In vitro biohydrogenation of ^{13}C -labeled α -linolenic acid in response to ruminal alterations associated with diet-induced milk fat depression in ewes

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ABSTRACT

The basis for marine lipid-induced milk fat depression (MFD) has not been established yet, but recent reports suggest the putative contribution of shifts in the ruminal metabolism of α -linolenic acid (ALA). To test this hypothesis, an isotopic tracer approach was used in batch cultures of rumen microorganisms with inoculum collected from cannulated ewes fed either a total mixed ration without lipid supplementation (control inoculum) or the same diet supplemented with 2% of fish oil, which is known to cause MFD in lactating sheep (FO-MFD inoculum). The $[1-^{13}\text{C}]$ ALA was added at a dose of 1% of incubated dry matter and the proportions of ^{13}C -labeled fatty acids (FA) were examined after 24 h of incubation, using complementary gas chromatography and gas chromatography-combustion isotope ratio mass spectrometry (GC-C-IRMS) analyses. Expected differences in FA profiles were confirmed between control and FO-MFD inocula (e.g., large decreases in 18:0 and increases in most 18:1 and 18:2 intermediates, particularly *trans* isomers, to fish oil supply). The biohydrogenation of ^{13}C -labeled ALA was extensive and yielded multiple metabolites, with a total of 48 chromatographic peaks showing ^{13}C enrichment, regardless of the inoculum type. However, although ALA was biohydrogenated through common pathways under standard or MFD conditions, large changes in the accumulation of ^{13}C -labeled FA suggest important differences in the relative contribution of each specific route. First, increased accumulation of *trans*-11-containing FA in FO-MFD incubations was accompanied by a general repression of the *trans*-13/14 pathway (supported by lower *trans*-13+14 18:1 or *trans*-11,*trans*-13 18:2 proportions), together with a lower production of *cis* FA (e.g., *cis*-9, -12, and -15 18:1 and some *cis,cis* 18:2). Results also downplayed the relevance of the *trans*-11 to *trans*-10 shift as an effective marker of diet-induced

MFD in sheep, and challenged the involvement of some *trans*-10 intermediates (e.g., *trans*-10 18:1 and *trans*-10,*cis*-15 18:2) in the low-fat milk syndrome in this species. Conversely, increased abundance of most 18:3 intermediates (including some unidentified isomers) might be representative of ruminal alterations related to fish oil supplementation in ewes. Further research is necessary to examine the potential association between these findings and MFD in lactating animals.

Key words: fish oil, lipid metabolism, sheep, stable isotope, *trans* fatty acid

INTRODUCTION

Ruminal biohydrogenation (BH), nutritional quality of milk fat, and performance traits are closely interrelated in dairy ruminants (Jenkins et al., 2008; Harvatine et al., 2009; Shingfield et al., 2010). It is well established that changes in ruminal fatty acid (FA) outflow modify milk fat composition (Chilliard et al., 2007). Concerning performance traits, certain feeding strategies that alter BH pathways detrimentally affect milk fat synthesis, the mechanism being probably mediated by increased formation of FA intermediates that inhibit mammary lipogenesis (Bauman and Griinari, 2001). A common example of this diet-induced milk fat depression (MFD) is found in dairy cows fed plant oils or high-concentrate diets that favor the accumulation of *trans*-10,*cis*-12 CLA in the rumen (Bauman and Griinari, 2001). However, the basis for the MFD observed in bovine and small ruminants supplemented with marine lipids has not been established yet (Harvatine et al., 2009; Carreño et al., 2016). In this regard, recent reports challenge the hypothesis of an involvement of the shortage of ruminal 18:0 and associated alterations in the maintenance of milk fat fluidity, and suggest the putative contribution of little known BH intermediates, such as *trans*-9,*cis*-11 CLA and *trans*-10,*cis*-15 18:2 (Kairenius et al., 2015; Toral et al., 2016; Frutos et al., 2018), which may derive from shifts in the ruminal metabolism of α -linolenic acid (*cis*-9,*cis*-12,*cis*-15 18:3, **ALA**; Lee and Jenkins, 2011; Alves and Bessa, 2014).

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Our understanding of ruminal BH has notably increased with the use of isotopic tracers, specifically ^{13}C -labeled FA, in *in vitro* assays (e.g., Mosley et al., 2002; Lee and Jenkins, 2011; Lee 2013). Early research postulated ruminal FA metabolism as a relatively straightforward process with few enzymatic reactions and products (Harfoot and Hazlewood, 1997), but multiple alternative pathways and intermediates are now recognized (Proell et al., 2002; Lee and Jenkins, 2011; Honkanen et al., 2016). In this context, microbial inoculum may be a crucial determinant to mimic the ruminal environment *in vitro* (Mould et al., 2005). However, *in vitro* BH assays have rarely compared the use of inocula derived from donors adapted to different diets (Vlaeminck et al., 2008; Laverroux et al., 2011) and no publications are available on the metabolic fate of ^{13}C -labeled FA under conditions of altered BH that would be at the core of marine lipid-induced MFD.

On this basis, an *in vitro* study was conducted to examine the changes in the BH of ALA in response to ruminal alterations associated with marine lipid-induced MFD in sheep. To meet this objective, an isotopic tracer approach was used in batch cultures of rumen microorganisms with inoculum collected from cannulated ewes fed either a diet without supplementation (control) or a diet supplemented with fish oil that was known to induce MFD (**FO-MFD**; Carreño et al., 2016; Toral et al., 2016).

MATERIALS AND METHODS

All experimental procedures were approved and completed in accordance with the Spanish Royal Decree 53/2013 for the protection of animals used for experimental purposes.

Batch Cultures of Rumen Microorganisms

Batch cultures of rumen microorganisms were conducted as outlined previously (Toral et al., 2017), with rumen fluid collected from 5 dry ewes (BW = 65.5 ± 2.28 kg), each equipped with a ruminal cannula of 40 mm of internal diameter. To obtain 2 different rumen inocula (i.e., experimental treatments), sheep were randomly divided in 2 groups that were fed either a basal TMR without lipid supplementation (control inoculum; 3 ewes) or the same TMR supplemented with 2% DM of fish oil (FO-MFD inoculum; 2 ewes). Table 1 reports the ingredients and chemical composition of both TMR, which were offered daily (0900 h) at 1.2 times the estimated maintenance energy requirements of the sheep (INRA, 2007). Clean drinking water was always accessible.

After an adaptation period of 21 d, individual rumen fluids were obtained via the cannula before feeding and immediately taken in thermal flasks to the laboratory, where they were strained through a nylon membrane (250 μm ; Fisher Scientific S.L., Madrid, Spain) while bubbled with CO_2 . For each group of ewes, equal volumes of the strained rumen fluids were combined and used as the inoculum in the incubations. Subsamples of the 2 inocula (ca. 50 mL) were also collected, immediately frozen at -80°C , freeze-dried, and stored again at -80°C until analyzed for FA composition.

The collection of rumen inocula and the batch cultures were repeated on 3 different days (3 runs). The incubation was conducted using 16-mL Hungate tubes that contained 12 mL of a mix (1:4) of the corresponding rumen inoculum and a phosphate-bicarbonate buffer (Goering and Van Soest, 1970). The basal TMR fed to the sheep of the control treatment was used as the substrate for incubation (6.25 mg of DM/mL of buffered rumen fluid), ground in a hammer mill fitted with a 0.5-mm screen. Each tube contained 0.75

Table 1. Ingredients, chemical composition, and fatty acid (FA) profile of the basal TMR without lipid supplementation or supplemented with 2% DM of fish oil fed to the ewes employed as donors of rumen inocula

Item	TMR	TMR + 2% DM fish oil
Ingredient, % of fresh matter		
Dehydrated alfalfa hay, particle size >4 cm	50.0	49.1
Whole corn grain	14.0	13.7
Whole barley grain	10.0	9.8
Soybean meal solvent, 440 g of CP/kg	15.0	14.7
Sugar beet pulp, pellets	5.0	4.9
Molasses, liquid	4.0	3.9
Fish oil ¹	0.0	1.9
Vitamin-mineral supplement ²	2.0	2.0
Chemical composition, % of DM		
OM	90.8	91.0
CP	18.7	18.3
NDF	29.2	28.6
ADF	19.8	19.4
Starch	12.9	12.7
Total FA	2.32	4.15
FA profile, % of total FA		
14:0	0.60	2.23
16:0	22.6	21.7
<i>cis</i> -9 16:1	0.00	2.13
18:0	3.95	4.67
<i>cis</i> -9 18:1	14.8	14.5
<i>cis</i> -11 18:1	0.85	1.67
<i>cis</i> -9, <i>cis</i> -12 18:2	40.1	22.8
<i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15 18:3	13.19	7.20
<i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17 20:5	0.00	3.34
<i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19 22:5	0.00	0.68
<i>cis</i> -4, <i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19 22:6	0.00	9.27

¹Semirefined tuna and sardine oil (Afampes 121 DHA; Afamsa, Mos, Spain) contained (% of total FA): 14:0 (4.2), 16:0 (20.7), *cis*-9 16:1 (4.7), 18:0 (5.5), *cis*-9 18:1 (14.0), *cis*-11 18:1 (2.7), 18:2n-6 (1.8), *cis*-11 20:1 (2.4), 20:5n-3 (7.4), 22:5n-3 (1.5), and 22:6n-3 (20.5).

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

mg of ^{13}ALA ($[1-^{13}\text{C}]$ *cis*-9,*cis*-12,*cis*-15 18:3, 99% atom % ^{13}C ; 694940, Sigma-Aldrich, Madrid, Spain), which was equivalent to 1% of substrate DM. The ^{13}ALA had been added to the tubes dissolved in hexane, which was evaporated to dryness under a steady stream of nitrogen before weighing the substrate. Another set of tubes in which ^{13}ALA was replaced by ^{12}ALA (10–1803–9, Larodan, Solna, Sweden) was prepared following the same methodology and served to determine the natural levels of ^{13}FA in digesta samples at the end of the incubation.

On each incubation, Hungate tubes with labeled and unlabeled ALA were incubated in duplicate [2 inocula (control and FO-MFD) $\times$ 2 ALA (labeled and unlabeled) $\times$ 2 duplicates $\times$ 3 d] under anaerobic conditions in an incubator set at 39.5°C. After 24 h of incubation, the reaction was stopped by placing the tubes into ice-water for approximately 5 min. They were then frozen at –80°C, freeze-dried, and stored again at –80°C until FA analysis.

Laboratory Analyses

The TMR samples were prepared (ISO 6498:2012) and analyzed for DM (ISO 6496:1999), ash (ISO 5984:2002), and CP (ISO 5983–2:2009). The NDF and ADF were determined using an Ankom²⁰⁰⁰ fiber analyzer (Ankom Technology Methods 13 and 12, respectively; Ankom Technology Corp., Macedon, NY); the former was assayed with sodium sulfite and α -amylase, and both were expressed with residual ash. The methyl esters of FA in 200 mg of freeze-dried TMR were prepared in duplicate following a 1-step extraction-transesterification procedure (Shingfield et al., 2003), and adding 1 mg of *cis*-12 13:1 (10–1301–9, Larodan) as an internal standard. The FAME were separated and quantified by GC-flame ionization detector (FID) as explained below for digesta samples. Peak identification was based on retention time comparisons with commercially available standard FAME mixtures (Nu-Chek Prep, Elysian, MN; and Sigma-Aldrich).

The lipids in 200 mg of freeze-dried rumen inocula and in the Hungate tubes (which also contained ~200 mg of freeze-dried digesta) were extracted using 4 mL of a mixture of hexane and isopropanol (3:2, vol/vol; Shingfield et al., 2003). Extractions were repeated and organic extracts were combined, dried at 45°C under nitrogen and converted to FAME by sequential base-acid catalyzed transesterification (Toral et al., 2010). Methyl esters were separated and quantified 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. 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 *trans*-11,*cis*-15 and *trans*-10,*cis*-15 18:2, and *cis*-9,*trans*-11 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 (from Sigma-Aldrich, Larodan, and Nu-Chek Prep), cross referencing with chromatograms reported in the literature (e.g., Gómez-Cortés et al., 2009; Lerch et al., 2012; de la Fuente et al., 2015; Toral et al., 2018), and comparison with reference ruminal digesta 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 (Toral et al., 2017, 2018).

Compound-specific isotope analysis was performed at the Stable Isotopes Laboratory of the Interdepartmental Research Service (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. The FAME were separated using the same CP-SIL 88 column and the isothermal program employed in GC-FID analysis, with helium as the carrier gas. The ^{13}C % in the methanol employed for FA transesterification was determined using an equipment (Thermo Scientific) composed of a FlashEA 1112-HT elemental analyzer coupled to a Delta V Advantage isotope ratio mass spectrometer through a ConFlo III interface. Methanol was placed in tin capsules and combustion was done at 1,020°C, with helium as the carrier gas.

Calculations and Statistical Analyses

The percentage of FA originating from the incubated ^{13}ALA was calculated as follows: First, the ^{13}C % in the methanol was used to correct for the isotopic shift due to the extra methyl group in ^{13}C -labeled FAME that were detected during GC-C-IRMS analysis. Second, to account for the natural levels of ^{13}C , the ^{13}C % of each FA measured in batch cultures with ^{12}ALA was subtracted from the respective ^{13}C % in incubations with ^{13}ALA . For each FA, the corrected ^{13}C % determined by GC-C-IRMS and its relative proportion over total FA measured by GC-FID were used to determine the

amount of the FA that would actually derive from the incubated ^{13}ALA .

Statistical analyses were performed using the SAS software package (version 9.4, SAS Institute Inc., Cary, NC). Data were analyzed by ANOVA to test the fixed effect of treatment (i.e., inoculum type: control vs. FO-MFD). The incubation run was designated as the random effect. Differences were declared significant at $P < 0.05$ and considered a trend toward significance at $0.05 \leq P < 0.10$.

RESULTS

Fatty Acid Profile of Rumen Inocula

The control and FO-MFD inocula showed marked differences in their lipid profile (Table 2). Responses to marine lipid supplementation were characterized by a large reduction in 18:0 concentration ($P < 0.001$) and increases in most 18:1 and 18:2 intermediates, such as

trans-9 to *trans*-15 + *cis*-10 18:1 and *trans*-11,*cis*-15 18:2 ($P < 0.05$). However, significant decreases to fish oil consumption were detected in *cis*-9 18:1 and 18:2n-6 (the latter coeluted with very low abundant 18:2 isomers).

Significant differences were also detected for medium-chain FA. For example, higher concentrations of 14:0 or 16:0 were observed in the FO-MFD inoculum, whereas the proportions of 15:0, *iso* 15:0, or *anteiso* 15:0 were lower than in the control ($P < 0.05$; Table 2). Some very long-chain PUFA present in the marine lipid (e.g., 20:5n-3 and 22:6n-3) could only be detected in FO-MFD inoculum.

Ruminal Biohydrogenation of ^{13}ALA

A total of 48 chromatographic peaks showed ^{13}C enrichment over the natural levels when ^{13}ALA was incubated, regardless of inoculum type (Table 3). All

Table 2. Fatty acid (FA) profile of the rumen inocula obtained from ewes fed a TMR without lipid supplementation (control inoculum) or supplemented with 2% DM of fish oil (FO-MFD inoculum)

FA, % of total FA	Control	FO-MFD	SEM	P-value
14:0	1.486	1.695	0.029	0.009
15:0	1.460	1.344	0.021	0.019
<i>iso</i> 15:0	0.481	0.281	0.007	<0.001
<i>anteiso</i> 15:0	1.061	0.453	0.022	<0.001
16:0	16.61	21.49	0.34	<0.001
<i>cis</i> -9 16:1	0.064	0.196	0.038	0.069
17:0	1.019	1.185	0.015	0.002
<i>iso</i> 17:0	0.555	0.649	0.017	0.017
<i>anteiso</i> 17:0	0.549	0.428	0.014	0.025
18:0	52.0	19.1	2.4	<0.001
<i>cis</i> -9 18:1	2.38	1.48	0.13	0.009
<i>cis</i> -11 18:1	0.278	0.610	0.093	0.066
<i>cis</i> -15 18:1	0.054	0.245	0.005	0.001
<i>trans</i> -9 18:1	0.149	1.093	0.075	<0.001
<i>trans</i> -10 18:1	0.339	1.544	0.117	0.002
<i>trans</i> -11 18:1	3.65	12.61	0.91	0.020
<i>trans</i> -12 18:1	0.340	1.898	0.046	0.002
<i>trans</i> -13+14 18:1	0.559	3.115	0.061	<0.001
<i>trans</i> -15 + <i>cis</i> -10 18:1	0.278	1.306	0.029	<0.001
<i>cis</i> -9, <i>cis</i> -12 + <i>cis</i> -12, <i>cis</i> -16 + <i>trans</i> -12, <i>cis</i> -15 18:2 ¹	3.233	0.761	0.068	0.002
<i>trans</i> -9, <i>cis</i> -12 18:2	0.053	0.109	0.009	0.014
<i>trans</i> -11, <i>cis</i> -15 + <i>trans</i> -10, <i>cis</i> -15 18:2 ²	0.078	0.307	0.041	0.016
<i>trans</i> -11, <i>trans</i> -15 + <i>trans</i> -9, <i>trans</i> -14 18:2	0.027	0.130	0.015	0.041
<i>cis</i> -9, <i>trans</i> -11 + <i>trans</i> -8, <i>cis</i> -10 CLA ³	0.092	0.132	0.004	0.023
<i>trans</i> -11, <i>trans</i> -13 CLA	0.021	0.019	0.001	0.220
<i>trans</i> -12, <i>trans</i> -14 CLA	0.005	0.018	0.003	0.085
<i>trans</i> -7, <i>trans</i> -9 + <i>trans</i> -8, <i>trans</i> -10 + <i>trans</i> -9, <i>trans</i> -11 CLA	0.019	0.057	0.009	0.033
<i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15 18:3 + <i>cis</i> -11 20:1	0.917	1.113	0.042	0.031
<i>cis</i> -9, <i>trans</i> -11, <i>trans</i> -15 18:3	0.035	0.045	0.003	0.131
20:0	1.09	1.18	0.08	0.528
<i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17 20:3	0.048	0.489	0.038	0.015
<i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17 20:5	—	0.138	—	—
22:0	0.661	0.618	0.008	0.067
<i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19 22:5	—	0.320	—	—
<i>cis</i> -4, <i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19 22:6	—	0.215	—	—

¹The *cis*-12,*cis*-16 and *trans*-12,*cis*-15 isomers coelute as minor components.

²The *trans*-10,*cis*-15 isomer coelutes as a minor component.

³The *trans*-8,*cis*-10 isomer coelutes as a minor component.

Table 3. Proportion of ¹³C-labeled fatty acids (FA) after 24 h of in vitro incubation of ¹³C α-linolenic acid with rumen inocula from ewes fed a TMR without lipid supplementation (control inoculum) or supplemented with 2% DM of fish oil (FO-MFD inoculum)

FA, % of incubated ¹³ C α-linolenic acid	Control	FO-MFD	SEM	P-value
18:0	63.4	41.5	3.5	0.012
Σ 18:1 isomers	30.0	49.4	3.1	0.011
<i>cis</i> -9 18:1	2.33	1.75	0.14	0.045
<i>cis</i> -11 18:1	0.215	0.430	0.056	0.113
<i>cis</i> -12 18:1	0.280	0.320	0.031	0.421
<i>cis</i> -13 18:1	0.153	0.263	0.027	0.100
<i>cis</i> -15 18:1	1.249	1.602	0.024	<0.001
<i>cis</i> -16 18:1	0.363	0.440	0.029	0.127
<i>trans</i> -4 18:1	0.090	0.120	0.005	0.011
<i>trans</i> -5 18:1	0.059	0.084	0.003	0.029
<i>trans</i> -6+7+8 18:1	0.809	1.457	0.081	0.005
<i>trans</i> -9 18:1	0.510	1.089	0.103	0.016
<i>trans</i> -10 18:1	0.811	1.351	0.139	0.051
<i>trans</i> -11 18:1	7.36	21.73	2.59	0.017
<i>trans</i> -12 18:1	1.46	2.53	0.23	0.028
<i>trans</i> -13+14 18:1	7.75	8.88	0.26	0.038
<i>trans</i> -15 + <i>cis</i> -10 18:1	2.529	3.471	0.073	0.012
<i>trans</i> -16 + <i>cis</i> -14 18:1	4.06	3.90	0.19	0.607
Σ 18:2 isomers	5.07	7.92	0.32	0.024
<i>cis</i> -7, <i>cis</i> -12 18:2	0.018	0.020	0.002	0.414
<i>cis</i> -9, <i>cis</i> -12 + <i>cis</i> -12, <i>cis</i> -16 + <i>trans</i> -12, <i>cis</i> -15 18:2	0.536	0.648	0.034	0.080
<i>cis</i> -10, <i>cis</i> -15 + <i>cis</i> -11, <i>cis</i> -15 18:2	0.068	0.091	0.002	0.010
<i>cis</i> -11, <i>cis</i> -14 18:2	0.121	0.078	0.020	0.206
<i>cis</i> -12, <i>cis</i> -15 18:2	0.074	0.059	0.017	0.538
<i>cis</i> -9, <i>trans</i> -12 18:2	0.060	0.063	0.004	0.578
<i>cis</i> -9, <i>trans</i> -13 18:2	0.189	0.238	0.019	0.211
<i>trans</i> -9, <i>cis</i> -12 18:2	0.163	0.339	0.020	0.024
<i>trans</i> -11, <i>cis</i> -15 + <i>trans</i> -10, <i>cis</i> -15 18:2	1.77	3.38	0.31	0.068
<i>trans</i> -9, <i>trans</i> -12 18:2	0.070	0.099	0.010	0.165
<i>trans</i> -10, <i>trans</i> -14 18:2	0.309	0.396	0.028	0.090
<i>trans</i> -11, <i>trans</i> -15 + <i>trans</i> -9, <i>trans</i> -14 18:2	0.579	1.450	0.172	0.070
<i>cis</i> -9, <i>cis</i> -11 CLA	0.006	0.030	0.004	0.052
<i>cis</i> -9, <i>trans</i> -11 + <i>trans</i> -8, <i>cis</i> -10 CLA	0.084	0.145	0.050	0.475
<i>cis</i> -11, <i>trans</i> -13 CLA	0.013	0.101	0.029	0.103
<i>trans</i> -9, <i>cis</i> -11 CLA	0.012	0.032	0.011	0.274
<i>trans</i> -10, <i>cis</i> -12 CLA	0.017	0.033	0.009	0.263
<i>trans</i> -11, <i>cis</i> -13 CLA	0.117	0.173	0.009	0.054
<i>trans</i> -11, <i>trans</i> -13 CLA	0.605	0.285	0.066	0.027
<i>trans</i> -12, <i>trans</i> -14 CLA	0.035	0.024	0.005	0.188
<i>trans</i> -7, <i>trans</i> -9 + <i>trans</i> -8, <i>trans</i> -10 + <i>trans</i> -9, <i>trans</i> -11 CLA	0.227	0.235	0.032	0.864
Σ 18:3 isomers	1.50	1.16	0.21	0.323
<i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15 18:3	1.07	0.27	0.13	0.012
<i>trans</i> -9, <i>trans</i> -12, <i>cis</i> -15 + <i>cis</i> -9, <i>cis</i> -12, <i>trans</i> -15 18:3	0.035	0.037	0.008	0.867
Unidentified #1, nonconjugated 18:3	0.010	0.008	0.002	0.597
Unidentified #2, nonconjugated 18:3	0.011	0.092	0.012	0.008
Unidentified #3, nonconjugated 18:3	0.031	0.116	0.018	0.027
Unidentified #4, nonconjugated 18:3	0.024	0.156	0.030	0.035
<i>cis</i> -9, <i>trans</i> -11, <i>cis</i> -15 18:3	0.247	0.303	0.049	0.468
<i>cis</i> -9, <i>trans</i> -11, <i>trans</i> -15 18:3	0.017	0.072	0.008	0.042
Unidentified #5, conjugated 18:3	0.027	0.066	0.006	0.008
Unidentified #6, conjugated 18:3	0.023	0.036	0.003	0.067

of them were identified as 18-carbon FA, although the position of double bonds could not be elucidated in six 18:3 isomers. Traces of enrichment were also detected at retention times compatible with the elution of few additional mono- and double-conjugated 18:3 intermediates, but their low signal intensity in GC-C-IRMS or GC-FID analyses precluded from obtaining conclusive results and they were therefore excluded from the

study. According to complementary GC-FID determinations using the SLB-IL111 column, approximately 88 and 92% of the *trans*-11,*cis*-15 + *trans*-10,*cis*-15 18:2 peak area belonged to the first isomer in the control and FO-MFD incubations, respectively, whereas *cis*-9,*trans*-11 CLA represented a minimum of 94% of the corresponding peak in both treatments, the remaining portion being identified as *trans*-8,*cis*-10 CLA.

Figure 1. Distribution of ^{13}C enrichment among 18:1 isomers (expressed as % of total 18:1 enrichment) after 24 h of in vitro incubation of ^{13}C α -linolenic acid with rumen inocula from ewes fed a TMR without lipid supplementation (control inoculum; white bars) or supplemented with 2% DM of fish oil [TMR supplemented with 2% DM of fish oil (FO-MFD) inoculum; gray bars]. For each isomer, error bars represent the SEM for the effect of rumen inoculum, * indicates significant differences ($P < 0.05$) and † indicates a tendency toward significance ($0.05 \leq P < 0.10$) between the experimental treatments. *c* = *cis*; *t* = *trans*.

Stearic acid (18:0) was the most abundant product of ^{13}C ALA, representing 63.4 and 41.5% of total ^{13}C enrichment in the control and FO-MFD cultures, respectively ($P = 0.012$; Table 3). This reduction was accompanied by the opposite change in the sum of 18:1 isomers, which rose from 30.0 to 49.4% of total ^{13}C enrichment ($P = 0.011$). Total 18:2 intermediates represented a much lower proportion of enriched FA (<8%), despite the 56% increase in the FO-MFD treatment ($P = 0.024$). On the other hand, the sum of ^{13}C -labeled 18:3 isomers was not modified by inoculum type ($P = 0.323$) and represented less than 1.5% of total ^{13}C enrichment.

Significant changes in individual 18:1 and 18:2 products of ^{13}C ALA often reflected the greater accumulation of these FA groups in the FO-MFD treatment, except for decreases in *cis*-9 18:1 and *trans*-11,*trans*-13 CLA ($P < 0.05$; Table 3). No variation was detected, however, for some less abundant products. The proportion of ^{13}C ALA decreased in incubations with the rumen inoculum from ewes fed the fish oil diet ($P = 0.012$), whereas other 18:3 isomers generally showed the opposite response.

Figures 1, 2, and 3 show the distribution of ^{13}C enrichment among the individual isomers of 18:1, 18:2, and 18:3, expressed as % of total enrichment within each FA group. These figures provide a direct comparison of the importance of each BH pathway in incubations

with either control or FO-MFD inocula, by removing potential bias due to treatment differences in the accumulation of each FA group. Starting with 18:1 isomers (Figure 1), only ^{13}C -labeled *trans*-6+7+8, *trans*-9 and, particularly, *trans*-11 18:1 were favored when FO-MFD inoculum was used (10, 30, and 76% increase, respectively; $P < 0.10$), whereas other major 18:1 isomers (e.g., *cis*-9, -15 and *trans*-13+14 18:1) and *cis*-12 18:1 decreased or tended to decrease.

As shown in Figure 2, the percentage of *trans*-11,*trans*-13 CLA and 5 less abundant 18:2 isomers (e.g., *cis*-11,*cis*-14 and *trans*-12,*trans*-14 18:2) was lower in the FO-MFD treatment ($P < 0.10$), which in turn favored the accumulation of major 18:2 intermediates (i.e., *trans*-11,*trans*-15 and *trans*-11,*cis*-15 18:2) and *cis*-9,*cis*-11 CLA ($P < 0.10$). No significant effects of inoculum type were observed for other 18:2 and CLA isomers.

Concerning the distribution of 18:3 isomers (Figure 3), the large drop in ^{13}C ALA in FO-MFD incubations (−67%; $P < 0.01$) was accompanied by increases in most 18:3 intermediates (the major one, *cis*-9,*trans*-11,*cis*-15 18:3, reached over 25% of total 18:3 enrichment; $P < 0.01$). Significant increments exceeded 400% for 3 non-conjugated 18:3 isomers of indeterminate double bond position and *cis*-9,*trans*-11,*trans*-15 18:3, and between 94 and 205% for 2 FA that eluted shortly after *cis*-

Figure 2. Distribution of ^{13}C enrichment among 18:2 isomers (expressed as % of total 18:2 enrichment) after 24 h of in vitro incubation of ^{13}C α -linolenic acid with rumen inocula from ewes fed a TMR without lipid supplementation (control inoculum; white bars) or supplemented with 2% DM of fish oil [TMR supplemented with 2% DM of fish oil (FO-MFD) inoculum; gray bars]. For each isomer, error bars represent the SEM for the effect of rumen inoculum, * indicates significant differences ($P < 0.05$) and † indicates a tendency toward significance ($0.05 \leq P < 0.10$) between the experimental treatments. *c* = *cis*; *t* = *trans*.

9,*trans*-11,*cis*-15 18:3 and were tentatively identified as conjugated 18:3.

DISCUSSION

Alterations in ruminal BH are at the core of diet-induced MFD, particularly in response to marine lipid consumption (Shingfield et al., 2003; Boeckeaert et al., 2007; Frutos et al., 2018). However, the fact that different UFA have common intermediates represents a major obstacle to advance our knowledge of changes in BH pathways (Jouany et al., 2007; Jenkins et al., 2008; Kairenius et al., 2018). To try to overcome this problem and provide new insights into BH under milk fat-depressing conditions, an isotopic tracer approach was used in the present in vitro study.

Rumen inocula were collected from cannulated ewes fed a TMR supplemented or not with fish oil, which resulted in clear differences in their FA profiles, consistent with in vivo changes in the ruminal BH in dairy sheep suffering from diet-induced MFD (Toral et al., 2016; Frutos et al., 2018). Inoculum from supplemented ewes had much lower 18:0 concentration than the control, which is consistent with the hypothesis relating the shortage of ruminal 18:0 available for mammary Δ^9 -desaturation to *cis*-9 18:1 with a higher milk fat melting point that would impair fat secretion (Chilliard et al., 2007; Shingfield et al., 2010). However, recent studies in ewes and cows fed fish oil have downplayed the relevance of this change in marine lipid-induced MFD (Kairenius et al., 2015; Toral et al., 2016). The

opposite difference was observed in most 18:1 isomers, specially *trans* 18:1, which is common in the digesta not only of ovine but also of bovine fed marine lipids (e.g., Boeckeaert et al., 2007; Or-Rashid et al., 2008; Toral et al., 2016). On the other hand, the associated shift from *trans*-11 to *trans*-10 18:1 is often less important in small ruminants than in cows (Boeckeaert et al., 2007; Frutos et al., 2018; Kairenius et al., 2018). In this study, the accumulation of the latter 18:1 isomer was favored but did not exceed that of vaccenic acid (which was on average 9.5 times more abundant). The low 18:2n-6 content observed in the FO-MFD inoculum reflected the effect of this marine lipid enhancing the extension of the BH of 18-carbon PUFA, which would be explained by changes in the microbial community (Shingfield et al., 2011; Frutos et al., 2018; Kairenius et al., 2018). Because odd- and branched-chain FA have a predominant bacterial origin (Fievez et al., 2012), observed differences between control and FO-MFD inocula would also support shifts in microbial populations. Overall, inoculum composition seems consistent with ruminal alterations related to marine lipid-induced MFD (Shingfield et al., 2003; Boeckeaert et al., 2007; Toral et al., 2016).

The level of ^{13}ALA inclusion was designed to be high enough to facilitate the identification of minor BH intermediates without detrimental effects on rumen function (Chikunya et al., 2004; Shingfield et al., 2011). This amount was similar to that provided by ALA-rich supplements (e.g., linseeds or linseed oil) in studies about marine lipid-induced MFD (≈ 0.8 – 1.8%

diet DM; AbuGhazaleh et al., 2003; Angulo et al., 2012; Kairenius et al., 2018).

About 99% of the incubated ^{13}C -labeled FA disappeared after 24 h of incubation, which agrees with the extensive in vivo BH of ALA in animals fed linseed products (usually $\geq 92\%$; Chikunya et al., 2004; Shingfield et al., 2011; Kairenius et al., 2018) or in batch cultures with ^{12}C - or ^{13}C -ALA (approximately 87–99%; Troegeler-Meynadier et al., 2003; Jouany et al., 2007; Lee and Jenkins, 2011). Its lower value in incubations with the FO-MFD inoculum (Table 3) would support the above-discussed increase in the extension of PUFA BH in response to this marine lipid (Shingfield et al., 2011; Kairenius et al., 2018). On the other hand, the opposite difference in *cis*-9,*cis*-12,*cis*-15 18:3 + *cis*-11 20:1 concentrations in the inocula (Table 2) was likely due to variation in the proportion of *cis*-11 20:1 over the total peak area. In this regard, the confounding effect of coelutions between 18- and 20- or 22-carbon FA represents a major challenge in the study of alterations in BH pathways associated with marine lipid supplementation (Kairenius et al., 2018; Toral et al., 2017, 2018). This problem, however, would not interfere with enrichment results in our trial (Table 3 and Figures 1, 2, and 3) because resolved 20- and 22-carbon FA showed no increase in $^{13}\text{C}/^{12}\text{C}$ ratio over basal levels.

Therefore, labeling in these coelutions was assumed to belong exclusively to the 18-carbon FA.

The in vitro BH of ^{13}C -ALA yielded multiple metabolites, but enrichment was found in the same 48 chromatographic peaks in all samples, regardless of inoculum type. However, although ALA may be biohydrogenated through common pathways under standard (control) or MFD conditions (Shingfield et al., 2010), large changes in the accumulation of ^{13}C -labeled FA suggest important differences in the relative contribution of each specific route. In the control, *trans*-11 18:1, *trans*-11,*cis*-15 18:2 and *cis*-9,*trans*-11,*cis*-15 18:3 were among the most abundant ALA products with 1, 2, or 3 double bonds (Figures 1, 2, and 3), consistent with the established major pathway of ALA BH (Jouany et al., 2007; Lee and Jenkins, 2011; Honkanen et al., 2016). The proportion of these 3 *trans*-11 intermediates was increased in FO-MFD incubations. On the contrary, the relevance of the *trans*-10 pathway did not seem to be affected by the inoculum type, according to the stable levels of labeled *trans*-10 18:1 and *trans*-10,*cis*-12 CLA, probably the most representative intermediates of this route (Shingfield et al., 2010; Alves and Bessa, 2014), or other *trans*-10-containing FA. The isomer *trans*-10,*cis*-15 18:2, a less known product of this pathway (Alves and Bessa, 2014), represented approxi-

Figure 3. Distribution of ^{13}C enrichment among 18:3 isomers (expressed as % of total 18:3 enrichment) after 24 h of in vitro incubation of ^{13}C α -linolenic acid with rumen inocula from ewes fed a TMR without lipid supplementation (control inoculum; white bars) or supplemented with 2% DM of fish oil [TMR supplemented with 2% DM of fish oil (FO-MFD) inoculum; gray bars]. For each isomer, error bars represent the SEM for the effect of rumen inoculum, * indicates significant differences ($P < 0.05$) and † indicates a tendency toward significance ($0.05 \leq P < 0.10$) between the experimental treatments. *c* = *cis*; *t* = *trans*.

mately 12 and 8% of the peak area in the control and FO-MFD incubations, respectively. This contrasts with a recent study showing that *trans*-10,*cis*-15 18:2 replaced *trans*-11,*cis*-15 18:2 as the major 18:2 isomer in the digesta of cows supplemented with fish and linseed oils (Kairenius et al., 2018), which might be explained by interspecies differences in ruminal BH. In this respect, Toral et al. (2017) noticed stronger increases of *trans*-11,*cis*-15 + *trans*-10,*cis*-15 18:2 in bovine than ovine digesta in response to very long-chain n-3 PUFA, which might be due to a greater accumulation of the *trans*-10-containing isomer in cows. On this basis, the suggested role of *trans*-10,*cis*-15 18:2 in fish oil-induced MFD in dairy ewes (Toral et al., 2016; Frutos et al., 2018) should be revisited.

In general, these results are consistent with previous reports in sheep downplaying the relevance of the *trans*-11 to *trans*-10 shift as an effective marker of ruminal alterations due to marine lipid supplementation in sheep, compared with its importance in cows (Toral et al., 2010; Frutos et al., 2018; Kairenius et al., 2018). The increase in vaccenic acid accumulation with FO-MFD inoculum might be due to either promotion of bacteria involved in the formation of this intermediate or inhibition of those saturating it (Jenkins et al., 2008). Nevertheless, isomerization of other 18:1 FA would have played a limited role, as suggested by previous in vitro studies using ¹³C-labeled *cis*-9 and *trans*-9 18:1 (Mosley et al., 2002; Proell et al., 2002).

As shown in Figure 1, promotion of the *trans*-11 pathway in MFD incubations seemed to be accompanied by inhibition of processes leading to the accumulation of *trans*-13 and -14 18:1, the major contributors to 18:1 enrichment in the control. This is in line with an in vitro study by Laverroux et al. (2011) supporting a relationship between *trans*-11 and *trans*-13+14 18:1; the latter FA were major 18:1 products of the isomerization of ¹³C-vaccenic acid in incubations with rumen inoculum from sheep fed only forage, whereas a high-concentrate diet hindered the conversion. Furthermore, a general repression of the *trans*-13/14 pathway would be favored by the marked decline in the proportion of *trans*-11 *trans*-13 CLA, and to a lower extent of *cis*-9,*trans*-13 and *trans*-12,*trans*-14 18:2 (Figure 2), potential precursors of *trans*-13 and -14 18:1. Similar results have previously been found in ewes fed fish oil (Toral et al., 2010; Frutos et al., 2018) and in cattle supplemented with fish oil alone or in combination with linseed oil (Shingfield et al., 2003, 2011; Kairenius et al., 2018). Although the biological implications of these shifts remain to be explored, a recent study has indicated that *trans*-11,*trans*-13 CLA would induce lipogenesis in the liver of mice (Pachikian et al., 2018). In this regard, much effort has been invested in finding antilipogenic

FA responsible for the low-fat milk syndrome (Bauman and Griinari, 2001; Shingfield et al., 2010), but the role of pro-lipogenic metabolites is less known.

The pathways of ALA BH that lead to *cis* FA accumulation also seemed to be inhibited in incubations with the FO-MFD inoculum, according to the lower abundance of ¹³C-labeled *cis*-9, -12, and -15 18:1 (Figure 1) and some nonconjugated *cis,cis* 18:2 isomers (Figure 2). Despite the inconsistent data on the production of *cis*-9 18:1 from ALA in cow digesta (Lee and Jenkins, 2011; Honkanen et al., 2016), our results would support this pathway in sheep, albeit ¹³C-labeled *cis*-9 18:1 represented only 5.6 ± 0.67 and $3.3 \pm 0.24\%$ of ¹²C-*cis*-9 18:1 in control and FO-MFD incubations, respectively (data not shown). High UFA contents in the ruminal environment can be toxic for microorganisms and stimulate the *cis* to *trans* isomerization as a protection mechanism (Jenkins et al., 2008; Heipieper et al., 2010). This response would be in line with a replacement of *cis* intermediates by *trans* FA in FO-MFD incubations, such as *trans*-7 and *trans*-9 18:1, 2 major products of *cis*-9 18:1 metabolism (Mosley et al., 2002). Increased ruminal accumulation of the potentially antilipogenic *cis*-11 18:1 (Burns et al., 2012) is a common feature of fish oil supplementation (Shingfield et al., 2003, 2011; Frutos et al., 2018) largely attributable to its presence in the oil and not to ruminal formation from ALA, as supported by its inconsistent variation in the inocula and the in vitro digesta. Other reported effects of marine lipids on the ruminal concentration of *cis* FA appear to be erratic in the literature (e.g., Or-Rashid et al., 2008; Vlaeminck et al., 2008; Toral et al., 2016), probably due, at least to some extent, to differences in dietary FA profiles (Jouany et al., 2007; Jenkins et al., 2008; Kairenius et al., 2018).

Early investigations assumed that ruminal metabolism of ALA did not lead to *cis*-9,*trans*-11 CLA formation (e.g., Harfoot and Hazlewood, 1997), but this hypothesis was challenged by the in vitro study by Lee and Jenkins (2011). Under our conditions, it is not possible to discern whether the low enrichment of the *cis*-9,*trans*-11 + *trans*-8,*cis*-10 CLA peak corresponds to just 1 or 2 intermediates (Lee and Jenkins, 2011; Lee, 2013; Honkanen et al., 2016). In contrast, the ¹³C-labeling in the *cis*-9,*cis*-12 + *trans*-12,*cis*-15 + *cis*-12,*cis*-16 18:2 peak would probably derive from the last 2 isomers, given that linoleic acid has not been described as a product of ¹³ALA (Lee and Jenkins, 2011; Lee, 2013; Honkanen et al., 2016). In any event, none of these isomers has been associated with marine lipid-induced MFD. On the other hand, *trans*-10,*cis*-12 and *trans*-9,*cis*-11 CLA (with confirmed and potentially antilipogenic effects, respectively; Shingfield et al., 2010) were not affected by the inoculum type in our trial.

Concerning 18:3 intermediates, the large and consistent increases in their accumulation might suggest an interesting role as biomarkers under conditions of altered ruminal BH. However, just as in other studies on 18:3 FA metabolism in ruminants (e.g., Lee and Jenkins, 2011; Lerch et al., 2012; Lee, 2013), double bond position and geometry could not be ascertained for some isomers. Their low abundance, the coelution with longer-chain FA, and the limited range of commercially available 18:3 standards represent major challenges for identifications, but the presence of conjugated double bond structures can be reasonably inferred based on elution order in the chromatogram (Lerch et al., 2012; Kairenius et al., 2015; Toral et al., 2018). Although evidence exists that some little known 18:3 isomers may regulate lipid metabolism (Yuan et al., 2014), we are aware of only one report testing the involvement of 18:3 intermediates (specifically, *cis*-9,*trans*-11,*cis*-15 + *cis*-9,*trans*-13,*cis*-15 18:3) in MFD in ruminants (Gervais and Chouinard, 2008). Further research would be advisable to examine the ruminal production of minor 18:3 FA and evaluate their effects on mammary lipogenesis.

Finally, the absence of enrichment in resolved 20- and 22-carbon FA is consistent with the hypothesis that rumen microbiota would not elongate dietary 18-carbon FA (Proell et al., 2002; Lee and Jenkins, 2011). Thus, ALA would not be a precursor of highly unsaturated FA (e.g., 20:5n-3 and 22:6n-3) in rumen digesta, at least under in vitro conditions.

CONCLUSIONS

Profiles of ^{13}C -labeled FA after 24 h of in vitro incubation of ^{13}ALA with inoculum collected from cannulated ewes fed a TMR without lipid supplementation (control) or supplemented with FO-MFD (which is known to induce MFD) show large changes in the accumulation of ^{13}C -labeled intermediates and important differences in the relative contribution of specific BH pathways. Increased accumulation of *trans*-11-containing FA in FO-MFD incubations are accompanied by a general repression of the *trans*-13/14 pathway, together with a lower production of *cis* FA. Results also downplay the relevance of the *trans*-11 to *trans*-10 shift as an effective marker of diet-induced MFD in sheep, and challenge the involvement of some *trans*-10 intermediates (e.g., *trans*-10 18:1 and *trans*-10,*cis*-15 18:2) in the low-fat milk syndrome in this species. Conversely, increased abundance of most 18:3 intermediates might be representative of ruminal alterations related to fish oil-induced MFD in ewes. Further research is necessary to examine the potential association between these findings and MFD in lactating animals.

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