INTERPRETIVE SUMMARY

Milk fat depression in dairy ewes fed fish oil: Might differences in rumen biohydrogenation, fermentation, or bacterial community explain the individual variation?

(by Frutos et al.)

Dairy ewes show large individual variation in the severity of milk fat depression (MFD), a syndrome caused by diet supplementation with marine lipids to modulate milk fatty acid profile. Understanding what is behind this variability might help to prevent the syndrome. We hypothesized that alterations in the processes of rumen biohydrogenation and fermentation, and in the bacterial community would explain differences in the extent of fish oil-induced MFD. However, these factors were not able to fully account for individual variations and further research must be pursued.

Milk fat depression in dairy ewes fed fish oil: Might differences in rumen biohydrogenation, fermentation, or bacterial community explain the individual variation?

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ABSTRACT

Dairy ewes show large individual variation in the extent of diet-induced milk fat depression (MFD) but reasons behind this variability remain uncertain. Previous results offered no convincing support for these differences being related to relevant changes in the milk fatty acid (FA) profile, including potentially antilipogenic FA, or in the transcript abundance of candidate genes involved in mammary lipogenesis. Therefore, we hypothesized that alterations in the processes of rumen biohydrogenation and fermentation, as well as in the bacterial community structure, might account for individual variation in fish oil-induced MFD severity. To test this explanation, 15 ewes received a total mixed ration without lipid supplementation (control; n = 5) or supplemented with 20 g of fish-oil/kg of dry matter [10 animals divided into those showing a strong (RESPON+; −25.4%; n = 5) or a mild (RESPON–; −7.7%; n = 5) decrease in milk fat concentration] for 5 weeks. Rumen fermentation parameters, biohydrogenation metabolites and bacterial structure and diversity were analyzed in rumen samples collected before and after treatments. Although the fish oil supplementation increased the concentration of demonstrated or putative antilipogenic FA (e.g., *cis*-9 16:1, *cis*-11 18:1 or

trans-10 *cis*-12 CLA), surprisingly, none of them differed significantly in relation to the extent of MFD (i.e., between RESPON− and RESPON+), and only did it few minor FA (e.g., *cis*-6+7 16:1 or 17:0 *anteiso*). Changes in total volatile FA, acetate and propionate concentrations were associated with MFD severity, with higher decreases in more susceptible animals. Individual responses were not related to shifts in rumen bacterial structure but some terminal restriction fragments compatible with *Clostridiales*, *Ruminococcaceae*, *Lachnospiraceae* and *Succiniclasticum* showed greater abundances in RESPON− whereas some others that may correspond to *Prevotella*, *Mogibacterium* and *Quinella*-related spp. were more abundant in RESPON+. Overall, the results suggest that individual variation in MFD severity in dairy ewes fed fish oil cannot be fully explained by differences in the processes of rumen biohydrogenation and fermentation or in the bacterial community, and further research would be necessary to elucidate the large variability in the responsiveness to MFD-inducing marine lipids.

Keywords: acetate, fatty acid, marine lipid, ruminal microbiota, sheep

INTRODUCTION

Diet-induced milk fat depression (**MFD**) is commonly observed in sheep when they are fed marine lipid supplements to modulate milk fatty acid (FA) composition (e.g., Toral et al., 2016b; Frutos et al., 2017). However, dairy ewes, as dairy cows, show large individual variation in the extent of this condition (Reynolds et al. 2006; Weimer et al., 2010). For example, Toral et al. (2016b) observed up to eight-fold differences in milk fat decreases within a group of lactating sheep fed the same MFD-inducing diet. Yet, reasons behind this variability are uncertain.

Elucidating the cause of this different responsiveness might help to understand diet-induced MFD, which continues to be an active research area given the economic value of milk fat and associated losses (Palmquist and Jenkins, 2017). With that aim, we conducted an experiment (Frutos et al., 2017) with dairy ewes displaying either strong or just mild MFD when fed a diet containing 2% fish oil. Unexpectedly, results offered no convincing support for individual variations being linked to relevant changes in the milk fatty acid (**FA**) profile, including potentially antilipogenic FA, or with the transcript abundance of candidate genes involved in mammary lipogenesis. Therefore, further research was necessary.

Bauman and Griinari (2001) postulated that MFD is related to active biohydrogenation (**BH**) intermediates that are produced under several feeding conditions that alter rumen function, and referred to this as the biohydrogenation theory of MFD. The production of these intermediates is primarily due to the rumen microbiota, especially bacteria, with no or limited contribution of other groups such as protozoa or fungi (Lourenço et al., 2010; Enjalbert et al., 2017). Nonetheless, it is still uncertain which populations are actually involved in the process (Buccioni et al., 2012; Enjalbert et al., 2017; Pitta et al., 2018). Although most studies focused on the *trans*-10 *cis*-12 18:2, whose role in marine lipid-induced MFD has been dismissed (Loor et al., 2005; Toral et al., 2012), other BH metabolites with potentially antilipogenic features

have then been connected to mammary lipogenesis (Alves and Bessa, 2014; Kairenius et al., 2015; Toral et al., 2016b). For this reason, we speculated that some minor BH metabolites possibly associated with BH-induced MFD might be better detected in rumen fluid than in milk as changes occurring in the mammary gland would be excluded.

Furthermore, early theories attributed the reduction in milk fat to an acetate deficiency, because this volatile FA is the main substrate for de novo synthesis of FA in dairy ruminants, but they were disregarded based on experiments infusing acetate to cows (see review by Bauman and Griinari, 2001). However, Urrutia and Harvatine (2017) have recently resumed research on the effect of acetate on mammary lipid synthesis and suggested that the subject would merit further investigation.

On this basis, this study was conducted to test the hypothesis that differences in the processes of rumen BH of unsaturated FA and fermentation, as well as in the bacterial community, would account for the individual variation in fish oil-induced MFD severity.

MATERIALS AND METHODS

All experimental procedures were approved and completed in accordance with EU and Spanish regulations (Council Directive 2010/63/EU and R.D. 53/2013) for the protection of animals used for experimental purposes.

Animals and Experimental Diets

Details of the experimental design and methodology were described in Frutos et al. (2017). Briefly, we used 15 lactating Assaf ewes (76.4 ± 2.66 kg of BW; 48 ± 1.4 DIM; 2.8 ± 0.15 kg of milk/d) that were selected from a total of 27 animals randomly allocated to 1 of 2 diets: a TMR based on alfalfa hay and a concentrate (50:50) without lipid supplementation (Control group; n = 5) or supplemented with 20 g of fish oil (Afampes 121 DHA; Afamsa, Mos,

Spain)/kg of diet DM to cause MFD (MFD group; n = 22). On average, experimental diets contained 138 g of starch, 180 g of crude protein and 315 g of NDF/kg DM [see Frutos et al. (2017) for further details about chemical composition, ingredients, and FA profile]. All ewes were fed the control diet for a 21-d adaptation period, and then both experimental diets for 36 more days. At the end of this latter period, 10 animals out of the 22 were selected and divided in those showing a strong MFD (**RESPON+**; −25.4% decrease in milk fat concentration; n = 5) or a mild MFD (**RESPON–**; −7.7% decrease in milk fat concentration; n = 5).

Measurements and Sampling Procedures

At the end of the adaptation period and after 36 d on the experimental diets, ewes were given free access to the diets for 1 h after morning milking. Then, feeds were removed and 3 h later, samples of rumen fluid were collected from each animal (ca. 150 mL) using an oral stomach probe (Ramos-Morales et al., 2014). Immediately after collection, the fluid was strained through a nylon membrane (400 μm; Fisher Scientific S.L., Madrid, Spain); 3 mL were acidified with 3 mL of 0.2 *M* HCl for ammonia analysis, and 0.8 mL were 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°C until analysis. Further aliquots of ruminal fluid were collected (approx. 50 mL), immediately frozen at −80ºC, freeze-dried, and stored again at −80ºC until analyzed for FA composition and bacterial community.

Laboratory Analysis

Ruminal Fermentation Parameters. Ammonia concentration was determined by a colorimetric method (Reardon et al., 1966) and VFA by gas chromatography, using crotonic acid as an internal standard (Ottenstein and Bartley, 1971), both in centrifuged samples.

Ruminal FA Composition. Fatty acid methyl esters (**FAME**) of lipid in 200 mg of freeze-

dried rumen digesta samples were extracted twice using 4 mL of a mixture (3:2, vol/vol) of hexane and isopropanol following the adjustment of digesta pH to 2 using 2 *M* HCl (Shingfield et al., 2003), and adding *cis*-12 13:1 (10-1301-9, Larodan Fine Chemicals AB, Solna, Sweden) as an internal standard. Organic extracts were combined and dried under nitrogen at 50°C. Lipid dissolved in 2 mL of hexane was 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°C followed by reaction with 1% (vol/vol) sulfuric acid in methanol at 50°C for 30 min (Toral et al., 2017). Methyl esters were separated and quantified using a gas chromatograph (Agilent 7890A GC System, Santa Clara, CA) equipped with a flame-ionisation detector and a 100-m fused silica capillary column (0.25 mm i.d., 0.2-μm film thickness; CP-SIL 88, CP7489, Varian Ibérica S.A., Madrid, Spain) and hydrogen as the carrier gas (207 kPa, 2.1 mL/min). Total FAME profile in a 2 μL sample volume at a split ratio of 1:20 was determined using a temperature gradient program, and isomers of 18:1 were further resolved in a separate analysis under isothermal conditions at 170°C (Shingfield et al., 2003). Peaks were identified based on retention time comparisons with commercially available standards (Larodan Fine Chemicals AB, Nu-Chek Prep., Elysian, MN; and Sigma–Aldrich, Madrid, Spain), cross referencing with chromatograms reported in the literature (e.g., Shingfield et al., 2003) and comparison with reference samples for which the FA composition was determined based on gas chromatography analysis of FAME and gas chromatography–mass spectrometry analysis of corresponding 4,4-dimethyloxazoline derivatives (Toral et al., 2017).

Ruminal Bacterial Community. ***Ruminal Bacterial Community.*** Freeze-dried rumen samples were thoroughly homogenized before DNA extraction, which was carried out following the protocol described by Yu and Morrison (2004), with the modification of a higher temperature (95ºC) to improve cell lysis. Duplicate DNA samples were combined and used as templates for terminal restriction fragment length polymorphism (**T-RFLP**) analysis of

bacterial 16S rRNA genes. This was performed using a universal bacteria specific primer pair set (6-carboxy-fluorescein (FAM)-labelled 27f = 5’-6-FAM-AGAGTTTGATCCTGGCTCAG-3’; and 1389r = 5’-ACGGGCGGTGTGTACAAG-3’; Hongoh et al., 2003). The PCR products were purified and then digested with *Hha*I, *Hae*III and *Msp*I in single-enzyme digestions. The labelled terminal restriction fragments (**T-RF**) were analyzed by capillary electrophoresis on an automatic sequence analyzer (MegaBace 500, GE Healthcare Life Sciences, Buckinghamshire, UK) and their lengths determined with the size standard ET-550-R (GE Healthcare Life Sciences, Buckinghamshire, UK) using the GeneMarker Analysis software (SoftGenetics, State College, PA). To infer the potential bacterial composition, in silico restriction for gut bacteria were obtained from the Ribosomal Database Project II website (http://rdp.cme.msu.edu/index.jsp). Data from T-RFLP (size, bp, and peak area for each T-RF) were analyzed as outlined by Abdo et al. (2006), and used to determine the relative abundance of each fragment and the diversity indices (number of T-RF or richness, Shannon-Wiener and Shannon evenness; Hill et al., 2003).

Calculations and Statistical Analysis

Statistical analyses were performed using the SAS software package (version 9.4; SAS Institute Inc., Cary, NC). Ruminal fermentation parameters, biohydrogenation metabolites and bacterial diversity indices were analyzed by one-way analysis of covariance (**ANCOVA**) with a model that included the fixed effect of treatments (control, RESPON– and RESPON+), and measurements at the end of the adaptation period as a covariate. Animals were nested within treatment. Previously defined orthogonal contrasts were used to confirm the effects of fish oil supplementation [i.e., control vs. MFD (RESPON+ and RESPON–)], as well as to examine differences in the response within ewes with diet-induced MFD (i.e., RESPON+ vs. RESPON–). For relative abundances of T-RF, because most of them did not satisfy the assumptions of

normality, a Blom rank-based nonparametric ANCOVA (with orthogonal contrast) was applied. Differences were declared significant at $P < 0.05$ and considered a trend towards significance at $0.05 \leq P < 0.10$. Least squares means are reported.

The relationship between ruminal fermentation parameters, biohydrogenation metabolites and T-RFLP data at the end of the adaptation period and after 36 days on treatments was assessed through a principal component analysis (**PCA**) using the 'R-project' software (version 3.2.3; http://www.r-project.org).

RESULTS

Rumen FA profile

Inclusion of fish oil in the diet altered the concentration of most FA identified in ruminal digesta and increased total FA content by 36% ($P < 0.05$; Table 1 and Supplementary Table S1). Changes in FA profile were characterized by strong decreases in 18:0 (on average, −85% compared with the control; $P < 0.001$) and large increases in *trans*-11 18:1 ($P < 0.001$), which was the most abundant FA in the rumen of supplemented ewes (≈21% of total FA). However, the few significant differences between RESPON− and RESPON+ were limited to minor FA. Specifically, dietary fish oil enhanced the concentration of *cis*-6+7 16:1, *cis*-9 *trans*-11 CLA, *cis*-15 22:1 and 22:4n-3 to a greater extent in ewes showing severe MFD ($P < 0.10$). Concentrations of 15:0 *iso*, 17:0 *anteiso*, *cis*-11 *cis*-14 18:2 and *trans*-9 *trans*-12 *cis*-15 + *cis*-9 *cis*-12 *trans*-15 18:3 also tended to be greater in RESPON+ than in RESPON− ($P < 0.10$), but none of their values differed significantly from the control. Increases to lipid supplementation in most demonstrated or putative antilipogenic FA (e.g., *cis*-9 16:1, 10-oxo-18:0, *cis*-11 18:1, *trans*-10 *cis*-12 CLA, *trans*-10 *cis*-15 18:2, which coeluted with *trans*-11 *cis*-15 18:2, or 22:6n-3), were not related to differences in MFD intensity (i.e., between RESPON− and RESPON+;

P > 0.10). Numerical variation in *trans*-10 18:1 did not reach the required level of significance (*P* = 0.16), and *trans*-9 *cis*-11 CLA could not be detected in digesta.

Dietary fish oil decreased the ruminal concentration of 18:2n-6 and 18:3n-3 (*P* < 0.001) and promoted the accumulation of most 18-carbon BH intermediates, including some *trans*-18:1, *cis*-18:1 and *trans*-18:2 isomers (*P* < 0.05). Some minor metabolites of ruminal BH and hydration were only detected in ewes receiving the marine lipid (e.g., *cis*-12 *cis*-15 + *cis*-12 *cis*-16 18:2). On the other hand, the proportion of few 18:1 and 18:2 isomers with at least one double bond at positions Δ13 to Δ16 (e.g., *cis*-16 18:1 and *trans*-11 *trans*-13 CLA) was lower in supplemented ewes (*P* < 0.10). None of these changes showed significant differences between RESPON− and RESPON+.

Consequences of fish oil feeding on ruminal odd- and branched-chain FA concentrations included increases (17:0 and 17:0 *iso*), no effects (15:0 and *trans*-5+6+7 15:1) and decreases (15:0 *anteiso* and 16:0 *iso*). Only 15:0 *iso* and 17:0 *anteiso* variations were associated with MFD extent. Most FA present in the marine lipid (such as 14:0, *cis*-9 16:1 and 22:6n-3) and their unsaturated BH metabolites were more abundant (*P* < 0.05) or could only be detected in the digesta of MFD sheep. However, 20:0 and 22:0 were reduced in both RESPON− and RESPON+, but without differences between them (*P* > 0.10).

Rumen Fermentation Characteristics

As shown in Table 2, feeding fish oil increased the concentration of ammonia and decreased total VFA, acetate, propionate and butyrate (*P* < 0.05). Changes in total VFA, acetate and propionate concentrations were linked to MFD severity (*P* ≤ 0.05) with the highest decreases in more responsive animals. The acetate:propionate ratio tended to be slightly lower in rumen digesta from supplemented ewes (*P* = 0.054) compared with the control, but without differences between RESPON− and RESPON+ (*P* = 0.73). Molar VFA proportions were also

similar in both supplemented groups (*P* > 0.10).

Bacterial Community Analysis

The PCA of bacterial profiles (Supplementary Figure S1) presented certain segregation by diet, with control ewes being separated from those fed the fish oil but without discrimination between RESPON− and RESPON+. Nevertheless, the first principal component accounted for only 14.5% of total variability.

Rumen bacterial T-RFLP analysis generated on average 37.9 ± 1.42, 25.0 ± 0.99 and 78.9 ± 1.54 fragments with the enzymes *Hha*I, *Msp*I and *Hae*III, respectively. No treatment effect was detected on the number of T-RF (richness; Table 3), but reductions of Shannon-Wiener and Shannon evenness indices due to fish oil consumption were observed with *Hae*III (*P* < 0.05) and *Msp*I (*P* < 0.10) digestions. There was no significant differences in bacterial diversity linked to MFD severity.

Several T-RF relative frequencies (Supplementary Table S2) showed irregular variations between control and MFD treatments (*P* < 0.10), with both greater (e.g., *Succinivibrio*-compatible 203 bp with *Hha*I) and lower (e.g., *Clostridiales*-compatible 383 bp with *Hha*I and 309 with *Hae*III) values with the marine lipids. The abundance of most generated fragments were not different between RESPON− and RESPON+.

Table 4 shows only relative frequencies of some T-RF that varied between both supplemented groups. Fragments compatible with *Prevotella* spp., *Quinella*-related bacteria and the genus *Mogibacterium* were more abundant or appeared only in ewes displaying a strong MFD, while the frequency of T-RF compatible with *Clostridiales*, *Ruminococcaceae* and *Lachnospiraceae* or *Succiniclasticum* was greater in animals with a mild MFD.

Principal Component Analysis

The score plot generated from PCA of all data (i.e., ruminal fermentation parameters, BH metabolites and relative frequencies of T-RF) from the adaptation period (Figure 1a) showed no a priori segregation of ewes. However, after 36 days on treatments (Figure 1b), control animals were clustered together and distant from the two groups of supplemented sheep (RESPON− and RESPON+), without separation between them.

DISCUSSION

Results from our companion study (Frutos et al., 2017), which compared changes in milk FA profile and transcript abundance of candidate genes involved in mammary lipogenesis in RESPON− vs. RESPON+, did not allow to discriminate dairy ewes based on their responsiveness to marine lipid consumption. Neither do results from this new investigation point to a particular rumen parameter as responsible for individual variations in MFD severity.

Following the BH theory (Bauman and Griinari, 2001), our first hypothesis pointed to the formation of antilipogenic FA in the rumen. However, although FO supplementation increased the concentration of demonstrated or putative antilipogenic FA (such as *cis*-9 16:1, 10-oxo-18:0, *cis*-11 18:1, *trans*-10 *cis*-12 CLA or *trans*-10 *cis*-15 18:2), surprisingly, none of them differed significantly in relation to the extent of MFD (i.e., between RESPON− and RESPON+).

In general, shifts in the rumen FA profile were consistent with those observed in milk, not only for antilipogenic FA, but there were also some differences. For instance, the unexpected higher content of *trans*-10 *cis*-12 CLA in the milk from RESPON− (Frutos et al., 2017) was not found in the rumen, which may be related to a coelution with 20-carbon metabolites (Toral et al., 2017) and would support a marginal role of this CLA isomer in marine lipid-induced MFD (Loor et al., 2005; Toral et al., 2012; Kairenius et al., 2015).

Differences between milk and rumen FA profiles also appeared in long chain n-3 PUFA.

The 22:6n-3 did not vary between RESPON− and RESPON+ in the rumen but did it in milk, and just the opposite occurred for 22:4n-3, which tended to be greater in the rumen of more susceptible ewes. It is still uncertain whether these or other very long-chain n-3 PUFA may be in the origin of MFD. However, lipogenic gene expression was inhibited in in vitro incubations of bovine mammary epithelial cells with 20:5n-3 (Kadegowda et al., 2009), and reductions in milk fat concentration after post-ruminal infusions of fish oil might be attributable to the combined action of these n-3 PUFA and some potentially antilipogenic FA present in FO, such as *cis*-9 16:1 and *cis*-11 18:1 (Loor et al., 2005; Burns et al., 2012; Dallaire et al., 2014).

Another metabolite of ruminal origin that has putatively been related to FO-induced MFD is the 10-oxo-18:0, which increased with the supplemented diet but regardless of sheep responsiveness. On the other hand, differences between RESPON− and RESPON+ were found for some T-RF compatibles with *Quinella*-related bacteria, microorganisms reported to be favoured in sheep suffering from MFD and perhaps associated with the formation of keto-acids during rumen BH (Toral et al., 2012).

Concerning *trans*-10 18:1, with an ambiguous involvement in MFD (Kadegowda et al., 2009; Shingfield et al., 2010), its changes did not reach the required level of significance because of considerable between-animal variability, something that is consistently observed (Kim et al., 2008; Or-Rashid et al., 2008; Toral et al., 2012). In our study, numerical differences between RESPON+ and RESPON− were mainly due to a very high value in a sheep displaying a strong MFD (10.4% of total FA), which contrasts with values ranging from 1.2 to 1.4% in the other 4 ewes in RESPON+ and from 1.2 to 3.8% in animals in RESPON−. The fact that *trans*-10 18:1 was not related with the extent of MFD, together with its increases in dairy ewes fed plant oils and not suffering from the low-fat milk syndrome (Mele and Banni, 2010; Shingfield et al., 2010), would challenge the actual usefulness of this 18:1 isomer as an indicator of alterations in rumen BH eliciting MFD in sheep. Looking into comprehensive FA profiles may

help to find better biomarkers of altered BH, such as probably those deriving from shifts in 18:3n-3 pathways (e.g., *trans*-13+14 18:1 and *trans*-10 *cis*-15 18:2; Shingfield et al., 2010; Alves and Bessa, 2014). Toral et al. (2017) noticed that alterations in some of these metabolites were stronger in ovine than bovine, which may strengthen their interest as biomarkers in this small ruminant species. However, once again, most changes were independent from degrees of MFD. Only *cis*-11 *cis*-14 18:2 and *trans*-18:3 isomers (i.e., *trans*-9 *trans*-12 *cis*-15 + *cis*-9 *cis*-12 *trans*-15 18:3) diverged in RESPON− and RESPON+, but without significant variation when compared with the control, which precludes from relating them confidently with MFD severity. Yet, because ewes are very little prone to other MFD conditions (Mele and Banni, 2010; Shingfield et al., 2010), further research would be advisable to determine if the promotion of alternative 18:3n-3 pathways may be associated with marine lipid-induced MFD in dairy ewes.

Another metabolite showing the greatest rumen concentration in RESPON+ was *cis*-6+7 16:1. This agrees with observations for *cis*-7 16:1 in milk, where it did not coelute with *cis*-6 16:1 (Frutos et al., 2017). Together with *cis*-9 16:1, which acts as antilipogenic in ruminants (Burns et al., 2012), the *cis*-7 16:1 has recently attracted much attention given its anti-inflammatory activity and potential as a biomarker of proatherogenicity (Guijas et al., 2016). Some common effects by *cis*-9 16:1 and *cis*-7 16:1 might tempt to speculate on certain involvement of the latter in lipid metabolism, but we are not aware of reports about this FA and ruminants or mammary lipogenesis. Something similar would occur with *cis*-15 22:1, a FA that comes from the fish oil and was enhanced in ewes showing severe MFD.

Differences with the extent of MFD in 15:0 *iso* and 17:0 *anteiso* were first considered to reflect changes in the rumen microbiota, because of their predominant origin (i.e., bacteria leaving the rumen; Fievez et al., 2012; Buccioni et al., 2012). Thus, the increase in RESPON+ of *Prevotella*, a bacteria with a substantial content of 15:0 *iso* (Fievez et al., 2012), would be consistent with this finding. However, statistical significance was mainly due to the lower

values found in RESPON− compared to the control, which does not allow to relate them confidently to responsiveness to marine lipid consumption. The apparent inconsistence with concentrations of these FA in milk, where they did not vary for the contrast RESPON− vs. RESPON+ (Frutos et al., 2017), may be due to the post-ruminal modification of the odd- and branched-chain FA profile (Fievez et al., 2012).

The possible role of ruminal VFA in the development of MFD is another issue of interest: this topic seemed closed, but has recently received considerable attention. The review by Bauman and Griinari (2001) offers a reasoned argument that a deficiency in acetate could not adequately explain the reduction caused in milk fat when dairy cows are fed diets rich in concentrates or plant lipids. Under those feeding conditions, reductions in the acetate/propionate ratio are often due to increases in propionate rather than decreases in acetate production. On the opposite, marine lipids usually reduce acetate and total VFA concentrations, despite frequent increases in propionate, as observed in many in vivo and in vitro investigations with dairy cows, ewes and goats suffering from this type of MFD (Fievez et al., 2007; AbuGhazaleh and Ishlak, 2014; Toral et al., 2016a). Recent studies have recommended to reconsider the effect of acetate supply in milk fat synthesis and depression (Maxin et al., 2011; Urrutia and Harvatine, 2017) as it may stimulate lipogenesis further than attributable to its role as substrate for de novo FA synthesis (Jacobs et al., 2013; Urrutia and Harvatine, 2017). The differences in total VFA and acetate concentrations that we observed between ewes displaying a mild or a strong MFD were in line with the molar yields of de novo FA in milk (Frutos et al., 2017), which might suggest an association between them. In fact, the mild fat depression in RESPON− was explained by a lower yield of de novo FA, which would agree with the hypothesis relating the deficiency in acetate and the reduction in milk fat. Conversely, the strong MFD in RESPON+ was due to decreases not only in de novo synthesized FA but also in preformed FA deriving from plasma uptake, which would downplay the role of acetate. Thus,

the milk fat fall in RESPON+ (25.4%) was above the range of reported positive responses to ruminal infusion of this VFA (e.g., Maxin et al., 2011; Urrutia and Harvatine, 2017).

The butyrate, a VFA with uncertain relevance in milk fat synthesis (Bauman and Griinari, 2001; Maxin et al., 2011), was also reduced with FO. Unlike acetate and total VFA, reported changes in its concentration in response to marine lipids are very inconsistent in the literature (Fievez et al., 2007; Shingfield et al., 2012; Toral et al., 2016a). This variability might be associated with shifts in butyrate-producing microorganisms within or phylogenetically close to the *Butyrivibrio* group, which includes the most active biohydrogenating species isolated from the rumen (Paillard et al., 2007). In this study, however, the lack of significant differences between RESPON− and RESPON+ in butyrate does not match with variations in T-RF compatible with the family *Lachnospiraceae*, where those populations belong.

With respect to the concentration of ammonia, this parameter is scarcely affected by the use of marine lipids (e.g., Kim et al., 2008; Shingfield et al., 2012; Toral et al., 2016a) and we found no differences related to the severity of MFD, which was the core issue of this study.

Regarding the rumen bacterial community, the strong host-microbiota specificity in ruminants (Weimer, 2015) might contribute to explain the large individual variation in the degree of FO-induced MFD in sheep. In cows suffering from MFD elicited by rapidly fermented starch and monensin, animals were grouped according to bacterial structures (Weimer et al., 2010), something that did not happen in our FO-supplemented ewes. Yet, differences in specific bacterial populations tentatively involved in rumen BH might help to identify some species linked to this syndrome. For example, T-RF compatible with *Mogibacterium,* species that was enriched in cows with MFD (Pitta et al., 2018), increased or appeared only in ewes displaying a strong MFD, and the same occurred with some *Prevotella* and *Quinella*-related spp., which may also participate in rumen BH (Huws et al., 2011; Toral et al., 2012; Cremonesi et al., 2018). On the contrary, some T-RF compatible with *Clostridiales*,

Ruminococcaceae, *Lachnospiraceae* and *Succiniclasticum,* and associated with rumen lipid metabolism (Huws et al., 2011; Patra and Yu, 2015; Cremonesi et al., 2018), showed greater abundances in less susceptible sheep. Therefore, it might be speculated that the latter bacteria could be favored at the expense of the former and somehow helped to minimize the extent of the low-fat milk syndrome in RESPON− ewes. In any event, it must be considered that samples collected through stomach tube contain mostly liquid, which obliges to be cautious when using this technique to assess the structure and diversity of the rumen microbial community (Ramos-Morales et al., 2014). Although the technique has been used in lipid metabolism studies (e.g., Or-Rashid et al., 2008; Toral et al., 2016a; Cremonesi et al., 2018), perhaps further differences between RESPON- and RESPON+ could be detected if the whole rumen content (liquid plus solid fractions) was analysed.

Finally, although it cannot be rule out that the individual variation in MFD severity could be due to the existence of underlying differences in the ruminal environment, the results from the PCA (see Figure 1) suggest that there was no evident previous condition predisposing the response of ewes to fish oil consumption. At the end of the experimental period, this analysis was able to discriminate clearly between control and low milk fat animals but not between dairy ewes displaying mild or strong MFD, which is in line with the small number of parameters differing significantly in RESPON− and RESPON+.

Overall, the results support that FO-induced MFD is most probably a multi-etiological syndrome with a number of causal factors (i.e., antilipogenic FA coming from the fish oil, rumen metabolites formed in the BH process, promotion or reduction of specific rumen bacteria, deficiency of acetate, etc.). Post-ruminal factors, particularly those acting at the mammary gland (e.g., milk FA composition or mammary transcriptome; Shingfield et al., 2010; Suárez-Vega et al., 2017), must be added to the list. All together, they could explain the gradual nature of the response, as different sensitivity of each animal to each particular factor would

come into play.

CONCLUSIONS

Individual variation in the extent of milk fat depression in ewes fed fish oil cannot be fully explained by the processes of rumen BH and fermentation or the bacterial community. Surprisingly, none of the demonstrated or putative antilipogenic FA varied significantly between ewes displaying different degrees of MFD (i.e., between RESPON− and RESPON+), and only did it few minor FA (e.g., *cis*-6+7 16:1 or 17:0 *anteiso*). Changes in total VFA, acetate and propionate concentrations were associated with MFD severity, with higher decreases in more susceptible animals. Individual variability in responsiveness was not related to rumen bacterial structure but some T-RF compatible with *Clostridiales*, *Ruminococcaceae*, *Lachnospiraceae* and *Succiniclasticum* showed greater abundances in RESPON− whereas some others that may correspond to *Prevotella, Mogibacterium* and *Quinella*-related spp. were more abundant in RESPON+. Overall, the results support that fish oil-induced MFD would be a multi-etiological condition with a number of causal factors, and further research is still necessary to explain individual variation in the extent of this syndrome.

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Table 1. Rumen fatty acid (FA) composition in dairy ewes fed a diet without (control) or with 2% fish oil and displaying mild (RESPON–) or strong (RESPON+) milk fat depression (data on additional FA are reported in Supplementary Table S1)

	Treatment				Contrast[2]	
	Control	RESPON–	RESPON+	SED[1]	Control vs. (RESPON– and RESPON+)	RESPON– vs. RESPON+
FA, g/100 g of total FA						
14:0	1.31	1.82	1.73	0.135	0.002	0.504
15:0	0.87	0.95	0.81	0.099	0.800	0.192
15:0 *anteiso*	0.91	0.60	0.60	0.092	0.002	0.942
15:0 *iso*	0.41	0.37	0.44	0.032	0.890	0.068
trans-5+6+7 15:1	0.39	0.30	0.34	0.059	0.207	0.486
16:0	17.28	19.35	19.12	0.537	0.001	0.646
16:0 *iso*	0.38	0.27	0.28	0.024	<0.001	0.494
cis-6+7 16:1	0.12	0.37	0.49	0.040	<0.001	0.012
cis-9 16:1	0.10	0.65	0.77	0.137	<0.001	0.384
trans-9 16:1	0.01	0.16	0.18	0.016	<0.001	0.272
17:0	0.66	0.78	0.75	0.036	0.005	0.372
17:0 *anteiso*	0.45	0.40	0.47	0.020	0.325	0.003
17:0 *iso*	0.36	0.57	0.61	0.045	<0.001	0.426
18:0	46.72	7.56	6.38	1.395	<0.001	0.403
9-oxo-18:0[3]	0.03	0.09	0.09	0.030	0.060	0.962
10-oxo-18:0	<0.01	2.04	2.06	0.358	<0.001	0.953
15-oxo-18:0	-	0.02	0.03	0.006	-	0.217
cis-9 18:1	4.36	6.01	6.90	0.528	<0.001	0.120
cis-11 18:1	0.55	1.72	1.73	0.127	<0.001	0.939
cis-12 18:1	0.42	0.33	0.28	0.045	0.014	0.264
cis-13 18:1	0.07	0.16	0.15	0.014	<0.001	0.469
cis-16 18:1	0.142	0.094	0.102	0.0066	<0.001	0.246
∑ *cis* 18:1	5.54	8.36	9.12	0.525	<0.001	0.171
trans-6+7+8 18:1	0.34	0.73	0.56	0.127	0.016	0.194
trans-9 18:1	0.24	0.91	0.91	0.092	<0.001	0.929

trans-10 18:1	0.49	1.87	3.08	1.539	0.163	0.447
trans-11 18:1	3.11	20.81	20.85	1.548	<0.001	0.981
trans-12 18:1	0.70	2.46	2.30	0.185	<0.001	0.393
trans-13+14 18:1	0.95	1.58	1.49	0.146	<0.001	0.564
trans-16 + *cis*-14 18:1	0.79	0.47	0.47	0.058	<0.001	0.993
∑ *trans* 18:1	7.06	29.66	30.52	1.221	<0.001	0.499
cis-9 *cis*-12 18:2	6.97	3.32	3.55	0.503	<0.001	0.659
cis-11 *cis*-14 18:2	0.050	0.041	0.049	0.0040	0.144	0.063
cis-12 *cis*-15 + *cis*-12 *cis*-16 18:2	-	0.131	0.135	0.0227	-	0.872
cis-9 *trans*-12 18:2	0.02	0.04	0.04	0.005	0.002	0.398
trans-9 *cis*-12 18:2	0.04	0.11	0.11	0.017	<0.001	0.803
trans-11 *cis*-15 + *trans*-10 *cis*-15 18:2	0.18	0.80	0.89	0.079	<0.001	0.209
trans-12 *cis*-15 + *cis*-11 *cis*-16 18:2	0.059	0.046	0.052	0.0064	0.092	0.352
cis-9 *trans*-11 CLA[4]	0.12	0.18	0.26	0.045	0.012	0.077
trans-10 *cis*-12 CLA[5]	0.01	0.06	0.04	0.013	0.004	0.180
cis-11 *trans*-13 CLA	0.041	0.029	0.021	0.0084	0.030	0.368
trans-11 *trans*-13 CLA	0.025	0.003	0.002	0.0042	<0.001	0.790
18:3n-3	1.48	0.75	0.79	0.126	<0.001	0.763
trans-9 *trans*-12 *cis*-15 18:3[6]	0.039	0.031	0.038	0.0031	0.107	0.033
20:0	0.70	0.54	0.53	0.033	<0.001	0.620
cis-5 + *trans*-9+10 20:1	0.004	0.070	0.055	0.0139	<0.001	0.276
cis-11 + *trans*-15+16 20:1	0.18	0.99	0.97	0.083	<0.001	0.776
20:4n-3	<0.01	0.15	0.12	0.024	<0.001	0.386
20:5n-3	-	0.33	0.33	0.060	-	0.971
∑ unsaturated C20	0.37	3.88	3.57	0.279	<0.001	0.292
22:0[7]	0.74	0.60	0.67	0.044	0.016	0.146
cis-13 22:1	0.05	0.20	0.27	0.061	0.004	0.326
cis-15 22:1	0.025	0.076	0.123	0.0127	<0.001	0.003
22:4n-3	0.032	0.039	0.127	0.0482	0.247	0.087
22:5n-6	-	0.86	0.66	0.209	-	0.367
22:5n-3	-	1.97	1.61	0.962	-	0.623
22:6n-3	-	0.83	1.27	0.369	-	0.269

∑ unsaturated C22	0.18	6.36	6.11	0.995	<0.001	0.809
Total FA, g/100 g of digesta DM	3.34	4.39	4.69	0.227	0.001	0.361

[1]SED = standard error of the difference.

[2]Probability of the orthogonal contrast.

[3]Coelutes with 10-hydroxy-18:0.

[4]Contains *trans*-7 *cis*-9 and *trans*-8 *cis*-10 CLA as minor components.

[5]Coelutes with a 20:2 isomer of indeterminate double bond position.

[6]Coelutes with *cis*-9 *cis*-12 *trans*-15 18:3.

[7]Coelutes with an unidentified dimethylacetal.

Table 2. Ruminal fermentation parameters in dairy ewes fed a diet without (control) or with 2% fish oil and displaying mild (RESPON−) or strong (RESPON+) milk fat depression

	Treatment				Contrast[2]	
	Control	RESPON−	RESPON+	SED[1]	Control vs. (RESPON− and RESPON+)	RESPON− vs. RESPON+
Ammonia, mg/L	134.3	165.4	181.7	10.18	0.001	0.137
VFA, mmol/L						
Total	117.2	107.1	92.3	7.12	0.014	0.051
Acetate	74.1	67.5	57.6	4.82	0.017	0.050
Propionate	22.7	21.4	17.9	1.50	0.041	0.039
Butyrate	16.1	14.3	13.0	1.17	0.028	0.270
Others[3]	4.33	4.00	3.65	0.445	0.217	0.450
Acetate:propionate ratio	3.35	3.15	3.19	0.097	0.054	0.726

[1]SED = standard error of the difference.

[2]Probability of the orthogonal contrast.

[3]Calculated as the sum of isobutyrate, isovalerate, valerate, and caproate.

Table 3. Diversity indices (richness, R; Shannon-Wiener, H; Shannon evenness, E) of rumen bacterial communities in dairy ewes fed a diet without (control) or with 2% fish oil and displaying mild (RESPON−) or strong (RESPON+) milk fat depression.

Index	Treatment			SED[1]	Contrast[2]	
	Control	RESPON−	RESPON+		Control vs. (RESPON− and RESPON+)	RESPON− vs. RESPON+
*Hha*I						
R	40.9	38.8	34.1	3.23	0.137	0.177
H	2.74	2.70	2.45	0.225	0.314	0.283
E	0.74	0.74	0.69	0.047	0.518	0.352
*Msp*I						
R	26.8	24.5	22.6	2.86	0.231	0.478
H	3.02	2.74	2.77	0.147	0.071	0.825
E	0.92	0.86	0.89	0.023	0.060	0.112
*Hae*III						
R	80.1	75.8	80.9	4.18	0.633	0.223
H	3.79	3.65	3.66	0.048	0.009	0.686
E	0.86	0.84	0.84	0.009	0.017	0.565

[1]SED = standard error of the difference.

[2]Probability of the orthogonal contrast.

Table 4. Relative frequencies (expressed as Blom rank-transformed values of % over the total peak area, with original values in parentheses) of some terminal restriction fragments (T-RF) detected in rumen digesta from dairy ewes fed a diet without (control) or with 2% fish oil and displaying mild (RESPON−) or strong (RESPON+) milk fat depression. Only T-RF differing in RESPON− vs. RESPON+ are shown (data on other relevant T-RF are reported in Supplementary Table S2).

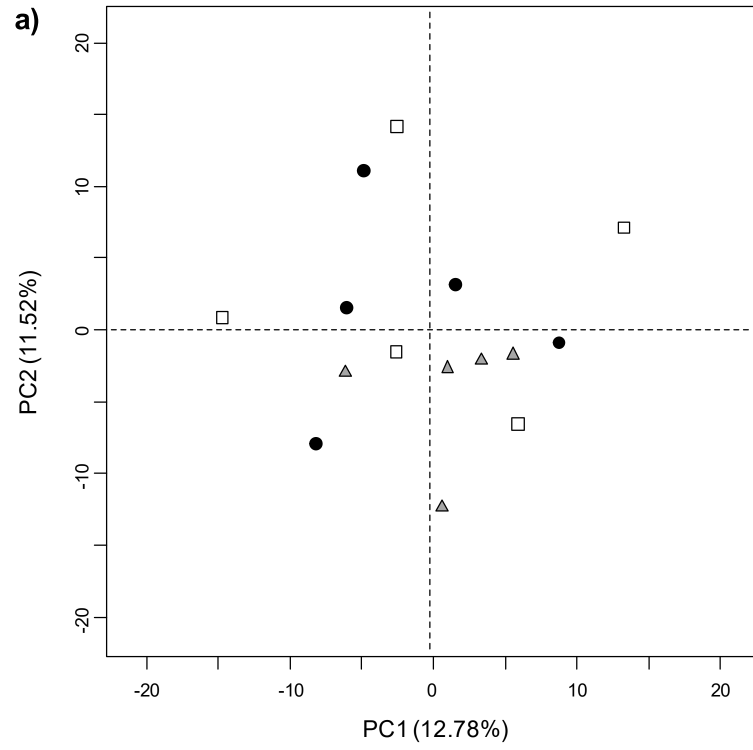
Putative taxonomic identification	T-RF (bp)	Treatment			SED[1]	Contrast[2]	
		Control	RESPON−	RESPON+		Control vs. (RESPON− and RESPON+)	RESPON− vs. RESPON+
Prevotella	102 (*Hha*I)	-0.033 (0.89)	-0.607 (0.41)	0.640 (1.28)	0.6251	0.930	0.042
	149 (*Msp*I)	0.133 (5.44)	-0.757 (2.90)	0.624 (6.49)	0.3856	0.539	0.004
Quinella-related	97 (*Hha*I)	-1.074 (4.20)	0.033 (6.20)	1.041 (9.94)	0.4816	0.002	0.049
	149+268 (*Msp*I)	0.105 (5.44)	-0.863 (2.90)	0.758 (8.07)	0.5265	0.706	0.011
	248 (*Hae*III)	-0.028 (0.36)	-0.734 (0.32)	0.762 (0.76)	0.4251	0.910	0.003
Mogibacterium	543 (*Msp*I)	-	-	(2.08)	-	-	-
	212 (*Hae*III)	-0.329 (1.21)	-0.372 (1.00)	0.701 (1.87)	0.4883	0.237	0.050
Clostridiales / Ruminococcaceae	521 (*Msp*I)	-	0.517 (1.90)	-0.517 (0.40)	0.4497	-	0.051
	256 (*Hae*III)	-0.591 (0.77)	0.931 (4.23)	-0.340 (1.14)	0.6346	0.075	0.070
Ruminococcaceae	300 (*HaeIII*)	0.913 (1.21)	0.074 (0.70)	-0.987 (0.54)	0.4734	0.008	0.009
Lachnospiraceae / Succiniclasticum	190 (*Hha*I)	0.609 (1.63)	0.290 (1.33)	-0.898 (0.46)	0.3784	0.017	0.009
	272 (*Hae*III)	-0.288 (1.23)	0.713 (2.22)	-0.425 (1.18)	0.5865	0.392	0.078

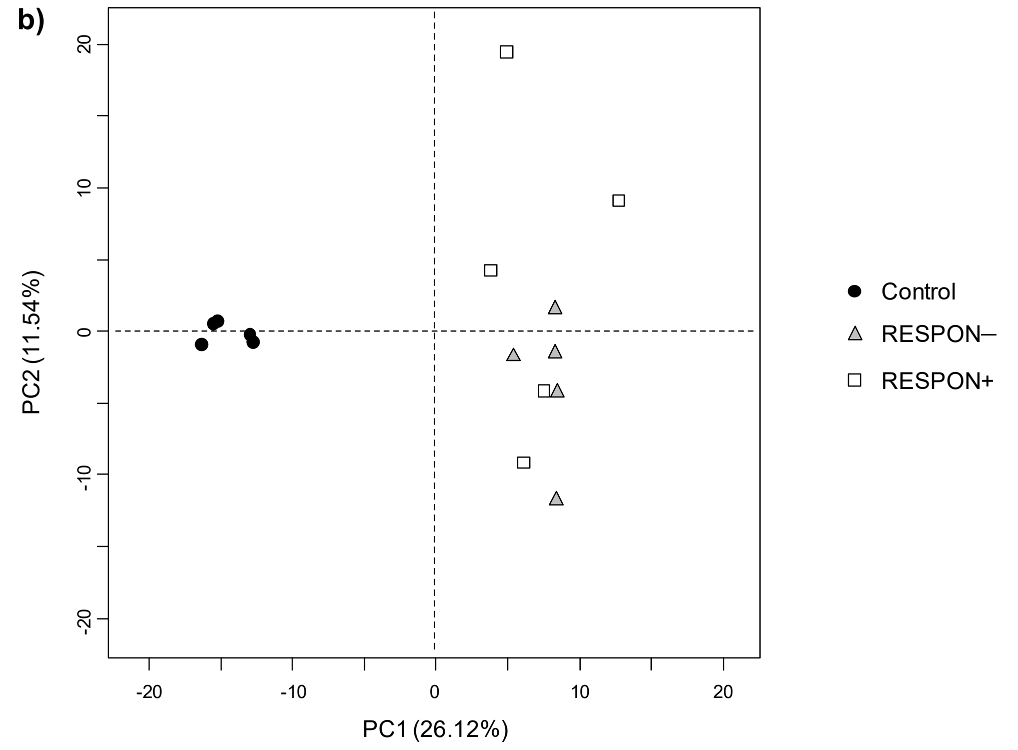
[1]SED = standard error of the difference.

[2]Probability of the orthogonal contrast.

Figure 1. Principal component analysis of ruminal fermentation parameters, biohydrogenation metabolites and relative frequencies of terminal restriction fragments in dairy ewes at the end of the adaptation period (a) and after 36 days (b) on a diet without (control; circles) or with 2% fish oil and displaying mild (RESPON−; triangles) or strong (RESPON+; squares) milk fat depression.

(FIGURE 1)





Supplementary Table S1. Other fatty acids (FA) in rumen digesta from lactating ewes fed a diet without (control) or with 2% fish oil and displaying mild (RESPON–) or strong (RESPON+) milk fat depression.

FA, g/100 g of total FA	Treatment			SED[1]	Contrast[2]	
	Control	RESPON–	RESPON+		Control vs. (RESPON– and RESPON+)	RESPON– vs. RESPON+
12:0	0.13	0.10	0.10	0.012	0.009	0.646
13:0	0.038	0.056	0.053	0.0171	0.279	0.863
13:0 *anteiso*	0.026	0.024	0.029	0.0054	0.885	0.349
13:0 *iso*	0.059	0.052	0.061	0.0076	0.724	0.245
14:0 *iso*	0.13	0.10	0.09	0.012	0.001	0.699
cis-11 16:1	0.041	0.049	0.059	0.0139	0.261	0.445
cis-13 16:1	0.007	0.031	0.035	0.0062	<0.001	0.586
trans-6+7+8 16:1	0.05	0.31	0.33	0.048	<0.001	0.605
trans-12+13 16:1	0.085	0.140	0.149	0.0105	<0.001	0.390
13-oxo 18:0	0.14	0.20	0.20	0.042	0.145	0.969
16-oxo-18:0[3]	0.21	0.26	0.24	0.018	0.017	0.228
18:0 *iso*	0.072	0.079	0.081	0.0136	0.490	0.833
cis-15 18:1[4]	0.17	0.21	0.25	0.031	0.036	0.178
trans-4 18:1	0.108	0.096	0.079	0.0120	0.076	0.178
trans-5 18:1	0.099	0.136	0.139	0.0310	0.105	0.917
trans-15 18:1	0.92	1.11	1.19	0.094	0.017	0.447
cis-9 *trans*-13 18:2[5]	0.083	0.120	0.131	0.0170	0.007	0.513
trans-9 *cis*-12 18:2	0.036	0.110	0.106	0.0167	<0.001	0.803
trans-9 *trans*-12 18:2	0.026	0.075	0.108	0.0356	0.061	0.314
∑ other *trans trans* CLA[6]	0.060	0.079	0.084	0.0079	0.011	0.526
19:0[7]	0.11	0.41	0.38	0.031	<0.001	0.407
cis-7 + *trans*-12+13 20:1	0.015	0.080	0.050	0.0221	0.025	0.178
cis-9 + *trans*-14 20:1	0.016	0.134	0.114	0.0355	0.005	0.598
cis-12 + *trans*-17 20:1	0.055	0.092	0.097	0.0121	0.002	0.698
cis-13 20:1	0.008	0.082	0.077	0.0106	<0.001	0.598
trans-11 20:1	0.005	0.071	0.061	0.0158	<0.001	0.510

20:2n-6	0.015	0.174	0.149	0.0243	<0.001	0.333
20:4n-6	0.001	0.067	0.178	0.0640	0.016	0.113
21:0	0.062	0.158	0.132	0.0148	<0.001	0.114
cis-12 21:1	0.002	0.099	0.126	0.0260	<0.001	0.316
cis-11 22:1[8]	-	0.48	0.46	0.051	-	0.669
22:2n-3	0.043	0.113	0.078	0.0280	0.056	0.199
22:2n-6	0.028	0.093	0.072	0.0146	0.001	0.146
22:4n-6	-	0.20	0.24	0.065	-	0.559
23:0	0.24	0.23	0.23	0.024	0.681	0.968
24:0[9]	1.34	1.20	1.35	0.100	0.486	0.171
cis-15 24:1	0.07	0.41	0.39	0.051	<0.001	0.752
29:0	0.037	0.055	0.065	0.0120	0.036	0.457
30:0	0.60	0.75	0.66	0.065	0.082	0.199

[1]SED = standard error of the difference.
[2]Probability of the orthogonal contrast.
[3]Coelutes with 28:0.
[4]Coelutes with *trans*-10 *trans*-14 18:2.
[5]Coelutes with 11-cyclohexyl-11:0 and *cis*-10 *trans*-14 + *trans*-10 *trans*-13 + *trans*-11 *trans*-14 18:2.
[6]Sum of *trans*-8 *trans*-10, *trans*-9 *trans*-11 and *trans*-10 *trans*-12 CLA.
[7]Coelutes with *trans*-11 *trans*-15 18:2.
[8]Coelutes with 20:3n-3.
[9]Coelutes with an unidentified dimethylacetal.

Supplementary Table S2. Relative frequencies (expressed as Blom rank-transformed values of % over the total peak area, with original values in parentheses) of relevant terminal restriction fragments (T-RF) detected in rumen digesta from dairy ewes fed a diet without (control) or with 2% fish oil and displaying mild (RESPON−) or strong (RESPON+) milk fat depression.

		Treatment				Contrast[2]	
Putative taxonomic identification	T-RF (bp)	Control	RESPON−	RESPON+	SED[1]	Control vs. (RESPON− and RESPON+)	RESPON− vs. RESPON+
Bacteroidetes	100 (*Hha*I)	0.029 (37.4)	-0.318 (33.3)	0.289 (42.4)	0.8220	0.948	0.437
	95 (*Msp*I)	0.269 (13.50)	-0.436 (8.54)	0.167 (12.15)	0.6214	0.467	0.352
	161 (*Hae*III)	0.848 (0.55)	-0.206 (0.34)	-0.642 (0.29)	0.4978	0.012	0.400
	182 (*Hae*III)	-0.663 (0.32)	0.527 (2.18)	0.135 (1.32)	0.6395	0.097	0.513
	263 (*Hae*III)	-0.279 (11.80)	0.093 (15.01)	0.186 (14.01)	0.6749	0.481	0.891
Succinivibrio	203 (*Hha*I)	-1.029 (0.63)	0.483 (4.85)	0.546 (4.12)	0.4957	0.005	0.893
Clostridiales	383 (*Hha*I)	0.590 (1.00)	-0.108 (0.69)	-0.483 (0.52)	0.4325	0.037	0.403
	545 (*Hha*I)	-	0.426 (1.46)	-0.426 (0.78)	0.5846	-	0.188
	309 (*Hae*III)	0.685 (0.57)	-0.266 (0.20)	-0.418 (0.18)	0.5749	0.059	0.783
	251 (*Hae*III)	-0.742 (0.95)	0.753 (3.00)	-0.012 (1.79)	0.5211	0.026	0.170
Lachnospiraceae	180 (*Hha*I)	0.310 (3.44)	-0.273 (3.30)	-0.037 (3.05)	0.6741	0.424	0.733
	212 (*Msp*I)	0.997 (4.98)	-0.616 (0.55)	-0.381 (1.15)	0.4402	0.002	0.563
	289 (*Msp*I)	0.671 (2.46)	-0.498 (0.54)	-0.173 (1.14)	0.5229	0.045	0.547
Prevotellaceae	450 (*Hha*I)	0.673 (0.54)	-0.015 (0.36)	-0.657 (0.10)	0.4973	0.039	0.216
Ruminococcaceae	94 (*Hha*I)	0.313 (2.69)	-0.496 (2.05)	-0.183 (2.43)	0.5757	0.354	0.262
	280 (*Msp*I)	0.008 (4.44)	-0.215 (3.89)	0.208 (5.42)	0.6059	0.983	0.500

[1]SED = standard error of the difference.
[2]Probability of the orthogonal contrast.

Supplementary Figure S1. Principal component analysis of the T-RFLP profiles of total bacteria in ruminal fluid samples collected from dairy ewes after 36 days on a diet without (control; circles) or with 2% fish oil and displaying mild (RESPON−; triangles) or strong (RESPON+; squares) milk fat depression.