Interpretive summary

Isolation of RNA from milk somatic cells as an alternative to biopsies of mammary tissue for nutrigenomic studies in dairy ewes

(by Toral et al.)

This study was conducted to validate the use of RNA isolated from milk somatic cells as an alternative in sheep to the performance of mammary tissue biopsies for nutrigenomic analyses. Diet-induced milk fat depression was elicited as a mean to modify the expression of candidate genes involved in mammary lipogenesis. Both types of samples (milk and mammary tissue) showed the consistent effects of the diet (i.e., downregulation of a number of analyzed genes), supporting the feasibility of milk as an RNA source. This would have positive implications in terms of animal welfare, economic cost, and possibility to conduct repeated samplings over time.

Isolation of RNA from milk somatic cells as an alternative to biopsies of mammary tissue for nutrigenomic studies in dairy ewes

P. G. Toral*, G. Hervás*[1], A. Suárez-Vega†, J. J. Arranz† and P. Frutos*

*Instituto de Ganadería de Montaña (CSIC-ULE), Finca Marzanas s/n, 24346 Grulleros, León, Spain

†Departamento de Producción Animal, Facultad de Veterinaria, Universidad de León, 24071, León, Spain

[1]Corresponding author: g.hervas@csic.es

ABSTRACT

Nutrigenomic studies of mammary lipogenesis in ruminants often rely on the use of mammary tissue (MT) collected either by biopsy or at slaughter. However, isolating RNA from milk would be a useful and cost-effective technique that may avoid distress to the animal and facilitate the collection of samples in time series experiments. This assay was therefore conducted to test the hypothesis that RNA extracted from milk somatic cells (MSC) in dairy sheep would be a feasible alternative to the performance of MT biopsies for nutrigenomic analyses. To meet this objective, 8 lactating Assaf ewes were divided in 2 groups and offered a total mixed ration without supplementation (control) or supplemented with 2.4% DM of fish oil (FO), which was known not only to elicit milk fat depression but also to downregulate the expression of some candidate genes involved in mammary

lipogenesis. Total RNA was extracted from MSC and biopsied MT to examine whether the potential changes in the abundance of transcripts was similarly detected with both RNA sources. Milk fatty acid (FA) profile was also analyzed, by gas chromatography, and related to variations in mRNA abundance, determined by reverse transcription quantitative PCR. Values of RIN were always ≥ 7.7. The expected and designed decrease of milk fat concentration with FO (–29%), was associated with a lower transcript abundance of genes coding for enzymes involved in FA activation (*ACSS1*), de novo synthesis (*ACACA* and *FASN*), uptake from plasma lipids (*LPL*) and esterification of FA to glycerol (*LPIN1*), as well as of a transcription factor that may regulate their expression (*INSIG1*). Stable mRNA levels were showed in other candidate genes, such as *FABP3*, *GPAT4* or *SCD*. Changes due to the dietary treatment were similarly detected with both RNA sources (MSC and MT biopsies), which supports the initial hypothesis and would validate the use of milk as an alternative RNA source for nutrigenomic analyses in dairy sheep.

Key words: fatty acid, fish oil, gene expression, RNA source, sheep

INTRODUCTION

Nutrigenomics is a novel and promising discipline that studies the impact of nutrition on physiological processes by altering gene expression (Bauman et al., 2011). Although the greater availability of DNA sequences in livestock has allowed for significant advances in this field (Shingfield et al., 2010; Bionaz et al., 2015), most aspects on molecular mechanisms involved in the nutritional regulation of mammary lipogenesis in ruminants are still uncertain.

In vivo studies have shown that milk fat depression (**MFD**) due to *trans*-10 *cis*-12 CLA administration is associated to downregulation of mammary genes encoding key lipogenic enzymes (e.g., *ACACA*, *FASN*, *LPL* and *SCD*; Harvatine and Bauman, 2006; Gervais et al., 2009; Hussein et al., 2013). On the contrary, scarce available data on the effects of plant oils or marine lipids on the mRNA abundance in the mammary gland of cows (Peterson et al., 2003; Invernizzi et al., 2010; Angulo et al., 2012) and ewes (Bichi et al., 2013a; Castro-Carrera et al., 2015; Carreño et al., in press) are inconsistent. Therefore, further in vivo research would be strictly necessary to advance the state-of-the-art in the interaction between nutrients, gene expression and milk fat synthesis.

Nutrigenomic studies often rely on the use of mammary tissue (**MT**) collected either by biopsy or at slaughter (e.g., Harvatine and Bauman, 2006; Invernizzi et al., 2010; Bichi et al., 2013a). Nonetheless, the validation of a technique of RNA extraction from milk would bring clear advantages in terms of animal welfare, economic cost, and feasibility to conduct repeated samplings over time. Yet, there are wide discrepancies among the very few reports on the comparative use of RNA isolated from either MT or milk for studying the nutritional regulation of mammary lipogenesis in cows (Murrieta et al., 2006; Angulo et al., 2012), which are probably related to the methodological approaches. In this regard, Cánovas et al. (2014) showed the suitability of an RNA extraction procedure from milk somatic cells (**MSC**) to characterize the lactating bovine mammary gland transcriptome. This protocol has recently

been adapted to ovine and used to examine the pattern of gene expression throughout the lactation in two dairy sheep breeds (Suárez-Vega et al., 2015).

The present assay was conducted to test the hypothesis that isolation of RNA from MSC in dairy ewes would be a feasible alternative to the performance of MT biopsies for nutrigenomic analyses. To meet this objective, we fed a group of sheep a diet supplemented with fish oil, which has been shown not only to elicit milk fat depression but also to decrease the mRNA abundance of some candidate genes involved in mammary lipogenesis (Carreño et al., in press). Then, we extracted RNA from milk somatic cells (MSC) and from biopsied MT to examine whether the potential changes in the expression of those genes may be similarly detected with both RNA sources. Milk fatty acid profile was also analyzed and related to transcript abundances.

MATERIALS AND METHODS

Animals, Experimental Design, 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. Eight lactating Assaf ewes (BW = 82.5 ± 2.45 kg; DIM = 62 ± 2.5; parity = 2 ± 0.3; milk yield = 1.8 ± 0.14 kg/d) were housed in individual tie stalls and divided in 2 groups (n = 4), balanced according to milk yield and composition, BW, DIM, and parity. Groups were assigned to one of 2 dietary treatments, consisting of a TMR based on alfalfa hay and concentrates (forage:concentrate ratio 40:60) without lipid supplementation (Control) or supplemented with 24 g of fish oil (Afampes 121 DHA; Afamsa, Mos, Spain)/kg of diet DM (**FO**). The ingredients of the experimental diets, which were prepared weekly, are presented in Table 1. All ewes were fed the control diet for a 21-d adaptation period, and then both experimental treatments for 40 more days. Fresh diets were offered daily ad libitum at approx.

0900 and 1900 h and refusals were removed and weighed each morning. Clean water was always available. Ewes were milked twice daily at 0830 and 1830 h in a single side milking parlor with 10 stalls (DeLaval, Madrid, Spain).

Measurements and Sampling Procedures

Milk. At the end of the adaptation period (days –2 and 0), and after 35 and 37 d on treatments, milk yield was recorded and individual milk samples were collected and composited according to morning and evening milk yield. One aliquot of composite milk was preserved with bronopol (D&F Control Systems Inc., San Ramon, CA) and stored at 4ºC until analyzed for fat concentration. Milk fatty acid (**FA**) composition was determined in untreated samples that were stored at –30ºC until analysis.

On days 38 and 39, milk samples were collected for RNA extraction. Following the protocol by Suárez-Vega et al. (2015), udders were cleaned with water and soap and then disinfected with povidone iodine. Nipples were also washed with RNAseZap (Ambion, Austin, TX). To maximize the number of somatic cells in milk, samples were collected approx. 1 h after morning milking (Gonzalo et al., 1994) and 10 minutes after the injection of oxytocin (5 IU/animal; Facilpart, Laboratorios SYVA, León, Spain). Individual samples were obtained by hand-milking both halves of the mammary gland into an RNAse-free 50-mL tube, which was covered with a sterile gauze to filter the milk. Samples were held in ice and transferred immediately to the laboratory for RNA extraction.

Biopsies. On day 40, mammary tissue samples were biopsied from each ewe. After 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 (≈20 mg) were collected using disposable needles (12 G × 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 and a wound-healing product. Ewes also received injections of antibiotic, anti-inflammatory and systemic hemostatic drugs. No incidences of mastitis were detected for any ewe in the study.

Laboratory Analysis

Diets and Orts. Dry matter concentration in diets and orts was determined according to the ISO 6496:1999 standard. Diets were also analyzed for ash (ISO 5984:2002) and CP (ISO 5983-2:2009). The NDF and ADF were determined using an Ankom^{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 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, 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). Total FAME profile in a 2 μL 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 (from Nu-Chek Prep., Elysian, MN; and Sigma-Aldrich, Madrid, Spain).

Milk. Fat concentration was determined by infrared spectrophotometry (ISO 9622:1999) using a MilkoScan FT6000 (Foss, Hillerød, Denmark), combined with a fluoro-opto-electronic counter (Fossomatic FC, Foss) for milk SCC (ISO 13366-2:2006). Lipid in 1 mL of milk was extracted using diethylether and hexane (5:4, vol/vol) and converted to FAME by base catalyzed transesterification (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 170ºC (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 (from Nu-Chek Prep.; Sigma-Aldrich; and Larodan), 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).

RNA Isolation and Quantitative PCR. Total RNA in 50 mL of fresh milk was extracted as described by Wickramasinghe et al. (2012), with modifications reported by Suárez-Vega et al. (2015). Briefly, MSC were pelleted by centrifugation at 650 × *g* for 10 min at 4°C in the presence of a final concentration of 0.5 mM of EDTA. The cell pellet was washed with 10 mL of PBS (pH 7.2 and 0.5 mM of EDTA) followed by another centrifugation at 650 × *g* for 10 min at 4°C. The washing and centrifugation procedure was repeated twice more by using 2 and 1.5 mL of the same PBS solution. Then, total RNA was extracted and purified from the milk cell pellet with TRIzol (Invitrogen, Carlsbad, CA), as outlined previously (Suárez-Vega et al., 2015).

Total RNA from the MT biopsies was isolated and purified using a commercially available kit (PureLink RNA Mini Kit, Invitrogen) as described in Bonnet et al. (2013), with the exception that the tissue was homogenized with a bead beater disruptor (Mini-bead Beater-8, BioSpec Products, Bartlesville, OK).

The RNA concentration was determined by fluorometry (Qubit 3.0; Life Technologies, Carlsbad, CA), and RNA integrity by capillary electrophoresis (Agilent 2100 Bioanalyzer, Agilent Technologies Inc.). For quantitative PCR (**qPCR**) analysis, cDNA was synthesized from 1.5 μg of RNA from each sample through the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA). The lack of DNA contamination was verified by a PCR amplification with *ACTB* primers flanking an intron. Specific primers were synthesized (Eurofins Genomics, Ebersberg, Germany) for the following genes: acyl-CoA synthetase short-chain family member 1 (*ACSS1*), involved in FA activation; acetyl-CoA carboxylase alpha (*ACACA*) and fatty acid synthase (*FASN*), involved in de novo FA synthesis; lipoprotein lipase (*LPL*) and fatty acid-binding protein 3 (*FABP3*), involved in the uptake and intracellular transport of FA; stearoyl-CoA desaturase (delta-9-desaturase; *SCD*), involved in Δ^9-desaturation of FA; glycerol-3-phosphate acyltransferase 4 (*GPAT4*) and lipin 1 (*LPIN1*), involved in the esterification of FA to glycerol; and the transcription factors: sterol regulatory element binding transcription factor 1 (*SREBF1*) and insulin induced gene 1 (*INSIG1*). The qPCR was carried out (Bonnet et al., 2013) using a 7500 Real-Time PCR System (Applied Biosystems). To account for variations in RNA integrity and quantification and cDNA synthesis, mRNA abundance was normalized using the geometric mean of 3 reference genes: eukaryotic translation initiation factor 3 subunit K (*EIF3K*), peptidylprolyl isomerase A (*PPIA*) and ubiquitiously expressed prefoldinlike chaperone (*UXT*), which have been identified as suitable internal controls (Bionaz and Loor, 2007; Bonnet et al., 2013). Gene stability was examined using the geNorm (Vandesompele et al., 2002) and BestKeeper

procedures (Pfaffl et al., 2004). The primer sequences and qPCR performance are shown in Table 2. The abundance of gene transcripts was calculated using a 5-point calibration curve generated from the serial dilution of a cDNA pool, prepared by mixing equal volumes of all MT and MSC samples, and was expressed as the $\log_2$ value of mRNA copy number relative to the geometric mean of the 3 reference genes.

Calculations and Statistical Analysis

All statistical analyses were performed using the MIXED procedure of the SAS software package (version 9.4, SAS Institute Inc., Cary, NC). Animal performance and FA composition data were analyzed by one-way ANCOVA with a model that included the fixed effect of experimental diet (mean values over d 35 and 37) and measurements at the end of the adaptation period (mean values over d −2 and 0) as a covariate. Thus, covariate-adjusted least square means are reported. Transcript mRNA abundance data were analyzed by ANOVA to test the fixed effects of experimental diet (**D**), the RNA source (**S**) and their interaction (**D** × **S**). In all cases, animals were nested within the treatment and used as the error term to contrast the effect of treatments. Means were separated using the "pdiff" option of the "lsmeans" statement of the MIXED procedure. The CORR procedure was used to generate the Pearson correlation coefficient (r) for the association between qPCR results (quantification cycles, Cq) for the whole set of genes studied in MT and MSC.

RESULTS

Animal Performance and Milk Composition

Compared with the control, feeding fish oil decreased milk fat concentration (5.87 vs. 4.19%; $P < 0.01$; Table 3). However, no significant differences were detected in milk fat yield

($P > 0.10$; Table 3) due to the 31% increase in milk production in ewes fed this lipid supplement. The experimental diet had no effect on DMI or milk SCC ($P > 0.10$; Table 3).

Milk FA Composition

As expected, milk FA composition (Table 4) was significantly affected by the addition of fish oil to the diet, although it hardly modified the concentration of milk FA synthesized de novo in the mammary gland (including *cis*-9 10:1, *cis*-9 12:1 and *cis*-9 14:1), except for a reduction in 15:0 ($P < 0.05$). However, changes in some 16- and in 18-carbon intermediate metabolites were notable and included an increase in the milk percentage of two CLA isomers with potential (*trans*-9 *cis*-11 18:2) or confirmed (*trans*-10 *cis*-12 18:2) antilipogenic effects ($P < 0.05$). Although numerical differences in the candidate milk fat inhibitor *trans*-10 18:1 did not attain statistical significance ($P > 0.10$), FO supplementation increased the concentration of most other *trans* FA (e.g., *trans*-11 18:1, *cis*-9 *trans*-11 CLA, or *trans*-11 *cis*-15 and *trans*-11 *trans*-15 18:2; $P < 0.05$). The concomitant rise ($P < 0.001$) in milk 10-O-18:0, found only in trace amounts in the control, was also relevant.

On the other hand, pronounced decreases ($P < 0.01$) were detected in 18:0 and *cis*-9 18:1, as well as in 18:2n-6 and 18:3n-3. Concentrations of unsaturated FA present in the fish oil, such as *cis*-9 16:1, *cis*-11 18:1, 20:5n-3 and 22:6n-3, and their intermediate metabolites (e.g., 20:1 and 22:1 isomers) were higher in the milk of ewes on the FO treatment ($P < 0.05$).

mRNA Abundance of Candidate Genes

All RIN values were ≥7.7 and slightly greater in samples from ewes fed FO than in the control (on average 8.2 vs. 7.9, respectively; $P < 0.05$). No differences were observed due to the source of RNA, either MT or MSC (8.1 vs. 8.0; $P > 0.05$). On average, 15.8 μg of RNA were isolated from milk samples and 32.0 μg from biopsies ($P < 0.01$). The experimental diet

had no effect on the quantity of RNA obtained from each type of sample (on average, 26.7 vs. 21.0 μg for the control and FO treatments, respectively; $P > 0.10$).

The selected reference genes (i.e., *PPIA*, *EIF3K* and *UXT*) were confirmed as the most stable genes among those quantified, using both the geNorm and the BestKeeper procedures. Their mRNA abundance was not affected by dietary treatment ($P > 0.10$), but was lower in MSC than in MT ($P < 0.05$). As shown in Figure 1, the abundance of other transcripts was also lower in MSC (e.g., *ACSS1*, *ACACA*, *FASN* and *SREBF1*; $P < 0.05$), but reductions due to the experimental diet were similarly detected in both RNA sources.

Dietary fish oil supplementation decreased ($P < 0.05$) the mRNA abundance of candidate genes coding for enzymes involved in FA activation (*ACSS1*), de novo synthesis (*ACACA* and *FASN*) and esterification of FA to glycerol (*LPIN1*), as well as the *INSIG1* transcription factor ($P < 0.05$; Figure 1). The *LPL* gene (involved in FA uptake from plasma lipoproteins) showed an erratic behavior and the interaction diet × type of sample did not reach the required *P*-level to be considered significant ($P = 0.074$). Addition of FO to the diet did not significantly affect the transcript abundance of *FABP3*, *GPAT4*, *SCD* genes and *SREBF1* transcription factor ($P > 0.05$).

DISCUSSION

By crossing traditional boundaries between academic disciplines, nutrigenomics represents a valuable tool to move forward our knowledge of the nutritional regulation of mammary lipogenesis (Bauman et al., 2011; Bionaz et al., 2015). In dairy ruminants, however, much work remains to be done to unravel the complex nutrient-gene interactions. In this regard, isolating RNA from milk would be a useful and cost-effective tool that may avoid distress to animals and facilitate the collection of samples in time series experiments (Boutinaud et al., 2002; Mura et al., 2013; Suárez-Vega et al., 2015).

In line with the milk fat depression syndrome induced in ewes fed the diet supplemented with FO, our results showed a downregulation of a number of analyzed genes related to mammary lipid synthesis (i.e., *ACSS1*, *ACACA*, *FASN*, *LPIN1* and *INSIG1*) in the two evaluated RNA sources, MSC and MT biopsies. The results also showed stable transcript abundances in other candidate genes, such as *FABP3*, *GPAT4* or *SCD*. These consistent effects of FO in MSC and MT biopsies, together with the relationship between qPCR results in both type of samples ($r = 0.914$; $P < 0.001$), would support the feasibility of MSC as an alternative RNA source for nutrigenomic analyses in sheep.

Overall, these similarities agree with those reported in dairy goats (Boutinaud et al., 2002), dairy cows (Cánovas et al., 2014) and lactating beef cows (Murrieta et al., 2006). However, Angulo et al. (2012) observed a clear discrepancy in dairy cows between samples of MT and antibody-captured mammary epithelial cells from milk. This latter non-invasive technique did not allow to detect any of the significant reductions in the transcript abundance of lipogenic genes found in MT during marine lipid-induced MFD. Because potential inter-species and -breed differences appear unlikely in this case, divergent results among trials might be explained, at least to some extent, by methodological features in RNA extraction from milk affecting its quality (Cánovas et al., 2014).

In our study, RNA isolated from MSC showed good RIN values (8.0 on average) that were slightly better in the FO samples (8.1), which contrasts with the decrease in this quality indicator observed by Angulo et al. (2012) in cows consuming marine lipids. Further research would be necessary to elucidate whether this inconsistency is attributable to laboratory techniques or to their interaction with dietary treatments. In goats, we only know one available study on this subject and it reports no effect of palm oil supplementation on the RIN value of RNA obtained from MSC (Tudisco et al., 2015). In sheep, we are not aware of other nutrigenomic studies using non-invasive sampling methods, the information on the use of

milk as an RNA source being still too scant in small ruminants (Mura et al., 2013; Suárez-Vega et al., 2015).

Besides yielding good quality RNA, the technique used to isolate RNA from MSC (Suárez-Vega et al., 2015) resulted in a greater extraction efficiency (on average, 0.32 μg of RNA/mL of milk) than that previously reported in dairy sheep (on average, 0.02 μg of RNA/mL of milk; Mura et al., 2013), which might be accounted for by the sampling protocol maximizing the presence of somatic cells in milk (Gonzalo et al., 1994). This would be in line with the reported increase in epithelial cell concentration and extracted RNA quantity in goat milk samples collected 30 min after regular milking (Boutinaud et al., 2002).

In any event, transcript abundances were generally lower in MSC than in MT, not only for candidate but also for reference genes, even though the same initial amount of RNA was used in both types of samples. This may be speculated to be due, at least in part, to isolation of bacterial RNA in milk. In experiments with MSC samples from cows, bacterial RNA has been reported to represent up to 20-25% of total reads in RNA-sequencing analysis (Cánovas et al., 2014; Medrano et al., 2014).

Concerning the milk fat depression syndrome, which was elicited in this experiment as a mean to cause differences to study the feasibility of MSC and MT as RNA sources, a number of experiments conducted by our team have already examined the association between diet supplementation with marine lipids and MFD in dairy ewes (e.g., Toral et al., 2010, 2015a, 2016; Bichi et al., 2013b) and, therefore, this part will only be discussed briefly.

Addition of FO is seldom accompanied by other relevant changes in ewe performance (Capper et al., 2007; Bichi et al., 2013b; Toral et al., 2016) but, unexpectedly, it increased milk yield in this trial. This response might be related to the energy spared in FO due to the inhibition of mammary lipogenesis, as previously shown in CLA-induced MFD (Lock et al., 2006; Weerasinghe et al., 2012) but not in marine lipid-induced MFD (Capper et al., 2007;

Bichi et al., 2013b; Carreño et al., in press) in sheep. The large increase in milk production would explain the lack of changes in milk fat output, even though the associated reduction in milk fat percentage behaved as expected (Capper et al., 2007; Toral et al., 2010; Bichi et al., 2013b) and designed.

The origin of diet-induced MFD, and the underlying downregulation of mammary lipogenic genes, appears to lie in altered ruminal biohydrogenation pathways leading to increased outflow of specific FA that inhibit milk fat synthesis (Shingfield et al., 2010; Bauman et al., 2011). In our case, neither the limited increase in the concentration of some CLA isomers in milk (namely *trans*-10 *cis*-12 and *trans*-9 *cis*-11 18:2) nor the lack of variation in *trans*-10 18:1 allow to support a major role of these biohydrogenation metabolites (Gervais et al., 2009; Shingfield et al., 2010; Hussein et al., 2013). Some recent studies (e.g., Kairenius et al., 2015; Toral et al., 2015a, 2016) have suggested the contribution of less well-known antilipogenic compounds, such as certain FA supplied with fish oil, intermediates of altered 18:3n-3 biohydrogenation pathways, or oxo-FA. Our findings, in particular the increase in *cis*-9 16:1, *cis*-11 18:1, *trans*-11 *cis*-15 and *trans*-11 *trans*-15 18:2, and 10-O-18:0, would support this speculation.

Regarding the downregulation of some candidate genes involved in the lipid metabolism in the mammary gland, our results agree with previous studies in dairy ewes (Hussein et al., 2013; Carreño et al., in press) and cows (Peterson et al., 2003; Harvatine and Bauman, 2006; Bionaz et al., 2015) establishing a causal relationship between increases in antilipogenic FA in milk and this downregulation. However, some variations in gene expression are hardly correlated with other changes in milk FA composition. For example, decreased mRNA abundance of genes coding for enzymes related with de novo FA synthesis, through activation of acetate to acetyl-CoA (*ACSS1*), its subsequent carboxylation to malonyl-CoA (*ACACA*) and the elongation of the hydrocarbon chain up to 16:0 (*FASN*), had little

impact on the relative proportions of FA with 16 or less carbon atoms, mostly derived from this metabolic pathway (Bernard et al., 2008; Bionaz and Loor, 2008b). Previous reports on the effects of FO in dairy ewes have also shown limited variation in de novo FA concentrations in milk (Toral et al., 2015a, 2016; Carreño et al., in press), in contrast with the marked reduction caused by the consumption of plant lipids (e.g., Mele et al., 2006; Castro-Carrera et al., 2015). Furthermore, increased availability of preformed long-chain FA supplied with FO does not appear to be accompanied by evident improvements in mammary FA uptake in sheep. The decrease in *LPL* mRNA abundance in the FO treatment would support the inhibition of this metabolic pathway during MFD in sheep (Hussein et al., 2013; Carreño et al., in press). The statistical analysis did not show significant differences in the ability of MT or MSC samples to detect this inhibition (*P*-value for the interaction = 0.074). However, it is probably worth mentioning that the mRNA abundances of this gene presented a high deviation in MT samples collected from ewes fed the diet supplemented with FO (see Figure 1). This may be related to the fact that *LPL* is also expressed in mammary cell populations other than epithelial secretory cells, such as macrophages and interstitial cells (Camps et al., 1990; Bernard et al., 2008), and its mRNA abundance has been observed to be decreased by fish oil n-3 PUFA in human and murine macrophages (Michaud and Renier, 2001). In this regard, Boutinaud et al. (2013) suggested that the nonspecific expression of *LPL* may account for the lack of variation in its mRNA abundance in response to the effect of once daily milking in antibody-captured mammary epithelial cells in cows, compared with reductions found in biopsied MT.

No difference due to FO was detected in the transcript abundance of the other studied candidate gene with a role in FA uptake and transport, the *FABP3*. Available information of the nutritional regulation of this gene is very scarce and inconsistent, with reductions (Carreño et al., in press), no changes (Hussein et al., 2013) or increases (Invernizzi et al., 2010) during

MFD, which would disallow from considering that it has a major role in the low-fat milk syndrome.

Neither were there variations due to the effect of experimental diets on *SCD* abundance in any of the two RNA sources. This gene, which codes for the enzyme Δ^9-desaturase, plays a key role in mammary lipogenesis (Bernard et al., 2013) and has been shown to be downregulated in cows and ewes during MFD (Ahnadi et al., 2002; Angulo et al., 2012; Carreño et al., in press). Still, other authors report no changes or even increases in *SCD* transcript abundance in marine lipid-induced MFD (Harvatine and Bauman, 2006; Invernizzi et al., 2010; Bichi et al., 2013a), which may be attributed to the particular relevance of post-transcriptional events in the nutritional regulation of its expression (Bernard et al., 2013). Consistent with the mRNA results, the milk fat concentration of *cis*-9 10:1, *cis*-9 12:1 and *cis*-9 14:1 (which are synthesized almost exclusively in the mammary gland through Δ^9-desaturase activity; Bernard et al., 2013; Toral et al., 2015b) were not affect by FO. Changes in other milk *cis*-9-containing FA may most probably be explained by their supply with the FO (as it contains, for instance, approx. 4.5% of *cis*-9 16:1) or by differences in the availability of substrates for the SCD enzyme (e.g., 18:0, and *trans*-11 and *trans*-12 18:1).

Following the pathways of mammary lipogenesis, FA derived from de novo synthesis, plasma uptake and Δ^9-desaturation are almost entirely esterified to glycerol prior to secretion in milk fat globules (Bernard et al., 2008). The effect of FO on the two candidate genes (*LPIN1* and *GPAT4*) involved in triacylglycerol synthesis that we studied, namely a decrease in the mRNA abundance of the former and no variation in the latter, contrast with the opposite changes recorded by Carreño et al. (in press; no changes in *LPIN1* and decreases in *GPAT4*). Nonetheless, it has been shown that alterations in either of the two enzymes would be rate limiting (Takeuchi and Reue, 2009), which would lead to similar consequences regardless of the gene affected. In addition to catalyze the penultimate step in triacylglycerol

synthesis, *LPIN1* appears to interact with some transcription factors, such as *PPARA*, and have a key role in the expression of other lipogenic genes (Takeuchi and Reue, 2009). However, its involvement in dairy ruminants remains to be determined.

Available information concurs with a prominent function of *SREBF1* signalling pathway in the transcriptional regulation of mammary lipogenesis (Harvatine and Bauman, 2006; Bionaz et al., 2015; Xu et al., 2016). This would be in agreement with the lower transcript abundance of *INSIG1* with FO in both MSC and MT, as simultaneous decreases in the mRNA levels of this transcription factor and *SREBF1* are common features of MFD (Harvatine and Bauman, 2006; Hussein et al., 2013; Carreño et al., in press). Nevertheless, reductions in the latter due to FO were above the threshold for statistical significance in the present study (*P*-value for the effect of diet = 0.068). According to the potential role of *INSIG1* as inhibitor of SREBP1 protein activation (Bernard et al., 2008; Bauman et al., 2011), a negative feedback regulation mechanism can not be ruled out (Xu et al., 2016). However, decreased *INSIG1* expression did not appear to counteract the decrease in SREBP1 activity, as suggested by the reductions in the mRNA abundance of its target genes, such as, for example, *ACACA* or *FASN* (Bernard et al., 2008; Xu et al., 2016).

CONCLUSIONS

In ewes fed the diet supplemented with FO, downregulation of a number of genes related to mammary lipid synthesis (i.e., *ACSS1*, *ACACA*, *FASN*, *LPIN1* and *INSIG1*) is consistent with the elicited milk fat depression syndrome, whereas stable transcript abundances are showed in other candidate genes (i.e., *FABP3*, *GPAT4* and *SCD*). The steady effects of FO in both types of samples, milk somatic cells and mammary tissue biopsies, support the feasibility of milk as an alternative RNA source for nutrigenomic analyses in sheep.

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REFERENCES

Ahnadi, C. E., N. Beswick, L. Delbecchi, J. J. Kennelly, and P. Lacasse. 2002. Addition of fish oil to diets for dairy cows. II. Effects on milk fat and gene expression of mammary lipogenic enzymes. J. Dairy Res. 69:521-531.

Angulo, J., L. Mahecha, K. Nuernberg, G. Nuernberg, D. Dannenberger, M. Olivera, M. Boutinaud, C. Leroux, E. Albrecht, and L. Bernard. 2012. Effects of polyunsaturated fatty acids from plant oils and algae on milk fat yield and composition are associated with mammary lipogenic and SREBF1 gene expression. Animal 6:1961-1972.

Bauman, D. E., K. J. Harvatine, and A. L. Lock. 2011. Nutrigenomics, rumen-derived bioactive fatty acids, and the regulation of milk fat synthesis. Ann. Rev. Nutr. 31:299-319.

Bernard, L., C. Leroux, and Y. Chilliard. 2008. Expression and nutritional regulation of lipogenic genes in the ruminant lactating mammary gland. Adv. Exp. Med. Biol. 606:67-108.

Bernard, L., C. Leroux, and Y. Chilliard. 2013. Expression and nutritional regulation of stearoyl-CoA desaturase genes in the ruminant mammary gland: Relationship with milk fatty acid composition. Pages 161-193 in Stearoyl-CoA Desaturase Genes in Lipid Metabolism. J. M. Ntambi, ed. Springer Science+Business Media, New York, NY.

Bichi, E., P. Frutos, P. G. Toral, D. Keisler, G. Hervás, and J. J. Loor. 2013a. Dietary marine algae and its influence on tissue gene network expression during milk fat depression in dairy ewes. Anim. Feed Sci. Technol. 186:36-44.

Bichi, E., G. Hervás, P. G. Toral, J. J. Loor, and P. Frutos. 2013b. Milk fat depression induced by dietary marine algae in dairy ewes: Persistency of milk fatty acid composition and animal performance responses. J. Dairy Sci. 96:524-532.

Bionaz, M., and J. J. Loor. 2007. Identification of reference genes for quantitative real-time PCR in the bovine mammary gland during the lactation cycle. Physiol. Genomics 29:312-319.

Bionaz, M., and J. J. Loor. 2008a. *ACSL1*, *AGPAT6*, *FABP3*, *LPIN1*, and *SLC27A6* are the most abundant isoforms in bovine mammary tissue and their expression is affected by stage of lactation. J. Nutr. 138:1019−1024.

Bionaz, M. and J. J. Loor. 2008b. Gene networks driving bovine milk fat synthesis during the lactation cycle. BMC Genomics 9:366.

Bionaz, M., J. Osorio, and J. J. Loor. 2015. Nutrigenomics in dairy cows: Nutrients, transcription factors, and techniques. J. Anim. Sci. 93:5531-5553.

Bonnet, M., L. Bernard, S. Bes, and C. Leroux. 2013. Selection of reference genes for quantitative real-time PCR normalisation in adipose tissue, muscle, liver and mammary gland from ruminants. Animal 7:1344-1353.

Bonnet, M., C. Leroux, Y. Faulconnier, J. F. Hocquette, F. Bocquier, P. Martin, and Y. Chilliard. 2000. Lipoprotein lipase activity and mRNA are up-regulated by refeeding in adipose tissue and cardiac muscle of sheep. J. Nutr. 130:749-756.

Boutinaud, M., L. Galio, V. Lollivier, L. Finot, S. Wiart, D. Esquerre, and E. Devinoy. 2013. Unilateral once daily milking locally induces differential gene expression in both

mammary tissue and milk epithelial cells revealing mammary remodeling. Physiol. Genomics 45:973-985.

Boutinaud, M., H. Rulquin, D. H. Keisler, J. Djiane, and H. Jammes. 2002. Use of somatic cells from goat milk for dynamic studies of gene expression in the mammary gland. J. Anim. Sci. 80:1258-1269.

Camps, L., M. Reina, M. Llobera, S. Vilaró, and T. Olivecrona. 1990. Lipoprotein lipase: cellular origin and functional distribution. Am. J. Physiol. 258:C673-681.

Capper, J. L., R. G. Wilkinson, A. M. Mackenzie, and L. A. Sinclair. 2007. The effect of fish oil supplementation of pregnant and lactating ewes on milk production and lamb performance. Animal 1:889-898.

Cánovas, A., G. Rincón, C. Bevilacqua, A. Islas-Trejo, P. Brenaut, R. C. Hovey, M. Boutinaud, C. Morgenthaler, M. K. VanKlompenberg, P. Martin, and J. F. Medrano. 2014. Comparison of five different RNA sources to examine the lactating bovine mammary gland transcriptome using RNA-Sequencing. Sci. Rep. 4:5297.

Carreño, D., G. Hervás, P. G. Toral, T. Castro-Carrera and P. Frutos. Fish oil-induced milk fat depression and associated downregulation of mammary lipogenic genes in dairy ewes. J. Dairy Sci. (in press).

Castro-Carrera, T., P. Frutos, C. Leroux, Y. Chilliard, G. Hervás, A. Belenguer, L. Bernard, and P. G. Toral. 2015. Dietary sunflower oil modulates milk fatty acid composition without major changes in adipose and mammary tissue fatty acid profile or related gene mRNA abundance in sheep. Animal 9:582-591.

Gervais, R., J. W. McFadden, A. J. Lengi, B. A. Corl, and P. Y. Chouinard. 2009. Effects of intravenous infusion of *trans*-10, *ci*s-12 18:2 on mammary lipid metabolism in lactating dairy cows. J. Dairy Sci. 92:5167-5177.

Gonzalo, C., J. A. Carriedo, J. D. Gomez, L. D. Gomez, and F. San Primitivo. 1994. Diurnal variation in the somatic cell count of ewe milk. J. Dairy Sci. 77:1856-1859.

Harvatine, K. J., and D. E. Bauman. 2006. SREBP1 and thyroid hormone responsive spot 14 (S14) are involved in the regulation of bovine mammary lipid synthesis during diet-induced milk fat depression and treatment with CLA. J. Nutr. 136:2468-2474.

Hussein, M., K. H. Harvatine, W. M. P. B. Weerasinghe, L. A. Sinclair, and D. E. Bauman. 2013. Conjugated linoleic acid-induced milk fat depression in lactating ewes is accompanied by reduced expression of mammary genes involved in lipid synthesis. J. Dairy Sci. 96:3825-3834.

Invernizzi, G., B. J. Thering, M. A. McGuire, G. Savoini, and J. J. Loor. 2010. Sustained upregulation of stearoyl-CoA desaturase in bovine mammary tissue with contrasting changes in milk fat synthesis and lipogenic gene networks caused by lipid supplements. Funct. Integr. Genomics 10:561-575.

Kadegowda, A. K. G., M. Bionaz, B. Thering, L. S. Piperova, R. A. Erdman, and J. J. Loor. 2009. Identification of internal control genes for quantitative polymerase chain reaction in mammary tissue of lactating cows receiving lipid supplements. J. Dairy Sci. 92:2007-2019.

Kairenius, P., A. Ärölä, H. Leskinen, V. Toivonen, S. Ahvenjärvi, A. Vanhatalo, P. Huhtanen, T. Hurme, J. M. Griinari, and K. J. Shingfield. 2015. Dietary fish oil supplements depress milk fat yield and alter milk fatty acid composition in lactating cows fed grass silage based diets. J. Dairy Sci. 98:5653-5672.

Lock, A. L., B. M. Teles, J. W. Perfield, D. E. Bauman, and L. A. Sinclair. 2006. A conjugated linoleic acid supplement containing *trans*-10, *cis*-12 reduces milk fat synthesis in lactating sheep. J. Dairy Sci. 89:1525-1532.

Medrano, J. F., A. Cánovas, and A. Islas-Trejo. 2014. RNA sequencing for the analysis of complex traits in milk: detection of bacteria. Abstract 137 in Proceedings of the 10th World Congress on Genetics Applied to Livestock Production. Vancouver, Canada.

Mele, M., A. Buccioni, F. Petacchi, A. Serra, S. Banni, M. Antongiovanni, and P. Secchiari. 2006. Effect of forage/concentrate ratio and soybean oil supplementation on milk yield, and composition from Sarda ewes. Anim. Res. 55:273-285.

Michaud, S. E. and G. Renier. 2001. Direct regulatory effect of fatty acids on macrophage lipoprotein lipase. Potential role of PPARs. Diabetes 20:660-666.

Mura, M. C., C. Daga, S. Bodano, M. Paludo, S. Luridiana, M. Pazzola, M. L. Dettori, G. M. Vacca, and V. Carcangiu. 2013. Development of a RNA extraction method from milk for gene expression study in the mammary gland of sheep. Mol. Biol. Rep. 40:2169-2173.

Murrieta, C. M., B. W. Hess, E. J. Scholljegerdes, T. E. Engle, K. L. Hossner, G. E. Moss, and D. C. Rule. 2006. Evaluation of milk somatic cells as a source of mRNA for study of lipogenesis in the mammary gland of lactating beef cows supplemented with dietary high-linoleate safflower seeds. J. Anim. Sci. 84:2399-2405.

Peterson, D. G., E. A. Matitashvili, and D. E. Bauman. 2003. Diet-induced milk fat depression in dairy cows results in increased *trans*-10, *cis*-12 CLA in milk fat and coordinate suppression of mRNA abundance for mammary enzymes involved in milk fat synthesis. J. Nutr. 133:3098-3102.

Pfaffl, M. W., A. Tichopad, C. Prgomet, and T. P. Neuvians. 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper-Excel-based tool using pair-wise correlations. Biotechnol. Lett. 26:509-515.

Shingfield, K. J., S. Ahvenjärvi, V. Toivonen, A. Äröla, K. V. V. Nurmela, P. Huhtanen, and J. M. Griinari. 2003. Effect of dietary fish oil on biohydrogenation of fatty acids and milk fatty acid content in cows. Anim. Sci. 77:165-179.

Shingfield, K. J., L. Bernard, C. Leroux, and Y. Chilliard. 2010. Role of *trans* fatty acids in the nutritional regulation of mammary lipogenesis in ruminants. Animal 4:1140-1166

Suárez-Vega, A., B. Gutiérrez-Gil, C. Klopp, C. Robert-Granie, G. Tosser-Klopp, and J. J. Arranz. 2015. Characterization and comparative analysis of the milk transcriptome in two dairy sheep breeds using RNA sequencing. Sci. Rep. 5:18399.

Sukhija, P. S., and D. L. Palmquist. 1988. Rapid method for determination of total fatty-acid content and composition of feedstuffs and feces. J. Agric. Food Chem. 36:1202-1206.

Takeuchi, K., and K. Reue. 2009. Biochemistry, physiology, and genetics of GPAT, AGPAT, and lipin enzymes in triglyceride synthesis. Am. J. Physiol. Endocrinol. Metab. 296:E1195-E1209.

Toral, P. G., G. Hervás, D. Carreño, A. Belenguer, and P. Frutos. 2015a. Comparison of milk fatty acid responses during fish oil- and *trans*-10 *cis*-12 18:2-induced milk fat depression in dairy ewes. Anim. Feed Sci. Technol. 210:66-73.

Toral, P. G., G. Hervás, D. Carreño, and P. Frutos. 2016. Does supplemental 18:0 alleviate fish oil-induced milk fat depression in dairy ewes? J. Dairy Sci. 99:1133-1144.

Toral, P. G., G. Hervás, and P. Frutos. 2015b. Reductions in milk Δ^9-desaturation ratios to oral dosing of cobalt-acetate are accompanied by the downregulation of *SCD1* in lactating ewes. J. Dairy Sci. 98:1961-1971.

Toral, P. G., G. Hervás, P. Gómez-Cortés, P. Frutos, M. Juárez, and M. A. de la Fuente. 2010. Milk fatty acid profile and dairy sheep performance in response to diet supplementation with sunflower oil plus incremental levels of marine algae. J. Dairy Sci. 93:1655-1667.

Tudisco, R., B. Chiofalo, L. Addi, V. Lo Presti, R. Rao, S. Calabro', N. Musco, M. Grossi, M. I. Cutrignelli, V. Mastellone, P. Lombardi, and F. Infascelli. 2015. Effect of hydrogenated palm oil dietary supplementation on milk yield and composition, fatty acids profile and Stearoyl-CoA desaturase expression in goat milk. Small Rumin. Res. 132:72-78.

Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, and F. Speleman. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3:RESEARCH0034.

Weerasinghe, W. M. P. B., R. G. Wilkinson, A. L. Lock, M. J. de Veth, D. E. Bauman, and L. A. Sinclair. 2012. Effect of a supplement containing *trans*-10,*cis*-12 conjugated linoleic acid on the performance of dairy ewes fed 2 levels of metabolizable protein and at a restricted energy intake. J. Dairy Sci. 95:109-116.

Wickramasinghe, S., G. Rincon, A. Islas-Trejo, and J. Medrano. 2012. Transcriptional profiling of bovine milk using RNA sequencing. BMC Genomics 13:45.

Xu, H. F., J. Luo, W. S. Zhao, Y. C. Yang, H. B. Tian, H. B. Shi, and M. Bionaz. 2016. Overexpression of SREBP1 (sterol regulatory element binding protein 1) promotes de novo fatty acid synthesis and triacylglycerol accumulation in goat mammary epithelial cells. J. Dairy Sci. 99:783-795.

Table 1. Formulation and chemical composition of the TMR without lipid supplementation (control) or supplemented with 24 g of fish oil/kg DM (FO)

	TMR	
	Control	FO
Ingredients, g/kg of fresh matter		
Dehydrated alfalfa hay, particle size >4 cm	400	391
Whole corn grain	180	176
Whole barley grain	130	127
Soybean meal solvent 440 g CP/kg	150	147
Sugar beet pulp, pellets	70	68
Molasses, liquid	50	49
Fish oil[1]	0	22
Vitamin-mineral supplement	20	20
Chemical composition, g/kg DM		
OM	902	901
CP	176	171
NDF	305	306
ADF	208	209
Starch	171	167
Total FA	18.2	40.3
14:0	0.24	1.14
16:0	3.91	8.20
cis-9 16:1	0.04	1.06
18:0	0.80	1.93
cis-9 18:1	2.89	6.66
cis-11 18:1	0.22	0.95
18:2n-6	6.78	7.26
18:3n-3	2.22	2.39
20:5n-3	0.00	1.49
22:5n-3	0.00	0.34
22:6n-3	0.00	4.55

[1]Semirefined tuna and sardine oil (Afampes 121 DHA; Afamsa, Mos, Spain); contained (g/100 g total FA): 14:0 (4.0), 16:0 (19.2), *cis*-9 16:1 (4.5), 17:0 (1.5), 18:0 (5.0), *cis*-9 18:1 (16.8), *cis*-11 18:1 (3.2), 18:2n-6 (2.8), 18:3n-3 (1.0), *cis*-11 20:1 (1.8), 20:5n-3 (6.5), 22:5n-3 (1.5) and 22:6n-3 (19.9).

Table 2. Primer sequences and quantitative PCR performance of candidate genes and internal controls

Gene	Accession#[1]	Primers[2]	Nucleotide sequence (5' → 3')	Source[3]	Cq[4]	Slope[5]	(R^2)[6]	Efficiency[7]
ACACA	NM_001009256.1	F.2201 R.2319	ACCATGCTGGGAGTTGTCTGT AGAAGTGTATGAGCAGAGAGGACTTG	(1)	20.9 ± 0.11	−3.453	0.996	1.95
ACSS1	XM_015099680.1	F.683 R.789	CGAAGCCATAAAGATCTGTCCAT CCATCTCCTGCTCAAGAGAAACA	(1)	25.8 ± 0.06	−3.464	0.998	1.94
FABP3	AY157617.1	F.1954 R.2054	AGGGCAAGAACCCCAATTAAA CTCATTCCCATTCCTCTAGTTTTTG	(1)	26.1 ± 0.07	−3.571	0.995	1.91
FASN	XM_015098375.1	F.6383 R.6474	ACCTCGTGAAGGCTGTGACTCA TGAGTCGAGGCCAAGGTCTGAA	(3)	25.3 ± 0.15	−3.230	0.993	2.04
GPAT4	XM_004021798.3	F.1383 R.1483	ACTTCCATTACATCAGCCTGAGGC CGTGAAAGCGAGAGCTATCCTG	(2)	23.6 ± 0.05	−3.439	0.999	1.95
INSIG1	XM_015095466.1	F.524 R.620	GTCATCGCCACCATCTTCTC GACTGTCGATGCAGGGGTA	(4)	24.2 ± 0.05	−3.432	0.999	1.96
LPIN1	NM_001280700.1	F.2041 R.2141	TGGCCACCAGAATAAAGCATG GCTGACGCTGGACAACAGG	(5)	25.8 ± 0.07	−3.418	0.998	1.96
LPL	NM_001009394.1	F.942 R.1041	TGGAGATGTGGACCAGCTAGTG CCGGTAGGCCTTACTTGGATT	(1)	18.2 ± 0.08	−3.505	0.993	1.93
SCD	FJ513370.1	F.1100 R.1200	GATGACATCTATGACCCAACTTACCA CCCAAGTGTAACAGACCCATGA	(1)	15.7 ± 0.08	−3.351	0.996	1.99
SREBF1	GU206528	F.152 R.267	GGGACAAGGTTTGCTCACATG GGCAGCTTGTCAGTGTCCACTA	(1)	20.1 ± 0.07	−3.468	0.997	1.94
EIF3K	XM_004015230.2	F.368 R.492	CCAGGCCCACCAAGAAGAA TTATACCTTCCAGGAGGTCCATGT	(3)	20.8 ± 0.06	−3.467	0.999	1.94
PPIA	NM_001308578.1	F.176 R.295	GGATTTATGTGTCCAGGGTGGTGA CAAGATGCCAGGACCTGTATG	(6)	18.3 ± 0.06	−3.552	0.997	1.91
UXT	XM_004022128.3	F.417 R.517	TGTGGCCCTTGGATATGGTT GGTTGTCGCTGAGCTCTGTG	(7)	24.9 ± 0.05	−3.500	0.997	1.93

[1]Correspond to the best alignments for sequencing results of PCR amplification products, using the BLASTN from NCBI against nucleotide collection (nr/nt) [accessed May 15, 2016. http://blast.ncbi.nlm.nih.gov/].

[2]Primer direction (F – forward; R – reverse) and hybridization position on the sequence. The PCR annealing temperature was 60°C in all cases.

[3](1) Bichi et al. (2013a); (2) adapted from Hussein et al. (2013); (3) Kadegowda et al. (2009); (4) Hussein et al. (2013); (5) Bionaz and Loor (2008a); (6) Bonnet et al. (2000); (7) Bionaz and Loor (2007).

[4]Quantification cycle.

[5]Slope of the calibration curve.

[6]Coefficient of determination of the calibration curve.

[7]Calculated as $[10^{(-1 / \text{slope})}]$.

Table 3. Intake, milk yield, milk fat concentration and yield and SCC in lactating ewes fed the TMR without lipid supplementation (control) or supplemented with 24 g of fish oil/kg DM (FO)[1]

	Treatment			
	Control	FO	SED[2]	*P*-value
DMI, g/d	2,797	2,651	264.2	0.601
Milk, g/d	1,465	1,921	123.9	0.014
Fat, %	5.87	4.19	0.262	0.001
g/d	84.4	81.6	4.77	0.578
SCC, log_{10}/mL	4.84	4.61	0.127	0.126

[1]Mean values (covariate-adjusted least square means) over d 35 and 37.

[2]SED = standard error of the difference.

Table 4. Milk fatty acid (FA) composition in lactating ewes fed the TMR without lipid supplementation (control) or supplemented with 24 g of fish oil/kg DM (FO)[1]

	Treatment			
FA, g/100 g total FA	Control	FO	SED[2]	*P*-value
4:0	2.44	2.38	0.180	0.741
6:0	2.77	2.91	0.205	0.517
8:0	3.03	3.35	0.311	0.344
10:0	10.62	10.63	0.570	0.984
cis-9 10:1	0.26	0.31	0.026	0.154
12:0	6.15	5.92	0.243	0.392
cis-9 12:1	0.10	0.09	0.019	0.516
14:0	12.27	12.51	0.276	0.433
cis-9 14:1	0.22	0.20	0.040	0.651
15:0	0.98	0.85	0.048	0.048
16:0	25.96	25.71	1.286	0.855
cis-9 16:1	0.79	1.08	0.145	0.097
trans-6+7+8 16:1	0.07	0.30	0.057	0.011
trans-9 16:1	0.09	0.35	0.067	0.012
17:0	0.59	0.73	0.035	0.010
18:0	7.89	1.53	0.444	<0.001
10-O-18:0	<0.01	0.57	0.052	<0.001
cis-9 18:1[3]	12.25	6.75	0.682	<0.001
cis-11 18:1	0.59	0.99	0.026	<0.001
Σ *cis* 18:1	13.26	7.91	0.712	<0.001
trans-10 18:1	0.94	1.86	0.474	0.109
trans-11 18:1	1.28	4.84	0.951	0.013
Σ *trans* 18:1	2.77	8.58	0.719	<0.001
cis-9 *cis*-12 18:2	2.59	1.54	0.097	<0.001
cis-9 *trans*-12 18:2	0.07	0.13	0.022	0.038
trans-9 *cis*-12 18:2	0.03	0.06	0.009	0.011
trans-11 *cis*-15 18:2	0.05	0.42	0.019	<0.001
trans-11 *trans*-15 18:2	0.02	0.07	0.004	<0.001
Σ nonconjugated 18:2	2.93	2.43	0.100	0.004
cis-9 *trans*-11 CLA[4]	0.54	2.19	0.492	0.020
trans-9 *cis*-11 CLA	0.03	0.10	0.018	0.012
trans-10 *cis*-12 CLA	0.003	0.012	0.0020	0.007
Σ CLA	0.70	2.40	0.480	0.017
cis-9 *cis*-12 *cis*-15 18:3[5]	0.60	0.37	0.042	0.003
cis-11 20:1	0.04	0.29	0.012	<0.001
Σ 20:1	0.09	0.39	0.017	<0.001
cis-5 *cis*-8 *cis*-11 *cis*-14 *cis*-17 20:5	0.04	0.45	0.034	<0.001
Σ unsaturated C20	0.38	1.32	0.070	<0.001
Σ 22:1	0.04	0.26	0.018	<0.001
cis-4 *cis*-7 *cis*-10 *cis*-13 *cis*-16 22:5	0.04	0.14	0.026	0.011
cis-7 *cis*-10 *cis*-13 *cis*-16 *cis*-19 22:5	0.11	0.91	0.093	<0.001
cis-4 *cis*-7 *cis*-10 *cis*-13 *cis*-16 *cis*-19 22:6	0.04	0.92	0.036	<0.001
Σ unsaturated C22	0.27	2.30	0.139	<0.001

[1]Mean values (covariate-adjusted least square means) over d 35 and 37.

[2]SED = standard error of the difference.

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

[4]Contains *trans*-8 *cis*-10 18:2 and *trans*-7 *cis*-9 18:2 as minor components.

[5]Contains *trans*-11 20:1 as a minor component.

Figure 1. mRNA relative abundance (log_2 transformed data) of candidate genes in the mammary tissue (MT) and milk somatic cells (MSC) of lactating ewes fed the TMR without lipid supplementation (control; □) or supplemented with 24 g of fish oil/kg DM (FO; ■). Vertical bars represent the SEM. The statistical probabilities (*P*) for the effects of experimental diet (D), RNA source (S), and their interaction (D × S) are shown below each panel.