Interpretive summary

Comparison of ruminal lipid metabolism in dairy cows and goats fed diets supplemented with starch, plant oil or fish oil

(by Toral et al.)

Alterations in rumen microbial biohydrogenation are probably at the core of diet-induced milk fat depression (MFD), but the underlying mechanisms might differ depending on diet or host animal species. In this study, direct comparison of rumen fatty acid composition and bacterial community in cows and goats demonstrates relevant differences in their response to dietary supplementation with either sunflower oil plus starch, which induces MFD in cows but not in goats, or 2% fish oil, which induces MFD in cows and to a lesser extent in goats. Results also suggest that distinct ruminal mechanisms lead to each type of MFD in cows, and may explain the negative effect of 2% fish oil on milk fat content in goats.

Comparison of ruminal lipid metabolism in dairy cows and goats fed diets supplemented with starch, plant oil or fish oil

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ABSTRACT

Direct comparison of cow and goat performance and milk fatty acid responses to diets known to induce milk fat depression (MFD) in the bovine points to relevant species by diet interactions in ruminal lipid metabolism. Thus, this study was conducted to infer potential mechanisms responsible for differences in the rumen microbial biohydrogenation (BH) due to diet and ruminant species. To meet this objective, 12 cows and 15 goats were fed a basal diet (Control), a similar diet supplemented with 2.2% fish oil (FO) or a diet containing 5.3% sunflower oil and additional starch (+38%; SOS) according to a 3 × 3 Latin square design

with 25 d experimental periods. On the last day of each period, fatty acid composition (by gas chromatography) and bacterial community (by terminal-RFLP), as well as fermentation characteristics, were measured in rumen fluid samples. Results showed significant differences in the response of cows and goats to dietary treatments, although variations in some fermentation parameters (e.g., decreases in the acetate:propionate ratio due to FO or SOS) were similar in both species. Main alterations in ruminal BH pathways potentially responsible for MFD on the SOS diet (i.e., the shift from *trans*-11 to *trans*-10 18:1 and related increases in *trans*-10,*cis*-12 18:2) tended to be more pronounced in cows, which is consistent with an associated MFD only in this species. However, changes linked to FO-induced MFD (e.g., decreases in 18:0 and increases in total *trans*-18:1) were stronger in caprine rumen fluid, which may explain their unexpected susceptibility (although less marked than in bovine) to the negative effect of FO on milk fat content. Altogether, these results suggest that distinct ruminal mechanisms lead to each type of diet-induced MFD and confirm a pronounced interaction with species. With regard to microbiota, differences between cows and goats in the composition of the rumen bacterial community might be behind the disparity in the microorganisms affected by the experimental diets (e.g., *Ruminococcaceae*, *Lachnospiraceae* and *Succinivibrionaceae* in the bovine, and *Pseudobutyrivibrio*, *Clostridium IV, Prevotella* and *Veillonellaceae* in the caprine), which hindered the assignation of bacterial populations to particular BH steps or pathways. Furthermore, most relevant variations in microbial groups corresponded to as yet uncultured bacteria and suggest that these microorganisms may play a predominant role in the ruminal lipid metabolism in both cows and goats.

Key words: biohydrogenation, fatty acid, rumen bacteria, milk fat depression

INTRODUCTION

Milk fat depression (**MFD**) represents a situation where milk fat concentration and yield can decrease up to 50%, often as a result of changes in diet composition (Bauman and Griinari, 2001; Shingfield et al., 2010a). Inducing MFD might have applications as a management tool in dairy cow farming (Bauman et al., 2011) but has a potential negative impact on the subsequent manufacture of cheese. Although goats were thought, for many years, to be less sensitive to diet-induced MFD (Shingfield et al., 2010a; Toral et al, 2014), more recent studies have demonstrated their susceptibility when fed marine lipids at a high dose (2.2% DM; Toral et al., 2015).

The effect of nutrition on milk FA composition is largely determined by rumen lipid metabolism, particularly by the biohydrogenation (**BH**) process (AbuGhazaleh et al., 2002; Loor et al., 2005; Toral et al., 2012). Alterations in rumen BH pathways may explain the basis for diet-induced MFD (Bauman and Griinari, 2001), but the underlying mechanisms remain uncertain and might differ depending on factors such as diet or host animal species.

Starting with the diet, despite differences in MFD induced by feeding either marine lipids or high-starch rations and plant oils (Chilliard et al., 2007; Shingfield and Griinari, 2007), there is not any direct comparative study testing the hypothesis that distinct ruminal mechanisms lead to each type of diet-induced MFD.

Regarding the host animal, inter-species variations in rumen digestion and microbiota (Moon et al., 2010; Lee et al., 2012) are well known in ruminant nutrition. On the other hand, knowledge of putative species-specific differences in BH pathways only arises from indirect comparisons (Loor et al., 2004; Boeckaert et al., 2008; Toral et al., 2012), often by extrapolating data from milk FA, due to the scarcity of available studies on this issue, particularly in the caprine (Chilliard et al., 2014; Li et al., 2014).

In line with that, a first direct comparison study was performed in cows and goats, both receiving diets known to induce MFD in bovine, to describe milk fat yield and FA composition (Toral et al., 2015). In that study, milk FA profiles evidenced a significant species by diet interaction in ruminal responses (Toral et al., 2015). Furthermore, the lack of MFD in goats fed a starch-rich diet supplemented with sunflower oil was attributed to a putatively greater stability in BH pathways compared with cows, while the decrease in milk fat content in both species when fed fish oil at 2.2% DM (–30 and –21% in cows and goats, respectively) was associated with a strong inhibition of ruminal 18:1 saturation (i.e., the same mechanism in cows and goats). Nevertheless, even though the reasons for this interaction are expected to be linked to species differences in the structure and composition of the rumen bacterial community (Moon et al., 2010; Lee et al., 2012), there is still a limited knowledge on the microbial ecology of FA metabolism, especially in goats (Huws et al., 2011; Toral et al., 2012; Zhu et al., 2012). Additionally, application of culture-independent molecular techniques, including next generation sequencing methodologies, has revealed that the effect of lipids on bacterial populations remains largely unknown (Castro-Carrera et al., 2014; Huws et al., 2014).

On this basis, a comparative study with lactating cows and goats was undertaken to test the following hypotheses: 1) ruminal mechanisms underlying MFD induced by marine lipid supplements or by diets containing high amounts of starch and plant oils are different, and 2) rumen microbial BH responses vary between animal species. To meet this objective, cows and goats were fed a basal diet (Control), a similar diet supplemented with fish oil (**FO**) or a diet containing sunflower oil and additional starch (**SOS**). Then, changes in rumen FA composition, bacterial community, and fermentation characteristics were measured and used to infer potential mechanisms responsible for differences in the regulation of ruminal lipid metabolism due to diet and ruminant species.

MATERIALS AND METHODS

Animals, Experimental Design, Diets, and Management

All procedures involving animals were approved by the Animal Care Committee of INRA in accordance with the guidelines established by the European Union Directive 2010/63/EU. Details of the experimental design have been described in Toral et al. (2015). Briefly, twelve multiparous non-pregnant Holstein cows and 15 multiparous non-pregnant Alpine goats were allocated to one of 3 groups (4 cows and 5 goats each) and used in a replicated 3 × 3 Latin square to test the effects of 3 treatments during three 25-d experimental periods. Unfortunately one goat had to be withdrawn from the experiment because it suffered diarrhea. All animals were offered grass hay ad libitum supplemented with concentrates containing no additional lipid (Control), fish oil (FO) or sunflower oil and wheat starch (SOS). The control concentrate was based on (% DM) cracked corn grain (54.9), pelleted dehydrated alfalfa (29.4), soybean meal (14.3) and a mineral-vitamin premix (1.4). In the FO and SOS concentrates, both fish oil (3.6% DM) and sunflower oil (9.0% DM) replaced alfalfa pellets on a proportionate basis and were mixed manually with other ingredients immediately before feeding out. The fish oil represented 2.2% and the sunflower oil 5.3% of total DMI. The remaining alfalfa pellets and part of the corn grain were replaced by flattened wheat grain (37.4% DM) in SOS concentrate. Hay refusals were weighed daily and used to adjust the amounts of concentrate offered the following day to maintain the targeted dietary forage to concentrate ratio (40:60 on a DM basis). Diets were offered as 2 equal meals at 0830 and 1600 h. Formulation, chemical composition and FA profile of the concentrates and hay have been reported previously (Toral et al., 2015). Experimental diets were formulated to be isoproteic (139 g CP/kg DM) and the mean starch concentrations (values for the control, FO, and SOS diets, respectively) were 232, 239, and 325 g/kg DM, and those of NDF 365, 349,

and 296 g/kg DM. Fish oil and sunflower oil supplied 400 and 953 g FA/d in cows and 48 and 114 g FA/d in goats, respectively. Animals had access to a constant supply of fresh water and were milked at 0800 and 1530 h.

Rumen Sample Collection

On d 25 of each experimental period, rumen fluid was collected by stomach tube from each animal after an overnight period without concentrate distribution (but with ad libitum access to hay) and before morning feeding. This technique has been validated as a feasible alternative to surgical rumen cannulation for obtaining representative rumen samples to examine diet and ruminant species effects (Ramos-Morales et al., 2014). Immediately after collection, the fluid was carefully checked to ensure that there was not saliva and strained through a nylon membrane (500 μm; Dominique Dutscher, Brumath, France). Then, the pH was measured, and a 4 mL subsample was acidified with 4 mL of 0.2 *M* HCl for ammonia determinations. Further 0.8 mL aliquots of strained ruminal fluid were taken for VFA analysis (deproteinized with 0.5 mL of 20 g/L metaphosphoric and 4 g/L crotonic acids in 0.5 *M* HCl). All these samples were stored at –30ºC until analysis. Additional subsamples (ca. 80 mL) of rumen fluid were also collected, immediately frozen at –80ºC, freeze-dried, and stored at –80ºC until analyzed for FA composition and bacterial community.

Chemical Analysis

Ruminal Fermentation Characteristics. Ammonia concentration was determined by colorimetric methods and VFA by gas chromatography, using crotonic acid as an internal standard (Frutos et al., 2004).

FA Composition. Lipid in 200 mg of freeze-dried rumen fluid was extracted using a mixture of hexane and isopropanol (3:2, vol/vol) and converted to FAME by sequential base-

acid catalyzed transesterification (Toral et al., 2010), using *cis*-12 13:1 as an internal standard (Larodan Fine Chemicals AB, Malmö, Sweden). The FAME recovered were quantified using a gas chromatograph (Agilent 7890A GC System, Santa Clara, CA) equipped with a flame-ionization 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 1:50 was determined using a temperature gradient programme (Shingfield et al., 2003). Isomers of 18:1 were further resolved in a separate analysis under isothermal conditions at 170ºC (Shingfield et al., 2003). Peaks were identified based on retention time comparisons with commercial authentic standards (Nu-Chek Prep., Elysian, MN; Sigma-Aldrich, Madrid, Spain; and Larodan Fine Chemicals AB), cross referencing with chromatograms reported in the literature (Shingfield et al., 2003; Toral et al., 2010) and comparison with reference samples for which the FA composition was determined based on gas chromatography analysis of FAME and GC-MS analysis of corresponding 4,4-dimethyloxazoline derivatives (Toral et al., 2010).

Bacterial Community. Freeze-dried samples of rumen fluid were thoroughly homogenized and, within each period and species, mixed per dietary group before DNA extraction (Belenguer et al*.,* 2010), which led to 6 samples per experimental diet and 9 samples per species. The DNA was extracted twice from each sample, and duplicates were combined and used as templates for terminal restriction fragment length polymorphism (**T-RFLP**) analysis of bacterial 16S rRNA genes. Concentrations of DNA were determined by spectrophotometry (NanoDrop ND-1000 Spectrophotometer, Nanodrop Technologies, Wilmington, DE). The T-RFLP analysis was performed using a universal bacteria-specific primer pair set and 3 restriction enzymes (*HhaI*, *MspI* and *HaeIII*; Belenguer et al., 2010). The lengths of the fluorescently labeled terminal restriction fragments (**T-RF**) were determined

with the size standard ET-900-R (GE Healthcare Life Sciences, Buckinghamshire, UK) using the GeneMarker Analysis software (SoftGenetics, State College, PA).

Data from T-RFLP (size, bp, and peak area for each T-RF) were analyzed for peak filtering and binning as outlined by Abdo et al. (2006), and used to determine the relative abundance of each fragment over the total peak area, as well as the diversity indices (number of T-RF or richness, and Shannon-Wiener and Shannon evenness indices; Hill et al., 2003). In silico restriction for the major rumen bacteria with the primers and enzymes used in the analysis, obtained from the Ribosomal Database Project II Web site (http://rdp.cme.msu.edu/index.jsp; Cole et al., 2014), was used to infer the potential bacterial composition of rumen fluid.

Statistical Analysis

Fermentation and FA data were subjected to ANOVA for a 3 × 3 Latin square design (Kaps and Lamberson, 2009) using the MIXED procedure of the SAS (version 9.4, SAS Institute Inc., Cary, NC). The statistical model included the fixed effects of period, species (**Sp**), experimental diet (**D**), the interaction Sp × D, the order in which treatments were allocated to each animal, and the random effect of animal nested within treatment order. For FA found exclusively in the rumen fluid of cows and goats fed the FO treatment, the fixed effects due to diet and diet by species interaction were removed. For relative abundances of each T-RF, the group (4 cows or 5 goats), instead of the animal, was considered the experimental unit (18 data per T-RF: 2 species × 3 diets × 3 periods). Because some of these T-RFLP results did not satisfy the assumptions of normality, data were transformed to $\log_{10}$ (n + c) before analysis. The constant "c" was used to avoid mathematically undefined computations in T-RF with zero values and was of the same order of magnitude as the variable (i.e, average value of relative abundances for each T-RF). The CORR procedure was

used to generate Pearson correlation coefficients (r) between rumen and milk FA percentages and milk fat content, in the same animals, derived from Toral et al. (2015). Differences were declared significant at $P < 0.05$ and considered a trend towards significance at $P < 0.10$. Means were separated through the "pdiff" option of the "lsmeans" statement of the MIXED procedure, and least square means are reported.

Hierarchical clustering analysis with Ward´s method based on Bray-Curtis distances was performed with R-project software (www.r-project.org, version 2.13.1) to build a dendrogram with relative abundance data derived from T-RFLP (T-RF). Distance-based permutational multivariate analysis of variance (Anderson, 2001) of the same data was conducted, using the PERMANOVA software (version 1.6), to assess the effect of diet, species, and their interaction on the whole rumen bacterial structure.

RESULTS

Rumen Fermentation Characteristics

The two ruminant species showed significant differences in rumen fermentation characteristics (Table 1). Thus, while the ammonia concentration was approximately twice higher in goats than in cows ($P < 0.001$), that of total VFA was 22% lower in caprine rumen fluid ($P < 0.01$). The molar proportion of acetate was slightly greater in the bovine ($P < 0.001$), whereas the converse was observed for the other reported VFA ($P < 0.001$ for propionate and minor VFA, and $P < 0.10$ for butyrate).

[Please, insert Table 1 near here]

In both species, ammonia concentration was decreased with SOS ($P < 0.001$), which also lowered that of total VFA when compared with the control ($P < 0.05$). Slight reductions in the molar proportion of acetate were also observed in FO and SOS in comparison with the control ($P < 0.001$), while propionate increased, particularly in goats offered the SOS diet

(interaction Sp × D; $P < 0.05$). Acetate:propionate ratio was reduced to the same extent in cows and goats on SOS and, less markedly, on FO diet ($P < 0.001$). Decreases in butyrate concentrations with SOS feeding also tended to be more pronounced in caprine rumen fluid (interaction Sp × D; $P < 0.10$), but no difference between species was found for the concomitant increase in minor VFA ($P < 0.001$). The rumen pH, measured before morning feeding, was marginally higher in SOS (mean value of 7.12 for control and FO vs. 7.28 for SOS; $P < 0.01$; data not shown in tables).

Fatty Acid Concentrations in the Rumen Fluid

The concentration of total FA was higher in the rumen fluid of goats ($P < 0.001$; Table 2) and increments due to SOS were significantly greater in this species than in the bovine (156 vs. 63%, respectively; $P < 0.001$ for the interaction Sp × D); these differences mirrored and were mainly due to those of saturated FA. In general, diet-induced variations in the major FA groups in the rumen were also more pronounced in goats than in cows (interaction Sp × D; $P < 0.01$), with the exception of the increase in total PUFA after FO supply ($P < 0.001$), which was similar in both species (interaction Sp × D; $P > 0.10$).

[Please, insert Table 2 near here]

Supplemented diets resulted in substantial variations in the concentration of individual FA, with numerous Sp × D interactions. The saturated 18:0 and 16:0 were the most abundant FA in the rumen fluid of both cows and goats fed the control diet; they were greater in goats when expressed in g/kg DM ($P < 0.001$), but 18:0 was similar in % total FA ($P > 0.10$; Supplemental Table 1). These FA were differently altered by FO and SOS diets ($P < 0.001$; Table 2). The amount of 16:0 increased with FO in both species, and to a minor extent with SOS in goats when expressed in g/kg DM (interaction Sp × D; $P < 0.05$) although it decreased with SOS and more markedly in goats when expressed in % total FA (Supplemental Table 1).

Concerning the 18:0, FO lowered its concentration in the rumen, but the proportionate decrease was greater in goats compared with cows (−85 vs. −63%, respectively). On the other hand, SOS increased by 2- and 3-fold the concentration of this FA in bovine and caprine rumen fluids, respectively (interaction Sp × D; $P < 0.001$). The sum of odd- and branched-chain fatty acids (**OBCFA**) was augmented by FO in both species (more markedly in goats; $P < 0.001$ for the interaction Sp × D) while SOS negatively affected their content. Although this general pattern was consistent for most individual OBCFA in goats, numerical differences did not always attain statistical significance in cows (Table 2). In addition, inter-species dissimilarities in OBCFA were observed in animals fed the control diet, both when concentrations were expressed as g/kg DM (e.g., *iso* 13:0, *iso* 14:0, 15:0, and *anteiso* 15:0; Table 2) and as % total FA (e.g., 15:0, *anteiso* 15:0, 17:0, and *iso* 18:0; Supplemental Table 1). Regarding oxygenated FA, the highest concentrations of 10-O-16:0 and 10-O-18:0 in the rumen fluid were detected in FO ($P < 0.001$), with a tendency towards greater increases of 10-O-18:0 in goats (interaction Sp × D; $P < 0.10$). Although SOS also favored the accumulation of this keto acid ($P < 0.001$), no differences due to animal species were observed. Lipid supplemented diets similarly increased 13-O-18:0 in the rumen of cows and goats ($P < 0.001$).

The abundance of identified MUFA was significantly affected by the experimental diets, with the exception of *trans*-9 14:1. Addition of FO led to the highest ($P < 0.05$) content of 16:1, 20:1, 23:1, and 24:1 intermediates in the rumen, while increments in the sum of *trans* 18:1 isomers were comparable for both FO and SOS, and 22:1 was detected only in FO (Tables 2 and 3). For all these MUFA, the response of goats was more pronounced than that of cows (interaction Sp × D; $P < 0.05$). As shown in Table 3, *trans*-11 18:1 was the most abundant MUFA on the control diet and its concentration augmented by 5.4 and 7.8 times, respectively, in cows and goats fed FO ($P < 0.001$). On the other hand, with SOS feeding, *trans*-10 18:1 expressed as % of total FA or as g/kg DM became the major *trans* FA in cows

($P < 0.001$) and reached similar concentrations to *trans*-11 18:1 in goats (Table 3 and Supplemental Table 1), whereas a high inter-individual variation precluded significant increases caused by FO, except for a significant increase in cows when expressed as % total FA. The amount of other *trans* 18:1 isomers was often greater in the SOS treatment, particularly in goats ($P < 0.05$), although *trans*-9 and *trans*-12 18:1 were similarly affected by FO and SOS treatments. Fish oil enhanced ($P < 0.05$) the abundance of *cis*-9 18:1 and *cis*-11 18:1, with increases in the latter being highest in the caprine ($P < 0.001$ for the interaction Sp × D).

[Please, insert Table 3 near here]

The concentration of 18:2 isomers varied substantially across diets and species (Table 3). The content of 18:2n-6 was lowered in cows on SOS and in goats on FO treatment ($P < 0.05$) but, on the contrary, FO favored the rumen accumulation of other non-conjugated 18:2, such as *cis*-9,*trans*-12 18:2, *trans*-9,*cis*-12 18:2, *trans*-11,*cis*-15 18:2, *trans*-9,*trans*-12 18:2, and *trans*-11,*trans*-15 18:2 in both species ($P < 0.05$). The rumen fluid of cows on SOS treatment tended to have the highest amount of *trans*-10,*cis*-12 CLA ($P < 0.10$ for the interaction Sp × D), an isomer that was increased to a minor extent in response to FO. Furthermore, the concentration (expressed as g/kg DM) of *trans*-9,*cis*-11 CLA augmented in FO compared with the other diets ($P < 0.001$), while *cis*-9,*trans*-11 CLA was not significantly affected by the experimental diets ($P > 0.10$; Table 3). However, *cis*-9,*trans*-11 CLA expressed as % total FA (Supplemental Table 1) was increased in cows fed FO and decreased in goats fed SOS. The accumulation of *trans*,*trans* CLA with supplemented diets was significantly greater in goats (interaction Sp × D; $P < 0.01$). *Trans*-10,*cis*-12 CLA (% total FA) was increased in cows by both lipid supplements, but not in goats (Supplemental Table 1). With the control diet, there were no inter-species differences in 18:2n-6 and CLA isomers when expressed as % total FA, including *cis*-9,*trans*-11 CLA, while 18:3n-3 percentage was

lower in goats than in cows (Supplemental Table 1). For both species, the lowest amount of 18:3n-3 was detected in SOS, but decreases of smaller extent were also found when feeding FO ($P < 0.001$; Table 3). Finally, FO increased the concentration of all 20- and 22-carbon PUFA identified in the rumen fluid (Table 3), but most of their concentrations were close or below detection limits in the Control and SOS animals (e.g., 20:3n-3, 20:5n-3, 22:4n-6, 22:5n-6, 22:5n-3, and 22:6n-3). These FA were often more abundant in goats ($P < 0.10$), although differences generally disappeared when values were expressed as % total FA (Supplemental Table 1).

Bacterial Community Analysis by T-RFLP

Regardless of the experimental diet and the species, rumen bacterial T-RFLP analysis generated on average 102.7 ± 3.03, 54.9 ± 4.36 and 100.6 ± 3.68 fragments with the enzymes *HhaI*, *MspI* and *HaeIII*, respectively. Results of the hierarchical clustering and PERMANOVA analyses indicated an effect of animal species on the bacterial structure ($P < 0.01$), with most ruminal fluid samples of the same species being allocated in the same cluster (Figure 1).Within each species, certain segregation by diet was clearer detected in goats ($P < 0.10$), especially due to the SOS treatment (Figure 1).

[Please, insert Figure 1 near here]

The diversity indices (Table 4) did not differ between ruminant species, with the exception that the Shannon evenness estimated with *HaeIII* was greater in cows ($P < 0.05$). Concerning the effect of diet, both FO and SOS seemed to reduce bacterial diversity, given that indices were numerically lower in many cases, although this was only significant for the Shannon-Wiener index with *HhaI* and the Shannon evenness with *HaeIII*. Moreover, an interaction was detected for the latter index with *MspI* ($P < 0.05$), with lower values in goats fed SOS and in cows fed FO.

[Please, insert Table 4 near here]

Differences between ruminant species were also observed in the relative frequency of many T-RF but only a few that might be potentially related to the changes detected in the rumen fatty acid profile are shown in Table 5. Once again, the effect of diet varied with the animal species, which was reflected in significant interactions. Thus, for instance, SOS diet induced an increased relative frequency of fragments compatible with species of *Succinivibrionaceae* (220 bp with *HhaI*, 492 with *MspI* and 202 with *HaeIII*), *Ruminococcaceae* (554 bp with *HhaI* and 284 with *MspI*) and *Lachnospiraceae* (149 with *MspI*) only in cows (interaction Sp × D; $P < 0.10$), whereas others that may correspond to *Pseudobutyrivibrio* (190 bp with *HhaI* and 315 with *MspI*) and *Prevotella* (102 bp with *HhaI*, and 162 and 266 with *HaeIII*) were stimulated with the same diet in goats (interaction Sp × D; $P < 0.10$). Terminal-restriction fragments potentially derived from other species of *Succinivibrionaceae* (495 with *MspI*) and *Clostridium IV* (269 with *MspI*) were greater with the FO diet in cows and goats, respectively ($P < 0.05$). Fragments putatively assigned to bacteria of the family *Veillonellaceae* (98 bp with *HhaI*, 150+268 with *MspI* and 244+287 with *HaeIII*) only changed significantly in goats, with values being favored by FO and SOS treatments ($P < 0.05$). A similar effect was observed in T-RF that might match with unclassified species of *Clostridia* (389 bp with *HhaI*, 70 with *MspI* and 262 with *HaeIII*) in both ruminant species.

[Please, insert Table 5 near here]

DISCUSSION

Nutritional strategies for modulating milk FA profile may cause alterations in microbial FA metabolism that, in certain cases, promote the formation of rumen BH intermediates with antilipogenic effects that can impair milk fat synthesis (Bauman and Griinari, 2001;

Shingfield et al., 2010a). In most cases, candidate inhibitors have been initially identified from variations in their milk concentrations and corresponding changes in milk fat content. However, up to date, only a few of these BH metabolites have been associated with diet-induced MFD (such as *trans*-10 18:1, *trans*-10,*cis*-12 CLA, or *trans*-9,*cis*-11 CLA; Shingfield and Griinari, 2007; Bauman et al., 2011) and, therefore, discussion of results will be focused on these intermediates and a selected number of other FA that may also be related to reductions in milk fat.

Direct comparison of cow and goat performance and responses to FO and SOS treatments (with mean milk fat contents of 3.34 vs. 3.11 for control, 2.34 vs. 2.47 for FO, and 2.29 vs. 2.90 for SOS in cows and goats, respectively; Toral et al., 2015) suggests inter-species differences in mammary lipogenesis. Furthermore, differences in milk FA composition in the two ruminant species in response to FO or SOS diets (e.g., lower *trans*-10 18:1/*trans*-11 18:1 ratios in goats than in cows; Toral et al., 2015) would point to a relevant role of ruminal BH pathways, which appear to be more stable and less prone to diet-induced shifts towards the formation of potential inhibitors of milk fat synthesis, such as *trans*-10 containing intermediates, in goats compared with cows (Chilliard et al, 2014).

In addition, examining together the associated changes in rumen FA concentrations and bacterial community could contribute to improve our knowledge of the microbiology of lipid metabolism and infer potential microorganisms involved in ruminal BH. However, divergences detected in the response of the bacterial populations of cows and goats to dietary treatments, which may be linked to functional differences in their gastrointestinal system (Ley et al., 2008), substantially hinders the assignation of bacterial groups to different lipid metabolic pathways.

Ruminal Fermentation

One of the earliest theories to explain diet-induced MFD in cows, based on the reduction in the ruminal production of acetate and butyrate and the increase in that of propionate, attributed this syndrome to an inadequate supply of lipid precursors for mammary lipogenesis. However, subsequent studies offered little support for this hypothesis (Bauman and Griinari, 2001). In line with this, the observed decrease in the molar ratio of acetate:propionate in cows and goats fed SOS was not accompanied by MFD in the caprine, while both species showed moderate reductions in this ratio and a decrease in milk fat content with FO (Toral et al., 2015). Similar shifts in the acetate:propionate ratio have been reported when feeding fish oil (Doreau and Chilliard, 1997; Shingfield et al., 2010b) or medium to high levels of sunflower oil (>4%; Shingfield et al., 2008; Zened et al., 2013a). In addition, the low NDF and high-starch content of the SOS diet might have contributed to decrease the acetate:propionate ratio through potentially negative effects on the abundance of cellulolytic bacteria (Beauchemin et al., 2008). Indeed, *iso* FA, which have been suggested as biomarkers of these microorganisms (Fievez et al., 2012), were reduced with the SOS diet. With regard to changes in specific bacterial groups, increased molar proportion of propionate with SOS might be related to variation in *Succinivibrionaceae* species in cows, some of which form succinate, a precursor of propionate (Russell and Hespell, 1981), and of *Prevotella* strains that promote propionate production (Bekele et al., 2010) in goats.

In both species, feeding SOS decreased the molar proportion of butyrate and the total concentration of VFA in the rumen fluid. Even though other assays in cattle fed plant oils or high concentrate diets have shown decreases, no effects and even increases in total VFA and butyrate (e.g., Bateman and Jenkins, 1998; Shingfield et al., 2008; Fuentes et al., 2009), the present results may reflect the combined effects of the high levels of starch and sunflower oil, given the importance of the basal diet on rumen fermentation parameters (Zened et al., 2013a; Ramos-Morales et al., 2014). Lower total VFA concentration for both species with SOS might

indicate decreases in either fermentation activity or microbial biomass (Fievez et al., 2012), which for the latter would be supported by reduced OBCFA concentration in the rumen fluid and milk fat (Toral et al., 2015) with the SOS diet. In any case, total VFA concentrations could also be explained by a potential interaction between diet and sampling time, reflecting a greater decrease in the fermentation substrate in the high-starch diet after the overnight period without concentrate distribution (Ramos-Morales et al., 2014).

Ammonia concentration was also lowered with SOS, which is consistent with other findings for high-starch diets or sunflower oil supplements (Shingfield et al., 2008; Fuentes et al., 2009). Ammonia concentration was about 2-fold greater in goats compared to cows, which could be related to differences in bacterial biomass or activity among species, which would need to be further explored. Although ruminal concentrations seem quite limited in cows, particularly with SOS, the calculated protein balance in the same animals was always positive and no differences in milk protein concentration and yield were observed (Toral et al., 2015). Low concentrations might then be explained, at least to some extent, by the sampling time (after an overnight fast; Ramos-Morales et al., 2014), which could also explain the high ruminal pH. In this regard, pH values have been shown to be usually greater when rumen fluid is collected by stomach tube but this does not preclude from detecting potential diet-induced effects (Ramos-Morales et al., 2014). In any event, the marginal differences in preprandial pH with the SOS diet would probably have no biological relevance, because pH is known to vary largely after meal intake.

Ruminal Fatty Acid Metabolism

Comparison of rumen FA concentrations (expressed as g/kg DM) in cows and goats fed the control diet provides evidence of inter-species differences in rumen BH that could be linked to differences in microbial composition. Thus, the much higher SFA concentration in

the rumen fluid of goats than cows (in particular, 16:0 and 18:0), together with the lack of differences in total PUFA, would suggest more extensive BH in this species. However, when expressed as % of total FA (Supplemental Table 1), the proportion of 18:0, total SFA, MUFA and PUFA, were similar in bovine and caprine. In other respects, the higher milk *cis*-9,*trans*-11 CLA/*trans*-11 18:1 ratio observed in goats when compared with cows fed the control diet (Toral et al., 2015) might be speculated to derive from potential inter-species differences in intestinal FA digestibility or tissue lipid metabolism, rather than in ruminal BH (Chilliard et al., 2007; Shingfield et al., 2010). Concerning the rumen microbiota, differences in the percentage of some specific OBCFA (e.g., 15:0, *anteiso* 15:0, 17:0, and *iso* 18:0) and similitudes in some others (e.g., *iso* 15:0, *iso* 16:0, *iso* 17:0, and *anteiso* 17:0), which derive mainly from de novo bacterial synthesis (Fievez et al., 2012), would support dissimilarities in the composition of the rumen microorganisms between cows and goats fed the control diet, as confirmed by T-RFLP. Furthermore, differences in the responses to lipid feeding of the ruminal microbiota of goats and cows hamper the identification of shared relevant bacteria associated with variations in the accumulation of specific BH intermediates. In other respects, species differences in OBCFA profile could also be due to differences in postruminal synthesis from the duodenum to the milk (Vlaeminck et al., 2015). Furthermore, the higher concentration of total FA observed in the rumen of goats compared to that of cows when both are fed the control diet might be related to inter-species differences in eating behavior, ruminal passage rates and digestion kinetics (Duncan and Poppi, 2008), which might also account for differences in rumen FA concentrations. However, to our knowledge, there is no published data on direct comparisons in goats and cows of either the total FA concentration or the rumen FA profile, which underlines the novelty of these data and the need for additional and targeted research to explain the observed differences.

For all diets, rumen FA composition was characterized by low PUFA concentrations (in particular, 18:2n-6), which might be explained by the sampling time in this experiment (before the morning meal) favoring a greater BH extent of the dietary FA (Loor et al., 2004). In any event, the effects of FO and SOS on rumen FA composition were consistent with reports in the literature with different rumen sampling times (e.g., means of five different time points in Boeckaert et al., 2008; 3 h after the meal in Toral et al., 2012; or 5 h after the meal in Zened et al., 2013a), supporting that, despite the relatively advanced stage of BH, results seem to reflect diet-induced effects on this process. Furthermore the links between rumen FA and daily milk FA (see below) suggest that morning sampling of rumen fluid gave an acceptable indication of the daily trend.

Indirect comparison of milk FA profiles from previous studies in goats and cows suggested similar effects on 18:0 concentrations when plant oil supplements are used (Chilliard et al., 2007), while the concentration of this FA was less affected in goats (Toral et al., 2014) than in cows (Offer et al., 1999; Shingfield et al., 2003) in response to fish oil. In the present study, however, diet-induced variations in rumen 18:0 contents were always more pronounced in goats, in agreement with milk FA composition in the same animals (r = 0.875 and 0.747 in goats [n = 41] and cows [n = 36], respectively, $P < 0.001$; Toral et al., 2015). In addition, greater increases in rumen 18:0 when fed SOS were observed in goats compared to cows (+209% vs. +97%, respectively), which suggests a more complete BH in goats of 18-carbon unsaturated FA from sunflower oil. On the other hand, a greater decrease in rumen 18:0 in goats than in cows when fed FO suggests a stronger inhibition of 18:0 formation due to FO (AbuGhazaleh et al., 2002; Loor et al., 2004; Shingfield et al., 2010b) in goats. This latter finding contrasts with indirect comparisons based on earlier works (Offer et al., 1999; Shingfield et al., 2003; Toral et al., 2014), although these might be biased by the scarcity of published studies in goats fed marine lipids and by differences in the percentage and

composition of fish oils and composition of basal diets (e.g., use of hay-based diets in caprine vs. silage in bovine). In line with this different response between ruminant species, the T-RF that changed similarly to rumen 18:0 concentration (namely that increased with SOS and decreased with FO) were not the same in bovine and caprine. Bacterial populations compatible with these T-RF, which were potentially linked to 18:1 saturation, would belong to *Ruminococcaceae* in cows and *Pseudobutryrivibrio* in goats. Microorganisms of both groups have been previously associated with 18:0 accumulation in the rumen of steers (Huws et al., 2011) and sheep (Castro-Carrera et al., 2014), whereas so far no data are available in goats.

Diet-induced variations in total *trans* 18:1 concentration were also more pronounced in goat rumen fluid, while milk FA responses had been observed to be greater in cows (Toral et al., 2015), which might be speculated to be due to inter-species differences in BH kinetics (without ruling out potential sampling time constraints), intestinal digestibility and/or in the mammary or body tissue uptake and utilization of these *trans* FA. On the other hand, effects of dietary treatments on the *trans* 18:1 isomer profile were relatively consistent in the rumen and milk of cows (e.g., $r = 0.923$ and 0.921 for concentrations of *trans*-10 18:1 and *trans*-11 18:1, respectively, $P < 0.001$, n = 36) and goats ($r = 0.858$ and 0.917 for the same correlations, $P < 0.001$, n = 41; Toral et al., 2015), even though samples might not be fully representative of rumen digestion over 24 hours. Thus, results from both types of samples (i.e., milk and rumen) indicated that SOS diet altered ruminal BH and resulted in *trans*-10 18:1 replacing *trans*-11 18:1 as the major intermediate in cows, in agreement with earlier studies examining the effect of high-starch diets and plant oils on cow milk or rumen digesta (e.g., Loor et al., 2005; Zened et al., 2013a). The fact that this shift was larger in the cow than in the goat suggests that BH pathways in caprine are more stable and robust in response to high-starch diets and plant oils (Chilliard et al., 2007; Shingfield et al., 2010a), which may

contribute to explain inter-species differences in the propensity to MFD on this type of diets (Roy et al., 2006; Bernard et al., 2009; Toral et al., 2015). Consistent with changes in rumen *trans*-10 18:1 concentrations, SOS increased the relative abundance of some T-RF compatible with species of *Lachnospiraceae* in cows and of *Prevotella* in goats, suggesting a potential relationship of these fragments with the *trans*-10 shift. In cows, species of both bacterial groups have been associated with lipid metabolism (Boeckaert et al., 2008; Huws et al., 2011) and strains of *Prevotella* were more abundant with a high-starch diet supplemented with sunflower oil (Zened et al., 2013b). Nonetheless, the available information on the relationship between these groups and dietary lipid BH in goats is very limited (Zhu et al., 2012).

Regarding specific BH intermediates that may be responsible for SOS-induced MFD, cows fed this diet showed the greatest ruminal content of *trans*-10,*cis*-12 CLA, which is remarkable because, as previously stated, the total FA concentration in the rumen fluid of cows was 60% lower than in goats. The *trans*-10,*cis*-12 CLA is the only BH metabolite shown unequivocally to inhibit milk fat synthesis (Shingfield and Griinari, 2007; Bauman et al., 2011) and the mammary gland of bovine is much more sensitive to its antilipogenic effect than that of caprine (Chilliard et al., 2014). In any case, as discussed in the companion paper (Toral et al., 2015), changes in the concentration of this CLA isomer in milk did not, in isolation, explain the extent of MFD in cows fed SOS and other rumen BH intermediates (such as *cis*-10,*trans*-12 CLA, *trans*-10 18:1, or other unidentified FA) would also be implicated. Furthermore, without a specific HPLC analysis, results on *trans*-10,*cis*-12 CLA should be taken with caution because our gas-chromatographic conditions might cause the coelution of minor CLA isomers and other low abundant intermediates. This fact may also be behind the observed increase in this FA in the rumen fluid of cows fed FO, which is not a common observation in digesta samples (e.g., Shingfield et al., 2003, 2010b; Loor et al., 2004). Something similar occurred with *trans*-9,*cis*-11 CLA concentrations, another BH

intermediate that has been reported as a potential milk fat inhibitor in cows (Perfield et al., 2007). Yet, its diet-induced proportion in milk is often lower than the level of enrichment used to demonstrate anti-lipogenic effects (Roy et al., 2006; Perfield et al., 2007; Bichi et al., 2013).

Induction of MFD with diets containing marine oils has often been related to the joint action of decreases in 18:0 availability and increases in *trans* 18:1 contributing to limit the synthesis of milk fat with an adequate fluidity to be secreted by mammary epithelial cells (Shingfield and Griinari, 2007). However, notwithstanding the greater extent of changes in these FA in the rumen of goats, these animals experienced a lower MFD than cows (21 vs. 30% reduction in milk fat content, respectively; Toral et al., 2015), which might point to either a major contribution of other FA to maintain milk fat fluidity, such as short-chain FA de novo synthesized in the mammary gland, or other mechanism explaining that goats are less prone to this syndrome (Bernard et al., 2010; Toral et al., 2014). In relation to specificities in the bacterial community composition, this response was linked to FO-induced increments of different T-RF in each species, such as those compatible with bacteria of *Succinivibrionaceae* in cows and *Clostridium IV* in goats. Although the role of these microorganisms in lipid metabolism is still uncertain, species of *Succinivibrionaceae* have been related to rumen BH in ewes (Castro-Carrera et al., 2014), and *Clostridium IV* has been recently associated with BH of n-3 PUFA in cattle (Petri et al., 2014).

As mentioned above, few individual FA have been directly associated with diet-induced MFD (Shingfield and Griinari, 2007; Bauman et al., 2011) but, in most cases, reports on their biological activity are still equivocal (e.g., *trans*-10 18:1; Shingfield et al., 2010a) and changes in their rumen and milk concentrations may not fully explain MFD (as mentioned above for *trans*-10,*cis*-12 CLA or *trans*-9,*cis*-11 CLA; Roy et al., 2006; Boeckaert et al., 2008; Bichi et al., 2013). Thus, other FA may also be involved and account for this syndrome.

For example, recent studies have indicated that *cis*-11 18:1, a FA commonly found in ruminant feedstuffs, was able to alter lipogenesis in bovine adipocyte cultures (Burns et al., 2012) and was associated with a decrease in milk fat content in goats fed FO (Bernard et al., 2015). In this regard, the FO diet increased the accumulation of this 18:1 isomer in the rumen, particularly in goats (5-fold) but also in cows (3-fold), whereas the greatest concentration in milk fat was observed in the latter (Toral et al., 2015), which would oblige to further investigate its potential role in mammary lipogenesis. Furthermore the role of BH intermediates from long-chain FA, such as 20- and 22- carbon FA, in particular those containing a *trans*-10 double bond, on the observed effects of FO on milk fat cannot be excluded (Toral et al., 2015).

Intermediate metabolites derived from sequential hydration and oxidation of dietary FA in the rumen, as alternative pathways (Jenkins et al., 2006), might also have biological activity in ruminants (Raphael et al., 2014), but their impact on milk fat synthesis has not been characterized yet. Concentrations of 10-O-16:0, 10-O-18:0, and 13-O-18:0 were particularly enhanced with FO, which is in agreement with earlier measurements in cows and ewes (Shingfield et al., 2010b; Toral et al., 2010, 2012). In the present study, the detection of these oxylipids could have been favored by the sampling time (i.e., after an overnight period without concentrate distribution), because they seem to be end-products of ruminal FA metabolism (Jenkins et al., 2006). Concerning rumen microbiota, specific T-RF that might be associated with hydration would correspond to *Veillonellaceae* in goats and to *Clostridia* in both species. Studies with sheep (Belenguer et al., 2010; Toral et al., 2012) suggested a putative participation of species of *Veillonellaceae*, such as *Quinella ovalis*, in this process. With respect to *Clostridia*, this is a broad and diverse group that includes microorganisms potentially related to lipid metabolism (Boeckaert et al., 2008; Huws et al., 2014), such as

those compatible with the 389 bp T-RF detected with *HhaI*, which has been previously shown to increase with marine lipids in ewes (Belenguer et al., 2010; Toral et al., 2012).

As discussed above for 18:0 and *trans*-18:1, the tendency towards greater effects of FO on 10-O-18:0 concentration in goats than in cows would further support a more potent impact of this supplement in the rumen of goats, while increases in keto acids in milk were more evident in cows (Toral et al., 2015). Overall, the reasons for the inconsistent results between diet-induced changes in rumen and milk FA are not readily apparent, but might be related, despite the possible interaction with the rumen sampling time, to inter-species differences in FA digestion and transport, and body tissues or mammary lipid metabolism (Chilliard et al., 2007; Shingfield et al., 2010a).

CONCLUSIONS

Direct comparison of ruminal responses to diets inducing MFD demonstrates relevant species by diet interactions on the rumen FA composition and bacterial community. However, changes in some ruminal fermentation characteristics due to FO or SOS (e.g., decreases in acetate:propionate ratio) were similar in cows and goats. The main alterations in ruminal BH pathways potentially responsible for MFD on the SOS treatment (i.e., the *trans*-10 shift and related increases in *trans*-10,*cis*-12 CLA concentration) tend to be more pronounced in cows than in goats, whereas changes linked to FO-induced MFD (e.g., decreases in 18:0 and increases in total *trans*-18:1 accumulation) are more evident in caprine rumen fluid. Results support the initial hypothesis that distinct ruminal mechanisms lead to each type of diet-induced MFD, and may also explain the unexpected susceptibility of dairy goats (although less marked than in cows) to the negative effect of FO on milk fat content. The bacterial populations affected by SOS or FO diets differ between cows and goats, in agreement with species-specific microbial community structures. Furthermore, most relevant variations in

microbial groups correspond to as yet uncultured bacteria and suggest that these microorganisms may play a predominant role in the ruminal lipid metabolism in both cows and goats.

ACKNOWLEDGMENTS

This study was supported by the INRA (UMR1213; France) and the Spanish Ministry of Economy and Competitiveness (MINECO; AGL2014-54587). P. G. Toral was granted a post-doctoral fellowship from *Fundación Alfonso Martín Escudero* (Madrid, Spain) and a research contract from the *Juan de la Cierva* programme. The authors gratefully acknowledge the staff of UERT 1354 (INRA, France) for the diligent care of the experimental animals, D. Bany, C. Labonne, and M. Tourret from UMR 1213 (INRA, France) for assistance in sampling collection, the staff of the *Instituto de Ganadería de Montaña* (CSIC; Spain) for assistance in laboratory analysis, and C. Martin and M. Sylberberg from UMR 213 (INRA, France) for helpful discussions. Thanks are also given to Neil R. McEwan from the Institute of Biological, Environmental and Rural Sciences (IBERS) of Aberystwyth University (Aberystwyth, UK) for kindly revising the manuscript.

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Table 1. Effect of dietary supplements of fish oil or sunflower oil and starch on ruminal fermentation characteristics in cows and goats[1]

	Cows			Goats				P^2		
	Control	FO	SOS	Control	FO	SOS	SEM	Sp	D	Sp × D
Ammonia (mg/L)	70.6	75.9	34.1	146.8	140.2	75.6	8.25	<0.001	<0.001	0.091
Total VFA (mmol/L)	66.8	62.2	50.8	50.4	47.1	42.9	4.91	0.009	0.039	0.596
Molar proportions (%)										
Acetate	70.83	68.63	68.62	66.90	64.02	62.37	0.687	<0.001	<0.001	0.208
Propionate	14.56^{d}	16.37^{c}	18.97^{b}	15.08^{cd}	18.73^{b}	22.84^{a}	0.642	<0.001	<0.001	0.026
Butyrate	11.14	11.09	7.87	12.46	11.72	7.69	0.351	0.092	<0.001	0.061
∑ Minor VFA[3]	3.47	3.91	4.54	5.56	5.53	7.09	0.261	<0.001	<0.001	0.195
Acetate:propionate	4.96	4.26	3.67	4.52	3.46	2.79	0.158	<0.001	<0.001	0.258

[1]Control, basal diet containing no additional oil; FO, diet supplemented with fish oil; SOS, diet containing sunflower oil and wheat starch.

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

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

[a-d]Within a row, different superscripts indicate significant differences ($P < 0.05$) due to the effect of Sp × D.

6 **Table 2.** Effect of dietary supplements of fish oil or sunflower oil and starch on FA concentration in the rumen fluid of cows and goats[1]

	Cows			Goats				P^2		
FA, g/kg DM	Control	FO	SOS	Control	FO	SOS	SEM	Sp	D	Sp × D
12:0	0.03	0.03	0.02	0.05	0.06	0.05	0.005	<0.001	0.304	0.821
13:0	0.04	0.03	0.04	0.05	0.05	0.06	0.007	0.005	0.750	0.714
anteiso 13:0	0.009^{c}	0.012^{b}	0.007^{c}	0.013^{ab}	0.015^{a}	0.007^{c}	0.0009	0.014	<0.001	0.018
iso 13:0	0.019^{cd}	0.024^{bc}	0.015^{de}	0.030^{b}	0.050^{a}	0.013^{e}	0.0021	<0.001	<0.001	<0.001
14:0	0.27^{d}	0.53^{b}	0.18^{d}	0.40^{c}	1.21^{a}	0.22^{d}	0.044	<0.001	<0.001	<0.001
iso 14:0	0.04^{c}	0.03^{cd}	0.02^{d}	0.07^{b}	0.10^{a}	0.04^{c}	0.006	<0.001	<0.001	0.002
trans-9 14:1	0.06	0.05	0.05	0.08	0.09	0.07	0.010	0.002	0.442	0.538
15:0	0.29^{c}	0.33^{c}	0.15^{d}	0.42^{b}	0.53^{a}	0.22^{d}	0.025	<0.001	<0.001	0.010
anteiso 15:0	0.28^{b}	0.31^{b}	0.17^{c}	0.28^{b}	0.54^{a}	0.13^{c}	0.022	0.000	<0.001	<0.001
iso 15:0	0.12^{c}	0.15^{bc}	0.06^{d}	0.19^{b}	0.33^{a}	0.05^{d}	0.015	<0.001	<0.001	<0.001
16:0	4.28^{d}	6.41^{c}	4.09^{d}	7.57^{c}	10.75^{a}	9.32^{b}	0.417	<0.001	<0.001	0.039
iso 16:0	0.12	0.11	0.04	0.18	0.19	0.14	0.016	<0.001	<0.001	0.558
10-O-16:0	0.02	0.13	0.02	0.02	0.14	0.02	0.008	0.371	<0.001	0.855
cis-9 16:1	0.02^{c}	0.12^{b}	0.01^{c}	0.03^{c}	0.25^{a}	0.02^{c}	0.014	0.001	<0.001	<0.001
trans-9 16:1	<0.01^{c}	0.04^{b}	<0.01^{c}	<0.01^{c}	0.07^{a}	<0.01^{c}	0.003	<0.001	<0.001	<0.001
17:0	0.20	0.21	0.17	0.30	0.35	0.31	0.019	<0.001	0.113	0.314
anteiso 17:0	0.16^{c}	0.18^{c}	0.07^{d}	0.23^{b}	0.27^{a}	0.08^{d}	0.013	<0.001	<0.001	0.011
iso 17:0	0.12^{c}	0.18^{b}	0.08^{d}	0.22^{b}	0.27^{a}	0.12^{c}	0.015	<0.001	<0.001	0.038
18:0	16.92^{c}	6.28^{d}	33.41^{b}	29.36^{b}	4.47^{d}	90.73^{a}	3.208	<0.001	<0.001	<0.001
iso 18:0	0.024^{bc}	0.023^{bcd}	0.018^{d}	0.027^{b}	0.033^{a}	0.020^{cd}	0.0019	0.028	<0.001	0.012
10-O-18:0	0.03	1.25	0.72	0.03	1.98	1.19	0.171	0.005	<0.001	0.093
13-O-18:0	0.04	0.15	0.14	0.04	0.16	0.17	0.016	0.307	<0.001	0.544
Σ *cis* 18:1	1.24	1.94	1.34	2.01	3.29	2.82	0.205	<0.001	<0.001	0.093
Σ *trans* 18:1	2.23^{c}	9.69^{b}	7.99^{b}	3.40^{c}	18.21^{a}	16.48^{a}	1.435	<0.001	<0.001	0.013
Σ non conjugated 18:2	1.18^{b}	1.40^{ab}	0.67^{c}	1.53^{ab}	1.67^{a}	1.74^{a}	0.177	<0.001	0.168	0.047
Σ conjugated 18:2	0.061	0.121	0.117	0.087	0.155	0.151	0.0110	0.004	<0.001	0.892
19:0	0.03^{d}	0.05^{bc}	0.04^{cd}	0.05^{bc}	0.06^{b}	0.08^{a}	0.005	<0.001	0.004	0.014

20:0	0.21^{c}	0.26^{bc}	0.24^{c}	0.33^{b}	0.28^{bc}	0.54^{a}	0.028	<0.001	<0.001	<0.001
3,7,11,15-tetramethyl 16:0	0.14^{c}	0.24^{b}	0.04^{d}	0.17^{bc}	0.42^{a}	0.06^{d}	0.027	0.008	<0.001	0.001
Σ 20:1	0.07	0.43	0.07	0.09	0.57	0.14	0.040	0.024	<0.001	0.257
Σ unsaturated C20	0.06^{c}	0.43^{b}	0.06^{c}	0.06^{c}	0.89^{a}	0.09^{c}	0.042	<0.001	<0.001	<0.001
21:0	<0.01^{c}	0.03^{b}	<0.01^{c}	<0.01^{c}	0.09^{a}	<0.01^{c}	0.007	0.002	<0.001	<0.001
22:0	0.15^{c}	0.17^{c}	0.24^{b}	0.19^{bc}	0.21^{bc}	0.55^{a}	0.023	<0.001	<0.001	<0.001
Σ 22:1	-	0.09	-	-	0.11	-	0.010	0.075	-	-
Σ unsaturated C22	0.02^{c}	0.43^{b}	0.02^{c}	0.04^{c}	0.79^{a}	0.03^{c}	0.050	0.002	<0.001	<0.001
23:0	0.031	0.039	0.027	0.039	0.054	0.049	0.0035	<0.001	0.004	0.131
24:0	0.10^{cd}	0.10^{cd}	0.09^{d}	0.12^{bc}	0.13^{b}	0.19^{a}	0.009	<0.001	0.001	<0.001
Summary										
Σ SFA	23.8^{c}	17.3^{c}	40.2^{b}	40.5^{b}	22.9^{c}	104.6^{a}	3.64	<0.001	<0.001	<0.001
Σ MUFA	3.91^{d}	13.02^{b}	10.01^{bc}	5.97^{cd}	23.72^{a}	20.94^{a}	1.619	<0.001	<0.001	0.008
Σ PUFA	1.68	2.78	1.07	2.00	4.01	2.24	0.249	<0.001	<0.001	0.123
Σ odd- and branched-chain FA	1.74^{d}	2.07^{c}	1.01^{e}	2.45^{b}	3.54^{a}	1.53^{d}	0.117	<0.001	<0.001	<0.001
Total FA	29.5^{c}	33.5^{c}	51.4^{b}	48.7^{b}	51.1^{b}	128.0^{a}	4.21	<0.001	<0.001	<0.001

[1]Control, basal diet containing no additional oil; FO, diet supplemented with fish oil; SOS, diet containing sunflower oil and wheat starch.

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

[a-e]Within a row, different superscripts indicate significant differences ($P < 0.05$) due to the effect of Sp × D.

Table 3. Effect of dietary supplements of fish oil or sunflower oil and starch on unsaturated long-chain FA concentration in the rumen fluid of cows and goats[1]

	Cows			Goats				P^2		
FA, g/kg DM	Control	FO	SOS	Control	FO	SOS	SEM	Sp	D	Sp × D
cis-9 18:1	0.90	1.20	0.87	1.59	2.04	1.72	0.157	<0.001	0.012	0.777
cis-11 18:1	0.14^{d}	0.42^{b}	0.17^{d}	0.18^{d}	0.88^{a}	0.32^{c}	0.034	<0.001	<0.001	<0.001
cis-12 18:1	0.086^{c}	0.112^{bc}	0.122^{bc}	0.092^{bc}	0.152^{b}	0.362^{a}	0.0231	<0.001	<0.001	<0.001
cis-13 18:1	0.030	0.055	0.040	0.052	0.090	0.089	0.0091	<0.001	<0.001	0.219
cis-15 18:1	0.05^{d}	0.11^{b}	0.08^{cd}	0.06^{d}	0.10^{bc}	0.19^{a}	0.011	<0.001	<0.001	<0.001
cis-16 18:1	0.03^{c}	0.04^{bc}	0.06^{b}	0.04^{bc}	0.04^{bc}	0.14^{a}	0.007	<0.001	<0.001	<0.001
trans-4 18:1	0.09^{c}	0.06^{c}	0.25^{b}	0.13^{c}	0.08^{c}	0.61^{a}	0.033	<0.001	<0.001	<0.001
trans-5 18:1	0.05^{c}	0.05^{c}	0.15^{b}	0.11^{b}	0.11^{bc}	0.38^{a}	0.022	<0.001	<0.001	<0.001
trans-6 + 7 + 8 18:1	0.14^{b}	0.29^{b}	0.51^{b}	0.19^{b}	0.43^{b}	1.21^{a}	0.136	0.007	<0.001	0.036
trans-9 18:1	0.11^{c}	0.30^{b}	0.26^{b}	0.13^{c}	0.45^{a}	0.53^{a}	0.038	<0.001	<0.001	0.008
trans-10 18:1	0.14	1.87	3.17	0.15	0.95	4.51	0.715	0.806	<0.001	0.271
trans-11 18:1	1.03^{c}	5.54^{b}	1.77^{c}	1.75^{c}	13.71^{a}	4.25^{b}	0.593	<0.001	<0.001	<0.001
trans-12 18:1	0.21^{c}	0.74^{b}	0.61^{b}	0.28^{c}	1.35^{a}	1.44^{a}	0.107	<0.001	<0.001	0.002
trans-13 + 14 18:1	0.28^{d}	0.68^{bc}	0.84^{b}	0.32^{cd}	0.93^{b}	2.29^{a}	0.144	<0.001	<0.001	<0.001
trans-15 18:1	0.25^{c}	0.42^{bc}	0.52^{b}	0.33^{c}	0.53^{b}	1.36^{a}	0.070	<0.001	<0.001	<0.001
trans-16 18:1	0.20^{cd}	0.16^{d}	0.45^{b}	0.34^{bc}	0.20^{cd}	1.25^{a}	0.059	<0.001	<0.001	<0.001
cis-9,*cis*-12 18:2	1.01^{ab}	0.59^{bc}	0.49^{c}	1.32^{a}	0.62^{bc}	1.35^{a}	0.162	0.003	0.003	0.039
cis-12,*cis*-15 18:2	0.025^{ab}	0.023^{b}	0.021^{b}	0.022^{b}	0.027^{a}	0.024^{ab}	0.0014	0.272	0.162	0.034
cis-9,*trans*-12 18:2	<0.01	0.02	<0.01	<0.01	0.03	<0.01	0.002	0.002	<0.001	0.116
trans-9,*cis*-12 18:2	0.01	0.04	0.02	0.01	0.06	0.03	0.004	<0.001	<0.001	0.140
trans-11,*cis*-15 18:2	0.05	0.46	0.03	0.06	0.55	0.04	0.037	0.238	<0.001	0.376
trans-9,*trans*-12 18:2	<0.01	0.07	<0.01	<0.01	0.07	<0.01	0.007	0.885	<0.001	0.633
trans-9,*trans*-13 18:2	0.02	0.03	0.02	0.03	0.04	0.04	0.006	0.007	0.149	0.582
trans-10,*trans*-14 18:2	0.03^{b}	0.03^{b}	0.04^{b}	0.03^{b}	0.04^{b}	0.11^{a}	0.006	<0.001	<0.001	<0.001
trans-11,*trans*-15 18:2	0.02^{c}	0.12^{b}	0.01^{c}	0.03^{c}	0.20^{a}	0.03^{c}	0.008	<0.001	<0.001	<0.001

cis-9,*trans*-11 CLA[3]	0.02	0.04	0.03	0.04	0.04	0.05	0.006	0.020	0.226	0.252
trans-9,*cis*-11 CLA	0.008	0.022	0.007	0.020	0.028	0.014	0.0042	0.035	<0.001	0.739
trans-10,*cis*-12 CLA	0.002	0.015	0.026	0.003	0.009	0.017	0.0028	0.042	<0.001	0.095
trans,*trans* CLA[4]	0.03^{c}	0.05^{b}	0.06^{b}	0.03^{c}	0.08^{a}	0.07^{a}	0.005	0.001	<0.001	0.003
cis-9,*cis*-12,*cis*-15 18:3	0.25	0.15	0.09	0.18	0.13	0.09	0.019	0.101	<0.001	0.135
cis-9,*trans*-11,*cis*-15 18:3	0.012^{bc}	0.016^{b}	0.009^{cd}	0.009^{cd}	0.028^{a}	0.006^{d}	0.0018	0.158	<0.001	<0.001
cis-11 20:1	0.05	0.23	0.04	0.05	0.29	0.07	0.021	0.077	<0.001	0.355
cis-11,*cis*-14 20:2	<0.01^{c}	0.04^{b}	<0.01^{c}	<0.01^{c}	0.08^{a}	<0.01^{c}	0.005	0.002	<0.001	<0.001
cis-8,*cis*-11,*cis*-14 20:3	0.041^{cd}	0.054^{bc}	0.046^{cd}	0.033^{d}	0.073^{a}	0.066^{ab}	0.0056	0.041	<0.001	0.017
cis-12,*cis*-15,*cis*-17 20:3	-	0.10	-	-	0.22	-	0.021	<0.001	-	-
Δ8,11,15 20:3	-	0.09	-	-	0.13	-	0.009	0.049	-	-
Δ6,11,14,17 20:4	-	0.05	-	-	0.14	-	0.023	0.009	-	-
cis-5,*cis*-8,*cis*-11,*cis*-14 20:4	<0.01	0.04	0.01	0.01	0.06	0.02	0.004	0.004	<0.001	0.071
cis-9,*cis*-12,*cis*-15,*cis*-17 20:4	<0.01	0.15	<0.01	<0.01	0.26	<0.01	0.029	0.140	<0.001	0.094
cis-5,*cis*-8,*cis*-11,*cis*-14,*cis*-17 20:5	-	0.07	-	-	0.19	-	0.049	0.085	-	-
cis-6,*cis*-9,*cis*-12,*cis*-15,*cis*-18 21:5	-	0.01	-	-	<0.01	-	0.003	0.834	-	-
cis-13 22:1	-	0.02	-	-	0.04	-	0.006	0.066	-	-
cis-11 22:1	-	0.05	-	-	0.05	-	0.004	0.838	-	-
cis-13,*cis*-16 22:2	0.006	0.014	0.005	0.011	0.019	0.008	0.0021	0.009	<0.001	0.883
cis-13,*cis*-16,*cis*-19 22:3	<0.01	0.02	<0.01	<0.01	0.03	<0.01	0.002	0.059	<0.001	0.224
cis-7,*cis*-10,*cis*-13,*cis*-16 22:4	-	0.01	-	-	0.02	-	0.002	0.046	-	-
cis-10,*cis*-13,*cis*-16,*cis*-19 22:4	0.01^{c}	0.06^{b}	<0.01^{c}	0.02^{c}	0.11^{a}	<0.01^{c}	0.011	0.016	<0.001	0.010
cis-4,*cis*-7,*cis*-10,*cis*-13,*cis*-16 22:5	-	0.14	-	-	0.24	-	0.033	0.047	-	-
cis-7,*cis*-10,*cis*-13,*cis*-16,*cis*-19 22:5	-	0.11	-	-	0.17	-	0.033	0.208	-	-
cis-4,*cis*-7,*cis*-10,*cis*-13,*cis*-16,*cis*-19 22:6	-	0.08	-	-	0.20	-	0.049	0.116	-	-
cis-14 23:1	<0.01^{c}	0.01^{b}	<0.01^{c}	<0.01^{c}	0.03^{a}	<0.01^{c}	0.002	0.004	<0.001	0.002
cis-15 24:1	0.02	0.03	<0.0	0.03	0.05	0.02	0.002	<0.001	<0.001	0.107

[1]Control, basal diet containing no additional oil; FO, diet supplemented with fish oil; SOS, diet containing sunflower oil and wheat starch.

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

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

[4]Sum of *trans*-8,*trans*-10 +*trans*-9,*trans*-11 + *trans*-10,*trans*-12 + *trans*-11,*trans*-13 CLA.

[a-d]Within a row, different superscripts indicate significant differences ($P < 0.05$) due to the effect of Sp × D.

Table 4. Effect of dietary supplements of fish oil or sunflower oil and starch on diversity indices (richness, R; Shannon-Wiener, H; Shannon evenness, E) of bacterial communities in the rumen fluid of cows and goats[1]

Restriction enzyme	Index	Cows			Goats			SEM	P^2		
		Control	FO	SOS	Control	FO	SOS		Sp	D	Sp×D
HhaI	R	107.0	99.7	89.0	113.0	107.3	100.0	7.54	0.315	0.199	0.945
	H	3.77	3.64	3.68	3.86	3.69	3.47	0.066	0.726	0.017	0.089
	E	0.81	0.79	0.83	0.82	0.79	0.75	0.018	0.391	0.328	0.078
MspI	R	57.7	64.7	43.7	59.0	53.0	51.3	13.00	0.959	0.399	0.562
	H	3.50	3.42	3.11	3.47	3.20	2.98	0.194	0.595	0.086	0.821
	E	0.88^{a}	0.82^{b}	0.87^{ab}	0.86^{ab}	0.81^{b}	0.76^{c}	0.016	0.110	0.015	0.026
HaeIII	R	92.0	93.7	106.6	103.3	113.3	94.3	10.51	0.620	0.814	0.257
	H	3.83	3.76	3.85	3.92	3.83	3.65	0.099	0.921	0.379	0.217
	E	0.86	0.83	0.83	0.85	0.81	0.80	0.007	0.014	0.001	0.597

[1]Control, basal diet containing no additional oil; FO, diet supplemented with fish oil; SOS, diet containing sunflower oil and wheat starch. For each experimental diet and species, n = 3.

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

[a-c]Within a row, different superscripts indicate significant differences ($P < 0.05$) due to the effect of Sp × D.

Table 5. Effect of dietary supplements of fish oil or sunflower oil and starch on the relative frequencies (expressed as $\log_{10}$ (n + c) of % over the total peak area, with original values in parentheses) of some terminal restriction fragments (T-RF) detected in the rumen fluid of cows and goats[1], and their putative taxonomic identification

Taxonomic identification	T-RF (bp)	Cows			Goats			SEM	*P*[2]		
		Control	FO	SOS	Control	FO	SOS		Sp	D	Sp×D
Succinivibrionaceae	220 (*HhaI*)	0.26^{b} (1.05)	0.07^{bc} (0.25)	0.67^{a} (4.00)	0.05^{bc} (0.22)	-0.04^{c} (0.00)	-0.04^{c} (0.00)	0.077	<0.001	0.014	0.010
	492 (*MspI*)	0.46 (1.28)	0.34 (0.44)	0.95 (8.46)	0.28 (0.16)	0.25 (0.00)	0.32 (0.38)	0.103	0.008	0.026	0.067
	202 (*HaeIII*)	0.19^{b} (0.86)	0.05^{b} (0.28)	0.63^{a} (3.77)	-0.02^{b} (0.11)	-0.02^{b} (0.11)	-0.07^{b} (0.00)	0.084	0.001	0.036	0.014
Ruminococcaceae	554 (*HhaI*)	0.34^{c} (0.65)	0.23^{d} (0.19)	0.51^{ab} (1.89)	0.47^{bc} (1.39)	0.71^{a} (3.78)	0.46^{bc} (1.34)	0.048	0.090	0.069	<0.001
	284 (*MspI*)	0.38 (1.45)	0.22 (0.73)	0.56 (3.00)	0.08 (0.34)	-0.03 (0.00)	0.03 (0.16)	0.051	<0.001	0.013	0.056
Pseudobutyrivibrio	190 (*HhaI*)	0.05 (1.13)	0.01 (1.02)	0.15 (1.43)	-0.19 (0.67)	-0.25 (0.57)	0.22 (1.73)	0.063	0.025	0.003	0.057
	315 (*MspI*)	-0.14^{ab} (0.50)	-0.06^{ab} (0.56)	-0.33^{bc} (0.18)	-0.47^{c} (0.00)	-0.47^{c} (0.00)	0.06^{a} (0.83)	0.096	0.293	0.254	0.009
Lachnospiraceae	149 (*MspI*)	0.66^{bc} (2.27)	0.73^{b} (3.04)	0.89^{a} (5.51)	0.54^{cd} (1.17)	0.56^{cd} (1.27)	0.49^{d} (0.86)	0.045	0.045	0.114	0.014
Prevotella	102 (*HhaI*)	-0.10^{b} (0.81)	0.16^{b} (1.50)	0.13^{b} (1.50)	-0.04^{b} (0.93)	0.06^{b} (1.17)	0.70^{a} (5.04)	0.096	0.056	0.003	0.020
	97 (*MspI*)	0.26 (0.37)	0.23 (0.18)	0.53 (2.79)	0.48 (1.04)	0.38 (0.88)	0.73 (3.90)	0.101	0.257	0.015	0.943
	162 (*HaeIII*)	-0.08^{a} (0.84)	0.11^{a} (1.28)	-0.01^{a} (1.01)	-0.63^{b} (0.25)	-0.62^{b} (0.25)	0.04^{a} (1.19)	0.094	<0.001	0.012	0.008
	266 (*HaeIII*)	0.38^{b} (0.89)	0.46^{b} (1.26)	0.32^{b} (0.60)	0.53^{b} (1.81)	0.41^{b} (0.94)	0.76^{a} (4.21)	0.059	0.109	0.109	0.004

Veillonellaceae (Quinella)	98 (*HhaI*)	0.80^{ab} (2.45)	0.71^{b} (1.50)	0.76^{b} (1.86)	0.84^{b} (3.07)	1.05^{a} (7.29)	1.04^{a} (7.26)	0.063	0.108	0.163	0.018
	150+268 (*MspI*)	0.50^{bc} (0.56)	0.43^{c} (0.00)	0.43^{c} (0.00)	0.64^{c} (1.73)	0.99^{a} (7.63)	0.93^{ab} (6.57)	0.087	0.076	0.019	0.002
	244+287 (*HaeIII*)	-0.01^{bc} (0.98)	-0.13^{c} (0.75)	-0.003^{bc} (1.05)	0.37^{b} (2.53)	0.70^{a} (5.27)	0.65^{a} (4.78)	0.098	0.039	0.087	0.016
Clostridia	389 (*HhaI*)	-0.28 (0.00)	0.10 (0.76)	0.15 (0.98)	-0.28 (0.00)	-0.02 (0.42)	0.18 (1.02)	0.076	0.729	<0.001	0.432
	70 (*MspI*)	-0.23 (0.15)	-0.09 (0.43)	-0.01 (0.62)	-0.33 (0.00)	0.02 (0.62)	0.15 (1.02)	0.103	0.690	0.003	0.162
	262 (*HaeIII*)	1.18 (6.51)	1.33 (12.02)	1.29 (10.06)	1.22 (7.16)	1.29 (10.17)	1.32 (11.45)	0.041	0.790	0.048	0.596
Succinivibrionaceae	495 (*MspI*)	-0.30^{b} (0.00)	-0.03^{a} (0.55)	-0.30^{b} (0.00)	0.15^{ab} (1.26)	-0.07^{ab} (0.66)	-0.01^{ab} (0.59)	0.136	0.312	0.337	0.029
Clostridium IV	269 (*MspI*)	-0.25^{b} (0.00)	-0.25^{b} (0.00)	-0.25^{b} (0.00)	-0.25^{b} (0.00)	0.47^{a} (3.37)	-0.25^{b} (0.00)	0.095	0.022	0.014	0.014
	287 (*HaeIII*)	-0.06 (0.89)	-0.17 (0.71)	-0.07 (0.91)	0.23 (1.95)	0.56 (4.05)	0.13 (1.49)	0.105	0.058	0.262	0.068

[1]Control, basal diet containing no additional oil; FO, diet supplemented with fish oil; SOS, diet containing sunflower oil and wheat starch .For each experimental diet and species, n = 3.

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

[a-d]Within a row, different superscripts indicate significant differences ($P < 0.05$) due to the effect of Sp × D.

Figure 1. Dendrograms of terminal restriction fragment length polymorphism (T-RFLP) profiles, based on Ward's method and the Bray-Curtis distances, of total bacteria in the rumen fluid of cows and goats fed a diet containing no additional oil (control) or supplemented with fish oil (FO) or containing sunflower oil and wheat starch (SOS).The experimental period is indicated in parentheses.