Effect of lipid supplementation on the endogenous synthesis of milk *cis*-9,*trans*-11 conjugated linoleic acid in dairy sheep and goats: A tracer assay with ¹³C-vaccenic acid

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ABSTRACT

A major proportion of milk rumenic acid (RA; *cis*-9,*trans*-11 CLA) is synthesized through mammary Δ^9 -desaturation of vaccenic acid (VA; *trans*-11 18:1). Diet composition may determine the relative contribution of this endogenous synthesis to milk RA content, with effects that might differ between ruminant species. However, this hypothesis is mostly based on estimated values, proxies of stearoyl-CoA desaturase (SCD) activity, and indirect comparisons between publications in the literature. With the aim of providing new insights into this issue, in vivo Δ^9 -desaturation of ¹³C-labeled VA (measured via milk ¹³C-VA and -RA secretion) was directly compared in sheep and goats fed a diet without lipid supplementation or including 2% of linseed oil. Four Assaf sheep and 4 Murciano-Granadina goats were used in a replicated 2 × 2 crossover design to test the effects of the 2 dietary treatments during 2 consecutive 25-d periods. On d 22 of each period, 500 mg of ¹³C-VA were i.v. injected to each animal. Dairy performance, milk fatty acid profile, including isotope analysis, and mammary mRNA abundance of genes coding for SCD were examined on d 21 to 25 of each period. Supplementation with linseed oil improved milk fat concentration and increased the content of milk VA and RA. However, the isotopic tracer assay suggested no variation in the relative proportion of VA desaturated to milk RA, and the percentage of this CLA isomer deriving from SCD activity would remain constant regardless of dietary treatment. These results put into question a major effect of lipid supplementation on the endogenous synthesis of milk RA and support that mammary Δ^9 -desaturation capacity would not represent a limiting factor when designing feeding strategies to increase milk RA content. The lack of diet-induced effects was common to caprines and ovines,

but inherent interspecies differences in mammary lipogenesis were found. Thus, the higher proportions of VA desaturation and endogenous synthesis of milk RA in sheep supported a greater SCD activity compared with goats, a finding that was not associated with the similar mRNA abundance of *SCD1* in the 2 species. On the other hand, transfer efficiency of the isotopic tracer to milk was 37% higher in caprine than in ovine, suggesting a greater efficiency in mammary fatty acid uptake from plasma in caprine.

Key words: caprine, desaturation, fatty acid, ovine, stable isotope

INTRODUCTION

Rumenic acid (RA; *cis*-9,*trans*-11 18:2) is the most abundant CLA isomer in milk and has attracted a great deal of attention in dairy science due to its potential benefits for human health (Dilzer and Park, 2012; Shingfield et al., 2013). Most of the RA found in milk is synthesized endogenously in the mammary gland through the action of the stearoyl-CoA desaturase (SCD) on *trans*-11 18:1 (vaccenic acid, VA; Griinari et al., 2000; Taugbøl et al., 2008; Hervás et al., 2020). Mammary uptake of circulating RA of ruminal origin or synthesized through VA desaturation in body tissues would represent the other source of milk RA (Palmquist et al., 2004; Shingfield et al., 2007; Glasser et al., 2008).

However, factors regulating the relative contribution of the mammary endogenous synthesis to total milk RA are still uncertain (Bernard et al., 2013), notwithstanding their relevance for designing convenient approaches to improve milk CLA content.

First, there is little information on the effect of diet composition on the actual proportion of endogenous RA in milk (Kay et al., 2004; Bernard et al., 2010), despite the effectiveness of certain feeding strategies, such as supplementation with unsaturated oils, to increase milk CLA concentration through changes in VA availability (Chilliard et al., 2007; Glasser et al., 2008; Nudda et al., 2020). In this regard, it could be hypothesized that the

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broad range of endogenous RA data in the literature (approximately 46–100% of total milk RA; Kay et al., 2004; Taugbøl et al., 2008; Hervás et al., 2020) may be determined, at least in part, by diet-induced variations in mammary SCD activity.

Another decisive factor in the contribution of endogenous synthesis to milk RA content might be the ruminant species, given the potential metabolic differences in ruminal and mammary fatty acid (FA) metabolism (Shingfield et al., 2013; Toral et al., 2016a; Nudda et al., 2020). Tsiplakou et al. (2009) conducted a direct comparative study and reported a greater mammary *SCD1* expression in sheep than in goats, which might explain the higher milk RA proportions described in early studies in ewes, relative to does and cows (Jahreis et al., 1999; Nudda et al., 2003; Tsiplakou and Zervas, 2008). Other authors have also suggested a major influence of diet composition in explaining interspecies variation in milk RA concentration, due to the more frequent use of diets that favor ruminal VA production in small ruminants (e.g., pasture and high-fiber rations; Cabiddu et al., 2005; Sanz Sampelayo et al., 2007; Bichi et al., 2012). Although between-species responses to feeding strategies aimed at modulating milk FA profile have also been described (Toral et al., 2016a; Fougère and Bernard, 2019; Nudda et al., 2020), the existing information is still too scarce to offer a full picture of the situation and, to our knowledge, there is no direct comparison of mammary RA synthesis between ruminant species.

On this basis, this study was conducted to examine the effect of lipid supplementation on the proportion of endogenous RA in the milk of dairy sheep and goats. The assay was conducted using ^{13}C -labeled VA to directly quantify SCD activity, and animals received a standard diet without lipid supplementation or a ration supplemented with linseed oil, which would improve milk RA by increasing the ruminal VA production (Glasser et al., 2008; Nudda et al., 2020). Our hypotheses were that (1) the broad range of endogenous milk RA proportions in the literature would partly be explained by the effect of diet composition, and (2) lipid-induced effects at this level may differ between sheep and goats.

MATERIALS AND METHODS

Ethics Statements

All experimental procedures were performed in accordance with European Union and Spanish legislations (Council Directive 2010/63/EU, EU, 2010; and R. D. 53/2013, BOE, 2013), being approved (no. 100102/2017–7) by the Research Ethics Committees of the Instituto de Ganadería de Montaña and the Spanish

National Research Council (CSIC), and authorized by the competent body (Junta de Castilla y León, Spain).

Animals, Experimental Diets, and Management

Four Assaf ewes (BW: 74.9 ± 0.5 kg) and 4 Murciano-Granadina does (BW: 33.1 ± 2.8 kg), at a similar stage of lactation (68.8 ± 5.1 and 70.8 ± 1.0 DIM, respectively), were housed in individual tiestalls and used in a replicated 2×2 change-over design to test the effects of 2 dietary treatments during 2 consecutive 25-d experimental periods (2 animals of each species per diet on each period). Diets were offered ad libitum and consisted of a TMR formulated from dehydrated alfalfa (particle size > 4 cm) and concentrates (forage: concentrate ratio 50:50), without lipid supplementation (control) or supplemented with 20 g of linseed oil (LIN)/kg of diet DM. The oil was included in the TMR and replaced other dietary ingredients on a proportionate basis. The ingredients and chemical composition of both diets, which were prepared weekly and contained molasses to avoid selection of dietary components, are reported in Table 1. Clean drinking water was always

Table 1. Ingredients and chemical composition of the TMR without lipid supplementation (control) or with 20 g of linseed oil/kg of DM (LIN)

Item	Diet	
	Control	LIN
Ingredient, g/kg of fresh matter		
Dehydrated alfalfa, particle size > 4 cm	500	491
Whole corn grain	140	138
Whole barley grain	100	98.3
Soybean meal solvent 440 g of CP/kg	150	147
Sugar beet pulp, pellets	50	49.1
Molasses, liquid	40	39.3
Linseed oil ¹	0	17.2
Vitamin-mineral supplement ²	20	19.7
Chemical composition, g/kg of DM		
OM	891	895
CP	194	186
NDF	298	301
ADF	210	211
Starch	116	113
Total fatty acids	20.2	38.8
16:0	4.52	5.64
18:0	0.85	1.81
<i>cis</i> -9 18:1	2.50	7.08
18:2n-6	7.19	9.76
18:3n-3	3.12	12.17

¹Linseed oil (Natursoy, Nutrition & Santé Iberia); contained [g/kg of total fatty acid (FA)]: 16:0 (63.7), 18:0 (51.3), *cis*-9 18:1 (243), *cis*-11 18:1 (9.1), 18:2n-6 (143), 18:3n-3 (479), and other FA (10.7).

²Macrofac Rumiantes (UP911755130; DSM Nutritional Products). Declared as containing: Ca (285 g/kg), Na (7.5 g/kg), Fe (3 g/kg), Mn (3 g/kg), Zn (2 g/kg), Mg (1 g/kg), P (910 mg/kg), Mo (100 mg/kg), Co (67 mg/kg), I (50 mg/kg), S (40 mg/kg), Se (7 mg/kg), vitamin A (200,000 IU/kg), vitamin D₃ (40,000 IU/kg), vitamin E (667 mg/kg), ethoxyquin (12 mg/kg), and propyl gallate (2 mg/kg).

available. All sheep and goats were milked once daily at approximately 0830 h in a 1 × 10-stall milking parlor (DeLaval).

On d 22 of each period, each animal received a dose of 500 mg of [$1\text{-}^{13}\text{C}$] *trans*-11 18:1 ([$1\text{-}^{13}\text{C}$]VA, 99% atom % ^{13}C ; 645516-SPEC; Sigma-Aldrich) suspended in an emulsion of 1 mL of Simulsol 5817 (Seppic), 0.5 mL of glycerol ($\geq 99\%$; G5516; Sigma-Aldrich), and 10 mL of 10% Intralipid (Fresenius Kabi), brought to a total volume of 120 mL using saline solution (0.9% NaCl, wt/vol; B. Braun Medical). This dose was selected to reach a minimum milk [$1\text{-}^{13}\text{C}$]VA enrichment of 1%, based on preliminary milk VA quantifications at the beginning of the trial, and our previous measurements of ^{13}C -labeled FA transference to ovine milk (17%; Hervás et al., 2020). The mixture was sonicated (at 24 kHz and 150 W) 6 times for 30 s with 20-s intervals between each sonication pulse. To ensure thorough mixing, the blend was further placed in an ultrasonic water bath for 30 min. The emulsion, tempered at 39°C, was delivered immediately after the morning milking by sequential infusion of 60 mL into each jugular vein over two 5-min periods, with a 15-min interval between them.

Measurements and Sampling Procedure

Diets. Representative samples of the TMR and the linseed oil were collected throughout the experiment, stored at -30°C , and then freeze-dried before chemical analysis. Feed intake was measured on the last 5 d of each period by weighing the amount of TMR offered and refused by each animal.

Milk. Milk yield was recorded, and individual milk samples were collected on d 21 to 25 of each period (-24 , 0, 24, 48, and 72 h postinjection (**p.i.**) of the [$1\text{-}^{13}\text{C}$]VA). Aliquots of 40 mL of milk were preserved with bronopol (D & F Control Systems Inc.) and stored at 4°C until analyzed for fat, protein, lactose, TS, and SCC. In addition, one set of aliquots of untreated milk was stored at -30°C for FA determination and compound-specific isotope analysis.

RNA from Milk Somatic Cells. Total RNA was isolated from milk somatic cells as detailed previously in Toral et al. (2016b). Milk samples were collected on d 25 on each period, 1 h after milking and 10 min after the injection of oxytocin i.v. (5 IU/ewe; Facipart, Laboratorios SYVA) to maximize the concentration of mammary epithelial cells. Udders were washed with water and soap and then disinfected with povidone iodine; nipples were also cleaned with RNaseZap (Ambion). Individual samples were obtained by hand-milking each half of the mammary gland into an RNase-free 50-mL tube (2 samples/ewe) that was covered with a sterile gauze to filter the milk. Samples were kept in ice and

immediately transferred to the laboratory. Milk somatic cells were pelleted by centrifugation at $650 \times g$ for 10 min at 4°C in the presence of a final concentration of 0.5 mM EDTA. The cell pellet was washed with 10 mL of PBS (pH 7.2, and 0.5 mM EDTA) followed by another centrifugation at $650 \times g$ for 10 min at 4°C . Washing and centrifugation procedures were repeated twice using 2 and 1.5 mL of the same PBS solution. Then, the pellet was suspended in 0.5 mL of RNeasy lysis buffer (Invitrogen) and frozen at -80°C until RNA extraction.

Laboratory Analysis

Diets and Orts. Dry matter concentration was determined in the TMR and Orts (ISO 6496:1999). The TMR was also analyzed for ash (ISO 5984:2002), and CP (ISO 5983-2:2009). The NDF and ADF were determined using an Ankom²⁰⁰⁰ fiber analyzer (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. Starch content was analyzed using a total starch assay kit (K-TSTA; Megazyme Intl. Ireland Ltd.). The FAME of lipid in freeze-dried samples of TMR and the linseed oil were prepared in a 1-step extraction-transesterification procedure (Shingfield et al., 2003) using tridecanoic acid (Sigma-Aldrich) as an internal standard. Separation and quantification of FAME was conducted using a gas chromatograph equipped with a flame ionization detector (**GC-FID**) as explained below for milk samples. Peaks were identified based on retention time comparisons with commercially available standards.

Milk. Concentrations of fat, protein, lactose, and TS were determined by infrared spectrophotometry (ISO 9622:1999) using a MilkoScan FT6000 (Foss), combined with a fluoro-opto-electronic counter (Fossomatic 5000, Foss) for SCC (ISO 13366-2:2006). Lipid in 1 mL of milk was extracted and converted to FAME by base-catalyzed transesterification (Shingfield et al., 2003). The total FAME profile was determined by GC-FID (Agilent 7890A GC System) using a 100-m fused silica capillary column (CP-SIL 88, Varian) and hydrogen as the carrier gas. Total FAME profile was determined using a temperature gradient program (Shingfield et al., 2003). Overlapping *cis*-9 + *trans*-13/14 18:1, *trans*-10, *cis*-15 + *trans*-11, *cis*-15 18:2, and *cis*-9, *trans*-11 + *trans*-7, *cis*-9 + *trans*-8, *cis*-10 CLA were resolved using a 100-m ionic liquid-coated capillary column (SLB-IL111, Sigma-Aldrich) and the temperature gradient program employed by de la Fuente et al. (2015), with hydrogen as the carrier gas. All peaks were identified based on retention time comparisons with commercially available FAME standards and cross-referencing chromatograms reported in the literature (e.g., Shingfield et al., 2003;

de la Fuente et al., 2015). Comparisons were also made with reference samples for which the FA composition was determined based on GC-FID analysis of FAME and gas chromatography-mass spectrometry analysis of corresponding 4,4-dimethyloxazoline derivatives (Bichi et al., 2013; Toral et al., 2017).

Compound-specific isotope analysis was performed using a gas chromatography-combustion isotope ratio mass spectrometry (GC-C-IRMS) system (Thermo Scientific) comprising a Trace GC Ultra gas chromatograph coupled to a Delta V Advantage isotope ratio mass spectrometer through a GC Combustion III interface maintained at 940°C (Toral et al., 2017). The FAME were separated using the same CP-SIL 88 column and the temperature gradient program employed in GC-FID analysis, with helium as the carrier gas. Peak identification was conducted as explained above for GC-FID analysis. The ^{13}C % in the methanol employed for FA transesterification was determined with a FlashEA 1112-HT elemental analyzer coupled to a Delta V Advantage isotope ratio mass spectrometer through a ConFlo III interface (Thermo Scientific). Methanol was placed in tin capsules, and combustion was performed at 1,020°C with helium as the carrier gas. The results were used to correct for the isotopic shift due to the extra methyl group in FAME.

RNA Isolation and Quantitative PCR. Total RNA was extracted and purified from the milk cell pellet with TRIzol (Invitrogen) as reported in Toral et al. (2016b). The RNA concentration was determined by fluorometry (Qubit 3.0; Life Technologies), and the RNA integrity number (RIN) by capillary electrophoresis (Agilent 2100 Bioanalyzer, Agilent Technologies Inc.). For quantitative PCR (qPCR) analysis, cDNA was synthesized using a High-Capacity RNA-to-cDNA Kit (Applied Biosystems). The qPCR was carried out as described in Bonnet et al. (2013) using a 7500 Real-Time PCR System (Applied Biosystems) and specific primers for genes encoding SCD isoforms in the mammary gland of ruminants (Supplemental Table S1; <http://hdl.handle.net/10261/240200>). 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 ubiquitously expressed prefoldin-like chaperone (*UXT*), all of which have been identified as suitable internal controls (Bonnet et al., 2013; Castro-Carrera et al., 2015; Toral et al., 2016b). Gene stability was examined using the geNorm and NormFinder procedures (Vandesompele et al., 2002; Andersen et al., 2004), and the lack of DNA contamination was verified by PCR amplification with primers flanking an intron (i.e., *EIF3K*). The abun-

dance 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 samples, and expressed as the $\log_2$ value of the mRNA copy number relative to the geometric mean of the 3 reference genes. The qPCR performance is shown in Supplemental Table S1.

Calculations and Statistical Analysis

Calculations to determine the endogenous synthesis of milk RA were conducted using the equations detailed in our previous study infusing $[1-^{13}\text{C}]\text{VA}$ to lactating ewes (Hervás et al., 2020) and reported in Supplemental Table S2 (<http://hdl.handle.net/10261/240200>). Because detailed kinetics of $[1-^{13}\text{C}]\text{VA}$ secretion have already been reported in goats and sheep (Bernard et al., 2010; Hervás et al., 2020), this study focused on examining the endogenous synthesis of milk RA for the 0- to 72-h p.i. period, within which the enrichment of $[1-^{13}\text{C}]\text{FA}$ in milk would have reached its maximum and subsequently declined to very low but still detectable levels using GC-C-IRMS.

To account for the natural levels of ^{13}C and determine the proportion of $[1-^{13}\text{C}]\text{VA}$ and $[1-^{13}\text{C}]\text{RA}$ that was actually derived from the injected labeled FA, the mean ^{13}C % of samples taken before $[1-^{13}\text{C}]\text{VA}$ injection (i.e., samples from -24 and 0 h) was subtracted from the ^{13}C % of each sample. The $[1-^{13}\text{C}]\text{RA}$ enrichments were also corrected for the coelution with *trans*-7,*cis*-9 CLA and *trans*-8,*cis*-10 CLA, assuming that these minor isomers had the same natural ^{13}C level as preinjection samples. The secretion of $[1-^{13}\text{C}]\text{VA}$ and $[1-^{13}\text{C}]\text{RA}$ in milk derived from the isotopic tracer was then calculated and employed to determine the proportion of VA desaturated to RA in vivo, the fraction of milk RA synthesized endogenously, and the percentage of injected labeled FA secreted into milk lipids.

Data were subjected to ANOVA for a replicated 2×2 change-over design (Kaps and Lamberson, 2009) using the MIXED procedure of the SAS software package (version 9.4; SAS Institute Inc.). The statistical model included the fixed effects of species (**Sp**; sheep vs. goat) dietary treatment (**D**; control vs. LIN), the interaction $\text{Sp} \times \text{D}$, the period, the order in which treatments were allocated to each animal, and the random effect of animal within order. Means were separated through the pairwise differences (pdiff) option of the least squares means (lsmeans) statement of the MIXED procedure, and adjusted for multiple comparisons using Bonferroni's method. Differences were declared significant at $P < 0.05$, and a trend toward significance at $0.05 \leq P < 0.10$. Least squares means are reported throughout the manuscript.

Table 2. Intake, milk production and composition, and SCC in dairy sheep and goats fed a TMR without lipid supplementation (control) or with 20 g of linseed oil/kg of DM (LIN)

Item	Sheep		Goats		SED ¹	P-value ²		
	Control	LIN	Control	LIN		Sp	D	Sp × D
DMI, kg/d	3.21	3.14	1.88	1.91	0.08	<0.001	0.607	0.207
Yield, g/d								
Milk	1,775	1,658	1,817	1,832	267	0.698	0.346	0.238
Fat	103.4	107.4	75.8	92.4	14.5	0.195	0.014	0.072
Protein	89.9	85.0	63.2	62.9	9.1	0.038	0.370	0.429
Lactose	85.4	79.9	79.5	81.8	12.2	0.872	0.571	0.201
TS	297	289	235	253	38	0.241	0.461	0.119
Composition, g/kg raw milk								
Fat	58.4	64.8	41.5	49.8	2.5	<0.001	0.002	0.615
Protein	50.9	51.3	34.8	34.2	1.9	<0.001	0.774	0.291
Lactose	48.0	48.3	43.7	44.5	1.1	0.012	0.178	0.457
TS	167	175	129	137	4	<0.001	0.015	0.838
SCC, log ₁₀ /mL	1.86	1.82	1.94	2.00	0.39	0.732	0.919	0.636

¹SED = standard error of the difference.²Probability of significant effects due to species (Sp), diet (D), and their interaction (Sp × D).

RESULTS

Animal Performance

Animal performance results are shown in Table 2. Despite inherent interspecies differences in some traits (e.g., greater DMI, and milk fat and protein concentration in ewes than in does; $P < 0.001$), linseed oil supplementation increased milk fat and TS concentration to a similar extent in both species ($P < 0.05$), and no treatment effects were detected for other performance features, including total milk yield or SCC ($P > 0.10$). However, a trend toward greater milk fat yield in LIN was only observed in goats ($P = 0.072$ for the interaction Sp × D).

Milk FA Composition

Milk FA profile was deeply affected by lipid supplementation (Table 3), but only a brief description of changes is made because this point does not represent an aim of the study. The addition of linseed oil to the diet decreased the concentrations of FA with 10 to 17 carbon atoms, *cis*-11 18:1, 18:2n-6, and the sums of C20–22 n-6 PUFA and odd- and branched-chain FA, whereas it had a positive effect on the proportions of 18:3n-3, most 18-carbon intermediate metabolites, and 18:0 in milk ($P < 0.05$). Some diet-induced changes were similar in both species (e.g., in *trans*-10 and *trans*-11 18:1, 18:2n-6, *cis*-9, *trans*-11 CLA, and 18:3n-3; $P > 0.10$ for the effect of Sp × D). However, increases in most other C18 biohydrogenation intermediates were greater in ewes than in does (e.g., *cis*-15 18:1, *trans*-12 18:1, *cis*-9, *trans*-13 18:2 or *trans*-11, *trans*-13 CLA; $P < 0.05$), or were only detected in the ovine (e.g., *cis*-9

18:1, *cis*-11, *cis*-14 18:2, *cis*-11, *trans*-13 CLA, and conjugated 18:3 of undetermined double bond position; $P < 0.05$ for the effect of Sp × D). On the contrary, LIN did not affect *trans*-10, *cis*-12 CLA and C20–22 n-3 PUFA concentrations in any species ($P > 0.10$). Supplemental Table S3 (<http://hdl.handle.net/10261/240200>) reports the changes in other milk FA, which are consistent with those described above (e.g., individual branched-chain FA and C20–22 FA, or other minor C18 metabolites).

In general, milk Δ^9 -desaturation ratios (see Table 3) were reduced by LIN, with frequent interspecies differences. Thus, with the exception of the decrease in milk *cis*-9 12:1/12:0 in sheep and goats ($P = 0.004$), ratios were only lowered by LIN in the caprine (i.e., *cis*-9 16:1/16:0, *cis*-9, *trans*-11 CLA/*trans*-11 18:1, and *cis*-9, *trans*-12 18:2/*trans*-12 18:1; $P < 0.05$), or showed a greater reduction in does than in ewes (i.e., *cis*-9 18:1/18:0; $P = 0.001$).

Only 3 minor FA reported in Supplemental Table S3 (i.e., *trans*-9, *cis*-12 18:2, *trans*-8, *cis*-10 CLA and *cis*-11 20:1) were significantly affected by the order in which treatments were allocated ($P < 0.05$). It is probably worth mentioning here that no other parameter of the study was affected by this factor.

Compound-Specific Isotope Analysis

The maximum enrichment of the 2 [1-¹³C] labeled FA (VA and RA) was detected in milk samples collected at 24 h after injection. (Figure 1). Greater enrichments were always found in the control than in LIN ($P < 0.05$), except for a lack of variation between treatments in [1-¹³C]VA in sheep at 24 h p.i. ($P = 0.014$ for Sp × D interaction). In general, [1-¹³C]RA reached lower

Table 3. Milk fatty acid (FA) composition (g/100 total FA) and Δ^9 -desaturation ratios in dairy sheep and goats fed a TMR without lipid supplementation (control) or with 20 g of linseed oil/kg of DM (LIN)

Milk FA	Sheep		Goats		SED ¹	P-value ²		
	Control	LIN	Control	LIN		Sp	D	Sp × D
4:0	3.22	3.34	2.21	2.32	0.16	0.001	0.114	0.926
6:0	2.81	2.75	2.47	2.59	0.13	0.059	0.663	0.302
8:0	2.83	2.65	2.94	3.08	0.18	0.142	0.866	0.127
10:0	9.97	7.92	11.00	10.46	0.57	0.017	0.005	0.038 ³
<i>cis</i> -9 10:1	0.316	0.259	0.257	0.219	0.026	0.099	0.004	0.388
12:0	6.05	4.20	5.39	4.18	0.38	0.321	0.001	0.221
<i>cis</i> -9 12:1	0.115	0.071	0.119	0.076	0.012	0.665	0.002	0.918
14:0	12.44 ^a	10.20 ^b	9.72 ^b	8.50 ^c	0.31	<0.001	<0.001	0.043
<i>cis</i> -9 14:1	0.209	0.165	0.148	0.106	0.017	0.010	0.003	0.899
15:0	1.061	0.867	0.873	0.715	0.067	0.016	0.013	0.717
<i>cis</i> -9 15:1	0.015	0.011	0.017	0.012	0.001	0.116	<0.001	0.204
16:0	29.2	22.8	31.1	25.8	1.3	0.099	<0.001	0.235
<i>cis</i> -9 16:1	0.780	0.610	0.764	0.533	0.061	0.458	<0.001	0.130
17:0	0.618	0.511	0.626	0.505	0.025	0.961	<0.001	0.698
<i>cis</i> -9 17:1	0.202	0.149	0.259	0.172	0.014	0.022	<0.001	0.064
18:0	6.24	10.74	6.15	9.93	0.64	0.484	<0.001	0.179
15-oxo-18:0	0.006	0.010	0.006	0.008	0.002	0.599	0.018	0.241
16-oxo-18:0	0.035 ^b	0.059 ^a	0.023 ^b	0.028 ^b	0.005	<0.001	0.003	0.025
<i>cis</i> -9 18:1	11.1 ^b	14.7 ^a	14.1 ^{ab}	15.2 ^{ab}	1.0	0.160	<0.001	0.007
<i>cis</i> -11 18:1	0.325	0.294	0.422	0.335	0.021	0.011	0.005	0.073
<i>cis</i> -12 18:1	0.207 ^b	0.496 ^a	0.168 ^b	0.261 ^b	0.049	0.012	0.002	0.034
<i>cis</i> -13 18:1	0.041 ^c	0.085 ^a	0.052 ^{bc}	0.065 ^b	0.005	0.226	<0.001	0.004
<i>cis</i> -15 18:1	0.065 ^c	0.364 ^a	0.069 ^c	0.223 ^b	0.033	0.015	<0.001	0.012
<i>cis</i> -16 18:1 ⁴	0.040 ^c	0.132 ^a	0.033 ^c	0.083 ^b	0.008	<0.001	<0.001	0.003
Σ <i>cis</i> 18:1	11.8 ^b	16.0 ^a	14.8 ^{ab}	16.0 ^{ab}	1.1	0.207	<0.001	0.003
<i>trans</i> -10 18:1	0.191	0.331	0.171	0.271	0.033	0.227	0.001	0.255
<i>trans</i> -11 18:1	0.740	1.380	0.760	2.358	0.485	0.250	0.013	0.168
<i>trans</i> -12 18:1	0.204 ^c	0.615 ^a	0.199 ^c	0.459 ^b	0.030	0.004	<0.001	0.005
<i>trans</i> -13 + 14 18:1	0.320 ^c	1.415 ^a	0.234 ^c	0.830 ^b	0.128	0.004	<0.001	0.020
<i>trans</i> -15 18:1	0.164 ^c	0.617 ^a	0.141 ^c	0.414 ^b	0.047	0.006	<0.001	0.022
Σ <i>trans</i> 18:1	1.91	5.06	1.83	5.00	0.50	0.862	<0.001	0.973
<i>cis</i> -9, <i>cis</i> -12 18:2 ⁵	2.45	1.92	2.47	1.81	0.15	0.716	0.001	0.467
<i>cis</i> -11, <i>cis</i> -14 18:2	0.011 ^b	0.181 ^a	0.008 ^b	0.051 ^b	0.024	0.012	0.001	0.012
<i>cis</i> -12, <i>cis</i> -15 18:2	0.017 ^{bc}	0.040 ^a	0.013 ^c	0.024 ^b	0.002	0.004	<0.001	0.006
<i>cis</i> -9, <i>trans</i> -13 18:2 ⁶	0.189 ^{bc}	0.550 ^a	0.156 ^c	0.342 ^b	0.041	0.012	<0.001	0.019
<i>cis</i> -9, <i>trans</i> -14 18:2	0.083 ^c	0.257 ^a	0.071 ^c	0.159 ^b	0.022	0.018	<0.001	0.040
<i>trans</i> -10, <i>cis</i> -15 18:2	0.013 ^c	0.099 ^a	0.011 ^c	0.044 ^b	0.009	<0.001	<0.001	0.001
<i>trans</i> -11, <i>cis</i> -15 18:2	0.052	0.320	0.040	0.286	0.041	0.464	<0.001	0.716
Σ nonconjugated 18:2	2.94 ^b	3.73 ^a	2.91 ^b	2.93 ^{ab}	0.19	0.044	0.015	0.018
<i>cis</i> -9, <i>trans</i> -11 CLA	0.385	0.612	0.431	0.910	0.177	0.255	0.028	0.324
<i>cis</i> -11, <i>trans</i> -13 CLA	0.001 ^b	0.006 ^a	0.002 ^b	0.003 ^b	0.001	0.066	0.001	0.005
<i>trans</i> -10, <i>cis</i> -12 CLA	0.006	0.006	0.005	0.004	0.001	0.046	0.801	0.145
<i>trans</i> -11, <i>cis</i> -13 CLA	0.010	0.048	0.012	0.066	0.014	0.386	0.005	0.456
<i>trans</i> -11, <i>trans</i> -13 CLA	0.007 ^{bc}	0.047 ^a	0.005 ^c	0.021 ^b	0.004	0.005	<0.001	0.004
<i>trans</i> -12, <i>trans</i> -14 CLA	0.003 ^c	0.021 ^a	0.003 ^c	0.013 ^b	0.002	0.024	<0.001	0.031
Σ CLA	0.52	0.89	0.54	1.12	0.19	0.430	0.013	0.416
<i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12 18:3	0.095 ^a	0.047 ^b	0.028 ^{bc}	0.018 ^c	0.007	<0.001	<0.001	0.002
<i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15 18:3	0.786	1.057	0.601	0.781	0.081	0.018	0.004	0.362
<i>trans</i> -9, <i>cis</i> -12, <i>cis</i> -15 18:3	0.004 ^b	0.020 ^a	0.004 ^b	0.008 ^b	0.002	0.019	0.001	0.009
<i>trans</i> -9, <i>trans</i> -12, <i>trans</i> -15 18:3	0.012	0.016	0.010	0.011	0.002	0.010	0.022	0.173
<i>cis</i> -9, <i>trans</i> -11, <i>cis</i> -15 18:3	0.016	0.064	0.008	0.046	0.006	0.013	<0.001	0.252
<i>cis</i> -9, <i>trans</i> -11, <i>trans</i> -15 18:3	0.006	0.038	0.005	0.034	0.005	0.483	<0.001	0.624
Unidentified #1, conjugated 18:3	0.002 ^b	0.011 ^a	0.001 ^b	0.003 ^b	0.001	0.009	0.001	0.009
Unidentified #2, conjugated 18:3	<0.001 ^b	0.005 ^a	0.002 ^b	0.002 ^b	<0.001	0.127	0.004	0.004
Σ odd- and branched-chain FA	5.04	4.11	4.49	3.59	0.24	0.028	0.002	0.909
Σ C20–22 n-6 PUFA	0.239	0.179	0.287	0.202	0.032	0.292	0.002	0.347
Σ C20–22 n-3 PUFA	0.141	0.145	0.085	0.092	0.013	0.005	0.245	0.794
Milk Δ^9 -desaturation ratio								
<i>cis</i> -9 10:1/10:0	0.032	0.033	0.024	0.021	0.003	0.028	0.329	0.040 ³
<i>cis</i> -9 12:1/12:0	0.019	0.017	0.022	0.018	0.002	0.296	0.004	0.281
<i>cis</i> -9 14:1/14:0	0.017	0.016	0.015	0.012	0.002	0.144	0.016	0.066
<i>cis</i> -9 15:1/15:0	0.014 ^c	0.013 ^c	0.020 ^a	0.017 ^b	0.000	<0.001	0.000	0.022
<i>cis</i> -9 16:1/16:0	0.027 ^{ab}	0.027 ^{ab}	0.025 ^a	0.021 ^b	0.002	0.088	0.019	0.016

Continued

Table 3 (Continued). Milk fatty acid (FA) composition (g/100 total FA) and Δ^9 -desaturation ratios in dairy sheep and goats fed a TMR without lipid supplementation (control) or with 20 g of linseed oil/kg of DM (LIN)

Milk FA	Sheep		Goats		SED ¹	P-value ²		
	Control	LIN	Control	LIN		Sp	D	Sp × D
<i>cis</i> -9 17:1/17:0	0.326	0.294	0.414	0.341	0.026	0.040	0.001	0.053
<i>cis</i> -9 18:1/18:0	1.788 ^{ab}	1.370 ^c	2.313 ^a	1.542 ^{bc}	0.197	0.134	<0.001	0.001
<i>cis</i> -9, <i>trans</i> -11 CLA/ <i>trans</i> -11 18:1	0.521 ^{ab}	0.443 ^{ab}	0.595 ^a	0.425 ^b	0.069	0.690	0.000	0.018
<i>cis</i> -9, <i>trans</i> -12 18:2/ <i>trans</i> -12 18:1	0.137 ^{ab}	0.112 ^{ab}	0.158 ^a	0.101 ^b	0.014	0.719	0.001	0.038

^{a-c}Within a row, different superscripts indicate significant differences ($P < 0.05$) due to Sp × D interaction.

¹SED = standard error of the difference.

²Probability of significant effects due to species (Sp), diet (D), and their interaction (Sp × D).

³In the pairwise analysis, no significant differences were found after adjustment for multiple comparisons using Bonferroni's method.

⁴Coelutes with *cis*-9,*trans*-15 18:2.

⁵Contains *cis*-9,*cis*-15 18:2 as a minor component.

⁶Coelutes with 11-cyclohexyl-11:0 and *cis*-10,*trans*-14 + *trans*-10,*trans*-13 + *trans*-11,*trans*-14 18:2.

enrichments than [1-¹³C]VA, and its decline at 48 and 72 h p.i. was also relatively slower.

Dairy performance and milk FA data used in calculations of endogenous RA synthesis are reported in Supplemental Table S4 (<http://hdl.handle.net/10261/240200>). Species and treatment differences in these traits (which correspond to overall values recorded from injection to 72 h p.i.) mirrored those described above for daily means.

Table 4 shows the final results of calculations to determine [1-¹³C]VA uptake and desaturation to RA. A trend toward species and treatment differences in labeled FA yield was found. Thus, [1-¹³C]VA secretion tended to be greater in goats than in ewes ($P = 0.069$), and that of [1-¹³C]RA in the control than in LIN, regardless of the species ($P = 0.011$). On average, 20.1% of the injected amount of [1-¹³C]VA was transferred to milk within 72 h p.i. in sheep, and 27.5% in goats ($P = 0.085$ for the effect of Sp), but no Sp × D interaction was detected. The proportion of VA desaturated to milk RA also showed between-species variation, being greater in sheep than in goats (on average, 30.3 and 22.3%, respectively, of VA taken up by the mammary gland; $P = 0.025$). Interspecies differences were even greater in the mean proportion of this CLA isomer deriving from Δ^9 -desaturation, which averaged 90.3 in ewes, and 58.9% in does ($P = 0.004$). Numerical differences in the proportion of endogenous milk RA between control and LIN treatments did not reach the required P -level to be considered significant ($P = 0.102$).

RNA Analysis

The average RIN of the milk somatic cell samples was 8.0 ± 0.1 . The geNorm and NormFinder procedures confirmed the 3 selected reference genes (i.e., *UXT*,

PPIA and *EIF3K*) as the most stable genes among those quantified.

As shown in Figure 2, dietary treatment did not affect the mRNA abundance of *SCD1* or *SCD5* ($P > 0.10$), but the quantity of the latter Δ^9 -desaturase was greater in sheep than in goats ($P = 0.004$).

DISCUSSION

Our hypotheses that diet composition may determine the relative contribution of endogenous synthesis to milk RA content, and that effects would differ between ruminant species, were mostly based on estimations of endogenous RA proportions and proxies of mammary SCD activity, as well as indirect comparisons between publications in the literature (e.g., Glasser et al., 2008; Tsiplakou et al., 2009; Bernard et al., 2013). With the aim of providing new insights into this issue, in vivo Δ^9 -desaturation of ¹³C-labeled VA was directly compared in sheep and goats fed a diet without supplementation or including linseed oil.

Animal Performance and Milk FA Composition

Because these results are not directly related to the objective of this work, they will only be discussed in passing. The dietary addition of small to moderate amounts of lipids from linseed is an effective means to improve dairy performance and milk FA profile in small ruminants, because it increases milk fat content or yield, and VA, RA, and 18:3n-3 concentrations, while decreases medium-chain SFA (Bernard et al., 2009; Gómez-Cortés et al., 2009; Martínez Marín et al., 2011). Our results confirmed the suitability of diet supplementation with 2% DM of linseed oil to achieve those improvements in the ovine and caprine and sug-

Figure 1. (A) Enrichment in $[1-^{13}\text{C}]$ in *trans*-11 18:1 (vaccenic acid; VA), and (B) *cis*-9,*trans*-11 CLA (ruminic acid; RA) in milk fat from dairy sheep and goats receiving an i.v. injection of 500 mg of $[1-^{13}\text{C}]$ VA at time 0 h, and fed a TMR without lipid supplementation (control) or with 20 g of linseed oil/kg of DM (LIN). For each fatty acid and time point, error bars represent the standard error of the difference. The statistical probabilities (P -value) for the effects of species (Sp), dietary treatment (D), and their interaction (Sp × D) are shown below each panel. Different letters (a,b) between values obtained at each postinjection (p.i.) time indicate differences ($P < 0.05$) due to Sp × D interaction. All values are corrected for the mean ^{13}C % of samples taken before $[1-^{13}\text{C}]$ VA injection (i.e., samples from -24 and 0 h).

Table 4. Calculations to determine the endogenous synthesis of milk *cis*-9,*trans*-11 CLA (ruminic acid; RA) in dairy sheep and goats fed a TMR without lipid supplementation (control) or with 20 g of linseed oil/kg of DM (LIN)¹

Item	Equation ²	Sheep		Goats		P-value ⁴	
		Control	LIN	Control	LIN	Sp	D
VA secreted into milk p.i., ⁵ mg	1	2,196	4,065	1,606	6,891	0.444	0.012
RA secreted into milk p.i., mg	2	1,134	1,815	873	2,514	0.611	0.016
[1- ¹³ C]VA enrichment, % of milk VA	3	3.55	1.55	7.21	3.02	0.050	0.001
[1- ¹³ C]RA enrichment, % of milk RA	4	3.10	1.43	3.98	1.93	0.254	0.006
[1- ¹³ C]VA secreted into milk p.i., mg	5	76.2	64.2	98.3	118.1	0.069	0.627
[1- ¹³ C]RA secreted into milk p.i., mg	6	34.5	26.3	30.6	28.2	0.627	0.011
[1- ¹³ C]FA secreted into milk, % of injected dose	7	22.1	18.1	25.8	29.3	0.085	0.865
VA desaturated to milk RA, % of VA taken up by the mammary gland	8	31.4	29.1	23.9	20.6	0.025	0.107
Mammary uptake of VA p.i., mg	9	3,194	5,731	2,084	8,432	0.626	0.010
VA desaturated to milk RA p.i., mg	10	998	1,666	478	1,542	0.188	0.006
Endogenous milk RA, % of milk RA	11	87.9	92.6	54.5	62.7	0.004	0.102

¹Animals received an i.v. injection of 500 mg of ¹³C-labeled *trans*-11 18:1 {vaccenic acid (VA); [1-¹³C]VA}.

²Equations detailed in Supplemental Table S2.

³SED = standard error of the difference.

⁴Probability of significant effects due to species (Sp), diet (D), and their interaction (Sp × D).

⁵p.i. = postinjection (0–72-h period).

⁶FA = fatty acid.

gested no evident variation between their responses, except for the trend toward greater milk fat yield only in goats.

We observed an overall consistence in the response of the 2 species to LIN in terms of lack of differences in milk *trans*-10,*cis*-12 CLA and limited increment in *trans*-10 18:1. Thus, despite the known relationship between *trans*-10 FA and milk fat synthesis (Lock et al., 2006; Shingfield et al., 2013; Palmquist and Jenkins, 2017), our results would rule out their association with milk fat yield changes in these small ruminant species. Furthermore, comparable milk 18:3n-3 concentrations in sheep and goats might call into question previously suggested interspecies differences in the transfer of n-3 PUFA from diet into milk, which were based on indirect comparisons under not identical feeding conditions (Chilliard et al., 2007; Boeckaert et al., 2008; Bichi et al., 2013). Supplementation with UFA-rich oils has been reported to increase the initial biohydrogenation rate of PUFA (Troegeler-Meynadier et al., 2003; Toral et al., 2016a; Kairenius et al., 2018); detected reductions in milk 18:2n-6 content with LIN would support no interspecies differences at this level either. In line with this, the reductions in milk C20 and C22 n-6 PUFA would likely be accounted for by a lower availability of 18:2n-6 for elongation and desaturation in body tissues (Miyazaki and Ntambi, 2008).

Interspecies differences were however observed in the effects of LIN increasing milk conjugated 18:3 and FA with double bonds at Δ^{13} to Δ^{16} positions (i.e., deriving from ruminal 18:3n-3 metabolism; Lee and Jenkins, 2011; Honkanen et al., 2016; Toral et al., 2019), which suggests a greater responsiveness of sheep than goats in terms of some alternative biohydrogenation pathways. Yet, milk content of 18:3n-3 and, particularly, 18:0 (the end product of ruminal C18 FA metabolism) were similar in ewes and does, and therefore, interspecies variation in the overall extent of biohydrogenation may be ruled out. In contrast, a direct comparison between cows and goats suggested a more complete process in the latter (Toral et al., 2016b).

Mammary Δ^9 -Desaturation

Mammary SCD activity has often been estimated stoichiometrically (i.e., using ratios between pairs of milk FA that represent substrates and products of the enzyme; Lock et al., 2006; Kgwatalala et al., 2009; Rincon et al., 2012). However, changes in milk Δ^9 -desaturation ratios might be misleading when using diets with different lipid composition, due to expected variations in the availability of preformed FA identified not only as substrates, but also as products of the enzyme (He et al., 2011; Bernard et al., 2013; Kairenius

Figure 2. mRNA relative abundance ($\log_2$ transformed data; arbitrary units) of genes coding for stearoyl-CoA desaturases (*SCD1* and *SCD5*) in milk somatic cells of dairy sheep and goats fed a TMR without lipid supplementation (control) or with 20 g of linseed oil/kg of DM (LIN). For each *SCD*, error bars represent the standard error of the difference. The statistical probabilities (*P*-value) for the effects of species (Sp), dietary treatment (D), and their interaction (Sp × D) are shown below each panel.

et al., 2018). The negative effects of the LIN treatment on Δ^9 -desaturation ratios in caprine, and to a lower extent in ovine, might lead to consider that goats were more sensitive in terms of nutritional regulation of SCD activity, whereas sheep would have more stable Δ^9 -desaturation regardless of the diet. In any event, differences in *cis*-9 14:1/14:0, which is considered the best estimator of SCD activity (Bernard et al., 2013) were subtle and only a trend toward a significant Sp × D effect was found. In addition, the hypothesis was neither supported by the isotopic tracer assay, where similar responses to LIN were found in both species.

In particular, LIN supplementation had significant effects on the proportion of VA desaturated to milk RA neither in ewes nor in goats. This agrees with the relatively narrow range of VA desaturation data (approximately 21–32% of VA taken up by the mammary gland) reported in earlier studies using isotopic tracers (Mosley et al., 2006; Bernard et al., 2010; Hervás et al., 2020), postprandial VA infusions (Shingfield et al.,

2007), and meta-analysis of milk FA yields in relation to duodenal flows (Glasser et al., 2008; Prado et al., 2019). Our results would therefore support that conversion of VA to RA in the mammary gland would remain stable in spite of presumed changes in VA supply due to LIN, and also that mammary Δ^9 -desaturation capacity would not represent a limiting factor when designing feeding strategies to increase milk RA content (Shingfield et al., 2007; Glasser et al., 2008).

On the other hand, observed interspecies differences in the percentage of VA desaturation would point to sheep showing a greater endogenous synthesis of milk RA than goats in response to identical increments in VA availability, but this hypothesis can hardly be tested against the scant data in the literature. Indeed, Bernard et al. (2010) reported that 32% of the VA taken up by the mammary gland was desaturated to RA in the caprine, which is similar to our results in ewes (29–31%) but higher than the proportion in does (21–24%). Given that similar methodology was employed in both

trials, a putative influence of lactation stage might contribute to explain this inconsistency in the caprine, because the study by Bernard et al. (2010) started at 266 DIM and our experiment at about 70 DIM. Previous studies have reported an effect of lactation stage on mRNA abundance of *SCD1* (Wickramasinghe et al., 2012; Suárez-Vega et al., 2016), but the relevance of posttranscriptional mechanisms (Bernard et al., 2013) might bias the comparison between SCD activity and gene expression. In this regard, we were not able to associate interspecies differences in SCD activity with the stable mRNA abundance of *SCD1*, in contrast with its greater expression in sheep than goats in a previous comparative study (Tsiplakou et al., 2009). The confounding effect of posttranscriptional regulation of mammary SCD activity in ruminants (Bernard et al., 2013) may also account for the lack of effects of LIN in our assay, and for the highly inconsistent effects of lipid supplements on *SCD1* expression in the literature (Dallaire et al., 2014; Palin et al., 2014; Fougère and Bernard, 2019). Although it is tempting to speculate about an association between mRNA abundance of *SCD5* and VA desaturation (both greater in the ovine), the actual relevance of this isoform in mammary lipid metabolism has not been elucidated yet (Bernard et al., 2013; Dallaire et al., 2014; Castro-Carrera et al., 2015), and its low expression in milk somatic cells might support a minor role.

Our first hypothesis was that dietary lipid composition may contribute to explain the broad range (approximately 46–100%) of endogenous synthesis of milk RA obtained through indirect comparisons of published studies (Grinari et al., 2000; Kay et al., 2004; Bichi et al., 2012). However, our isotopic analysis would also rule out an effect of LIN, both in sheep and goats. This observation is consistent with 2 direct comparisons of diet-induced effects on the proportion of endogenous RA in milk (Kay et al., 2004; Bernard et al., 2010). However, they were inconclusive because total milk RA content also remained unaffected by treatments (i.e., supplementation with sunflower oil in cows, and with sunflower oil alone or combined with fish oil and additional starch in goats). We are not aware of other direct comparative studies on this issue. Overall, these results put into question a major effect of lipid supplementation on the endogenous synthesis of milk RA, but further research is required to elucidate whether other differences in feeding conditions (e.g., in forage type and proportion) may be responsible for reported variations in the literature. In addition, methodology might also be a confounding factor, as shown in a recent study to evaluate the use of an SCD inhibition assay via oral administration of cobalt through comparison with the

reference technique (i.e., stable isotopic labeling), with values of endogenous RA ranging from 46 to 75% with the first technique (using different calculation methods) to 82 to 90% in the isotopic tracer assay (Hervás et al., 2020).

Concerning species differences in the endogenous synthesis of RA, results in ovine (88–93% of milk RA) and caprine (55–63%) species were on the same and the lower range relative to previous quantifications using ^{13}C -labeled VA (82–90 and 63–73% in ewes and does, respectively; Bernard et al., 2010; Hervás et al., 2020). Results applying the same methodology to cows averaged 83% of endogenous milk RA (Mosley et al., 2006). Underlying mechanisms may be associated with a greater RA outflow from the rumen of goats than sheep, but also with a hypothetically greater efficiency in mammary RA uptake from plasma lipids, which would require confirmation. Nevertheless, this second proposition appears to be consistent with transfer rates of the isotopic tracer from blood to milk, which were 37% higher in caprine than in ovine (26–29 vs. 18–22% of the injected dose, respectively). Although no additional information seems to be available in goats, indirect comparisons of published data of milk ^{13}C -labeled VA suggest a lower transfer efficiency in ewes (17–22%; Hervás et al., 2020; and the present study) than in cows (33%; Mosley et al., 2006), supporting inherent interspecies differences in mammary FA uptake. Further research is warranted to examine whether the greater VA desaturation in ovine than in caprine represents an adaptation mechanism to counteract this lower transfer efficiency from plasma.

Finally, time-dependent variations in both ^{13}C -labeled FA were consistent with previously described kinetics of appearance of isotopic tracers in ewes and goats (Bernard et al., 2010; Hervás et al., 2020), supporting slower declines in ^{13}C enrichment than in cows (baseline levels were recovered 24 h p.i.; Mosley et al., 2006).

CONCLUSIONS

Diet supplementation with linseed oil modulates milk FA profile in dairy sheep and goats, increasing the concentration of VA and RA. According to the isotopic tracer assay, these changes are not accompanied by variations in the proportion of VA desaturated to milk RA, and the percentage of this CLA isomer deriving from SCD activity remains constant regardless of dietary treatment. These results would not support our first hypothesis and suggest that mammary SCD activity would not represent a limiting factor when designing feeding strategies to increase milk RA content. The sec-

ond hypothesis must also be rejected because the lack of diet-induced effects is common to caprine and ovine, although inherent interspecies differences in mammary lipogenesis exist. Thus, the higher proportions of VA desaturation and endogenous synthesis of milk RA in sheep support a greater SCD activity compared with goats, a finding that could not be associated with the similar mRNA abundance of *SCD1* in the 2 species. On the other hand, transfer rates of the isotopic tracer from blood to milk were 37% higher in caprine than in ovine, suggesting a greater efficiency in mammary FA uptake from plasma lipids in goats.

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REFERENCES

- Andersen, C. L., J. L. Jensen, and T. F. Ørntoft. 2004. Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Canc. Res.* 64:5245–5250. <https://doi.org/10.1158/0008-5472.CAN-04-0496>.
- 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.
- Bernard, L., J. Mouriot, J. Rouel, F. Glasser, P. Capitan, E. Pujos-Guillot, J. M. Chardigny, and Y. Chilliard. 2010. Effects of fish oil and starch added to a diet containing sunflower-seed oil on dairy goat performance, milk fatty acid composition and in vivo Δ^9 -desaturation of [^{13}C] vaccenic acid. *Br. J. Nutr.* 104:346–354. <https://doi.org/10.1017/S0007114510000486>.
- Bernard, L., K. J. Shingfield, J. Rouel, A. Ferlay, and Y. Chilliard. 2009. Effect of plant oils in the diet on performance and milk fatty acid composition in goats fed diets based on grass hay or maize silage. *Br. J. Nutr.* 101:213–224. <https://doi.org/10.1017/S0007114508006533>.
- Bichi, E., G. Hervás, P. G. Toral, J. J. Loo, and P. Frutos. 2013. 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. <https://doi.org/10.3168/jds.2012-5875>.
- Bichi, E., P. G. Toral, G. Hervás, P. Frutos, P. Gómez-Cortés, M. Juárez, and M. A. de la Fuente. 2012. Inhibition of Δ^9 -desaturase activity with stercularic acid: Effect on the endogenous synthesis of *cis*-9 18:1 and *cis*-9,*trans*-11 18:2 in dairy sheep. *J. Dairy Sci.* 95:5242–5252. <https://doi.org/10.3168/jds.2012-5349>.
- BOE. 2013. Royal Decree 53/2013, of 8 February, on the protection of animals used for experimental purposes. Pages 11370–11421 in *Boletín Oficial del Estado*. Spanish Government Publishing.
- Boeckaert, C., B. Vlaeminck, J. Dijkstra, A. Issa-Zacharia, T. Van Nespén, W. Van Straalen, and V. Fievez. 2008. Effect of dietary starch or micro algae supplementation on rumen fermentation and milk fatty acid composition of dairy cows. *J. Dairy Sci.* 91:4714–4727. <https://doi.org/10.3168/jds.2008-1178>.
- 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. <https://doi.org/10.1017/S1751731113000475>.
- Cabiddu, A., M. Decandia, M. Addis, G. Piredda, A. Pirisi, and G. Molle. 2005. Managing Mediterranean pastures in order to enhance the level of beneficial fatty acids in sheep milk. *Small Rumin. Res.* 59:169–180. <https://doi.org/10.1016/j.smallrumres.2005.05.005>.
- 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. <https://doi.org/10.1017/S1751731114002882>.
- Chilliard, Y., F. Glasser, A. Ferlay, L. Bernard, J. Rouel, and M. Doreau. 2007. Diet, rumen biohydrogenation and nutritional quality of cow and goat milk fat. *Eur. J. Lipid Sci. Technol.* 109:828–855. <https://doi.org/10.1002/ejlt.200700080>.
- Dallaire, M. P., H. Taga, L. Ma, B. A. Corl, R. Gervais, Y. Lebeuf, F. J. Richard, and P. Y. Chouinard. 2014. Effects of abomasal infusion of conjugated linoleic acids, *Sterculia foetida* oil, and fish oil on production performance and the extent of fatty acid Δ^9 -desaturation in dairy cows. *J. Dairy Sci.* 97:6411–6425. <https://doi.org/10.3168/jds.2013-7853>.
- de la Fuente, M. A., V. Rodríguez-Pino, and M. Juárez. 2015. Use of an extremely polar 100-m column in combination with a cyano-alkyl polysiloxane column to complement the study of milk fats with different fatty acid profiles. *Int. Dairy J.* 47:52–63. <https://doi.org/10.1016/j.idairyj.2015.02.010>.
- Dilzer, A., and Y. Park. 2012. Implication of conjugated linoleic acid (CLA) in human health. *Crit. Rev. Food Sci. Nutr.* 52:488–513. <https://doi.org/10.1080/10408398.2010.501409>.
- EU (European Union). 2010. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. *Off. J. Eur. Union.* 276:33–79.
- Fougère, H., and L. Bernard. 2019. Effect of diets supplemented with starch and corn oil, marine algae, or hydrogenated palm oil on mammary lipogenic gene expression in cows and goats: A comparative study. *J. Dairy Sci.* 102:768–779. <https://doi.org/10.3168/jds.2018-15288>.
- Glasser, F., A. Ferlay, M. Doreau, P. Schmidely, D. Sauvant, and Y. Chilliard. 2008. Long-chain fatty acid metabolism in dairy cows: A meta-analysis of milk fatty acid yield in relation to duodenal flows and de novo synthesis. *J. Dairy Sci.* 91:2771–2785. <https://doi.org/10.3168/jds.2007-0383>.
- Gómez-Cortés, P., A. Bach, P. Luna, M. Juárez, and M. A. de la Fuente. 2009. Effects of extruded linseed supplementation on n-3 fatty acids and conjugated linoleic acid in milk and cheese from ewes. *J. Dairy Sci.* 92:4122–4134. <https://doi.org/10.3168/jds.2008-1909>.
- Griinari, J. M., B. A. Corl, S. H. Lacy, P. Y. Chouinard, K. V. V. Nurmela, and D. E. Bauman. 2000. Conjugated linoleic acid is synthesized endogenously in lactating dairy cows by Δ^9 -desaturase. *J. Nutr.* 130:2285–2291. <https://doi.org/10.1093/jn/130.9.2285>.
- He, M. L., Y.-H. Chung, T. A. McAllister, K. A. Beauchemin, P. S. Mir, J. L. Aalhus, and M. E. R. Dugan. 2011. Inclusion of flaxseed

- in hay- and barley silage diets increases alpha-linolenic acid in cow plasma independent of forage type. *Lipids* 46:577–585. <https://doi.org/10.1007/s11745-011-3534-4>.
- Hervás, G., P. Frutos, and P. G. Toral. 2020. Endogenous synthesis of milk *cis*-9, *trans*-11 conjugated linoleic acid in dairy ewes: Quantification using ¹³C-labeled vaccenic acid and comparison with estimates based on cobalt administration. *J. Dairy Sci.* 103:368–378. <https://doi.org/10.3168/jds.2019-17050>.
- Honkanen, A. M., H. Leskinen, V. Toivonen, N. McKain, R. J. Wallace, and K. J. Shingfield. 2016. Metabolism of α -linolenic acid during incubations with strained bovine rumen contents: Products and mechanisms. *Br. J. Nutr.* 115:2093–2105. <https://doi.org/10.1017/S0007114516001446>.
- Jahreis, G., J. Fritsche, and J. Kraft. 1999. Species-dependent, seasonal, and dietary variation of conjugated linoleic acid in milk. Pages 215–225 in *Advances in Conjugated Linoleic Acid Research* Vol. 1. M. P. Yurawecz, M. M. Mossoba, J. K. G. Kramer, M. W. Pariza, and G. Nelson, ed. AOCS Press.
- Kairenius, P., H. Leskinen, V. Toivonen, S. Muetzel, S. Ahvenjärvi, A. Vanhatalo, P. Huhtanen, R. J. Wallace, and K. J. Shingfield. 2018. Effect of dietary fish oil supplements alone or in combination with sunflower and linseed oil on ruminal lipid metabolism and bacterial populations in lactating cows. *J. Dairy Sci.* 101:3021–3035. <https://doi.org/10.3168/jds.2017-13776>.
- Kaps, M., and W. R. Lamberson. 2009. *Biostatistics for Animal Science*, 2nd edition. CABI Publishing.
- Kay, J. K., T. R. Mackle, M. J. Auldist, N. A. Thomson, and D. E. Bauman. 2004. Endogenous synthesis of *cis*-9, *trans*-11 conjugated linoleic acid in dairy cows fed fresh pasture. *J. Dairy Sci.* 87:369–378. [https://doi.org/10.3168/jds.S0022-0302\(04\)73176-8](https://doi.org/10.3168/jds.S0022-0302(04)73176-8).
- Kgwatalala, P. M., E. M. Ibeagha-Awemu, J. F. Hayes, and X. Zhao. 2009. Stearoyl-CoA desaturase 1 3'UTR SNPs and their influence on milk fatty acid composition of Canadian Holstein cows. *J. Anim. Breed. Genet.* 126:394–403. <https://doi.org/10.1111/j.1439-0388.2008.00796.x>.
- Lee, Y.-J., and T. C. Jenkins. 2011. Biohydrogenation of linolenic acid to stearic acid by the rumen microbial population yields multiple intermediate conjugated diene isomers. *J. Nutr.* 141:1445–1450. <https://doi.org/10.3945/jn.111.138396>.
- Lock, A. L., B. M. Teles, J. W. Perfield II, 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. [https://doi.org/10.3168/jds.S0022-0302\(06\)72220-2](https://doi.org/10.3168/jds.S0022-0302(06)72220-2).
- Martínez Marín, A. L., P. Gómez-Cortés, A. G. Gómez Castro, M. Juárez, L. M. Pérez Alba, M. Pérez Hernández, and M. A. de la Fuente. 2011. Animal performance and milk fatty acid profile of dairy goats fed diets with different unsaturated plant oils. *J. Dairy Sci.* 94:5359–5368. <https://doi.org/10.3168/jds.2011-4569>.
- Miyazaki, M., and J. M. Ntambi. 2008. Fatty acid desaturation and chain elongation in mammals. Pages 191–211 in *Biochemistry of Lipids, Lipoproteins and Membranes*. 5th ed. D. E. Vance and J. E. Vance, ed. Elsevier.
- Mosley, E. E., B. Shafii, P. J. Moate, and M. A. McGuire. 2006. *cis*-9, *trans*-11 Conjugated linoleic acid is synthesized directly from vaccenic acid in lactating dairy cattle. *J. Nutr.* 136:570–575. <https://doi.org/10.1093/jn/136.3.570>.
- Nudda, A., A. Cannas, F. Correddu, A. S. Atzori, M. F. Lunesu, G. Battaccone, and G. Pulina. 2020. Sheep and goats respond differently to feeding strategies directed to improve the fatty acid profile of milk fat. *Animals (Basel)* 10:1290. <https://doi.org/10.3390/ani10081290>.
- Nudda, A., M. Mele, G. Battaccone, M. G. Usai, and N. P. P. Macciotta. 2003. Comparison of conjugated linoleic acid (CLA) content in milk of ewes and goats with the same dietary regimen. *Ital. J. Anim. Sci.* 2:515–517.
- Palin, M.-F., C. Côrtes, C. Benchaar, P. Lacasse, and H. V. Petit. 2014. mRNA Expression of lipogenic enzymes in mammary tissue and fatty acid profile in milk of dairy cows fed flax hulls and infused with flax oil in the abomasum. *Br. J. Nutr.* 111:1011–1020. <https://doi.org/10.1017/S0007114513003589>.
- Palmquist, D. L., and T. C. Jenkins. 2017. A 100-Year review: Fat feeding of dairy cows. *J. Dairy Sci.* 100:10061–10077. <https://doi.org/10.3168/jds.2017-12924>.
- Palmquist, D. L., N. St-Pierre, and K. McClure. 2004. Tissue fatty acid profiles can be used to quantify endogenous rumenic acid synthesis in lambs. *J. Nutr.* 134:2407–2414. <https://doi.org/10.1093/jn/134.9.2407>.
- Prado, L. A., P. Schmidely, P. Nozière, and A. Ferlay. 2019. Milk saturated fatty acids, odd- and branched-chain fatty acids, and isomers of C18:1, C18:2, and C18:3n-3 according to their duodenal flows in dairy cows: A meta-analysis approach. *J. Dairy Sci.* 102:3053–3070. <https://doi.org/10.3168/jds.2018-15194>.
- Rincon, G., A. Islas-Trejo, A. R. Castillo, D. E. Bauman, B. J. German, and J. F. Medrano. 2012. Polymorphisms in genes in the SREBP1 signalling pathway and SCD are associated with milk fatty acid composition in Holstein cattle. *J. Dairy Res.* 79:66–75. <https://doi.org/10.1017/S002202991100080X>.
- Sanz Sampelayo, M. R., Y. Chilliard, P. Schmidely, and J. Boza. 2007. Influence of type of diet on the fat constituents of goat and sheep milk. *Small Rumin. Res.* 68:42–63. <https://doi.org/10.1016/j.smallrumres.2006.09.017>.
- Shingfield, K. J., S. Ahvenjärvi, V. Toivonen, A. Äröla, K. V. V. Nurmela, P. Huhtanen, and J. M. Grinari. 2003. Effect of dietary fish oil on biohydrogenation of fatty acids and milk fatty acid content in cows. *Anim. Sci.* 77:165–179. <https://doi.org/10.1017/S1357729800053765>.
- Shingfield, K. J., S. Ahvenjärvi, V. Toivonen, A. Vanhatalo, and P. Huhtanen. 2007. Transfer of absorbed *cis*-9, *trans*-11 conjugated linoleic acid into milk is biologically more efficient than endogenous synthesis from absorbed vaccenic acid in lactating cows. *J. Nutr.* 137:1154–1160. <https://doi.org/10.1093/jn/137.5.1154>.
- Shingfield, K. J., M. Bonnet, and N. D. Scollan. 2013. Recent developments in altering the fatty acid composition of ruminant-derived foods. *Animal* 7(Suppl 1):132–162. <https://doi.org/10.1017/S1751731112001681>.
- Suárez-Vega, A., B. Gutiérrez-Gil, and J. J. Arranz. 2016. Transcriptome expression analysis of candidate milk genes affecting cheese-related traits in 2 sheep breeds. *J. Dairy Sci.* 99:6381–6390. <https://doi.org/10.3168/jds.2016-11048>.
- Taugbøl, O., I. J. Karlengen, T. Bolstad, A. H. Aastveit, and O. M. Harstad. 2008. Cobalt supplied per os reduces the mammary Δ^9 -desaturase index of bovine milk. *J. Anim. Sci.* 86:3062–3068. <https://doi.org/10.2527/jas.2007-0372>.
- Toral, P. G., L. Bernard, A. Belenguer, J. Rouel, G. Hervás, Y. Chilliard, and P. Frutos. 2016a. Comparison of ruminal lipid metabolism in dairy cows and goats fed diets supplemented with starch, plant oil, or fish oil. *J. Dairy Sci.* 99:301–316. <https://doi.org/10.3168/jds.2015-10292>.
- Toral, P. G., G. Hervás, D. Carreño, H. Leskinen, A. Belenguer, K. J. Shingfield, and P. Frutos. 2017. In vitro response to EPA, DPA, and DHA: Comparison of effects on ruminal fermentation and biohydrogenation of 18-carbon fatty acids in cows and ewes. *J. Dairy Sci.* 100:6187–6198. <https://doi.org/10.3168/jds.2017-12638>.
- Toral, P. G., G. Hervás, and P. Frutos. 2019. In vitro biohydrogenation of ¹³C-labeled α -linolenic acid in response to ruminal alterations associated with diet-induced milk fat depression in ewes. *J. Dairy Sci.* 102:1213–1223. <https://doi.org/10.3168/jds.2018-15536>.
- Toral, P. G., G. Hervás, and P. Frutos. 2021. Dataset: Effect of lipid supplementation on the endogenous synthesis of milk *cis*-9, *trans*-11 CLS in dairy sheep and goats. A tracer assay with ¹³C-vaccenic acid. [Dataset]. Digital CSIC. <https://doi.org/https://dx.doi.org/10.20350/digitalCSIC/13862>.
- Toral, P. G., G. Hervás, A. Suárez-Vega, J. J. Arranz, and P. Frutos. 2016b. Isolation of RNA from milk somatic cells as an alternative to biopsies of mammary tissue for nutrigenomic studies in dairy ewes. *J. Dairy Sci.* 99:8461–8471. <https://doi.org/10.3168/jds.2016-11184>.

- Troegeler-Meynadier, A., M. C. Nicot, C. Bayourthe, R. Moncoulon, and F. Enjalbert. 2003. Effects of pH and concentrations of linoleic and linolenic acids on extent and intermediates of ruminal biohydrogenation in vitro. *J. Dairy Sci.* 86:4054–4063. [https://doi.org/10.3168/jds.S0022-0302\(03\)74017-X](https://doi.org/10.3168/jds.S0022-0302(03)74017-X).
- Tsiplakou, E., E. Flemetakis, C. Kalloniati, G. Papadomichelakis, P. Katinakis, and G. Zervas. 2009. Sheep and goats differences in CLA and fatty acids milk fat content in relation with mRNA stearyl-CoA desaturase and lipogenic genes expression in their mammary gland. *J. Dairy Res.* 76:392–401. <https://doi.org/10.1017/S0022029909990100>.
- Tsiplakou, E., and G. Zervas. 2008. Comparative study between sheep and goats on rumenic acid and vaccenic acid in milk fat under the same dietary treatments. *Livest. Sci.* 119:87–94. <https://doi.org/10.1016/j.livsci.2008.03.009>.
- 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 Biol.* 3:research0034.1. <https://doi.org/10.1186/gb-2002-3-7-research0034>.
- Wickramasinghe, S., G. Rincon, A. Islas-Trejo, and J. F. Medrano. 2012. Transcriptional profiling of bovine milk using RNA sequencing. *BMC Genomics* 13:45. <https://doi.org/10.1186/1471-2164-13-45>.

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