



## Susceptibility to milk fat depression in dairy sheep and goats: Individual variation in ruminal fermentation and biohydrogenation

Antonella Della Badia, , Pilar Frutos, , Pablo G. Toral, , and Gonzalo Hervás\* 

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

### ABSTRACT

Small ruminants are susceptible to milk fat depression (MFD) induced by marine lipid supplementation. However, as observed in dairy cows, there is wide individual variation in the response to MFD-inducing diets, which may be due to individual differences in ruminal processes. Therefore, we compared the ruminal responses of goats and sheep with varying degrees of MFD extent to improve our understanding of this complex syndrome. Our specific aims were to attempt to elucidate whether pre-existing variations in ruminal fermentation and biohydrogenation determine a higher tolerance or susceptibility to MFD, and whether the severity of MFD depends exclusively on the response to the diet. The trial was conducted with 25 does and 23 ewes fed a basal diet without lipid supplementation for 3 wk (control period). Then, 2% fish oil (FO) was added to the same diet for 5 additional weeks (MFD period). Based on the extent of the elicited MFD (i.e., the percentage variation between milk fat concentrations recorded at the end of the control and MFD periods), the 5 most responsive (RESPON+) and the 5 least responsive (RESPON-) animals were selected within each species. On the last day of each period, ruminal fluid samples were collected to examine fermentation parameters and fatty acid profiles. In general, the individual degree of MFD in sheep and goats did not seem to be predetermined by traits related to ruminal fermentation and biohydrogenation, including fatty acids that may serve as biomarkers of microorganisms. Regarding differences in the response to FO, the results suggest no link between MFD susceptibility and concentration of biohydrogenation intermediates such as *trans*-10-containing C18, C20, and C22 metabolites.

The explanation for individual responses based on a shortage of ruminal acetate and 18:0 for mammary uptake also seems to be dismissed, based on the lack of variation in these compounds between RESPON+ and RESPON-. However, the concentration of unsaturated fatty acids provided by FO (e.g., *cis*-9 16:1, *cis*-11 18:1, and 20:5n-3) was higher in the rumen of RESPON+ than RESPON- ewes and does. Thus, although further research is needed, the extent of biohydrogenation of these fatty acids might be associated with tolerance or susceptibility to MFD.

**Key words:** caprine, fatty acid, fish oil, lipid, ovine

### INTRODUCTION

Over the last decade, several reports have contributed to the elucidation of interspecies differences in susceptibility to diet-induced milk fat depression (MFD) in dairy ruminants (Shingfield et al., 2013; Dewanckele et al., 2020). In contrast to the earlier perception that small ruminants are resistant to this syndrome (Chilliard et al., 2007; Sanz Sampelayo et al., 2007), the development of MFD has been confirmed when sheep and goats are fed supplemental fish oil (FO) or marine microalgae to modulate the milk fatty acid (FA) profile (Torral et al., 2016; Bernard et al., 2017). However, much less is known about intraspecies variation in response to MFD-inducing diets (i.e., individual differences in the degree of reduction in milk fat concentration and yield when animals consume the same diet; Frutos et al., 2017; Della Badia et al., 2021).

According to some studies, individual variation in the extent of MFD might be predetermined by differences in certain traits (e.g., the milk production level or the milk fat concentration), but the information on this is scarce and inconsistent (Baldin et al., 2018b; Dewanckele et al., 2019; Della Badia et al., 2021). Some such pre-existing traits may be related to ruminal processes. Ruminal fermentation provides substrates for milk fat synthesis, and biohydrogenation (BH) provides not only substrates for milk fat synthesis but also bioactive C18 FA with antilipogenic activity (Bauman et al., 2011; Enjalbert et al., 2017; Dewanckele et al., 2020).

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\*Corresponding author: [g.hervas@csic.es](mailto:g.hervas@csic.es)

Therefore, individual differences in ruminal function might account for individual differences in the response to MFD-inducing diets.

However, in a recent study in sheep and goats (Della Badia et al., 2021), we found no solid relationship between MFD severity and milk concentrations of antilipogenic C18 FA formed in the rumen (e.g., *trans*-10 18:1, *trans*-10,*cis*-12 CLA, or *trans*-9,*cis*-11 CLA; Shingfield et al., 2013). In that study (Della Badia et al., 2021), we were also interested in certain C20–22 BH intermediates because they had been suggested as candidate milk fat inhibitors (Kairenius et al., 2018; Toral et al., 2018). Nevertheless, their very low proportion in milk precluded their quantification, which might be overcome by analyzing their concentration in ruminal samples. Moreover, Della Badia et al. (2021) showed a possible link between milk *cis*-9 16:1 concentration and the extent of MFD. Yet, the endogenous synthesis of this putative antilipogenic FA (Burns et al., 2012a; Bernard et al., 2013; Duckett et al., 2014) precluded firm conclusions about its actual origin (i.e., from the diet or from mammary  $\Delta^9$ -desaturation) and involvement in MFD susceptibility.

For these reasons, we propose that individual differences derived from ruminal processes might be more easily detected in ruminal fluid than in milk. It must be considered that the milk FA profile is only examined in fat that has been successfully secreted, which may not be an accurate representation of FA leaving the rumen and reaching the mammary gland. On this basis, this study was conducted to examine (1) whether the individual responses of dairy goats and sheep to FO, in terms of the extent of MFD, are predetermined by variations in ruminal fermentation and BH under normal (i.e., non-MFD) conditions, and (2) whether individual differences in these ruminal processes contribute to explain tolerance or susceptibility to diet-induced MFD. An investigation of variations similarly detected in both ruminant species is expected to strengthen our knowledge of the mechanisms underlying MFD syndrome.

## MATERIALS AND METHODS

### Ethics Statements

All procedures involving animals were completed in accordance with European Union and Spanish regulations [Council Directive 2010/63/EU (EU, 2010) and Royal Decree 53/2013 (BOE, 2013)] and granted prior approval by the Research Ethics Committees of the Instituto de Ganadería de Montaña, the Spanish National

Research Council (CSIC), and the Junta de Castilla y León (Spain).

### Animals and Experimental Treatments

This assay is part of a larger study conducted to provide insight into MFD in small ruminants. A detailed description of the experimental design and composition of the diets was reported in a previous article (Della Badia et al., 2021). In brief, 25 Murciano-Granadina does ( $30.5 \pm 3.6$  kg of BW;  $40.1 \pm 5.9$  DIM;  $1.49 \pm 0.06$  kg of milk/d) and 23 Assaf ewes ( $64.5 \pm 9.5$  kg of BW;  $38.3 \pm 4.7$  DIM;  $1.11 \pm 0.07$  kg of milk/d) were housed in individual pens and milked once daily (0830 h). They were fed a TMR composed of dehydrated alfalfa, whole corn and barley grains, soybean meal, sugar beep pulp and molasses, and a vitamin-mineral supplement (50:50 forage:concentrate ratio; 184 g of CP and 276 g of NDF per kilogram of DM) without lipid supplementation for 3 wk (control period). Then, this basal TMR was supplemented with 20 g of FO (providing 66 g of 20:5n-3 and 204 g of 22:6n-3 per kilogram of total FA) per kilogram of diet DM for 5 additional weeks (MFD period). At the end of this second period, based on the extent of the elicited MFD (i.e., the percentage of variation between milk fat concentrations recorded at the end of the control and MFD periods), the 5 most responsive (**RESPON+**) and the 5 least responsive (**RESPON-**) animals were selected within each species (10 does and 10 ewes in total). The mean reductions in milk fat concentration were 25.4% in RESPON+ and 7.3% in RESPON- animals (Della Badia et al., 2021; Supplemental Figure S1, <https://digital.csic.es/handle/10261/272926>, Della Badia et al., 2022).

### Rumen Sampling Procedure

On the last day of each period, animals were given free access to the TMR for 1 h after milking. Orts were then removed and, 3 h later, individual samples of ruminal fluid (approximately 150 mL) were obtained using an oral stomach probe (Ramos-Morales et al., 2014). The fluid was immediately strained through a nylon membrane (400  $\mu$ m; Fisher Scientific S.L.). Then, 3 mL of ruminal fluid were acidified with 3 mL of 0.2 M HCl for ammonia analysis, and 0.8 mL was deproteinized with 0.5 mL of 20 g of metaphosphoric acid/L and 4 g of crotonic acid/L in 0.5 M HCl for VFA determinations. These samples were stored at  $-30^\circ\text{C}$  until laboratory analyses. Aliquots of ruminal fluid (approximately 50 mL) were also collected for FA analysis,

immediately frozen at  $-80^{\circ}\text{C}$ , freeze-dried, and stored again at  $-80^{\circ}\text{C}$ .

### Chemical Analyses

The ruminal fluid concentration of ammonia was determined using a colorimetric method (Reardon et al., 1966) and that of VFA by GC, using crotonic acid as an internal standard (Ottenstein and Bartley, 1971), both in centrifuged samples.

Total lipids were extracted twice from 200 mg of freeze-dried ruminal digesta samples using 4 mL of a hexane-isopropanol mixture (3:2, vol/vol) following adjustment of the digesta pH to 2 using 2 M HCl (Shingfield et al., 2003) and the addition of *cis*-12 13:1 (10-1301-9, Larodan Fine Chemicals AB) as an internal standard. Organic extracts were combined and dried under nitrogen at  $50^{\circ}\text{C}$ . Lipids dissolved in 2 mL of hexane were converted to FAME using a sequential base-acid catalyzed transesterification procedure with freshly prepared 0.5 M sodium methoxide in methanol for 5 min at  $20^{\circ}\text{C}$  followed by reaction with 1% (vol/vol) sulfuric acid in methanol at  $50^{\circ}\text{C}$  for 30 min (Toral et al., 2017). Methyl esters were separated and quantified using a gas chromatograph (Agilent 7890A GC System) equipped with a flame-ionization detector (FID) and a 100-m fused silica capillary column (0.25 mm i.d., 0.2- $\mu\text{m}$  film thickness; CP-SIL 88, CP7489, Varian Ibérica S.A.), with hydrogen as the fuel and carrier gas. The total FAME profile in a 2- $\mu\text{L}$  sample volume at a split ratio of 1:20 was determined using a temperature gradient program, and isomers of 18:1 were resolved in a separate analysis under isothermal conditions at  $170^{\circ}\text{C}$  (Shingfield et al., 2003). Overlapping *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 further 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 fuel and carrier gas. All peaks were identified by comparison of their retention times with those of commercially available FAME standards (GLC463, U-37-M, U-43-M, U-45-M, and U-64-M, Nu-Chek Prep.; 18919-1AMP Supelco, L6031, L8404, and O5632, Sigma-Aldrich; and 11-1600-8, 20-2024-1, 20-2210-9, 20-2305-1-4, 21-1211-7, 21-1413-7, 21-1614-7, 21-1615-7, and BR mixtures 2 and 3, Larodan Fine Chemical AB), cross-referencing chromatograms reported in the literature (e.g., Shingfield et al., 2003; de la Fuente et al., 2015), and comparison 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-dimethylloxazoline derivatives (Toral et al., 2017, 2018).

### Statistical Analysis

Statistical analyses were performed using the MIXED procedure of the SAS software package (version 9.4, SAS Institute Inc.) and focused on the following 2 aims of the study: (1) to examine pre-existing variations in ruminal fermentation and BH between RESPON+ and RESPON- goats and sheep in the control period (i.e., under non-MFD conditions), and (2) to examine the ruminal responses of these goats and sheep to FO supplementation (i.e., to explain the tolerance or susceptibility to diet-induced MFD). All differences, both in the control period and in the response to FO supplementation, were analyzed by 2-way ANOVA according to the following model:

$$Y_{ijk} = \mu + Sp_i + Res_j + Sp \times Res_{ij} + \xi_{ijk},$$

where  $Y_{ijk}$  is the individual value of each dependent variable;  $\mu$ , the overall mean;  $Sp_i$ , the fixed effect of the species (**Sp**;  $i$  = caprine vs. ovine),  $Res_j$ , the fixed effect of the response (**Res**;  $j$  = RESPON- vs. RESPON+);  $Sp \times Res_{ij}$ , their interaction; and  $\xi_{ijk}$ , the residual error. 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 a Bonferroni correction. Differences were declared significant at  $P < 0.05$  and considered a trend toward significance at  $0.05 \leq P < 0.10$ . Least squares means are reported.

## RESULTS

Table 1 reports ruminal fermentation parameters, whereas the FA profile is displayed in 3 tables: SFA (Table 2), MUFA (Table 3), and PUFA (Table 4). Each table reports pre-existing variations during the control period, as well as the difference in the response to FO supplementation ( $\Delta\text{MFD}$ ). These topics will be described in 2 independent subsections.

### Pre-Existing Variations (Control Period)

No differences between the least and most responsive goats and sheep were observed in ammonia and VFA concentration and molar proportions during the control period ( $P > 0.10$ ). Similarly, ruminal SFA and MUFA profiles showed almost no pre-existing variation between the RESPON- and RESPON+ groups, except for the tendency toward a lower accumulation of *cis*-9 17:1 in animals that displayed more severe MFD ( $P = 0.097$ ; Table 3). Regarding PUFA (Table 4), the minor 18:3n-6 was more abundant in RESPON- than RESPON+ goats ( $P = 0.005$  for the interaction Sp

**Table 1.** Ruminal concentrations of ammonia (mg/L) and total VFA (mmol/L), molar proportions (mol/100 mol) of VFA, and acetate:propionate ratio in dairy sheep and goats with a mild (RESPON–) or strong (RESPON+) response to a diet inducing milk fat depression (MFD)

Variable	Item <sup>1</sup>	Goats		Sheep		SED <sup>2</sup>	P <sup>3</sup>		
		RESPON–	RESPON+	RESPON–	RESPON+		Sp	Res	Sp × Res
Ammonia	Control	64.6	141.4	123.8	156.7	61.9	0.355	0.181	0.582
	ΔMFD	130.1	25.3	–21.8	–24.9	54.3	0.011	0.137	0.159
Total VFA	Control	106.2	92.1	108.1	103.3	10.4	0.385	0.218	0.532
	ΔMFD	–28.6	–16.5	2.56	28.3	9.59	<0.001	0.013	0.329
Molar proportions									
Acetate	Control	65.9	66.3	65.0	65.6	0.90	0.238	0.909	0.419
	ΔMFD	–1.86	–2.40	–0.64	–2.86	1.207	0.661	0.127	0.341
Propionate	Control	17.1	15.9	16.7	17.5	1.61	0.584	0.857	0.382
	ΔMFD	–2.11	–0.97	1.88	4.50	2.031	0.005	0.209	0.613
Butyrate	Control	14.1	15.1	14.1	13.0	1.28	0.272	0.964	0.254
	ΔMFD	2.83	2.02	–1.17	–0.20	1.640	0.016	0.943	0.456
Isobutyrate	Control	0.882	0.804	1.06	1.34	0.265	0.076	0.594	0.351
	ΔMFD	0.40	0.43	–0.09	–0.55	0.247	<0.001	0.240	0.177
Valerate	Control	1.03	0.97	1.10	1.26	0.133	0.070	0.558	0.258
	ΔMFD	0.08	0.18	–0.01	–0.03	0.141	0.171	0.716	0.537
Isovalerate	Control	0.76	0.67	1.04	1.55	0.405	0.059	0.483	0.308
	ΔMFD	0.72	0.75	–0.01	–0.80	0.352	<0.001	0.149	0.118
Caproate	Control	0.25	0.23	0.31	0.26	0.076	0.408	0.470	0.799
	ΔMFD	–0.05	–0.01	0.03	–0.07	0.068	0.776	0.493	0.179
Acetate:propionate ratio	Control	3.87	4.33	3.98	3.79	0.375	0.428	0.625	0.233
	ΔMFD	0.44	0.05	–0.48	–0.91	0.439	0.008	0.208	0.944

<sup>1</sup>Control = data obtained when animals were fed a TMR without lipid supplementation; ΔMFD = difference between the data obtained after diet supplementation with 20 g of fish oil/kg of DM (to induce MFD) and those previously recorded in the control period.

<sup>2</sup>SED = standard error of the difference.

<sup>3</sup>Probability of significant effects due to species (Sp), response (Res), and their interaction (Sp × Res).

× Res), but other C18 and C20-C22 PUFA showed comparable concentrations in ewes and does.

It was not the aim of our study to examine inter-species differences. In brief, variations in fermentation were scant: only the concentrations of isobutyrate, valerate, and isovalerate tended to be greater in sheep than in goats ( $P < 0.10$ ; Table 1). Ewes also showed a higher proportion ( $P < 0.05$ ) of most SFA (e.g., 16:0 and odd- and branched-chain FA; Table 2), 16:1 isomers (Table 3), and some BH intermediates of 18:3n-3 (e.g., *trans*-10,*cis*-15 and *trans*-11,*cis*-13 18:2; Table 4). However, goats had greater percentages of 18:1 isomers ( $P < 0.05$ ; Table 3) or *cis*-9,*trans*-11 CLA ( $P = 0.073$ ; Table 4).

### Differences in the Response to FO Supplementation

Total VFA concentrations showed differences due to both Sp and Res ( $P < 0.001$  and  $P = 0.013$ , respectively), but the interaction Sp × Res was not significant ( $P = 0.329$ ; Table 1). Thus, ΔMFD values were always lower in goats than in sheep, and in RESPON– than in RESPON+ animals.

Overall, 3 major types of response to FO supplementation were observed in ruminal FA concentrations: increases in both species were the most prevalent, fol-

lowed by decreases in both species and, finally, by few inconsistent changes in goats and sheep.

Beginning with FA that increased in abundance in both species during the MFD period, no difference due to susceptibility to MFD ( $P > 0.10$ ) was detected for some ruminal metabolites that were previously associated with MFD, such as 10-O-18:0 (Table 2), *trans*-10 18:1, and *trans*-10,*cis*-15 18:2 (Tables 3 and 4, respectively, and Supplemental Figure S2; <https://digital.csic.es/handle/10261/272926>; Della Badia et al., 2022). However, increases in FA directly supplied with the FO were significantly greater ( $P < 0.05$ ) or tended to be greater ( $P < 0.10$ ) in RESPON+ than RESPON– groups of both sheep and goats. These included the *cis*-9 and *cis*-11 16:1, *cis*-11 18:1, *cis*-11 20:1, and *cis*-13 22:1 MUFA (Table 3), and the 20:5n-3 and 22:6n-3 PUFA (Table 4). For a graphic representation of variations in these FA, please see Supplemental Figure S1. Furthermore, RESPON+ animals showed the greatest increments in 7-methyl-hexadec-7-enoate ( $P = 0.092$ ; Table 2), *cis*-11,*cis*-15, *trans*-9,*trans*-12, and *cis*-9,*trans*-12 18:2 ( $P < 0.05$ ; Table 4), and *cis*-4,*cis*-7,*cis*-10,*trans*-14,*trans*-17 22:5 ( $P = 0.051$ ; Table 4) in both species, and in *cis*-4,*cis*-7,*cis*-10,*trans*-14,*cis*-19 22:5 in ewes ( $P = 0.008$  for the interaction Sp × Res; Table 4). By contrast, FA with more pronounced increases in RESPON– ani-

**Table 2.** Ruminal SFA profile (g/100 g fatty acids) in dairy sheep and goats with a mild (RESPON–) or strong (RESPON+) response to a diet inducing milk fat depression (MFD)<sup>1</sup>

Variable	Item <sup>2</sup>	Goats		Sheep		SED <sup>3</sup>	P <sup>4</sup>		
		RESPON–	RESPON+	RESPON–	RESPON+		Sp	Res	Sp × Res
12:0	Control	0.11	0.084	0.17	0.16	0.015	<0.001	0.123	0.652
	ΔMFD	–0.046	–0.015	–0.059	–0.032	0.026	0.423	0.133	0.905
<i>anteiso</i> -13:0	Control	0.005	0.005	0.012	0.014	0.002	<0.001	0.654	0.705
	ΔMFD	0.002	0.002	–0.005	–0.007	0.003	<0.001	0.647	0.701
<i>iso</i> -13:0	Control	0.037	0.043	0.066	0.061	0.011	0.006	0.950	0.483
	ΔMFD	0.011	0.005	–0.019	–0.026	0.011	0.002	0.229	0.786
14:0	Control	0.78	0.58	0.81	0.80	0.108	0.119	0.193	0.220
	ΔMFD	0.55	0.79	0.37	0.34	0.149	0.008	0.311	0.212
<i>iso</i> -14:0	Control	0.12	0.14	0.18	0.16	0.035	0.153	0.850	0.494
	ΔMFD	–0.033	–0.044	–0.067	–0.091	0.028	0.060	0.401	0.736
15:0	Control	0.64	0.64	0.95	1.07	0.150	0.003	0.599	0.578
	ΔMFD	0.15	0.17	–0.090	–0.34	0.134	0.001	0.251	0.153
<i>anteiso</i> -15:0	Control	0.55	0.53	0.89	0.89	0.089	<0.001	0.898	0.807
	ΔMFD	0.076	0.074	–0.37	–0.41	0.091	<0.001	0.753	0.780
<i>iso</i> -15:0	Control	0.38	0.37	0.49	0.49	0.052	0.006	0.776	0.898
	ΔMFD	0.083	0.049	–0.12	–0.18	0.063	<0.001	0.321	0.785
16:0	Control	15.8	16.7	18.4	17.6	0.949	0.020	0.891	0.208
	ΔMFD	4.68	3.13	0.39	1.53	1.044	0.001	0.788	0.088
<i>iso</i> -16:0	Control	0.30	0.42	0.52	0.39	0.082	0.114	0.909	0.050 <sup>5</sup>
	ΔMFD	0.066	–0.078	–0.25	–0.21	0.092	0.003	0.429	0.176
17:0	Control	0.54	0.55	0.72	0.81	0.079	0.001	0.418	0.504
	ΔMFD	0.23	0.24	0.096	–0.046	0.087	0.004	0.296	0.233
<i>anteiso</i> -17:0	Control	0.38	0.44	0.59	0.57	0.077	0.008	0.741	0.519
	ΔMFD	0.21	0.077	–0.23	–0.25	0.081	<0.001	0.195	0.313
<i>iso</i> -17:0 <sup>6</sup>	Control	0.32	0.37	0.54	0.55	0.104	0.015	0.675	0.778
	ΔMFD	0.48	0.31	0.14	–0.007	0.104	<0.001	0.049	0.882
7-methyl-hexadec-7-enoate 17:0	Control	0.018	0.014	0.021	0.022	0.004	0.064	0.591	0.362
	ΔMFD	0.28	0.34	0.30	0.35	0.040	0.555	0.092	0.910
18:0	Control	45.8	44.7	44.3	47.3	3.479	0.865	0.719	0.428
	ΔMFD	–40.9	–39.2	–37.6	–41.2	3.637	0.799	0.710	0.321
10-O-18:0	Control	0.048	0.025	0.048	0.031	0.019	0.824	0.158	0.840
	ΔMFD	1.46	1.77	2.15	1.69	0.337	0.221	0.739	0.124
<i>iso</i> -18:0	Control	0.007	0.005	0.011	0.011	0.003	0.064	0.589	0.672
	ΔMFD	0.064 <sup>ab</sup>	0.068 <sup>ab</sup>	0.073 <sup>a</sup>	0.055 <sup>b</sup>	0.006	0.584	0.079	0.014
Σ OCFA	Control	1.83	1.73	2.46	2.72	0.213	<0.001	0.593	0.239
	ΔMFD	1.53	1.69	0.50	0.084	0.248	<0.001	0.484	0.119
Σ BCFA	Control	2.53	2.78	3.94	3.78	0.279	<0.001	0.800	0.314
	ΔMFD	1.56	0.98	–0.51	–0.94	0.291	<0.001	0.027	0.710

<sup>a,b</sup>Within a row, different superscripts indicate significant differences ( $P < 0.05$ ) due to the effect of Sp × Res.

<sup>1</sup>Other SFA are reported in Supplemental Table S1 (<http://hdl.handle.net/10261/272926>; Della Badia et al., 2022). OCFA = odd-chain fatty acids; BCFA = branched-chain fatty acids.

<sup>2</sup>Control = data obtained when animals were fed a TMR without lipid supplementation; ΔMFD = difference between the data obtained after diet supplementation with 20 g of fish oil/kg of DM (to induce MFD) and those previously recorded in the control period.

<sup>3</sup>SED = standard error of the difference.

<sup>4</sup>Probability of significant effects due to species (Sp), response (Res), and their interaction (Sp × Res).

<sup>5</sup>In the pairwise analysis, no significant differences were found after adjustment for multiple comparisons using a Bonferroni correction.

<sup>6</sup>Contains a 16:1 isomer of indeterminate double bond position as minor component.

mals were, for example, *iso*-17:0 ( $P = 0.049$ ; Table 2), *trans*-9 + 10 20:1 and *trans*-11 18:1 ( $P < 0.05$ ; Table 3). Several polyunsaturated C20–22 metabolites were also more abundant in RESPON–, particularly in sheep ( $P < 0.05$  for the interaction Sp × Res), including some *trans*-10-containing FA, such as *trans*-10, *cis*-17 and *trans*-10, *trans*-16 20:2, *trans*-10, *trans*-14, *cis*-19 22:3 and *trans*-10, *trans*-13, *cis*-16, *cis*-19 22:4 (Table 4).

Fewer FA showed negative responses to the FO supply in both species. Among them, decreases in 18:0 and

*cis*-9, *cis*-12 18:2 were similar in the RESPON– and RESPON+ groups ( $P > 0.10$ ; Tables 2 and 4, respectively). In fact, significant differences due to the response to MFD were only observed in *cis*-9, *cis*-12, *cis*-15 18:3, which decreased to a greater extent in RESPON– animals ( $P = 0.044$ ; Table 4 and Supplemental Figure S2). A similar trend was found for *trans*-9, *trans*-12, *cis*-15 18:3 ( $P = 0.094$ ; Table 4).

Ruminal FA showing inconsistent changes (i.e., either increases or decreases depending on the species)

**Table 3.** Ruminal MUFA profile (g/100 g fatty acids) in dairy sheep and goats with a mild (RESPON–) or strong (RESPON+) response to a diet inducing milk fat depression (MFD)<sup>1</sup>

Variable	Item <sup>2</sup>	Goats		Sheep		SED <sup>3</sup>	P <sup>4</sup>		
		RESPON–	RESPON+	RESPON–	RESPON+		Sp	Res	Sp × Res
<i>cis</i> -7 16:1	Control	0.25	0.14	0.23	0.28	0.097	0.405	0.631	0.278
	ΔMFD	0.29	0.42	0.21	0.14	0.108	0.031	0.688	0.198
<i>cis</i> -9 16:1	Control	0.091	0.072	0.083	0.096	0.016	0.479	0.800	0.182
	ΔMFD	0.63	0.75	0.53	0.84	0.116	0.976	0.021	0.249
<i>cis</i> -11 16:1	Control	0.006	0.005	0.009	0.009	0.002	0.019	0.875	0.526
	ΔMFD	0.034	0.039	0.026	0.034	0.005	0.096	0.078	0.640
<i>cis</i> -13 16:1	Control	0.023	0.008	0.006	0.014	0.009	0.362	0.551	0.075
	ΔMFD	0.014	0.023	0.024	0.007	0.012	0.746	0.674	0.145
<i>trans</i> -5 16:1	Control	0.020	0.021	0.030	0.025	0.003	0.010	0.381	0.172
	ΔMFD	0.025	0.019	0.001	0.008	0.005	<0.001	0.859	0.091
<i>trans</i> -6 + 7 + 8 16:1	Control	0.15	0.092	0.076	0.093	0.034	0.154	0.413	0.143
	ΔMFD	0.40	0.40	0.28	0.30	0.065	0.034	0.844	0.801
<i>trans</i> -9 16:1	Control	0.008	0.006	0.015	0.013	0.002	0.001	0.213	0.969
	ΔMFD	0.11	0.11	0.13	0.13	0.012	0.065	0.720	0.669
<i>cis</i> -9 17:1	Control	0.029	0.021	0.041	0.031	0.007	0.048	0.097	0.846
	ΔMFD	–0.008	–0.005	–0.022	–0.006	0.011	0.339	0.236	0.397
<i>cis</i> -9 18:1	Control	6.20	6.79	5.05	4.40	0.961	0.019	0.968	0.375
	ΔMFD	0.028 <sup>b</sup>	–0.51 <sup>b</sup>	0.94 <sup>b</sup>	3.50 <sup>a</sup>	0.778	<0.001	0.085	0.013
<i>cis</i> -11 18:1	Control	0.37	0.35	0.40	0.39	0.064	0.447	0.747	0.866
	ΔMFD	0.92	1.04	0.94	1.15	0.132	0.483	0.092	0.613
<i>cis</i> -12 18:1	Control	0.45	0.47	0.37	0.45	0.079	0.321	0.365	0.618
	ΔMFD	–0.27	–0.29	–0.13	–0.26	0.082	0.143	0.218	0.347
Σ <i>cis</i> -18:1	Control	7.33	7.87	6.05	5.50	1.092	0.031	0.998	0.493
	ΔMFD	0.82 <sup>b</sup>	0.46 <sup>b</sup>	2.07 <sup>ab</sup>	4.69 <sup>a</sup>	0.894	0.001	0.093	0.032
<i>trans</i> -9 18:1	Control	0.37	0.43	0.27	0.28	0.053	0.004	0.395	0.557
	ΔMFD	0.54	0.50	1.03	0.67	0.141	0.005	0.067	0.121
<i>trans</i> -10 18:1	Control	0.71	0.72	0.46	0.51	0.103	0.006	0.678	0.822
	ΔMFD	0.51	1.11	1.39	2.50	0.979	0.120	0.233	0.715
<i>trans</i> -11 18:1	Control	4.42	4.95	3.68	3.24	0.648	0.017	0.916	0.304
	ΔMFD	21.6	18.4	19.5	17.4	1.63	0.196	0.033	0.649
<i>trans</i> -12 18:1	Control	0.84	0.86	0.55	0.61	0.129	0.010	0.642	0.830
	ΔMFD	0.84	0.83	1.77	1.11	0.254	0.004	0.082	0.089
<i>trans</i> -13 + 14 18:1	Control	1.28	1.24	0.83	1.01	0.181	0.016	0.575	0.382
	ΔMFD	–0.005	0.079	1.23	0.34	0.338	0.006	0.111	0.059
Σ <i>trans</i> -18:1	Control	9.31	9.91	6.91	6.92	1.21	0.006	0.725	0.736
	ΔMFD	23.7	21.2	26.5	22.7	2.24	0.201	0.064	0.697
<i>cis</i> -7 + <i>trans</i> -13 20:1	Control	0.010	0.008	0.008	0.009	0.002	0.579	0.622	0.147
	ΔMFD	0.093	0.069	0.085	0.039	0.021	0.203	0.027	0.454
<i>cis</i> -11 20:1 <sup>5</sup>	Control	0.12	0.11	0.11	0.11	0.018	0.673	0.585	0.908
	ΔMFD	0.63	0.71	0.63	0.72	0.058	0.982	0.057	0.925
<i>cis</i> -13 + <i>trans</i> -17 20:1	Control	0.008	0.006	0.009	0.009	0.001	0.059	0.411	0.411
	ΔMFD	0.062	0.072	0.056	0.071	0.009	0.574	0.068	0.754
<i>cis</i> -17 20:1	Control	—	—	—	—	—	—	—	—
	ΔMFD	0.036 <sup>b</sup>	0.039 <sup>b</sup>	0.092 <sup>a</sup>	0.034 <sup>b</sup>	0.009	0.001	0.001	<0.001
<i>trans</i> -9 + 10 20:1	Control	—	—	—	—	—	—	—	—
	ΔMFD	0.081	0.061	0.040	0.020	0.011	<0.001	0.023	0.967
<i>trans</i> -11 20:1	Control	0.014	0.012	0.018	0.018	0.005	0.160	0.837	0.680
	ΔMFD	0.021	0.029	0.053	0.026	0.011	0.083	0.239	0.035 <sup>6</sup>
<i>trans</i> -12 20:1	Control	—	—	—	—	—	—	—	—
	ΔMFD	0.026 <sup>b</sup>	0.037 <sup>ab</sup>	0.073 <sup>a</sup>	0.037 <sup>ab</sup>	0.012	0.019	0.174	0.017
<i>cis</i> -13 22:1	Control	0.027	0.015	0.009	0.027	0.014	0.760	0.769	0.147
	ΔMFD	0.13	0.17	0.039	0.26	0.065	0.959	0.011	0.076
<i>cis</i> -19 22:1	Control	—	—	—	—	—	—	—	—
	ΔMFD	0.013 <sup>ab</sup>	0.012 <sup>ab</sup>	0.016 <sup>a</sup>	0.007 <sup>b</sup>	0.002	0.800	0.009	0.031

<sup>a,b</sup>Within a row, different superscripts indicate significant differences ( $P < 0.05$ ) due to the effect of Sp × Res.

<sup>1</sup>Other MUFA are reported in Supplemental Table S1 (<http://hdl.handle.net/10261/272926>; Della Badia et al., 2022).

<sup>2</sup>Control = data obtained when animals were fed a TMR without lipid supplementation; ΔMFD = difference between the data obtained after diet supplementation with 20 g of fish oil/kg of DM (to induce MFD) and those previously recorded in the control period.

<sup>3</sup>SED = standard error of the difference.

<sup>4</sup>Probability of significant effects due to species (Sp), response (Res), and their interaction (Sp × Res).

<sup>5</sup>Coelutes with *trans*-15 + 16 20:1.

<sup>6</sup>In the pairwise analysis, no significant differences were found after adjustment for multiple comparisons using a Bonferroni correction.

during the MFD period included *trans*-10,*cis*-12 CLA and *trans*-9,*cis*-11 CLA, but no effect of the response was detected ( $P > 0.10$ ; Table 4). Although the interaction Sp  $\times$  Res was significant for the latter CLA isomer ( $P = 0.016$ ; Table 4), no significant differences were found after adjustment for multiple comparisons using a Bonferroni correction. The abundance of *cis*-9 18:1 was increased in some groups, with a greater increment in RESPON+ sheep ( $P = 0.013$  for the interaction Sp  $\times$  Res; Table 3).

Finally, some interspecies differences in the MFD period included opposite changes ( $P < 0.05$ ) in the concentration of ammonia, in the molar proportions of butyrate, isobutyrate and isovalerate, and in the acetate:propionate ratio in goats (increases) and ewes (decreases). On the contrary, total VFA and the molar proportion of propionate increased in sheep and decreased in goats ( $P < 0.05$ ; Table 1). Goats showed stronger responses to FO ( $P < 0.05$ ) in the concentrations of 14:0 and 16:0 (Table 2) and most 16:1 isomers (Table 3). By contrast, changes in most 18:1 isomers (Table 3), and in *cis*-9,*cis*-12,*cis*-15 18:3, and C20–22 PUFA (Table 4) were more dramatic in sheep ( $P < 0.05$ ). All individual FA were detected in the 2 species, and no unique metabolites were identified in goats or sheep.

## DISCUSSION

The biohydrogenation theory proposes that diet-induced MFD is caused by alterations in ruminal FA metabolism leading to the production of antilipogenic BH intermediates (Bauman and Griinari, 2001). However, we are still uncertain as to whether a higher tolerance or susceptibility to MFD is determined by certain pre-existing traits or whether the severity of MFD depends only on the response to the diet (Baldin et al., 2018b; Dewanckele et al., 2019; Della Badia et al., 2021). In this study, we examined the ruminal response of goats and sheep with varying extents of MFD to attempt to answer that question and improve our understanding of this complex syndrome.

### Pre-Existing Variations (Control Period)

In dairy cows, production has been associated with individual responses to MFD (although inconsistently, because animals with both high and low production have been suggested as more sensitive to the syndrome; Bradford and Allen, 2004; Baldin et al., 2018b; Dewanckele et al., 2019). Our 2 previous reports on this subject (Frutos et al., 2017; Della Badia et al., 2021) agree that sheep and goats with a lower energy balance before consuming the MFD-inducing diet displayed

greater decreases in milk fat concentration and yield. Those individual variations in energy balance were not accompanied by differences in DMI (Della Badia et al., 2021), leaving room for speculation about a putative role of the digestive or metabolic utilization of the diet. In the present study, however, ruminal fermentation parameters indicative of energy metabolism (i.e., VFA or the acetate:propionate ratio) could not be related to MFD susceptibility.

Between-animal differences in the response to diet might also be predetermined by the rumen microbial composition (Weimer et al., 2010). Concentrations of odd- and branched-chain FA may serve as potential markers of microorganisms (Fievez et al., 2012), although functional redundancy of the microbiota and endogenous FA synthesis might represent limitations (Fievez et al., 2012; Weimer, 2015). In the control period, MFD-tolerant and MFD-susceptible animals showed similar proportions of odd- and branched-chain FA, except for the difference in *cis*-9 17:1. Nonetheless, this FA appears to have no known role as a biomarker of specific microbial populations (Fievez et al., 2012) or as a regulator of mammary lipogenesis in ruminants (Shingfield and Griinari, 2007; Dewanckele et al., 2020). Therefore, this observation could be a chance finding, without a cause-effect relationship with MFD. Another FA that differed between RESPON– and RESPON+ animals, 18:3n-6, was only affected in goats, which also refuted a major role in the MFD susceptibility.

Concerning BH, despite the comprehensive FA profile of ruminal fluid analyzed, it was not possible to find any trend in BH explaining the individual susceptibility to MFD. Overall, traits related to ruminal fermentation and BH did not seem to predetermine the individual degree of MFD in sheep and goats. More conclusive results might be obtained through a meta-analysis of more trials, which would help to increase the statistical power compared with single experiments.

Regarding interspecies differences, the scant variations between goats and sheep suggest only a minor effect of the species in animals fed a standard hay-based TMR. Such small differences in ruminal fermentation and BH might derive from interspecies variations in the microbial composition, which would be supported by divergences in odd- and branched-chain FA (Fievez et al., 2012).

### Differences in the Response to FO Supplementation

Starting with ruminal fermentation, the hypothesis relating the deficiency in ruminal acetate and the reduction in milk fat has been reconsidered in recent years (Maxin et al., 2011; Urrutia and Harvatine, 2017). Nevertheless, the present results suggest no link

**Table 4.** Ruminal PUFA profile (g/100 g fatty acids) in dairy sheep and goats with a mild (RESPON–) or strong (RESPON+) response to a diet inducing milk fat depression (MFD)<sup>F</sup>

Variable <sup>2</sup>	Item <sup>3</sup>	Goats		Sheep			P <sup>5</sup>		
		RESPON–	RESPON+	RESPON–	RESPON+	SED <sup>4</sup>	Sp	Res	Sp × Res
<i>cis</i> -9, <i>cis</i> -12 18:2	Control	8.49	8.47	7.71	6.21	1.43	0.153	0.465	0.474
	ΔMFD	–5.29	–5.34	–4.78	–2.67	1.45	0.142	0.332	0.306
<i>cis</i> -11, <i>cis</i> -15 + <i>cis</i> -10, <i>cis</i> -15 18:2	Control	0.034	0.031	0.029	0.026	0.006	0.292	0.491	0.968
	ΔMFD	0.010	0.016	0.018	0.037	0.007	0.007	0.022	0.191
<i>cis</i> -9, <i>trans</i> -12 18:2	Control	0.031	0.029	0.039	0.030	0.006	0.307	0.219	0.492
	ΔMFD	0.015	0.017	0.011	0.025	0.005	0.666	0.041	0.120
<i>trans</i> -10, <i>cis</i> -15 18:2	Control	0.011	0.010	0.028	0.027	0.005	<0.001	0.823	0.994
	ΔMFD	0.17	0.37	0.030	0.15	0.149	0.105	0.144	0.721
<i>trans</i> -11, <i>cis</i> -15 18:2	Control	0.22	0.21	0.23	0.17	0.053	0.782	0.359	0.472
	ΔMFD	0.51	0.50	0.32	0.74	0.154	0.800	0.085	0.069
<i>trans</i> -9, <i>trans</i> -12 18:2	Control	0.016	0.012	0.011	0.011	0.003	0.150	0.321	0.343
	ΔMFD	0.032	0.062	0.057	0.090	0.024	0.138	0.085	0.919
<i>cis</i> -9, <i>trans</i> -11 CLA <sup>6</sup>	Control	0.13	0.25	0.11	0.11	0.057	0.073	0.162	0.131
	ΔMFD	–0.020	–0.113	0.002	0.029	0.049	0.031	0.355	0.100
<i>trans</i> -9, <i>cis</i> -11 CLA	Control	0.037	0.017	0.011	0.038	0.012	0.800	0.670	0.015 <sup>7</sup>
	ΔMFD	–0.003	0.013	0.011	–0.015	0.011	0.357	0.529	0.016 <sup>7</sup>
<i>trans</i> -10, <i>cis</i> -12 CLA	Control	0.022	0.021	0.018	0.026	0.008	0.944	0.501	0.431
	ΔMFD	–0.010	–0.009	0.003	–0.009	0.008	0.260	0.283	0.264
<i>trans</i> -11, <i>cis</i> -13 CLA	Control	0.008	0.009	0.012	0.012	0.002	0.063	0.848	0.783
	ΔMFD	0.029 <sup>b</sup>	0.035 <sup>b</sup>	0.084 <sup>a</sup>	0.037 <sup>b</sup>	0.013	0.007	0.037	0.011
<i>trans</i> -12, <i>trans</i> -14 CLA <sup>8</sup>	Control	0.005	0.006	0.010	0.016	0.006	0.0685	0.431	0.549
	ΔMFD	0.057 <sup>b</sup>	0.057 <sup>b</sup>	0.099 <sup>a</sup>	0.025 <sup>c</sup>	0.010	0.492	<0.001	<0.001
<i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12 18:3	Control	0.013 <sup>b</sup>	0.008 <sup>c</sup>	0.018 <sup>a</sup>	0.020 <sup>a</sup>	0.001	<0.001	0.222	0.005
	ΔMFD	–0.002	0.006	–0.006	–0.007	0.002	<0.001	0.027	0.005 <sup>7</sup>
<i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15 18:3 <sup>9</sup>	Control	1.34	1.05	1.63	1.33	0.268	0.160	0.138	0.979
	ΔMFD	–0.46	–0.19	–0.95	–0.43	0.254	0.057	0.044	0.502
<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	Control	0.023	0.019	0.028	0.023	0.004	0.114	0.110	0.789
	ΔMFD	–0.007	–0.004	–0.014	–0.006	0.004	0.190	0.094	0.396
<i>cis</i> -11, <i>cis</i> -14 20:2	Control	0.050	0.023	0.014	0.015	0.011	0.014	0.136	0.092
	ΔMFD	0.084	0.12	0.13	0.12	0.017	0.126	0.313	0.073
<i>trans</i> -9, <i>cis</i> -17 + <i>trans</i> -14, <i>cis</i> -17 20:2	Control	—	—	—	—	—	—	—	—
	ΔMFD	0.008	0.011	0.017	0.008	0.003	0.154	0.207	0.016 <sup>7</sup>
<i>trans</i> -10, <i>cis</i> -17 20:2	Control	—	—	—	—	—	—	—	—
	ΔMFD	0.036 <sup>b</sup>	0.039 <sup>b</sup>	0.078 <sup>a</sup>	0.038 <sup>b</sup>	0.007	0.001	0.001	<0.001
<i>trans</i> -13, <i>cis</i> -17 20:2	Control	—	—	—	—	—	—	—	—
	ΔMFD	0.022 <sup>b</sup>	0.033 <sup>b</sup>	0.093 <sup>a</sup>	0.036 <sup>b</sup>	0.012	0.001	0.017	0.001
<i>trans</i> -10, <i>trans</i> -16 20:2	Control	—	—	—	—	—	—	—	—
	ΔMFD	0.070 <sup>b</sup>	0.077 <sup>b</sup>	0.16 <sup>a</sup>	0.085 <sup>b</sup>	0.014	<0.001	0.003	0.001
<i>trans</i> -11, <i>trans</i> -17 + <i>trans</i> -12, <i>trans</i> -17 20:2	Control	—	—	—	—	—	—	—	—
	ΔMFD	0.006 <sup>b</sup>	0.006 <sup>b</sup>	0.021 <sup>a</sup>	0.009 <sup>b</sup>	0.002	<0.001	0.001	0.001
<i>cis</i> -11, <i>trans</i> -14, <i>cis</i> -17 20:3	Control	—	—	—	—	—	—	—	—
	ΔMFD	0.017 <sup>ab</sup>	0.018 <sup>ab</sup>	0.025 <sup>a</sup>	0.017 <sup>b</sup>	0.003	0.096	0.068	0.035
<i>trans</i> -10, <i>trans</i> -14, <i>trans</i> -17 20:3	Control	—	—	—	—	—	—	—	—
	ΔMFD	0.039	0.038	0.052	0.035	0.007	0.342	0.088	0.099
<i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17 20:5	Control	—	—	—	—	—	—	—	—
	ΔMFD	0.40	0.56	0.33	0.74	0.135	0.574	0.008	0.229
<i>trans</i> -15, <i>cis</i> -19 22:2	Control	—	—	—	—	—	—	—	—
	ΔMFD	0.035	0.028	0.065	0.035	0.009	0.009	0.009	0.078
<i>trans</i> -11, <i>trans</i> -17 + <i>trans</i> -13, <i>trans</i> -18 22:2	Control	—	—	—	—	—	—	—	—
	ΔMFD	0.049 <sup>b</sup>	0.058 <sup>ab</sup>	0.085 <sup>a</sup>	0.058 <sup>ab</sup>	0.011	0.034	0.261	0.033
<i>cis</i> -11, <i>cis</i> -16, <i>cis</i> -19 + <i>trans</i> -13, <i>cis</i> -16, <i>trans</i> -19 22:3	Control	—	—	—	—	—	—	—	—
	ΔMFD	0.064 <sup>b</sup>	0.063 <sup>b</sup>	0.17 <sup>a</sup>	0.10 <sup>b</sup>	0.021	<0.001	0.030	0.036
<i>cis</i> -13, <i>cis</i> -16, <i>trans</i> -17 22:3 <sup>10</sup>	Control	—	—	—	—	—	—	—	—
	ΔMFD	0.030 <sup>b</sup>	0.035 <sup>b</sup>	0.070 <sup>a</sup>	0.025 <sup>b</sup>	0.011	0.074	0.022	0.005
<i>trans</i> -10, <i>trans</i> -14, <i>cis</i> -19 + <i>trans</i> -12, <i>trans</i> -15, <i>cis</i> -19 22:3	Control	—	—	—	—	—	—	—	—
	ΔMFD	0.020 <sup>b</sup>	0.029 <sup>b</sup>	0.074 <sup>a</sup>	0.024 <sup>b</sup>	0.013	0.014	0.034	0.004
<i>trans</i> -11, <i>trans</i> -14, <i>trans</i> -17 22:3	Control	—	—	—	—	—	—	—	—
	ΔMFD	0.011 <sup>b</sup>	0.017 <sup>b</sup>	0.033 <sup>a</sup>	0.018 <sup>b</sup>	0.004	0.001	0.071	0.001
<i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19 22:4	Control	—	—	—	—	—	—	—	—
	ΔMFD	0.40 <sup>b</sup>	0.50 <sup>b</sup>	0.72 <sup>a</sup>	0.48 <sup>b</sup>	0.056	0.002	0.099	0.001
<i>trans</i> -9, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19 + <i>trans</i> -8, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19 22:4	Control	—	—	—	—	—	—	—	—
	ΔMFD	0.15	0.15	0.21	0.15	0.024	0.104	0.068	0.080
<i>trans</i> -10, <i>trans</i> -13, <i>cis</i> -16, <i>cis</i> -19 22:4	Control	—	—	—	—	—	—	—	Continued



**Table 4 (Continued).** Ruminal PUFA profile (g/100 g fatty acids) in dairy sheep and goats with a mild (RESPON–) or strong (RESPON+) response to a diet inducing milk fat depression (MFD)<sup>1</sup>

Variable <sup>2</sup>	Item <sup>3</sup>	Goats		Sheep		SED <sup>4</sup>	P <sup>5</sup>		
		RESPON–	RESPON+	RESPON–	RESPON+		Sp	Res	Sp × Res
<i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19 22:5	ΔMFD	0.073 <sup>b</sup>	0.077 <sup>b</sup>	0.20 <sup>a</sup>	0.057 <sup>b</sup>	0.017	0.001	<0.001	<0.001
	Control	—	—	—	—	—	—	—	—
<i>cis</i> -4, <i>cis</i> -7, <i>cis</i> -10, <i>trans</i> -14, <i>cis</i> -19 22:5	ΔMFD	0.58	0.70	1.39	1.49	0.265	0.001	0.558	0.980
	Control	—	—	—	—	—	—	—	—
<i>cis</i> -4, <i>cis</i> -7, <i>cis</i> -10, <i>trans</i> -14, <i>trans</i> -17 22:5	ΔMFD	0.11 <sup>ab</sup>	0.073 <sup>ab</sup>	0.046 <sup>b</sup>	0.14 <sup>a</sup>	0.032	0.906	0.211	0.008
	Control	—	—	—	—	—	—	—	—
<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	ΔMFD	0.079	0.081	0.008	0.085	0.026	0.088	0.051	0.058
	Control	—	—	—	—	—	—	—	—
	ΔMFD	1.64	2.35	0.68	2.84	0.667	0.631	0.008	0.146

<sup>a-c</sup>Within a row, different superscripts indicate significant differences ( $P < 0.05$ ) due to the effect of Sp × Res.

<sup>1</sup>Other PUFA are reported in Supplemental Table S1 (<http://hdl.handle.net/10261/272926>; Della Badia et al., 2022).

<sup>2</sup>Except for n-3 and n-6 PUFA, the tentative geometry of double bonds was inferred from the retention times and elution order of FAME during GC analysis (Toral et al., 2018).

<sup>3</sup>Control = data obtained when animals were fed a TMR without lipid supplementation; ΔMFD = difference between the data obtained after diet supplementation with 20 g of fish oil/kg of DM (to induce MFD) and those previously recorded in the control period.

<sup>4</sup>SED = standard error of the difference.

<sup>5</sup>Probability of significant effects due to species (Sp), response (Res), and their interaction (Sp × Res).

<sup>6</sup>Contains *trans*-8,*cis*-10 CLA as minor component, representing on average  $5.87 \pm 1.14\%$  of the peak in the control period. In the MFD period, *trans*-8,*cis*-10 CLA could not be resolved due to coelution with a 20:2 isomer of indeterminate double bond position. No traces of *trans*-7 *cis*-9 CLA were detected in Control or MFD periods.

<sup>7</sup>In the pairwise analysis, no significant differences were found after adjustment for multiple comparisons using a Bonferroni correction.

<sup>8</sup>Coelutes with a 20:2 isomer of indeterminate double bond position.

<sup>9</sup>Contains *cis*-9 + *trans*-14 20:1 as minor components.

<sup>10</sup>Coelutes with *cis*-16,*cis*-19 22:2.

between this VFA and susceptibility to FO-induced MFD. Some studies report that marine lipids often decrease acetate and total VFA concentrations in the rumen (e.g., Fievez et al., 2007; Zhu et al., 2016), and we observed in a previous trial with dairy ewes (Frutos et al., 2018) that these reductions were related to MFD severity. However, inconsistent changes in sheep and goats were found in the present study, which would rule out a clear relationship with MFD susceptibility.

Regarding BH, no variation in ruminal *trans*-10,*cis*-12 CLA was detected (Supplemental Figure S2), which would also downplay the role of this CLA isomer in determining the individual response to FO consumption, consistent with available information in dairy sheep and goats (Frutos et al., 2018; Della Badia et al., 2021). By contrast, the milk FA profiles of RESPON– and RESPON+ animals reported previously by Della Badia et al. (2021) drew attention to *trans*-10 18:1 and *trans*-10,*cis*-15 18:2 as potential determinants of the extent of MFD. A similar relationship with *trans*-10 18:1 may exist in dairy cows (Baldin et al., 2018b; Dewanckele et al., 2019), although the few studies that examined the biological effects of both *trans*-10 FA in cattle were inconclusive (Lock et al., 2007; Kadegowda et al., 2009; Shingfield et al., 2009; Vahmani et al., 2016). In the present trial, ruminal concentrations of *trans*-10 18:1

and *trans*-10,*cis*-15 18:2 showed numerical variations between RESPON– and RESPON+ animals, both in goats and in sheep, but the differences did not reach the required level of significance (Supplemental Figure S2). This is not surprising because *trans*-10 FA usually show higher individual variations than other BH intermediates, in particular when diet-induced increases take place (e.g., Bernard et al., 2015; Baldin et al., 2018b; Frutos et al., 2018).

The BH of very-long-chain PUFA from FO also leads to the production of other *trans*-10 isomers with a putative role in MFD (Kairenius et al., 2018; Toral et al., 2018). In general, the very low amounts of most C20–22 BH intermediates would hinder their determination in milk samples, but not that in ruminal fluid. Specifically, digesta samples were collected 4 h after the morning feeding, when ruminal processes were very active and accumulation of BH metabolites was probably favored (Aldai et al., 2018; Baldin et al., 2018a). In any event, ruminal concentrations of *trans*-10-containing C20–22 FA observed in this trial were not associated with the individual MFD susceptibility of sheep and goats.

Neither could changes in ruminal 18:0 be related to MFD severity, even though its large reductions during FO-induced MFD suggested a role in the syndrome and motivated an extension of the biohydrogenation

theory some years ago (Loor et al., 2005; Shingfield and Griinari, 2007; Gama et al., 2008). The shortage of ruminal 18:0 for mammary uptake and  $\Delta^9$ -desaturation was presumed to impair the capacity to achieve adequate milk fat fluidity for efficient secretion, but this hypothesis was then challenged as a major determinant of MFD (Toral et al., 2016). The present results also dismiss it as an explanation for the individual response to dietary FO.

Interestingly, most UFA provided by FO (i.e., *cis*-9 and *cis*-11 16:1, *cis*-11 18:1, *cis*-11 20:1, *cis*-13 22:1, 20:5n-3, and 22:6n-3) were more abundant in the ruminal fluid of sheep and goats suffering more severe MFD (Supplemental Figure S1). Duodenal infusion of FO has been shown to cause MFD in cows (Loor et al., 2005; Dallaire et al., 2014), which could be attributed to a direct antilipogenic effect of certain FA from this lipid supplement (Kadegowda et al., 2009; Burns et al., 2012a; Duckett et al., 2014). In particular, 20:5n-3 was reported to downregulate the expression of lipoprotein lipase (*LPL*) and sterol regulatory element-binding transcription factor 1 (*SREBF1*) in mammary epithelial cell cultures (Kadegowda et al., 2009). The first gene is involved in preformed FA uptake by mammary epithelial cells, whereas the transcription factor has a central role in the regulation of mammary lipogenesis, and both of them are often downregulated during MFD (Bauman et al., 2011; Shingfield et al., 2013).

In vitro studies suggested that *cis*-9 16:1 and *cis*-11 18:1 also impair lipogenesis in bovine adipocytes (Burns et al., 2012a,b). Likewise, in vivo administration of an oil rich in *cis*-9 16:1 reduced the intramuscular lipid percentage in sheep (Duckett et al., 2014, 2019), but duodenal infusion of a similar lipid source did not impair milk fat synthesis in cows (Plante-Dubé et al., 2021). Therefore, further research in dairy ruminants is needed to characterize the biological effects of *cis*-9 16:1, which has raised interest in human nutrition due to its identification as a lipokine (i.e., a FA that regulates systemic metabolism; de Souza et al., 2018). Additional studies are also required to examine the link between MFD severity and other FA from FO (such as *cis*-11 16:1, which was previously correlated with milk fat concentration; Bernard et al., 2015, 2017) and to clarify if they exert inhibitory effects or just co-vary with milk fat due to FO intake.

Overall, the association between the MFD susceptibility and ruminal concentrations of presumably antilipogenic UFA provided by FO suggests lower BH extent in the rumen of RESPON+ goats and sheep. However, this seems to disagree with the greater reduction in ruminal *cis*-9,*cis*-12,*cis*-15 18:3 in these more responsive animals. This apparent contradiction may

be explained by the specificity of bacterial enzymes involved in FA metabolism (Wallace et al., 2007; Enjalbert et al., 2017). In this regard, dietary marine lipids are known to favor the ruminal disappearance of *cis*-9,*cis*-12,*cis*-15 18:3 and *cis*-9,*cis*-12 18:2 (Kim et al., 2008; Zhu et al., 2016; Kairenius et al., 2018), the most abundant PUFA in plants and therefore in standard ruminant diets. Their BH process seems mostly initiated by *cis*-12 isomerase activity (Wallace et al., 2007; Honkanen et al., 2012), which may be mediated by a higher abundance or activity of bacteria in RESPON+ animals. However, these microbial populations would be irrelevant in the initial BH of all the above-mentioned FA derived from FO (i.e., *cis*-9 16:1, *cis*-11 16:1, *cis*-11 18:1, *cis*-11 20:1, *cis*-13 22:1, 20:5n-3, and 22:6n-3) because none of them contains a *cis*-12 double bond. Therefore, bacteria showing greater isomerase or hydrogenase activity on double bonds other than *cis*-12 (e.g., *cis*-4, *cis*-5, *cis*-9, or *cis*-11; Honkanen et al., 2012; Aldai et al., 2018; Toral et al., 2018) could be less abundant in RESPON+ animals and may explain the apparent inconsistency in the extent of BH of UFA from vegetable or marine lipids.

Finally, differences between ruminant species were more evident in the MFD than in the control period. Again, interspecies variation in the ruminal microbial community might be at the core of these differences, as suggested by changes in odd- and branched-chain FA (Fievez et al., 2012). As previously reported for sheep and cattle (Toral et al., 2017, 2018), the same FA were found in ewes and does, indicating that BH followed similar pathways in both species, although an influence on BH kinetics existed (e.g., a slower process for C20–22 PUFA in sheep than in goats).

## CONCLUSIONS

The individual responses of dairy goats and sheep to FO-induced MFD would not be predetermined by variations in ruminal fermentation and BH. Based on the commonality of the responses in both ruminant species, the tolerance or susceptibility to MFD may depend predominantly on individual differences in the extent of BH of certain potentially antilipogenic UFA provided by FO (e.g., *cis*-9 16:1, *cis*-11 18:1, and 20:5n-3). Further research is needed to establish the actual biological activity of these FA in dairy ruminants.

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## ORCID

- Antonella Della Badia <https://orcid.org/0000-0002-2685-5328>  
 Pilar Frutos <https://orcid.org/0000-0002-4919-5094>  
 Pablo G. Toral <https://orcid.org/0000-0002-1913-7707>  
 Gonzalo Hervás <https://orcid.org/0000-0002-0013-7459>