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

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ABSTRACT

Isotopic tracers are used to directly quantify the effect of mammary Δ^9 -desaturation on milk fatty acid (FA) composition, but very few studies have applied this method to measuring the endogenous synthesis of rumenic acid (RA; *cis*-9,*trans*-11 conjugated linoleic acid) in cows and goats, and no publications exist in ewes. In sheep, knowledge about the contribution of stearoyl-coenzyme A desaturase (SCD) to milk FA secretion is derived mostly from indirect estimates based on inhibition of the enzyme by oral administration of cobalt, a cost-effective method that has not been validated to date. To fill both gaps, we conducted an isotopic tracer assay in sheep to quantify the proportion of endogenous RA in milk for the first time in this species. We then compared the results with estimates derived from a Co administration assay performed on the same animals. First, 5 lactating ewes received an intravenous injection of 200 mg of [¹³C]*trans*-11 18:1 (vaccenic acid, VA), the precursor for RA production by SCD activity. At -24, -15, 0, 9, 24, 33, 48, 57, 72, 81, and 96 h post-injection, we recorded milk yield and collected milk samples to examine fat concentration and FA profile. We conducted compound-specific isotope analysis of VA and RA by gas chromatography-combustion isotope ratio mass spectrometry. Afterward, in the Co administration assay, ewes received a daily dose of 7 mg of Co/kg of body weight for 5 d. We analyzed milk samples for composition before and on the last days of cobalt dosing. On average, 17% of the injected amount of [¹³C]VA was transferred to milk within 96 h post-injection, and up to 29% of the VA taken up by the mammary gland was desaturated to milk RA. Under our conditions, the mean proportion of this conjugated linoleic acid isomer deriving from Δ^9 -desaturation represented 82 to 90% of the amount

secreted in milk. However, the proportion estimated in the Co assay with calculations based on individual FA concentrations was lower (on average, 46%). When we calculated the same estimates based on changes in Δ^9 -desaturation ratios after Co dosing, the higher values of endogenous RA (75%) did not differ from the results of the isotopic tracer assay. Nevertheless, correlation analysis between the results obtained through [¹³C] VA or Co administration revealed no significant relationship, which would prevent acceptance of the latter as a reliable alternative to isotopic labeling to examine mammary Δ^9 -desaturation in dairy ewes.

Key words: Δ^9 -desaturation, rumenic acid, sheep, stable isotope, stearoyl-CoA desaturase

INTRODUCTION

Ruminant-derived foods, particularly milk and dairy products, represent the major source of CLA in the human diet (Palmquist et al., 2005; Shingfield and Wallace, 2014). The most abundant isomer in milk is *cis*-9,*trans*-11 18:2, commonly known as rumenic acid (RA), and its consumption has been associated with potential benefits for human health because of its anti-carcinogenic, antiatherogenic, and immunomodulatory activities (Pariza, 2004; Dilzer and Park, 2012). In recent years, there has been great interest in increasing the concentration of this fatty acid (FA) in dairy products (Nudda et al., 2014; Shingfield and Wallace, 2014).

Milk RA has a dual origin: ruminal or endogenous (Palmquist et al., 2005). The first source relates to the biohydrogenation of dietary lipids, because RA is an intermediate metabolite of 18-carbon PUFA. The second source is essentially explained by the action of mammary stearoyl-coenzyme A desaturase (SCD) converting vaccenic acid (VA; *trans*-11 18:1), another product of ruminal FA metabolism, to RA (Palmquist et al., 2005; Bernard et al., 2013). To design the most appropriate strategies to increase milk RA content, the specific contribution of these 2 sources must be

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characterized, which requires the quantification of SCD activity (Bernard et al., 2013).

Numerous studies use SCD products/substrates ratios as proxies for mammary Δ^9 -desaturation, but these calculations may not be sufficiently robust or representative of enzyme activity (Tsiplakou et al., 2009; Bernard et al., 2013). Although radioactive tracers were initially applied to directly quantifying this activity (e.g., Annison et al., 1967; Bickerstaffe and Annison, 1970), indirect estimates based on the inhibition of SCD with stercularic acid or cobalt represent safer alternatives and have been used more frequently in recent years (Griinari et al., 2000; Shingfield et al., 2006; Dallaire et al., 2014). In this regard, oral administration of Co-EDTA or Co-acetate would be particularly cost-effective and easy to implement (Taugbøl et al., 2008; Frutos et al., 2014; Leskinen et al., 2016), but resulting estimates have never been validated by direct comparison with a reference method.

At present, stable isotope labeling constitutes the reference technique for directly, accurately, and safely quantifying the effect of mammary Δ^9 -desaturation on milk FA composition (Mosley et al., 2006; Bernard et al., 2010; Toral et al., 2017). Nevertheless, its application is not widespread (Ecker and Liebisch, 2014). Its high cost and the need for specialized equipment and staff might explain the limited number of publications that have applied this method to quantify the proportion of endogenous RA in milk (which seems limited to one study in cows and another study in goats; Mosley et al., 2006; Bernard et al., 2010). We are not aware of any reports in dairy sheep.

With the main aim of quantifying the contribution of Δ^9 -desaturation to the synthesis of milk RA in dairy ewes, we conducted an isotopic tracer assay using ^{13}C -labeled VA. Then, we compared the results from this first approach with indirect estimates obtained through the inhibition of SCD activity with oral administration of Co-acetate to validate this alternative method of estimation.

MATERIALS AND METHODS

Animals, Experimental Design, and Management

All experimental procedures were approved by the Research Ethics Committee of the Instituto de Ganadería de Montaña, the Spanish National Research Council (CSIC), and the Junta de Castilla y León (Spain), following proceedings described in Spanish and European Union legislation (Royal Decree 53/2013 and Council Directive 2010/63/EU).

We used 5 lactating Assaf ewes with similar milk yield (2.15 ± 0.125 kg/d), milk fat concentration (5.46

$\pm 0.271\%$), BW (78.4 ± 4.24 kg), and DIM (105 ± 1.2 d) at the beginning of the study. Sheep were housed in individual tie stalls and fed a TMR ad libitum, formulated from alfalfa hay and concentrates (Table 1). Ewes were milked daily at 0830 and 1800 h in a milking parlor, and clean drinking water was always available.

The isotopic tracer assay began after 21 d of adaptation to the diet (see Figure 1 for a diagram of the experimental schedule). Each sheep received a single dose of 200 mg of $[1-^{13}\text{C}]trans\text{-}11\ 18:1$ ($[1-^{13}\text{C}]VA$, 99% atom $\%^{13}\text{C}$; 645516-SPEC; Sigma-Aldrich, Madrid, Spain) suspended in an emulsion of 0.4 g of Simulsol 5817 (Seppic, Paris, France), 0.2 mL of glycerol ($\geq 99\%$; G5516; Sigma-Aldrich), and 10 mL of 10% Intralipid (Fresenius Kabi SA, Barcelona, Spain) in 140 mL of saline solution (0.9% NaCl, wt/vol; B. Braum Medical SA, Barcelona, Spain). This dose was selected based on our previous measurements of ^{13}C -labeled FA transfer to ovine milk (Toral et al., 2017); it represented 3.6% of the mean VA + RA yield for 72 h in lactating ewes fed a TMR similar to the one used in the present study (Frutos et al., 2014; Toral et al., 2017). The mixture was sonicated (24 kHz, 150 W) 6 times for 30 s, with 20-s intervals between each sonication pulse. To ensure thorough mixing, the blend was placed in an ultrasonic water bath for 30 min. The emulsion, tempered at 39°C ,

Table 1. Formulation and chemical composition of the experimental diet

Item	TMR ¹	SEM
Ingredient, g/kg of fresh matter		
Dehydrated alfalfa hay (particle size >4 cm)	500	—
Whole corn grain	140	—
Whole barley grain	100	—
Soybean meal solvent 44% CP	150	—
Sugar beet pulp (pellets)	50	—
Molasses (liquid)	40	—
Vitamin–mineral supplement ²	20	—
Chemical composition, g/kg of DM		
OM	907	1.4
CP	182	4.0
NDF	288	15.0
ADF	203	18.2
Total fatty acids	23.0	1.63
16:0	5.21	0.369
18:0	0.91	0.065
<i>cis</i> -9 18:1	3.42	0.242
18:2n-6	9.25	0.655
18:3n-3 ³	3.04	0.215

¹n = 4.

²Macrofac Rumiantes (UP911755130; DSM Nutritional Products S.A., Madrid, Spain). 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).

³Coelutes with 20:1 *cis*-11.

Figure 1. Diagram of the experimental schedule. [1-¹³C]VA = ¹³C-labeled vaccenic acid; RA = rumenic acid; Co = cobalt.

was delivered immediately after the morning milking by continuous jugular infusion for 30 min.

For the Co administration assay, ewes received a daily dose of 7 mg of Co/kg BW from d 5 to 10 of the experiment (see Figure 1). Cobalt supplied in the form of cobalt(II) acetate tetrahydrate ($\geq 98\%$; 403024; Sigma-Aldrich) was dissolved in 450 mL of water and administered orally. Sheep were drenched with one-third of the daily dose at 8-h intervals (0800, 1600, and 2400 h). The dose and protocol of Co administration were based on our previous studies applying this method to examine Δ^9 -desaturation in dairy ewes (see Frutos et al., 2014, and Toral et al., 2015 for details).

Measurements and Sampling Procedure

Representative samples of the TMR were collected throughout the experiment, stored at -30°C , and freeze-dried before chemical analysis. Feed intake was measured daily by weighing the amount of DM offered and refused by each animal.

Milk yield was recorded, and individual milk samples were collected at each milking. For the isotopic tracer assay, aliquots of milk collected at -24 , -15 , 0, 9, 24, 33, 48, 57, 72, 81, and 96 h post-injection (**p.i.**) with [1-¹³C]VA (d -1 to 4 of experiment; see Figure 1) were preserved with bronopol (D&F Control Systems Inc., San Ramon, CA) and stored at 4°C until analyzed for fat concentration. In addition, 1 set of aliquots of untreated milk was stored at -30°C for FA determination and compound-specific isotope analysis. For this latter analysis, and for each ewe, samples collected at -24 and -15 h p.i. were mixed according to yield, and the pool was used as the sample from -24 h.

For the Co administration assay, individual milk samples were collected before and on the last days of cobalt administration (pre-Co and Co periods, respectively). Specifically, milk samples were collected over 4 consecutive milkings starting at 1800 h on d 2 and 8 of the experiment (Figure 1), composited daily according to evening and next morning milk yield, and stored until fat and milk FA composition analyses, as described above.

Laboratory Analysis

Diets and Orts. Dry matter concentration was determined in the TMR and Orts (ISO 6496; ISO, 1999a). The TMR was also analyzed for ash (ISO 5984; ISO, 2002) and CP (ISO 59832; ISO, 2009). The NDF and ADF were determined using an Ankom 2000 fiber analyzer (methods 13 and 12, respectively; Ankom Technology Corp., Macedon, NY). The former was assayed with sodium sulfite and α -amylase, and both were expressed with residual ash. The FAME of lipids in freeze-dried TMR samples were prepared in a 1-step extraction-transesterification procedure (Shingfield et al., 2003) using tridecanoic acid (Sigma-Aldrich) as an internal standard. Then, FAME were separated and quantified by gas chromatography–flame ionization detector (**GC-FID**) as explained below for milk samples. Peaks were identified based on retention time comparisons with commercially available standards (GLC463, Nu-Chek Prep., Elysian, MN; and 18919-1AMP Supelco, Sigma-Aldrich).

Milk. Fat concentration was determined by infrared spectrophotometry (ISO 9622:1999; ISO, 1999b) using a MilkoScan FT6000 (Foss, Hillerød, Denmark). Lipids

in 1 mL of milk were 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, Santa Clara, CA) using a 100 m fused silica capillary column (CP-SIL 88, CP7489; Varian Ibérica S.A., Madrid, Spain) and hydrogen as the carrier gas. The total FAME profile was determined using a temperature gradient program, and then isothermal conditions at 170°C to further resolve 18:1 isomers (Shingfield et al., 2003). Overlapping *cis*-9,*trans*-11, *trans*-7,*cis*-9, and *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 (GLC463, U-37-M, U-39-M, U-43-M, U-45-M, U-54-M, U-64-M, U-85-M, U-87-M, N-21-M, and N-23-M, Nu-Chek Prep.; 18919-1AMP Supelco, L6031, L8404, H6389, H6639, T1902, O4129, and O5632, Sigma-Aldrich; and 10-1620, 10-1840, 10-2001-1, 11-1600-8, 20-1606-4, 20-1611, 20-1869-2, 10-2014-5, 20-2024-1, 20-2103-4, 20-2105-4, 20-2210-9, 20-2265-7, 20-2305-1-4, 20-2405-4, 20-2500-1, 20-2500-7, 20-2900-7, 21-1211-7, 21-1413-7, 21-1614-7, 21-1615-7, and BR mixtures 2 and 3, Larodan, Solna, Sweden) 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 GC-MS analysis of corresponding 4,4-dimethyloxazoline derivatives (Bichi et al., 2013).

Compound-specific isotope analysis was performed at the Stable Isotopes Laboratory of the Interdepartmental Research Service (SIIdI, Autonomous University of Madrid, Spain) using a gas chromatography-combustion isotope ratio mass spectrometry (GC-C-IRMS) system (Thermo Scientific, Bremen, Germany) 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 isothermal program used in the 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.

Calculations and Statistical Analysis

In the isotopic tracer assay, calculations to determine the endogenous synthesis of milk RA (see Supplemental Table S1; <https://doi.org/10.3168/jds.2019-17050>) were based on the equations detailed in a previous report infusing ^{13}C -labeled FA to lactating ewes (Toral et al., 2017). First, we computed total milk FA yield as outlined by Glasser et al. (2007). In brief, we used the mean molecular weight of the FA to estimate the proportion of FA in milk triglycerides, which we then multiplied by milk fat yield. We used milk FA yield to calculate the amount in grams of VA and RA secreted in milk at each post-injection sampling period. To account for the natural levels of ^{13}C and determine the proportions of $[1-^{13}\text{C}]\text{VA}$ and $[1-^{13}\text{C}]\text{RA}$ that were actually derived from the injected labeled FA, we subtracted the mean ^{13}C % of samples taken before $[1-^{13}\text{C}]\text{VA}$ injection (i.e., samples from -24 and 0 h) from the ^{13}C % of each sample. We also corrected the $[1-^{13}\text{C}]\text{RA}$ enrichments for the coelution with *trans*-7,*cis*-9 and *trans*-8,*cis*-10 CLA, assuming that these minor isomers had the same natural ^{13}C level as pre-injection samples. We then calculated the secretion of $[1-^{13}\text{C}]\text{VA}$ and $[1-^{13}\text{C}]\text{RA}$ in milk derived from the isotopic tracer and used that to determine the proportion of VA desaturated to RA in vivo, the fraction of milk RA synthesized endogenously, and the percent of the injected labeled FA secreted into milk lipids.

In the Co administration assay, we estimated the endogenous synthesis of milk RA based on previous reports (e.g., Griinari et al., 2000; Frutos et al., 2014). Equations are detailed in Supplemental Table S2 (<https://doi.org/10.3168/jds.2019-17050>). First, because complete inhibition of the enzyme is unlikely, we estimated the extent of this inhibition in response to our dose of Co by examining the reduction in milk concentrations of FA that are synthesized almost exclusively in the mammary gland through SCD activity (Bauman and Davis, 1974; Mosley and McGuire, 2007). Specifically, we used the concentrations of *cis*-9 10:1, *cis*-9 12:1, and *cis*-9 14:1 in milk in the pre-Co and Co periods to this aim. Then, we applied these results as correction factors to adjust the decrease in milk RA concentration after Co dosing and re-estimate it in the theoretical situation of complete inhibition of the enzyme. The estimated proportion, relative to total milk RA concentration in the pre-Co period, would represent the endogenous synthesis of this FA. We conducted an alternative estimation using the same calculations but replacing individual FA

concentrations (i.e., *cis*-9 10:1, *cis*-9 12:1, *cis*-9 14:1, and RA) by milk Δ^9 -desaturation ratios (i.e., *cis*-9 10:1/10:0, *cis*-9 12:1/12:0, *cis*-9 14:1/14:0, and RA/VA, which represent SCD product/SCD substrate).

Animal performance and FA composition data in the pre-Co and Co periods were analyzed by repeated measurement analysis using the MIXED procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC). The statistical model included the fixed effect of period (pre-Co vs. Co), animal was considered a random effect, and day (nested within period) appeared in the repeated statement. We tested different covariance structures (e.g., compound symmetric, first-order autoregressive, heterogeneous first-order autoregressive, first-order antedependence, or unstructured) and for each individual variable, we selected the best model based on Schwarz's Bayesian information model fit criterion (Kaps and Lamberson, 2009).

Milk ^{13}C enrichments of the substrate (VA) and its desaturation product (RA) at different post-injection samplings were also subjected to repeated measurement analysis using the MIXED procedure of SAS. The statistical model included the fixed effect of post-injection time and the random effect of animal. We used the pre-injection value (mean of samples from -24 and 0 h) as the covariate. For each FA, we selected the covariance structure as described above. 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 the Bonferroni method.

We modeled the decay in milk ^{13}C enrichment over time for VA and RA from the observed maximum enrichment (9 h p.i.) using an exponential decay function, as reported by Mosley et al. (2006):

$$E(t) = A \times e^{[-B \times (t - L)]},$$

where $E(t)$ is the predicted enrichment at time t (h), A is the maximum enrichment, B is the rate of decay, and L is the lag term set to 9 h. Parameter estimation was accomplished using the iterative DUD method via the NLIN procedure of SAS.

Data from endogenous FA synthesis derived from each method and the estimated inhibition of Δ^9 -desaturase in the Co administration assay were submitted to 1-way ANOVA using the MIXED procedure of SAS with a statistical model that included the fixed effect of calculation method and the random effect of animal. We conducted pairwise comparisons as described above and, for data for endogenous RA synthesis, adjusted for multiple comparisons using the Bonferroni method.

We then examined correlations between the different results of endogenous FA synthesis in milk and of the inhibition of Δ^9 -desaturase using the CORR procedure of SAS.

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 article.

RESULTS

Isotopic Tracer Assay

As shown in Supplemental Figure S1 (<https://doi.org/10.3168/jds.2019-17050>), feed intake, milk yield, and milk fat concentration were relatively stable throughout the isotopic tracer assay. Milk FA concentrations also remained constant during this period, and mean daily values for the 5 ewes ranged from 0.62 ± 0.078 to 0.66 ± 0.069 for VA, and from 0.30 ± 0.026 to 0.31 ± 0.026 for RA. A detailed FA profile of milk collected over d -1 to 4 of the experiment is presented in Supplemental Table S3 (<https://doi.org/10.3168/jds.2019-17050>). According to GC-FID analysis using the SLB-IL111 column, milk RA represented $91.4 \pm 0.25\%$ of the coelution between CLA isomers measured with the CP-SIL 88 column on average, and the remaining portion was identified as *trans*-7,*cis*-9 CLA ($6.9 \pm 0.15\%$) and *trans*-8,*cis*-10 CLA ($1.7 \pm 0.26\%$).

Figure 2 reports the ^{13}C enrichment of milk VA and RA in milk fat, and parameter estimates for the exponential models describing the decay in milk ^{13}C enrichment over time are shown in Supplemental Table S4 (<https://doi.org/10.3168/jds.2019-17050>). In both FA, maximum enrichments were observed at 9 h p.i. ($P \leq 0.047$) and followed by a relatively rapid decline between 9 and 33 h p.i. (Figure 2). Afterward, the decrease in $[1-^{13}\text{C}]\text{VA}$ and $[1-^{13}\text{C}]\text{RA}$ concentration was less pronounced, with no statistical differences ($P \geq 0.56$) between 33 and 96 h p.i. after Bonferroni correction. From 57 h p.i., mean ^{13}C enrichments were always numerically higher (11 to 47%) for RA than for VA. Thus, we conducted calculations of VA uptake and desaturation to RA using animal performance and milk FA data recorded from injection to 33, 57, and 96 h p.i.. These data are compiled in Table 2, and Table 3 reports the final results of the equations. On average, 17.1% of the injected amount of $[1-^{13}\text{C}]\text{VA}$ was transferred to milk within 96 h p.i., and 27.7 to 29.4% of the VA taken up by the mammary gland would be desaturated to milk RA in vivo. The mean proportion of this CLA isomer derived from Δ^9 -desaturation was 82.3, 85.8, and 89.9% for the 0 to 33, 0 to 57, and 0 to 96 h p.i. periods, respectively.

Co Administration Assay

Table 4 reports the effects of oral administration of Co-acetate on animal performance and milk FA composition. Compared with the pre-Co period, milk fat content remained unaffected ($P = 0.97$), but we found trends toward lower feed intake (-12.9% ; $P = 0.056$) and milk yield (-5.8% ; $P = 0.099$). The concentration of *cis*-9-containing FA was decreased because of Co dosing ($P \leq 0.01$), with a different pattern of variation depending on carbon chain length. Short- and medium-

chain MUFA showed the greatest reductions (40 to 46%; $P \leq 0.01$), whereas *cis*-9 18:1 and 18:2 isomers were least affected (16 to 27% decrease; $P \leq 0.01$). We found intermediate values for the minor *cis*-9,*trans*-12,*cis*-15 18:3 (35% decline, always in comparison with the pre-Co period; $P = 0.009$). Milk Δ^9 -desaturation ratios were also lower due to Co-acetate, decreasing by 42 to 50% for FA with 10, 12, and 14 carbons ($P < 0.001$), and by 33 to 34% for *cis*-9 18:1/18:0 and *cis*-9,*trans*-11 18:2/*trans*-11 18:1 ($P < 0.001$). In contrast, variations in SCD substrates did not follow a consistent

Figure 2. Enrichment of ^{13}C in (A) *trans*-11 18:1 (vaccenic acid; VA), and (B) *cis*-9,*trans*-11 18:2 (rumenic acid; RA), in milk fat from dairy ewes receiving the intravenous injection of 200 mg of $[1-^{13}\text{C}]\text{VA}$ at time 0. Error bars represent the SEM. For each fatty acid, different letters (a–c) indicate differences ($P < 0.05$) between values obtained at each post-injection time. The samples from -24 h correspond to the pool of milk collected at -24 and -15 h (i.e., for each animal, samples collected at both milkings were mixed according to yield). All values are corrected for the mean ^{13}C % of samples taken before $[1-^{13}\text{C}]\text{VA}$ injection (i.e., -24 and 0 h samples).

Table 2. Animal performance and milk fatty acid (FA) data used to determine the endogenous synthesis of milk *cis*-9,*trans*-11 18:2 (ruminic acid; RA) in dairy ewes receiving an i.v. injection of 200 mg ¹³C-labeled 18:1 *trans*-11 (vaccenic acid; [¹³C]VA)

Item	Injection to 33 h post-injection		Injection to 57 h post-injection		Injection to 96 h post-injection	
	Mean	SEM	Mean	SEM	Mean	SEM
Total milk yield, g	2,927	248.7	4,985	429.9	8,214	691.3
Milk fat, % of raw milk	5.75	0.248	5.78	0.237	5.56	0.216
Total milk fat yield, g	167	12.9	286	21.5	453	34.9
Total FA, % of milk fat	94.0	0.02	94.0	0.02	94.0	0.01
Total FA yield, g	157	12.1	269	20.2	426	32.8
VA, % of total milk FA	0.664	0.0778	0.658	0.0716	0.657	0.0675
RA, % of total milk FA	0.307	0.0232	0.302	0.0221	0.303	0.0204
[¹³ C]VA, % of milk VA	3.03	0.352	2.44	0.242	2.00	0.164
[¹³ C]RA, % of milk RA	2.62	0.209	2.19	0.143	1.88	0.099

pattern, and increases in the Co period were significant only for 14:0, 18:0, and *trans*-11 18:1 concentrations (10, 26, and 22%, respectively; $P < 0.05$).

The estimated inhibition of Δ^9 -desaturase by Co administration was similar ($P = 0.58$), regardless of the method of calculation; it averaged 43.6% when using the mean concentration of *cis*-9 10:1, *cis*-9 12:1, and *cis*-9 14:1, and 45.5% with the respective Δ^9 -desaturation ratios (Table 5). Both results were also highly correlated ($r = 0.99$; $P = 0.001$). However, the estimated proportion of endogenous milk RA varied substantially ($P < 0.001$), from 46.3% in calculations based on individual FA concentrations to 75.2% in those derived from Δ^9 -desaturation ratios (Table 5). These latter estimations were not correlated ($P = 0.65$).

Comparison of Methods to Determine Endogenous RA Synthesis

We compared data for endogenous RA synthesis from each method using pairwise analysis (Table 5).

The proportion obtained in the Co administration assay (calculations based on individual FA concentrations) was lower ($P < 0.001$) than that based on isotope analysis, regardless of the time period used (0 to 33, 0 to 57 or 0 to 96 h p.i.). However, estimations based on changes in Δ^9 -desaturation ratios after Co dosing resulted in higher values of endogenous RA synthesis ($P = 0.007$), which did not differ ($P \geq 0.66$) from those found with the isotopic tracer technique. In any event, we noted no correlation ($P \geq 0.24$) between the results of the 2 assays.

DISCUSSION

Isotopic tracers are used to directly measure in vivo mammary SCD activity, but very few studies have applied this technique to quantify the endogenous synthesis of milk RA in cows and goats (Mosley et al., 2006; Bernard et al., 2010), and we are not aware of any reports in ewes. In sheep, our knowledge about the contribution of Δ^9 -desaturation to milk FA secretion

Table 3. Calculations to determine the endogenous synthesis of milk *cis*-9,*trans*-11 18:2 (ruminic acid; RA) in dairy ewes receiving an i.v. injection of 200 mg of ¹³C-labeled *trans*-11 18:1 (vaccenic acid; [¹³C]VA)

Item	Equation ¹	Injection to 33 h post-injection		Injection to 57 h post-injection		Injection to 96 h post-injection	
		Mean	SEM	Mean	SEM	Mean	SEM
VA secreted into milk post-injection, mg	1	1,019	77.8	1,736	135.1	2,750	211.6
RA secreted into milk post-injection, mg	2	476	30.4	804	56.0	1,277	86.3
[¹³ C]VA enrichment, % of milk VA	3	1.96	0.352	1.36	0.242	0.93	0.164
[¹³ C]RA enrichment, % of milk RA	4	1.54	0.209	1.11	0.143	0.79	0.099
[¹³ C]VA secreted into milk, mg	5	19.0	2.27	22.5	2.38	24.3	2.47
[¹³ C]RA secreted into milk, mg	6	7.25	0.829	8.83	0.797	10.01	0.810
[¹³ C]VA secreted into milk, % of injected dose	7	13.1	1.53	15.7	1.56	17.1	1.61
VA desaturated to milk RA, % of VA taken up by the mammary gland	8	27.7	0.78	28.4	1.04	29.4	1.04
Mammary uptake of VA post-injection, mg	9	1,414	115.5	2,431	200.7	3,906	315.8
VA desaturated to milk RA post-injection, mg	10	394	38.6	695	70.8	1,156	112.6
Endogenous milk RA, % of milk RA	11	82.3	4.96	85.8	4.80	89.9	4.88

¹Equations detailed in Supplemental Table S1 (<https://doi.org/10.3168/jds.2019-17050>).

Table 4. Animal performance, and fatty acid (FA) composition and Δ^9 -desaturation ratios in milk of dairy ewes before Co dosing (pre-Co period) and on the last days of administration of 7 mg of Co/kg of BW per day (Co period)

Item	Period ¹		SEM	P-value
	Pre-Co	Co		
Animal performance				
Feed intake, kg of DM/d	3.03	2.64	0.095	0.056
Milk yield, kg/d	2.07	1.95	0.040	0.099
Milk fat, % of raw milk	5.56	5.55	0.237	0.966
Milk FA, % of total FA				
10:0	10.9	11.1	0.10	0.240
12:0	6.28	6.20	0.069	0.467
14:0	12.2	13.4	0.04	<0.001
16:0	27.2	26.9	0.46	0.681
17:0	0.602	0.614	0.0065	0.269
18:0	7.02	8.81	0.221	0.005
<i>cis</i> -9 10:1	0.287	0.169	0.0052	<0.001
<i>cis</i> -9 12:1	0.100	0.054	0.0039	0.001
<i>cis</i> -9 14:1	0.153	0.086	0.0066	0.002
<i>cis</i> -9 16:1	0.650	0.391	0.0158	<0.001
<i>cis</i> -9 17:1	0.187	0.142	0.0038	0.001
<i>cis</i> -9 18:1 ²	11.6	9.79	0.151	0.001
<i>trans</i> -11 18:1	0.655	0.798	0.0326	0.036
<i>trans</i> -12 18:1	0.272	0.274	0.0094	0.932
<i>cis</i> -9, <i>trans</i> -11 18:2	0.299	0.240	0.0066	0.003
<i>cis</i> -9, <i>trans</i> -12 18:2	0.028	0.021	0.0010	0.008
<i>cis</i> -9, <i>trans</i> -13 18:2	0.173	0.127	0.0052	0.003
<i>trans</i> -7, <i>cis</i> -9 18:2	0.023	0.018	0.0005	0.003
<i>cis</i> -9, <i>trans</i> -12, <i>cis</i> -15 18:3	0.017	0.011	0.0008	0.009
Milk Δ^9 -desaturation ratios				
<i>cis</i> -9 10:1/10:0	0.026	0.015	0.0005	<0.001
<i>cis</i> -9 12:1/12:0	0.016	0.009	0.0004	<0.001
<i>cis</i> -9 14:1/14:0	0.012	0.006	0.0004	<0.001
<i>cis</i> -9 18:1/18:0	1.66	1.12	0.017	<0.001
<i>cis</i> -9, <i>trans</i> -11 18:2/ <i>trans</i> -11 18:1	0.461	0.306	0.0023	<0.001

¹Values represent the mean over 2 d of milk sample collection (see Figure 1).²Contains *trans*-13+14 18:1 as minor components.

is derived mostly from indirect estimates based on Co administration (Frutos et al., 2014; Toral et al., 2015), a method that has not been validated to date. To fill both gaps, we employed ¹³C-labeled VA to quantify the proportion of endogenous RA in ewe milk, and then

compared the results with estimates derived from Co dosing in the same animals.

The transfer efficiency of [1-¹³C]VA to milk (17% in 96 h) was similar to the value obtained after the i.v. injection of [1-¹³C]18:0 to lactating ewes under simi-

Table 5. Endogenous synthesis of milk *cis*-9,*trans*-11 18:2 (rumenic acid; RA) in dairy ewes in the 2 assays, and estimated inhibition of Δ^9 -desaturase in the Co administration assay

Item	Endogenous milk RA, % of milk RA	Inhibition of Δ^9 -desaturase, %
Isotopic tracer assay		
Injection to 33 h post-injection	82.3 ^a	NA ¹
Injection to 57 h post-injection	85.8 ^a	NA
Injection to 96 h post-injection	89.9 ^a	NA
Co administration assay		
Calculations based on individual fatty acid concentrations	46.3 ^b	43.6
Calculations based on Δ^9 -desaturation ratios ²	75.2 ^a	45.5
SEM	5.05	2.28
P-value	<0.001	0.582

^{a,b}Means within a column with different superscripts differed significantly ($P < 0.05$).¹Not applicable.²Determined as Δ^9 -desaturase product/substrate.

lar experimental conditions (15% in 72 h; Toral et al., 2017). However, in cows receiving an abomasal infusion of this tracer (Mosley et al., 2006), it was estimated that 33% of the absorbed $[1-^{13}\text{C}]$ VA appeared in milk within 24 h. These inconsistent results might be attributed to differences in the mammary FA uptake of each species or in the route of $[1-^{13}\text{C}]$ VA administration (i.v. vs. abomasal), but the scarcity of data in the literature makes it difficult to elucidate the relevance of each component. In any event, a meta-analysis in dairy cows indicated that the transfer efficiency of VA + RA from duodenum to milk can be highly variable (1.3 to 43.3%; Glasser et al., 2008), suggesting that several factors might be involved.

The kinetics of the appearance of $[1-^{13}\text{C}]$ VA and $[1-^{13}\text{C}]$ RA in ewe milk (Figure 2) had similarities to previous observations in cows (Mosley et al., 2006) and goats (Bernard et al., 2010). In the 3 species, a clear peak of ^{13}C enrichment was found in the first 8 to 12 h p.i. for both the tracer and its SCD product, even though the subsequent decline in enrichment was faster in cows (baseline levels were recovered 24 h p.i.; Mosley et al., 2006) than in goats (slow decline until 72 h p.i.; Bernard et al., 2010) and ewes (Figure 2). When these data were fitted to an exponential model, rates of decay in ^{13}C enrichment of milk VA and RA in ewes were approximately 25 and 21% of those obtained by Mosley et al. (2006) in dairy cows. The curve from the present study (Figure 2) also differs from that obtained after administration of ^{13}C -labeled 18:0 to dairy sheep (Toral et al., 2017), when the maximum concentration of the tracer was found later (16 h p.i.), and no peak of enrichment was detected for its SCD product (*cis*-9 18:1). However, the kinetics of $[1-^{13}\text{C}]$ VA secretion in cows (Mosley et al., 2006) were comparable to that described for ^{13}C -labeled 18:0, 16:0, and 14:0 in a preliminary test using a single cow (Mosley and McGuire, 2007). Given the very low number of reports on the use of isotopic tracers to examine FA metabolism in dairy ruminants, further research is necessary to determine the contribution of interspecies variations in mammary lipogenesis or in the transport kinetics of each FA.

Milk ^{13}C enrichments of VA and RA were still beyond detection levels at 96 h p.i., a longer period than that reported in earlier trials in cows (up to 24 h p.i.; Mosley et al., 2006; Mosley and McGuire, 2007) and goats (72 h p.i.; Bernard et al., 2010), which may have been partly related to the higher resolution and sensitivity of the GC-C-IRMS used in our assay compared with the more frequent GC-MS method (Meier-Augenstein, 1999). In this regard, our results support that GC-C-IRMS would represent the technique of choice when low ^{13}C enrichments are expected, and it allows for reductions in the dose and therefore the cost of the isotopic tracer,

minimizing the potential interference with in vivo FA pools.

The proportion of VA desaturated by SCD action (28 to 29% of the amount taken up by the mammary gland) was within the range obtained by means of isotopic tracers in cows (26%; Mosley et al., 2006) and goats (32%; Bernard et al., 2010) and indirect estimates in dairy ewes (22 to 29%; Bichi et al., 2012; Frutos et al., 2014; Toral et al., 2015). This limited variation across ruminant species and experimental conditions was consistent with the results of 2 meta-analyses (Glasser et al., 2008; Prado et al., 2019), which suggested that mammary SCD activity may not be a limiting factor for milk RA synthesis. Therefore, the VA-to-RA ratio in lipids absorbed in the duodenum and available for mammary uptake might be more relevant in determining the proportion of endogenous RA in milk.

The VA-to-RA ratio in digesta is expected to be greater when diets are richer in 18:3n-3 than in 18:2n-6, given that RA is not a major biohydrogenation metabolite of the former PUFA (Lee and Jenkins, 2011; Kairenius et al., 2018; Toral et al., 2019). This would be consistent with comparisons between assays based on SCD inhibition, which suggested greater endogenous RA proportions in the milk of cows and sheep fed pasture or linseed oil supplements (74 to 91%; Kay et al., 2004; Bichi et al., 2012; Toral et al., 2015) relative to those that received hay and concentrates (46 to 78%; Griinari et al., 2000; Corl et al., 2001; Frutos et al., 2014; and the present study). However, our isotopic tracer assay indicated that the latter feeding conditions resulted in a higher percentage of endogenous RA in ewes (82 to 90%). In cows and goats receiving rations rich in 18:2n-6, large proportions of milk RA (83 and 73%, respectively) derived from $[1-^{13}\text{C}]$ VA desaturation (Mosley et al., 2006; Bernard et al., 2010). Comparative isotopic tracer assays in animals fed diets with high 18:3n-3 or 18:2n-6 contents are warranted to confirm these differences. Nevertheless, Co dosing has been suggested as a cost-effective alternative to study SCD activity (Taugbøl et al., 2010; Frutos et al., 2014; Leskinen et al., 2016), but the comparison with the isotopic tracer assay raises doubts about its validity, at least in sheep.

In our study, we found large differences in endogenous RA between methodologies when estimations based on Co administration were calculated with changes in individual FA concentrations, a standard procedure in SCD inhibition trials (Corl et al., 2001; Kay et al., 2004; Bichi et al., 2012). This result agreed with the lower contribution of Δ^9 -desaturation to milk RA production observed in a previous trial dosing Co-acetate to ewes fed a similar high-concentrate TMR (51% of endogenous RA; Frutos et al., 2014) and challenges the

accuracy of estimates based on this method, at least in lactating ewes.

However, replacing individual FA concentrations by corresponding milk Δ^9 -desaturation ratios markedly increased the estimated proportion of endogenous RA in the Co assay (to 75%), removing the difference with the isotopic tracer assay. When the 2 calculation methods were applied to *cis*-9 18:1, a FA that had been the focus of our previous research (Toral et al., 2017), we observed large differences in the amounts derived from SCD activity in estimates based individual FA data or Δ^9 -desaturation ratios (36 vs. 72%, respectively). Consistent variations in endogenous RA and oleic acid proportions were also found when both estimates were conducted with data from earlier studies dosing Co to cows or sheep (Shingfield et al., 2008; Karlengen et al., 2012; Frutos et al., 2014), highlighting the importance of the mathematical approach. Nonetheless, the lack of correlation between the results obtained in the isotopic tracer or the Co assay prevents acceptance of the latter as a reliable alternative to examining mammary Δ^9 -desaturation in ewes. Available information is not sufficient to determine the origin of the inconsistency. In this regard, Co administration may disturb performance and mammary lipogenesis beyond SCD inhibition (e.g., decreasing DMI, milk yield, and de novo FA synthesis; Karlengen et al., 2013; Frutos et al., 2014; Leskinen et al., 2016), although the association between these effects and estimates of endogenous RA remains uncertain.

CONCLUSIONS

According to the isotopic tracer assay, an average of 29% of the VA ($[1-^{13}\text{C}]trans$ -11 18:1) transferred to the mammary gland of dairy sheep is desaturated in vivo to RA ($[1-^{13}\text{C}]cis$ -9, *trans*-11 CLA). Under our experimental conditions, the endogenous synthesis represented 82 to 90% of the RA secreted in ewe milk. However, when the same animals were used in the Co administration assay, the estimated proportions of endogenous RA ranged from 46 to 75%, depending on the calculation method (derived from the use of individual FA or from Δ^9 -desaturation ratios, respectively). The inconsistencies and lack of correlation in the results from the assays using $[1-^{13}\text{C}]VA$ or oral dosing of Co would prevent acceptance of the latter as an accurate and reliable alternative for examining mammary Δ^9 -desaturation in dairy ewes.

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