Changes in milk fatty acid profile and animal performance in response to fish oil supplementation, alone or in combination with sunflower oil, in dairy ewes

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ABSTRACT

Ruminant diet supplementation with sunflower oil (SO) and fish oil (FO) has been reported as a good strategy for enhancing some milk fat compounds such as conjugated linoleic acid (CLA) and n-3 polyunsaturated fatty acids in dairy cows, but no information is available regarding dairy sheep. In this work, ewe diet was supplemented with FO, alone or in combination with SO, with the aim of improving milk nutritional value and evaluating its effect on animal performance. Sixty-four Assaf ewes in mid lactation, fed a high-concentrate diet, were distributed in 8 lots of 8 animals each and assigned to 4 treatments (2 lots/treatment): no lipid supplementation (control) or supplementation with 20 g of SO/kg (SO), 10 g of FO/kg (FO), or 20 g of SO plus 10 g of FO/kg (SOFO). Milk production and composition, including a complete fatty acid profile, were analyzed on d 0, 3, 7, 14, 21, and 28 of treatments. Supplementation with FO tended to reduce dry matter intake compared with the control treatment (–15%), and its use in combination with SO (SOFO) resulted in a significant decrease in milk yield as well (–13%). All lipid supplements reduced milk protein content, and FO also reduced milk fat content by up to 21% alone (FO) and 27% in combination with SO (SOFO). Although the mechanisms involved in FO-induced milk fat depression are not yet well established, the observed increase in some milk *trans*-FA that are putative inhibitors of milk fat synthesis, such as *trans*-9,*cis*-11 CLA, and the 63% decrease in C18:0 (consistent with the theory of reduced milk fat fluidity) may be involved. When compared with the control, lipid supplementation remarkably improved the milk content of rumenic acid (*cis*-9,*trans*-11 CLA; up to 4-fold increases with SO and SOFO diets), whereas FO-containing diets also increased milk n-3 polyunsaturated fatty acids, mainly docosahexaenoic acid (with mean contents of 0.29 and 0.38% of total fatty acids for SOFO and FO, respective-

ly), and reduced the n-6:n-3 FA ratio to approximately half the control value. All lipid supplements resulted in high levels of some *trans*-FA, mainly *trans*-11 C18:1 (vaccenic acid) but also *trans*-10 C18:1.

Key words: conjugated linoleic acid, milk fat depression, n-3, sheep

INTRODUCTION

Marine lipids are rich in long-chain n-3 polyunsaturated fatty acids (PUFA) that, as potent inhibitors of *trans*-C18:1 ruminal reduction, promote the outflow of *trans*-11 C18:1 (vaccenic acid, VA), that is subsequently desaturated to *cis*-9,*trans*-11 C18:2 (rumenic acid, RA) in the mammary gland (Lock and Bauman, 2004). A combination of a high linoleic vegetable oil such as sunflower oil (SO), substrate for VA formation in the rumen, and marine lipids, such as fish oil (FO), is then considered a good nutritional strategy for enhancing *cis*-9,*trans*-11 conjugated linoleic acid and n-3 PUFA in milk fat (Palmquist and Griinari, 2006; Shingfield et al., 2006). Although FO inclusion in dairy cow diets has been associated with milk fat depression (MFD), initial objections to fat reduction have been put aside by consumers' increasing interest in healthier food products (Lock and Bauman, 2004; Shingfield et al., 2008) and the decreasing market value of milk fat, as well as the additional benefits to animal health (Griinari and Bauman, 2006). However, in contrast to dairy cow milk, ovine milk is almost entirely destined to cheese production and decreases in the solid fractions or alterations to the fat:protein ratio caused by lipid supplementation may have a negative effect on cheese yield and quality (Bocquier and Caja, 2001). Notwithstanding, some authors have reported that sheep seem less prone to MFD than cows when fed high-concentrate diets plus free vegetable oils (Pulina et al., 2006; Hervás et al., 2008).

Despite the promising results obtained for lipid inclusion in dairy ewe diets (Pulina et al., 2006) and the ample background about FO supplementation in dairy cows (Doreau and Chilliard, 1997; Palmquist and Griinari, 2006; Shingfield et al., 2006), few stud-

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Table 1. Ingredients and chemical composition of the experimental diets¹

Item	Control	SO	FO	SOFO
Ingredient (g/kg of fresh matter)				
Dehydrated alfalfa hay (particle size >4 cm)	200	196	198	194
Whole corn grain	250	245	247	242
Soybean meal	200	196	198	194
Whole barley grain	150	147	149	146
Beet pulp	90	88	89	87
Molasses	65	64	64	63
Salts ²	40	39	40	39
Minerals and vitamins ³	5	5	5	5
Sunflower oil ⁴	0	20	0	20
Fish oil ⁵	0	0	10	10
Chemical composition (g/kg of DM)				
OM	893	891	891	892
CP	205	208	203	203
NDF	226	222	220	219
ADF	125	121	122	120
Ether extract	31	54	41	67

¹Diets: control = with no oil supplementation; SO = supplemented with 20 g of sunflower oil/kg of fresh matter; FO = supplemented with 10 g of fish oil/kg of fresh matter; SOFO = supplemented with 20 g of sunflower oil plus 10 g of fish oil/kg of fresh matter.

²Contained: NaHCO₃ (375 g/kg), CaCO₃ (350 g/kg), Ca₂HPO₄ (150 g/kg), and mine salt (125 g/kg).

³INA OV1 (Evalis, Madrid, Spain).

⁴Contained (% total fatty acid methyl esters): C16:0 (7.5), C18:0 (4.3), C18:1 (26.3), and C18:2 (60.5).

⁵Semirefined tuna and sardine oil (Afampes 121 DHA, Afamsa, Vigo, Spain); contained (% total fatty acid methyl esters): C16:0 (21.4), C18:0 (5.9), C18:1 (14.9), C18:2 (1.7), C20:5 n-3 (6.3), and C22:6 n-3 (17.8).

ies have examined the effects of FO inclusion in the diet of lactating ewes (Kitessa et al., 2003; Capper et al., 2007) and, to date, the study by Reynolds et al. (2006) provides the only available information about diet supplementation with marine lipids (marine algae biomass oil) plus a high linoleic vegetable oil (soybean oil) in this species. On this basis and taking into account our previous works (Gómez-Cortés et al., 2008a; Hervás et al., 2008), we hypothesized that the combined use of SO and FO would result in a healthier milk fatty acid (FA) profile in dairy ewes, with the potential to increase CLA and n-3 PUFA concentrations. Therefore, this work was conducted with the aim of studying the effects of diet supplementation with FO, SO, and their combination (SOFO) on animal performance and milk FA composition in dairy ewes.

MATERIALS AND METHODS

Animals and Experimental Diets

Sixty-four multiparous Assaf ewes (BW = 86.4 ± 1.21 kg) in mid lactation (at wk 12 at the beginning of the experiment) were used. The ewes were distributed in 8 lots of 8 animals each, balanced for milk yield, BW, days postpartum, and number of lactation, and allocated at random to 4 experimental treatments (2 lots per treatment): control, supplemented with SO, supplemented with FO, and supplemented with SOFO.

The diets, prepared in a feed mixer every week, consisted of a TMR based on alfalfa hay and a concentrate with no supplementation (control) or supplemented with 20 g of SO/kg of fresh matter (SO diet), 10 g of FO/kg of fresh matter (FO diet), and 20 g of SO plus 10 g of FO/kg of fresh matter (SOFO diet). The ingredients and chemical composition of the 4 experimental diets, which included molasses to avoid selection of dietary components, are given in Table 1. During a 3-wk adaptation period (before the beginning of the trial), all the animals received the control diet. Clean water and a vitamin and mineral supplement were always available and fresh diets were offered daily ad libitum at 0900 and 1900 h.

The ewes were milked at approximately 0830 and 1830 h in a 1 × 10 stall milking parlor (DeLaval, Madrid, Spain). The experiment lasted for 4 wk and was carried out in accordance with Spanish Royal Decree 1201/2005 for the protection of animals used for experimental purposes.

Measurements, Sample Collection, and Chemical Analyses

Samples of offered and refused diets were collected once a week, stored at -30°C, and then freeze-dried. The DMI was therefore recorded weekly for each experimental lot. Samples were analyzed for DM (ISO, 1999a), ash (ISO, 2002a), and CP (ISO, 2005). Neutral

detergent fiber and ADF were determined by the methods described by Ankom Technology (2006a,b). Neutral detergent fiber was assayed with sodium sulfite and α -amylase and expressed with residual ash (the latter also for ADF). The content of ether extract in the diets was determined by the Ankom filter bag technology (American Oil Chemists' Society Official Procedure Am 5-04; AOCS, 2008).

Individual milk yield was recorded on d 0, 3, 7, 14, 21, and 28, both at morning and evening milkings. With the same frequency, chosen to study time-dependent responses to lipid supplementation, milk samples for the analysis of fat, protein, and TS were collected from each animal and treated with natamycin. The protein, fat, and TS concentrations were determined by infrared spectrophotometry (ISO, 1999b) using a MilkoScan 255 A/S N (Foss Electric, Hillerød, Denmark).

Milk FA composition was determined in untreated samples from each experimental lot, composited according to individual milk production. Milk fat was extracted following the method described by Luna et al. (2005) and fatty acid methyl esters (**FAME**) were prepared by base-catalyzed methanolysis of the glycerides (ISO, 2002b). Analysis of FAME in hexane was performed on a gas chromatograph (Agilent 6890 N Network System, Palo Alto, CA) with an autoinjector and fitted with a flame ionization detector. The FAME profile was determined by split injection (1:100) onto a CP-Sil 88 fused silica capillary column (100 m $\times$ 0.25 mm i.d., 0.20 μ m film thickness; Varian, Midlandburg, the Netherlands) using the same programmed temperature gradient method described in Hervás et al. (2008). Finally, discrimination between *trans*-7,*cis*-9, and *cis*-9,*trans*-11 CLA isomers was achieved by silver ion HPLC according to the procedure described by Gómez-Cortés et al. (2008a).

At the end of the experiment, on the morning of d 29, ewes were milked and then given free access to the diets as on other days. After 2 h, the feed was removed, and 3 h later, samples of ruminal fluid were individually collected from 4 animals per treatment using a stomach tube and visually checked to ensure that the samples did not contain saliva. Samples were strained through 2 layers of muslin, the pH was immediately measured, and aliquots were collected for ammonia and VFA analysis (see Toral et al., 2009).

Statistical Analyses

All analyses were performed using SAS (version 9.1, SAS Institute Inc., Cary, NC). Data on DMI, milk yield and composition, and FA composition were analyzed by repeated measurement analysis using the MIXED procedure of SAS and assuming a covariance structure on

the basis of Schwarz's Bayesian information model fit criteria. The statistical model included the fixed effects of diet, time, their interaction, and the initial record measured at 0 wk (covariate). For all data collected either individually (milk yield and composition) or per lot (DMI and FA composition), the lot was nested within the diet to contrast the effect of lipid supplementation. Pearson correlation coefficients were generated for associations between some FA, milk yield, and fat content using the CORR procedure of SAS. Data from ruminal fermentation parameters were analyzed by one-way ANOVA using the MIXED procedure. Differences were declared significant at $P < 0.05$, with values of $P < 0.10$ being interpreted as a trend toward significance. Least squares means (adjusted for the covariance) are reported throughout.

RESULTS AND DISCUSSION

Ewe Performance and Ruminal Fermentation

As shown in Table 2, FO supplementation (both individually and in combination with SO) tended to reduce DMI in comparison with the control diet ($P < 0.10$), whereas there were no differences between the control and SO diets. Previous studies in sheep have reported significant decreases in feed intake when using unprotected FO, either as a supplement in the diet (30 g/kg of DM; Kitessa et al., 2001) or by rumen injection (42 g/kg of DM; Fievez et al., 2003), but no decrease when using SO (60 g/kg of DM; Hervás et al., 2008) or protected FO (30 g/kg of DM; Kitessa et al., 2001). The depression in DMI because of unprotected FO supplementation has been attributed to increases in some biohydrogenation intermediaries that are potentially toxic for rumen microbiota (Kitessa et al., 2001). In the present work, the greatest reduction in DMI was observed for the SOFO treatment (-19%), which might have been produced not only by the FO itself but also by the higher level of total oil inclusion in a low-forage diet. However, in this trial, decreased DMI was not associated with any impairment of ruminal fermentation (Table 3). Thus, for instance, rumen pH was not significantly affected by oil supplementation ($P > 0.10$), in agreement with other studies in sheep fed FO (Fievez et al., 2003), SO (Hervás et al., 2008), and SOFO (Toral et al., 2009), and which may be related to an acceptable level of effective fiber in the 4 diets (Mertens, 1997) because of the particle size of the alfalfa hay (>4 cm) and the supply of cereals as whole grains. Furthermore, lipid supplementation did not significantly affect either ammonia or VFA ruminal concentrations ($P > 0.10$). The SO diet results are consistent with other findings for this oil reported in sheep (6% DM; Hervás et al., 2008).

Table 2. Dry matter intake, milk yield, and milk composition in dairy ewes fed different oil supplements

Item	Diet ¹				SED ²	P-value ³		
	Control	SO	FO	SOFO		D	T	D × T
DMI (g/d)	3,103	2,849	2,750	2,521	157.7	†	***	†
Yield (g/d)								
Milk	2,661 ^a	2,633 ^a	2,626 ^a	2,299 ^b	65.1	*	***	NS
Protein	133.9 ^a	127.8 ^a	128.1 ^a	109.3 ^b	2.51	**	***	†
Fat	151.4 ^a	153.3 ^a	134.8 ^b	119.7 ^c	3.31	**	***	***
TS	443.1 ^a	434.6 ^{ab}	418.1 ^b	363.4 ^c	6.16	***	***	**
Composition (g/100 g of raw milk)								
Protein	5.17 ^a	4.89 ^b	4.88 ^b	4.73 ^b	0.064	*	***	NS
Fat	5.89 ^a	5.90 ^a	5.15 ^b	5.14 ^b	0.197	*	***	***
TS	16.91 ^a	16.66 ^{ab}	15.98 ^{bc}	15.73 ^c	0.247	*	***	***

^{a-c}Means within a row with different superscripts differ significantly ($P < 0.05$).

¹Diets: control = with no oil supplementation; SO = supplemented with 20 g of sunflower oil/kg of fresh matter; FO = supplemented with 10 g of fish oil/kg of fresh matter; SOFO = supplemented with 20 g of sunflower oil plus 10 g of fish oil/kg of fresh matter.

²SED = standard error of the difference.

³Probability of significant effects of experimental diet (D), time on diet (T), and their interaction (D × T). NS = not significant ($P > 0.10$).

† $P < 0.10$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

and cows (up to 3.4% DM doses; Shingfield et al., 2008). Regarding FO, rumen injection of moderate amounts of this oil in sheep (4.2% DM; Fievez et al., 2003) did not cause any significant variation in total VFA either but induced a decrease in the acetate:propionate ratio. The lower level of oil inclusion in the present study might explain why ruminal fermentation was not negatively affected because the amount of supplemental lipids in the diet has been shown to be a determinant factor in producing these effects (Shingfield et al., 2008). Similar levels of SO plus FO to those used in our work (different combinations supplying 3% DM) were studied in dairy cows and sheep (Palmquist and Griinari, 2006; Toral et al., 2009), with transient variations in fermentation parameters that disappeared 6 h postfeeding being attributed to rumen microorganism adaptation and development of tolerance to potentially toxic FA. This

would have been facilitated in the present work by the longer period on treatments.

Concerning milk production and composition (Table 2), the lower DMI observed for the SOFO diet (−19%) would explain the lower milk yield of the animals on this treatment (−13%; $P < 0.05$). The reductions in protein content caused by all oil supplements ($P < 0.05$) is something frequently observed in dairy ewes (Bocquier and Caja, 2001; Pulina et al., 2006). In the present study, this decrease cannot be related to a dilution effect resulting from an increase in milk yield but rather can probably be related to reduced AA availability to the mammary gland or an induction of insulin resistance (Pulina et al., 2006). It is worthy of note that this decrease in milk protein concentration balances the natural low fat content of milk from high-production Assaf ewes under intensive production systems and

Table 3. Ruminal pH and ammonia and VFA concentrations in ewes fed different oil supplements

Item	Diet ¹				SED ²	P-value ³
	Control	SO	FO	SOFO		
pH	6.6	6.6	6.7	6.5	0.20	NS
Ammonia (mg/L)	109.2	92.5	93.1	98.9	38.69	NS
Total VFA (mmol/L)	125.2	110.4	108.3	130.5	16.64	NS
Acetate	75.2	61.4	61.0	75.1	12.67	NS
Propionate	30.3	25.0	27.1	27.1	4.02	NS
Butyrate	16.2	19.7	17.3	23.2	4.19	NS
Acetate:propionate	2.5	2.5	2.3	2.8	0.53	NS

¹Diets: control = with no oil supplementation; SO = supplemented with 20 g of sunflower oil/kg of fresh matter; FO = supplemented with 10 g of fish oil/kg of fresh matter; SOFO = supplemented with 20 g of sunflower oil plus 10 g of fish oil/kg of fresh matter.

²SED = standard error of the difference.

³Probability of significant effects of experimental diet. NS = not significant ($P > 0.10$).

improves the fat:protein ratio, an important parameter in achieving adequate fat content in cheese for manufacturing processes and ripening properties (Bocquier and Caja, 2001).

In relation to milk fat, the significant interaction diet $\times$ time showed the different responses to diet oil addition (Figure 1); supplementation with SO and other unprotected vegetable oils has often been observed to have either no effect or positive effects on milk fat content (Pulina et al., 2006; Gómez-Cortés et al., 2008a; Hervás et al., 2008), whereas unprotected FO supply would reduce both milk fat content and yield (Capper et al., 2007). Although the reasons for these different responses are still uncertain, some authors have reported that lactating sheep might be less sensitive than cows to some MFD-inducing factors, such as high-concentrate diets, probably because of their ability to maintain normal rumen function (Pulina et al., 2006).

Saturated and Monoenoic FA

Table 4 shows the FA profile of milk in ewes fed either the control or the supplemented diets. Milk fat composition was markedly changed by the type of feed and the time on the diet, with significant diet $\times$ time interactions for numerous FA.

All supplemented diets decreased the proportion of even-chain saturated FA but via different mechanisms. Diets containing SO reduced C6:0 to C16:0 FA (Figure 2), probably because of the potential inhibitory effect of the dietary PUFA or its metabolites on de novo FA synthesis in the mammary gland (Palmquist and Griinari, 2006; Kadegowda et al., 2009) or a dilution effect.

As observed in Figure 2, FO affected only C18:0 content, inducing a sharp decrease that was maintained throughout the whole period monitored ($P < 0.001$). This was probably caused by the action of the long-chain n-3 PUFA present in FO, mainly docosahexaenoic acid (DHA), a potent inhibitor of *trans*-C18:1 ruminal reduction, that would have induced the accumulation of VA in the rumen (Lor et al., 2005; Palmquist et al., 2005). Thus, FO treatment resulted in increased milk VA content ($P < 0.01$), with further improvements when combined with SO (SOFO). However, the latter produced a greater increase in milk VA only during the first week of lipid supplementation but then stabilized at levels similar to those corresponding to the SO diet (i.e., almost 4 times higher than the control) throughout the rest of the experiment (Figure 1).

The interest in increasing VA concentration comes from its role as substrate for RA endogenous synthesis, not only in the ruminant mammary gland but also in human tissues (Palmquist et al., 2005). It has been reported that high levels of this FA in cow milk could

be better sustained with high levels of forage, or low lipid supplementation, or both (Palmquist et al., 2005; Cruz-Hernandez et al., 2007). Nevertheless, it is worth noting that, in ewes, the addition of lipids to a high-concentrate diet produced an increased and stable level of milk VA after 2 wk of supplementation (Figure 1).

As shown in Figure 1, *trans*-10 C18:1 behaved differently; its largest increase was observed in wk 2 for FO and SOFO treatments and it tended to reach a plateau afterward ($P < 0.01$). Large increases in this *trans*-C18:1 concentration would suggest important changes in ruminal biohydrogenation pathways in FO-containing diets, as previously observed in cows (Shingfield et al., 2006; Cruz-Hernandez et al., 2007). The increases observed for other *trans*-C18:1 isomers (*trans*-6+7+8, *trans*-9, and *trans*-12; Figure 3) in the SO diet were similar to those found with olive oil supplementation in ewes (Gómez-Cortés et al., 2008b) and could derive from the biohydrogenation of SO oleic acid (26% of total FA) involving the formation of several positional isomers of *trans*-monoene (Mosley et al., 2002; Jenkins et al., 2008). A potential inhibitory effect of FO on the oleic acid biohydrogenation pathway might explain the lower milk content of these *trans*-monoene in the FO treatment. Notwithstanding, treatments supplying FO did also result in a significant diminution of *cis*-C18:1 FA content, the reduction in oleic acid (*cis*-9 C18:1) content being more pronounced when this marine oil was fed alone ($P < 0.01$; Figure 2), probably because of lower supply of stearic acid for endogenous oleic acid synthesis in the mammary gland (Shingfield and Griinari, 2007).

A significant ($P < 0.05$) reduction of most odd- and branched-chain FA concentrations was found for all lipid-supplemented diets, but it was quantitatively of small magnitude.

Conjugated and Nonconjugated Linoleic Acid

Rumenic acid was the most abundant CLA isomer and its changes clearly mirrored those of VA (Figure 1), with no differences between SO and SOFO diets after the second week of supplementation, when the RA content in the milk appeared to be stabilized and represented, on average, a 3.5-fold increase compared with the control. Though not as extensively investigated as in dairy cows, where direct measurements have evidenced the importance of its endogenous synthesis in the mammary gland (Palmquist et al., 2005), the strong relationship observed between RA and VA contents ($r = 0.97$; $P < 0.001$; Table 5) might support the hypothesis that dairy ewes do not differ from cows in this regard (Pulina et al., 2006). After 7 d on SOFO treatment, the milk RA content was among the high-

Figure 1. Temporal changes in milk fat content, *trans-10,cis-12* C18:2, *trans-9,cis-11* C18:2, *cis-9,trans-11* C18:2, *trans-10* C18:1, and *trans-11* C18:1 [g/100 g of fatty acids methyl esters (FAME)] in ewes fed diets with no supplementation (control) or supplemented with 20 g of sunflower oil/kg (SO), 10 g of fish oil/kg (FO), and 20 g of sunflower oil plus 10 g of fish oil/kg (SOFO). Values represent the mean from 2 lots of 8 animals per lot. SE of the difference = 0.197, 0.002, 0.008, 0.141, 0.248, and 0.591 for milk fat content, *trans-10,cis-12* C18:2, *trans-9,cis-11* C18:2, *cis-9,trans-11* C18:2, *trans-10* C18:1, and *trans-11* C18:1, respectively.

est (4.3% total FA) reported for ewes (Pulina et al., 2006; Reynolds et al., 2006; Gómez-Cortés et al., 2008a; Hervás et al., 2008), but after 2 wk of supplementation, SO and SOFO diets resulted in average milk RA increases (2.7% of total FA).

Changes in the mean concentrations of other CLA isomers differed substantially from that of RA, and only *trans-7,cis-9* CLA exceeded 0.10% of total FA in the case of the SO diet (Figure 3). The biological importance of this FA has received little attention but

its synthesis in cows seems to be endogenous in the mammary gland because its precursor, *trans-7* C18:1, is a substrate for Δ^9 desaturase (Palmquist et al., 2005), in contrast to other CLA isomers that appear to be exclusively of rumen origin (Lock and Bauman, 2004). *Trans-7,cis-9* CLA was highly correlated with the unresolved GC peak grouping *trans-6+7+8* C18:1 isomers ($r = 0.98$; $P < 0.001$), suggesting that *trans-7* C18:1 conversion might represent a major pathway for its formation in ewes. Both *trans-7,cis-9* CLA and *trans-7*

Table 4. Fatty acid profile of the milk from ewes fed different oil supplements

Fatty acid (g/100 g of total fatty acid methyl esters)	Diet ¹				SED ²	P-value ³		
	Control	SO	FO	SOFO		D	T	D × T
Saturated								
4:0	3.26 ^c	3.68 ^a	3.38 ^b	3.58 ^a	0.026	**	***	**
6:0	3.34 ^{ab}	3.15 ^{bc}	3.52 ^a	2.97 ^c	0.070	*	***	***
8:0	3.24 ^a	2.75 ^b	3.50 ^a	2.68 ^b	0.080	**	***	***
10:0	10.69 ^a	8.02 ^b	10.86 ^a	7.87 ^b	0.279	**	***	***
12:0	6.10 ^a	4.26 ^b	5.90 ^a	4.41 ^b	0.195	**	***	***
13:0 <i>anteiso</i>	0.07 ^a	0.04 ^b	0.06 ^a	0.05 ^b	0.003	*	NS	NS
13:0	0.23 ^a	0.14 ^c	0.20 ^b	0.14 ^c	0.008	**	***	***
14:0 <i>iso</i>	0.09 ^a	0.08 ^b	0.07 ^b	0.06 ^c	0.002	**	***	NS
14:0	12.18	10.72	11.74	10.80	0.365	NS	**	NS
15:0 <i>iso</i>	0.19	0.18	0.15	0.16	0.010	NS	**	NS
15:0 <i>anteiso</i>	0.41 ^a	0.32 ^b	0.32 ^b	0.28 ^c	0.010	**	***	*
15:0	0.95 ^a	0.73 ^c	0.86 ^b	0.71 ^c	0.009	***	***	**
16:0 <i>iso</i>	0.35 ^a	0.28 ^b	0.28 ^b	0.23 ^c	0.007	**	**	NS
16:0	27.03 ^a	24.05 ^b	26.53 ^a	24.15 ^b	0.399	*	***	***
17:0	0.50 ^a	0.42 ^b	0.50 ^a	0.45 ^b	0.011	*	***	**
18:0	5.80 ^a	6.21 ^a	2.04 ^b	2.45 ^b	0.334	**	***	***
19:0	0.06 ^a	0.04 ^b	0.05 ^b	0.04 ^b	0.003	*	NS	NS
20:0	0.15 ^{ab}	0.16 ^a	0.13 ^b	0.14 ^b	0.004	*	*	NS
21:0	0.04	0.04	0.04	0.04	0.001	†	NS	NS
22:0	0.08 ^b	0.10 ^a	0.08 ^b	0.10 ^a	0.004	*	***	***
Monounsaturated								
10:1	0.47 ^a	0.33 ^b	0.42 ^a	0.30 ^b	0.016	**	***	***
<i>cis</i> 14:1	0.22	0.26	0.25	0.23	0.012	NS	***	NS
15:1	0.11 ^b	0.09 ^c	0.14 ^a	0.11 ^b	0.004	**	NS	†
<i>trans</i> -8 16:1	0.06 ^d	0.10 ^c	0.16 ^b	0.24 ^a	0.007	***	***	***
<i>trans</i> -9 16:1 + 17:0 <i>iso</i>	0.41 ^b	0.76 ^a	0.54 ^b	0.82 ^a	0.037	**	***	***
<i>cis</i> -7 16:1	0.28	0.27	0.29	0.28	0.011	NS	†	**
<i>cis</i> -9 16:1 + 17:0 <i>anteiso</i>	1.39 ^b	1.26 ^c	1.66 ^a	1.43 ^b	0.023	***	***	NS
<i>cis</i> -13 16:1	0.17 ^a	0.09 ^c	0.12 ^b	0.08 ^c	0.008	**	***	***
17:1	0.18 ^a	0.14 ^c	0.17 ^b	0.16 ^b	0.003	**	NS	NS
<i>trans</i> -6+7+8 18:1	0.30 ^b	0.56 ^a	0.18 ^d	0.26 ^c	0.004	***	*	***
<i>trans</i> -9 18:1	0.27 ^c	0.56 ^a	0.25 ^c	0.32 ^b	0.006	***	***	***
<i>trans</i> -10 18:1	0.88 ^d	2.11 ^c	4.34 ^b	6.48 ^a	0.248	***	***	**
<i>trans</i> -11 18:1 (vaccenic acid)	1.58 ^c	5.86 ^{ab}	4.47 ^b	8.05 ^a	0.591	**	***	***
<i>trans</i> -12 18:1	0.33 ^c	0.64 ^a	0.32 ^c	0.48 ^b	0.014	***	***	***
<i>cis</i> -9 18:1	12.43 ^a	13.53 ^a	9.02 ^c	10.96 ^b	0.363	**	***	***
<i>cis</i> -11 + <i>trans</i> -15 18:1	0.52 ^b	0.64 ^a	0.65 ^a	0.67 ^a	0.016	*	***	***
<i>cis</i> -12 18:1	0.36 ^b	0.66 ^a	0.09 ^c	0.11 ^c	0.049	**	NS	***
<i>cis</i> -13 18:1	0.08 ^{ab}	0.09 ^a	0.06 ^c	0.06 ^{bc}	0.004	*	*	NS
<i>cis</i> -14 + <i>trans</i> -16 18:1	0.34 ^b	0.40 ^a	0.11 ^c	0.14 ^c	0.014	***	***	***
<i>cis</i> -15 18:1	0.07 ^b	0.10 ^a	0.07 ^b	0.07 ^b	0.004	*	*	NS
<i>cis</i> -11 20:1	0.07 ^d	0.11 ^c	0.33 ^a	0.29 ^b	0.006	***	**	NS
<i>cis</i> -13 22:1	0.02 ^b	0.01 ^b	0.10 ^a	0.09 ^a	0.006	***	***	***
Nonconjugated C18:2								
<i>trans</i> -9, <i>cis</i> -13	0.09 ^a	0.09 ^a	0.07 ^b	0.06 ^b	0.003	**	***	***
<i>trans</i> -8, <i>cis</i> -12	0.16 ^a	0.20 ^a	0.08 ^b	0.10 ^b	0.012	**	***	***
<i>trans</i> -8, <i>cis</i> -13	0.09 ^a	0.10 ^a	0.04 ^c	0.06 ^b	0.004	**	**	***
<i>cis</i> -9, <i>trans</i> -12	0.08 ^b	0.12 ^a	0.05 ^c	0.07 ^b	0.004	**	***	***
<i>trans</i> -9, <i>cis</i> -12	0.03 ^c	0.06 ^b	0.06 ^b	0.09 ^a	0.002	***	***	***
<i>trans</i> -11, <i>cis</i> -15	0.05 ^b	0.10 ^b	0.46 ^a	0.44 ^a	0.012	***	***	***
<i>cis</i> -9, <i>cis</i> -12	2.64	2.77	2.47	2.49	0.065	†	***	***
Conjugated C18:2								
<i>cis</i> -9, <i>trans</i> -11 (rumenic acid)	0.64 ^c	2.31 ^a	1.66 ^b	2.83 ^a	0.141	**	***	***
<i>trans</i> -7, <i>cis</i> -9	0.06 ^b	0.12 ^a	0.03 ^d	0.04 ^c	0.002	***	**	**
<i>trans</i> -9, <i>cis</i> -11	0.02 ^c	0.04 ^{bc}	0.06 ^b	0.10 ^a	0.008	**	***	***
<i>trans</i> -10, <i>cis</i> -12	0.01	0.01	0.01	0.01	0.002	NS	NS	NS
<i>trans</i> -11, <i>cis</i> -13	0.01	0.01	0.01	0.01	0.001	NS	NS	NS
<i>trans</i> -11, <i>trans</i> -13	0.03 ^a	0.02 ^c	0.02 ^b	0.01 ^d	0.001	***	NS	NS
Other <i>trans-trans</i>	0.02 ^b	0.04 ^a	0.02 ^b	0.04 ^a	0.002	*	***	**
Other polyunsaturated								
18:3 n-6	0.08 ^a	0.05 ^b	0.04 ^b	0.03 ^c	0.002	***	***	***
18:3 n-3	0.33 ^a	0.27 ^b	0.34 ^a	0.26 ^b	0.008	**	***	*
20:2 n-6	0.02 ^c	0.02 ^c	0.04 ^a	0.04 ^b	0.002	**	***	***

Continued

Table 4 (Continued). Fatty acid profile of the milk from ewes fed different oil supplements

Fatty acid (g/100 g of total fatty acid methyl esters)	Diet ¹				SED ²	P-value ³		
	Control	SO	FO	SOFO		D	T	D × T
20:3 n-6	0.02 ^b	0.02 ^b	0.03 ^a	0.03 ^a	0.001	*	***	**
20:3 n-3	0.02 ^c	0.02 ^c	0.05 ^a	0.05 ^b	0.001	***	***	***
20:4 n-6	0.13	0.11	0.15	0.15	0.007	†	***	***
20:5 n-3 (eicosapentaenoic acid)	0.03 ^c	0.02 ^c	0.15 ^a	0.10 ^b	0.005	***	***	***
22:5 n-3 (docosapentaenoic acid)	0.06 ^c	0.04 ^c	0.18 ^a	0.15 ^b	0.006	***	***	***
22:6 n-3 (docosahexaenoic acid)	0.02 ^c	0.02 ^c	0.38 ^a	0.29 ^b	0.013	***	***	***
n-6:n-3 ratio	6.69 ^b	8.14 ^a	2.97 ^d	3.47 ^c	0.104	***	***	***

^{a-d}Means within a row with different superscripts differ significantly ($P < 0.05$).

¹Diets: control = with no oil supplementation; SO = supplemented with 20 g of sunflower oil/kg of fresh matter; FO = supplemented with 10 g of fish oil/kg of fresh matter; SOFO = supplemented with 20 g of sunflower oil plus 10 g of fish oil/kg of fresh matter.

²SED = standard error of the difference.

³Probability of significant effects of experimental diet (D), time on diet (T), and their interaction (D × T). NS = not significant ($P > 0.10$).

† $P < 0.10$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

C18:1 were decreased in FO and SOFO treatments, whereas *trans*-9,*cis*-11 CLA was increased ($P < 0.01$), as is more extensively discussed below. The milk fat content of *trans*-10,*cis*-12 C18:2 was negligible in all

treatments and remained so throughout the experiment ($P > 0.10$; Figure 1).

Regarding nonconjugated C18:2 FA, linoleic acid concentration was little modified and did not increase

Figure 2. Temporal changes in saturated fatty acids (C6:0 + C8:0 + C10:0 + C12:0 + C14:0), C18:0, C16:0, and *cis*-9 C18:1 [g/100 g of fatty acids methyl esters (FAME)] in ewes fed diets with no supplementation (control) or supplemented with 20 g of sunflower oil/kg (SO), 10 g of fish oil/kg (FO), and 20 g of sunflower oil plus 10 g of fish oil/kg (SOFO). Values represent the mean from 2 lots of 8 animals per lot. SE of the difference = 0.674, 0.334, 0.399, and 0.363 for saturated fatty acids (C6:0 + C8:0 + C10:0 + C12:0 + C14:0), C18:0, C16:0, and *cis*-9 C18:1, respectively.

Table 5. Pearson correlation coefficients for relationships between concentrations of *trans*-C18:1 fatty acids, conjugated linoleic acid isomers (g/100 g of total fatty acid methyl esters), milk fat yield (g/d), and milk fat content (g/100 g of raw milk) from ewes fed different oil supplements¹

Item	Fat content	<i>trans</i> -6+7+8	<i>trans</i> -9	<i>trans</i> -10	<i>trans</i> -11	<i>trans</i> -12	<i>cis</i> -9, <i>trans</i> -11	<i>trans</i> -7, <i>cis</i> -9	<i>trans</i> -9, <i>cis</i> -11	<i>trans</i> -10, <i>cis</i> -12	<i>trans</i> -11, <i>cis</i> -13	<i>trans</i> -11, <i>trans</i> -13
Fat yield	0.843***	0.384**	0.156	−0.910***	−0.205	0.174	−0.216	0.280	−0.918***	−0.446**	0.257	0.534***
Fat content		0.527***	0.357*	−0.735***	−0.016	0.395**	−0.037	0.427*	−0.740***	−0.362*	−0.222	0.314*
<i>trans</i> -6+7+8			0.933***	0.372**	0.161	0.795***	0.251	0.976***	−0.339*	0.283	−0.107	−0.190
<i>trans</i> -9				−0.123	0.402**	0.892***	0.491***	0.933***	−0.079	0.420**	0.026	−0.455**
<i>trans</i> -10					0.333*	0.094	0.290*	−0.307*	0.986***	0.506***	0.324*	−0.619***
<i>trans</i> -11						0.690***	0.973***	0.094	0.374**	0.261	0.300*	−0.810***
<i>trans</i> -12							0.740***	0.729***	0.045	0.333*	0.108	0.581***
<i>cis</i> -9, <i>trans</i> -11								0.210	0.349*	0.310*	0.295*	−0.822***
<i>trans</i> -7, <i>cis</i> -9									−0.269	0.334*	−0.098	−0.197
<i>trans</i> -9, <i>cis</i> -11										0.519***	0.322*	−0.655***
<i>trans</i> -10, <i>cis</i> -12											0.431**	−0.505***
<i>trans</i> -11, <i>cis</i> -13												−0.389**

¹Diets: control = with no oil supplementation; SO = supplemented with 20 g of sunflower oil per kg of fresh matter; FO = supplemented with 10 g of fish oil per kg of fresh matter; SOFO = supplemented with 20 g of sunflower oil plus 10 g of fish oil per kg of fresh matter.

P* < 0.05, *P* < 0.01, ****P* < 0.001.

Figure 3. Temporal changes in *trans*-6+7+8 C18:1, *trans*-9 C18:1, and *trans*-7,*cis*-9 C18:2 [g/100 g of fatty acids methyl esters (FAME)] in ewes fed diets with no supplementation (control) or supplemented with 20 g of sunflower oil/kg (SO), 10 g of fish oil/kg (FO), and 20 g of sunflower oil plus 10 g of fish oil/kg (SOFO). Values represent the mean from 2 lots of 8 animals per lot. SE of the difference = 0.004, 0.006, and 0.002 for *trans*-6+7+8 C18:1, *trans*-9 C18:1, and *trans*-7,*cis*-9 C18:2, respectively.

with the addition of SO even if it represented 60% of its total FA, which may be attributed to its more extensive biohydrogenation when its content in the diet increases (Slingsfield et al., 2008).

Another nonconjugated C18:2 that should be considered is *trans*-11,*cis*-15 C18:2, an intermediate of α -linolenic acid metabolism whose milk content was

Figure 4. Temporal changes in C20:5 n-3, C22:5 n-3, and C22:6 n-3 [g/100 g of fatty acids methyl esters (FAME)] in ewes fed diets with no supplementation (control) or supplemented with 20 g of sunflower oil/kg (SO), 10 g of fish oil/kg (FO), and 20 g of sunflower oil plus 10 g of fish oil/kg (SOFO). Values represent the mean from 2 lots of 8 animals per lot. SE of the difference = 0.005, 0.006, and 0.013 for C20:5 n-3; C22:5 n-3, and C22:6 n-3, respectively. EPA = eicosapentaenoic acid; DPA = docosapentaenoic acid; DHA = docosahexaenoic acid.

multiplied 9 times with FO supplementation ($P < 0.001$), in agreement with previous studies (Shingfield et al., 2006; Cruz-Hernandez et al., 2007), and supposedly as a result of the FO inhibitory effect on

biohydrogenation of dietary α -linolenic acid (Loor et al., 2005). However, the strong correlation observed between *trans*-11,*cis*-15 C18:2 and some n-3 PUFA, such as DHA ($r = 0.95$; $P < 0.001$) or docosapentaenoic acid ($r = 0.94$; $P < 0.001$), suggests that this FA might also be an intermediate of other n-3 PUFA less clear pathways (Jenkins et al., 2008).

Putative Inhibitors of Milk Fat Synthesis

The inhibitory effects of FO on milk fat secretion does not seem to be attributable to the same putative depressors as those of MFD induced by more traditional diets (Loor et al., 2005; Shingfield and Griinari, 2007; Gama et al., 2008). For example, *trans*-10,*cis*-12 CLA concentration showed no change during FO-induced MFD (Figure 1), as previously reported by Whitlock et al. (2002) or Griinari and Bauman (2006), and so other biohydrogenation intermediates with antilipogenic effects may be involved (Griinari and Bauman, 2006; Harvatine et al., 2009). Accordingly, *trans*-9,*cis*-11 CLA, which showed increased values in FO diets and a high correlation with fat yield ($r = 0.92$; $P < 0.001$; Table 5) but very low concentrations, has also been identified as a potential inhibitor of milk fat synthesis (Perfield et al., 2007).

The strong relationship observed between fat yield and *trans*-10 C18:1 content ($r = 0.91$; $P < 0.001$; Table 5) may suggest that this FA is another putative inhibitor of lipogenesis, although previous studies in ewes have shown that oil supplementation resulting in similar concentrations did not cause MFD (Gómez-Cortés et al., 2008a,b; Hervás et al., 2008). Furthermore, in FO-containing diets, the reduction in C18:0 for endogenous oleic acid synthesis and the increase in *trans*-C18:1 isomers probably played a role in MFD, related to maintenance of milk fat fluidity (Gama et al., 2008).

Long-Chain n-3 FA

The long chain n-3 PUFA contents in FO and SOFO diets increased sharply during the first days of supplementation, followed by a plateau (Figure 4), as previously observed in the few studies conducted in dairy sheep (Papadopoulos et al., 2002; Kitessa et al., 2003). These increases reached their highest values with the FO treatment. The greatest increase corresponded to DHA ($P < 0.001$), and those of eicosapentaenoic acid and docosapentaenoic acid (Figure 4) were of minor magnitude. Their apparent transfer efficiencies into milk after 4 wk of supplementation were relatively low (0.135 vs. 0.086 for EPA, and 0.114 vs. 0.075 for DHA in FO and SOFO diets, respectively) and within the

same range as that calculated for marine oils fed to ewes (Papadopoulos et al., 2002; Reynolds et al., 2006; Capper et al., 2007), but considerably higher than that usually reported for dairy cows (Lock and Bauman, 2004; Looor et al., 2005; Shingfield et al., 2006). This low transference would be caused not only by extensive biohydrogenation in the rumen (Doreau and Chilliard, 1997; Jenkins et al., 2008) but also by preferential partitioning of these FA into some plasma lipid fractions such as plasma cholesterol ester and phospholipids, which are poorly taken up by the mammary gland (Kittessa et al., 2001; Lock and Bauman, 2004). The ratio of n-6:n-3 FA in milk (Table 4) was augmented with the use of SO, whereas SOFO almost halved (−48%) the value observed for the control treatment, and the FO diet further reduced it (−55%; $P < 0.001$). The ratios lower than 4 observed in the FO-containing treatments would suggest a milk having a potentially suppressive effect on the pathogenesis of highly prevalent chronic diseases (Simopoulos, 2008).

CONCLUSIONS

Although supplementation of ewe diet with FO significantly reduced milk fat content, the addition of SO did not, and SOFO further decreased milk yield and TS content. The increase in some milk *trans*-FA that are putative inhibitors of milk fat synthesis, such as *trans*-9,*cis*-11 CLA and perhaps *trans*-10 C18:1, and the theory of reduced milk fat fluidity (consistent with changes in rumen biohydrogenation pathways) may provide a suitable explanation for FO-induced MFD. On the other hand, *trans*-10,*cis*-12 CLA was detected in very low concentrations and showed no changes with time. Lipid supplementation with SO, FO, and SOFO proved to be an effective nutritional strategy for altering milk FA profile and improving the content of some bioactive components, such as RA, VA, and, to a minor extent, n-3 PUFA.

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