentration and yield in dairy ewes consuming 1.7% DM of fish oil were not detected on d 7 but reached up to 25 and 22%, respectively, after 30 d on the diet. However, increases in potentially antilipogenic FA in milk already appeared on the early stage of treatment and lasted until the end of the feeding period. These increments were accompanied by decreases in the mRNA abundance of candidate genes encoding key lipogenic enzymes, which supports a relationship between them and suggests that FO-induced milk fat depression in dairy ewes would be mediated by transcriptional mechanisms. The coordinated nature of the downregulation, which tended to affect most studied metabolic pathways, including FA activation (*ACSS1*), de novo synthesis (*ACACA* and *FASN*), uptake and transport (*LPL* and *FABP3*), desaturation (*SCD1*), and esterification (*AGPAT6*), suggests the involvement of a central regulator of milk fat synthesis. Without ruling out the potential contribution of *PPARG*, our results suggest that the *SREBF1* transcription factor might play a relevant role in the MFD syndrome in lactating sheep fed FO.

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