Instituto de Ganadería de Montaña (IGM)

SUPLEMENTATION OF THE DIET OF DAIRY EWES WITH SUNFLOWER OIL AND MARINE LIPIDS TO MODULATE MILK FAT COMPOSITION.

EFFECT ON ANIMAL PERFORMANCE, RUMEN MICROBIOTA AND FERMENTATION CHARACTERISTICS, AND MILK AND DIGESTA FATTY ACID PROFILES

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Leon, April 2010

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SUPLEMENTACIÓN DE LA DIETA DE OVEJAS LECHERAS CON ACEITE DE GIRASOL Y LÍPIDOS MARINOS PARA MODIFICAR LA COMPOSICIÓN DE LA GRASA DE LA LECHE.

EFECTO SOBRE EL RENDIMIENTO PRODUCTIVO DE LOS ANIMALES, LA MICROBIOTA Y LAS CARACTERÍSTICAS DE LA FERMENTACIÓN RUMINAL Y EL PERFIL DE ÁCIDOS GRASOS DE LA LECHE Y LA DIGESTA

Memoria de Tesis Doctoral presentada por

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para acceder al grado de Doctor, dentro del programa de Doctorado "Producción y Sanidad Ovina" de la Universidad de León.

León, abril de 2010

El autor de esta memoria ha disfrutado de una ayuda del Programa I3P (Itinerario Integrado de Inserción Profesional) del Consejo Superior de Investigaciones Científicas (CSIC) cofinanciado por el Fondo Social Europeo.

Los trabajos que componen esta memoria han sido financiados por el Ministerio de Ciencia e Innovación (MICINN; Proyecto AGL2008-04805) y la Junta de Castilla y León (Proyecto GR158).

Deseo expresar mi agradecimiento a todas las personas e instituciones que han puesto a nuestra disposición sus conocimientos y los medios necesarios para la realización de este trabajo.

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ABBREVIATIONS

A..................asymptotic gas production
ADF ....acid detergent fibre
AFR.............average fermentation rate
BH ....biohydrogenation
c...................fractional rate of gas production
CLA ....conjugated linoleic acid
CP................crude protein
CPD ....crude protein disappearance
D..................diet
DHA ....docosahexaenoic acid
DM ....dry matter
DMD...........dry matter disappearance
DMI ....dry matter intake
DMOX 4,4-dimethyloxazoline
DNA ....deoxyribonucleic acid
DPA ....docosapentaenoic acid
E ....Shannon evenness (diversity index)
EPA.............eicosapentaenoic acid
FA ....fatty acid
FAME ....fatty acid methyl ester
FISH............fluorescence in situ hybridization
FM ....fresh matter
FO ....fish oil
G..................gas production
GC...............gas chromatography
GC-MS.........gas chromatography-mass spectrometry
GLC ....gas-liquid chromatography
H ....Shannon-Wiener diversity index
HPLC...........high performance liquid chromatography
LW ....live weight
$LW^{0.75}$ ....metabolic weight
MA ....marine algae
MFD............milk fat depression
NDF ....neutral detergent fibre
NDFD..........neutral detergent fibre disappearance
ns.................not significant
OM..............organic matter
P ....probability
PCR.............polymerase chain reaction
PUFA...........polyunsaturated fatty acid
qPCR...........quantitative real-time polymerase chain reaction
r...................Pearson correlation coefficient
R..................richness (diversity index)

rRNA........... ribosomal ribonucleic acid
RA................ rumenic acid
SA .... stearic acid
SED .... standard error of the difference
SFO.............. sunflower oil plus fish oil (Chapters II, III and IV)
SO .... sunflower oil
SOMA .... sunflower oil plus marine algae
SOFO .... sunflower oil plus fish oil (Chapters I, IV and V)
T................... time
TMR .... total mixed ration
T-RF............. terminal restriction fragment
T-RFLP........ terminal restriction fragment length polymorphism
VA .... vaccenic acid
VFA .... volatile fatty acid

SUMMARY

Potential benefits to human health have led to great interest in developing nutritional strategies for enhancing the concentration of some bioactive fatty acids, such as *cis*-9, *trans*-11 conjugated linoleic acid (CLA), in ruminant milk.

Diet supplementation with plant oils and marine lipids has been reported to be a good strategy for modulating milk fat composition in dairy cows, but very few studies have examined the effects in dairy sheep. Previous reports have shown that the inclusion of an oil rich in 18:2*n*-6, such as sunflower oil, in the diet of ewes increases the milk content of *cis*-9, *trans*-11 CLA with no detrimental effects on animal performance. In cows, the use of sunflower oil in combination with marine lipids rich in long-chain *n*-3 FA is known to induce larger increases in that CLA isomer and transference of 20:5*n*-3 and 22:6*n*-3 to milk, but this strategy has also been consistently associated with the syndrome of low-fat milk. On the other hand, sheep seem less prone to milk fat depression, but the information available is scant and further research would be necessary.

A series of five studies, corresponding to the five chapters into which this dissertation is divided, was therefore carried out with sheep to investigate the effects of supplementation with sunflower oil and either fish oil or marine algae on milk and digesta FA composition, rumen microbiota and fermentation characteristics, and animal performance.

In the study in Chapter I, a high-concentrate diet formulated for highly productive lactating sheep was supplemented with sunflower oil (SO) and fish oil (FO), either alone or in combination, to evaluate the impact of these nutritional strategies on milk FA profile and animal performance. Sixty-four Assaf ewes were distributed in 8 lots of 8 animals each and assigned to 4 treatments (2 lots/treatment): no lipid supplementation (control) or supplementation with 20 g of SO/kg (SO), 10 g of FO/kg (FO), or 20 g of SO plus 10 g of FO/kg (SOFO). Milk production and composition, including a complete FA profile, determined by complementary gas-liquid chromatography and Ag^{+}-HPLC analysis of FA methyl esters, were analyzed on days 0, 3, 7, 14, 21, and 28 on treatments. Supplementation with FO tended to reduce feed intake compared to the control

treatment (−15%), and its use in combination with SO (i.e., SOFO) resulted in a significant decrease in milk yield as well (−13%). All lipid supplements reduced milk protein content, and FO also reduced milk fat content by up to 21% when fed alone (FO) and 27% in combination with SO (SOFO). Although the mechanisms responsible for FO-induced milk fat depression are not yet well established, the fact that the *trans*-10, *cis*-12 CLA was always detected in very low concentrations and showed no changes with time would rule out this CLA isomer as the principal factor responsible for the reduction observed in milk fat content. On the other hand, the reduction might have been related to the observed increase in some milk *trans* FA that are putative inhibitors of milk fat synthesis, such as *trans*-9, *cis*-11 CLA, together with the almost 63% decrease in 18:0 (consistent with the theory of reduced milk fat fluidity). When compared with the control, supplementation with SO or SOFO improved the milk content of *cis*-9, *trans*-11 CLA up to 4-fold, whereas FO-containing diets also increased milk long-chain *n*-3 FA, mainly 22:6*n*-3 (with mean contents of 0.29 and 0.38% of total FA for SOFO and FO, respectively), and reduced the *n*-6:*n*-3 FA ratio to approximately half the control value, which appears to be of great importance for human health. All lipid supplements resulted in high levels of *trans* 18:1, mainly *trans*-11 18:1. However, there was also a concomitant enhancement of *trans*-10 18:1 that would disallow from considering that this milk has a healthier fat profile before determining the specific role of each individual FA and ensuring that this *trans* FA is at least innocuous in relation to cardiovascular disease risk.

Since the joint use of SO and FO induced the greatest modifications in animal performance and milk FA composition, the studies described in Chapters II, III, and IV refer only to this combination and were designed to examine its effects on rumen function and bacterial community.

The second study (Chapter II) was conducted with cannulated ewes and included in vivo, in situ and in vitro assays with the aim of determining whether the disturbances in animal performance observed in the first experiment could be attributed to a negative impact of lipid supplements on ruminal fermentation.

Four ruminally cannulated ewes were fed the same control diet than in the first experiment, for a 14-day adaptation period. Afterwards, they were fed the SOFO diet for a further 11 days. On days 0, 3 and 10 on the experimental diet, rumen fluid was sampled at 0, 1.5, 3, 6 and 9 h after the morning feeding, for analysis of pH, and ammonia, lactate and total volatile fatty acid (VFA) concentrations. Alfalfa hay was incubated in situ, using the nylon bag technique, for 12 and 24 h to examine the effect of oil supplementation on ruminal disappearance of DM, crude protein (CP) and neutral-detergent fibre (NDF). On days 0 and 11, rumen fluid was collected just before the morning feeding and used to incubate alfalfa hay and the control and SOFO diets by means of the in vitro gas production technique. The mean concentrations of acetate and butyrate were reduced by oil supplementation and the total VFA showed a tendency to be lower with the SOFO diet (139.0 vs. 122.1 mmol/L). However, none of the other in vivo ruminal fermentation parameters were affected by the treatment. The oil supplementation affected neither in situ rumen disappearance of alfalfa hay, nor rates of gas production. On the other hand, a significant, but little reduction in cumulative gas production was observed when the experimental diets were incubated with rumen fluid derived from animals fed the oil-rich diet. Overall, the results suggest that the supplementation of high-concentrate diets with SOFO had little effect on ruminal fermentation and therefore its use to improve the nutritional value of ruminant-derived products cannot be precluded.

The third study (Chapter III) was also carried out with cannulated ewes, following the same experimental design as in Chapter II, to examine time-dependent changes in the accumulation of biohydrogenation intermediates in the rumen after the inclusion of SOFO (20 g of SO plus 10 g of FO/kg DM) in a high-concentrate diet. Detailed analysis of the FA composition of rumen digesta collected on days 0, 3, and 10 on diet was performed and, although it was not a prime objective of the study, nutritional assays were also included to provide further information on the influence of these oils on ruminal fermentation characteristics. Identification of biohydrogenation intermediates was conducted

by gas chromatography-mass spectrometry analysis of 4,4-dimethyloxazoline derivatives of FA methyl esters. The results confirmed that the inclusion of SOFO in the diet had no effect on rumen pH, VFA concentrations or nutrient digestion. However, it altered the FA composition of ruminal digesta, changes that were characterised by time-dependent decreases in 18:0 and 18:2*n*-6 and accumulation of *trans* 16:1, *trans* 18:1, 10-O-18:0, and *trans* 18:2. Lipid supplements also enhanced the proportion of 20:5*n*-3 and 22:6*n*-3 in digesta and resulted in numerical increases in *cis*-9, *trans*-11 CLA concentrations, but decreased the relative abundance of *trans*-10, *cis*-12 CLA, with no evidence of a shift in ruminal biohydrogenation pathways towards *trans*-10 18:1 formation. Furthermore, detailed analysis revealed the appearance of several unique 20:1, 20:2, 22:1, 22:3, and 22:4 metabolites in ruminal digesta that accumulated over time, providing the first indications that ruminal metabolism of 20:5*n*-3, 22:5*n*-3, and 22:6*n*-3 may proceed via the reduction of the double bond closest to the carboxyl group.

In Chapter IV, changes in the rumen bacterial community of the sheep used in the experiments reported in Chapters I and III (i.e., lactating and cannulated ewes, respectively) were monitored to provide an insight into the effect of SO and FO on rumen microbial populations. It is remarkable that, despite the impact of the microbiota on the biohydrogenation of dietary unsaturated FA, and subsequently on the development of healthier dairy products, there is no in vivo study on this subject in sheep. First, in the experiment with lactating ewes, rumen fluid samples were taken after 21 days on treatments from 4 animals per lot, for DNA extraction and fluorescence in situ hybridization (FISH) analysis. Then, in the experiment with cannulated ewes, rumen content samples were taken for DNA extraction and FISH analysis (fluid) after 0, 3 and 10 days on the oil-rich diet. The total bacteria and the *Butyrivibrio* group were studied in microbial DNA by terminal restriction fragment length polymorphism (T-RFLP), and real time PCR (qPCR) was used to quantify *Butyrivibrio* bacteria producing *trans*-11 18:1 or 18:0. In rumen fluid samples, total bacteria, and clostridial clusters IX and XIV were analysed by FISH. The dietary supplementation with SOFO induced important

changes in the total bacteria and *Butyrivibrio* population, but there was a high inter-individual variation and the speed of the effects depended on the individual microbial composition. Analyses by T-RFLP and FISH showed increases in cluster IX bacteria with SOFO, presumably *Quinella*-like microorganisms. The T-RFLP also showed a variable effect of lipid supplementation on different microorganisms of the family *Lachnospiraceae*, which includes the cultured bacteria known to be actively involved in rumen biohydrogenation. The abundance of *trans*-11 18:1- and 18:0-producing *Butyrivibrio* relative to total bacteria, estimated by qPCR, were low (0.28 and 0.18%, respectively, in rumen fluid, and 0.86 and 0.81% in rumen contents), and only the concentration of the latter (i.e., 18:0-producing bacteria) was reduced by diets containing FO, although only significantly in lactating ewes, while the numbers of *trans*-11 18:1-producing *Butyrivibrio* remained always unchanged. These results suggest that the bacteria commonly thought to carry out the biohydrogenation would not play a dominant role in this process, and therefore other yet-uncultivated microorganisms might be more relevant.

Once these studies with SO and FO were completed, a last experiment (Chapter V) was conducted to examine the effect of the joint use of SO and marine algae (MA) on dairy sheep performance and milk FA profile. The same experimental procedures as in the first experiment (i.e., that reported in Chapter I) were followed, although in this case, three incremental levels of MA were tested. Fifty Assaf ewes were distributed in 10 lots of 5 animals each and allocated to 5 treatments (2 lots/treatment): no lipid supplementation (control) or supplementation with 25 g of SO/kg DM plus 0, 8, 16 or 24 g of MA/kg DM (SO, $SOMA_1$, $SOMA_2$, and $SOMA_3$, respectively). Neither DM intake nor milk yield were significantly affected by lipid addition, but all MA supplements decreased milk fat content from day 14 onward, reaching a 30% reduction after 28 days on $SOMA_3$. As mentioned above for FO-induced milk fat depression, these reductions in milk fat content might be related not only to the joint action of some putative fat synthesis inhibitors, but also to the limited ability of the mammary gland to

maintain a desirable milk fat fluidity, that would have been caused by the noticeable increase in *trans* 18:1 together with the lowered availability of 18:0 for *cis*-9 18:1 synthesis through Δ^9-desaturase. Supplementation with SO plus MA resulted in larger increases in *cis*-9, *trans*-11 CLA than those observed with SO alone, achieving a mean content of 3.22% of total FA, which represents more than a 7-fold increase compared with the control. *Trans*-11 18:1 was also significantly enhanced (on average +794% in SOMA treatments), as was 22:6*n*-3 content, although the transfer efficiency of the latter, from the diets to the milk, was very low (5%). All the same, the highest levels of MA inclusion ($SOMA_2$ and $SOMA_3$) reduced the milk *n*-6:*n*-3 ratio, achieving a value as low as 1.4 after 28 days on diet. On the other hand, MA supplements caused an important enhancement of *trans*-10 18:1, as it had been observed with FO-containing diets (Chapter I).

Finally, the dissertation includes a General discussion integrating all the chapters. An important consideration in that section is the comparison between results obtained in Chapters I and V, together with the potential associations between changes in ruminal lipid metabolism and variations in milk FA profile.

RESUMEN

Los posibles efectos beneficiosos para la salud humana de algunos ácidos grasos con propiedades bioactivas, como es el caso del isómero *cis*-9, *trans*-11 del ácido linoleico conjugado (CLA), han motivado un gran interés en el desarrollo de estrategias nutricionales que permitan aumentar su concentración en la leche de los rumiantes.

La suplementación de la dieta con aceites vegetales y lípidos de origen marino es una estrategia efectiva para modificar la composición de la grasa láctea en el ganado vacuno lechero. Sin embargo, el número de estudios llevados a cabo en el ovino es aún muy limitado, aunque algunos trabajos han mostrado que la inclusión en la dieta de ovejas de un aceite rico en 18:2*n*-6, como es el de girasol, aumenta el contenido del *cis*-9, *trans*-11 CLA de la leche sin perjudicar al rendimiento productivo de los animales. En las vacas, se sabe que el uso de aceite de girasol junto con lípidos de origen marino (ricos en ácidos grasos *n*-3 de cadena larga) induce aumentos incluso mayores en la concentración de este isómero del CLA en la leche, así como la transferencia a esta de 20:5*n*-3 y 22:6*n*-3. No obstante, dicha estrategia de suplementación ha sido sistemáticamente relacionada con el denominado síndrome de baja grasa en la leche, al cual parece que las ovejas son menos propensas. De todos modos, en el ovino no existe suficiente información al respecto y sería necesaria más investigación.

Por ello, en esta tesis se llevó a cabo una serie de cinco experimentos con ovejas, correspondientes a los cinco capítulos en los cuales se divide la memoria, con el objetivo de investigar el efecto de la suplementación con aceite de girasol y, o bien aceite de pescado, o bien microalgas marinas, sobre la composición de los ácidos grasos de la leche y de la digesta, la microbiota y la fermentación ruminal, y el rendimiento productivo de los animales.

En el estudio descrito en el Capítulo I, una dieta rica en alimentos concentrados, formulada para ovejas lecheras de alta producción, se suplementó con aceites de girasol (SO) y de pescado (FO), tanto de forma individual como conjunta, para evaluar su impacto sobre el perfil de ácidos grasos de la leche y el rendimiento productivo de los animales. Sesenta y cuatro ovejas de raza assaf se

distribuyeron en 8 lotes de 8 animales cada uno y se asignaron a 4 tratamientos (2 lotes/tratamiento): dieta sin suplementación lipídica (control) o suplementada con 20 g de SO/kg (SO), con 10 g de FO/kg (FO), o con 20 g de SO más 10 g de FO/kg (SOFO). Los días 0, 3, 7, 14, 21 y 28 de experimento se registró la producción de leche y se recogió una muestra para analizar su composición, incluyendo un perfil completo de ácidos grasos mediante análisis complementarios de cromatografía de gas-líquido y Ag^+-HPLC. La ingestión de alimento tendió a ser menor con la suplementación con FO, en comparación con el tratamiento control (−15%) y el uso combinado de SO y FO (es decir, SOFO) provocó, además, una disminución significativa de la producción de leche (−13%). Todos los suplementos lipídicos redujeron el contenido proteico de la leche y, en el caso del FO, también disminuyó el contenido graso, hasta un 21% cuando se administró solo (FO) y hasta un 27% cuando se hizo en combinación con SO (SOFO). Aunque los mecanismos responsables del síndrome de baja grasa en la leche causado por el aceite de pescado aún no están bien definidos, la concentración de *trans*-10, *cis*-12 CLA en la leche fue siempre baja y no mostró variaciones importantes con el tiempo, lo cual descartaría que este isómero del CLA fuera el principal responsable de la disminución del contenido graso de la leche. Por el contrario, la reducción podría haber estado relacionada con el aumento de algunos ácidos grasos *trans* que son supuestos inhibidores de la síntesis grasa, como por ejemplo el *trans*-9, *cis*-11 CLA, junto con la caída de un 63% del contenido de 18:0 (lo cual coincidiría con la teoría de la disminución de la fluidez de la grasa de la leche). Comparada con el control, la suplementación con SO o SOFO mejoró el contenido lácteo de ácidos grasos *n*-3 de cadena larga, principalmente el 22:6*n*-3 (que mostró contenidos medios de 0,29 y 0,38% sobre el total de ácidos grasos en las dietas SOFO y FO, respectivamente), y redujo la relación de ácidos grasos *n*-6:*n*-3 hasta aproximadamente la mitad del valor obtenido para el control, lo cual podría ser de gran importancia para la salud humana. Todos los suplementos lipídicos dieron lugar a niveles elevados de *trans* 18:1, principalmente *trans*-11 18:1. Sin embargo, produjeron también un incremento del *trans*-10 18:1, lo cual hace que no se pueda

considerar que esta leche tiene un perfil más saludable de ácidos grasos hasta que se determine el papel específico de cada uno de ellos y se asegure que este *trans* 18:1 es, al menos, inocuo en relación al riesgo de sufrir enfermedades cardiovasculares.

Dado que el uso conjunto de SO y FO provocó las mayores modificaciones en el rendimiento productivo de los animales y en la composición de los ácidos grasos de la leche, los estudios descritos en los Capítulos II, III y IV se refieren solo a esta combinación de aceites, y fueron diseñados para examinar su efecto sobre la función ruminal (incluida la fermentación de la dieta y la biohidrogenación de los ácidos grasos) y la comunidad bacteriana.

El segundo trabajo (Capítulo II) se realizó con ovejas canuladas e incluyó estudios in vivo, in situ e in vitro, con el objetivo de determinar si las alteraciones observadas en el rendimiento productivo de los animales durante el primer experimento pudieron ser debidas a un impacto negativo de los aceites sobre la fermentación ruminal. Cuatro ovejas canuladas en el rumen fueron alimentadas con la misma dieta control utilizada en el primer estudio, durante un periodo de adaptación de 14 días, y a continuación, se les ofertó la dieta SOFO durante 11 días más. Los días 0, 3 y 10 del experimento, se tomaron muestras de fluido ruminal tras 0, 1,5, 3, 6 y 9 h desde la oferta matinal del alimento, para medir su pH y analizar la concentración de amoniaco, lactato y ácidos grasos volátiles (VFA). También se incubó in situ un heno de alfalfa, mediante la técnica de las bolsas de nailon y durante 12 y 24 h, para examinar el efecto de la suplementación con aceites sobre la desaparición de materia seca, proteína bruta y fibra neutro detergente en el rumen. Los días 0 y 11 del experimento, se recogió fluido ruminal justo antes de la oferta de alimento de la mañana para ser usado como inóculo en la incubación in vitro de un heno de alfalfa y de las dietas control y SOFO, siguiendo la técnica de producción de gas. Las concentraciones medias de acetato y butirato se redujeron tras la suplementación con aceites y la de VFA totales mostró una tendencia a ser también menor con la dieta SOFO (139.0 contra 122.1 mmol/L). Sin embargo, ninguno de los otros parámetros indicativos de la

fermentación ruminal in vivo se vio afectado por el tratamiento. La adición de aceites tampoco afectó a la desaparición in situ del heno de alfalfa, ni a los ritmos de producción de gas. Sí se observó, sin embargo, una reducción estadísticamente significativa, pero pequeña, de la producción acumulada de gas en aquellos casos en los que las dietas experimentales fueron incubadas con el fluido ruminal obtenido de los animales alimentados con la dieta que contenía aceites. En conjunto, estos resultados sugieren que la suplementación de una dieta rica en alimentos concentrados con SOFO apenas afectó a la fermentación ruminal y, por lo tanto, no se podría descartar su uso para mejorar el valor nutricional de los productos derivados de los rumiantes.

El tercer estudio (Capítulo III) también se llevó a cabo con ovejas canuladas y siguió el mismo diseño experimental del Capítulo II. Su objetivo fue examinar los cambios en la acumulación de metabolitos intermedios de la biohidrogenación a lo largo del tiempo, como consecuencia de la inclusión de SOFO (20 g de SO más 10 g de FO/kg MS) en una dieta rica en alimentos concentrados. Los días 0, 3 y 10 del experimento, se tomaron muestras del contenido ruminal de las ovejas para el análisis detallado de la composición de sus ácidos grasos y, aunque no era el objetivo principal de este trabajo, estudiar algunos parámetros que proporcionaran más información acerca de la influencia de estos aceites sobre la fermentación ruminal. La identificación de los metabolitos intermedios de la biohidrogenación se llevó a cabo mediante el análisis por cromatografía de gases-espectrometría de masas de los derivados 4,4-dimetiloxazolínicos de los ésteres metílicos de los ácidos grasos. Los resultados obtenidos confirmaron que la inclusión de SOFO en la dieta no tuvo un efecto negativo sobre el pH, las concentraciones de VFA o la digestión ruminal. Por el contrario, la composición de los ácidos grasos de la digesta ruminal sí se vio alterada, caracterizándose los cambios observados por una reducción a lo largo del tiempo del 18:0 y el 18:2*n*-6, así como por la acumulación de *trans* 16:1, *trans* 18:1, 10-O-18:0 y *trans* 18:2. Los suplementos lipídicos también aumentaron la proporción de 20:5*n*-3 y de 22:6*n*-3 en el contenido digestivo y se observaron aumentos numéricos en las

concentraciones de *cis*-9, *trans*-11 CLA y una disminución en la abundancia relativa de *trans*-10, *cis*-12 CLA, sin que hubiera evidencia de un cambio en las rutas de biohidrogenación hacia la formación de *trans*-10 18:1. Además, el análisis minucioso reveló la presencia de varios metabolitos 20:1, 20:2, 22:1, 22:3 y 22:4 únicamente en el contenido digestivo, proporcionando las primeras indicaciones existentes de que el metabolismo ruminal del 20:5*n*-3, 22:5*n*-3 y 22:6*n*-3 podría comenzar con la reducción del doble enlace más cercano al grupo carboxilo.

En el Capítulo IV, se analizaron los cambios de las comunidades bacterianas del rumen en las ovejas utilizadas durante los experimentos descritos en los Capítulos I y III (es decir, las ovejas en lactación y las canuladas, respectivamente) para investigar el efecto del SO y del FO sobre dichas poblaciones microbianas. En este sentido, resulta sorprendente que, a pesar del conocido impacto de la microbiota ruminal sobre la biohidrogenación de los ácidos grasos de la dieta y, por consiguiente, sobre el desarrollo de productos lácteos más saludables, en el ovino no existiera ningún estudio in vivo sobre dicho tema. En primer lugar, en el experimento con ovejas en lactación, se tomaron muestras del fluido ruminal de 4 animales de cada lote, tras 21 días de tratamiento, para la extracción del ADN y su análisis mediante hibridación fluorescente in situ (FISH). Después, en el experimento con ovejas canuladas, se tomaron muestras del contenido ruminal tras 0, 3 y 10 días de suplementación con SOFO, para la extracción del ADN y su análisis mediante FISH (en el fluido). La abundancia de bacterias totales y del grupo de *Butyrivibrio* en el ADN microbiano se estudiaron mediante la técnica del polimorfismo de la longitud de los fragmentos terminales de restricción (T-RFLP), mientras que se utilizó la PCR a tiempo real (qPCR) para cuantificar aquellas bacterias del grupo de *Butyrivibrio* productoras de *trans*-11 18:1 o 18:0. En las muestras de fluido ruminal, se usó la técnica FISH para analizar las bacterias totales y de los clusters IX y XIV de los Clostridiales. La suplementación de la dieta con SOFO indujo cambios importantes en las bacterias totales y en las poblaciones de *Butyrivibrio*, aunque hubo una alta variación interindividual y la velocidad de los cambios dependió de la composición microbiana individual. Los

análisis mediante T-RFLP y FISH mostraron aumentos en unas bacterias del cluster IX, supuestamente microorganismos del género *Quinella* o similares, con la dieta SOFO. La T-RFLP también mostró un efecto variable de los aceites sobre diversos microorganismos de la familia *Lachnospiraceae*, que incluye aquellas bacterias cultivadas que se sabe que están implicadas activamente en la biohidrogenación ruminal. La abundancia de las bacterias del género *Butyrivibrio* productoras de *trans*-11 18:1 y de 18:0 respecto al total de bacterias, estimada por qPCR, fue siempre baja (0,28 y 0,18%, respectivamente, en el fluido, y 0,86 y 0,81% en el contenido ruminal), viéndose solo la concentración de las últimas (es decir, de las bacterias productoras de 18:0) reducida en las dietas que contenían FO, aunque de forma significativa únicamente en las ovejas en lactación; mientras que la abundancia de las bacterias del género *Butyrivibrio* productoras de *trans*-11 18:1 no se vio modificada en ningún caso. Estos resultados sugieren que las bacterias que comúnmente se consideraba que eran las principales responsables de la biohidrogenación ruminal, parecen no tener un papel dominante en este proceso y, por lo tanto, otras bacterias aún no cultivadas podrían ser más relevantes.

Tras haber finalizado los trabajos con SO y FO, se llevó a cabo un último experimento (Capítulo V) con el objetivo de estudiar el efecto de la suplementación de la dieta con SO más microalgas marinas (MA) sobre el rendimiento productivo de las ovejas y el perfil de ácidos grasos de su leche. Se siguieron los mismos procedimientos experimentales que en la primera prueba (es decir, la descrita en el Capítulo I) aunque, en este caso, se evaluaron tres niveles crecientes de MA. Así, cincuenta ovejas de raza assaf se distribuyeron en 10 lotes de 5 animales cada uno y se asignaron a 5 tratamientos (2 lotes/tratamiento): dieta sin suplementación lipídica (control) o suplementada con 25 g de SO/kg MS más 0, 8, 16 o 24 g de MA/kg MS (SO, $SOMA_1$, $SOMA_2$ y $SOMA_3$, respectivamente). La adición de lípidos no afectó ni a la ingestión de alimento ni a la producción de leche, si bien todas las dietas que incluyeron MA redujeron el contenido de grasa de la leche desde el día 14 en adelante, alcanzándose una disminución del 30% tras 28 días con el tratamiento $SOMA_3$. Tal y como se mencionó en el caso del

síndrome de baja grasa en la leche inducido por FO, la reducción del contenido de grasa láctea podría estar relacionado no solo con la acción sinérgica entre algunos metabolitos supuestamente inhibidores de la síntesis grasa, sino también con la limitada capacidad de la glándula mamaria para mantener la fluidez adecuada de la grasa láctea, lo que estaría relacionado, a su vez, con el marcado aumento de los *trans* 18:1 y con la menor disponibilidad de 18:0 para la síntesis de *cis*-9 18:1. Los aumentos del contenido lácteo de *cis*-9, *trans*-11 CLA fueron mayores en respuesta a la suplementación con SOMA que cuando se usó solo SO, alcanzándose un contenido medio del 3,22% del total de ácidos grasos, lo cual supone un valor 7 veces mayor que el del control. También se mejoró el contenido de *trans*-11 18:1 (de media, +794% para los tratamientos SOMA) y el de 22:6*n*-3, si bien la tasa de transferencia de este último fue muy baja (5%). Aun así, los niveles más altos de SOMA ($SOMA_2$ y $SOMA_3$) resultaron muy eficaces para disminuir la relación de ácidos grasos *n*-6:*n*-3 en la leche, lográndose valores tan bajos como un 1,4 tras 28 días de tratamiento. Por el contrario, los suplementos de MA causaron un aumento importante del *trans*-10 18:1, tal y como se había observado con el uso del FO (Capítulo I).

Por último, esta memoria incluye una Discusión general que integra todos los capítulos anteriores. Un aspecto importante de esa sección es la comparación entre los resultados obtenidos en los Capítulos I y V, así como la posible relación entre los cambios del metabolismo lipídico en el rumen y las variaciones del perfil de ácidos grasos de la leche.

GENERAL INTRODUCTION

Changes in dietary and lifestyle patterns over the past decades have deeply modified the health and nutritional status of the human population in developed and developing countries. Improvements in standards of living and food availability have been accompanied, however, by a growing incidence of diet-related chronic diseases, such as obesity, diabetes, some types of cancer, and cardiovascular disorders (WHO, 2003). However, besides being a major cause of chronic disease, diet can also play a key role in its prevention, so research into so-called functional foods has been driven by increasing health care costs and mounting scientific evidence on their ability to reduce the risk of disease and improve quality of life (Craig-Schmidt, 2006; Shingfield et al., 2008; Siró et al., 2008).

Although functional foods have not as yet been characterized by legislation in Europe, the European Food Information Council has defined them as those foods intended to be consumed as part of the normal diet and containing biologically active components offering the potential of enhanced health or reduced risk of disease (EUFIC, 2010). Functional foods, only marketed in industrialized countries, have always been well received in Japan, but were initially rejected in USA and Europe because of the importance that consumers give to taste. The production of new foods that combine potential health benefits with the taste of traditional products has therefore been a major challenge in these countries (Siró et al., 2008).

Much of the interest in these foods concerns the development of functional milk and dairy products, not least because their consumption is on the rise (Saxelin, 2008; Siró et al., 2008). It has been shown that the nutritional value of milk can be naturally enhanced through changes in the feeding systems of lactating ruminants (Lock and Bauman, 2004; Pulina et al., 2006; Chilliard et al., 2007) and, consequently, especial attention has been given to research in this field over the last few years, in particular in dairy cows (Lock and Bauman, 2004; Dewhurst et al., 2006). In goats, a number of studies have been made of the response to dietary changes (Bouattour et al., 2008; Luna et al., 2008; Bernard et al.,

2009). In sheep however, despite possible nutritional advantages of their milk over that of cows (Haenlein, 2001; Park et al., 2007; Recio et al., 2009), it has all too often been accepted that knowledge gained from dairy cattle can be directly extrapolated down to them, on the assumption that the only difference between these species is one of size. Ewes, therefore, have undeservedly received very little research attention (Haenlein, 2001; Sanz Sampelayo et al., 2007; Shingfield et al., 2010).

1. BIOACTIVE COMPONENTS IN MILK

Milk is intended to meet the nutritional requirements of young mammals, and constitutes an important supplier of energy, high-quality protein, vitamins and minerals for human consumers as well. While milk is widely recognized as a valuable natural source of beneficial amino acids, such as carnitine and taurine, and bioactive peptides with potential antihypertensive, antithrombotic, and antimicrobial activities (Park et al., 2007), the role of dairy fats is often less well understood (Elwood et al., 2008; Lock et al., 2008). In fact, milk fat has been a target of dieticians' criticisms owing to its significant contribution to total saturated fatty acid (FA) consumption (WHO, 2003), despite the presence of a number of health-promoting bioactive lipids, such as butyrate, branched-chain FA, conjugated linoleic acid (CLA), *trans*-11 18:1 (vaccenic acid, VA) and sphingomyelin (Shingfield et al., 2008).

Because of their potential to affect long-term human health, either beneficially or detrimentally, a great deal of attention has been given to dairy fats over the past two decades, in particular to saturated FA, long chain *n*-3 polyunsaturated fatty acids (PUFA), CLA, and *trans* FA (Pariza et al., 2001; Simopoulos, 2002; Kühlsen et al., 2005; Parodi, 2009). A brief overview of their reported biological properties is given below.

1.1. Saturated fatty acids

For over half a century, dietary guidelines aimed at reducing the risk of coronary heart disease have recommended a diet low in saturated fats, since these have been consistently associated with elevated serum cholesterol levels (Shingfield et al., 2008; Parodi, 2009). Thus, it is not surprising that milk fat, containing about 60% of the FA as saturated (Jensen, 2002), has been perceived to be detrimental to health (Lock et al., 2008). Nevertheless, it is worth noting that probably only 12:0, 14:0 and 16:0 would be cholesterol-raising saturates, while other saturated FA in milk (4:0, 6:0, 8:0, 10:0, and 18:0) might suppose no risk of cardiovascular disease (Lock et al., 2008; Parodi, 2009).

Recently, a review of Parodi (2009) has disputed the collective perception that reduced saturated FA intake decreases the risk of coronary heart disease. As mentioned above, not all saturated fats behave in the same way and, since most studies are based on epidemiological studies, concerted and targeted research effort would be necessary to unravel the potential relationship between particular saturated FA and human disease (Elwood et al., 2008; Parodi, 2009). In any event, the recognized beneficial effect of milk and dairy products on human health (Elwood et al., 2008) provides further support for the importance of recognizing that dairy saturated FA are not consumed as a single dietary entity but along with other milk nutrients that appear to counteract any potential negative effect (Lock et al., 2008; Parodi, 2009).

1.2. Long chain n*-3 fatty acids*

A considerable amount of evidence indicates that *n*-3 PUFA are essential for neuronal development in the foetus and the new-born, as well as for adequate neurological and cognitive functions in adults (Salem et al., 2001; Bourre, 2005). Docosahexaenoic acid (DHA; 22:6*n*-3), eicosapentaenoic acid (EPA; 20:5*n*-3), and α-linolenic acid (18:3*n*-3) would also have beneficial cardiovascular and anti-inflammatory properties, so opportunities to increase their content in foods are being explored (Lock and Bauman, 2004; Bourre, 2005). In addition, the balance of

n-6:*n*-3 FA in the diet is an important determinant of health, and a ratio lower than 4 is related to a reduction in the risk of highly prevalent chronic diseases, including stroke, cancer, and inflammatory and autoimmune diseases (Simopoulos, 2002). In some Western diets, where the average *n*-6:*n*-3 FA ratio is higher than 15, both the increase of *n*-3 FA and the reduction of *n*-6 FA would thus be necessary to achieve a healthier diet (Simopoulos, 2002).

1.3. Conjugated linoleic acid

The term conjugated linoleic acid (CLA) refers to a mixture of positional and geometric isomers of linoleic acid (18:2*n*-6) with conjugated double bonds. The predominant sources of CLA in human diet are dairy products (representing more than 60% of total daily intake), with the *cis*-9, *trans*-11 isomer (rumenic acid, RA) constituting a 70-85% of total CLA in milk (Luna et al., 2005; Palmquist et al., 2005). Since the discovery of the inhibitory effect of RA on the growth of a number of cancer cell lines (Ha et al., 1987; Pariza, 2001), numerous biological properties have been attributed to CLA isomers, such as antiobesitic, anticarcinogenic, antiatherogenic, antidiabetogenic, immunomodulatory, apoptotic and osteosynthetic effects (Pariza et al., 2001; Benjamin and Spener, 2009). However, it is becoming increasingly apparent that these actions are isomer-specific and, for example, that *trans*-9, *trans*-11 CLA seems to have a more potent antiproliferative and apoptotic effect than RA (Shingfield et al., 2008). *Trans*-10, *cis*-12 CLA appears to have interesting health-promoting properties too, such as decreasing body fat, but some recent studies have reported its potential negative effects, namely induction of colon carcinogenesis, fatty liver and insulin resistance (Benjamin and Spener, 2009; Navarro et al., 2010). Nevertheless, it is important to emphasize that ruminant-derived foods contain very small amounts of *trans*-10, *cis*-12 CLA (Palmquist et al., 2005) and that the administration of mixtures of CLA isomers or their combination with long chain *n*-3 FA could offset the few adverse effects observed so far (Benjamin and Spener, 2009). Overall, results from human clinical

studies indicate more beneficial health effects of CLA, but more biomedical trials are required to provide conclusive evidence for particular isomers.

1.4. Trans *fatty acids*

The European Food Safety Authority defines *trans* FA as unsaturated FA with at least one double bond in the *trans* configuration (EFSA, 2004). Although this definition would include PUFA with conjugated double bonds (e.g., CLA), legislation in some countries (e.g., Denmark, U.S.A. and Canada) explicitly excludes this class of FA (Kühlsen et al., 2005).

There are two major sources of dietary *trans* FA: partially hydrogenated vegetable oils found in many industrially prepared foods and ruminant-derived products, mainly dairy fats, with *trans* 18:1 isomers being quantitatively the predominant *trans* FA in both cases (Craig-Schmidt, 2006; Shingfield et al., 2008). Legislation or advice on limiting *trans* FA has, in many instances, been restricted to those from industrial foods (Kühlsen et al., 2005), since their consumption has been consistently associated with cardiovascular disease, whereas natural *trans* FA in dairy products have been considered innocuous (Mozaffarian et al., 2006; Chardigny et al., 2008). However, when direct comparison is possible, feeding large amounts of ruminant *trans* FA has been shown to have similar adverse effects on cholesterol homeostasis to industrial fats, but it should be noted that the levels tested were unlikely to be found in conventional dairy foods (Motard-Bélanger et al., 2008). Furthermore, ruminant and industrial fats contain the same *trans* 18:1 isomers, but their relative proportion differs significantly (Figure 1; Kühlsen et al., 2005; Craig-Schmidt, 2006), which is of crucial importance regarding the unfavourable effects mentioned for particular FA. For instance, studies with animal models suggest that *trans*-10 18:1, a relatively abundant isomer in industrial fats, may contribute to an increased risk of cardiovascular disease (Roy et al., 2007), while *trans*-11 18:1 (vaccenic acid; VA), the major *trans* 18:1 in dairy foods, might be protective (Tyburczy et al., 2009). This latter FA has also been reported to have anti-carcinogenic properties, through its conversion to

RA, while the association of other *trans* FA with cancer risk is poorly understood and remains controversial (Smith et al., 2009). Finally, *trans* FA with more than one double bond (e.g., *trans*-9, *trans*-12 18:2 and *trans*-9, *cis*-12 18:2) have recently been related to an increased risk of myocardial infarction (Shingfield et al., 2008).

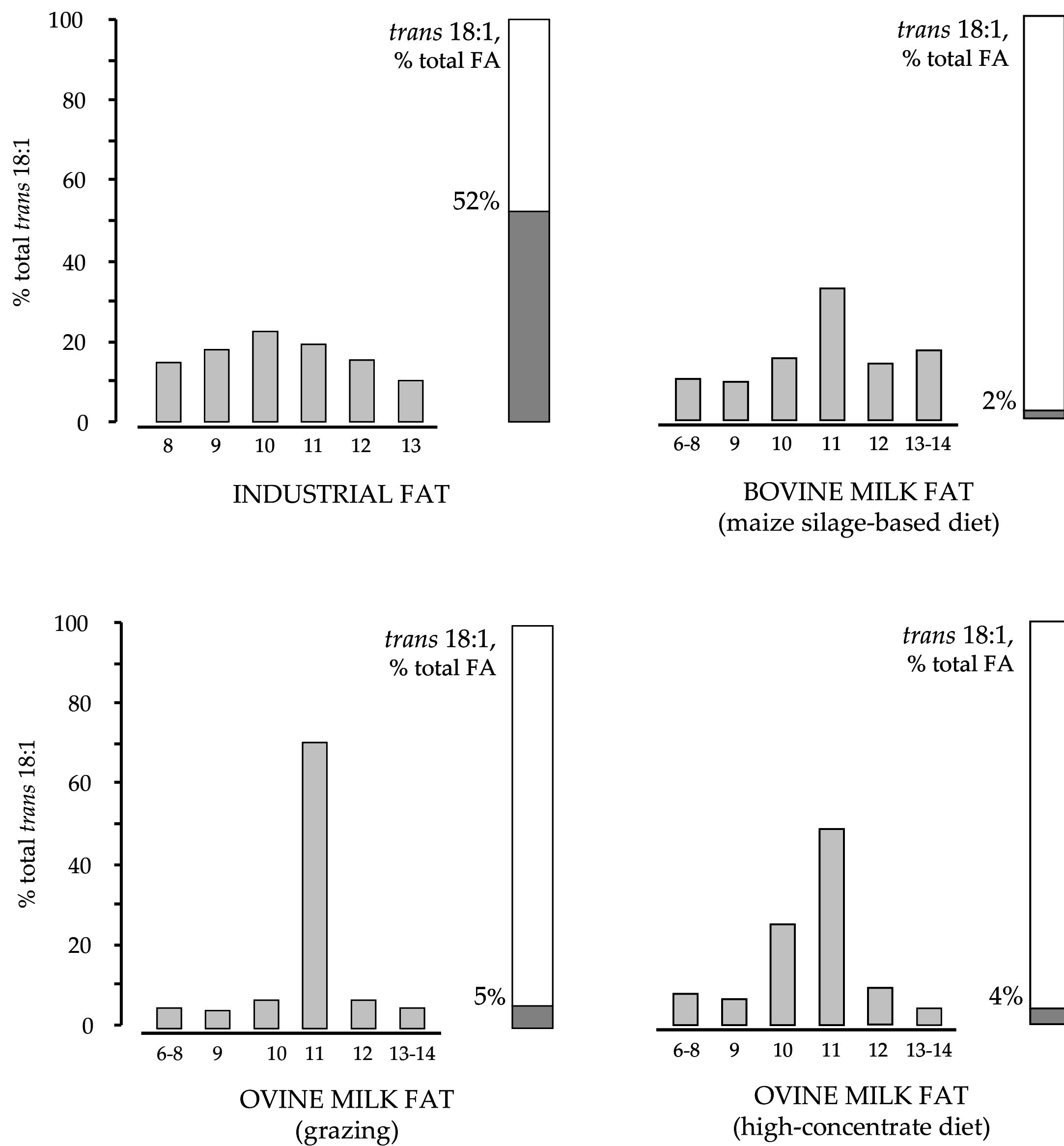

Figure 1. Distribution of *trans* 18:1 isomers in industrial and ruminant dairy fats. Values on the x-axis indicate the position of the double bond. (Data from Shingfield et al., 2006, Gómez-Cortés et al., 2009, and Tyburczy et al., 2009)

2. METABOLIC ORIGINS OF MILK LIPIDS

2.1. Mammary lipid synthesis

Milk fat consists predominantly of triacylglycerides (> 95% of total milk lipids) containing over 400 individual FA (Jensen, 2002), only a few of which are present in amounts greater than 1% (Table 1). Ovine milk typically contains a higher fat content (3.6-10%) than caprine (3.5-5.6%) and bovine (3-5%) milks (Jensen, 2002; Park et al., 2007; Raynal-Ljutovac et al., 2008). Nevertheless, the main characteristic of small ruminant milk fat is the higher content in the metabolically valuable caproic (6:0), caprylic (8:0) and capric (10:0) acids (Park et al., 2007; Raynal-Ljutovac et al., 2008; Table 1), which, like other short- and medium-chain FA, arise almost exclusively from de novo mammary synthesis from acetate and β-hydroxybutyrate (Van Soest, 1994). Long-chain FA (18 or more carbons) are derived from the uptake of circulating plasma lipids, and FA with 16 carbons originate from both sources (Van Soest, 1994).

The distribution of FA in milk triacylglycerides is species-specific and not random, since the necessity for liquidity of the milk fat globule requires the esterification of FA to triacylglycerides in combinations that have a melting point at or below body temperature (Timmen and Patton, 1989; Jensen, 2002). The principal means of assuring this liquidity is the incorporation of short-chain FA (4:0-10:0, melting point: –8 to 31 ºC) and the conversion of 18:0 (stearic acid) to *cis*-9 18:1 (oleic acid; melting point: 69 and 14 ºC, respectively) through Δ^9-desaturase activity (Timmen and Patton, 1989).

Besides stearic acid, a number of other FA can serve as substrates for Δ^9-desaturase in the mammary gland and other animal tissues. In this respect, the ability of this enzyme to convert *trans*-11 18:1 to *cis*-9, *trans*-11 CLA has generated much interest in the scientific community (Palmquist et al., 2005) because between 64 and 97% of the milk RA is estimated to result from endogenous synthesis in the mammary gland from VA (Lock and Garnsworthy, 2002; Mouriot et al., 2009; Palmquist et al., 2005).

Table 1. *Main fatty acids (g/100 g total FA) in milk fat of sheep, goats and cows under similar feeding practices (high-concentrate diet).*

	Sheep[a]	Goat[b]	Cow[c]
4:0	4.12	2.38	3.28
6:0	3.71	2.47	2.70
8:0	3.45	2.74	1.62
10:0	10.24	10.58	4.26
cis-9 10:1	0.42	0.27	0.43
12:0	5.10	5.72	5.14
14:0	10.18	12.07	12.79
cis-9 14:1	0.16	0.23	1.49
15:0	0.83	1.20	1.70
16:0	25.06	29.85	28.73
cis-9 16:1	1.41	0.78	1.81
17:0	0.58	0.57	0.79
18:0	6.23	4.88	5.77
cis-9 18:1	13.84	13.70	14.90
trans-10 18:1	0.84	0.44	2.96
trans-11 18:1	2.23	1.17	1.04
Total 18:1	19.35	16.86	21.11
cis-9, *cis*-12 18:2	2.87	2.41	2.96
Total non-conjugated 18:2	3.67	2.73	3.09
cis-9, *trans*-11 18:2	0.94	0.82	0.60
trans-10, *cis*-12 18:2	0.01	<0.01	<0.01
Total CLA	1.10	0.93	0.70
cis-9, *cis*-12, *cis*-15 18:3	0.46	0.19	0.09
Σ saturates	70.79	71.26	70.80
Σ monounsaturates	22.46	22.39	25.09
Σ polyunsaturates	5.23	5.03	3.81
cis-9, *trans*-11 18:2/(*cis*-9, *trans*-11 18:2 + *trans*-11 18:1)	0.30	0.41	0.37

[a]Hervás et al. (2008).
[b]Bernard et al. (2009).
[c]Roy et al. (2006).

2.2. Rumen lipid metabolism

Extensive lipid metabolism results in marked differences between the FA profile of fats entering (mostly unsaturated; 18:2*n*-6 and 18:3*n*-3) and leaving (mostly saturated; 18:0) the rumen (Harfoot and Hazlewood, 1997; Jenkins et al., 2008). On entering the rumen, dietary fat is subject to hydrolysis by microbial lipases,

releasing free FA (Harfoot and Hazlewood, 1997). To reduce the toxic effect of unsaturated FA on microbial growth (Maia et al., 2007), some rumen microorganisms are able to biohydrogenate them to saturated FA (Harfoot and Hazlewood, 1997; Paillard et al., 2007). At present, there is a consensus that bacteria are largely responsible for ruminal biohydrogenation, protozoa having this activity owing to ingested bacteria, whilst the contribution of anaerobic fungi is practically negligible (Yáñez-Ruiz et al., 2006; Maia et al., 2007; Jenkins et al., 2008).

The impact of microbial biohydrogenation on the development of healthier dairy products, by changing the profile of FA available for absorption and incorporation into milk fat, has renewed interest in ruminal lipid metabolism (Wallace et al., 2007; Jenkins et al., 2008; Kim et al., 2008). In this regard, considerable progress has been made in establishing the pathways of linoleic acid (18:2*n*-6) biohydrogenation, which was previously thought to involve only three steps: an initial isomerization to *cis*-9, *trans*-11 CLA, followed by hydrogenation to *trans*-11 18:1 and, subsequently, to 18:0 (Figure 2; Harfoot and Hazlewood, 1997). However, more recently, other 18:2 and 18:1 metabolites have also been identified, demonstrating that linoleic acid biohydrogenation can also occur via the formation of 10,12 18:2 intermediates (Figure 2; Wallace et al., 2007) and other less known pathways, putatively via action on the *cis*-9 double bond (Honkanen et al., 2009). As outlined below, it has now been demonstrated that, despite the frequently lower concentration of these less prevalent intermediates (e.g., *trans*-10, *cis*-12 and *trans*-9, *cis*-11 CLA), their biological effects on the host ruminant may be of relevance (Shingfield and Griinari, 2007; Harvatine et al., 2009). As a consequence, recent work has been directed toward characterizing the additional alternative pathways of FA biohydrogenation and understanding the structure and physiological effects of their respective intermediates (Wallace et al., 2007; Jenkins et al., 2008).

As mentioned above, optimizing ruminal outflow of VA is of great interest because of its important contribution to the mammary synthesis of RA (Palmquist

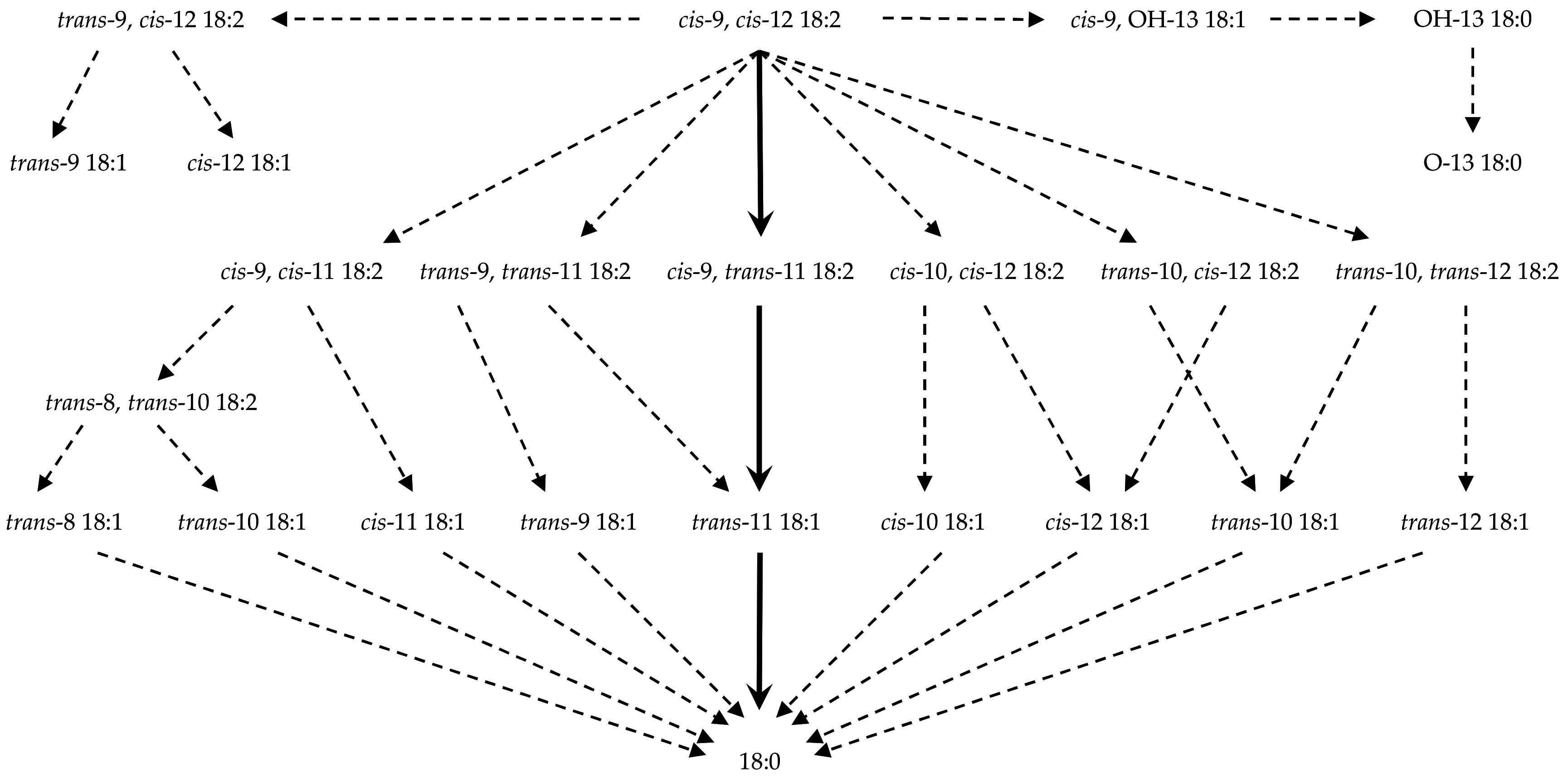

Figure 2. *Putative pathways describing 18:2*n*-6 metabolism in the rumen. Arrows with solid lines highlight the major biohydrogenation pathway while arrows with dashed lines describe the formation of minor fatty acid metabolites (from Shingfield et al., 2010).*

et al., 2005). Although the intermediates of 18:2*n*-6 biohydrogenation are relatively well characterized (Wallace et al., 2007; Jenkins et al., 2008; Honkanen et al., 2009), as yet little is known about ruminal metabolism of 18:3*n*-3 and other *n*-3 PUFA, such as 20:5*n*-3 and 22:6*n*-3 (Jenkins et al., 2008). Nevertheless, long-chain *n*-3 FA also contribute to VA accumulation, but via inhibition of the reduction of *trans* 18:1 intermediates to 18:0 (AbuGhazaleh and Jenkins, 2004). As this is thought to be caused by changes in the rumen bacterial populations, understanding which microbes are involved in FA metabolism is a key to developing novel strategies for improving the quality of ruminant-derived products by increasing the content of potentially beneficial unsaturated FA (Paillard et al., 2007; Kim et al., 2008).

As has already been pointed out, the biohydrogenation process has long been known to occur in the rumen as a mechanism of reducing the toxicity of dietary unsaturated FA to certain microorganisms (Maia et al., 2007). Traditionally, the bacteria responsible for the biohydrogenation of PUFA had been categorized as group A, capable of hydrogenating 18:2*n*-6 and 18:3*n*-3 to *trans*-11 18:1, and group B, which convert the same FA to 18:0 (Harfoot and Hazlewood, 1997). However, most of the information on the role of different microbial species in ruminal lipid metabolism predates the application of modern molecular techniques and, unfortunately, recent results have revealed that the explanation of rumen biohydrogenation in terms of early microbiological studies was quite weak (Jenkins et al., 2008). Modern phylogenetical analyses have shown that virtually all the bacteria reported to be actively involved in 18-carbon FA biohydrogenation belong to the *Butyrivibrio* group, which includes the genera *Butyrivibrio* and *Pseudobutyrivibrio* (Paillard et al., 2007; Jenkins et al., 2008). Remarkably, although a number of bacteria falling within the *Butyrivibrio* group are known to produce RA, VA or both from linoleic acid, only a few strains also have the ability to produce 18:0, forming a tight group clustering together close to the species *Butyrivibrio proteoclasticus*, the only cultured stearate producer (Wallace et al., 2006; Jenkins et al., 2008). In this regard, isolation of individual strains and pure-culture studies could provide further insight into the bacteria involved in rumen

biohydrogenation (Maia et al., 2007; Paillard et al., 2007), although it should be stressed that in vitro findings do not always reflect in vivo mechanisms (Boeckaert et al., 2008a; Kim et al., 2008) and so, to date, the effect of dietary unsaturated FA on the microbial community structure still remains largely unknown.

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3. EFFECT OF FEEDING ON MILK FAT COMPOSITION

Ruminant milk FA composition is related to intrinsic (species, breed, genotype, and physiological stage) and extrinsic (nutrition) factors. Of all these factors, feeding changes appear to affect it the most and constitute a natural and fast strategy to modulate milk fat composition in response to consumers' demand, which has led to an increasing number of studies on their effect on bovine, caprine and ovine milk FA profiles (Lock and Bauman, 2004; Dewhurst et al., 2006; Pulina et al., 2006; Chilliard et al., 2007; Sanz Sampelayo et al., 2007).

3.1. Effect of basal diet composition

Forages contain relatively low amounts of lipids but are often the greatest source of FA in ruminant diets (Palmquist and Jenkins, 1980; Dewhurst et al., 2006). In addition, both level and type of forage are important determinants of ruminal lipid metabolism and, consequently, of milk FA composition (Dewhurst et al., 2006; Mele et al., 2006; Roy et al., 2006).

Pasture is a rich source of 18:3*n*-3 (Palmquist and Jenkins, 1980) and, compared to conserved forages and total mixed rations (TMR), results in increased levels of milk 18:3*n*-3, RA and VA, and decreased 10:0-16:0 content (Chilliard et al., 2007; Gómez-Cortés et al., 2009). Higher pasture quality in spring (young and growing) than in summer (senescence) has a positive influence on milk FA composition, the physiological stage of forages being therefore of significance when making hay or silage (Dewhurst et al., 2006).

It has been speculated that dairy sheep production systems based on pasture are responsible for the usually higher levels of RA in ovine milk compared with

that of other ruminants (Park et al., 2007; Sanz Sampelayo et al., 2007). However, some direct comparisons between goats and sheep under the same feeding practices have still shown higher milk RA content in the latter (Tsiplakou et al., 2006; Tsiplakou and Zervas, 2008), which could be attributed to differences in Δ^9-desaturase gene expression (Tsiplakou et al., 2009). Furthermore, inherent inter-species differences may also exist in ruminal lipid metabolism. For example, low forage:concentrate ratios (< 40:60) are known to induce a shift toward *trans*-10 18:1 at the expense of *trans*-11 18:1 in rumen digesta lipids and milk in dairy cows, often accompanied by reductions in milk fat content (Bauman and Griinari, 2001; Roy et al., 2006). These effects are, however, of much smaller magnitude in dairy sheep and goats (Park et al., 2007; Gómez-Cortés et al., 2008; Shingfield et al., 2010) although information on small ruminants is still too scant to offer a whole picture.

3.2. Effect of diet supplementation with plant lipids

For decades, dietary lipid supplementation has been used to meet the energy requirements in dairy farming in unfavourable areas, where food supply can be temporarily scarce, and in high productive lactating ruminants (Palmquist and Jenkins, 1980; Gargouri et al., 2006; Sanz Sampelayo et al., 2007). In most cases, protected oils (e.g., calcium soaps of palm oil) were supplied with the aim of sustaining high yields of milk or fat (Casals et al., 2006; Gargouri et al., 2006). However, nowadays, particular emphasis is laid on the effect of lipid supplementation on milk FA profile.

Plant lipids can be fed either as whole oilseeds, protected oils, or free oils, but results indicate that the latter, more accessible to rumen microorganisms, bring about the most notable changes in milk fat composition (Lock et al., 2004; Chilliard et al., 2007). When the goal is to enhance the CLA content of milk fat, diet supplementation with vegetable oils rich in 18:2*n*-6 and 18:3*n*-3, which provide the substrates for the production of VA or RA in the rumen, has proved very effective in cows, sheep and goats (Lock and Garnsworthy, 2002; Hervás et al., 2008; Bernard et al., 2009). Furthermore, this supplementation is an effective way of

decreasing milk fat saturated FA, in particular 12:0, 14:0, and 16:0, while resulting in only small, if any, increases in linoleic or linolenic acid in milk (Mele et al., 2006; Luna et al., 2008; Rego et al., 2008). This is due not only to the extensive biohydrogenation of PUFA in the rumen (Lock and Garnsworthy, 2002; Glasser et al., 2008), but also to their preferential incorporation into plasma lipid fractions, such as phospholipids and cholesterol esters, poorly used by the mammary gland (Kitessa et al., 2001; Loor et al., 2005; Chilliard et al., 2007).

Some studies have indicated that linoleic-rich oils (e.g., sunflower or soybean oils) increase milk RA more than those rich in linolenic acid (e.g., linseed or rapeseed oils), but results are not always consistent (Kelly et al., 1998; Zhang et al., 2006; Bernard et al., 2009). In this regard, responses to both types of oils are in some cases comparable, which could be accounted for by the influence of the dietary forage (Lock and Garnsworthy, 2002; Loor et al., 2005; Bernard et al., 2009). Besides, similar increases in milk RA content with linoleic- and linolenic-rich oils provide further evidence that VA supply for endogenous synthesis in the mammary gland is the most important determinant of RA concentration in milk fat (Palmquist et al., 2005), since only VA, and not RA, is formed in the rumen during linolenic acid biohydrogenation (Harfoot and Hazlewood, 1997; Jenkins et al., 2008).

Despite the positive effects of vegetable oil supplementation on milk FA profile (i.e., increased VA and RA content) mentioned above, it should be pointed out that, in some cases, relatively high concentrations of *trans*-10 18:1 are indeed observed (Roy et al., 2006; Gómez-Cortés et al., 2008). *Trans*-10 18:1 may have potentially negative effects on consumers' health (Kühlsen et al., 2005; Roy et al., 2007) and, when plant lipids are added to high-concentrate diets, progressive increases in the milk content of this 18:1 isomer are often accompanied by reductions in *trans*-11 18:1 and, subsequently, in *cis*-9, *trans*-11 18:2 (Bauman and Griinari, 2001; Roy et al., 2006; Shingfield et al., 2008). Under similar conditions, cows appear more prone to these alterations in the biohydrogenation pathways than small ruminants. Thus, relatively low contents of *trans*-10 18:1 are always

found in caprine milk (Luna et al., 2008; Bernard et al., 2009; Gagliostro et al., 2009), and high levels of this 18:1 isomer can be accompanied by notable increases in VA and RA in ovine milk (Reynolds et al., 2006; Gómez-Cortés et al., 2008; Hervás et al., 2008).

3.3. Effect of diet supplementation with marine oils

Ruminant dairy products are poor sources of long-chain *n*-3 FA, such as 20:5*n*-3 and 22:6*n*-3, in the human diet (Simopoulos, 2002; Bourre, 2005). For this reason, a number of studies attempted to enrich their content in milk fat through the supplementation of dairy cows' diet with marine lipids, including fish oil and marine algae (Franklin et al., 1999; Donovan et al., 2000; Lock and Bauman, 2004), but very little work of a similar nature has been published for small ruminants, in particular for ewes (Mozzon et al., 2002; Papadopoulos et al., 2002; Capper et al., 2007).

Marine oils are rich in 20:5*n*-3 and 22:6*n*-3 (although their contents can vary with season and species of origin) but the transfer efficiency of these FA from diet into milk is always low and usually lower in cows (on average, 2-4%; Shingfield et al., 2003; Lock and Bauman, 2004; Cruz-Hernandez et al., 2007) and goats (on average, 4-5%; Chilliard et al., 2007) than in sheep (3-10 and 6-18% for EPA and DHA, respectively; Papadopoulos et al., 2002; Reynolds et al., 2006; Capper et al., 2007). Low transferences would be explained not only by their extensive biohydrogenation in the rumen (Shingfield et al., 2003; Kim et al., 2008), but also by the fact that these FA are not transported in the plasma lipid fractions that serve as major sources for mammary uptake (triacylglycerols and nonesterified FA; Kitessa et al., 2001; Lock and Bauman, 2004).

Interestingly, research on the inclusion of marine lipids in the diet has more often been directed toward their use as modulators of rumen fermentation, with the aim of improving CLA content in ruminant derived products. For a given incorporation level to the ration, marine oils seem more effective at increasing milk RA content than plant oils (Whitlock et al., 2002; Lock and Bauman, 2004), as

a result of their potent inhibitory effect on the final biohydrogenation step, where *trans* 18:1 are converted to 18:0 (AbuGhazaleh and Jenkins, 2004). Studies in dairy cows have shown that the addition of marine lipids to a diet supplemented with linoleic-rich oils, as a substrate for VA formation in the rumen, can further increase milk RA (Whitlock et al., 2002; AbuGhazaleh et al., 2003). In lactating ewes, the report by Reynolds et al. (2006) provides the only available information on the combined use of marine oils (microalgae biomass oil) and a linoleic-rich plant oil (soybean oil).

As previously stated for other lipid supplements, notable differences in the response to marine oils might also exist among ruminants, and while in dairy cattle increases in milk VA and RA appear to be transient and their content tends to decline with time (inversely to *trans*-10 18:1 level; Shingfield et al., 2006; Cruz-Hernandez et al., 2007; Boeckaert et al., 2008b), stable increases have been observed in the few studies conducted with small ruminants (Chilliard et al., 2007; Gagliostro et al., 2009).

4. IMPACT OF DIET SUPPLEMENTATION WITH LIPIDS ON ANIMAL PERFORMANCE

In past decades, the possible negative effect of lipid-rich feeds on ruminal digestion was a major concern for dairy nutritionists. Improved potential for milk production led to an ever-increasing demand for higher intakes of energy (Palmquist and Jenkins, 1980) and fat supplementation was considered an effective and economic way of achieving it. Nevertheless, early studies indicated that this nutritional strategy could result in depressed fibre digestibility and volatile fatty acids (VFA) production, and shifts towards lower acetate:propionate ratio (Palmquist and Jenkins, 1980; Jenkins, 1993). Further research demonstrated, however, that these disturbances largely depended on the amount of fat, its nature, and the basal diet composition (Jenkins, 1993; Doreau and Chilliard, 1997; Kucuk et al., 2004), mainly as a consequence of changes in the rumen microbial

community (Boeckaert et al., 2008a; Kim et al., 2008). Considering that unsaturated FA could be toxic to certain microorganisms (Maia et al., 2007), different techniques of lipid protection were tested in order to avoid negative effects on the rumen microbial ecosystem and, eventually, on ruminal digestion (Jenkins, 1993; Doreau and Chilliard, 1997; Gargouri et al., 2006). Nevertheless, when the goal is to modify the FA profile of ruminant-derived foods, the levels of inclusion are often too low to interfere significantly with ruminal digestion (Doreau and Chilliard, 1997; Kucuk et al., 2004). For example, even if reduced feed intake is frequently observed with fish oil supplementation (Donovan et al., 2000; Kitessa et al., 2001; Shingfield et al., 2006), it appears to be attributable to an increase in the ruminal outflow of unsaturated FA rather than to a decrease in ruminal fibre digestibility (Shingfield et al., 2003; Kim et al., 2008).

With regard to other parameters indicative of animal performance, milk production seldom varies even if feed intake is reduced (Franklin et al., 1999; Donovan et al., 2000; Papadopoulos et al., 2002), which may be due to an increase in diet energy concentration or the mobilization of body reserves. On the other hand, changes in milk composition are more variable and largely depend on ruminant species (Shingfield et al., 2010). To date, the major concern of lipid supplementation would be, in fact, its effect on milk protein and fat contents, which is of particular importance in the case of small ruminants, since most caprine and ovine milk is processed into cheese (Caja and Bocquier, 2000; Pulina et al., 2006; Raynal-Ljutovac et al., 2008).

Decreased milk protein content is frequently observed with oil-rich supplements in ewes (Gargouri et al., 2006; Pulina et al., 2006) and cows (DePeters and Cant, 1992; Franklin et al., 1999; Shingfield et al., 2006), but not in goats (Sanz Sampelayo et al., 2007; Luna et al., 2008; Bernard et al., 2009). These reductions have been attributed to nutritional and endocrine factors, such as reduced amino acid availability to the mammary gland and insuline resistance (DePeters and Cant, 1992; Mackle et al., 2000), which appear to be linked. Recent studies demonstrate a decisive role of endocrine signals (especially from the lactogenic

hormones hydrocortisone, insulin, and prolactin) integrating information on nutrient availability and hence modulating protein translation (Burgos et al., 2010).

Although decreases in milk protein content are relatively small, the effect of lipid supplementation on milk fat content can be more dramatic in the particular case of dairy cattle and has consequently received much attention (Bauman and Griinari, 2001; Shingfield and Griinari, 2007; Harvatine et al., 2009). In this species, feeding unprotected vegetable or marine oils has frequently been associated with milk fat depression (MFD), which Bauman and Griinari (2001) defined as a decrease in milk fat yield of up to 50%, with no change in milk yield or in the yield of other components. Varying levels of MFD have, however, been observed, and are commonly characterized as a level of milk fat production below the genetic potential of the animal (Harvatine et al., 2009). Although this syndrome has been consistently observed in dairy cows (Donovan et al., 2000; Roy et al., 2006; Shingfield et al., 2006), the scant data available for small ruminants indicate a lower propensity to MFD in lactating sheep (Mele et al., 2006; Pulina et al., 2006; Hervás et al., 2008), and no occurrence in the case of goats (Bouattour et al., 2008; Bernard et al., 2009; Gagliostro et al., 2009).

The biohydrogenation theory proposed by Bauman and Griinari (2001) established that MFD relates to an inhibition of milk fat synthesis by specific biohydrogenation intermediates that are only produced under certain conditions of altered rumen fermentation, such as feeding with plant oils and high proportions of rapidly fermented carbohydrate, or with marine lipid supplements (Shingfield et al., 2006; Cruz-Hernandez et al., 2007; Gama et al., 2008). Since response patterns to lipid supplements are deeply influenced by the basal diet composition (Dewhurst et al., 2006; Roy et al., 2006), different susceptibilities to variations in the ratio of starch to fibre in the diet (and associated rumen pH) between ruminant species (Pulina et al., 2006; Chilliard et al., 2007) could partially contribute to explaining the differences observed.

Trans-10, *cis*-12 CLA was the first biohydrogenation intermediate shown to exert anti-lipogenic effects (Shingfield and Griinari, 2007), but recent studies have reported that *cis*-10, *trans*-12 CLA and probably *trans*-9, *cis*-11 would also inhibit milk fat synthesis (Shingfield and Griinari, 2007; Harvatine et al., 2009), while the contribution of *trans*-10 18:1 appears controversial (Shingfield et al., 2010). Although the biohydrogenation theory provides a basis for explaining most cases of diet-induced MFD, some particular situations (i.e., feeding marine oils) require further explanation other than direct inhibition (Loor et al., 2005; Boeckaert et al., 2008b; Gama et al., 2008). Recently, Shingfield and Griinari (2007) proposed an extension of this theory based on changes of preformed long-chain FA available to the mammary gland when marine lipids are fed (Shingfield et al., 2003; Kim et al., 2008). Thus, a shortage of 18:0 for oleic acid synthesis in the mammary gland, together with an increase in *trans* FA originating in the rumen (with melting points higher than body temperature; Timmen and Patton, 1989; Jensen, 2002), would have a negative impact on the maintenance of milk fat fluidity and, therefore, on the rate of milk fat synthesis, causing MFD (Shingfield and Griinari, 2007; Harvatine et al., 2009).

5. OUTLINE OF THE THESIS

On the basis of the above, and considering the economic importance of ovine milk production in Spain (441,400 tonnes in 2008, 60% of this in the region of Castile and Leon; MARM, 2010), the development of a nutritional strategy to further improve the nutritional value of sheep milk could make its products more attractive to health-conscious consumers and provide worthwhile support to dairy sheep breeders. Thus, based on the promising results of earlier studies supplementing the diet of lactating ewes with linoleic-rich vegetable oils (Gómez-Cortés et al., 2008; Hervás et al., 2008), and the few available studies for this species on the effects of marine lipid supplementation on the milk FA composition (Papadopoulos et al., 2002; Reynolds et al., 2006; Capper et al., 2007), we

hypothesized that the addition of a combination of sunflower oil and marine lipids to the diet of dairy ewes would improve the FA profile of their milk, in particular its CLA and long-chain *n*-3 FA content, with no detrimental effects on animal performance.

A series of five studies, corresponding to the five chapters into which this dissertation is divided, was carried out to investigate the effects of the supplementation with sunflower oil and either fish oil or marine algae on ewe performance, FA composition of milk and ruminal digesta lipids, and rumen microbiota and fermentation characteristics.

In the study in Chapter I, a high-concentrate diet formulated for highly productive lactating sheep was supplemented with sunflower oil and fish oil, either alone or in combination, to evaluate the impact of these nutritional strategies on milk FA profile and ewe performance. Since the joint use of both lipids induced the greatest modifications in animal performance and milk FA composition, the studies described in Chapters II, III, and IV refer only to this combination and were designed to examine its effects on rumen function and bacterial community.

The second study (Chapter II) was conducted with cannulated ewes and included in vivo, in situ and in vitro assays with the aim of determining whether the disturbances in animal performance observed in the first experiment could be attributed to a negative impact of lipid supplements on ruminal fermentation.

The third study (Chapter III) was also carried out with cannulated ewes to examine time-dependent changes in the accumulation of biohydrogenation intermediates in the rumen after the inclusion of sunflower plus fish oils in the diet. Detailed analysis of the FA composition of rumen digesta lipids was performed and, although it was not a prime objective of this study, nutritional assays were also included to provide further information on the influence of these oils on ruminal fermentation characteristics.

In Chapter IV, changes in the rumen bacterial community of sheep were monitored to provide an insight into the effect of sunflower and fish oils on

microbial populations because, despite the impact of the microbiota on the biohydrogenation of dietary unsaturated FA, there is no in vivo study on this subject in sheep.

Once these studies with sunflower and fish oils were completed, a last experiment (Chapter V) was conducted to examine the effect of the joint use of sunflower oil and marine algae on dairy sheep performance and milk FA profile. The same experimental procedures as in the first experiment were followed although, in this case, three incremental levels of marine algae were tested. This decision was based on the fact that the limited reports on the addition of marine algae to the diet of dairy ewes (Papadopoulos et al., 2002; Reynolds et al., 2006) or cows (Franklin et al., 1999; Offer et al., 2001; Boeckaert et al., 2008b) showed inconsistent results, making it difficult to decide on an appropriate algae dosage to obtain the highest improvements in milk VA and RA and, at the same time, limit the potential negative effects on animal performance and on the increase of some non-desired *trans* FA, such as *trans*-10 18:1.

Finally, there is a General discussion integrating all the chapters. An important consideration in that section is the comparison between results obtained in Chapters I and V, together with the potential associations between changes in ruminal lipid metabolism and variations in milk FA profile.

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CHAPTER I

CHANGES IN MILK FATTY ACID PROFILE AND ANIMAL PERFORMANCE IN RESPONSE TO FISH OIL SUPPLEMENTATION, ALONE OR IN COMBINATION WITH SUNFLOWER OIL, IN DAIRY EWES

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Published in: Journal of Dairy Science 93 (2010) 1604-1615

doi:10.3168/jds.2009-2530

Presented in part at the *XIII Jornadas sobre Producción Animal AIDA*, 12–13 May 2009, Saragossa, Spain

CHANGES IN MILK FATTY ACID PROFILE AND ANIMAL PERFORMANCE IN RESPONSE TO FISH OIL SUPPLEMENTATION, ALONE OR IN COMBINATION WITH SUNFLOWER OIL, IN DAIRY EWES

P. G. Toral, P. Frutos, G. Hervás, P. Gómez-Cortés, M. Juárez,

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Abstract. Ruminant diet supplementation with sunflower oil (SO) and fish oil (FO) has been reported as a good strategy for enhancing some milk fat compounds such as conjugated linoleic acid (CLA) and *n*-3 polyunsaturated fatty acids in dairy cows, but no information is available regarding dairy sheep. In this work, ewe diet was supplemented with FO, alone or in combination with SO, with the aim of improving milk nutritional value and evaluating its effect on animal performance. Sixty-four Assaf ewes in mid lactation, fed a high-concentrate diet, were distributed in 8 lots of 8 animals each and assigned to 4 treatments (2 lots/treatment): no lipid supplementation (control) or supplementation with 20 g of SO/kg (SO), 10 g of FO/kg (FO), or 20 g of SO plus 10 g of FO/kg (SOFO). Milk production and composition, including a complete fatty acid profile, were analyzed on days 0, 3, 7, 14, 21, and 28 of treatments. Supplementation with FO tended to reduce dry matter intake compared to the control treatment (−15%), and its use in combination with SO (SOFO) resulted in a significant decrease in milk yield as well (−13%). All lipid supplements reduced milk protein content, and FO also reduced milk fat content by up to 21% alone (FO) and 27% in combination with SO (SOFO). Although the mechanisms involved in FO-induced milk fat depression are not yet well established, the observed increase in some milk *trans* FA that are putative inhibitors of milk fat synthesis, such as *trans*-9, *cis*-11 CLA, and the almost 63% decrease in 18:0 (consistent with the theory of reduced milk fat fluidity) may be involved. When compared with the control, lipid supplementation remarkably improved the milk content of rumenic acid (*cis*-9,

trans-11 CLA; up to 4-fold increases with SO and SOFO diets), whereas FO-containing diets also increased milk *n*-3 polyunsaturated fatty acids, mainly docosahexaenoic acid (with mean contents of 0.29 and 0.38% of total fatty acids for SOFO and FO, respectively), and reduced the *n*-6:*n*-3 FA ratio to approximately half the control value. All lipid supplements resulted in high levels of some *trans* FA, mainly *trans*-11 18:1 (vaccenic acid) but also *trans*-10 18:1.

Key words: conjugated linoleic acid, milk fat depression, *n*-3, sheep

1. INTRODUCTION

Marine lipids are rich in long-chain *n*-3 polyunsaturated fatty acids (PUFA) that, as potent inhibitors of *trans* 18:1 ruminal reduction, promote the outflow of *trans*-11 18:1 (vaccenic acid, VA), that is subsequently desaturated to *cis*-9, *trans*-11 18:2 (rumenic acid, RA) in the mammary gland (Lock and Bauman, 2004). A combination of a high linoleic vegetable oil such as sunflower oil (SO), substrate for VA formation in the rumen, and marine lipids, such as fish oil (FO), is then considered a good nutritional strategy for enhancing RA and *n*-3 PUFA in milk fat (Palmquist and Griinari, 2006; Shingfield et al., 2006). Although FO inclusion in dairy cow diets has been associated with milk fat depression (MFD), initial objections to fat reduction have been put aside by consumers' increasing interest in healthier food products (Lock and Bauman, 2004; Shingfield et al., 2008) and the decreasing market value of milk fat, as well as the additional benefits to animal health (Griinari and Bauman, 2006). However, in contrast to dairy cow milk, ovine milk is almost entirely destined to cheese production and decreases in the solid fractions or alterations to the fat:protein ratio caused by lipid supplementation may have a negative effect on cheese yield and quality (Bocquier and Caja, 2001). Notwithstanding, some authors have reported that sheep seem less prone to MFD than cows when fed high-concentrate diets plus free vegetable oils (Pulina et al., 2006; Hervás et al., 2008).

Despite the promising results obtained for lipid inclusion in dairy ewe diets (Pulina et al., 2006) and the ample background about FO supplementation in dairy cows (Doreau and Chilliard, 1997; Palmquist and Griinari, 2006; Shingfield et al., 2006), few studies have examined the effects of FO inclusion in the diet of lactating ewes (Kitessa et al., 2003; Capper et al., 2007) and, to date, the study by Reynolds et al. (2006) provides the only available information about diet supplementation with marine lipids (marine algae biomass oil) plus a high linoleic vegetable oil (soybean oil) in this species. On this basis and taking into account our previous works (Gómez-Cortés et al., 2008a; Hervás et al., 2008), we hypothesized that the combined use of SO and FO would result in a healthier milk fatty acid (FA) profile in dairy ewes, with the potential to increase CLA and *n*-3 PUFA concentrations. Therefore, this work was conducted with the aim of studying the effects of diet supplementation with FO, SO, and their combination (SOFO) on animal performance and milk FA composition in dairy ewes.

2. MATERIALS AND METHODS

2.1. Animals and experimental diets

Sixty-four multiparous Assaf ewes (LW = 86.4 ± 1.21 kg) in mid lactation (at week 12 at the beginning of the experiment) were used. The ewes were distributed in 8 lots of 8 animals each, balanced for milk yield, LW, days postpartum, and number of lactation, and allocated at random to 4 experimental treatments (2 lots per treatment): control, supplemented with SO, supplemented with FO, and supplemented with SOFO.

The diets, prepared in a feed mixer every week, consisted of a total mixed ration (TMR) based on alfalfa hay and a concentrate with no supplementation (control) or supplemented with 20 g of SO/kg of fresh matter (SO diet), 10 g of FO/kg of fresh matter (FO diet), and 20 g of SO plus 10 g of FO/kg of fresh matter (SOFO diet). The ingredients and chemical composition of the 4 experimental diets, which included molasses to avoid selection of dietary components, are given

in Table 1. During a 3-week adaptation period (before the beginning of the trial), all the animals received the control diet. Clean water and a vitamin and mineral supplement were always available and fresh diets were offered daily ad libitum at 09:00 and 19:00 h.

The ewes were milked at approximately 08:30 and 18:30 h in a 1 × 10 stall milking parlour (DeLaval, Madrid, Spain). The experiment lasted for 4 weeks and was carried out in accordance with Spanish Royal Decree 1201/2005 for the protection of animals used for experimental purposes.

Table 1. *Ingredients and chemical composition of the experimental diets.*

	Diet[1]			
	Control	SO	FO	SOFO
Ingredients, g/kg fresh matter				
Dehydrated alfalfa hay (particle size > 4 cm)	200	196	198	194
Whole corn grain	250	245	247	242
Soybean meal	200	196	198	194
Whole barley grain	150	147	149	146
Beet pulp	90	88	89	87
Molasses	65	64	64	63
Salts[2]	40	39	40	39
Minerals and vitamins[3]	5	5	5	5
Sunflower oil[4]	0	20	0	20
Fish oil[5]	0	0	10	10
Chemical composition, g/kg DM				
Organic matter	893	891	891	892
Crude protein	205	208	203	203
Neutral-detergent fiber	226	222	220	219
Acid-detergent fiber	125	121	122	120
Ether extract	31	54	41	67

[1] Diets: control = with no oil supplementation; SO = supplemented with 20 g of sunflower oil/kg of fresh matter); FO = supplemented with 10 g of fish oil/kg of fresh matter; SOFO = supplemented with 20 g of sunflower oil plus 10 g of fish oil/kg of fresh matter.

[1] Contained (g/kg): $NaHCO_3$ (375), $CaCO_3$ (350), Ca_2HPO_4 (150) and mine salt (125).

[2] INA OV1 (EVIALIS, Madrid, Spain).

[3] Contained (% total fatty acid methyl esters): 16:0 (7.5), 18:0 (4.3), 18:1 (26.3), and 18:2 (60.5).

[4] Semi-refined tuna and sardine oil (Afampes 121 DHA, Afamsa, Vigo, Spain); contained (% total fatty acid methyl esters) 16:0 (21.4), 18:0 (5.9), 18:1 (14.9), 18:2 (1.7), 20:5*n*-3 (6.3), and 22:6*n*-3 (17.8).

2.2. Measurements, sample collection, and chemical analyses

Samples of offered and refused diets were collected once a week, stored at −30 ºC and then freeze-dried. The dry matter intake (DMI) was therefore recorded weekly for each experimental lot. Samples were analyzed for DM (ISO 6496:1999), ash (ISO 5984:2002), and CP (ISO 5983–2:2005). Neutral detergent fiber and ADF were determined by the methods described by Ankom technology (Ankom, 2006a,b). Neutral detergent fiber was assayed with sodium sulfite and α-amylase and expressed with residual ash (the latter also for ADF). The content of ether extract in the diets was determined by the Ankom filter bag technology (American Oil Chemists' Society Official Procedure Am 5–04; AOCS, 2008).

Individual milk yield was recorded on days 0, 3, 7, 14, 21, and 28, both at morning and evening milkings. With the same frequency, chosen to study time-dependent responses to lipid supplementation, milk samples for the analysis of fat, protein, and total solids were collected from each animal and treated with natamycin. The protein, fat, and total solids concentrations were determined by infrared spectrophotometry (ISO 9622:1999) using a MilkoScan 255 A/S N (Foss Electric, Hillerød, Denmark).

Milk FA composition was determined in untreated samples from each experimental lot, composited according to individual milk production. Milk fat was extracted following the method described by Luna et al. (2005) and fatty acid methyl esters (FAME) were prepared by base-catalyzed methanolysis of the glycerides (ISO 15884:2002). Analysis of FAME in hexane was performed on a gas chromatograph (Agilent 6890 N Network System, Palo Alto, CA, USA) with an autoinjector and fitted with a flame ionization detector. The FAME profile was determined by split injection (1:100) onto a CP-Sil 88 fused silica capillary column (100 m × 0.25 mm i.d., 0.20 μm film thickness; Varian, Middelburg, the Netherlands) using the same programmed temperature gradient method described in Hervás et al. (2008). Finally, discrimination between *trans*-7, *cis*-9, and *cis*-9, *trans*-11 CLA isomers was achieved by Ag^{+}-HPLC according to the procedure described by Gómez-Cortés et al. (2008a).

At the end of the experiment, on the morning of day 29, ewes were milked and then given free access to the diets as on other days. After 2 h, the feed was removed, and 3 h later, samples of ruminal fluid were individually collected from 4 animals per treatment using a stomach tube and visually checked to ensure that the samples did not contain saliva. Samples were strained through 2 layers of muslin, the pH was immediately measured, and aliquots were collected for ammonia and volatile fatty acids (VFA) analysis (see Toral et al., 2009).

2.3. Statistical analyses

All analyses were performed using SAS (version 9.1, SAS Institute Inc., Cary, NC, USA). Data on DMI, milk yield and composition, and FA composition were analyzed by repeated measurement analysis using the MIXED procedure of SAS and assuming a covariance structure on the basis of Schwarz's Bayesian information model fit criteria. The statistical model included the fixed effects of diet, time, their interaction, and the initial record measured at 0 week (covariate). For all data collected either individually (milk yield and composition) or per lot (DMI and FA composition), the lot was nested within the diet to contrast the effect of lipid supplementation. Pearson correlation coefficients (r) were generated for associations between some FA, milk yield, and fat content using the CORR procedure of SAS. Data from ruminal fermentation parameters were analyzed by one-way ANOVA using the MIXED procedure. Differences were declared significant at $P < 0.05$, with values of $P < 0.10$ being interpreted as a trend toward significance. Least squares means (adjusted for the covariance) are reported throughout.

3. RESULTS AND DISCUSSION

3.1. Ewe performance and ruminal fermentation

As shown in Table 2, FO supplementation (both individually and in combination with SO) tended to reduce DMI in comparison with the control diet ($P < 0.10$),

whereas there were no differences between the control and SO diets. Previous studies in sheep have reported significant decreases in feed intake when using unprotected FO, either as a supplement in the diet (30 g/kg of DM; Kitessa et al., 2001) or by rumen injection (42 g/kg of DM; Fievez et al., 2003), but no decrease when using SO (60 g/kg of DM; Hervás et al., 2008) or protected FO (30 g/kg of DM; Kitessa et al., 2001). The depression in DMI because of unprotected FO supplementation has been attributed to increases in some biohydrogenation intermediaries that are potentially toxic for rumen microbiota (Kitessa et al., 2001). In the present work, the greatest reduction in DMI was observed for the SOFO treatment (−19%), which might have been produced not only by the FO itself but also by the higher level of total oil inclusion in a low-forage diet. However, in this trial, decreased DMI was not associated with any impairment of ruminal fermentation (Table 3). Thus, for instance, rumen pH was not significantly affected by oil supplementation ($P > 0.10$), in agreement with other studies in sheep fed FO (Fievez et al., 2003), SO (Hervás et al., 2008), and SOFO (Toral et al., 2009), and which may be related to an acceptable level of effective fiber in the 4 diets (Mertens, 1997) because of the particle size of the alfalfa hay (> 4 cm) and the supply of cereals as whole grains. Furthermore, lipid supplementation did not significantly affect either ammonia or VFA ruminal concentrations ($P > 0.10$). The SO diet results are consistent with other findings for this oil reported in sheep (6% DM; Hervás et al., 2008) and cows (up to 3.4% DM doses; Shingfield et al., 2008). Regarding FO, rumen injection of moderate amounts of this oil in sheep (4.2% DM; Fievez et al., 2003) did not cause any significant variation in total VFA either but induced a decrease in the acetate:propionate ratio. The lower level of oil inclusion in the present study might explain why ruminal fermentation was not negatively affected because the amount of supplemental lipids in the diet has been shown to be a determinant factor in producing these effects (Shingfield et al., 2008). Similar levels of SO plus FO to those used in our work (different combinations supplying 3% DM) were studied in dairy cows and sheep (Palmquist and Griinari, 2006; Toral et al., 2009), with transient variations in

fermentation parameters that disappeared 6 h postfeeding being attributed to rumen microorganism adaptation and development of tolerance to potentially toxic FA. This would have been facilitated in the present work by the longer period on treatments.

Table 2. *Dry matter intake, milk yield, and milk composition in dairy ewes fed different oil supplements.*

	Diet[1]				SED[2]	P-value[3]		
	Control	SO	FO	SOFO		D	T	D × T
Dry matter intake, g/d	3,103	2,849	2,750	2,521	157.7	†	***	†
Yield, g/d								
Milk	2,661^{a}	2,633^{a}	2,626^{a}	2,299^{b}	65.1	*	***	ns
Protein	133.9^{a}	127.8^{a}	128.1^{a}	109.3^{b}	2.51	**	***	†
Fat	151.4^{a}	153.3^{a}	134.8^{b}	119.7^{c}	3.31	**	***	***
Total solids	443.1^{a}	434.6^{ab}	418.1^{b}	363.4^{c}	6.16	***	***	**
Composition, g/100 g raw milk								
Protein	5.17^{a}	4.89^{b}	4.88^{b}	4.73^{b}	0.064	*	***	ns
Fat	5.89^{a}	5.90^{a}	5.15^{b}	5.14^{b}	0.197	*	***	***
Total solids	16.91^{a}	16.66^{ab}	15.98^{bc}	15.73^{c}	0.247	*	***	***

[a–c] Means within a row with different superscripts differ significantly ($P < 0.05$).
[1] Diets: control = no oil supplementation; SO = supplemented with 20 g of sunflower oil/kg of fresh matter; FO = supplemented with 10 g of fish oil/kg of fresh matter; SOFO = supplemented with 20 g of SO plus 10 g of FO/kg of fresh matter.
[2] SED = standard error of the difference.
[3] Probability of significant effects due to experimental diet (D), time on diet (T), and their interaction (D × T). ns, not significant ($P > 0.10$); †$P < 0.10$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 3. *Ruminal pH and ammonia and volatile fatty acid (VFA) concentrations in ewes fed different oil supplements.*

	Diet[1]				SED[2]	P-value[3]
	Control	SO	FO	SOFO		
pH	6.6	6.6	6.7	6.5	0.20	ns
Ammonia, mg/L	109.2	92.5	93.1	98.9	38.69	ns
Total VFA, mmol/L	125.2	110.4	108.3	130.5	16.64	ns
Acetate	75.2	61.4	61.0	75.1	12.67	ns
Propionate	30.3	25.0	27.1	27.1	4.02	ns
Butyrate	16.2	19.7	17.3	23.2	4.19	ns
Acetate:propionate	2.5	2.5	2.3	2.8	0.53	ns

[1] Control = no oil supplementation; SO = supplemented with 20 g of sunflower oil/kg of fresh matter; FO = supplemented with 10 g of fish oil/kg of fresh matter; SOFO = supplemented with 20 g of SO plus 10 g of FO/kg of fresh matter.
[2] SED = standard error of the difference.
[3] Probability of significant effects due to experimental diet (D). ns, not significant ($P > 0.10$).

Concerning milk production and composition (Table 2), the lower DMI observed for the SOFO diet (−19%) would explain the lower milk yield of the animals on this treatment (−13%; $P < 0.05$). The reductions in protein content caused by all oil supplements ($P < 0.05$) is something frequently observed in dairy ewes (Bocquier and Caja, 2001; Pulina et al., 2006). In the present study, this decrease cannot be related to a dilution effect resulting from an increase in milk yield but rather can probably be related to reduced amino acid availability to the mammary gland or an induction of insulin resistance (Pulina et al., 2006). It is worthy of note that this decrease in milk protein concentration balances the natural low fat content of milk from high-production Assaf ewes under intensive production systems and improves the fat:protein ratio, an important parameter in achieving adequate fat content in cheese for manufacturing processes and ripening properties (Bocquier and Caja, 2001).

In relation to milk fat, the significant interaction diet × time showed the different responses to diet oil addition (Figure 1); supplementation with SO and other unprotected vegetable oils has often been observed to have either no effect or positive effects on milk fat content (Pulina et al., 2006; Gómez-Cortés et al., 2008a; Hervás et al., 2008), whereas unprotected FO supply would reduce both milk fat content and yield (Capper et al., 2007). Although the reasons for these different responses are still uncertain, some authors have reported that lactating sheep might be less sensitive than cows to some MFD-inducing factors, such as high-concentrate diets, probably because of their ability to maintain normal rumen function (Pulina et al., 2006).

3.2. Saturated and monoenoic fatty acids

Table 4 shows the FA profile of milk in ewes fed either the control or the supplemented diets. Milk fat composition was markedly changed by the type of feed and the time on the diet, with significant diet × time interactions for numerous FA.

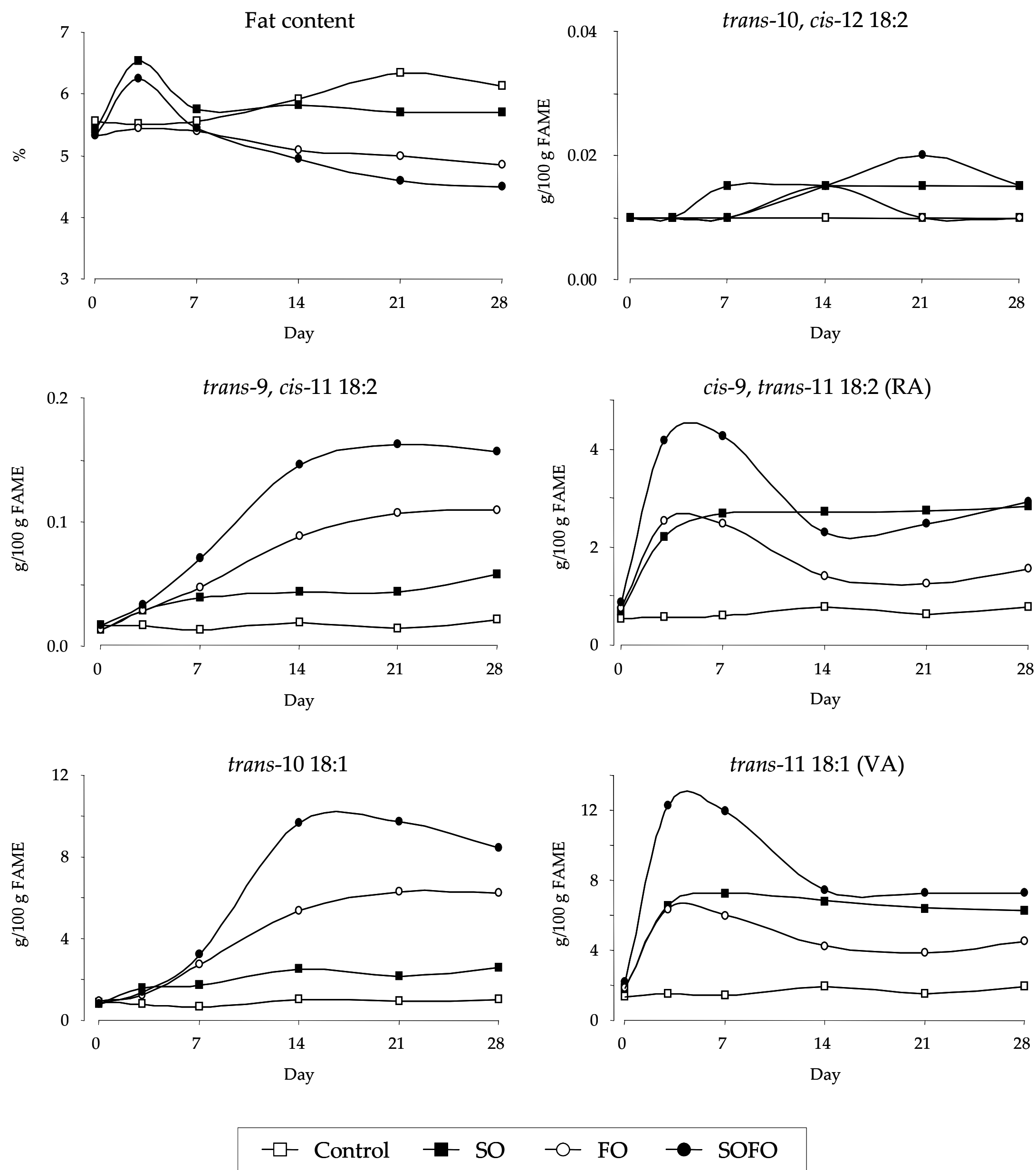

Figure 1. *Temporal changes in milk fat content,* trans-*10,* cis-*12 18:2,* trans-*9,* cis-*11 18:2,* cis-*9,* trans-*11 18:2,* trans-*10 18:1, and* trans-*11 18:1 [g/100 g fatty acids methyl esters (FAME)] in ewes fed diets with no supplementation (control), or supplemented with 20 g of sunflower oil/kg (SO), 10 g of fish oil/kg (FO), and 20 g of sunflower oil plus 10 g of fish oil/kg (SOFO). Values represent the mean from 2 lots of 8 animals per lot. Standard error of the difference = 0.197, 0.002, 0.008, 0.141, 0.248 and 0.591 for milk fat content,* trans-*10,* cis-*12 18:2,* trans-*9,* cis-*11 18:2,* cis-*9,* trans-*11 18:2,* trans-*10 18:1, and* trans-*11 18:1, respectively.*

All supplemented diets decreased the proportion of even-chain saturated FA but via different mechanisms. Diets containing SO reduced 6:0 to 16:0 FA (Figure 2), probably because of the potential inhibitory effect of the dietary PUFA or its metabolites on de novo FA synthesis in the mammary gland (Palmquist and Griinari, 2006; Kadegowda et al., 2009) or a dilution effect.

As observed in Figure 2, FO affected only 18:0 content, inducing a sharp decrease that was maintained throughout the whole period monitored ($P < 0.001$). This was probably caused by the action of the long-chain *n*-3 PUFA present in FO, mainly docosahexaenoic acid (DHA), a potent inhibitor of *trans* 18:1 ruminal reduction, that would have induced the accumulation of VA in the rumen (Loor et al., 2005; Palmquist et al., 2005). Thus, FO treatment resulted in increased milk VA content ($P < 0.01$), with further improvements when combined with SO (SOFO). However, the latter produced a greater increase in milk VA only during the first week of lipid supplementation but then stabilized at levels similar to those corresponding to the SO diet (i.e., almost 4 times higher than the control) throughout the rest of the experiment (Figure 1).

The interest in increasing VA concentration comes from its role as substrate for RA endogenous synthesis, not only in the ruminant mammary gland but also in human tissues (Palmquist et al., 2005). It has been reported that high levels of this FA in cow milk could be better sustained with high levels of forage, or low lipid supplementation, or both (Palmquist et al., 2005; Cruz-Hernandez et al., 2007). Nevertheless, it is worth noting that, in ewes, the addition of lipids to a high-concentrate diet produced an increased and stable level of milk VA after 2 week of supplementation (Figure 1).

As shown in Figure 1, *trans*-10 18:1 behaved differently; its largest increase was observed in week 2 for FO and SOFO treatments and it tended to plateau afterward ($P < 0.01$). Large increases in this *trans* 18:1 concentration would suggest important changes in ruminal biohydrogenation pathways in FO-containing diets, as previously observed in cows (Shingfield et al., 2006; Cruz-Hernandez et al., 2007). The increases observed for other *trans* 18:1 isomers (*trans*-6, -7, -8, *trans*-9,

and *trans*-12; Figure 3) in the SO diet were similar to those found with olive oil supplementation in ewes (Gómez-Cortés et al., 2008b) and could derive from the biohydrogenation of SO oleic acid (26% of total FA) involving the formation of several positional isomers of *trans*-monoenes (Mosley et al., 2002; Jenkins et al., 2008). A potential inhibitory effect of FO on the oleic acid biohydrogenation pathway might explain the lower milk content of these *trans*-monoenes in the FO treatment. Notwithstanding, treatments supplying FO did also result in a significant diminution of *cis*-18:1 FA content, the reduction in oleic acid (*cis*-9 18:1) content being more pronounced when this marine oil was fed alone ($P < 0.01$; Figure 2), probably because of lower supply of stearic acid for endogenous oleic acid synthesis in the mammary gland (Shingfield and Griinari, 2007).

A significant ($P < 0.05$) reduction of most odd- and branched-chain FA concentrations was found for all lipid-supplemented diets, but it was quantitatively of small magnitude.

3.3. Conjugated and non-conjugated linoleic acid

Rumenic acid (*cis*-9, *trans*-11) was the most abundant isomer of CLA and its changes clearly mirrored those of VA (Figure 1), with no differences between SO and SOFO diets after the second week of supplementation, when the RA content in the milk appeared to be stabilized and represented, on average, a 3.5-fold increase compared with the control. Though not as extensively investigated as in dairy cows, where direct measurements have evidenced the importance of its endogenous synthesis in the mammary gland (Palmquist et al., 2005), the strong relationship observed between RA and VA contents ($r = 0.97$; $P < 0.001$; Table 5) might support the hypothesis that dairy ewes do not differ from cows in this regard (Pulina et al., 2006). After 7 days on SOFO treatment, the milk RA content was among the highest (4.3% total FA) reported for ewes (Pulina et al., 2006; Reynolds et al., 2006, Gómez-Cortés et al., 2008a; Hervás et al., 2008), but after 2 week of supplementation, SO and SOFO diets resulted in average milk RA increases (2.7% of total FA).

Changes in the mean concentrations of other CLA isomers differed substantially from that of RA, and only *trans*-7, *cis*-9 CLA exceeded 0.10% of total FA in the case of the SO diet (Figure 3). The biological importance of this FA has received little attention but its synthesis in cows seems to be endogenous in the mammary gland because its precursor, *trans*-7 18:1, is a substrate for Δ^9-desaturase (Palmquist et al., 2005), in contrast to other CLA isomers that appear to be exclusively of rumen origin (Lock and Bauman, 2004). *Trans*-7, *cis*-9 CLA was highly correlated with the unresolved gas chromatography peak grouping *trans*-6,

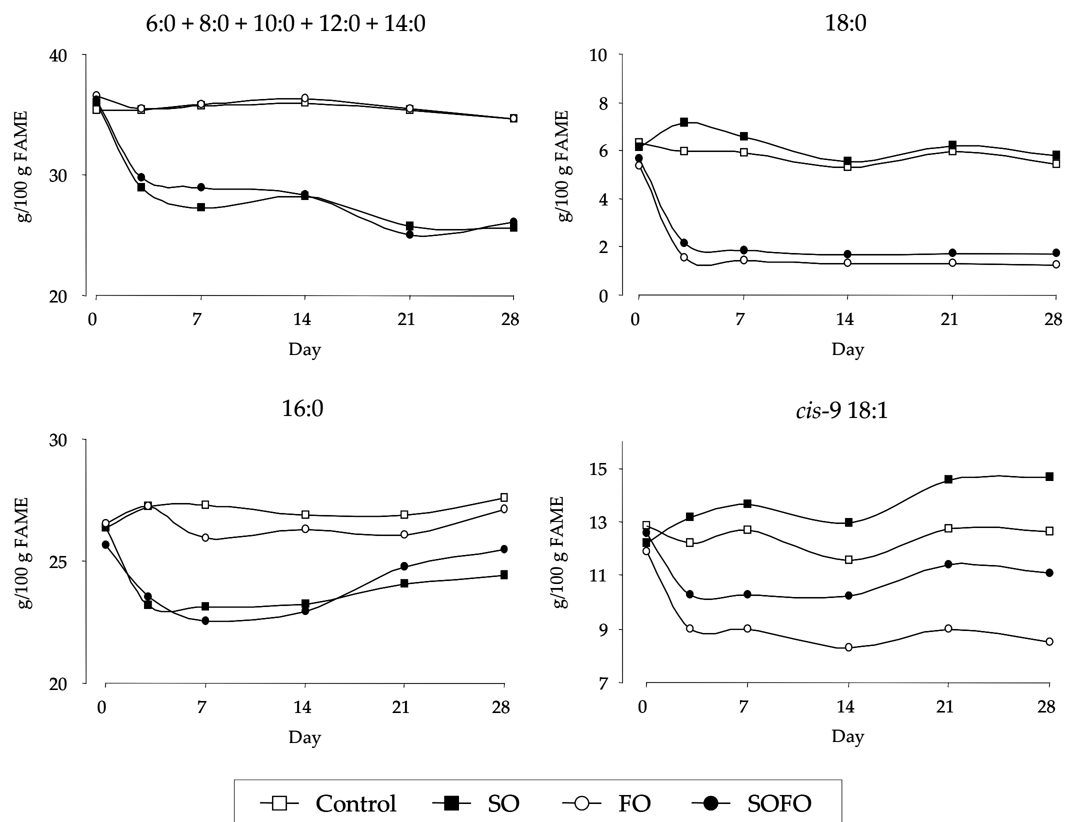

Figure 2. *Temporal changes in saturated fatty acids (6:0 + 8:0 + 10:0 + 12:0 + 14:0), 18:0, 16:0, and* cis-*9 18:1 [g/100 g fatty acids methyl esters (FAME)] in ewes fed diets with no supplementation (control), or supplemented with 20 g of sunflower oil/kg (SO), 10 g of fish oil/kg (FO), and 20 g of sunflower oil plus 10 g of fish oil/kg (SOFO). Values represent the mean from 2 lots of 8 animals per lot. Standard error of the difference = 0.674, 0.334, 0.399 and 0.363 for saturated fatty acids (6:0 + 8:0 + 10:0 + 12:0 + 14:0), 18:0, 16:0, and* cis-*9 18:1, respectively.*

Table 4. *Fatty acid profile of the milk from ewes fed different oil supplements.*

Fatty acid, g/100 g of total fatty acid methyl esters	Treatment[1]				SED[2]	P-value[3]		
	Control	SO	FO	SOFO		D	T	D × T
Saturated								
4:0	3.26^{c}	3.68^{a}	3.38^{b}	3.58^{a}	0.026	**	***	**
6:0	3.34^{ab}	3.15^{bc}	3.52^{a}	2.97^{c}	0.070	*	***	***
8:0	3.24^{a}	2.75^{b}	3.50^{a}	2.68^{b}	0.080	**	***	***
10:0	10.69^{a}	8.02^{b}	10.86^{a}	7.87^{b}	0.279	**	***	***
12:0	6.10^{a}	4.26^{b}	5.90^{a}	4.41^{b}	0.195	**	***	***
13:0 *anteiso*	0.07^{a}	0.04^{b}	0.06^{a}	0.05^{b}	0.003	*	ns	ns
13:0	0.23^{a}	0.14^{c}	0.20^{b}	0.14^{c}	0.008	**	***	***
14:0 *iso*	0.09^{a}	0.08^{b}	0.07^{b}	0.06^{c}	0.002	**	***	ns
14:0	12.18	10.72	11.74	10.80	0.365	ns	**	ns
15:0 *iso*	0.19	0.18	0.15	0.16	0.010	ns	**	ns
15:0 *anteiso*	0.41^{a}	0.32^{b}	0.32^{b}	0.28^{c}	0.010	**	***	*
15:0	0.95^{a}	0.73^{c}	0.86^{b}	0.71^{c}	0.009	***	***	**
16:0 *iso*	0.35^{a}	0.28^{b}	0.28^{b}	0.23^{c}	0.007	**	**	ns
16:0	27.03^{a}	24.05^{b}	26.53^{a}	24.15^{b}	0.399	*	***	***
17:0	0.50^{a}	0.42^{b}	0.50^{a}	0.45^{b}	0.011	*	***	**
18:0	5.80^{a}	6.21^{a}	2.04^{b}	2.45^{b}	0.334	**	***	***
19:0	0.06^{a}	0.04^{b}	0.05^{b}	0.04^{b}	0.003	*	ns	ns
20:0	0.15^{ab}	0.16^{a}	0.13^{b}	0.14^{b}	0.004	*	*	ns
21:0	0.04	0.04	0.04	0.04	0.001	†	ns	ns
22:0	0.08^{b}	0.10^{a}	0.08^{b}	0.10^{a}	0.004	*	***	***

(continued)

Table 4 *(continued)*.

Fatty acid, g/100 g of total fatty acid methyl esters	Treatment[1]				SED[2]	*P*-value[3]		
	Control	SO	FO	SOFO		D	T	D × T
Monounsaturated								
10:1	0.47[a]	0.33[b]	0.42[a]	0.30[b]	0.016	**	***	***
cis-14:1	0.22	0.26	0.25	0.23	0.012	ns	***	ns
15:1	0.11[b]	0.09[c]	0.14[a]	0.11[b]	0.004	**	ns	†
trans-8 16:1	0.06[d]	0.10[c]	0.16[b]	0.24[a]	0.007	***	***	***
trans-9 16:1 + 17:0 *iso*	0.41[b]	0.76[a]	0.54[b]	0.82[a]	0.037	**	***	***
cis-7 16:1	0.28	0.27	0.29	0.28	0.011	ns	†	**
cis-9 16:1 + 17:0 *anteiso*	1.39[b]	1.26[c]	1.66[a]	1.43[b]	0.023	***	***	ns
cis-13 16:1	0.17[a]	0.09[c]	0.12[b]	0.08[c]	0.008	**	***	***
17:1	0.18[a]	0.14[c]	0.17[b]	0.16[b]	0.003	**	ns	ns
trans-6, -7, -8 18:1	0.30[b]	0.56[a]	0.18[d]	0.26[c]	0.004	***	*	***
trans-9 18:1	0.27[c]	0.56[a]	0.25[c]	0.32[b]	0.006	***	***	***
trans-10 18:1	0.88[d]	2.11[c]	4.34[b]	6.48[a]	0.248	***	***	**
trans-11 18:1 (VA)	1.58[c]	5.86[ab]	4.47[b]	8.05[a]	0.591	**	***	***
trans-12 18:1	0.33[c]	0.64[a]	0.32[c]	0.48[b]	0.014	***	***	***
cis-9 18:1	12.43[a]	13.53[a]	9.02[c]	10.96[b]	0.363	**	***	***
cis-11 + *trans*-15 18:1	0.52[b]	0.64[a]	0.65[a]	0.67[a]	0.016	*	***	***
cis-12 18:1	0.36[b]	0.66[a]	0.09[c]	0.11[c]	0.049	**	ns	***
cis-13 18:1	0.08[ab]	0.09[a]	0.06[c]	0.06[bc]	0.004	*	*	ns
cis-14 + *trans*-16 18:1	0.34[b]	0.40[a]	0.11[c]	0.14[c]	0.014	***	***	***
cis-15 18:1	0.07[b]	0.10[a]	0.07[b]	0.07[b]	0.004	*	*	ns
cis-11 20:1	0.07[d]	0.11[c]	0.33[a]	0.29[b]	0.006	***	**	ns
cis-13 22:1	0.02[b]	0.01[b]	0.10[a]	0.09[a]	0.006	***	***	***

(continued)

Table 4 *(continued)*.

Fatty acid, g/100 g of total fatty acid methyl esters	Treatment [1]				SED [2]	*P*-value [3]		
	Control	SO	FO	SOFO		D	T	D × T
Non-conjugated 18:2								
trans-9, *cis*-13	0.09 [a]	0.09 [a]	0.07 [b]	0.06 [b]	0.003	**	***	***
trans-8 *cis*-12	0.16 [a]	0.20 [a]	0.08 [b]	0.10 [b]	0.012	**	***	***
trans-8 *cis*-13	0.09 [a]	0.10 [a]	0.04 [c]	0.06 [b]	0.004	**	**	***
cis-9, *trans*-12	0.08 [b]	0.12 [a]	0.05 [c]	0.07 [b]	0.004	**	***	***
trans-9, *cis*-12	0.03 [c]	0.06 [b]	0.06 [b]	0.09 [a]	0.002	***	***	***
trans-11, *cis*-15	0.05 [b]	0.10 [b]	0.46 [a]	0.44 [a]	0.012	***	***	***
cis-9, *cis*-12	2.64	2.77	2.47	2.49	0.065	†	***	***
Conjugated 18:2								
cis-9, *trans*-11 (RA)	0.64 [c]	2.31 [a]	1.66 [b]	2.83 [a]	0.141	**	***	***
trans-7, *cis*-9	0.06 [b]	0.12 [a]	0.03 [d]	0.04 [c]	0.002	***	**	**
trans-9, *cis*-11	0.02 [c]	0.04 [bc]	0.06 [b]	0.10 [a]	0.008	**	***	***
trans-10, *cis*-12	0.01	0.01	0.01	0.01	0.002	ns	ns	ns
trans-11, *cis*-13	0.01	0.01	0.01	0.01	0.001	ns	ns	ns
trans-11, *trans*-13	0.03 [a]	0.02 [c]	0.02 [b]	0.01 [d]	0.001	***	ns	ns
Other *trans-trans*	0.02 [b]	0.04 [a]	0.02 [b]	0.04 [a]	0.002	*	***	**

(continued)

Table 4 *(continued)*.

Fatty acid, g/100 g of total fatty acid methyl esters	Treatment[1]				SED[2]	*P*-value[3]		
	Control	SO	FO	SOFO		D	T	D × T
Other polyunsaturated								
18:3*n*-6	0.08[a]	0.05[b]	0.04[b]	0.03[c]	0.002	***	***	***
18:3*n*-3	0.33[a]	0.27[b]	0.34[a]	0.26[b]	0.008	**	***	*
20:2*n*-6	0.02[c]	0.02[c]	0.04[a]	0.04[b]	0.002	**	***	***
20:3*n*-6	0.02[b]	0.02[b]	0.03[a]	0.03[a]	0.001	*	***	**
20:3*n*-3	0.02[c]	0.02[c]	0.05[a]	0.05[b]	0.001	***	***	***
20:4*n*-6	0.13	0.11	0.15	0.15	0.007	†	***	***
20:5*n*-3 (EPA)	0.03[c]	0.02[c]	0.15[a]	0.10[b]	0.005	***	***	***
22:5*n*-3 (DPA)	0.06[c]	0.04[c]	0.18[a]	0.15[b]	0.006	***	***	***
22:6*n*-3 (DHA)	0.02[c]	0.02[c]	0.38[a]	0.29[b]	0.013	***	***	***
n-6:*n*-3 ratio	6.69[b]	8.14[a]	2.97[d]	3.47[c]	0.104	***	***	***

[a–d] Means within a row with different superscripts differ significantly ($P < 0.05$).

[1] Control = no oil supplementation; SO = supplemented with 20 g of sunflower oil/kg of fresh matter; FO = supplemented with 10 g of fish oil/kg fresh matter; SOFO = supplemented with 20 g of SO plus 10 g of FO/kg of fresh matter.

[2] SED = standard error of the difference.

[3] Probability of significant effects due to experimental diet (D), time on diet (T), and their interaction (D × T). ns, not significant ($P > 0.10$); †$P < 0.10$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 5. *Pearson correlation coefficients for relationships between concentrations of* trans-*18:1 fatty acids, conjugated linoleic acid isomers (g/100 of total fatty acid methyl esters), milk fat yield (g/day) and milk fat content (g/100 g raw milk) from ewes fed control, sunflower oil, fish oil and sunflower oil plus fish oil diets.*

	Fat content	*trans*-6, -7, -8	*trans*-9	*trans*-10	*trans*-11	*trans*-12	*cis*-9, *trans*-11	*trans*-7, *cis*-9	*trans*-9, *cis*-11	*trans*-10, *cis*-12	*trans*-11, *cis*-13	*trans*-11, *trans*-13
Fat yield	0.843***	0.384**	0.156	-0.910***	-0.205	0.174	-0.216	0.280	-0.918***	-0.446**	0.257	0.534***
Fat content		0.527***	0.357*	-0.735***	-0.016	0.395**	-0.037	0.427*	-0.740***	-0.362*	-0.222	0.314*
trans-6, -7, -8			0.933***	0.372**	0.161	0.795***	0.251	0.976***	-0.339*	0.283	-0.107	-0.190
trans-9				-0.123	0.402**	0.892***	0.491***	0.933***	-0.079	0.420**	0.026	-0.455**
trans-10					0.333*	0.094	0.290*	-0.307*	0.986***	0.506***	0.324*	-0.619***
trans-11						0.690***	0.973***	0.094	0.374**	0.261	0.300*	-0.810***
trans-12							0.740***	0.729***	0.045	0.333*	0.108	0.581***
cis-9, *trans*-11								0.210	0.349*	0.310*	0.295*	-0.822***
trans-7, *cis*-9									-0.269	0.334*	-0.098	-0.197
trans-9, *cis*-11										0.519***	0.322*	-0.655***
trans-10, *cis*-12											0.431**	-0.505***
trans-11, *cis*-13												-0.389**

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

-7, -8 18:1 isomers (r = 0.98; P < 0.001), suggesting that *trans*-7 18:1 conversion might represent a major pathway for its formation in ewes. Both *trans*-7, *cis*-9 CLA and *trans*-7 18:1 were decreased in FO and SOFO treatments, whereas *trans*-9, *cis*-11 CLA was increased (P < 0.01), as is more extensively discussed below. The milk fat content of *trans*-10, *cis*-12 18:2 was negligible in all treatments and remained so throughout the experiment (P > 0.10; Figure 1).

Regarding non-conjugated 18:2 FA, linoleic acid concentration was little modified and did not increase with the addition of SO even if it represented 60% of its total FA, which may be attributed to its more extensive biohydrogenation when its content in the diet increases (Shingfield et al., 2008).

Another non-conjugated 18:2 that should be considered is *trans*-11, *cis*-15 18:2, an intermediate of α-linolenic acid metabolism whose milk content was multiplied 9 times with FO supplementation (P < 0.001), in agreement with previous studies (Shingfield et al., 2006; Cruz-Hernandez et al., 2007), and supposedly as a result of the FO inhibitory effect on biohydrogenation of dietary α-linolenic acid (Loor et al., 2005). However, the strong correlation observed between *trans*-11, *cis*-15 18:2 and some n-3 PUFA, such as DHA (r = 0.95; P < 0.001) or docosapentaenoic acid (r = 0.94; P < 0.001), suggests that this FA might also be an intermediate of other n-3 PUFA less clear pathways (Jenkins et al., 2008).

3.4. Putative inhibitors of milk fat synthesis

The inhibitory effects of FO on milk fat secretion does not seem to be attributable to the same putative depressors as those of MFD induced by more traditional diets (Loor et al., 2005; Shingfield and Griinari, 2007; Gama et al., 2008). For example, *trans*-10, *cis*-12 CLA concentration showed no change during FO-induced MFD (Figure 1), as previously reported by Whitlock et al. (2002) or Griinari and Bauman (2006), and so other biohydrogenation intermediates with antilipogenic effects may be involved (Griinari and Bauman, 2006; Harvatine et al., 2009). Accordingly, *trans*-9, *cis*-11 CLA, which showed increased values in FO diets and a high correlation with fat yield (r = −0.92; P < 0.001; Table 5) but very low concentrations,

has also been identified as a potential inhibitor of milk fat synthesis (Perfield et al., 2007).

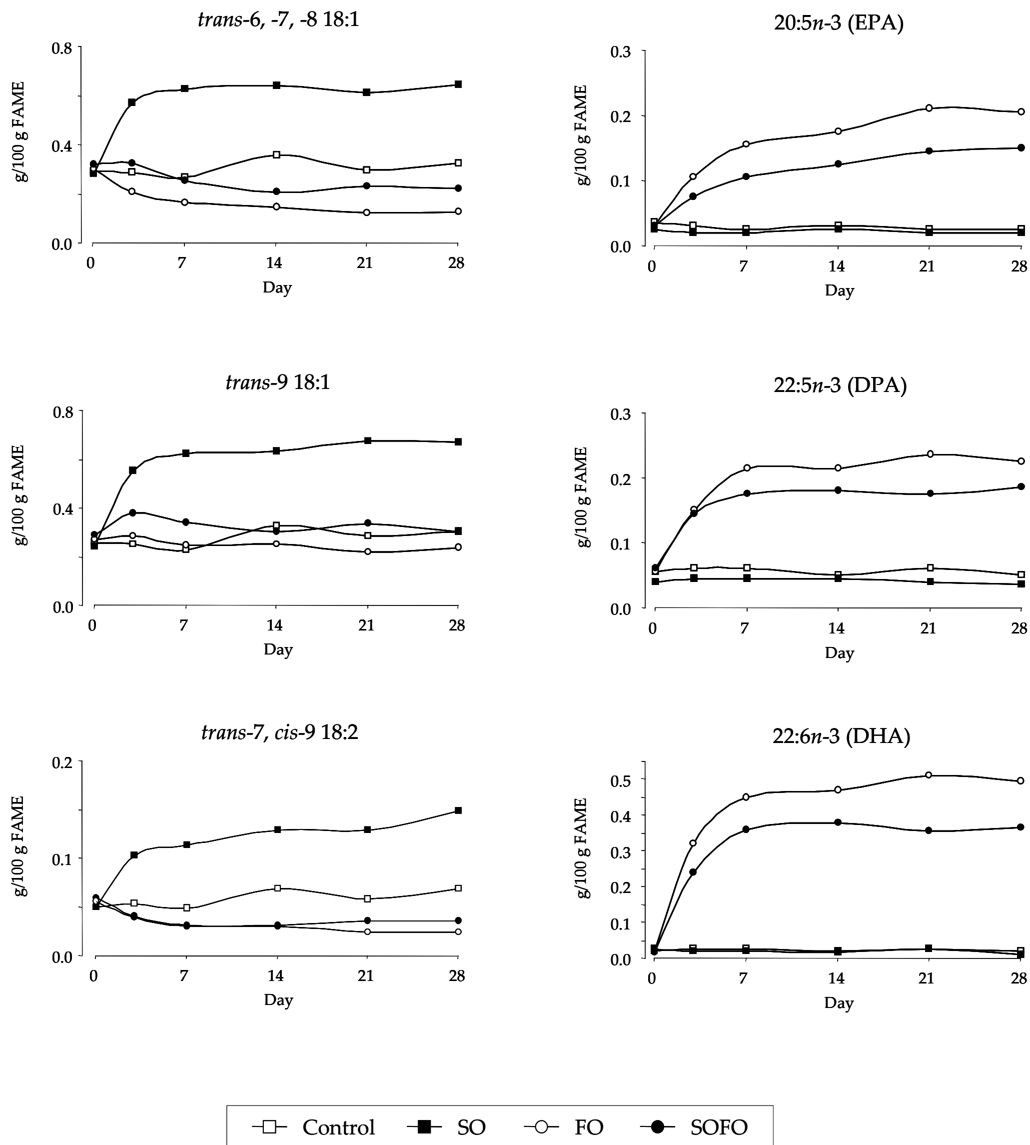

Figure 3. *Temporal changes in* trans-*6, -7, -8 18:1,* trans-*9 18:1,* trans-*7,* cis-*9 18:2, 20:5*n-*3, 22:5*n-*3, and 22:6*n-*3 [g/100 g fatty acids methyl esters (FAME)] in ewes fed diets with no supplementation (control), or supplemented with 20 g of sunflower oil/kg (SO), 10 g of fish oil/kg (FO), and 20 g of sunflower oil plus 10 g of fish oil/kg (SOFO). Values represent the mean from 2 lots of 8 animals per lot. Standard error of the difference = 0.004, 0.006, 0.002, 0.005, 0.006 and 0.013 for* trans-*6, -7, -8 18:1,* trans-*9 18:1,* trans-*7,* cis-*9 18:2, 20:5*n-*3; 22:5*n-*3, and 22:6*n-*3 respectively.*

The strong relationship observed between fat yield and *trans*-10 18:1 content ($r = -0.91$; $P < 0.001$; Table 5) may suggest that this FA is another putative inhibitor of lipogenesis, although previous studies in ewes have shown that oil supplementation resulting in similar concentrations did not cause MFD (Gómez-Cortés et al., 2008a,b; Hervás et al., 2008). Furthermore, in FO-containing diets, the reduction in 18:0 for endogenous oleic acid synthesis and the increase in *trans* 18:1 isomers probably played a role in MFD, related to maintenance of milk fat fluidity (Gama et al., 2008).

3.5. Long chain n-*3 fatty acids*

The long chain *n*-3 PUFA contents in FO and SOFO diets increased sharply during the first days of supplementation, followed by a plateau (Figure 3), as previously observed in the few studies conducted in dairy sheep (Papadopoulos et al., 2002; Kitessa et al., 2003). These increases reached their highest values with the FO treatment. The greatest increase corresponded to DHA ($P < 0.001$), and those of eicosapentaenoic acid and docosapentaenoic acid (Figure 3) were of minor magnitude. Their apparent transfer efficiencies into milk after 4 week of supplementation were relatively low (0.135 vs. 0.086 for EPA, and 0.114 vs. 0.075 for DHA in FO and SOFO diets, respectively) and within the same range as that calculated for marine oil fed to ewes (Papadopoulos et al., 2002; Reynolds et al., 2006; Capper et al., 2007), but considerably higher than that usually reported for dairy cows (Lock and Bauman, 2004; Loor et al., 2005; Shingfield et al., 2006). The reasons for this low transference would not only be due to extensive biohydrogenation in the rumen (Doreau and Chilliard, 1997; Jenkins et al., 2008) but also to preferential partitioning of these FA into some plasma lipid fractions such as plasma cholesterol ester and phospholipids, which are poorly taken up by the mammary gland (Kitessa et al., 2001; Lock and Bauman, 2004). The ratio of *n*-6:*n*-3 FA in milk (Table 4) was augmented with the use of SO, whereas SOFO almost halved (−48%) the value observed for the control treatment, and the FO diet further reduced it (−55%; $P < 0.001$). The ratios lower than 4 observed in the

FO-containing treatments would suggest a milk having a potentially suppressive effect on the pathogenesis of highly prevalent chronic diseases (Simopoulos, 2008).

4. CONCLUSIONS

While supplementation of ewe diet with FO significantly reduced milk fat content, the addition of SO did not, and SOFO further decreased milk yield and total solids content. The increase in some milk *trans* FA that are putative inhibitors of milk fat synthesis, such as *trans*-9, *cis*-11 CLA and perhaps *trans*-10 18:1, and the theory of reduced milk fat fluidity (consistent with changes in rumen biohydrogenation pathways) may provide a suitable explanation for FO-induced MFD. On the other hand, *trans*-10, *cis*-12 CLA was detected in very low concentrations and showed no changes with time. Lipid supplementation with SO, FO, and SOFO proved to be an effective nutritional strategy for altering milk FA profile and improving the content of some bioactive components, such as RA, VA, and, to a minor extent, *n*-3 PUFA.

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CHAPTER II

EFFECT OF THE SUPPLEMENTATION OF A HIGH-CONCENTRATE DIET WITH SUNFLOWER AND FISH OILS ON RUMINAL FERMENTATION IN SHEEP

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Published in: Small Ruminant Research 81 (2009): 119-125

doi:10.1016/j.smallrumres.2008.12.009

EFFECT OF THE SUPPLEMENTATION OF A HIGH-CONCENTRATE DIET WITH SUNFLOWER AND FISH OILS ON RUMINAL FERMENTATION IN SHEEP

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Abstract. This study was conducted to test the hypothesis that the supplementation of a high-concentrate diet with lipids, reportedly a good strategy for improving the nutritional value of ruminant-derived products, may not necessarily be associated with detrimental effects on ruminal fermentation in sheep. Four ruminally cannulated adult ewes were fed a high-concentrate diet, with no oil (Control diet), for a 14-day adaptation period. Afterwards, they were fed the same basal diet but supplemented with sunflower oil [20 g/kg fresh matter (FM)] and fish oil (10 g/kg FM) (SOFO diet) for a further 11 days, to investigate the impact of the addition of oils on the ruminal fermentation of the diet. On days 0 (Control), 3 and 10 of the experimental period rumen fluid was sampled at 0, 1.5, 3, 6 and 9 h after the morning feeding, for analysis of pH, and ammonia, lactate and total volatile fatty acid (VFA) concentrations. Alfalfa hay was incubated in situ, using the nylon bag technique, for 12 and 24 h to examine the effect of oil supplementation on ruminal disappearance of dry matter (DM), crude protein (CP) and neutral detergent fibre (NDF). On days 0 and 11, rumen fluid was collected just before the morning feeding and used to incubate alfalfa hay and the Control and SOFO diets by means of the in vitro gas production technique. The mean concentrations of acetate (87.8 mmol/L vs. 73.7 mmol/L) and butyrate (21.2 mmol/L vs. 17.7 mmol/L) were reduced by oil supplementation ($P < 0.05$) and the total VFA showed a tendency ($P = 0.098$) to be lower with the SOFO diet (139.0 mmol/L vs. 122.1 mmol/L). However, none of the other in vivo ruminal fermentation parameters were affected by the treatment ($P > 0.10$). The oil supplementation affected neither in situ rumen disappearance of DM, CP and NDF of alfalfa hay, nor rates of gas production ($P > 0.10$). On the other hand, a

little, but significant reduction in cumulative gas production was observed when the experimental diets were incubated with rumen fluid derived from animals fed the oil-rich diet ($P < 0.05$).

Overall, the results suggest that the supplementation of high-concentrate diets with sunflower oil (20 g/kg FM) plus fish oil (10 g/kg FM) had little effect on ruminal fermentation and therefore its use to improve the nutritional value of ruminant-derived products cannot be precluded.

Key words: lipid, rumen degradation, linoleic acid, *n*-3 polyunsaturated fatty acids

1. INTRODUCTION

Ruminant-derived products are the major source of conjugated linoleic acid (CLA) in the diet (Lawson et al., 2001). Feeding ruminants with vegetable oils rich in linoleic acid, such as sunflower oil, or fish oils, which are rich in *n*-3 polyunsaturated fatty acids (PUFA), has proved to be an effective strategy for increasing CLA in milk (Shingfield et al., 2006). Benefits of some isomers of CLA, such as rumenic acid (*cis*-9, *trans*-11 C18:2), and *n*-3 PUFA to human health (Williams, 2000) have motivated the current interest in lipid supplementation to ruminants.

Nevertheless, earlier studies on the addition of lipids to ruminant diets as an energy source raised concerns about detrimental effects of fatty acids (FA) on ruminal fermentation (Jenkins, 1993). Lipids are extensively hydrolysed in the rumen, rendering FA that have bacteriostatic and bacteriocidal effects. Among them, unsaturated FA are more antimicrobial than saturated ones (Harfoot and Hazlewood, 1997), and a differential toxicity of different PUFA to rumen microorganisms has also been observed (Maia et al., 2007). Dietary supplementation with oils has given inconsistent results on ruminal fermentation, with detrimental consequences (Fievez et al., 2003), no effects (Keady and Mayne, 1999; Beauchemin et al., 2007) and even positive responses (Sinclair et al., 2005).

Controversial results may be due not only to the type (Wachira et al., 2000; Fievez et al., 2003) and amount (Doreau and Chilliard, 1997; Shingfield et al., 2008) of oil, but also to the basal diet composition. Palmquist and Griinari (2006) observed in cattle that a high-forage diet supplemented with a combination of sunflower oil and fish oil implied no adverse effects on rumen fermentation. However, Ueda et al. (2003) indicated that cattle fed high-concentrate diets were more prone to the detrimental effects of unsaturated FA, although our previous studies in lactating sheep (e.g., Gómez-Cortés et al., 2008) suggested that the supplementation of a concentrate-rich diet with 60 g/kg dry matter (DM) of soybean oil did not affect in vitro ruminal fermentation.

On the basis of the latter studies, we hypothesized that the supplementation of a high-concentrate diet with lipids is not necessarily associated with detrimental effects on ruminal fermentation in sheep. The aim of this study was therefore to investigate the impact of the supplementation of a high-concentrate diet with a combination of sunflower oil (20 g/kg), rich in linoleic acid, and fish oil (10 g/kg), rich in *n*-3 PUFA, on ruminal fermentation in ewes.

2. MATERIAL AND METHODS

2.1. Animals and diets

Four individually penned Merino ewes (mean LW 61.3 ± 10.80 kg), fitted with a ruminal cannula (40 mm internal diameter), were used. The diets, prepared in a feed mixer every week, consisted of a total mixed ration, based on alfalfa hay (particle size > 4 cm) and concentrate (forage:concentrate ratio 20:80) supplemented with 0 (Control diet) or 20 g of sunflower oil plus 10 g of fish oil/kg fresh matter (FM) (SOFO diet), whose ingredients and chemical composition are given in Table 1. Clean water and vitamin-mineral supplement were always available.

The experiment was performed in accordance with Spanish Royal Decree 1201/2005 for the protection of animals used for experimental and other scientific purposes.

Table 1. *Ingredients and chemical composition of the experimental diets: Control diet (with no oil supplementation) and SOFO diet (supplemented with 20 g of sunflower oil plus 10 g of fish oil per kg of fresh matter).*

	Diets	
	Control	SOFO
Ingredients, g/kg FM		
Dehydrated alfalfa hay	200.0	194.0
Whole maize grain	250.0	242.5
Soybean meal	200.0	194.0
Whole barley grain	150.0	145.5
Beet pulp	90.0	87.2
Molasses	65.0	63.1
Salts[1]	40.0	38.8
Minerals and vitamins[2]	5.0	4.9
Sunflower oil[3]	0.0	20.0
Fish oil[4]	0.0	10.0
Composition, g/kg DM		
Organic matter	891	887
Crude protein	185	191
Neutral detergent fibre	226	219
Acid detergent fibre	138	129
Ether extract	25	66

[1] Containing (g/kg) $NaHCO_3$ (375), $CaCO_3$ (350), Ca_2HPO_4 (150) and mine salt (125).
[2] INA OV1 (EVIALIS, Madrid, Spain).
[3] Sunflower oil contained (% total fatty acid methyl esters) C16:0 (7.5), C18:0 (4.3), C18:1 (26.3), and C18:2 (60.5).
[4] Fish oil (semi-refined tuna and sardine oil; Afampes 121 DHA, Afamsa, Spain) contained (% total fatty acid methyl esters) C16:0 (21.4), C18:0 (5.9), C18:1 (14.9), C18:2 (1.7), C20:5 (6.3), and C22:6 (17.8).

3.2. Experimental design

All the animals were offered the experimental diets (37 g DM/kg $LW^{0,75}$ and day) in two meals (60% at 9:00 h and 40% at 18:00 h) at 0.8 times the voluntary feed intake previously determined ad libitum, over two consecutive periods. First, the Control diet was supplied for a 14-day adaptation period, and afterwards the animals were switched to the SOFO diet for 11 more days. In vivo, in situ and in

vitro assays were conducted immediately before starting the administration of the SOFO diet, when the ewes were adapted to the consumption of the basal diet (day 0, Control), and after 3 days ($SOFO_3$; to study a possible prompt response) and 10 days ($SOFO_{10}$) of the administration of the SOFO diet. Even though the treatment might be confounded with the experimental period, this approach was used rather than a switch-back design in order to allow the adaptation of the sheep to the basal diet (i.e., the diet without supplementation) and to avoid a carry over effect of the oils on the rumen microbiota known to influence the ruminal fermentation (Wąsowska et al., 2006). Similar experimental designs have been previously used for similar studies (e.g., Shingfield et al., 2003).

2.3. Experimental procedures

In vivo studies. Feed intake was monitored daily by weighing and drying the refusals. After 0 (Control), 3 ($SOFO_3$) and 10 ($SOFO_{10}$) days of sunflower plus fish oil supply, rumen fluid samples were collected via the cannula from each sheep at 0, 1.5, 3, 6 and 9 h after the morning feeding. Rumen fluid was strained through two layers of muslin, pH was measured and 4 mL were acidified with 4 mL of 0.2 M HCl for ammonia determination. Further aliquots of 4 and 0.8 mL of ruminal fluid were taken respectively for lactic acid and volatile fatty acid (VFA; deproteinized with 0.5 mL of 20 g/L metaphosphoric and 4 g/L crotonic acids in 0.5 M HCl) determinations. All samples were stored at –30 ºC until analysis.

In situ studies. With the same frequency, i.e., after 0 (Control), 3 ($SOFO_3$) and 10 ($SOFO_{10}$) days of SOFO supply, in situ rumen degradation of a reference feedstuff, alfalfa hay, was estimated. Nylon bags (150 mm × 100 mm; Ankom, Macedon, NY, USA) made of filter cloth with an approximate pore size of 50 μm of diameter were filled with 4 g of alfalfa hay [902 g organic matter/kg DM; 173 g crude protein (CP)/kg DM; 419 g neutral detergent fibre (NDF)/kg DM], previously ground through a 2 mm screen. Bags were incubated in duplicate for 12 and 24 h (two bags/time, four bags in total per animal), by suspending them in the rumen

of each ewe just before the morning feeding. After removal from the rumen, the bags were washed with cold tap water and frozen (–30 ºC) to help remove microbial attachment to feed particles. Once defrosted, bags were washed again with cold water in a commercial washing machine and dried in a forced-air oven at 60 ºC to constant weight to determine DM disappearance (DMD). Concentrations of CP and NDF in the residues were measured to determine CP and NDF disappearances, respectively (CPD and NDFD).

In vitro studies. After 0 (Control) and 11 ($SOFO_{11}$) days of SOFO supply, in vitro ruminal fermentation was assessed using a modification of the gas production technique described by Theodorou et al. (1994), as adapted by Mauricio et al. (1999). Two rumen inocula were obtained from the ewes (2 + 2), pre-feeding, through the ruminal cannula. Rumen fluid was collected and transferred to the laboratory in pre-warmed thermos flasks and then strained through a double layer of muslin and kept under CO_2 flushing. The incubated substrates were alfalfa hay and the two experimental diets (Control and SOFO diets). Six samples per substrate (500 mg) [2 inocula (replicates) × 3 flask/inoculum] were incubated in sealed serum flasks at 39 ºC with 10 mL rumen fluid and 40 mL phosphate-bicarbonate buffer. Buffer solution was prepared as described by Goering and Van Soest (1970), with the exception that no trypticase was added, and pH was adjusted to 6.5 with orthophosphoric acid. Accumulated head-space gas pressures were measured with a pressure transducer at 2, 4, 6, 8, 10, 15, 18, 21, 24, 30, 36, 48, 72 and 96 h post-inoculation. Pressure values, corrected for the quantity of substrate organic matter incubated and gas released from blanks (i.e., rumen fluid plus buffer medium, without substrate; three flasks/inoculum), were used to generate gas volume estimates using a predictive equation derived from earlier simultaneous pressure and volume measurements (Hervás et al., 2005).

2.4. Chemical analyses

Experimental diets and in situ incubation residues were analysed for DM (ISO 6496:1999), ash (ISO 5984:2002) and CP (ISO 5983-2:2005). NDF and acid (ADF) detergent fibre were determined by the methods described by Mertens (2002) and the AOAC (2006; Official Method 973.18), respectively, using the Ankom technology (Ankom, Macedon, NY, USA). NDF was assayed with sodium sulphite and α-amylase and expressed with residual ash (the latter also for ADF). The content of ether extract in the diets was determined by the filter bag technology (AOCS, 2008; Official Procedure Am 5-04), using an Ankom XT15 extraction system (Ankom, Macedon, NY, USA). Ammonia and lactic acid concentrations were determined by colorimetric methods (Weatherburn, 1967, and Taylor, 1996; respectively) and VFA by gas chromatography, with crotonic acid as internal standard (Ottenstein and Bartley, 1971), in centrifuged samples.

2.5. Calculations and statistical analysis

Gas production (G) data were fitted with time to the exponential model:

$$G = A \cdot (1 - e^{-c \cdot t})$$

where A represents the asymptotic gas production (mL/g organic matter), c is the fractional rate of gas production (/h), and t is the gas reading time (h). Parameters A and c were estimated by an iterative least squares procedure using the NLIN procedure of the Statistical Analysis System package (SAS, 2003).

The average fermentation rate (AFR; mL gas/h) was defined as the average gas production rate between the start of the incubation and the time when the cumulative gas production was half of its asymptotic value, and was calculated according to the equation proposed by France et al. (2000):

$$AFR = A \cdot c / (2 \cdot \ln 2)$$

All data were analysed by one-way analysis of variance, using the MIXED procedure of the SAS (2003). The statistical model included the fixed effect of treatment (Control, $SOFO_3$ and $SOFO_{10}$ in the in vivo and the in situ data, and Control vs. $SOFO_{11}$ in the in vitro gas production parameters). In vivo data taken

over time (hours post-feeding) were analysed by repeated measures, using the MIXED procedure of the SAS (2003). The statistical model included the fixed effects of treatment, hours post-feeding and their interaction. Means were separated using the 'pdiff' option of the 'lsmeans' statement of the MIXED procedure. Significant differences were declared at $P < 0.05$, and tendencies at $P < 0.10$.

3. RESULTS

3.1. In vivo studies

The daily feed intake was not significantly affected by the experimental treatment and averaged 36 ± 0.3 g DM/kg $LW^{0.75}$. Rumen pH, and ammonia, lactate and VFA concentrations are presented in Table 2. Supplementation with SOFO had no effect ($P > 0.10$) on any of these ruminal fermentation parameters when analysed separately for each time post-feeding, except for a lower acetate concentration ($P < 0.05$) observed at 3 h post-feeding in $SOFO_3$ sampling (i.e., after 3 days of SOFO supplementation). However, mean values of acetate and butyrate concentrations were significantly lower when animals were fed the SOFO diet ($P < 0.05$), and the mean total VFA concentration showed a statistical tendency to be also lower with the oil supplementation ($P = 0.098$). The interaction between the treatment and the hours post-feeding was never significant, and therefore is not included in the table. The acetate:propionate ratio (data not shown) was not affected by the experimental treatment, the average value being 3.64 ± 0.139 ($P > 0.10$). Nor were there any changes in molar proportions of acetate, propionate, butyrate or other VFA (calculated as the sum of isobutyrate, isovalerate, valerate and caproate) with oil supplementation, with average values of 0.61, 0.20, 0.14 and 0.05 mol/mol, respectively (data not shown).

3.2. In situ studies

Disappearances of DM, CP and NDF of alfalfa hay in the rumen were not affected by the dietary supplementation with SOFO, giving mean values of 0.729, 0.904,

Table 2. *Post-feeding changes (at 0, 1.5, 3, 6 and 9 h after the morning feeding) in pH, and ammonia (mg/L), volatile fatty acid (VFA; mmol/L) and lactic acid (mmol/L) concentrations in the rumen liquid of animals on treatments Control, $SOFO_3$ and $SOFO_{10}$.*

		Treatment[1]			SED	Significance (*P*)	
	Time (h)	Control	$SOFO_3$	$SOFO_{10}$		Treatment	Hours
pH	0	6.64	6.74	6.80	0.284	0.860	
	1.5	6.16	6.34	6.32	0.141	0.412	
	3	6.07	6.18	6.13	0.182	0.836	
	6	6.19	6.24	6.22	0.240	0.980	
	9	6.37	6.41	6.64	0.220	0.460	
	Mean	*6.28*	*6.38*	*6.42*	*0.069*	*0.382*	*<0.001*
Ammonia	0	238.7	354.5	310.0	55.93	0.167	
	1.5	342.0	378.9	362.2	49.76	0.765	
	3	312.9	290.1	360.9	61.61	0.527	
	6	205.8	200.0	258.8	47.41	0.428	
	9	193.4	272.7	233.0	49.02	0.317	
	Mean	*258.5*	*299.3*	*305.0*	*16.50*	*0.108*	*<0.001*
Total VFA	0	89.4	78.2	68.5	21.84	0.646	
	1.5	177.0	146.4	154.9	15.47	0.180	
	3	174.7	144.5	166.0	16.18	0.214	
	6	127.4	118.7	127.6	21.20	0.916	
	9	126.2	116.1	100.2	20.16	0.459	
	Mean	*139.0*	*120.8*	*123.4*	*6.28*	*0.098*	*<0.001*
Acetate	0	55.6	46.2	39.1	13.60	0.507	
	1.5	110.8	89.0	91.2	10.39	0.124	
	3	110.1[a]	88.1[b]	102.1[a]	6.17	0.018	
	6	81.7	73.0	76.6	12.85	0.796	
	9	81.0	71.2	60.4	12.00	0.282	
	Mean	*87.8*[a]	*73.5*[b]	*73.9*[b]	*3.58*	*0.009*	*<0.001*
Propionate	0	13.5	11.9	12.7	5.56	0.961	
	1.5	30.0	29.0	30.9	7.01	0.965	
	3	30.8	31.2	34.6	9.17	0.905	
	6	22.6	25.1	28.6	10.41	0.850	
	9	21.4	21.9	21.4	7.33	0.998	
	Mean	*23.7*	*23.8*	*25.6*	*2.56*	*0.836*	*<0.001*
Butyrate	0	14.6	13.7	11.5	3.47	0.668	
	1.5	28.1	21.2	24.5	3.45	0.184	
	3	26.6	18.8	22.2	3.92	0.194	
	6	18.0	15.9	17.4	2.84	0.749	
	9	18.8	17.3	13.8	3.14	0.312	
	Mean	*21.2*[a]	*17.4*[b]	*17.9*[b]	*1.07*	*0.029*	*<0.001*
Lactic acid	0	0.66	0.63	0.77	0.168	0.711	
	1.5	1.15	1.17	1.16	0.477	0.999	
	3	0.66	0.57	0.70	0.067	0.174	
	6	0.56	0.48	0.58	0.114	0.687	
	9	0.86	0.61	0.74	0.326	0.747	
	Mean	*0.78*	*0.69*	*0.79*	*0.087*	*0.692*	*0.021*

Different letters indicate significant differences within a row ($P < 0.05$).

[1] Control = after 0, $SOFO_3$ = after 3, and $SOFO_{10}$ = after 10 days of sunflower and fish oil supplementation.

and 0.452 g/g incubated, respectively after 24 h of incubation (Table 3). After 12 h, approximately 0.90 of the DM and CP and 0.78 of the NDF that disappeared in the 24 h incubation period had been degraded.

3.3. In vitro gas production

In vitro gas production parameters are shown in Table 4. No significant differences between treatments were observed in the fractional rate of degradation (c) and the average fermentation rate (AFR) for any of the incubated substrates. However, the incubations with inocula obtained after 11 days of SOFO supply ($SOFO_{11}$) showed a slight reduction in the cumulative gas production (A; $P < 0.05$) compared with those with the inocula derived from animals fed the Control diet. This effect was not observed when alfalfa hay was incubated ($P > 0.10$).

4. DISCUSSION

There is a widespread idea that PUFA can detrimentally affect ruminal fermentation. However, in the present study ewes were fed a high-concentrate diet supplemented with a combination of sunflower oil (20 g/kg) and fish oil (10 g/kg), and rumen fermentation was not significantly impaired, as it may have been expected from the low oil intake.

Lipids are hydrolysed extensively in the rumen by microbial lipases, releasing long-chain fatty acids that may inhibit bacterial activity. Among long-chain FA, unsaturated ones are more antimicrobial than saturated ones (Harfoot and Hazlewood, 1997), biohydrogenation therefore serving to protect microbes from their toxic effect. Microbial toxicity of *n*-3 PUFA, which are present in high amounts in fish oil, has been reported to be greater than the toxicity of linoleic acid (Maia et al., 2007), which is abundant in sunflower oil. However, in this study they seemed to exert no negative effects on the ruminal fermentation parameters studied in vivo.

First of all, the diet supplementation with oils had no effect on the feed consumption, contrarily to what was observed by Shingfield et al. (2006) when

supplementing dairy cows with sunflower and fish oils. Nevertheless, the fact that the feed supply was fixed (at 0.80 times ad libitum intake, as above explained) precludes any conclusion on the lack of effect to be withdrawn.

Table 3. *Disappearance (g/g incubated) of dry matter (DMD), crude protein (CPD) and neutral detergent fibre (NDFD) from alfalfa hay incubated for 12 and 24 h in the rumen of animals on treatments Control, $SOFO_3$ and $SOFO_{10}$.*

		Treatment[1]			SED	Significance
		Control	$SOFO_3$	$SOFO_{10}$		(*P*)
DMD	12 h	0.653	0.659	0.662	0.0254	0.943
	24 h	0.729	0.726	0.732	0.0209	0.965
CPD	12 h	0.828	0.847	0.843	0.0125	0.298
	24 h	0.900	0.908	0.905	0.0075	0.586
NDFD	12 h	0.341	0.358	0.361	0.0482	0.904
	24 h	0.452	0.441	0.464	0.0422	0.965

[1] Control = after 0, $SOFO_3$ = after 3, and $SOFO_{10}$ = after 10 days of sunflower and fish oil supplementation.

Table 4. *In vitro gas production parameters (*A*, mL/g organic matter and *c*, /h) and average fermentation rate (AFR, mL/h) for each substrate (alfalfa hay, Control diet and SOFO diet) incubated with rumen inoculum derived from animals on treatments Control and $SOFO_{11}$.*

	Treatment[1]		SED	Significance
	Control	$SOFO_{11}$		(*P*)
Cumulative gas production (*A*)				
Alfalfa hay	258.0	249.9	4.74	0.232
Control diet	346.8[a]	334.3[b]	2.64	0.041
SOFO diet	346.2[a]	322.2[b]	5.49	0.049
Fractional rate of gas production (*c*)				
Alfalfa hay	0.097	0.091	0.0084	0.528
Control diet	0.080	0.081	0.0019	0.632
SOFO diet	0.078	0.082	0.0057	0.533
Average fermentation rate (AFR)				
Alfalfa hay	18.02	16.33	1.212	0.297
Control diet	19.95	19.48	0.575	0.499
SOFO diet	19.33	19.00	1.102	0.790

Different letters indicate significant differences within a row ($P < 0.05$).
[1] Control = after 0, and $SOFO_{11}$ = after 11 days of sunflower and fish oil supplementation.

Lactate was always present at normal concentrations (i.e., less than 5 mM; Owens et al., 1998), and the mean pH remained always within a physiological range (Krause and Oetzel, 2006). The pH was not affected by oil supplementation, in agreement with previous in vivo studies using different lipid sources, including fish and sunflower oils (Fievez et al., 2003; Beauchemin et al., 2007). Shingfield et al. (2003) reported a higher pH when fish oil was included in the diet of cows, which was attributed to associated decreases in DM intake that were not observed in our study.

Ammonia concentrations were always greater than the 100 mg/L reported by Van Soest (1994) as optimal for the efficiency of amino acid synthesis and microbial growth, and were not affected by oil supplementation. Previous experiments with rumen fluid from sheep show inconsistent results, with significant increases or decreases in ammonia with linolenic or linoleic sources (Gómez-Cortés et al., 2008; Zhang et al., 2008). According to Shingfield et al. (2008), sunflower oil supplementation tends to reduce ammonia concentration in the rumen of cattle, whereas fish oil supplementation has been reported to increase it (Keady and Mayne, 1999). The lack of a significant effect on the concentrations of either ammonia or those VFA originating from the deamination of some amino acids (valerate and branched-chain VFA) would indicate that N metabolism was not altered in our study.

Although an inhibition of microbial activity by oil supplementation might lead to a reduction in total VFA concentration in the rumen, as Lee et al. (2005) observed with their highest level of fish oil inclusion, other studies involving sheep (Fievez et al., 2003) and cattle (Keady and Mayne, 1999; Shingfield et al., 2008) reported no significant effect of oil supplementation on total VFA concentration. In the current study, total VFA concentration was not affected at any specific time post-feeding, and only its mean value showed a trend (P = 0.098) to be lower with oil supplementation.

Concerning particular VFA, the addition of fish oil to the diet has often been reported to result in an increase in the molar proportion of propionate

concentration and a decrease in acetate (Doreau and Chilliard, 1997; Keady and Mayne, 1999; Wachira et al 2000; Fievez et al 2003). Similar results were observed when linoleic acid was incubated in vitro with rumen fluid from sheep (Zhang et al., 2008), whereas supplementation with sunflower oil in cattle did not affect the rumen VFA proportions (Beauchemin et al., 2007). The propionate concentration was not significantly changed in this study, whereas the average acetate concentration showed a reduction with oil supplementation. This suggests that acetate-producing bacteria, such as *Fibrobacter succinogenes* and *Ruminococcus flavefaciens*, which are considered to be predominant cellulolytic bacteria in the rumen, may have been more inhibited by PUFA (Maia et al., 2007; Zhang et al., 2008).

From a physiological point of view, a shift in the rumen microbial communities may result in changes in biohydrogenation and, consequently, in the milk FA profile (Palmquist et al., 2005). Furthermore, a decrease in acetate concentration might contribute to a reduction in mammary de novo fatty acid synthesis, which requires acetate as a precursor (Doreau and Chilliard, 1997).

Regarding butyrate, the effect of oil supplementation is very inconsistent, with reductions with fish oil or linoleic acid (Fievez et al., 2003; Zhang et al., 2008), no effects with linoleic-rich sources or fish oil (Keady and Mayne, 1999; Beauchemin et al., 2007; Shingfield et al., 2008) and even increases with a combination of sunflower oil and fish oil (Palmquist and Griinari, 2006) having been reported. In the current study, the reduction in the mean butyrate concentration might be explained by variations in butyrate-producing bacteria, such as *Eubacterium ruminantium* and *Butyrivibrio fibrisolvens*. The former bacteria is known to be inhibited by PUFA (Maia et al., 2007), whereas the numbers of the latter species, which are tolerant to high concentrations of unsaturated fatty acids (Maia et al., 2007), are usually reduced in animals receiving high-concentrate diets (Harfoot and Hazlewood, 1997).

Ruminal degradation of DM, CP and NDF was not affected by oil supplementation in our study, in agreement with Keady and Mayne (1999), who

observed no effect of fish oil supplementation, even when a shift in the rumen fermentation pattern was observed. The consequences of oil supplementation reported elsewhere, however, include reductions, no effects or even increases in fibre degradation (Wachira et al., 2000; Sinclair et al., 2005). In the present study, although a high-concentrate diet could have limited the number of predominant cellulolytic bacteria, which are usually more affected by oil supplementation (Doreau and Chilliard, 1997), other bacteria, able to degrade fibre, might have occupied their niches, which would explain the absence of any effect on the degradation of fibre.

In a previous study in milking ewes supplemented with sunflower and fish oils (results to be published elsewhere), significant changes in milk composition and fatty acid profile after only 3 days of lipid inclusion suggested a prompt response of the rumen microbiota. However, this hypothetic prompt modification of the microbial communities could not be confirmed in the current experiment because it was not reflected in remarkable differences between the two samplings carried out during the SOFO treatment ($SOFO_3$ and $SOFO_{10}$) on the studied ruminal fermentation parameters.

The effect of lipid supplementation on ruminal fermentation relies mainly on three factors, as previously mentioned: first, the type of oil (Wachira et al., 2000), second, the level of oil inclusion in the diet (Shingfield et al., 2008) and third, the dietary forage:concentrate ratio, since animals fed a concentrate-rich diet might be more likely to be adversely affected by oil supplementation (Ueda et al., 2003). In lactating cows, a high-forage diet supplemented with a combination of sunflower and fish oils only induced a transient reduction in total VFA concentration at 2 h post-feeding, but no effect was observed at 6 h (Palmquist and Griinari, 2006). In our study, although the ewes were fed a high-concentrate diet, the dietary value of physically effective fibre (Mertens, 1997), with the concentrate including whole cereal grains and the alfalfa hay being supplied with a particle size greater than 4 cm, may explain the lack of a negative effect of oil supplementation on pH,

ammonia, lactate and VFA concentrations, and in situ ruminal degradation of DM, CP and NDF, suggesting that the rumen function was probably adequate.

In vitro gas production was slightly, although significantly, reduced when inocula from the animals fed the oil-rich diet were used to incubate either the Control (–3.6 %) or the SOFO diet (–6.9 %). The inocula derived from the animals fed the SOFO diet induced a decrease in in vitro gas production when both experimental diets were used as substrates compared to the inocula from the animals fed the Control diet, probably due to changes in the rumen microbial communities produced by the oil supplementation, as previously thought. The fact that the batch cultures of rumen microorganisms are closed systems renders them especially reliable for the detection of small changes in fermentation, which might explain why little reductions in gas production were found statistically significant.

5. CONCLUSIONS

The supplementation of a high-concentrate diet with a combination of sunflower oil (20 g/kg) and fish oil (10 g/kg), reportedly a good strategy for increasing the nutritional quality of ruminant-derived products, did not seem to remarkably impair ruminal fermentation in ewes. However, a shift in the VFA profile was observed, with decreases in acetate and butyrate concentrations, probably due to variations in the rumen microbial composition. Nonetheless, rumen function seemed to be maintained with the oil-rich diet, suggesting that microorganisms can adapt rapidly to fat supplementation and degrade the diet normally. It is probably worth mentioning that these results are based on a relatively small number of observations, due to the complexity of using cannulated animals, and further research involving longer-term experiments and molecular-based studies on rumen microbiota would be advisable.

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CHAPTER III

EFFECT OF FISH OIL AND SUNFLOWER OIL ON RUMEN FERMENTATION CHARACTERISTICS AND FATTY ACID COMPOSITION OF DIGESTA IN EWES FED A HIGH-CONCENTRATE DIET

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Submitted to: Journal of Dairy Science

Presented in part at the XIth International Symposium on Ruminant Physiology,
6–9 September 2009, Clermont-Ferrand, France

EFFECT OF FISH OIL AND SUNFLOWER OIL ON RUMEN FERMENTATION CHARACTERISTICS AND FATTY ACID COMPOSITION OF DIGESTA IN EWES FED A HIGH-CONCENTRATE DIET

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Abstract. Based on the potential benefits on human health there is interest in developing effective nutritional strategies for enhancing milk fat *cis*-9, *trans*-11 conjugated linoleic acid (CLA), 20:5*n*-3 and 22:6*n*-3 concentrations. Studies in ruminants have shown that supplementing the diet with a mixture of fish oil (FO) and sunflower oil (SO) enhances the concentration of these beneficial fatty acids in milk due to alterations in ruminal biohydrogenation but the intermediates formed under these conditions are not well characterised. Five ewes fitted with rumen cannula and fed a high-concentrate diet were used to examine the effect of a mixture (30 g/kg DM) of FO and SO (1:2, w/w) on temporal changes in rumen fermentation characteristics and the relative abundance of biohydrogenation intermediates in ruminal digesta collected on days 0, 3, and 10 on diet. Appearance and identification of biohydrogenation intermediates was determined based on complimentary gas-liquid chromatography and Ag^+-high performance liquid cromatography analysis of fatty acid methyl esters and gas chromatography-mass spectrometry analysis of corresponding 4,4-dimethyloxazoline derivatives. Inclusion of FO and SO in the diet had no effect on rumen pH, VFA concentrations or nutrient digestion, but altered the fatty acid composition of ruminal digesta, changes that were characterised by time-dependent decreases in 18:0 and 18:2*n*-6 and the accumulation of *trans* 16:1, *trans* 18:1, 10-O-18:0, and *trans* 18:2. Lipid supplements enhanced the proportion of 20:5*n*-3 and 22:6*n*-3 in digesta and resulted in numerical increases in *cis*-9, *trans*-11 CLA concentrations, but decreased the relative abundance of *trans*-10, *cis*-12 CLA. Furthermore, detailed analysis revealed the appearance of several unique 20:1,

20:2, 22:1, 22:3, and 22:4 metabolites in ruminal digesta that accumulated over time, providing the first indications of products formed during the biohydrogenation of long chain fatty acids in sheep. In conclusion, FO and SO in a high-concentrate diet caused a time-dependent inhibition of the complete biohydrogenation of 18 carbon unsaturated fatty acids resulting in the accumulation of *trans* 16:1, *trans* 18:1, *trans* 18:2, 20, and 22 carbon metabolites in ruminal digesta of sheep, with no evidence of a shift in ruminal biohydrogenation pathways towards *trans*-10 18:1 formation.

Key words: biohydrogenation, fish oil, sheep, *trans* fatty acid

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1. INTRODUCTION

Due to the potential beneficial effects on human health, numerous studies have examined various nutritional strategies for enhancing the concentration of *cis*-9, *trans*-11 conjugated linoleic acid (CLA) and long chain *n*-3 fatty acids in ruminant milk (Palmquist et al., 2005; Chilliard et al., 2007). Studies in lactating ruminants have shown that inclusion of fish oil (FO) in the diet alone or in combination with plant oils increases *cis*-9, *trans*-11 CLA, 20:5*n*-3, and 22:6*n*-3 content in milk (Shingfield et al., 2003, 2006; Toral et al., 2010a), with the level of enrichment being related to the metabolism of dietary unsaturated fatty acids in the rumen

Plant oils rich in 18:2*n*-6 are known to enhance the accumulation and ruminal outflow of *trans* 18:1 and *cis*-9, *trans*-11 CLA (Kucuk et al., 2004; Atkinson et al., 2006; Shingfield et al., 2008). Inclusion of FO containing 20:5*n*-3 and 22:6*n*-3 inhibits the complete biohydrogenation of 18 carbon unsaturated fatty acids (FA) causing an increase in the supply of *trans* 18:1 available for incorporation in milk fat triacylglycerides (Shingfield et al., 2003; Lee et al., 2008; Shingfield et al., 2010b). Even though 20:5*n*-3 and 22:6*n*-3 are extensively metabolized in the rumen, the products formed and the mechanisms involved are not known.

Measurements of temporal changes in milk FA composition have provided evidence that the inclusion of plant oils in high-concentrate diets (Bauman et al.,

2000; Roy et al., 2006) or FO and sunflower oil (SO) in total mixed rations (Shingfield et al., 2006; Cruz-Hernandez et al., 2007) result in time-dependent changes in ruminal biohydrogenation. Recent studies in sheep have also shown that supplementing a high-concentrate diet with 10 g of FO and 20 g of SO/kg diet increases milk fat *cis*-9, *trans*-11 CLA, 20:5*n*-3 and 22:6*n*-3 concentrations. However, enrichment of *cis*-9, *trans*-11 CLA in milk increased within 7 days on diet but declined thereafter (Toral et al., 2010a). Transient increases in milk *cis*-9, *trans*-11 CLA content were also associated with a progressive decrease in dry matter intake and milk fat synthesis over time (Toral et al., 2010a), effects that may, at least in part, be related to the impact of lipid supplements on nutrient digestion in the rumen (Wachira et al., 2000; Fievez et al., 2003; Shingfield et al., 2003). Furthermore, the decreases in milk fat content were not explained by significant increases in milk fat concentrations of *trans*-9, *cis*-11 CLA or *trans*-10, *cis*-12 CLA, biohydrogenation intermediates known to inhibit milk fat synthesis in cows (Shingfield and Griinari, 2007), suggesting that other metabolites and/or mechanisms are involved. In the current experiment, the effect of FO and SO on temporal changes in rumen fermentation patterns and the accumulation of biohydrogenation intermediates in sheep fed high-concentrate diets were examined based on the analysis of samples collected on days 0, 3 and 10 on diet. Fatty acid composition of ruminal digesta was analyzed using gas-liquid chromatography (GLC) and Ag^{+}-high performance liquid chromatography analysis of fatty acid methyl esters and gas chromatography-mass spectrometry analysis of corresponding 4,4-dimethyloxazoline derivatives to provide further insight into the metabolic fate of long-chain fatty acids in the rumen.

2. MATERIAL AND METHODS

2.1. Animals and diets

All experimental procedures were performed in accordance with the Spanish Royal Decree 1201/2005 for the protection of animals used for experimental and

other scientific purposes. Five individually penned Merino ewes (mean LW = 63.0 ± 5.99 kg), fitted with rumen cannulae were used. Diets were comprised of alfalfa hay (mean particle size > 4 cm) and a concentrate supplement (forage:concentrate ratio 35:65, DM basis) containing 0 (Control diet) or 30 g/kg DM of a mixture (1:2 w/w) of fish oil and sunflower oil (SFO). Oils replaced concentrate ingredients on a proportionate basis. Ingredients and chemical composition of the experimental diets are shown in Table 1. Diets were fed as a total mixed ration (TMR) to avoid selection of dietary components as two meals (60% at 9:00 h and 40% at 18:00 h) at a rate of 41 g DM/kg $LW^{0.75}$ equivalent to proportionately 0.8 of ad libitum intake measured over a 15 day period immediately before the start of the experiment. Ration mixes were prepared every 4 days and adjusted for changes in component DM content. Semi-refined tuna and sardine oil (Afampes 121 DHA, Afamsa, Vigo, Spain) and sunflower oil (Carrefour S.A., Madrid, Spain) were stored in the dark at 4 ºC prior to incorporation into daily rations. Animals had continuous access to fresh clean water.

2.2.Experimental design

The experiment was conducted over two consecutive 11 days periods in which all ewes were offered the control diet during the first experimental period followed by the same basal ration containing FO and SO in the second period. In vivo, in situ, and in vitro determinations were conducted immediately before the start of oil supplementation (day 0, Control), and on day 3 (SFO_3) and day 10 (SFO_{10}) on the SFO diet. Even though treatment was confounded with experimental period, this approach was used rather than a more robust switch-back or change-over design to avoid known residual carry-over effects of FO and SO on rumen fermentation and lipid metabolism (Cruz-Hernandez et al., 2006). The same experimental design has been used previously to examine the effects of FO on ruminal biohydrogenation and milk fat composition (Kitessa et al., 2001; Shingfield et al., 2003; Roy et al., 2006).

Table 1. *Ingredients and chemical composition of the experimental diets: Control diet (with no oil supplementation) and SFO diet (supplemented with 20 g of sunflower oil plus 10 g of fish oil per kg of fresh matter).*

	Diets	
	Control	SFO
Ingredients (g/kg fresh matter)		
Dehydrated alfalfa hay	350	341
Whole corn grain	203	198
Soybean meal	163	158
Whole barley grain	122	119
Beet pulp	73	71
Molasses	53	51
Salt premix[2]	33	32
Minerals and vitamins[3]	4	4
Sunflower oil[4]	0	18
Fish oil[5]	0	9
Composition (g/kg DM)		
Organic matter	882	891
Crude protein	172	163
Neutral detergent fiber	269	279
Acid detergent fibre	156	172
Starch	274	253
Fatty acids	46	75

[1] Control = no oil supplementation; SFO = supplemented with 20 g of sunflower oil plus 10 g of fish oil/kg DM.

[2] Containing (g/kg) $NaHCO_3$ (375); $CaCO_3$ (350); Ca_2HPO_4 (150), and NaCl (125).

[3] INA OV1 (EVIALIS, Madrid, Spain) declared as containing (g/kg): S (60); Mg (30); Fe (12); Zn (10); Mn (10); choline (6); as well as (in mg/kg) Co (200); I (200); Se (36); vitamin B1 (200); vitamin B2 (200); and (in UI/g) vitamin A (1250); vitamin D3 (250); DL-α-tocopheryl acetate (3).

[4] Sunflower oil (Carrefour S.A., Madrid, Spain) contained (g/kg) 12:0 (0.04), 14:0 (0.59), 16:0 (52.7), *cis*-9 16:1 (0.83), 18:0 (42.1), *cis*-9 18:1 (347), *cis*-11 18:1 (7.72), 18:2*n*-6 (479), 18:3*n*-3 (0.61), 20:0 (2.73), *cis*-11 20:1 (1.55), 22:0 (7.08), 24:0 (2.16), other (8.89), and total fatty acids (953).

[5] Semi-refined tuna and sardine oil (Afampes 121 DHA, Afamsa, Vigo, Spain) contained (g/kg) 12:0 (0.53), 14:0 (38.9), 15:0 (8.31), 16:0 (185), *cis*-9 16:1 (47.3), *cis*-11 16:1 (2.25), *trans*-6 16:1 (5.71), 16:2*n*-4 (2.72), 16:3*n*-4 (2.17), 16:4*n*-1 (2.06), 16:4*n*-3 (0.40), 17:0 (9.39), *cis*-9 17:1 (5.38), 18:0 (48.7), *cis*-9 18:1 (140), *cis*-11 18:1 (25.5), *cis*-12 18:1 (0.74), *cis*-13 18:1 (1.58), 18:2*n*-6 (21.3), 18:3*n*-3 (6.20), 18:3*n*-6 (1.04), 18:4*n*-3 (8.71), 19:0 (2.60), 20:0 (3.36), *cis*-9 20:1 (2.33), *cis*-11 20:1 (21.0), *cis*-13 20:1 (2.00), 20:2*n*-6 (2.94), 20:3*n*-3 (1.75), 20:3*n*-6 (1.11), 20:4*n*-3 (4.86), 20:4*n*-6 (14.0), 20:5*n*-3 (57.6), 21:5*n*-3 (1.98), 22:0 (2.04), *cis*-11 22:1 (15.9), *cis*-13 22:1 (3.19), 22:4*n*-6 (2.08), 22:5*n*-3 (12.5), 22:5*n*-6 (10.1), 22:6*n*-3 (164), *cis*-15 24:1 (6.49), 24:5*n*-3 (0.72), 24:6*n*-3 (0.92), other (48.4), unidentified (8.25), and total fatty acids (954).

2.3. Experimental procedures

In vivo studies. Dry matter intake (DMI) was measured daily. Rumen fluid was collected from each sheep on day 0 (Control), day 3 (SFO_3), and day 10 (SFO_{10}) on the SFO diet at 0, 1.5, 3, 6, and 9 h after morning feeding for the measurement of pH, and ammonia, lactic acid, and VFA concentrations (Toral et al., 2009). In addition, samples of ruminal digesta were collected at 0, 3, 6, 12, 18, and 24 h post-feeding and stored at –30 ºC immediately after collection. Digesta was pooled to provide composite samples for each animal and sampling day, freeze-dried, and stored at –80 ºC until submitted for FA determinations.

In situ studies. On day 0 (Control), day 3 (SFO_3), and day 10 (SFO_{10}) on the SFO diet, in situ rumen degradation of alfalfa hay used as a reference feed was estimated using nylon bags that were incubated in duplicate for 12 and 24 h. Concentrations of DM, CP, and NDF in incubation residues were assessed according to standard procedures (Toral et al., 2009).

In vitro studies. After 0 (Control) and 11 (SFO_{11}) days on the SFO diet, in vitro ruminal fermentation was assessed by the gas production technique using rumen inocula collected from the experimental ewes (Toral et al., 2009). Six samples (500 mg) per substrate (alfalfa hay, and control and SFO diets) were incubated in sealed serum flasks at 39 ºC with 10 mL rumen fluid and 40 mL phosphate-bicarbonate buffer [2 inocula (replicates) × 3 flask/inoculum]. Accumulated head-space gas pressures were measured at 2, 4, 6, 8, 10, 15, 18, 21, 24, 29, 35, 48, and 72 h post-inoculation. Pressure values, corrected for the amount of OM incubated and gas released from blanks (i.e., rumen fluid plus buffer medium, without substrate), were used to generate gas volume estimates using a predictive equation derived from earlier simultaneous measurements of pressure and volume (Hervás et al., 2005). In vitro DM disappearance after 72 h incubations was estimated by filtering fermentation residues.

2.4. Chemical analysis

Samples of diets were collected, stored at –30 ºC, and freeze-dried. Diet samples were analysed for DM (ISO 6496:1999), ash (ISO 5984:2002), CP (ISO 5983-2:2005), and starch (ISO 6493:2000). Concentrations of NDF and ADF were determined according to the instructions of the manufacturer (Ankom Technology Corp., Macedon, NY, USA) that included residual ash. Determinations of NDF were made using sodium sulphite and α-amylase.

Fatty acid methyl esters (FAME) of lipid in FO, SO, and freeze-dried samples of TMR were prepared in a one-step extraction-transesterification procedure using chloroform (Sukhija and Palmquist, 1988) and 2% (v/v) sulphuric acid in methanol (Shingfield et al., 2003). Fatty acid content was determined using tritridecanoin (T-135; Nu-Chek Prep Inc., Elysian, MN, USA) as an internal standard. Lipid in 200 mg of freeze dried ruminal digesta was extracted in duplicate using 4 mL of a mixture (3:2, v/v) of hexane and isopropanol following the adjustment of digesta pH to 2.0 using 2 M hydrochloric acid (Shingfield et al., 2003). Organic extracts were combined and dried under nitrogen at 50 ºC. Lipid dissolved in 2 mL of hexane was converted to FAME using a base-acid catalyzed transesterification procedure with freshly prepared 0.5 M sodium methoxide in methanol for 5 min at 20 ºC followed by reaction with 1% (v/v) sulphuric acid in methanol at 50 ºC for 30 min.

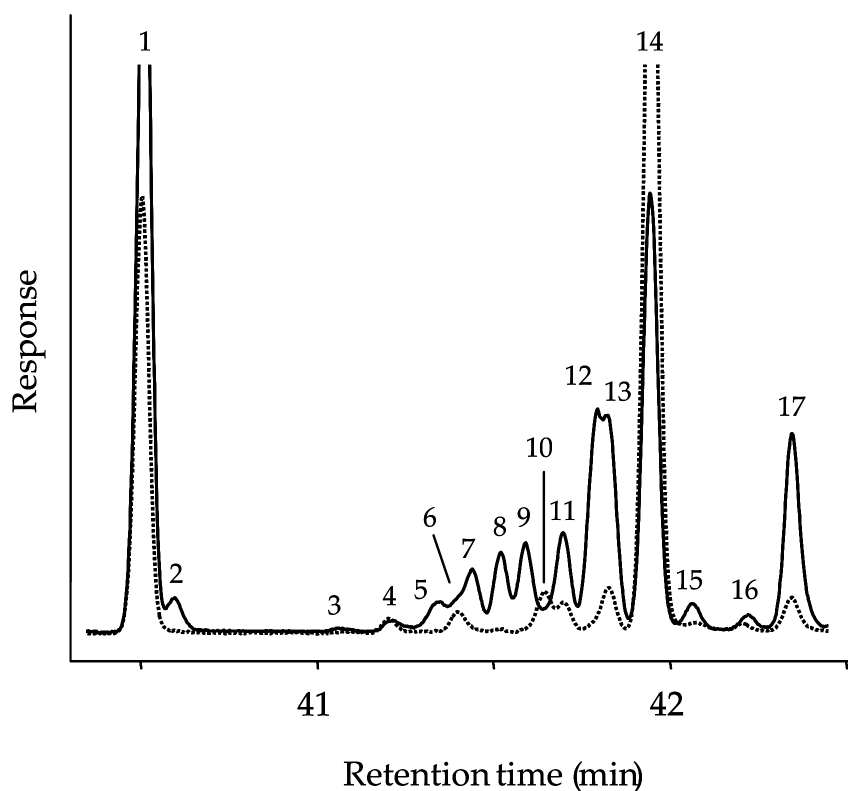
Methyl esters were quantified by GLC using a gas chromatograph (model 6890; Hewlett-Packard, Wilmington, DE, USA) equipped with a flame-ionization detector, selective quadrupole mass detector (model 5973N; Agilent Technologies Inc., Wilmington, DE, USA) and 100 m fused silica capillary column (i.d. 0.25 mm) coated with a 0.2 μm film of cyanopropyl polysiloxane (CP-Sil 88, Chrompack 7489; Middelburg, the Netherlands) using hydrogen as the carrier and fuel gas. Total FAME profile in a 2 μL sample at a split ratio of 1:50 was determined using a temperature gradient program (Shingfield et al., 2003). Isomers of 18:1 and 18:2 methyl esters were further resolved in a separate analysis under isothermal conditions at 170 ºC (Shingfield et al., 2003). Peaks were routinely identified using

a mixture of authentic FAME standards (GLC #463, N-21-M, N-23-M, N-24-M, U-37-M, U-39-M, U-43-M, U-54-M, U-64-M, U-85-M, and U-87-M, Nu-Chek Prep Inc., Elysian, MN, USA; 10-1840, 11-1600-8, 20-2001-1-4, 20-2024-1, 20-2103-1-4, 20-2105-1-4, 20-2210-9, 20-2265-7, 20-2305-1-4, 20-2405-4, 2000-2500-7, 20-2900-7, BR2, and BR3; Larodan Fine Chemicals, Malmö, Sweden; H-6389, H-6639, L-6031, O-4129, O-5632, and T-1902, Sigma-Aldrich, St. Louis, MO, USA). Identification was verified by GC-MS analysis of FAME and corresponding 4,4-dimethyloxazoline (DMOX) derivatives prepared according to reference procedures (Shingfield et al., 2006). The mass spectrometer was operated at 230°C in the electron impact ionization mode and mass spectra were recorded under an ionization energy of 70 eV using helium as the carrier gas and the same temperature gradient used for routine analysis of FAME (Shingfield et al., 2006). Electron impact ionization spectra of DMOX derivatives was used to locate double bonds based on atomic mass unit distances, with an interval of 12 atomic mass units between the most intense peaks of clusters of ions containing n and n-1 carbon atoms being interpreted as cleavage of the double bond between carbon n and n+1 in the FA moiety. Identification was verified based on comparisons with an online reference library of DMOX derivative electron impact ionization spectra (http://lipidlibrary.aocs.org/ms/masspec.html) and previous determinations of FA composition in omasal digesta of cows fed FO identified based on fractionation of FAME by argentation silver-ion thin-layer chromatography and GC-MS analysis of corresponding DMOX derivatives (Kairenius et al., submitted). Geometric configuration of polyunsaturated methyl ester double bonds was inferred based on the elution order of authentic methyl ester standards containing geometric isomers of 9,12 18:2 and 9,12,15 18:3. Partial gas chromatograms indicating the separation of 20 and 22 carbon FAME are shown in Figures 1 and 2, respectively.

The distribution of CLA isomers in ruminal digesta was determined by HPLC using four silver-impregnated silica columns (ChromSpher 5 lipids, 250 × 4.6 mm; 5 μm particle size; Varian Ltd., Walton-on-Thames, UK) coupled in series and 0.1% (v/v) acetonitrile in heptane as the mobile phase (Shingfield et al., 2003).

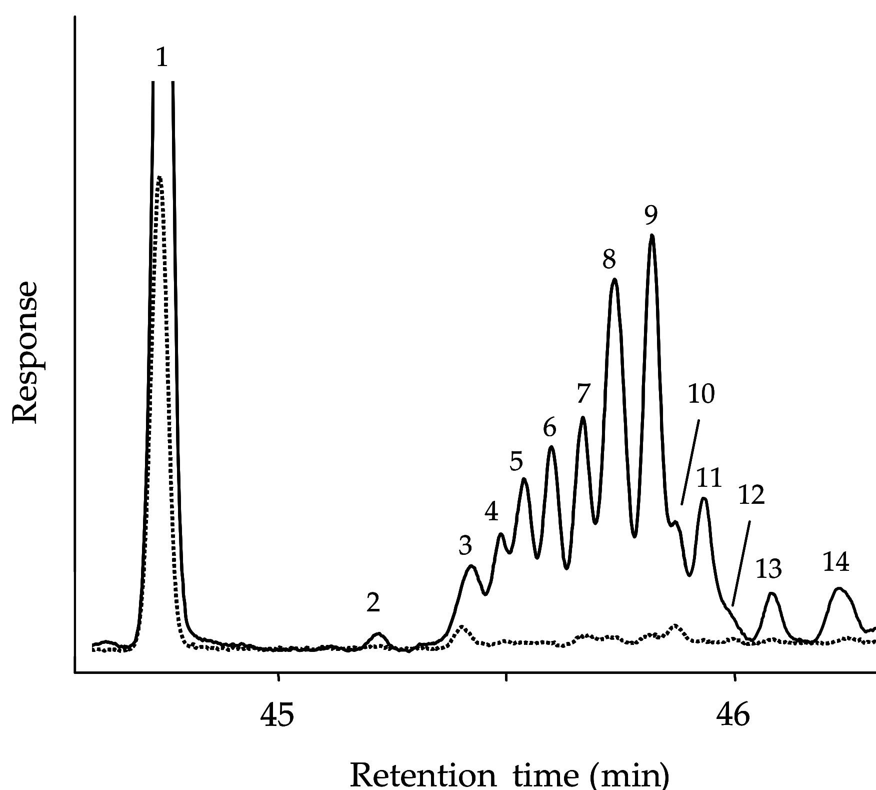
Isomers were identified using an authentic CLA methyl ester standard and chemically synthesized *trans*-9, *cis*-11 CLA (Shingfield et al., 2005) using *cis*-9, *trans*-11 CLA as a landmark isomer.

Figure 1. *Partial gas chromatogram indicating the separation of 20:1 isomers obtained using a temperature gradient for fatty acid methyl esters (FAME) prepared from lipid in ruminal digesta of ewes fed a high-concentrate diet supplemented with 20 g sunflower oil plus 10 g fish oil (SFO)/kg DM, after 0 (-----), and 3 days (—) on diet. The y-axis represents arbitrary response units. Identification was verified based on argentation silver-ion thin-layer chromatography fractionation of FAME and electron impact ionization spectra recorded during GC-MS analysis of methyl esters and corresponding 4,4-dimethyloxazoline (DMOX) derivatives.*



Peak identification: 1 = 20:0; 2 = 7,9 17:2 (double bond geometry not determined); 3 = cis-*6,* cis-*9,* cis-*12 18:3; 4 = unresolved* trans-*6, -7, -8 20:1; 5 = unresolved* trans-*9, -10 20:1; 6 =* cis-*5 20:1; 7 =* trans-*11 20:1; 8 =* trans-*12 20:1; 9 =* trans-*13 20:1; 10 =* cis-*8 20:1; 11 = unresolved* trans-*14 20:1 and* cis-*9 20:1; 12 =* trans-*15 20:1; 13 =* cis-*11 20:1; 14 = unresolved* cis-*9,* cis-*12,* cis-*15 18:3 and* cis-*12 20:1; 15 =* cis-*13 20:1; 16 =* cis-*14 20:1; 17 = unresolved* trans-*7,* cis-*9 CLA,* trans-*8,* cis-*10 CLA, and* cis-*9,* trans-*11 CLA.*

Figure 2. *Partial gas chromatogram indicating the separation of 22:1 isomers obtained using a temperature gradient for fatty acid methyl esters (FAME) prepared from lipid in ruminal digesta of ewes fed a high-concentrate diet supplemented with 20 g sunflower oil plus 10 g fish oil (SFO)/kg DM, after 0 (-----), and 3 days (—) on diet. The y-axis represents arbitrary response units. Identification was verified based on argentation silver-ion thin-layer chromatography fractionation of FAME and electron impact ionization spectra recorded during GC-MS analysis of methyl esters and corresponding 4,4-dimethyloxazoline (DMOX) derivatives.*



Peak identification: 1 = unresolved cis-*9,* trans-*14,* trans-*17 20:3 and 22:0; 2 =* cis-*8,* cis-*11,* cis-*14 20:3; 3 = unresolved* trans-*10, -11 22:1; 4 =* trans-*12 22:1; 5 =* trans-*13 22:1; 6 = unresolved* trans-*14 22:1 and* cis-*9 22:1; 7 =* trans-*15 22:1; 8 = unresolved* cis-*11 22:1 and* trans-*16 22:1; 9 = unresolved* trans-*17 22:1 and* cis-*12 22:1; 10 =* cis-*13 22:1; 11 =* cis-*14 22:1; 12 =* cis-*11,* cis-*14,* cis-*17 20:3; 13 =* cis-*15 22:1; 14 = unresolved* cis-*5,* cis-*8,* cis-*11,* cis-*14 20:4 and* cis-*16 22:1.*

2.5. Calculations and statistical analysis

Gas production data were fitted to an exponential model [$G = A \cdot (1 - e^{-c \cdot t})$] to provide parameters describing gas release (G) in terms of cumulative gas production (A; mL gas/g OM incubated) and fractional rate of gas production (c,

/h). Calculations were made using the NLIN procedure of SAS (Version 9.1, SAS Institute Inc., Cary, NC , USA).

Data were evaluated by a one-way ANOVA using the MIXED procedure of SAS with a model that included the fixed effect of treatment (Control, SFO_3, and SFO_{10} for in vivo and in situ measurements, and Control vs. SFO_{11} for in vitro gas production data). Measurements of rumen fermentation parameters were analysed by ANOVA for repeated measures using the MIXED procedure of SAS with a model that included the fixed effects of treatment, hours post-feeding, and their interaction. Differences between treatment means were evaluated using the 'pdiff' option of the 'LS means' statement in the MIXED procedure and declared significant at $P < 0.05$. Treatment effects at $P < 0.10$ were considered to reflect a trend towards significance.

3. RESULTS AND DISCUSSION

3.1. Intake and rumen fermentation parameters

Inclusion of a mixture of sunflower oil and fish oil in a high-concentrate diet had no effect on nutrient ingestion, with ewes consuming all feeds offered throughout the experiment. Previous studies in sheep have reported that oils rich in 18:2*n*-6 have no negative effects on intake (Kucuk et al., 2004; Atkinson et al., 2006; Hervás et al., 2008), whereas FO often decreases DMI (Kitessa et al., 2001; Wachira et al., 2002; Fievez et al., 2003). Measurements in cattle have indicated that decreases in DMI to FO in the diet are not associated with a reduction in the extent of NDF digestion occurring in the rumen, suggesting that the negative effects on intake may be mediated via increases in ruminal outflow of unsaturated FA (Shingfield et al., 2003; Lee et al., 2008; Shingfield et al., 2010b). Inclusion of SO and FO in the diet had no effect on DMI in this experiment, which is probably related to ewes being fed at a restricted level, rather than an indication of lipid supplements having no role in the regulation of intake in sheep. A recent study has

demonstrated that SO and FO tended to lower DMI of lactating ewes fed a high-concentrate diet ad libitum (Toral et al., 2010a).

Supplementation of the diet with SFO had no effect ($P > 0.10$) on mean rumen pH, and ammonia, lactic acid, or VFA concentrations (Table 2), with no evidence of significant changes in these parameters when experimental data were analysed separately for each sampling time post-feeding ($P > 0.10$; data not shown). Inclusion of SFO in the diet had no effect ($P > 0.10$) on molar VFA proportions, or on the molar ratio of acetate:propionate and acetate plus butyrate:propionate (Table 2), other than a transient increase ($P = 0.038$) in molar butyrate proportions on day 3.

Supplements of FO are known to alter rumen fermentation characteristics in cattle, often causing an increase in the molar proportions of propionate at the expense of acetate, butyrate or both lipogenic VFA (Keady and Mayne, 1999; Shingfield et al., 2003; Shingfield et al., 2010b). In ewes, FO has been reported to decrease the molar acetate:propionate ratio in some (Wachira et al., 2000; Fievez et al., 2003) but not in all cases (Chikunya et al., 2004). The effects of FO on butyrate appear the most variable., with reports of a decrease, no change or increase in the molar proportions of this VFA in cows (Keady and Mayne, 1999; Shingfield et al., 2003; Shingfield et al., 2010b) and sheep (Fievez et al., 2003; Chikunya et al., 2004; Toral et al., 2009). The underlying reasons for the variable effects of FO on rumen VFA profiles both between and within ruminant species are not readily apparent but may be related to selective changes in rumen microbial populations (Belenguer et al., 2010), including the *Butyrivibrio*-like bacteria, which produce butyrate and are known to be sensitive to the toxic effects of unsaturated fatty acids (Jenkins et al., 2008).

Consistent with previous results, SFO supplementation had no effect ($P > 0.05$) on in vitro ruminal fermentation characteristics, irrespective of the substrate incubated, with mean asymptotic values of 206, 314, and 299 mL/g OM, for the reference alfalfa hay, control, and SFO diets, respectively (Figure 3). The fractional rate of gas production averaged 0.08, 0.08, and 0.09 /h for alfalfa hay, control and

Table 2. *Mean rumen fermentation characteristics and disappearance of dry matter (DMD), crude protein (CPD), and neutral detergent fibre (NDFD) from alfalfa hay incubated for 24 h in the rumen of sheep fed a high-concentrate diet supplemented with 20 g sunflower oil plus 10 g fish oil (SFO)/kg DM, after 0 (Control), 3 (SFO_3), and 10 days (SFO_{10}) on diet.*

	Treatment			SED[1]	*P*-value		
	Control	SFO_3	SFO_{10}		Treatment	Hours	Treatment × hours
pH	6.26	6.22	6.30	0.071	0.501	<0.001	0.972
Ammonia (mg/L)	326	404	425	50.9	0.131	<0.001	0.999
Lactic acid (mmol/L)	0.66	0.76	0.81	0.091	0.256	<0.001	0.015
VFA (mmol/L)	175	189	167	14.4	0.294	<0.001	0.876
Molar proportion (mmol/mmol)							
Acetate	65.2	63.9	64.6	0.89	0.350	0.285	0.985
Propionate	16.5	16.4	17.0	0.73	0.641	<0.001	0.769
Butyrate	13.5[b]	15.1[a]	14.2[ab]	0.59	0.038	<0.001	0.823
Others[2]	4.78	4.59	4.17	0.320	0.160	<0.001	0.950
Molar ratio							
Acetate:propionate	4.09	3.99	4.01	0.228	0.894	<0.001	0.927
(Acetate+butyrate):propionate	4.97	4.93	4.91	0.284	0.980	<0.001	0.886
Disapperance (g/g incubated)							
DMD	0.579	0.616	0.610	0.0168	0.099	-	-
CPD	0.727[b]	0.767[a]	0.753[a]	0.0108	0.009	-	-
NDFD	0.302	0.360	0.357	0.0278	0.108	-	-

[1] SED = standard error of the difference.

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

[a,b] Different superscripts indicate significant differences within a row ($P < 0.05$).

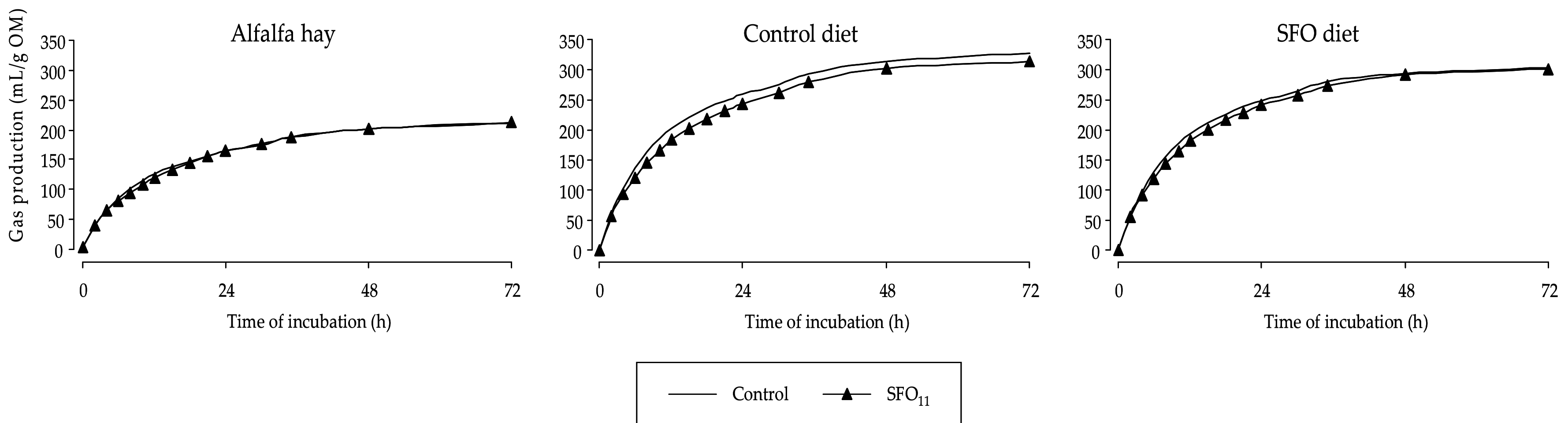


Figure 3. *Cumulative gas production profiles (mL/g OM) for substrates (alfalfa hay, control diet, and sunflower and fish oil –SFO– diet) incubated with rumen inocula from ewes fed a high-concentrate diet supplemented with 20 g sunflower oil plus 10 g fish oil (SFO)/kg DM, after 0 (Control), and 11 days (SFO_{11}) on diet.*

SFO diets, respectively, whereas mean DM disappearance after 72 h incubation was 579, 773, and 789 g/kg, for alfalfa hay, control and SFO diets. In situ measurements indicated that the SFO diet was associated with a numerical increase in the extent of ruminal DM, NDF, and CP degradation (Table 2), but the effects on DM and NDF disappearance (P values of 0.099 and 0.108) were not considered significant.

Overall, current data suggest that inclusion of SO and FO in the diet has no negative impact on rumen function, consistent with earlier studies indicating that moderate amounts of FO (Sinclair et al., 2005; Lee et al., 2008; Shingfield et al., 2010b) or plant oils (Kucuk et al., 2004; Atkinson et al., 2006; Shingfield et al., 2008) do not depress nutrient digestion in the rumen of sheep or cattle.

3.2. Fatty acid composition of ruminal digesta

Inclusion of FO and SO in the diet resulted in time-dependent alterations in the FA composition of ruminal digesta, changes characterized by a decrease ($P < 0.001$) in 18:0 and 18:2 intermediates, and a concomitant increase ($P < 0.001$) in the accumulation of 16:1, 10-O-18:0, 18:1, 20, and 22 carbon metabolites (Table 3).

Supplementation with SFO was associated with a time-dependent accumulation of 10-O-18:0 in ruminal digesta (Table 3). Early studies reported that FO promoted the formation of 10-OH-18:0 in the rumen of sheep (Kitessa et al., 2001), whereas more recent studies have indicated that 10-OH-18:0 formed during the dehydration of *cis*-9 18:1 and *trans*-11 18:1 in vitro is subsequently reduced to 10-O-18:0 (Jenkins et al., 2006; McKain et al., 2010). Ruminal digesta was also found to contain 13-O-18:0 and 15-O-18:0 (Table 3), suggesting that double bonds at other positions along the carbon chain may also be hydrated in the rumen (Hudson et al., 2000).

Relative concentrations of most odd- and branched-chain FA with 17 or less carbon atoms were decreased ($P < 0.05$) on the SFO diet (Table 3). Despite no change or increase in the abundance of longer chain odd numbered carbon FA, the total content of odd- and branched-chain FA was a 35% lower ($P < 0.001$) in

Table 3. *Fatty acid composition of lipid in ruminal digesta of sheep fed a high-concentrate diet supplemented with 20 g sunflower oil plus 10 g fish oil (SFO)/kg DM, after 0 (Control), 3 (SFO_3), and 10 days (SFO_{10}) on diet.*

Fatty acid (g/100 g fatty acids)	Treatment			SED[1]	*P*-values
	Control	SFO_3	SFO_{10}		
11-cyclohexyl 11:0	0.06[a]	0.03[b]	0.04[ab]	0.009	0.020
12:0	<0.01	0.01	0.01	0.004	0.341
13:0	0.07[a]	0.04[b]	0.03[b]	0.011	0.011
13:0 *iso*	0.09[a]	0.03[b]	0.03[b]	0.013	0.001
13:0 *anteiso*	0.01	0.01	0.01	0.005	0.552
cis-12 13:1	0.33[a]	0.14[b]	0.12[b]	0.051	0.002
14:0	0.68	0.64	0.71	0.098	0.779
14:0 *iso*	0.16[a]	0.05[b]	0.04[b]	0.027	0.001
15:0	0.87[a]	0.59[b]	0.59[b]	0.033	<0.001
15:0 *iso*	0.47[a]	0.17[b]	0.21[b]	0.050	<0.001
15:0 *anteiso*	0.84[a]	0.27[b]	0.26[b]	0.053	<0.001
trans-5 15:1	0.01[b]	0.01[b]	0.03[a]	0.005	0.005
trans-11 15:1	0.44[a]	0.15[b]	0.11[b]	0.039	<0.001
trans-13 15:1	0.03[a]	0.02[b]	0.02[b]	0.005	0.050
16:0	17.93[a]	14.92[b]	15.22[b]	0.399	<0.001
16:0 *iso*	0.41[a]	0.14[b]	0.13[b]	0.085	0.010
Σ unsaturated 16	0.77[b]	0.78[b]	1.01[a]	0.081	0.019
17:0	0.58	0.53	0.50	0.032	0.069
17:0 *iso*[2]	0.32	0.28	0.38	0.039	0.061
7-methyl-hexadec-7-enoate	0.01[c]	0.12[b]	0.14[a]	0.006	<0.001
trans-10 17:1	0.00[c]	0.02[b]	0.03[a]	0.001	<0.001
18:0	43.66[a]	29.05[b]	10.39[c]	4.691	<0.001
18:0 iso[3]	0.08[a]	0.03[b]	0.03[b]	0.013	0.004
10-O-18:0	0.04[b]	0.74[b]	3.11[a]	0.471	<0.001
13-O-18:0	0.05	0.08	0.06	0.019	0.277
15-O-18:0	0.01	0.01	0.02	0.005	0.272
Σ 18:1 *cis*	6.12	7.94	9.71	1.370	0.066
Σ 18:1 *trans*	7.06[c]	26.72[b]	41.57[a]	2.745	<0.001
Σ 18:1	13.18[c]	34.66[b]	51.28[a]	3.291	<0.001
Σ 18:2[4]	11.48[a]	5.98[b]	6.04[b]	1.500	0.004
Σ CLA[5]	0.23	0.43	0.51	0.177	0.300
20:0	0.73[a]	0.88[a]	0.49[b]	0.098	0.006
3,7,11,15-tetramethyl 16:0	0.07[a]	0.18[b]	0.18[b]	0.035	0.009
Σ unsaturated 20	1.20[b]	2.15[a]	2.20[a]	0.077	<0.001
21:0	0.06[b]	0.11[a]	0.10[a]	0.008	<0.001
cis-6, *cis*-9, *cis*-12, *cis*-15, *cis*-18 21:5	0.00[b]	0.03[a]	0.03[a]	0.006	<0.001
22:0[6]	0.48[b]	0.77[a]	0.59[b]	0.060	0.001
Σ unsaturated 22	0.25[b]	2.20[a]	2.26[a]	0.120	<0.001
23:0	0.19[c]	0.30[a]	0.24[b]	0.024	0.003
cis-14 23:1	0.01[b]	0.03[ab]	0.04[a]	0.009	0.007
cis-11 24:1	0.02[a]	<0.01[b]	0.01[b]	0.003	0.002
cis-15 24:1	0.07[c]	0.15[b]	0.18[a]	0.010	<0.001

(continued)

Table 3 *(continued)*.

Fatty acid (g/100 g fatty acids)	Treatment			SED[1]	*P*-values
	Control	SFO_3	SFO_{10}		
cis-16 24:1	<0.01	0.01	0.02	0.004	0.077
25:0	0.08	0.07	0.07	0.015	0.915
26:0	0.27[a]	0.23[b]	0.23[b]	0.018	0.043
cis-17 26:1	<0.01[c]	0.01[b]	0.03[a]	0.003	<0.001
27:0	0.02	0.01	0.02	0.006	0.535
28:0	0.16[a]	0.12[b]	0.12[b]	0.009	0.001
29:0	0.05	0.03	0.04	0.018	0.519
30:0	0.34	0.40	0.44	0.066	0.370
Summary					
Σ Saturates	68.97[a]	51.10[b]	34.40[c]	4.553	<0.001
Σ Monounsaturates	15.37[c]	38.29[b]	54.61[a]	3.306	<0.001
Σ Polyunsaturates	15.40[a]	9.79[b]	10.45[b]	2.020	0.033
Σ *n*-6 Polyunsaturates	11.29[b]	5.64[a]	5.41[a]	1.479	0.003
Σ *n*-3 Polyunsaturates	2.83	2.05	2.33	0.392	0.174
Σ Odd- and branched-chain fatty acids	5.31[a]	3.43[b]	3.45[b]	0.178	<0.001

[1] SED = standard error of the difference.
[2] Coelutes with *trans*-9 16:1.
[3] Coelutes with *trans*-11 17:1.
[4] Sum of 18:2 fatty acids excluding isomers of conjugated linoleic acid.
[5] CLA = conjugated linoleic acid.
[6] Contains *cis*-9, *trans*-14, *trans*-17 20:3 as a minor component.
[a,b,c] Different superscripts indicate significant differences within a row ($P < 0.05$).

digesta in ewes fed the SFO diet compared with the control These findings are consistent with previous reports examining the impact of diets containing FO and plant oils rich in 18:2*n*-6 on the concentration of these FA in ovine milk (Toral et al., 2010a), bovine milk (Shingfield et al., 2006; Cruz-Hernandez et al., 2007) and ruminal digesta in lactating cows (AbuGhazaleh et al., 2002).

Increases in the relative abundance in 16 carbon intermediates were isomer dependent and due in the most part to the accumulation of *trans*-6, -7, -10 and -13 (Table 4). Few investigations have examined the formation of 16 carbon metabolites in the rumen, but a recent study reported that FO in the diet increases the flow of *trans* 16:1 isomers with double bonds from Δ6-13 at the duodenum in growing cattle, an effect attributed to incomplete ruminal metabolism of 16:2*n*-4, 16:3*n*-4, 16:4*n*-1, and 16:4*n*-3 (Shingfield et al., 2010b).

Table 4. *Concentration of 16 carbon unsaturated fatty acids of lipid in ruminal digesta of sheep fed a high-concentrate diet supplemented with 20 g sunflower oil plus 10 g fish oil (SFO)/kg DM, after 0 (Control), 3 (SFO₃), and 10 days (SFO₁₀) on diet.*

Fatty acid	Treatment			SED[1]	*P*-value
(g/100 g fatty acids)	Control	SFO_3	SFO_{10}		
cis-9 16:1[2]	0.51	0.43	0.56	0.071	0.242
cis-11 16:1	0.08[a]	0.05[b]	0.03[b]	0.014	0.018
cis-13 16:1	0.00[b]	0.03[a]	0.04[a]	0.006	<0.001
trans-5 16:1	0.00[b]	0.02[a]	0.01[a]	0.006	0.022
trans-6, -7 16:1	0.01[c]	0.06[b]	0.10[a]	0.012	<0.001
trans-8 16:1	0.00[c]	0.02[b]	0.03[a]	0.003	<0.001
trans-10 16:1	0.00[c]	0.03[b]	0.05[a]	0.007	<0.001
trans-11 16:1	0.08	0.07	0.10	0.018	0.298
trans-12 16:1	0.08[a]	0.04[b]	0.04[b]	0.012	0.002
trans-13 16:1	<0.01[c]	0.02[b]	0.03[a]	0.005	<0.001
cis-9, *cis*-12 16:2	<0.01[b]	0.01[a]	0.01[a]	0.002	0.002

[1] SED = standard error of the difference.
[2] Coelutes with 17:0 *anteiso*.
[a,b,c] Different superscripts indicate significant differences within a row ($P < 0.05$).

Table 5. *Concentration of 18:1 isomers of lipid in ruminal digesta of sheep fed a high-concentrate diet with 20 g sunflower oil plus 10 g fish oil (SFO)/kg DM, after 0 (Control), 3 (SFO₃), and 10 days (SFO₁₀) on diet.*

Fatty acid	Treatment			SED[1]	*P*-value
(g/100 g fatty acids)	Control	SFO_3	SFO_{10}		
cis-9 18:1	5.02	6.30	7.82	1.322	0.148
cis-11 18:1	0.56[b]	0.64[b]	1.01[a]	0.099	0.002
cis-12 18:1	0.29	0.35	0.29	0.047	0.394
cis-13 18:1	0.04[b]	0.15[a]	0.13[a]	0.028	0.005
cis-15 18:1[2]	0.14[b]	0.33[a]	0.31[a]	0.033	<0.001
cis-16 18:1	0.07[b]	0.18[a]	0.15[a]	0.031	0.013
trans-4 18:1	0.09[b]	0.25[a]	0.24[a]	0.040	0.003
trans-5 18:1	0.05[b]	0.21[a]	0.24[a]	0.034	<0.001
trans-6, -7, -8 18:1	0.32[b]	1.74[a]	1.72[a]	0.166	<0.001
trans-9 18:1	0.19[c]	1.20[b]	1.57[a]	0.166	<0.001
trans-10 18:1	0.46[c]	1.54[b]	2.11[a]	0.251	<0.001
trans-11 18:1	4.07[c]	14.36[b]	28.45[a]	2.339	<0.001
trans-12 18:1	0.46[b]	1.88[a]	2.20[a]	0.211	<0.001
trans-13, -14 18:1	0.63[b]	3.05[a]	3.01[a]	0.413	<0.001
trans-15 18:1[3]	0.38[b]	1.35[a]	1.22[a]	0.175	0.003
trans-16 18:1[4]	0.41[b]	1.14[a]	0.81[ab]	0.185	0.007

[1] SED = standard error of the difference.
[2] Coelutes with 19:0.
[3] Contains *cis*-10 18:1 as a minor component.
[4] Contains *cis*-14 18:1 as a minor component.
[a,b,c] Different superscripts indicate significant differences within a row ($P < 0.05$).

Supplementing the diet with FO and SO increased the accumulation of all *trans* 18:1 intermediates over time (Table 5). It is well established that FO (Kitessa et al., 2001; Wachira et al., 2002; Shingfield et al., 2003) and plant oils rich in 18:2*n*-6 (Kucuk et al., 2004; Atkinson et al., 2006; Shingfield et al., 2008) in the diet increase the flow of *trans* 18:1 leaving the rumen in sheep and cattle, indicating that the collection of rumen digesta samples in this experiment provided a reliable insight into the impact of SO and FO on ruminal lipid metabolism. However, inferences drawn on the effect of oils on ruminal biohydrogenation are based on the measurement of relative proportions of FA in digesta, but it is possible that ruminal outflow of some intermediates may not be altered on the SFO diet despite of measured changes in their relative abundance.

Even though plant oils rich in 18:2*n*-6 and FO cause *trans* 18:1 to accumulate in the rumen, the mode of action explaining these effects differs. Studies in vitro have shown that 20:5*n*-3 and 22:6*n*-3 in FO specifically inhibit the reduction of *trans* 18:1 to 18:0 (AbuGhazaleh and Jenkins, 2004; Wąsowska et al., 2006), whereas the higher ruminal outflow of *trans* 18:1 in ruminants fed sources of 18:2*n*-6 is accompanied by an increase in ruminal outflow of 18:0 (Kucuk et al., 2004; Atkinson et al., 2006; Shingfield et al., 2008). Incubations with rumen inocula indicate that metabolism of both 18:2*n*-6 and 22:6*n*-3 causes *trans* 18:1 to accumulate in the absence of changes in 18:0 formation in vitro (AbuGhazaleh and Jenkins, 2004; Chow et al., 2004; Boeckaert et al., 2007). In this experiment, feeding SFO resulted in elevated *trans* 18:1 concentrations and a corresponding decrease in 18:0 in digesta over time, suggesting that the long chain *n*-3 fatty acids in FO exert more potent effects on ruminal biohydrogenation of 18 carbon unsaturated FA than 18:2*n*-6. Data from this and earlier studies (Wachira et al., 2000; Shingfield et al., 2006; Cruz-Hernandez et al., 2007) highlight that inclusion of small amounts of FO in combination with moderate or relatively high quantities of plant oils represents a very effective nutritional strategy for inhibiting the reduction of *trans* 18:1 to 18:0 in the rumen.

Isomers of *trans* 18:1 were found to accumulate over time on the SFO diet but there was no evidence of a significant shift in ruminal biohydrogenation towards *trans*-10 18:1 at the expense of *trans*-11 18:1 (Table 5). Previous studies in cows have demonstrated that diets containing FO and SO results in the rapid enrichment of *trans*-11 18:1 in milk fat that declines over time that is associated with concomitant increases in *trans*-10 18:1 concentrations (Shingfield et al., 2006; Cruz-Hernandez et al., 2007). Measurements of milk fat composition or FA flow at the duodenum also indicate that plant oils enhance ruminal formation of *trans*-10 18:1 in cows fed high-concentrate diets (Griinari et al., 1998; Loor et al., 2004), in direct relation to time on diet (Roy et al., 2006). Indirect comparisons of milk FA composition between ruminants fed diets containing moderate amounts of 18:2*n*-6 rich oils tend to suggest that the propensity for a shift in ruminal biohydrogenation pathways towards *trans*-10 18:1 is lower in sheep and goats compared with cows (Shingfield et al., 2010a). However, recent experiments demonstrated that concentrations of *trans*-10 18:1 were marginally higher than *trans*-11 18:1 in milk from ewes fed high-concentrate diets supplemented with SO and marine lipids (Toral et al., 2010a,b). The reasons underlying differences in the accumulation of *trans* 18:1 metabolites in ruminal digesta in this experiment compared with changes in the *trans* 18:1 isomer profile in milk reported previously are not obvious. It is possible that changes in ruminal biohydrogenation to SO and FO occur over a longer period in dry compared with lactating ewes, although evidence from previous studies in cows (Roy et al., 2006; Shingfield et al., 2006) suggest that alterations in ruminal biohydrogenation pathways would be expected within 10 days on diet.

Relative to the control, the SFO diet altered the composition of 18:2 isomers in ruminal digesta resulting in time dependent decreases in the relative abundance of 18:2*n*-6 and 18:3*n*-3 and the accumulation of *cis*-9, *trans*-12 18:2, *cis*-11, *trans*-15 18:2, *trans*-9, *cis*-12 18:2, *trans*-11, *cis*-15 18:2, *trans*-12, *cis*-15 18:2, *trans*-11, *trans*-14 18:2, and *trans*-11, *trans*-15 18:2 (Table 6). Studies in cattle have indicated that the flow of *trans*-9, *cis*-12 18:2 at the omasum is enhanced on diets containing SO

Table 6. *Concentration of 18 carbon polyunsaturated fatty acids of lipid in ruminal digesta of sheep fed a high-concentrate diet supplemented with 20 g sunflower oil plus 10 g fish oil (SFO)/kg DM, after 0 (Control), 3 (SFO_3), and 10 days (SFO_{10}) on diet.*

Fatty acid	Treatment			SED[1]	*P*-value
(g/100 g fatty acids)	Control	SFO_3	SFO_{10}		
cis-9, *cis*-12 18:2	11.25[a]	5.47[b]	5.17[b]	1.465	0.002
cis-11, *cis*-14 18:2	0.03	0.02	0.01	0.006	0.092
cis-12, *cis*-15 18:2	0.01	0.01	0.01	0.001	0.321
cis-9, *trans*-12 18:2	0.03[b]	0.03[ab]	0.05[a]	0.008	0.035
cis-11, *trans*-15 18:2[2]	0.01[b]	0.02[b]	0.04[a]	0.005	<0.001
trans-9, *cis*-12 18:2	0.03[c]	0.14[b]	0.19[a]	0.022	<0.001
trans-11, *cis*-15 18:2	0.10[b]	0.17[b]	0.34[a]	0.037	<0.001
trans-12, *cis*-15 18:2	0.00[c]	0.02[b]	0.05[a]	0.007	<0.001
trans-11, *trans*-14 18:2[3]	0.02[b]	0.03[b]	0.05[a]	0.007	0.002
trans-11, *trans*-15 18:2	0.02[c]	0.06[b]	0.12[a]	0.013	<0.001
cis-9, *trans*-11 CLA[4]	0.09	0.33	0.43	0.167	0.170
cis-12, *trans*-14 CLA	0.001[a]	<0.001[b]	<0.001[b]	0.0001	0.010
trans-8, *cis*-10 CLA	<0.01	<0.01	<0.01	0.001	0.744
trans-9, *cis*-11 CLA	<0.01	<0.01	<0.01	0.001	0.714
trans-10, *cis*-12 CLA	0.04[a]	0.02[b]	0.01[c]	0.004	<0.001
trans-11, *cis*-13 CLA	0.02	0.02	0.01	0.006	0.213
trans-8, *trans*-10 CLA	<0.01	<0.01	<0.01	0.002	0.889
trans-9, *trans*-11 CLA	0.01	0.02	0.03	0.007	0.128
trans-10, *trans*-12 CLA	0.02	0.01	<0.01	0.003	0.083
trans-11, *trans*-13 CLA	0.02[a]	0.01[ab]	<0.01[b]	0.004	0.016
trans-12, *trans*-14 CLA	0.008[a]	0.007[a]	0.005[b]	0.001	0.015
cis-6, *cis*-9, *cis*-12 18:3	0.01	0.01	0.01	0.001	0.413
cis-9, *cis*-12, *cis*-15 18:3[5]	2.38[a]	0.87[b]	0.69[b]	0.278	<0.001
cis-9, *trans*-11, *cis*-15 18:3	0.02	0.04	0.05	0.018	0.340

[1] SED = standard error of the difference.
[2] Contains *trans*-9, *trans*-12 18:2 as a minor component.
[3] Contains *trans*-10, *trans*-13 18:2 as a minor component.
[4] CLA = conjugated linoleic acid.
[5] Contains *cis*-12 20:1 as a minor component.
[a,b,c] Different superscripts indicate significant differences within a row ($P < 0.05$).

(Shingfield et al., 2008), whilst FO causes an increase in ruminal outflow of *trans*-11, *cis*-15 18:2 formed during 18:3*n*-3 metabolism (Shingfield et al., 2003; Lee et al., 2008; Shingfield et al., 2010b). Observed reductions in the concentrations of 18:2*n*-6 and 18:3*n*-3 in digesta are most probably explained by increases in the percentages of other FA, rather than reflecting an absolute decrease in the abundance in the rumen. Despite extensive biohydrogenation in the rumen (AbuGhazaleh et al.,

2002; Shingfield et al., 2008), the flow of 18:2*n*-6 and 18:3*n*-3 at the duodenum can be expected to be higher in direct relation to the increases in dietary supply (Kucuk et al., 2004; Loor et al., 2004; Atkinson et al. 2006).

Inclusion of FO and SO in the diet had no effect on *cis*-9, *trans*-11 CLA (P = 0.17) or total CLA concentrations in ruminal digesta (Table 6). These findings are consistent with reports of fatty acid flow at the duodenum in sheep fed high-concentrate diets containing 18:2*n*-6 rich plant oils or FO (Kucuk et al., 2004; Sinclair et al., 2005; Atkinson et al., 2006) and with recent studies indicating that the concentration of *cis*-9, *trans*-11 CLA is increased in milk of ewes supplemented fed the same amount of SO and FO as evaluated in this experiment (Toral et al., 2010a). Current data indicate that the increase in this milk FA can be explained by increased endogenous synthesis in the mammary gland using *trans*-11 18:1 as a substrate (Palmquist et al., 2005), rather than increased outflow of *cis*-9, *trans*-11 CLA from the rumen.

Relatively low concentrations of *trans*-10, *cis*-12 CLA in the rumen digesta on the control diet were found to decrease (P < 0.001) in response to the SFO treatment, whilst abundance of *trans*-9, *cis*-11 CLA was not affected (P = 0.71). Ruminal digesta was devoid of *cis*-10, *trans*-12 CLA. Studies in cattle have shown that typically FO has no effect on the amount of *trans*-10, *cis*-12 CLA leaving the rumen (Shingfield et al., 2003; Lee et al., 2008; Shingfield et al., 2010b), but plant oils rich in 18:2*n*-6 often enhance ruminal *trans*-10, *cis*-12 CLA outflow in sheep and cattle (Kucuk et al., 2004; Atkinson et al., 2006; Shingfield et al., 2008). Previous studies reported that the progressive decline in the milk fat content in lactating ewes fed high-concentrate diets containing FO and SO (Toral et al., 2010a), were not accompanied by an increase in milk *trans*-10, *cis*-12 CLA content or substantial change in *trans*-9, *cis*-11 CLA concentrations. Overall available data are consistent with the view that other biohydrogenation intermediates and/or mechanisms are involved in milk fat depression on diets containing FO (Chilliard et al., 2007; Shingfield and Griinari, 2007; Shingfield et al., 2010a).

Table 7. *Concentration of 20 carbon unsaturated fatty acids of lipid in ruminal digesta of sheep fed a high-concentrate diet supplemented with 20 g sunflower oil plus 10 g fish oil (SFO)/kg DM, after 0 (Control), 3 (SFO$_3$), and 10 days (SFO$_{10}$) on diet.*

Fatty acid	Treatment			SED[1]	*P*-value
(g/100 g fatty acids)	Control	SFO$_3$	SFO$_{10}$		
cis-5 20:1	0.02	0.03	0.02	0.009	0.411
cis-8 20:1	0.04	0.03	0.04	0.016	0.786
cis-11 20:1	0.11^{c}	0.24^{b}	0.49^{a}	0.061	<0.001
cis-13 20:1	0.02^{c}	0.04^{b}	0.05^{a}	0.003	<0.001
cis-14 20:1	<0.01^{b}	0.02^{a}	0.01^{b}	0.003	0.005
trans-6, -7, -8 20:1	0.03^{a}	0.02^{b}	0.02^{b}	0.003	0.007
trans-9, -10 20:1	0.00^{c}	0.04^{a}	0.03^{b}	0.004	<0.001
trans,-11 20:1	<0.01^{b}	0.07^{a}	0.06^{a}	0.007	<0.001
trans-12 20:1	<0.01^{b}	0.07^{a}	0.06^{a}	0.009	<0.001
trans-13 20:1	<0.01^{c}	0.09^{a}	0.06^{b}	0.009	<0.001
trans-14 20:1[2]	0.03^{c}	0.12^{a}	0.09^{b}	0.010	<0.001
trans-15 20:1	<0.01^{b}	0.22^{a}	0.06^{b}	0.048	0.002
cis-11, *cis*-14 20:2	0.03^{b}	0.04^{b}	0.06^{a}	0.008	0.005
cis-10, *trans*-15 20:2	0.00^{b}	0.03^{a}	0.03^{a}	0.005	<0.001
trans-11, *cis*-17 20:2	0.02	0.03	0.02	0.005	0.385
trans-13, *cis*-17 20:2	<0.01^{c}	0.03^{a}	0.02^{b}	0.006	<0.001
trans-14, *cis*-17 20:2	<0.01^{b}	0.02^{a}	<0.01^{b}	0.003	0.020
cis-8, *cis*-11, *cis*-14 20:3	<0.01^{c}	0.01^{b}	0.03^{a}	0.003	<0.001
cis-11, *cis*-14, *cis*-17 20:3	<0.01^{b}	0.02^{a}	0.03^{a}	0.006	0.009
cis-5, *cis*-8, *cis*-11, *cis*-14 20:4[3]	<0.01^{b}	0.06^{a}	0.07^{a}	0.010	<0.001
cis-8, *cis*-11, *cis*-14, *cis*-17 20:4	0.38^{a}	0.33^{b}	0.27^{c}	0.020	<0.001
cis-5, *cis*-8, *cis*-11, *cis*-14, *cis*-17 20:5[4]	0.48^{b}	0.57^{a}	0.64^{a}	0.036	0.003

[1] SED = standard error of the difference.
[2] Coelutes with *cis*-9 20:1.
[3] Contains *cis*-16 22:1 as a minor component.
[4] Coelutes with 24:0.
[a,b,c] Different superscripts indicate significant differences within a row ($P < 0.05$).

Inclusion of a source of 20:5*n*-3 in the diet resulted in time-dependent increases in concentration of 20 carbon FA in digesta (Table 7). Detailed analysis revealed the occurrence of *cis*-14 20:1, *trans*-9 to -15 20:1, *cis*-10, *trans*-15 20:2, *trans*-13, *cis*-17 20:2, and *trans*-14, *cis*-17 20:2 in ruminal digesta, FA not contained in FO, indicating that these must have been formed during ruminal biohydrogenation of FO and SO. No 20 carbon FA containing a conjugated double bond or other unique 20:4 or 20:5 products were identified in digesta, indicating that the initial metabolism of 20:5*n*-3 occurs at a faster rate than the reduction of 20 carbon

products containing fewer double bonds. Owing to the complex composition of FO it is not possible to elucidate unequivocally the pathways of 20:5*n*-3 metabolism in the rumen. However, characterization of unique 20 carbon metabolites in ruminal digesta in this experiment suggests that the first committed steps of 20:5*n*-3 biohydrogenation involve the reduction of the *cis*-5 and *cis*-8 double bonds.

Table 8. *Concentration of 22 carbon unsaturated fatty acids of lipid in ruminal digesta of sheep fed a high-concentrate diet supplemented with 20 g sunflower oil plus 10 g fish oil (SFO)/kg DM, after 0 (Control), 3 (SFO_3), and 10 days (SFO_{10}) on diet.*

Fatty acid	Treatment			SED[1]	*P*-value
(g/100 g fatty acids)	Control	SFO_3	SFO_{10}		
cis-11 22:1[2]	<0.01[b]	0.28[a]	0.29[a]	0.005	<0.001
cis-13 22:1	0.02[c]	0.05[b]	0.10[a]	0.013	<0.001
cis-14 22:1	0.00[b]	0.09[a]	<0.01[b]	0.020	<0.001
cis-15 22:1	<0.01[b]	0.03[a]	0.03[a]	0.003	<0.001
trans-10, -11 22:1	0.02[c]	0.07[a]	0.04[b]	0.009	<0.001
trans-12 22:1	<0.01[b]	0.05[a]	0.01[b]	0.008	<0.001
trans-13 22:1	0.00[b]	0.09[a]	0.02[b]	0.013	<0.001
trans-14 22:1[3]	<0.01[b]	0.10[a]	0.01[b]	0.016	<0.001
trans-15 22:1	<0.01[b]	0.12[a]	0.02[b]	0.018	<0.001
trans-17 22:1[4]	<0.01[b]	0.25[a]	0.03[b]	0.047	<0.001
cis-13, *cis*-16 22:2	0.03	0.04	0.04	0.006	0.184
10,13,17 22:3[5]	0.00[c]	0.04[b]	0.07[a]	0.011	<0.001
cis-10, *trans*-14, *cis*-19 22:3	0.08[a]	0.06[b]	0.05[b]	0.007	0.002
cis-10, *cis*-13, *cis*-16 22:3	0.00[b]	<0.01[b]	0.02[a]	0.004	0.003
trans-12, *cis*-16, *cis*-19 22:3	<0.01[b]	0.04[a]	0.06[a]	0.011	<0.001
cis-13, *cis*-16, *cis*-19 22:3	0.01[c]	0.02[b]	0.04[a]	0.006	0.001
cis-7, *trans*-13, *cis*-16, *cis*-19 22:4	0.01[b]	0.02[a]	0.02[a]	0.002	<0.001
trans-8, *cis*-13, *cis*-16, *cis*-19 22:4	0.00[b]	0.02[b]	0.04[a]	0.010	0.008
cis-10, *cis*-13, *cis*-16, *cis*-19 22:4	0.03[b]	0.13[ab]	0.22[a]	0.050	0.009
cis-4, *cis*-7, *cis*-10, *cis*-13, *cis*-16 22:5	0.00[c]	0.04[b]	0.08[a]	0.014	<0.001
cis-7, *cis*-10, *cis*-13, *cis*-16, *cis*-19 22:5	<0.01[c]	0.09[b]	0.18[a]	0.018	<0.001
cis-4, *cis*-7, *cis*-10, *cis*-13, *cis*-16, *cis*-19 22:6	<0.01[b]	0.55[a]	0.88[a]	0.158	<0.001

[1] SED = standard error of the difference.
[2] Contains *trans*-16 22:1 as a minor component.
[3] Coelutes with *cis*-9 22:1.
[4] Contains *cis*-12 22:1 as a minor component.
[5] Double bond geometry not determined.
[a,b,c] Different superscripts indicate significant differences within a row ($P < 0.05$).

Supplementing the diet with SO and FO also caused an increase ($P < 0.01$) in the accumulation of *cis*-14, -15 22:1, *trans*-10 to -15 22:1, *trans*-17 22:1, 10,13,17 22:3, *cis*-10, *cis*-13, *cis*-16 22:3, *trans*-12, *cis*-16, *cis*-19 22:3, *cis*-13, *cis*-16, *cis*-19 22:3, *cis*-7, *trans*-13, *cis*-16, *cis*-19 22:4, *trans*-8, *cis*-13, *cis*-16, *cis*-19 22:4, and *cis*-10, *cis*-13, *cis*-16, *cis*-19 22:4 in ruminal digesta (Table 8). Since these FA were not supplied from the diet, their appearance in the rumen confirms that these are products formed during biohydrogenation of 22 carbon unsaturated FA. Digesta was devoid of 22:5 and 22:6 intermediates or 22 carbon metabolites containing a conjugated bond. This observation provides further evidence that the metabolism of 22:5*n*-3 and 22:6*n*-3, as in the case of 20:5*n*-3, differs from that of 18:2*n*-6 and 18:3*n*-3 which under typical conditions proceeds via the isomerization of the *cis*-12 double bond (Palmquist et al., 2005; Jenkins et al., 2008; Shingfield et al., 2010a). Based on the number of double bonds, *trans*-8, *cis*-13, *cis*-16, *cis*-19 22:4 appears to be the first possible metabolite of 22:5*n*-3 and/or 22:6*n*-3 identified in digesta (Table 8). These findings suggest that biohydrogenation of 22:6*n*-3 is initiated by the reduction of the *cis*-4 double bond and that biohydrogenation of both 22:5*n*-3 and 22:6*n*-3 may involve the isomerization of the *cis*-7 double bond. Current data would also tend to indicate that the initial rates of reduction and isomerization of 22:5*n*-3 and 22:6*n*-3 are higher than the metabolism of 22:3 and 22:4 intermediates. It is possible that this sequence of events is related to the necessity of specific rumen bacteria to lower the toxic effects of unsaturated FA on growth, effects that are known to be more potent for more highly unsaturated FA (Maia et al., 2007).

In conclusion, supplementing a high-concentrate diet with FO and SO had no effect on rumen fermentation characteristics in sheep, but altered the fatty acid composition of lipid in rumen digesta consistent with a time-dependent inhibition of the complete biohydrogenation of 16, 18, 20 and 22 carbon unsaturated fatty acids. Furthermore, temporal changes in the accumulation of *trans* 18:1 intermediates offered no support that FO and SO in the diet induced a shift in ruminal biohydrogenation pathways towards the formation of *trans*-10 18:1. Several unique 20:1, 20:2, 22:1, 22:3, and 22:4 metabolites were detected in digesta

and provided the first indications that ruminal metabolism of 20:5*n*-3, 22:5*n*-3, and 22:6*n*-3 may proceed via the reduction of the double bond closest to the carboxyl group.

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CHAPTER IV

CHANGES IN THE RUMEN BACTERIAL COMMUNITY IN RESPONSE TO SUNFLOWER OIL AND FISH OIL SUPPLEMENTS IN THE DIET OF DAIRY SHEEP

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Published in: Journal of Dairy Science 93 (2010) - *in press*

doi:10.3168/jds.2010-3101

Presented in part at the *II Simposio sobre Metodologías Aplicadas al Estudio de la Microbiología Digestiva*, 14 May 2009, Saragossa, Spain,

and at the 13th Seminar of the FAO-CIHEAM Sub-Network of Sheep and Goat Nutrition, 14–16 October 2009, Leon, Spain

CHANGES IN THE RUMEN BACTERIAL COMMUNITY IN RESPONSE TO SUNFLOWER OIL AND FISH OIL SUPPLEMENTS IN THE DIET OF DAIRY SHEEP

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Abstract. Rumen microbial biohydrogenation of dietary unsaturated fatty acids has a major impact on the process of developing healthier dairy products. This study aimed to investigate in vivo the effect of diet supplementation with sunflower (SO) and fish (FO) oils on the rumen bacterial community in dairy sheep. First, 32 lactating ewes, divided in 8 lots of 4 animals each (2 lots/treatment), were fed a high-concentrate total mixed ration supplemented with 0, 2% SO, 1% FO or 2% SO plus 1% FO. After 21 days, rumen fluid samples were taken from each lot for DNA extraction and fluorescence in situ hybridization (FISH) analysis. In a second experiment, 5 cannulated ewes were first fed the same total mixed ration, with the exception of a higher forage level, and then changed to the same diet supplemented with 2% SO plus 1% FO. After 0, 3 and 10 days, rumen content samples were taken for DNA extraction and FISH analysis (fluid). Total bacteria and the *Butyrivibrio* group were studied in microbial DNA by terminal restriction fragment length polymorphism (T-RFLP), and real time PCR was used to quantify *Butyrivibrio* bacteria that produce vaccenic acid (VA) or stearic acid (SA). In rumen fluid samples, total bacteria, and clostridial clusters IX and XIV were analysed by FISH. The dietary supplementation with SO plus FO seemed to induce important changes in the total bacteria and *Butyrivibrio* population, but a high inter-individual variation was observed and the speed of the effect of the lipid supplementation depended on the individual microbial composition. The T-RFLP and FISH showed increases in cluster IX bacteria with SO plus FO supplementation, presumably *Quinella*-like microorganisms. The abundances of VA- and SA-producing *Butyrivibrio* relative to total bacteria,

estimated by real time PCR, were low (0.28 and 0.18%, respectively, in rumen fluid, and 0.86 and 0.81% in rumen contents) and only that of SA-producing bacteria seemed to be reduced by diets containing FO, although differences were only significant in lactating ewes. The T-RFLP showed also a variable effect of lipid supplementation on different bacteria of the family *Lachnospiraceae*, which includes the cultured bacteria known to be actively involved in rumen biohydrogenation. These results suggest that the latter bacteria do not play a dominant role in this process, and therefore other yet uncultivated microorganisms might be more relevant.

Key words: ewe, lipid supplementation, molecular technique, rumen microbiota.

1. INTRODUCTION

Dairy products are the major source of potentially health-promoting conjugated linoleic acid (CLA) in the human diet (Palmquist et al., 2005). Among CLA isomers, *cis*-9, *trans*-11 18:2 (rumenic acid) is the most abundant in ruminant-derived foods, and can be formed in the rumen from dietary linoleic acid via microbial biohydrogenation (BH) of fatty acids (FA) (Jenkins et al., 2008). This process may accumulate a wide range of intermediates (Palmquist et al., 2005), including rumenic acid, which is mostly reduced to *trans*-11 18:1 (vaccenic acid, VA), and finally to 18:0 (stearic acid, SA). Identifying which bacterial species have a role in biohydrogenation should aid in the process of developing healthier dairy products.

The ruminal formation of VA is also desirable because this FA can be desaturated to rumenic acid in the mammary gland and in other tissues (Palmquist et al., 2005). Vegetable oils rich in linoleic acid, such as sunflower oil (SO), provide the substrate for rumen VA synthesis, and marine products rich in long chain polyunsaturated fatty acids, such as fish oil (FO), are potent inhibitors of VA reduction to SA (Lee et al., 2008). Thus, a combination of both oils (SO and

FO) has been described as a good nutritional strategy to improve the milk FA profile in cows and sheep (Shingfield et al., 2006, Toral et al., 2010). Unsaturated FA can, however, be toxic to certain microorganisms, although some rumen bacteria are able to detoxify them through BH (Maia et al., 2007).

Thus far, the main bacterial species identified to be involved in the BH process belong to the *Butyrivibrio* group, and includes the genera *Butyrivibrio* and *Pseudobutyrivibrio* (Paillard et al., 2007a) and phylogenetically related bacteria (Devillard et al., 2007). Within this group, the only cultured SA-producing bacteria, initially identified as *Fusocillus* spp. (Kemp et al., 1975), are closely related to *Clostridium proteoclasticum* isolated by Wallace et al. (2006) and lately proposed for reclassification as *Butyrivibrio proteoclasticus* (Moon et al., 2008). Recently, Boeckaert et al. (2008) suggested that other, as-yet-uncultivated bacteria, which cluster closely to the *Butyrivibrio* and *Pseudobutyrivibrio* genera may be more important for SA production than *B. proteoclasticus*. Other less abundant rumen bacteria, such as strains of *Megasphaera elsdenii*, may also have a role in BH (Jenkins et al., 2008).

In vitro studies have provided fundamental insight into the effect of lipids on rumen bacteria and the identification of microorganisms involved in rumen BH. The relevance of individual strains in the in vivo ruminal lipid metabolism and the effect of dietary unsaturated FA on the bacterial community structure, however, remain largely unknown (Boeckaert et al., 2008). Complete in vivo BH is believed to be a synergistic process involving a bacterial consortium with each microorganism having a share in the conversion of unsaturated to relatively more saturated FA (Harfoot and Hazlewood, 1997). Despite a number of studies examining the microbiology of FA metabolism, there is a lack of in vivo studies in sheep, which might have different biohydrogenating bacteria based on the suggested inter-species differences in lipid metabolism (Shingfield et al., 2009). The current investigation aimed to study the effect of dietary supplementation with SO and FO on the composition of the rumen bacterial community in sheep

using cultivation-independent techniques, with a particular focus on bacterial populations previously reported to play a role in BH.

2. MATERIALS AND METHODS

2.1. Animals, diets and experimental design

Two experiments were carried out in accordance with Spanish Royal Decree 1201/2005 for the protection of animals used for experimental purposes.

Experiment 1. Thirty-two lactating ewes were fed a total mixed ration (TMR) based on (% DM basis) alfalfa hay (20%) and a concentrate (25% corn grain, 20% soybean meal, 15% barley grain, 9% beet pulp, 4.5% vitamin-mineral supplement, and 6.5% molasses to avoid selection of dietary components) for a 3-week adaptation period (before the beginning of the trial). Animals were distributed in 8 lots of 4 animals each and allocated at random to 4 experimental treatments (2 lots per treatment): a TMR supplemented with 0 (control diet), 2% sunflower oil (SO diet), 1% fish oil (FO diet), and a combination of 2% SO and 1% FO (SOFO diet). Experimental diets were consumed for 3 weeks before taking the samples.

Experiment 2. Detailed studies were also conducted on lipid supplementation on the rumen bacteria over time. Five adult ewes, fitted with a ruminal cannula (40 mm internal diameter), were allocated in individual cages and fed the same TMR described for Experiment 1, with the exception that it contained 35% alfalfa hay (forage:concentrate ratio 35:65), for a 10-day adaptation period. Afterwards, animals received the same TMR but supplemented with 2% SO plus 1% FO (SFO diet) for 11 days.

In both experiments, fresh TMR was offered daily ad libitum at 09:00 and 19:00 h, and clean water was always available.

2.2. Rumen sample collection

Experiment 1. After 21 days on treatments, animals were milked (08:30 h) and given free access to the diets as on other days. After 2 h the feed was removed and 3 h later, samples of ruminal fluid were individually collected using a stomach tube. Samples were strained through 2 layers of muslin, mixed for each lot, and immediately frozen at –80 ºC for DNA extraction. Aliquots of strained rumen fluid (1 mL), from each lot, were diluted with filter-sterilized phosphate-buffered saline (0.14 M NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4), then fixed by being mixed 1:3 in 4% (wt/vol) paraformaldehyde at 4°C for 16 h and finally stored at –20°C for fluorescence in situ hybridization (FISH) analysis.

Experiment 2. Samples of the rumen contents were collected in the morning before feed administration via the cannula 3 times along the experiment, immediately before starting the administration of the SFO diet, when the ewes were adapted to the consumption of the basal diet (day 0, control), and after 3 (SFO_3; to study a possible prompt response) and 10 (SFO_{10}) days on the SFO diet. Even though the treatment may be confounded with the experimental period, this approach was used rather than a switch-back design in order to allow the adaptation of the sheep to the basal diet (i.e., the diet without supplementation) and to avoid a carry over effect of the oils on the rumen microbiota (Wąsowska et al., 2006). Similar experimental designs have been previously used (e.g., Shingfield et al., 2003, Boeckaert et al., 2008). Total rumen contents were sampled and immediately frozen at –80 ºC for DNA extraction. Another fraction of rumen contents was strained through 2 layers of muslin and the rumen fluid (1 mL) was diluted with phosphate-buffered saline, then processed as described in Experiment 1, and finally stored at –20°C for FISH analysis.

2.3. DNA extraction and quantification

Frozen rumen samples were freeze-dried and thoroughly mixed before DNA extraction, which was performed in duplicate by physical disruption using a bead

beater (Mini-bead Beater, BioSpec Products, Bartlesville, OK, USA), following the protocol described by Yu and Morrison (2004), with the modification of a higher temperature (95 ºC) to improve cell lysis. Duplicate DNA samples were combined and used as templates for quantitative real-time PCR (qPCR) amplification and terminal restriction fragment length polymorphism (T-RFLP) analysis. The DNA concentrations were measured by spectrophotometry (NanoDrop® ND-1000 Spectrophotometer, Nanodrop Technologies, Wilmington, DE, USA).

2.4. qPCR analysis

Quantitative real-time PCR was carried out using the Applied Biosystems StepOne Plus™ Real Time PCR system (Applied Biosystems, Foster City, CA, USA) to investigate the abundance of 2 bacterial groups potentially able to carry out the rumen BH: *Butyrivibrio* VA- and *Butyrivibrio* SA-producing bacteria. The 16S rRNA gene-targeted primer sets used for qPCR analysis are presented in Table 1. The PCR reactions were performed in duplicate or triplicate with SYBR Green Supermix (Takara Bio Inc., Otsu, Shiga, Japan) as previously described (Maeda et al., 2003), using a 20 µL reaction mixture and 0.2 µL of each primer (10 µM) for total bacteria and 0.4 µL for *Butyrivibrio* VA-producing bacteria. For the detection of the *Butyrivibrio* SA-producing bacteria (*B. proteoclasticus* group) using qPCR, the molecular beacon approach with the primers and probe designed by Paillard et al. (2007b) was employed.

The primers used to detect the *Butyrivibrio* VA-producing bacteria were tested for their specificity with *Butyrivibrio fibrisolvens* JW11 as positive control, whereas *Mitsuokella multiacidus* 46/5, *B. proteoclasticum* P18, *Ruminococcus albus* SY3, *M. elsdenii* J1 and *Selenomonas ruminantium* Z108 were used as negative controls. Genomic DNA was extracted from pure cultures of the bacterial strains, most of them obtained from R. J. Wallace and N. McKain (Rowett Institute of Health and Nutrition, Aberdeen, UK), except the strains J1 and SY3 that were gifts from C. J. Newbold (Institute of Biological, Environmental and Rural Sciences, Aberystwyth, UK).

Table 1. *Primers used in this study for real time PCR (qPCR) and terminal restriction fragment length polymorphism (T-RFLP), and probes employed for fluorescence in situ hybridization (FISH) analysis.*

Primer or probe	Sequence (5′ → 3′)	Target	Source of primer
qPCR			
TotBacf	GTGSTGCAYGGYTGTCGTCA	Total Bacteria	Maeda et al., 2003
TotBacr	ACGTCRTCCMCACCTTCCTC		
VAf	GCCTCAGCGTCAGTAATCG	*Butyrivibrio* VA-producing bacteria	R. J. Wallace (Rowett Institute, Aberdeen, UK), personal communication
VAr	GGAGCGTAGGCGTTTTAC		
SAf	TCCGGTGGTATGAGATGGGC	*Butyrivibrio* SA-producing bacteria	Paillard et al., 2007b
SAr	GTCGCTGCATCAGAGTTTCCT		
MBP[1]	CCGCTTGGCCGTCCGACCTCTCAGTCCGAGCGG		
T-RFLP			
27f	AGAGTTTGATCCTGGCTCAG	Universal bacteria	Hongoh et al., 2003
1389r	ACGGGCGGTGT GTACAAG		
B395f	GYGAAGAAGTATTTCGGTAT	*Butyrivibrio* group	Boeckaert et al., 2008
B812r	CCAACACCTAGTA TTCATC		
FISH			
Eub338	GCTGCCTCCCGTAGGAGT	Domain *Bacteria*	Amann et al., 1990
Prop853	ATTGCGTTAACTCCGGCAC	Clostridial cluster IX	Walker et al., 2005
Erec482	GCTTCTTAGTCAGGTACCG	Clostridial clusters XIVa and XIVb	Franks et al., 1998

[1] Molecular beacon probe.

Dilutions of purified genomic DNA from control strains (*B. fibrisolvens* JW11 as a *Butyrivibrio* VA-producer, and *B. proteoclasticus* P18 as a representative of the *Butyrivibrio* SA-producing bacteria) were used to construct species-specific calibration curves and check the PCR amplification efficiency for the relative quantification of specific DNA in rumen DNA preparations, which was performed following Pfaffl (2001). The qPCR efficiency ranged between 86 and 100% for the analysis of rumen samples for both biohydrogenating groups and universal bacteria. Negative controls without DNA template were run with every assay to assess the overall specificity.

2.5. T-RFLP analysis

Two different T-RFLP analyses were performed, the first one using a universal bacteria-specific primer pair set (Hongoh et al. 2003) and the second analysis was conducted with *Butyrivibrio* group-specific primers (Boeckaert et al. 2008; Table 1). Both forward primers were labelled with 6-carboxy-fluorescein and procedures were as described previously (Castillo et al., 2007), using 2 restriction enzymes for the total bacteria analysis (*HhaI* and *MspI*) and only 1 for the *Butyrivibrio* group T-RFLP (*HhaI*). The fluorescently labelled terminal restriction fragments (T-RF) were analyzed by capillary electrophoresis on an automatic sequence analyzer (MegaBace 500, GE Healthcare Life Sciences, Buckinghamshire, UK). Determination of the sizes of T-RF was performed with the size standard ET 500-R (GE Healthcare Life Sciences, Buckinghamshire, UK), and data were analyzed using the GeneMarker Analysis software (SoftGenetics, State College, PA, USA).

Sample data consisted of size (bp) and peak area for each T-RF, and were analysed as described by Castillo et al. (2007). Based on the binary data generated, the number of T-RF (richness) and the Shannon-Wiener and Shannon evenness indices (Hill et al., 2003) were determined.

In order to infer the potential bacterial composition in the samples, in silico restriction for the major rumen bacteria with the primers and the enzymes used

were obtained from the Ribosomal Database Project II website (URL: http://rdp.cme.msu.edu/index.jsp; Cole et al., 2009).

2.6. FISH analysis

For FISH analysis diluted cell suspensions were applied to gelatin-coated slides. Samples were prepared as described previously (Belenguer et al., 2007). Images were captured with an epifluorescence microscope equipped with a digital camera (Nikon Eclipse TE2000-E, Nikon Instruments, Melville, NY, USA) using the Metamorph imaging software version 7.1 (Universal Imaging, Downington, PA, USA). Cells were counted in 25 microscopic fields for each sample. The samples were assessed with the probes Prop853 and Erec482 (Table 1). Total bacterial numbers were estimated using the universal probe Eub338 (Amann et al., 1990).

2.7. Statistical analysis

All data were analysed by one-way analysis of variance, using the MIXED procedure of the SAS software package, version 9.1 (SAS Inst. Inc., Cary, NC, USA). Some qPCR data did not meet the ANOVA requirement of normality and were $\log_{10}$ transformed for the analysis. The statistical models included the fixed effect of treatment (control, SO, FO and SOFO in Experiment 1, and control, SFO_3 and SFO_{10} in Experiment 2), and the lot (Experiment 1) or animal (Experiment 2) as a random effect that was nested within the treatment and used as the error term to contrast the effect of oil supplementation. Means were separated using the ‘pdiff’ option of the ‘lsmeans’ statement of the MIXED procedure. Significant differences were declared at $P < 0.05$, and tendencies at $P < 0.10$.

Data from T-RFLP were analysed using hierarchical clustering, with the Ward’s method based on Jaccard distances (1 – Jaccard coefficient). This statistic (Jaccard coefficient) measures similarity between samples and is defined as the ratio of the number of T-RF in common between two profiles to the total number of T-RF present in those profiles. This agglomerative hierarchical clustering was applied to obtain dendograms from the data obtained by the 2 single enzyme

(*HhaI* and *MspI*) digestions for the total bacteria and by the *HhaI* digestion for the *Butyrivibrio* group with the Community Analysis Package 4 software (Pisces Conservation Ltd, Lymington, Hampshire, UK).

3. RESULTS

3.1. Bacterial community analysis by T-RFLP

The T-RFLP analysis of 16S rRNA genes using universal primers for bacteria indicated complex communities in all samples, with over 40 peaks after *HhaI* digestion and more than 60 peaks after *MspI* digestion. The diversity indices (richness, Shannon-Wiener and Shannon evenness) were similar for all experimental treatments either from *HhaI* or *MspI* digestions (Table 2), except in cannulated ewes after 10 days of SFO administration, when bacterial diversity estimated in data derived from *HhaI* digestion decreased, as reflected by the Shannon-Wiener ($P = 0.04$) and Shannon evenness indices ($P < 0.01$).

In lactating ewes (Experiment 1), the samples derived from the SOFO-fed animals were grouped together in a separate subcluster by the cluster analysis (Figure 1a), suggesting that the combination of both lipid sources together (SOFO) induced similar and important shifts in bacterial communities.

In cannulated ewes (Experiment 2), the SFO_{10} T-RFLP pattern showed in general greater differences with the control treatment (average percent similarity 53%) than the SFO_3 (> 55%), although a variable response depending on the individual rumen microbiota was observed. It is remarkable that samples from each animal are clustered together, except those collected after 10 days (SFO_{10}) from animals 2 and 3 (Figure 1b).

The relative frequency out of the total peak area for several matching T-RF, which showed a similar effect with either *HhaI* or *MspI*, were different among treatments. In silico restriction allows to assign potential bacterial species to these fragments (Table 3). One of these T-RF (66 bp with *HhaI*) may correspond to bacteria of the order *Clostridiales* and was reduced by the SOFO diet in lactating

ewes. In Experiment 2, two *Clostridiales*-compatible fragments (181 bp and 361 bp with *HhaI*), which might be affiliated to uncultured microorganisms of the family *Lachnospiraceae* (Table 3), showed a reduction with SFO consumption. Different T-RF (99 bp and 390 bp with *HhaI*), which may correspond to bacteria belonging to cluster IX of the *Clostridium* subphylum (Collins et al., 1994), increased their abundance when both SO and FO were combined in the diet of lactating ewes. Similar clostridial cluster IX-compatible fragments (390 bp with *HhaI*, and 275 bp with *MspI*) increased its relative frequency in cannulated sheep on the SFO_{10} treatment. Conversely, the relative frequency of a *Prevotella*-compatible T-RF (102 bp with *HhaI*) was strongly reduced with lipid supplementation in lactating ewes, although it was hardly detected in the cannulated sheep.

3.2. Butyrivibrio population analysis by T-RFLP

The T-RFLP analysis of 16S rRNA genes for members of the *Butyrivibrio* group showed a less complex pattern than total bacteria. Diversity indices did not change with any of the dietary treatments (Table 2), except in lactating ewes, which tended to show lower Shannon-Wiener (P = 0.09) and Shannon evenness (P = 0.08) indices in samples derived from animals on the SO treatment than in those obtained from sheep receiving the control diet.

Hierarchical analysis on data from Experiment 1 resulted in 2 major clusters, with most samples extracted from animals fed a lipid supplemented diet being located in the same cluster, except one from the FO treatment that grouped with the control diet (Figure 1c). The SOFO profiles were the least similar to the control group (percent similarity < 30%).

In Experiment 2, the DNA extracted from the sample obtained from animal 5 on the SFO_3 treatment failed to amplify with the *Butyrivibrio* group specific primers and could not be analysed. No clusters due to the dietary treatment or to differences in the individual microbiota composition were observed (Figure 1d). Within each animal, percent similarities between T-RFLP patterns after 0 (control) and 3 (SFO_3) days on the SFO diet were already lower than 44%.

Table 2. *Diversity indices (richness, R; Shannon-Wiener, H; and Shannon evennnes, E) calculated from the total bacteria and the* Butyrivibrio *group-specific T-RFLP profiles obtained from rumen fluid of lactating ewes receiving a diet with no lipid supplementation (control) or supplemented with 2% sunflower oil (SO), 1% fish oil (FO) or 2% SO plus 1% FO (SOFO) in Experiment 1, and from rumen contents of cannulated ewes collected after 0 (control), 3 (SFO_3) and 10 (SFO_{10}) days on a diet supplemented with 2% SO plus 1% FO in Experiment 2.*

	Total bacteria						*Butyrivibrio*		
	HhaI			*MspI*			*HhaI*		
	R	H	E	R	H	E	R	H	E
Experiment 1									
Control	45.0	2.40	0.63	62.0	2.92	0.71	37.0	2.96	0.82
SO	50.0	2.40	0.61	71.0	2.98	0.70	23.5	2.08	0.66
FO	44.5	2.28	0.60	67.5	2.87	0.68	28.5	2.33	0.70
SOFO	46.5	2.44	0.64	70.0	2.99	0.70	32.5	2.49	0.72
SED[1]	7.40	0.361	0.070	14.67	0.392	0.061	5.28	0.241	0.044
P-value[2]	0.87	0.97	0.95	0.92	0.99	0.98	0.21	0.09	0.08
Experiment 2									
Control	59.2	3.07^{a}	0.75^{a}	82.4	3.61	0.82	21.4	1.83	0.61
SFO_3	55.2	2.97^{ab}	0.74^{a}	75.8	3.53	0.82	24.7	2.08	0.65
SFO_{10}	58.0	2.73^{b}	0.67^{b}	74.6	3.38	0.78	21.8	1.94	0.63
SED[1]	4.70	0.112	0.018	3.73	0.104	0.018	4.37	0.158	0.047
P-value[2]	0.70	0.04	<0.01	0.14	0.14	0.15	0.72	0.35	0.66

[a,b] For each experiment, means within a column with different superscripts differ significantly ($P < 0.05$).
[1] SED = standard error of the difference.
[2] Probability of significant effects of experimental diet.

Table 3. *Range of frequencies over the total peak area (%) of some fragments (T-RF) identified by terminal restriction fragment length polymorphism in rumen samples collected from ewes receiving diets supplemented with sunflower oil or fish oil or both, and their potential compatible bacteria.*

	T-RF length (bp)							
HhaI	66	99	102	127	181	361	390	538
MspI	225	267	498	297	136	468	299	275
Compatible bacteria	*Clostridiales*	*Quinella*	*Prevotella*	*Clostridiales*	*Lachnospiraceae*	*Lachnospiraceae*	Cluster IX	*Quinella*
Experiment 1								
HhaI	1.8 - 2.5	10.1 - 26.2	0 – 5.9	0.3 - 1.7	ND[1]	ND	0.4 - 7.5	NE[2]
MspI	0.8 - 6.2	4.4 - 14.5	3.7 - 8.6	0 - 5.9	ND	ND	2.3 - 7.7	1.3 - 2.7
Experiment 2								
HhaI	11.7 - 13.4	3.5 - 5.4	0.3 - 0.4	0.6 - 1.6	2.5 - 4.0	0.4 - 1.8	0.6 - 4.8	NE
MspI	1.6 - 2.3	0.5 - 0.9	1.0 - 1.4	4.4 - 6.4	0.5 - 1.6	0.5 - 3.3	2.4 - 4.4	1.7 - 6.1

[1] ND = not detected.

[2] NE = not estimated.

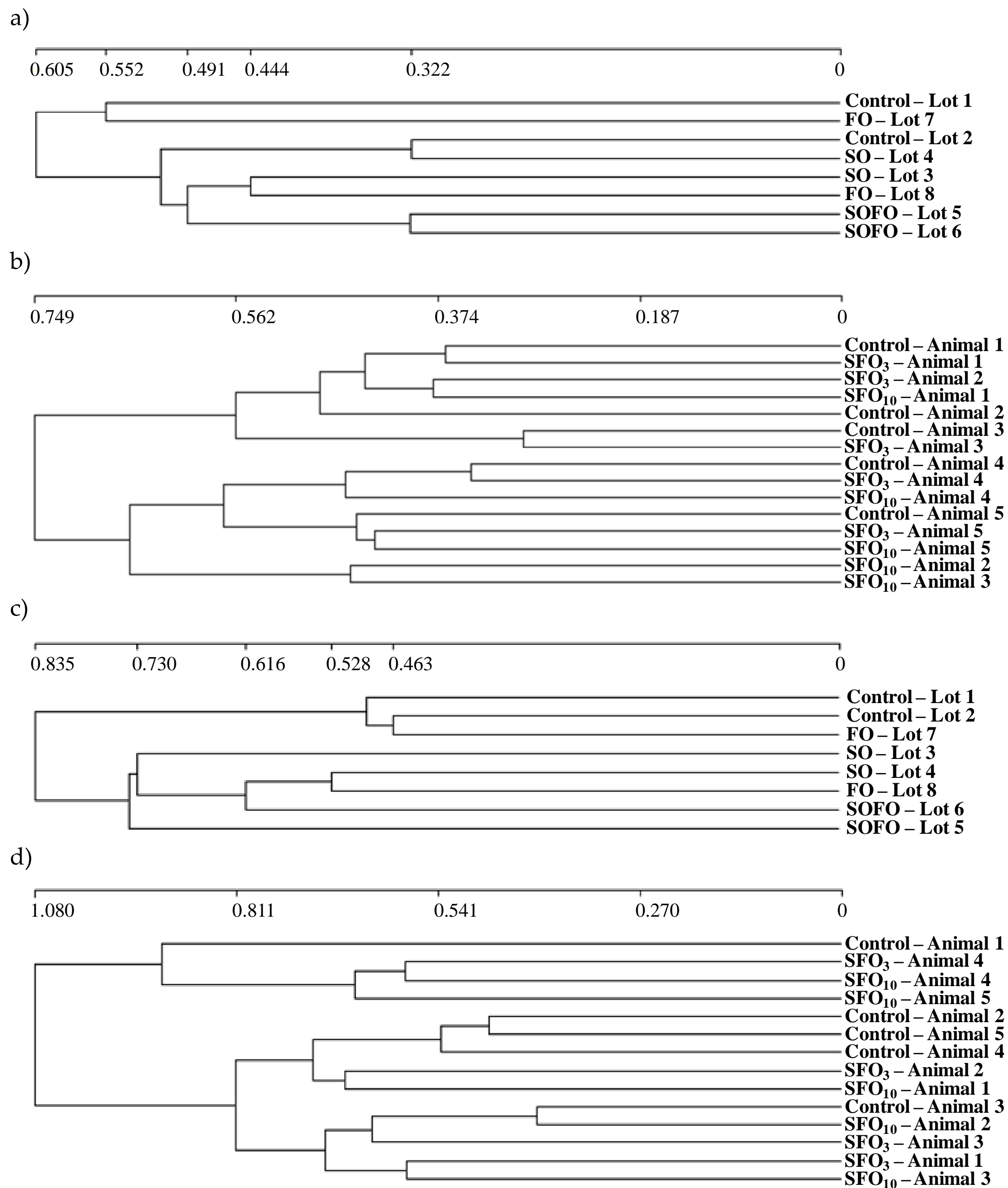

Figure 1. *T-RFLP-derived Ward's method with Jaccard distances dendrograms showing the effect on the total bacteria (a, b) and* Butyrivibrio *population (c, d) in the DNA extracted from rumen fluid of lactating ewes receiving a diet with no lipid supplementation (control) or supplemented with 2% sunflower oil (SO), 1% fish oil (FO) or 2% SO plus 1% FO (SOFO) in Experiment 1 (a, c), and from rumen contents of cannulated ewes collected after 0 (control), 3 (SFO_3) and 10 (SFO_{10}) days on a diet supplemented with 2% SO plus 1% FO in Experiment 2 (b, d).*

In the samples derived from the SOFO-fed lactating ewes 3 unique T-RF (182, 185 and 189 bp) were detected. Interestingly, the 176 bp T-RF disappeared in the samples derived from the SOFO group. Furthermore, the relative frequency of the 299 bp T-RF was stimulated by the dietary combination of the oils in both experiments.

3.3. Microbial population analysis by qPCR

Quantification of the 16S rRNA gene copy number of the *Butyrivibrio* VA-producing bacteria showed no changes due to the experimental treatments in their relative proportion of the total bacteria (Table 4), average values being 0.28 and 0.86% in the samples derived from lactating ewes (Experiment 1) and cannulated sheep (Experiment 2), respectively. When expressed as DNA concentration (pg/ng total DNA), however, the abundance of the *Butyrivibrio* VA-producing bacteria tended to be lower ($P = 0.09$) in lactating ewes fed diets supplemented with lipid sources, especially with FO (FO and SOFO diets), although that effect was not observed in Experiment 2.

The relative abundances of the *Butyrivibrio* SA-producing bacteria (*B. proteoclasticus* group) 16S rRNA gene copy number averaged 0.18% of total bacteria in Experiment 1 and 0.81% in Experiment 2, and it was not affected by dietary treatments (Table 4). However, in terms of 16S rRNA gene concentration (pg/ng total DNA), the *B. proteoclasticus* group was reduced when FO was included in the diet, their abundance being lower ($P < 0.01$) in the samples derived from the FO or SOFO-fed lactating ewes, whereas in cannulated sheep on the SFO_{10} treatment their concentration was only numerically smaller ($P = 0.15$), but not different from the control or SFO_3 treatments.

3.4. Enumeration of bacteria in rumen samples by FISH analysis

Total numbers of bacteria, estimated with the Eub338 probe, were similar in all groups in Experiment 1 (Table 5), with an average value of 9.45 log_{10} cells/mL. However, in Experiment 2, total numbers of bacteria were reduced ($P = 0.01$) after

3 days of SFO consumption, although they were recovered on day 11 (SFO_{10}) and showed numbers similar to day 0 (control).

Probes targeted to clostridial clusters IX (Prop853) and XIVa and XIVb (Erec482) accounted for 14.4 to 40.0% of the bacteria present in the rumen fluid of lactating ewes, the proportion of the bacteria detected by the Erec482 probe being greater than the proportion of the cluster IX representatives only with the control and SO diets. In Experiment 2, a high interindividual variability was observed and these probes accounted for 8.2 to 56.5% of the total bacteria. Unlike Experiment 1, the proportion of cluster IX representatives was always greater than the bacteria detected by the Erec482 probe.

Bacteria belonging to the clostridial clusters XIVa and XIVb, detected with the Erec482 probe, showed average numbers of 8.50 and 8.65 $\log_{10}$ cells/mL in Experiments 1 and 2, respectively, with no variations due to the experimental treatments. The numbers of bacteria belonging to the clostridial cluster IX, detected with the Prop853 probe, were not affected either in Experiment 1 or in Experiment 2. Interestingly, within the latter group large oval bacteria were observed and counted. The numbers of these oval microorganisms tended to be higher ($P = 0.09$) in lactating ewes fed the FO and SOFO diets, and showed values higher than 7 $\log_{10}$ cells/mL in all groups receiving a lipid-supplemented diet and proportions up to 2.5% of total bacteria. In Experiment 2, their numbers were greater after 10 days of SFO supplementation ($P = 0.01$).

4. DISCUSSION

There is a great interest in the study of the ruminal microorganisms involved in lipid metabolism due to their impact on the quality of ruminant-derived products (Palmquist et al., 2005). Several studies have investigated the effect of lipid supplementation on the rumen microbiota in cattle, using molecular techniques (Boeckaert et al., 2007, 2008, Kim et al., 2008). However, inter-species differences between ruminants in lipid metabolism have been suggested (Shingfield et al.,

2009) and no similar in vivo studies have been reported in sheep. This study showed that lipid supplementation with a combination of SO plus FO promoted changes in the rumen microbial communities in vivo in sheep, including variations within the *Butyrivibrio* population and increases in the abundance of a bacterial subgroup of the clostridial cluster IX.

Table 4. *Quantity of bacterial DNA of Butyrivibrio vaccenic acid (VA) and stearic acid (SA) producing bacteria, determined by real-time PCR, expressed as a log_{10} of the percentage (%; no transformed values in brackets) of the total genomic bacterial DNA and of the specific DNA concentration (pg/ng total DNA; no transformed values in brackets), in total DNA extracted from rumen fluid of lactating ewes receiving a diet with no lipid supplementation (control) or supplemented with 2% sunflower oil (SO), 1% fish oil (FO) or 2% SO plus 1% FO (SOFO) in Experiment 1, and from rumen contents of cannulated ewes collected after 0 (control), 3 (SFO_3) and 10 (SFO_{10}) days on a diet supplemented with 2% SO plus 1% FO in Experiment 2.*

	VA-producing bacteria (log_{10})		SA-producing bacteria (log_{10})	
	%	pg DNA/ng total DNA	%	pg DNA/ng total DNA
Experiment 1				
Control	-0.309	0.326	-0.937	0.073[a]
	(0.525)	(2.967)	(0.120)	(1.186)
SO	-0.675	-0.377	-0.679	-0.017[a]
	(0.213)	(0.452)	(0.238)	(0.962)
FO	-0.687	-0.597	-0.657	-0.277[b]
	(0.206)	(0.254)	(0.222)	(0.533)
SOFO	-0.772	-0.606	-0.921	-0.456[c]
	(0.176)	(0.252)	(0.122)	(0.351)
SED[1]	0.1473	0.2980	0.1875	0.0544
P-value[2]	0.11	0.09	0.39	<0.01
Experiment 2				
Control	-0.106	0.292	-0.108	0.626
	(0.886)	(2.159)	(0.836)	(4.502)
SFO_3	-0.061	0.237	-0.055	0.674
	(0.922)	(1.839)	(0.953)	(4.970)
SFO_{10}	-0.191	0.072	-0.236	0.361
	(0.777)	(1.482)	(0.639)	(2.903)
SED[1]	0.1144	0.1305	0.1305	0.1525
P-value[2]	0.54	0.27	0.40	0.15

[a-c]For each experiment, means within a column with different superscripts differ significantly ($P < 0.05$).

[1] SED = standard error of the difference.

[2] Probability of significant effects of experimental diet.

Table 5. *Counts (log$_{10}$; no transformed values in brackets) of total bacteria per mL rumen fluid (using the universal probe Eub338) and the bacterial groups clostridial cluster IX (probe Prop853), clostridial clusters XIVa and XIVb (probe Erec482), and a subgroup of the clostridial cluster IX (large oval bacteria) in lactating ewes receiving a diet with no lipid supplementation (control) or supplemented with 2% sunflower oil (SO), 1% fish oil (FO) or 2% SO plus 1% FO (SOFO) in Experiment 1, and in cannulated ewes after 0 (control), 3 (SFO$_3$) and 10 (SFO$_{10}$) days on a diet supplemented with 2% SO plus 1% FO in Experiment 2.*

	Eub338	Erec482	Prop853	Oval bacteria
Experiment 1				
Control	9.41	8.56	8.24	6.97
	(2.60×10^9)	(3.65×10^8)	(1.77×10^8)	(9.36×10^6)
SO	9.45	8.59	8.33	7.34
	(2.80×10^9)	(3.96×10^8)	(3.50×10^8)	(2.60×10^7)
FO	9.47	8.38	8.64	7.69
	(2.95×10^9)	(2.45×10^8)	(4.51×10^8)	(5.27×10^7)
SOFO	9.47	8.47	8.79	7.71
	(2.96×10^9)	(2.93×10^8)	(6.22×10^8)	(5.18×10^7)
SED[1]	0.029	0.089	0.351	0.232
P-value[2]	0.32	0.21	0.46	0.09
Experiment 2				
Control	9.89^a	8.58	8.78	7.44^b
	(9.01×10^9)	(4.06×10^8)	(6.41×10^8)	(2.75×10^7)
SFO$_3$	9.56^b	8.68	8.83	7.28^b
	(4.17×10^9)	(5.11×10^8	(7.11×10^8)	(1.91×10^7)
SFO$_{10}$	9.74^{ab}	8.70	8.91	7.87^a
	(5.64×10^9)	(5.54×10^8)	(9.12×10^8)	(7.41×10^7)
SED[1]	0.084	0.121	0.107	0.158
P-value[2]	0.01	0.61	0.52	0.01

[a,b]For each experiment, means within a column with different superscripts differ significantly ($P < 0.05$).
[1] SED = standard error of the difference.
[2] Probability of significant effects of experimental diet.

A concomitant study showed that the milk FA profile responded within a week to SO and FO diet supplementation in lactating ewes (Toral et al., 2010). The combination of both lipids together induced the greatest modifications in the milk FA profile (Toral et al., 2010) with no impairment of rumen fermentation (Toral et al., 2009a,b). Therefore, a diet supplemented with both SO and FO was offered to the animals for a shorter period in the second experiment.

It is noteworthy that some experimental and sampling differences existed between Experiments 1 and 2, which might contribute to explain the differences

detected in microbial profiles. The forage:concentrate ratio in the diet was higher in the second experiment (20:80 vs. 35:65 in Experiments 1 and 2, respectively), which may have a relevant effect on the rumen microbiota and consequently on the BH process (Fuentes et al., 2009). The sampling procedure (stomach tube vs. direct collection through the cannula) and sampling time (3 h post-feeding vs. pre-feeding) were also different. The rumen samples collected with a stomach tube relied solely on the fluid fraction (Egan, 2005), even though differences between liquid and solid associated bacteria, including those involved in the ruminal biohydrogenation process, have been reported (Tajima et al., 1999; Boeckaert et al., 2009).

In the samples collected 3 h after the morning feeding (lactating ewes) there might be a dilution of rumen microbes with feed, water and saliva (Mould et al., 2005). Nonetheless, the time of sample collection appeared to have little impact on the assessment of bacterial diversity in the rumen (Li et al., 2009), with the microbial taxonomy affected by host animal to a greater extent than by sampling time. Although in Experiment 1 different animals were used for each diet, the superior microbial growth rates expected at 3 h post-feeding may help to detect the treatment effects rather than individual differences (Van Gylswyk et al., 1992).

The extent of the effect of the dietary lipid supplementation on the rumen bacterial communities seemed to depend on the level of oil supplementation, in agreement with a previous report where the effect of FO was concentration-dependent (Kim et al., 2008). In addition, the speed of the response to lipid supplementation appeared to rely on the individual bacterial composition. In fact, after 3 days of SFO supplementation differences in bacterial composition among animals remained even larger than the treatment effect.

In silico restriction was used to deduce potential ecological changes by lipid supplementation, using Ribosomal Database Project II (Cole et al., 2009). Nevertheless, it should be noted that diverse phylogenetic groups can share similar or identically-sized T-RF and, then, caution should be taken in the interpretation of results. However, the utilization of more than 1 restriction

enzyme in different single digestions (*HhaI* and *MspI*) enhances the accuracy in the identification from database matches (Kitts, 2001).

An increase in the relative abundance of clostridial cluster IX-compatible fragments was found in both trials with SO plus FO supplementation. Some of these T-RF may match rumen bacteria related to *Quinella ovalis* (Deng et al., 2007), which is a large oval-shaped bacterium that belong to the *Selenomonas-Megasphaera-Sporomusa* group (clostridial cluster IX) (Krumholz et al., 1993) and has been found in the rumen of sheep (Vicini et al., 1987) and steers (Tolosa, 2006) fed diets with molasses, as in the present experiments. Furthermore, FISH results showed increases in the numbers of large oval bacteria of the clostridial cluster IX in the samples from animals fed lipid supplemented diets, specially with both oils together, which seems to reinforce the hypothesis that the favored microorganisms are presumably *Quinella*-like bacteria. *Q. ovalis* is known to show a metabolism similar to that of *S. ruminantium* (Orpin, 1972; Krumholz et al., 1993). Regarding their function in relation to BH, further research is necessary to investigate if these bacteria can metabolise unsaturated FA or are simply not sensitive to their toxic effect.

So far, all bacteria that have been shown to be actively involved in rumen BH belong to the *Butyrivibrio* group (Devillard et al., 2007, Paillard et al., 2007a, Jenkins et al., 2008). An effect of lipid supplementation on the *Butyrivibrio* population has been also previously observed, using FO (Kim et al., 2008) and DHA-rich marine algae (Boeckaert et al., 2008), although the lower levels of supplementation did not seem to affect largely this bacterial group, as previously observed (Kim et al., 2008). The relative abundances of the *Butyrivibrio* VA- and SA-producing bacteria were much lower than those reported in cattle (Paillard et al., 2007b), which suggests inter-species differences among ruminants. These bacteria might be more abundant in the solid-associated bacterial fraction, specially *B. proteoclasticus* (Boeckaert et al., 2009), which might explain the lower relative abundances of both bacterial groups in lactating ewes, (whose rumen samples were obtained by stomach tube) than in the cannulated sheep.

Within the *Butyrivibrio* group, strains of the family *Lachnospiraceae* that have been recently identified in the rumen and are potentially able to biohydrogenate FA (Boeckaert et al., 2009), would match a fragment detected by T-RFLP that increased its relative frequency with the combination of SO and FO in both trials. However, the lipid supplementation did not affect significantly the abundance of the *Butyrivibrio* VA-producing bacteria. Conversely, *B. proteoclasticus* concentration seemed to be reduced with the dietary inclusion of FO, although this effect was not significant in Experiment 2 when the rumen contents were analysed. In steers, a lack of a significant effect of FO on this bacterium has also been observed (Kim et al., 2008). The reported inhibitory effect of DHA, present in FO, on SA-producing bacteria belonging to the *B. proteoclasticus* group (Maia et al., 2007) was not significant in cannulated sheep, although in a concomitant study VA was accumulated in the rumen with SFO supplementation (Toral et al., 2009b). These results, therefore, would suggest that the *B. proteoclasticus* group may not play a dominant role in the conversion of VA to SA and that other bacteria could be involved. Alternatively, it may occur that the metabolic activity of the *B. proteoclasticus* group may not be proportional to 16S rRNA gene concentration, as previously indicated (Kim et al., 2008, Fuentes et al., 2009).

In conclusion, supplementation of the ewe diet with 2% SO and 1% FO promoted important qualitative changes in the total bacteria and *Butyrivibrio* population. The results from the Experiment 2 suggested that the speed of this effect relied on the individual microbial composition, although after 10 days of supplementation bacterial profiles in all animals appeared to be altered, despite a high interindividual variation. The changes caused by SO plus FO administration might be partly due to variations in microorganisms of the family *Lachnospiraceae*, and to an increase in the abundance of members of the clostridial cluster IX, presumably *Quinella*-like bacteria. Within the *Butyrivibrio* population, a few uncultured strains of the family *Lachnospiraceae* seemed to be stimulated. Although great attention has been paid to some identified strains potentially involved in rumen BH, mostly within the *Butyrivibrio* group, the present results suggest that

those reported bacteria do not seem to play a dominant role in this process, whereas yet uncultured bacteria of other groups might be stimulated by lipid supplementation and be more relevant. Further research is necessary to evaluate the potential role of all these bacteria in the rumen FA metabolism.

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CHAPTER V

MILK FATTY ACID PROFILE AND DAIRY SHEEP PERFORMANCE IN RESPONSE TO DIET SUPPLEMENTATION WITH SUNFLOWER OIL PLUS INCREMENTAL LEVELS OF MARINE ALGAE

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Published in: Journal of Dairy Science 93 (2010) 1655-1667

doi:10.3168/jds.2009-2769

Presented in part at the 13th Seminar of the FAO-CIHEAM Sub-Network of Sheep and Goat Nutrition, 14–16 October 2009, Leon, Spain

MILK FATTY ACID PROFILE AND DAIRY SHEEP PERFORMANCE IN RESPONSE TO DIET SUPPLEMENTATION WITH SUNFLOWER OIL PLUS INCREMENTAL LEVELS OF MARINE ALGAE

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Abstract. In an attempt to develop strategies for enhancing the nutritional value of sheep milk fat, dairy ewe diet was supplemented with three incremental levels of marine algae (MA), in combination with sunflower oil, to evaluate the effects of these marine lipids on milk fatty acid (FA) profile and animal performance. Fifty Assaf ewes in mid lactation were distributed in 10 lots of 5 animals each and allocated to 5 treatments (2 lots/treatment): no lipid supplementation (control) or supplementation with 25 g of sunflower oil/kg of DM plus 0 (SO), 8 ($SOMA_1$), 16 ($SOMA_2$), or 24 ($SOMA_3$) g of MA (56.7% ether extract)/kg of DM. Milk production and composition, including FA profile, were analyzed on days 0, 3, 7, 14, 21, and 28 of treatment. Neither intake nor milk yield were significantly affected by lipid addition, but all MA supplements decreased milk fat content from day 14 onward, reaching a 30% reduction after 28 days on $SOMA_3$. This milk fat depression might be related not only to the joint action of some putative fat synthesis inhibitors, such as *trans*-9, *cis*-11 18:2 and probably *trans*-10 18:1, but also to the limited ability of the mammary gland to maintain a desirable milk fat fluidity, that would have been caused by the noticeable increase in *trans* 18:1 together with the lowered availability of stearic acid for oleic acid synthesis through Δ^9-desaturase. Furthermore, all lipid supplements, and mainly MA, reduced the secretion of de novo FA (6:0–14:0) without increasing the yield of preformed FA (> 16 carbons). Supplementation with sunflower oil plus MA resulted in larger increases in *cis*-9, *trans*-11 18:2 than those observed with sunflower oil alone, achieving a mean content as high as 3.22% of total FA and representing a more than 7-fold increase compared with the control. Vaccenic acid (*trans*-11 18:1) was also significantly

enhanced (on average +794% in SOMA treatments), as was 22:6*n*-3 (DHA) content, although the transfer efficiency of the latter, from the diets to the milk, was very low (5%). All the same, the highest levels of MA inclusion (SOMA$_2$ and SOMA$_3$) reduced the milk *n*-6:*n*-3 ratio. On the other hand, MA supplements caused an important increase in *trans*-10 18:1, which would disallow from considering that this milk has a healthier fat profile before determining the specific role of each individual FA and ensuring that this *trans* FA is at least innocuous in relation to cardiovascular disease risk.

Key words: conjugated linoleic acid, milk fat depression, *n*-3, *trans* fatty acid

1. INTRODUCTION

A growing epidemic of chronic disease related to dietary and lifestyle changes afflicts both developed and developing countries, with cardiovascular disease, cancer, and diabetes nowadays being among the most important causes of premature death (WHO, 2003). The discovery of potential anticarcinogenic, antiatherosclerotic, and antidiabetic effects of conjugated linoleic acid (CLA; Pariza et al., 2001; Shingfield et al., 2008) and the recognized role in human health of the *n*-3 fatty acids (FA; Simopoulos, 2008) have led to an increasing number of studies over the past decade seeking to enhance the content of these bioactive compounds in ruminant-derived products, mainly in cow milk. Even though consumption of ovine milk might have several nutritional advantages over bovine milk consumption, such as its higher mineral (e.g., Ca, P, and Mg), and caprylic (8:0) and capric (10:0) acid contents and its easier digestibility (Recio et al., 2009), research in ewes is still scarce.

In a previous study in dairy sheep, inclusion in the diet of sunflower oil (SO), rich in linoleic acid, induced a 4-fold increase in milk CLA content (Hervás et al., 2008), presumably through increased ruminal formation of vaccenic acid (VA; *trans*-11 18:1; Palmquist et al., 2005; Chilliard et al., 2007), which serves as a

substrate for endogenous synthesis of the major isomer of CLA, rumenic acid (RA; *cis*-9, *trans*-11 CLA) not only in the ruminant mammary gland but also in some human tissues (Palmquist et al., 2005). The use of SO in combination with long-chain *n*-3 polyunsaturated fatty acids (PUFA) of marine lipids, which are inhibitors of the ruminal reduction of *trans* 18:1 to stearic acid (18:0; Loor et al., 2005; Or-Rashid et al., 2008), would induce further increases in VA ruminal outflow and subsequently RA mammary synthesis, as reported in cows (Shingfield et al., 2006; Cruz-Hernandez et al., 2007). However, the nutritional strategy of supplying a source of linoleic acid together with marine lipids may also increase some *trans* 18:1 in ruminant milk fat (Reynolds et al., 2006; Shingfield et al., 2006; Cruz-Hernandez et al., 2007), whose potential specific role for human health is still unclear (Shingfield et al., 2008).

In addition to the effects addressed to modify milk FA profile, the inclusion of fish oil in the diet appears to affect animal performance, reducing milk fat content in both dairy ewes (Capper et al., 2007) and cows (Griinari and Bauman, 2006; Cruz-Hernandez et al., 2007; Gama et al., 2008). Addition of marine algae (MA), on the contrary, has been reported to induce milk fat depression (MFD) in the latter (Franklin et al., 1999; Offer et al., 2001; Boeckaert et al., 2008) but not in sheep (Papadopoulos et al., 2002; Reynolds et al., 2006) and is responsible for a greater transfer efficiency of long-chain *n*-3 PUFA into the milk in this species than in cattle (Papadopoulos et al., 2002; Reynolds et al., 2006; Chilliard et al., 2007). Notwithstanding, effects of MA inclusion described in the literature are quite inconsistent and might depend on several factors, such as basal diet composition and algae dosage (Reynolds et al., 2006). This fact, together with the scarcity of published studies on this issue, makes it difficult to establish an appropriate level of MA inclusion in the diet of sheep to obtain a healthier milk FA profile for human consumers, with no detrimental effects on animal performance.

The objective of this study was therefore to investigate the effect of the dietary inclusion of incremental levels of MA, in combination with SO, on dairy ewes' performance and milk fatty acid profile.

2. MATERIALS AND METHODS

2.1. Animals, experimental diets and management

Fifty multiparous Assaf ewes (LW = 84.9 kg; standard deviation = 11.75) in mid lactation (at week 14 at the beginning of the experiment; standard deviation = 1.0) were stratified according to milk production, LW, days postpartum, and number of lactation, and randomly distributed in 10 lots of 5 animals each, and allocated to 5 experimental treatments (2 lots/treatment): no lipid supplementation or supplementation with SO (Carrefour S.A., Madrid, Spain), either alone or in combination with 3 increasing levels of MA (DHA Gold Animal Feed Ingredient, Martek Biosciences Corp., Columbia, MD, USA; 567 g of ether extract/kg of DM).

The diets, prepared weekly, consisted of a total mixed ration (TMR) based on alfalfa hay (particle size > 4 cm) and a concentrate (50:50) either without lipid supplementation (control diet; negative control) or supplemented with 25 g of SO/kg of DM plus 0 (SO diet; positive control), 8 ($SOMA_1$), 16 ($SOMA_2$), or 24 ($SOMA_3$) g of MA/kg of DM. The ingredients and chemical composition of the 5 experimental diets, which included molasses to avoid selection of dietary components, are given in Table 1. During a 3-week adaptation period (prior to commencing the trial), all animals received the control diet. Clean water and a vitamin-mineral supplement were always available and fresh diets were offered daily ad libitum at 09:00 and 19:00 h.

The ewes were milked at approximately 08:30 and 18:30 h in a 1 × 10 stall-milking parlour (DeLaval, Madrid, Spain). The experiment lasted for 4 weeks and was carried out in accordance with Spanish Royal Decree 1201/2005 for the protection of animals used for experimental purposes.

2.2. Measurements, sample collection, and chemical analyses

Samples of offered and refused diets were collected once a week, stored at −30 ºC, and then freeze-dried. The DMI was recorded weekly for each experimental lot. Diet samples were analyzed for DM (ISO 6496:1999,), ash (ISO 5984:2002), and CP

Table 1. *Ingredients and chemical composition of the experimental diets.*

	Diet[1]				
	Control	SO	$SOMA_1$	$SOMA_2$	$SOMA_3$
Ingredients, g/kg fresh matter					
Dehydrated alfalfa hay	484	474	470	466	462
Whole corn grain	136	133	131	130	129
Whole barley grain	175	170	169	168	167
Soybean meal	97	95	94	93	92
Beet pulp	49	47	47	47	46
Molasses	37	36	36	36	36
Feed supplement[2]	22	21	21	21	21
Sunflower oil[3]	0	24	24	24	24
Marine algae[4]	0	0	8	15	23
Chemical composition, g/kg DM					
Organic matter	896	900	897	893	899
Crude protein	161	159	158	159	158
Neutral-detergent fiber	308	304	296	300	293
Acid-detergent fiber	198	195	190	191	187
Ether extract	26	50	54	57	63

[1] Diets included a total mixed ration without lipid supplementation (control) or supplementation with 25 g of sunflower oil/ kg of DM plus 0 (SO), 8 ($SOMA_1$), 16 ($SOMA_2$), or 24 ($SOMA_3$) g of marine algae (56.7% ether extract)/kg of DM.

[2] INA OV1 (EVIALIS, Madrid, Spain). Contained (g/kg): $NaHCO_3$ (333), $CaCO_3$ (311), Ca_2HPO_4 (133), mine salt (111), and mineral and vitamins (111).

[3] Contained (% total fatty acid methyl esters): 16:0 (7.5), 18:0 (4.3), 18:1 (26.3), and 18:2 (60.5).

[4] As declared by the supplier (Martek Biosciences Corp., Columbia, MD, USA), marine algae (DHA GOLD Animal Feed Ingredient) contained (g/kg of DM), organic matter (910), crude protein (170), crude fibre (46), and ether extract (557). Fatty acid composition (% free fatty acids) 14:0 (8.5), 16:0 (23.2), 20:3*n*-6 (1.6), 20:5*n*-3 (1.9), 22:5*n*-6 (17.7) and 22:6*n*-3 (42.3).

(ISO 5983-2:2005). Neutral detergent fiber and ADF were determined as described by Ankom technology (Ankom, 2006a,b). Neutral detergent fiber was assayed with sodium sulphite and α-amylase and expressed with residual ash (the latter also for ADF). The content of ether extract in the diets was determined by the Ankom filter bag technology (American Oil Chemists' Society Official Procedure Am 5–04; AOCS, 2008).

Individual milk yield was recorded on days 0, 3, 7, 14, 21, and 28, both at morning and evening milkings. With the same frequency, milk samples for the analysis of fat, protein, and total solids were collected from each animal, composited according to morning and evening milk yield, and treated with

natamycin. The protein, fat, and total solids concentrations were determined by infrared spectrophotometry (ISO 9622:1999), using a MilkoScan 255 A/S N (Foss Electric, Hillerød, Denmark).

Milk FA composition was determined in untreated samples from each experimental lot and composited according to individual milk production within day. Milk fat was extracted as described by Luna et al. (2005), and fatty acid methyl esters (FAME) were prepared by base-catalyzed methanolysis of the glycerides (ISO 15884:2002). Analysis of FAME in hexane was performed on a gas chromatograph (Agilent 6890 N Network System, Palo Alto, CA, USA) with auto injector and fitted with a flame ionization detector. The FAME profile was determined by split injection (1:100) onto a CP-Sil 88 fused silica capillary column (100 m × 0.25 mm i.d., 0.20 μm film thickness; Varian, Middelburg, the Netherlands) using the same programmed temperature gradient method as described in Hervás et al. (2008). Separation of CLA methyl esters was carried out using an HPLC (Shimadzu, model SPE-MA10AVP, Kyoto, Japan) equipped with a diode array detector operated at 233 nm. Three ChromSpher 5 Lipid analytical silver-impregnated columns (250 mm × 4.6 mm i.d. stainless steel; 5 μm particle size; Varian, Middelburg, the Netherlands) were used in series. The mobile phase was 0.1% acetonitrile and 0.5% diethyl ether in hexane and was operated isocratically at a flow-rate of 1.0 mL/min. Quantification of individual FAME was made by reference to a milk fat with a known composition (CRM 164; European Community Bureau of Reference, Brussels, Belgium). Individual CLA isomers were identified by comparison with standard mixtures distributed by Nu-Chek Prep Inc. (Elysian, MN, USA). GLC-461 from Nu-Chek Prep Inc. was also used to identify other FA.

2.3. Calculations and statistical analyses

Milk fat melting point was calculated as described by Jensen and Patton (2000) using FA with a molar fraction > 0.1% in milk fat (97% of total FA). For each individual FA, melting point was obtained from Gunstone et al. (1994). Transfer

efficiency of 22:6*n*-3 (docosahexaenoic acid; DHA) from diet to milk was calculated as [(g of milk fat yield · % DHA in milk fat)/(DMI · % DHA in the TMR)] · 100. Desaturase index was calculated as product of Δ^9-desaturase/(product of Δ^9-desaturase + substrate of Δ^9-desaturase).

All analyses were performed using the SAS software package (version 9.1, SAS Institute Inc., Cary, NC, USA). Data on DMI, and milk yield and composition, as well as FA composition, were analyzed by repeated measurement analysis using the MIXED procedure of SAS and assuming a covariance structure on the basis of Schwarz's Bayesian information model fit criteria. The statistical model included the fixed effects of diet, time, their interaction, and the initial record measured at day 0 (covariate). For all data collected either individually (milk yield and composition) or per lot (DMI and FA composition), the lot was nested within the diet to contrast the effect of the lipid supplementation. Least squares means (adjusted for the covariance) are reported throughout. They were generated and separated using the PDIFF option of SAS incorporating the Tukey test for pairwise comparison of treatment means, both for main or interactive effects when they were significant. Pearson correlation coefficients (r) were generated for associations between some FA, milk yield, and fat content with the CORR procedure of SAS using the lot as the experimental unit. Differences were declared significant at $P < 0.05$, and values of $P < 0.10$ were interpreted as tendencies toward significance.

3. RESULTS

3.1. Ewe performance and milk composition

Neither DMI nor milk yield was significantly affected by lipid supplementation ($P > 0.10$; Table 2), and, although there were no changes in milk protein yield ($P = 0.166$), its content was slightly reduced when feeding MA (−4.7%; $P = 0.030$). Both milk fat content and yield, however, were significantly affected by the inclusion of lipids in the diet. Thus, all MA supplements decreased milk fat content from day

Table 2. *Dry matter intake, and milk yield and composition in ewes fed the experimental diets.*

	Treatment[1]					SED[2]	*P*-value[3]		
	Control	SO	$SOMA_1$	$SOMA_2$	$SOMA_3$		D	T	D × T
Dry matter intake, g/d	3,280	3,585	3,608	3,436	3,459	211.7	ns	**	ns
Yield, g/d									
Milk	2,059	2,153	2,010	2,110	1,928	134.0	ns	***	ns
Protein	107.1	110.1	98.7	103.4	95.5	5.62	ns	***	ns
Fat	125.9^{ab}	140.9^{a}	115.7^{bc}	118.4^{bc}	101.7^{c}	6.80	*	***	**
Total solids	349.1	372.5	328.7	340.3	306.0	17.65	†	***	†
Composition, g/100 g raw milk									
Protein	5.22^{a}	5.16^{a}	4.93^{b}	4.95^{b}	4.96^{b}	0.081	*	ns	ns
Fat	6.15^{ab}	6.51^{a}	5.75^{bc}	5.67^{bc}	5.29^{c}	0.212	**	***	***
Total solids	16.98^{a}	17.35^{a}	16.33^{b}	16.22^{b}	15.88^{b}	0.251	**	***	***

[a–c] Means within a row with different superscripts differ significantly.

[1] Diets included a total mixed ration without lipid supplementation (control) or supplementation with 25 g of sunflower oil/ kg of DM plus 0 (SO), 8 ($SOMA_1$), 16 ($SOMA_2$), or 24 ($SOMA_3$) g of marine algae (56.7% ether extract)/kg of DM.

[2] SED = standard error of the difference.

[3] Probability of significant effects due to experimental diet (D), time on diet (T), and their interaction (D × T). ns, not significant ($P > 0.10$); †$P < 0.10$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

14 onward (Figure 1), reaching a 30% reduction after 28 days on the highest level of MA inclusion ($SOMA_3$; $P < 0.001$). Changes in total solid content mirrored those reported for milk fat, and all SOMA diets reduced the percentage of milk solids ($P = 0.006$).

3.2. Saturated and monoenoic fatty acids

As shown in Table 3, percentages (g/100 g of total FA) of most short- and medium-chain saturates were significantly reduced in supplemented diets, except for 4:0 (slightly increased; $P = 0.009$) and 14:0 (unmodified, $P = 0.149$). Feeding SO significantly decreased 16:0 concentration, but only when fed alone or combined with the lowest dose of MA ($SOMA_1$). As observed in Figure 2, stearic acid content was increased with the SO (+35%), whereas MA induced a decrease ($P < 0.001$). After only 7 days on SOMA diets, its average levels were as low as 2.27% of total FAME in $SOMA_1$ (−66%; $P < 0.001$) and 1.38% of total FAME in $SOMA_2$ and $SOMA_3$ (−79%; $P < 0.001$). Percentages of most odd- and branched-chain FA also diminished with lipid supplementation ($P < 0.05$; Table 3).

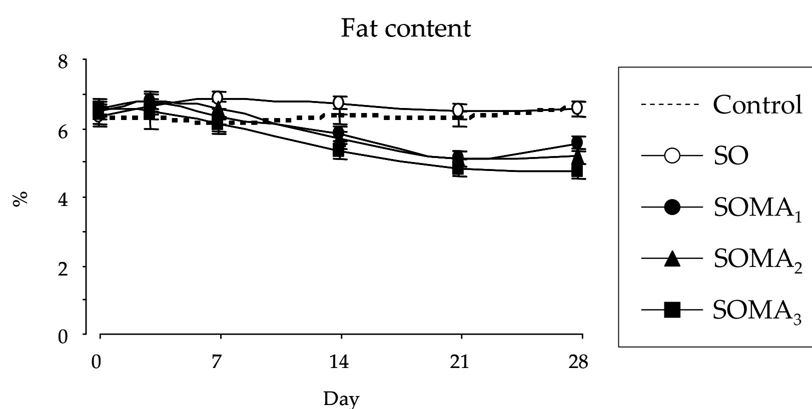

Figure 1. *Temporal changes in milk fat content in ewes fed a total mixed ration without lipid supplementation (control) or supplemented with 25 g of sunflower oil/kg of DM plus 0, 8, 16, or 24 g of marine algae/kg DM (SO, $SOMA_1$, $SOMA_2$, and $SOMA_3$ diets, respectively). Values are the mean from 2 lots of 5 animals per lot; vertical bars represent the standard error of the means.*

Table 3. *Fatty acid (FA) profile of the milk from ewes fed the experimental diets.*

Fatty acid, g/100 g of total FA methyl esters	Treatment[1]					SED[2]	*P*-value[3]		
	Control	SO	$SOMA_1$	$SOMA_2$	$SOMA_3$		D	T	D × T
Saturated FA									
4:0	3.71^{c}	4.00^{a}	4.06^{a}	4.00^{ab}	3.84^{bc}	0.096	**	ns	*
6:0	3.49^{a}	3.34^{b}	3.02^{d}	3.29^{bc}	3.22^{c}	0.040	***	***	*
8:0	3.47^{a}	3.08^{b}	2.77^{c}	3.02^{b}	2.92^{bc}	0.085	**	***	***
10:0	10.90^{a}	8.80^{b}	8.38^{b}	8.73^{b}	8.27^{b}	0.390	*	***	***
12:0	5.99^{a}	4.70^{b}	4.83^{b}	4.61^{b}	4.40^{b}	0.279	*	***	***
13:0 *anteiso*	0.07^{a}	0.04^{b}	0.05^{b}	0.04^{b}	0.05^{b}	0.005	**	ns	*
13:0	0.21^{a}	0.13^{b}	0.14^{b}	0.12^{b}	0.13^{b}	0.019	*	***	***
14:0 *iso*	0.11^{a}	0.10^{ab}	0.09^{bc}	0.08^{c}	0.08^{c}	0.006	**	***	***
14:0	11.79	10.80	11.76	11.40	11.11	0.385	ns	***	*
15:0 *iso*	0.20^{a}	0.18^{b}	0.18^{b}	0.16^{c}	0.18^{b}	0.007	*	**	ns
15:0 *anteiso*	0.43^{a}	0.39^{ab}	0.36^{bc}	0.32^{c}	0.33^{c}	0.020	*	***	*
15:0	0.95^{a}	0.79^{b}	0.80^{b}	0.74^{c}	0.73^{c}	0.010	***	***	***
16:0 *iso*	0.40^{a}	0.29^{b}	0.30^{b}	0.23^{d}	0.25^{c}	0.020	***	***	ns
16:0	25.94^{a}	22.44^{c}	24.29^{b}	24.84^{ab}	25.91^{a}	0.578	**	***	**
17:0	0.53^{a}	0.43^{b}	0.44^{b}	0.42^{b}	0.41^{b}	0.025	*	***	**
18:0	6.57^{b}	8.84^{a}	3.16^{c}	2.37^{d}	2.33^{d}	0.207	***	***	***
18:0 *iso*	0.06	0.06	0.06	0.06	0.06	0.006	ns	**	ns
20:0	0.17^{a}	0.17^{a}	0.15^{b}	0.14^{b}	0.14^{b}	0.007	*	*	*
21:0	0.07^{a}	0.06^{b}	0.05^{b}	0.05^{b}	0.05^{b}	0.004	*	***	†
22:0	0.13^{b}	0.15^{a}	0.17^{a}	0.17^{a}	0.17^{a}	0.008	*	***	*
23:0	0.06^{b}	0.05^{b}	0.09^{a}	0.09^{a}	0.09^{a}	0.005	**	***	***
24:0	0.04^{c}	0.04^{c}	0.06^{b}	0.07^{a}	0.07^{a}	0.004	***	***	**

(continued)

Table 3 *(continued.)*

Fatty acid, g/100 g of total methyl esters	Treatment[1]					SED[2]	P-value[3]		
	Control	SO	SOMA1	SOMA2	SOMA3		D	T	D × T
Monounsaturated FA									
10:1 + 12:0 *iso*	0.49^{a}	0.38^{b}	0.34^{b}	0.31^{b}	0.32^{b}	0.034	*	***	***
cis-9 14:1	0.23^{a}	0.20^{bc}	0.23^{ab}	0.19^{c}	0.18^{c}	0.013	*	*	†
15:1	0.14	0.11	0.13	0.13	0.15	0.010	†	**	**
trans-8 16:1	0.06^{b}	0.09^{b}	0.22^{a}	0.21^{a}	0.18^{a}	0.019	**	***	*
trans-9 16:1 + 17:0 *iso*	0.39^{d}	0.49^{c}	0.80^{b}	0.75^{b}	0.88^{a}	0.023	***	***	***
cis-7 16:1	0.29	0.30	0.28	0.27	0.27	0.016	ns	*	†
cis-9 16:1 + 17:0 *anteiso*	1.41^{a}	1.15^{c}	1.22^{b}	1.05^{d}	1.04^{d}	0.030	***	***	*
cis-13 16:1	0.17^{a}	0.09^{b}	0.08^{bc}	0.07^{c}	0.08^{bc}	0.008	***	ns	**
17:1	0.20^{a}	0.16^{b}	0.15^{bc}	0.13^{d}	0.13^{cd}	0.008	**	***	**
trans-6, -7, -8 18:1	0.19^{b}	0.41^{a}	0.43^{a}	0.20^{b}	0.17^{b}	0.020	***	***	***
trans-9 18:1	0.18^{c}	0.38^{a}	0.42^{a}	0.26^{b}	0.26^{b}	0.017	***	***	***
trans-10 18:1	0.34^{c}	0.65^{c}	4.04^{b}	4.99^{a}	3.62^{b}	0.239	***	***	***
trans-11 18:1 (VA)	0.93^{d}	2.80^{c}	6.91^{b}	7.25^{b}	7.98^{a}	0.169	***	***	***
trans-12 18:1	0.26^{d}	0.47^{b}	0.57^{a}	0.34^{c}	0.34^{c}	0.025	***	***	***
trans-15 + *cis*-11 18:1	0.32^{b}	0.37^{b}	0.48^{a}	0.45^{a}	0.43^{a}	0.021	**	***	***
trans-16 + *cis*-14 18:1	0.33^{b}	0.48^{a}	0.33^{b}	0.22^{c}	0.22^{c}	0.015	***	***	***
cis-9 18:1	13.32^{b}	16.01^{a}	9.84^{c}	9.34^{c}	9.19^{c}	0.283	***	***	***
cis-12 18:1	0.34^{b}	0.70^{a}	0.19^{c}	0.13^{cd}	0.12^{d}	0.025	***	*	***
cis-13 18:1	0.05^{c}	0.06^{b}	0.07^{a}	0.08^{a}	0.07^{ab}	0.004	**	**	*
cis-15 18:1	0.08^{c}	0.11^{a}	0.10^{ab}	0.09^{bc}	0.09^{bc}	0.009	*	*	*
cis-11 20:1	0.07^{b}	0.07^{b}	0.10^{a}	0.11^{a}	0.10^{a}	0.003	***	***	*
cis-13 22:1	0.02^{d}	0.02^{d}	0.03^{c}	0.05^{b}	0.06^{a}	0.002	***	***	***
24:1	0.02	0.03	0.02	0.03	0.03	0.004	ns	**	ns

(continued)

Table 3 *(continued)*.

Fatty acid, g/100 g of total methyl esters	Treatment[1]					SED[2]	*P*-value[3]		
	Control	SO	SOMA1	SOMA2	SOMA3		D	T	D × T
Non-conjugated 18:2									
trans-9, *cis*-12	0.03^{c}	0.04^{c}	0.10^{ab}	0.11^{a}	0.09^{b}	0.005	***	***	***
trans-11, *cis*-15	0.05^{c}	0.04^{c}	0.21^{b}	0.28^{a}	0.30^{a}	0.019	***	***	***
cis-9, *cis*-12	2.37^{a}	2.23^{ab}	2.03^{c}	2.04^{bc}	1.86^{c}	0.075	**	***	***
cis-9, *cis*-15	0.08^{a}	0.06^{b}	0.05^{c}	0.05^{c}	0.05^{bc}	0.005	***	**	*
Other 18:2[4]	0.20^{c}	0.28^{a}	0.24^{b}	0.16^{d}	0.15^{d}	0.010	***	*	*
Other 18:2[5] + *cis*-16 18:1	0.19^{bc}	0.27^{a}	0.23^{b}	0.18^{c}	0.17^{c}	0.013	**	***	***
Conjugated 18:2									
cis-9, *trans*-11 (RA)	0.44^{d}	1.23^{c}	2.78^{b}	2.58^{b}	3.22^{a}	0.114	***	***	***
trans-7, *cis*-9	0.04^{d}	0.08^{b}	0.09^{a}	0.05^{c}	0.04^{cd}	0.002	***	***	***
trans-8, *cis*-10	0.02^{c}	0.03^{b}	0.04^{a}	0.03^{ab}	0.03^{a}	0.003	***	***	***
trans-9, *cis*-11	0.02^{d}	0.03^{c}	0.10^{b}	0.11^{a}	0.10^{b}	0.005	***	***	***
trans-10, *cis*-12	$<0.01^{c}$	$<0.01^{bc}$	0.01^{ab}	0.01^{a}	0.01^{abc}	0.002	*	***	ns
trans-11, *cis*-13	0.01^{b}	0.01^{b}	0.02^{a}	0.02^{a}	0.02^{a}	0.002	**	***	ns
trans-12, *trans*-14	0.01	0.01	0.01	0.01	0.01	0.001	ns	***	ns
trans-11, *trans*-13	0.03^{a}	0.03^{a}	0.02^{b}	0.02^{b}	0.02^{b}	0.002	**	***	*
Other *trans*-*trans*	0.01^{c}	0.02^{b}	0.03^{a}	0.03^{a}	0.03^{ab}	0.002	***	***	*

(continued)

Table 3 *(continued)*.

Fatty acid, g/100 g of total methyl esters	Treatment[1]					SED[2]	P-value[3]		
	Control	SO	SOMA1	SOMA2	SOMA3		D	T	D × T
Other polyunsaturated FA									
16:2*n*-6	0.02^{b}	0.02^{b}	0.05^{a}	0.06^{a}	0.06^{a}	0.005	***	***	*
18:3*n*-6	0.08^{a}	0.06^{b}	0.06^{b}	0.05^{b}	0.05^{b}	0.004	***	***	*
18:3*n*-3	0.53^{a}	0.41^{b}	0.37^{c}	0.36^{c}	0.34^{d}	0.013	***	†	ns
cis-9, *trans*-11, *cis*-15 18:3	0.03	0.03	0.03	0.03	0.03	0.003	ns	**	ns
18:4*n*-3	0.01^{c}	0.02^{b}	0.02^{b}	0.02^{ab}	0.03^{a}	0.002	*	***	ns
20:2*n*-6	0.02	0.02	0.02	0.03	0.03	0.003	ns	†	ns
20:3*n*-3	0.02^{bc}	0.01^{c}	0.02^{ab}	0.02^{ab}	0.03^{a}	0.003	*	*	†
20:4*n*-6	0.16^{c}	0.12^{d}	0.17^{bc}	0.20^{b}	0.24^{a}	0.014	**	***	***
20:5*n*-3 (EPA)	0.05^{c}	0.04^{c}	0.05^{c}	0.09^{b}	0.10^{a}	0.004	***	***	***
22:4*n*-6	0.03^{d}	0.03^{d}	0.04^{c}	0.05^{b}	0.05^{a}	0.001	***	***	**
22:5*n*-6	0.01^{d}	0.02^{d}	0.11^{c}	0.28^{b}	0.33^{a}	0.014	***	***	***
22:5*n*-3 (DPA)	0.10^{c}	0.07^{d}	0.10^{c}	0.13^{b}	0.15^{a}	0.004	***	***	***
22:6*n*-3 (DHA)	0.03^{d}	0.02^{d}	0.17^{c}	0.46^{b}	0.57^{a}	0.021	***	***	***

[a–d] Means within a row with different superscripts differ significantly($P < 0.05$).

[1] Diets included a total mixed ration without lipid supplementation (control) or supplementation with 25 g of sunflower oil/ kg of DM plus 0 (SO), 8 (SOMA1), 16 (SOMA2), or 24 (SOMA3) g of marine algae (56.7% ether extract)/kg of DM.

[2] SED = standard error of the difference.

[3] Probability of significant effects due to experimental diet (D), time on diet (T), and their interaction (D × T). ns, not significant ($P > 0.10$); †$P < 0.10$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

[4] *trans*-9, *trans*-12 + *cis*-9, *trans*-13 + *trans*-8, *cis*-12 18:2.

[5] *trans*-8, *cis*-13 + *cis*-9, *trans*-12 18:2.

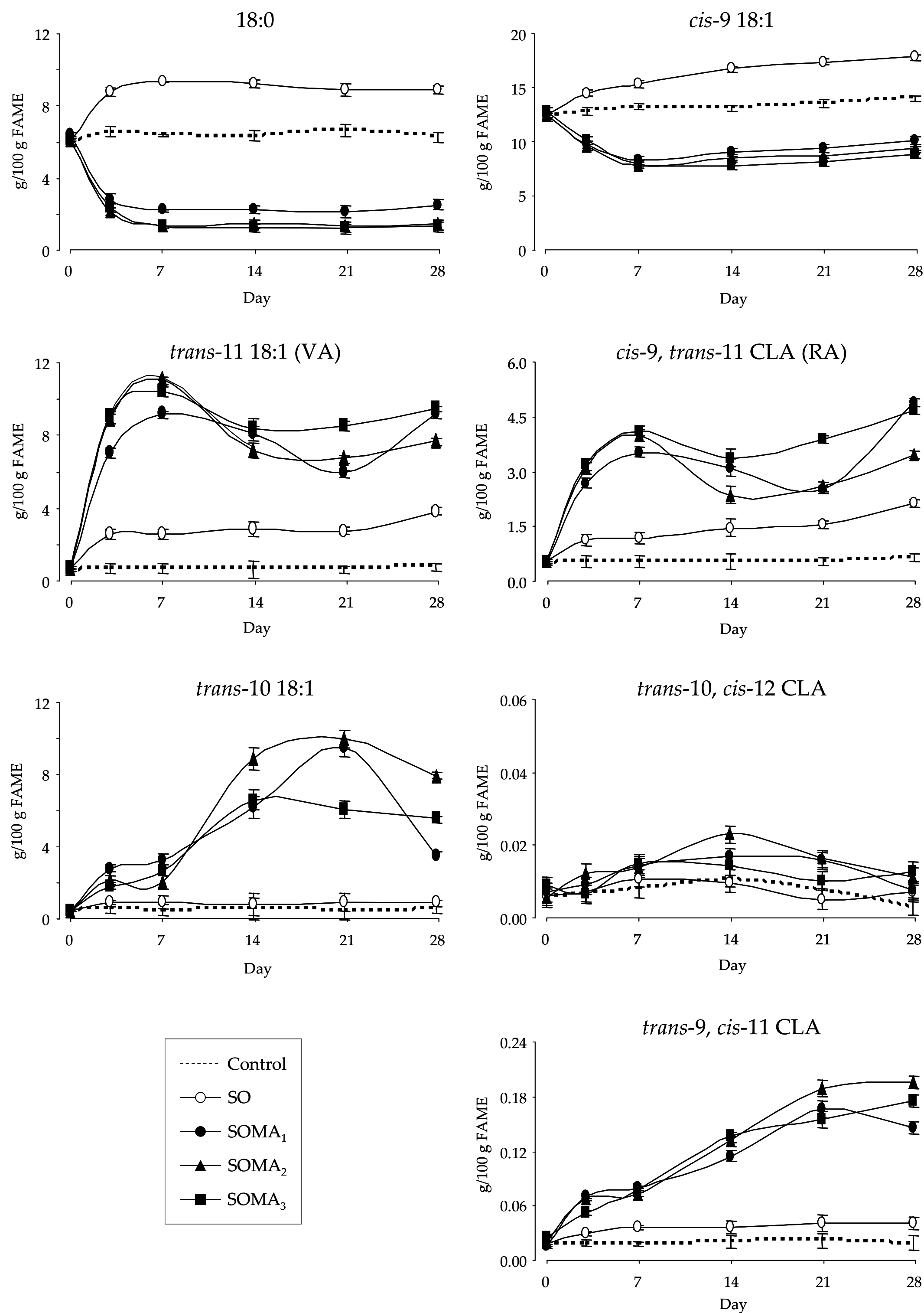

Figure 2. *Temporal changes in 18:0,* cis*-9 18:1,* trans*-11 18:1,* cis*-9,* trans*-11 CLA, trans-10 18:1, trans-10, cis-12 CLA and trans-9, cis-11 CLA (g/100 g of fatty acids methyl esters) in ewes fed a total mixed ration without lipid supplementation (control) or supplemented with 25 g of sunflower oil/kg DM plus 0, 8, 16, or 24 g of marine algae/kg DM (SO, SOMA$_1$, SOMA$_2$, and SOMA$_3$ diets, respectively). Values are the mean from 2 lots of 5 animals per lot; vertical bars represent the standard error of the mean. CLA = conjugated linoleic acid.*

There were also important changes in milk content of almost all monounsaturated FA, with significant diet × time interactions. Oleic acid (*cis*-9 18:1) level was significantly increased with SO diet (+20%), while inclusion of MA diminished it (−29%; Figure 2). All lipid supplements resulted in greater *trans* 18:1 percentages, mainly *trans*-10 and *trans*-11, which were remarkably enhanced in SOMA diets ($P < 0.001$). The content of VA increased by 8.6 times in $SOMA_3$ treatment (averaging 8% of total FA; Figure 2), with this elevated level being sustained throughout the whole monitoring period. Milk content of *trans*-10 18:1 remained statistically unmodified when SO was fed alone, but inclusion of MA induced large increases, especially on day 14 of supplementation ($P < 0.001$).

Finally, the milk fat concentration of other *trans* 18:1 isomers (*trans*-6, -7, -8, *trans*-9, and *trans*-12) was significantly increased with SO and $SOMA_1$ treatments (Table 3), except for *trans*-9, which was also augmented with $SOMA_2$ and $SOMA_3$ ($P < 0.001$).

3.3. Conjugated and non-conjugated linoleic acid

The addition of MA to SO-containing diets resulted in large increases in milk fat RA, much higher than those observed when SO was supplemented alone ($P < 0.001$; Table 3), with the highest concentration corresponding to $SOMA_3$ treatment. As shown in Figure 2, RA content increased sharply during the first 7 days on SOMA treatments and, after a transient declination, the highest levels were achieved in $SOMA_1$ and $SOMA_3$ on day 28 ($P < 0.001$). Addition of SO alone induced a constant increase of RA content over the whole period. *Trans*-7, *cis*-9 CLA doubled its level with SO and $SOMA_1$ diets ($P < 0.001$; Table 3). A 9-fold increase in *trans*-9, *cis*-11 CLA ($P < 0.001$; Table 3) was reached after 28 days on $SOMA_2$ and $SOMA_3$ treatments (Figure 2). Other CLA isomers, such as *trans*-10, *cis*-12, were present only in very low concentrations (Table 3; Figure 2).

The most abundant non-conjugated PUFA was linoleic acid (18:2*n*-6), but its level was little modified except for the slight decrease observed with MA

(P = 0.006). This marine additive significantly augmented the concentration of other, less abundant non-conjugated dienes, such as *trans*-11, *cis*-15 18:2.

3.4. Other polyunsaturated fatty acids

Lipid inclusion significantly decreased α-linolenic acid (18:3*n*-3) in milk fat, whereas 22:6n-3 (DHA) content showed a sharp increase after the start of MA supplementation (P < 0.001), with relatively stable concentration afterward. However, calculated DHA transfer efficiencies from SOMA diets to milk were very low (0.040–0.058). Increases in other long-chain *n*-3 PUFA, such as 22:5*n*-3 (docosapentaenoic acid) or 20:5*n*-3 (eicosapentaenoic acid), were observed only with the medium and high levels of MA inclusion (P < 0.001) and, in any event, their content was always below 0.2% of total FAME. Changes in milk content of *n*-6 PUFA were of lower magnitude with the exception of the higher levels of 22:5*n*-6 obtained with SOMA diets (Table 3).

4. DISCUSSION

4.1. Ewe performance and milk composition

Inclusion of free vegetable oils in dairy ewe diet has been reported to have no apparent detrimental effect on DMI and milk yield (Pulina et al., 2006; Gómez-Cortés et al., 2008a; Hervás et al., 2008), and although decreases in feed consumption are frequently observed when feeding marine algae to sheep or cows, this reduction does not always affect milk yield (Franklin et al., 1999; Offer et al. 2001; Papadopoulos et al., 2002).

In relation to milk composition, the 6% reduction of the total solid content found with SOMA treatments was the consequence of the significant decrease in milk protein and fat contents caused by the inclusion of these lipids in the diet. Reductions in milk protein are frequently observed when the diet of dairy ewes is supplemented with oil (Pulina et al., 2006; Gómez-Cortés et al., 2008b) and are generally attributed to reduced amino acid availability. Previous studies have

shown no evidence of MFD in dairy ewes fed free vegetable oils (Pulina et al., 2006; Gómez-Cortés et al., 2008a,b; Hervás et al., 2008), but few experiments have examined the effect of unprotected marine lipids in this species. Furthermore, the reported effects of the latter on ewe milk fat content are very inconsistent, with reductions, no changes, and even increases (Papadopoulos et al., 2002; Reynolds et al., 2006; Capper et al., 2007), which could be mostly accounted for by differences in basal diet composition and lipid dosage.

4.2. Milk fat depression and putative inhibitors

In dairy cows, MFD induced by the use of free vegetable oils and high-concentrate diets is characterized by increased milk fat concentration of *trans*-10, *cis*-12 CLA, a biohydrogenation (BH) intermediate with antilipogenic effect not only in this ruminant species (Chilliard et al., 2007; Shingfield et al., 2009) but also in sheep (Lock et al., 2006). However, the very low levels and the lack of variation in this isomer (Table 3; Figure 2) would suggest that other inhibitors of milk fat synthesis should also be involved. Furthermore, greater increases in *trans*-10, *cis*-12 CLA in the absence of MFD have previously been reported in dairy sheep fed either low- or high-concentrate diets (Reynolds et al., 2006; Gómez-Cortés et al., 2008a; Hervás et al., 2008).

Post-ruminal infusion experiments have provided tentative evidence that *cis*-10, *trans*-12 and *trans*-9, *cis*-11 CLA also exert antilipogenic effects (Perfield et al., 2007; Harvatine et al., 2009). The former FA was not detected in the present study, but the remarkable increases in *trans*-9, *cis*-11 18:2 could partially explain the MFD arising in ewes consuming SOMA diets. Previous evidence in cows (Roy et al., 2006; Perfield et al., 2007) indicates that this CLA isomer inhibits milk fat synthesis, but with a much lower efficacy than *trans*-10, *cis*-12 18:2.

The inverse relationship between milk fat yield and *trans*-10 18:1 ($r = -0.71$; $P < 0.001$) might suggest that this FA is another candidate for the inhibition of milk fat synthesis. However, a 4-day abomasal infusion of 40 g/day of this *trans* 18:1 isomer was not effective in reducing the milk fat percentage in cows (Lock et al.

2007), which may be the result of the low enrichment of *trans*-10 18:1 during infusions (Kadegowda et al., 2008). A more recent study (Kadegowda et al., 2009) supports the idea that *trans*-10 18:1 reaching the mammary gland could potentially induce MFD by decreasing lipogenic gene expression. Furthermore, infusions of approximately 90 g/day of *trans*-10 18:1 for a 5-day period reduced milk fat secretion, although the authors (Shingfield et al., 2009) suggest that this *trans* FA was about 40- to 50-fold less effective than *trans*-10, *cis*-12 18:2. Interestingly, in that study, the level of milk *trans*-10 18:1 was about 4%, similar to that observed here for SOMA diets (Table 3). However, it is noteworthy that higher levels (about 6%) have previously been found in dairy ewes supplemented with vegetable oils without concomitant reductions in milk fat (Gómez-Cortés et al., 2008a; Hervás et al., 2008), which would rule out *trans*-10 18:1 as the principal factor responsible for the reduction observed in milk fat content.

The high correlation between *trans*-10 18:1 and *trans*-9, *cis*-11 18:2 ($r = 0.93$; $P < 0.001$) might support the hypothesis that they have a common precursor or are produced by a common rumen bacteria (Roy et al., 2006; Shingfield et al., 2006; Gama et al., 2008), and although the low concentration of *trans*-9, *cis*-11 CLA makes it unlikely that it may be the principal component responsible for MFD (Perfield et al., 2007), *trans*-9, *cis*-11 CLA and milk fat content were also negatively correlated ($r = -0.78$; $P < 0.001$). Accordingly, a joint action of *trans*-9, *cis*-11 18:2 and *trans*-10 18:1, together with that of other unidentified intermediates, would seem to be a feasible explanation for MFD in SOMA treatments (Perfield et al., 2007; Kadegowda et al., 2008, 2009; Shingfield et al., 2009), but mechanisms other than direct inhibition may also be involved. In this regard, several authors have speculated about an effect of the maintenance of milk fat fluidity on milk fat secretion (Shingfield et al., 2006; Chilliard et al., 2007; Gama et al., 2008) related to the incorporation of oleic acid (*cis*-9 18:1) and short-chain FA (4:0–10:0) into triglycerides as the principal means of ensuring milk fat liquidity at body temperature (Timmen and Patton, 1988). Thus, the known inhibition of marine lipids on 18:1 ruminal saturation (Or-Rashid et al., 2008), reducing therefore the

availability of 18:0 for endogenous mammary synthesis of oleic acid, plus the contribution of decreased de novo FA synthesis, would detrimentally affect the maintenance of milk fat liquidity at body temperature. Moreover, the augmentation in *trans* FA (mainly 18:1 isomers), with higher melting points than their equivalent *cis*-isomers (Gunstone et al., 1994), might also contribute to reduce milk fat fluidity because milk fat globules with melting points higher than body temperature could not be secreted (Timmen and Patton, 1988; Gama et al., 2008). Major changes in milk FA composition induced numerically minor variations in calculated milk fat melting point (between −0.7 and 0.7 ºC; Table 4), even lower than those reported in the milk of cows fed fish oil (Gama et al., 2008), which gives an idea of the high regulatory capacity of the mammary gland.

Furthermore, the activity of the Δ^9-desaturase in the mammary gland of ruminants is also thought to occur as a mechanism to maintain and regulate the fluidity of milk fat for efficient secretion. Although significant differences were observed for all the calculated desaturase indices (Table 4), only changes in *cis*-9 18:1/(18:0 + *cis*-9 18:1) were of quantitative importance. According to Gama et al. (2008), this could be attributable to either an increase in the specific activity of Δ^9-desaturase for stearic acid when feeding marine lipids or a higher uptake of oleic acid coming from adipose tissues. Both responses may result from a physiological adaptation to the increased supply of *trans* 18:1 FA and the decline in the stearic acid uptake and availability for mammary oleic acid synthesis.

In addition, as previously reported for CLA-induced MFD (Lock et al., 2006; Harvatine et al., 2009), lipid supplements reduced the milk content of FA with fewer than 16 carbons (i.e., synthesized de novo in the mammary gland) and increased those with more than 16 carbon (i.e., originated from mammary uptake of circulating FA), suggesting further mechanisms by which the mammary gland can adapt to altered supply of FA. To give a clearer insight into changes in mammary fat secretion, data of de novo and preformed FA yields are shown in Figure 3. Thus, when SO was fed alone, reduced de novo FA yield (from day 14 onward; $P < 0.05$) was alleviated by an increase in the secretion of > 16 carbon FA

($P < 0.01$). However, the greater decrease in < 16 carbon FA yield induced by the addition of MA ($P < 0.05$) was not compensated for by the transient increase in long-chain FA yield.

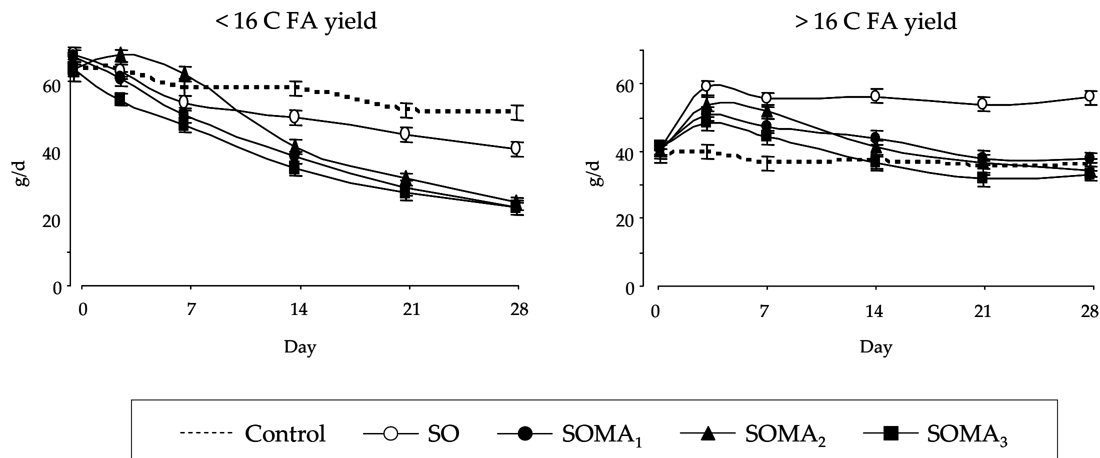

Figure 3. *Temporal changes in < 16 C and > 16 C fatty acid (FA) yield (g/d) in ewes fed a total mixed ration without lipid supplementation (control) or supplemented with 25 g of sunflower oil/kg of dry matter plus 0, 8, 16, or 24 g of marine algae/kg dry matter (SO, SOMA$_1$, SOMA$_2$, and SOMA$_3$ diets, respectively). Values are the mean from 2 lots of 5 animals per lot; vertical bars represent the standard error of the mean.*

4.3. Saturated and monoenoic fatty acids

It is now well established that increases in the supply of long-chain FA to the mammary gland alter the synthesis of short- and medium-chain saturates (Pulina et al., 2006; Chilliard et al., 2007; Kadegowda et al., 2009). Since high intakes of saturated FA could be related to an increased risk of cardiovascular disease and development of the metabolic syndrome (Shingfield et al., 2008), the effect of SO and MA supplementation (Table 4) would have a positive effect on the nutritional value of milk fat. It is, however, noteworthy that the specific role of saturated FA should be considered individually in relation to their putative effect on human health (see review by Shingfield et al., 2008).

Table 4. *Fatty acid (FA) composition, indices of desaturase activity,* n-6:n-3 *ratio, and melting point of the milk from ewes fed the experimental diets*[1].

	Treatment					SED[2]	*P*-value[3]		
	Control	SO	SOMA1	SOMA2	SOMA3		D	T	D × T
According to degree of saturation, g/100 g of FAME									
Saturated FA	75.40^{a}	69.00^{b}	65.07^{c}	65.01^{c}	64.91^{c}	0.383	***	***	***
Monounsaturated FA	19.73^{d}	25.48^{c}	26.98^{a}	26.54^{ab}	25.90^{bc}	0.312	***	***	***
Polyunsaturated FA	4.50^{d}	5.47^{c}	7.82^{b}	8.03^{b}	8.91^{a}	0.120	***	***	***
According to origin[4], g/100 g of FAME									
<16 carbons	42.60^{a}	37.18^{b}	37.07^{b}	37.25^{b}	36.14^{b}	0.977	**	***	***
16 carbons	28.65^{ab}	24.92^{c}	27.31^{b}	27.51^{ab}	28.79^{a}	0.524	**	***	***
>16 carbon	29.00^{c}	37.80^{a}	35.61^{b}	35.24^{b}	35.08^{b}	0.581	***	***	***
Desaturase index									
cis-9 14:1/(14:0 + *cis*-9 14:1)	0.019^{a}	0.019^{a}	0.019^{a}	0.017^{b}	0.015^{b}	0.0009	**	***	†
cis-9 16:1/(16:0 + *cis*-9 16:1)	0.051^{a}	0.049^{ab}	0.046^{bc}	0.044^{c}	0.040^{d}	0.0114	**	***	*
cis-9 18:1/(18:0 + *cis*-9 18:1)	0.670^{c}	0.645^{d}	0.768^{b}	0.817^{a}	0.820^{a}	0.0043	***	***	***
cis-9,*trans*-11 18:2/(*trans*-11 18:1+ *cis*-9, *trans*-11 18:2)	0.321^{a}	0.287^{b}	0.264^{c}	0.267^{c}	0.327^{a}	0.0073	***	***	***
n-6:*n*-3 ratio	3.47^{b}	4.21^{a}	3.27^{b}	2.42^{c}	2.12^{c}	0.119	***	***	***
Milk melting point, ºC	34.6^{b}	34.0^{c}	35.1^{a}	34.5^{b}	34.6^{b}	0.14	***	***	*

[a–d] Means within a row with different superscripts differ significantly.

[1] Diets included a total mixed ration without lipid supplementation (control) or supplementation with 25 g of sunflower oil/ kg DM plus 0 (SO), 8 (SOMA1), 16 (SOMA2), or 24 (SOMA3) g of marine algae (56.7% ether extract)/kg DM.

[2] SED = standard error of the difference.

[3] Probability of significant effects due to experimental diet (D), time on diet (T), and their interaction (D × T). ns, not significant ($P > 0.10$); †$P < 0.10$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

[4] < 16 carbons represent de novo synthesized FA, > 16 carbons represent preformed FA taken up from circulation, and 16 carbons FA are derived from both sources.

The decrease in 18:0 and *cis*-9 18.1 together with the increase in *trans* 18:1 in SOMA diets milk fat would reflect the inhibitory action of the long-chain *n*-3 PUFA present in MA, mainly DHA (AbuGhazaleh and Jenkins, 2004), on *trans* 18:1 ruminal reduction (Loor et al., 2005; Sinclair et al., 2005; Or-Rashid et al., 2008). As a consequence, SOMA treatments resulted in the greatest percentage of *trans* 18:1, mainly VA and *trans*-10, two intermediaries of linoleic acid BH pathways (Jenkins et al., 2008). Other minor *trans* 18:1 (*trans*-6, -7, -8, *trans*-9, and *trans*-12) could also derive from isomerization of oleic acid (26% of total FA in sunflower oil) because BH of this latter FA involves the formation of several isomers of *trans*-monoenes further to *trans*-10 and *trans*-11 18:1 (Jenkins et al., 2008).

High contents of VA in bovine milk are better maintained with high levels of forage, or low lipid supplementation, or both (Palmquist et al., 2005; Chilliard et al., 2007; Cruz-Hernandez et al., 2007), whereas addition of marine lipids or linoleic oil- and starch-rich diets have been related to a shift in ruminal BH toward increased formation of *trans*-10 18:1 at the expense of VA (Roy et al., 2006; Chilliard et al., 2007; Or-Rashid et al., 2008). It is remarkable that high levels of *trans*-10 18:1 in ovine milk appear to be accompanied by similar or even higher contents of VA (Reynolds et al., 2006), whereas in dairy cows, there is a pronounced inverse relationship between these two FA, so the *trans*-10 18:1 content may amply exceed that of VA (Roy et al., 2006; Shingfield et al., 2006; Boeckaert et al., 2008). This highlights the importance of interspecific differences among dairy ruminants, as has been stated in the case of goats and cows (Chilliard et al., 2007). Even though notable and greater *trans*-10 18:1 levels were previously achieved in dairy ewes with the inclusion of linoleic-rich oils in high-starch diets (Gómez-Cortés et al., 2008a; Hervás et al., 2008), an adequate fibre content probably prevented altered ruminal biohydrogenation toward *trans*-10 18:1 ruminal formation in the SO treatment but not in MA-containing diets (Roy et al., 2006; Shingfield et al., 2006), where rumen environment might be altered by MA. This hypothesis would be in line with the decrease of branched- and odd-chain FA contents observed mainly in MA treatments. Unexpectedly, the high *trans*-10 18:1

concentration observed in SOMA treatments decreased significantly in SOMA1 on day 28, which might indicate adaptation of the rumen microbiota to MA consumption. This speculation could be supported by the concomitant variations in *trans*-9, *cis*-11 CLA (decrease) and VA and RA (increases) and the slight recovery in milk fat content.

4.4. Conjugated and non-conjugated linoleic acid

Because of the transient nature of RA formed in the rumen, endogenous synthesis from VA through mammary Δ^9-desaturase is considered the principal origin of this CLA isomer in ruminant milk (Palmquist et al., 2005; Pulina et al., 2006; Shingfield et al., 2008). Thus, those diets inducing the largest accumulation of VA resulted also in the highest milk RA concentrations ($r = 0.97$; $P < 0.001$). Temporal variations in RA (Figure 1) contrast with the situation in dairy cows, where a progressive, time-dependent reduction in this FA is frequently found after initial increases following lipid supplementation of starch-rich diets (Shingfield et al., 2006; Chilliard et al., 2007).

Changes in non-conjugated 18:2 were less remarkable, and although mean intake of linoleic acid increased by 53 g/day with lipid supplementation, its concentration in milk fat was unmodified with SO and even decreased with SOMA treatments, probably because the usually high ruminal BH of linoleic acid can be further increased with marine lipid supplements (up to 90% according to Sinclair et al., 2005). The notable rise in milk *trans* 18:1 would also support this hypothesis.

Regarding other non-conjugated 18:2 FA detected in milk fat, *trans*-11, *cis*-15 18:2 was the most extensively modified by lipid supplementation, reaching on average a 6-fold increase with $SOMA_2$ and $SOMA_3$ (0.30% of total FA). Identified as an intermediate of 18:3*n*-3 (α-linolenic acid) metabolism (Jenkins et al., 2008), its content is frequently greater in milk fat when marine oils are fed (Shingfield et al., 2006; Chilliard et al., 2007), which suggests a putative inhibitory effect of MA long-chain *n*-3 PUFA on the complete BH process of α-linolenic acid (Loor et al., 2005).

4.5. Other polyunsaturated fatty acids

Diet supplementation with SO plus MA offered some additional changes pointing toward a healthier milk FA profile, such as the dose-dependent improvement in DHA concentration and the decrease in the *n*-6:*n*-3 FA ratio, of great importance in reducing the risk of cardiovascular diseases (Simopoulos, 2008). However, despite the high DHA content of the MA supplement (42% of total FA), these increases appear limited and represented an average transfer efficiency of only 5%, in line with transferences observed by other authors in cows and sheep (Papadopoulos et al., 2002; Loor et al., 2005; Chilliard et al., 2007) but lower than some previous reports in dairy ewes (Reynolds et al., 2006; Capper et al., 2007). The ruminal BH of long-chain *n*-3 PUFA (Loor et al., 2005; Sinclair et al., 2005; Castañeda-Gutiérrez et al., 2007) may partially account for their low transfer efficiencies into milk. Preferential partitioning of *n*-3 PUFA into some plasma lipid fractions that are poorly taken up by the mammary gland, such as plasma cholesterol ester and phospholipids (Offer et al., 1999; Kitessa et al., 2001; Chilliard et al., 2007), would also help to explain these results.

5. CONCLUSIONS

Supplementation of the diet of ewes with three incremental levels of MA and a moderate amount of SO affected animal performance by reducing the milk content of fat and protein, even with the lowest level of MA inclusion. Although all lipid supplements lowered the yield of FA originating from de novo synthesis (< 16 carbons), sustained increase in the output of long-chain FA prevented MFD when only SO was added. Marine algae-induced MFD might have been related to the joint action of some putative fat synthesis inhibitors, such as *trans*-9, *cis*-11 CLA and probably *trans*-10 18:1, as well as to the effect on milk fat fluidity of the decrease in 18:0 and subsequently in *cis*-9 18:1 content together with the rise in *trans* FA contents. The combination of SO and MA, especially with the highest level of the latter, proved to be very effective for improving the milk concentration

of some bioactive components, such as RA, VA, and DHA, and reducing the *n*-6:*n*-3 FA ratio. However, despite changes induced by SOMA supplementation in the milk FA profile that pointed to an enhanced nutritional value of the milk fat, large increases in other *trans* FA, mainly *trans*-10 18:1, were also observed.

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GENERAL DISCUSSION

Over recent years, the supplementation of ruminant diet with lipid sources has received special attention owing to its potential to improve the FA profile of milk to meet health-conscious consumers' demands (Lock and Bauman, 2004; Shingfield et al., 2008). Nevertheless, most studies have involved cows while very little work of a similar nature has been published concerning dairy sheep (Papadopoulos et al., 2002; Mele et al., 2006; Capper et al., 2007; Hervás et al., 2008). In the series of studies making up this dissertation, the combination of SO and marine lipids (either FO or MA) proved to be very effective in increasing milk fat concentration of RA, VA, and long-chain *n*-3 FA, supporting the first part of our hypothesis. However, it also enhanced the accumulation of *trans*-10 18:1 in milk and affected animal performance by reducing milk content in fat and protein (Chapters I and V), and even the DMI and milk yield in the case of the SOFO (Chapter I), invalidating the second half of our hypothesis. These changes were accompanied by important modifications in ruminal lipid metabolism, specifically a marked inhibition of the complete biohydrogenation of unsaturated FA (Chapter III), and variations in the microbial community (Chapter IV), but no apparent detrimental effects on ruminal fermentation were observed (Chapters II and III).

All these results are analysed and discussed in the corresponding chapter of the dissertation, so this brief section is only intended to summarise and integrate the major findings and discuss the main differences detected.

1. ANIMAL PERFORMANCE

1.1. Dry matter intake, milk production and ruminal fermentation

Of all the diets tested, only the combination of SO and FO resulted in a decrease in milk yield (Chapter I), which was related to the lower feed intake observed in the ewes on this treatment. Reduced DMI is often observed in response to the addition of fats, especially marine lipids, to the diet and has often been explained by potential disturbances of the rumen fermentation by PUFA (Jenkins, 1993). However, no apparent detrimental effects on rumen digestion were observed in

cannulated ewes fed SFO (Chapters II and III). Furthermore, previous studies have reported that FO reduces feed intake not only when included in the diet (Kitessa et al., 2001; Wachira et al., 2002; Shingfield et al., 2010), but also in response to its duodenal administration (Hagemeister et al., 1988; Fievez et al., 2003), which supports the idea that the negative effect on intake may be mediated by mechanisms other than disturbed ruminal fermentation, and probably related to increased duodenal flow of unsaturated FA (Shingfield et al., 2003; Lee et al., 2008; Shingfield et al., 2010).

Remarkably, no decrease in DMI was observed in MA-containing treatments, in contrast to all the previous reports in sheep (Papadopoulos et al., 2002; Reynolds et al., 2006) and cows (Franklin et al., 1999; Offer et al., 2001; Boeckaert et al., 2008a). The reasons for this discrepancy remain uncertain, but the role of factors beyond our control, such as weather conditions, must not be ruled out.

1.2. Milk composition

The inclusion of either FO or MA in the diet of dairy ewes decreased the total solid content of milk, because of the significant reduction in protein and, mainly, fat concentrations. However, no variation in total solids was observed with SO supplementation, regardless of the different forage:concentrate ratio of the basal diets (Chapters I and V). A reduction in total solid content could be of great relevance in dairy sheep and therefore of interest to breeders because it is well known that most ovine milk is processed into cheese.

1.2.1. Milk protein content

Reductions in protein content are frequently observed in sheep and cows fed lipid supplements (Schingoethe, 1996; Cant, 2003; Pulina et al., 2006). In the studies in Chapters I and V, this decrease averaged 6%, which is within the range commonly observed for ewes fed oil-rich diets (Mele et al., 2006; Pulina et al., 2006; Hervás et al., 2008).

Early studies suggested that, when diets were supplemented with lipids, adequate amounts of fibre and fermentable energy were required to prevent milk protein reduction by ensuring that microbial protein synthesis was not disturbed (DePeters and Cant, 1992; Schingoethe, 1996). In this regard, the higher fibre content of the 50:50 TMR used in the last experiment (Chapter V) could be argued to be the responsible for the lack of milk protein depression observed in that assay for the SO diet (2.5% of SO on a DM basis), whereas a reduction was elicited by a very similar treatment (2.3% DM of SO) in the experiment using the 20:80 TMR (Chapter I). However, more recent work has shown that decreased milk protein concentration after the inclusion of oils in the diet is not necessarily associated with disturbed protein metabolism in the rumen (Wachira et al., 2002; Kucuk et al., 2004) but probably with reduced protein synthesis in the mammary gland (Cant, 2003). In fact, it has been proposed that, when lipid supplements are fed, changes in the concentration of plasma metabolites and increases in the energy supply to the mammary gland could reduce blood flow rates to this organ, limiting the uptake of some critical amino acids (Cant, 2003). Furthermore, Burgos et al. (2010) have recently indicated that variations in the lactogenic hormones hydrocortisone, insulin, and prolactin, together with amino acid and energy cellular availability, would also play a key role in this process through direct regulation of protein translation in the mammary tissue. Nevertheless, the mechanisms mediating these effects remain largely unknown.

1.2.2. Milk fat content

Milk fat depression is uncommon in sheep, even when fed starch-rich diets and relatively high amounts of plant oils (Mele et al., 2006; Gómez-Cortés et al., 2008; Hervás et al., 2008). However, the inclusion of FO or MA in the diet of dairy ewes (Chapters I and V) resulted in important decreases in milk fat content, which reached values of up to 30% after 28 days on $SOMA_3$ treatment (see Figure 1). Despite the differences in the forage:concentrate ratio of the basal diets, comparable reductions over time were observed with SOFO and $SOMA_3$

treatments, which supplied similar amounts of fat (Figure 1). In dairy cows, direct comparison of several FO-containing diets with different proportions of forage and concentrate did also show similar responses in milk fat content (Shingfield et al., 2005; Gama et al., 2008), which suggests that the mechanisms causing marine oil-induced MFD would be independent of the basal diet composition, in contrast to vegetable oil-induced MFD.

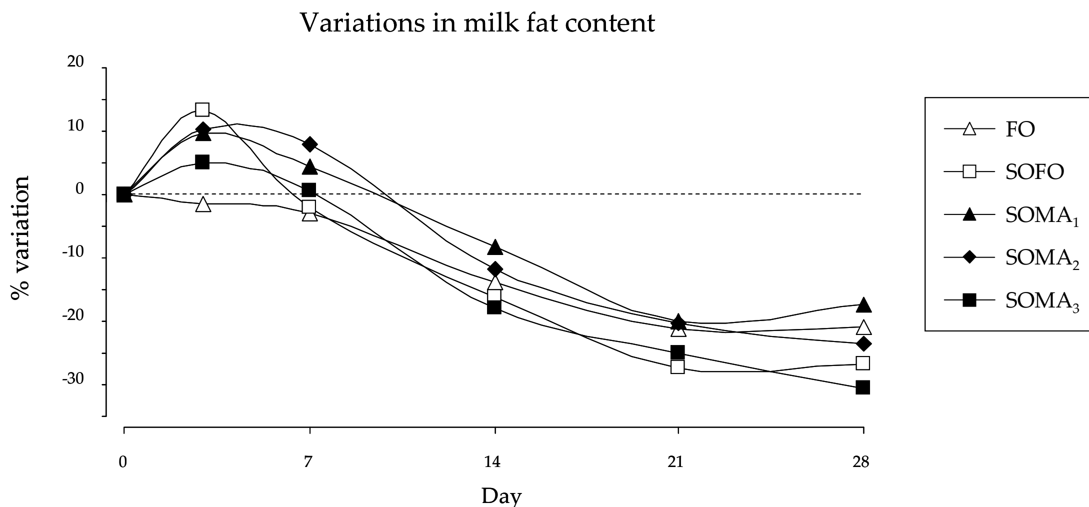

Figure 1. *Percentage of variation in milk fat content in ewes on high-concentrate diets supplemented with marine lipids (Chapters I and V).*

The discrepancy between the results observed in Chapters I and V (i.e., MFD) and those reported previously for sheep fed plant oils (i.e., non MFD; Mele et al., 2006; Hervás et al., 2008; Luna et al., 2008) may be due to a distinct susceptibility of the ovine species to the specific causal agents of MFD. Besides, Papadopoulos et al. (2002) and Reynolds et al. (2006) did not find MFD in ewes fed marine oil supplements, which may, at least in part, be accounted for by differences in the genetic potential of the ewes used in the different trials (highly productive dairy vs. non-dairy or dual-purpose breeds), and animal management (suckling vs. milking).

The inhibitory effects of marine lipids on milk fat secretion recorded in the present study (Chapters I and V) were not associated with increases in *trans*-10, *cis*-12 CLA, the only biohydrogenation intermediate demonstrated so far to exert antilipogenic effects in sheep (Lock et al., 2006). Nor were there sufficiently substantial changes in *cis*-10, *trans*-12 and *trans*-9, *cis*-11 CLA to justify a role of these putative inhibitors of milk fat synthesis (Shingfield et al., 2010) in the MFD observed in the ewes. Despite the negative relationship between *trans*-9, *cis*-11 CLA and sheep milk fat content ($r = -0.71$; $P < 0.001$; Figure 2), increases in this FA are not always associated with MFD in this species (Gómez-Cortés et al., 2008; Hervás et al., 2008).

In like manner, large increases in *trans*-10 18:1 in the absence of MFD (Gómez-Cortés et al., 2008; Hervás et al., 2008) would offer no support for a decisive role of this intermediate in the regulation of milk fat synthesis in ewes. The effect of *trans*-10 18:1 on milk fat secretion is controversial (Lock et al., 2007; Shingfield et al., 2009), although a recent study using molecular-based techniques (Kadegowda et al., 2009) has demonstrated that this FA inhibits lipogenic gene expression in bovine mammary epithelial cells through a mechanism similar to that of the *trans*-10, *cis*-12 CLA. Thus, the reasons underlying the different response to large quantities of *trans*-10 18:1 in ewes (Chapters I and V vs. Gómez-Cortés et al., 2008 and Hervás et al., 2008) are not obvious but may lie in inter-species differences between ruminants in the molecular mechanisms regulating milk fat synthesis. In this regard, a recent review has suggested that feeding milk fat depressing diets substantially alters lipogenic gene expression in cows but not in goats, while no information is currently available for sheep (Shingfield et al., 2010).

Milk contents of *trans*-10 18:1 and *trans*-9, *cis*-11 CLA were closely associated (Figure 2), and have frequently been observed in cows (Shingfield et al., 2005, 2006; Gama et al., 2008), although the reasons for such a close relationship are not well understood. *Trans*-10 18:1 is known to be an intermediate formed during the metabolism of *cis*-9 18:1, and, mainly, 18:2*n*-6 (Mosley et al., 2002; Jouany et al.,

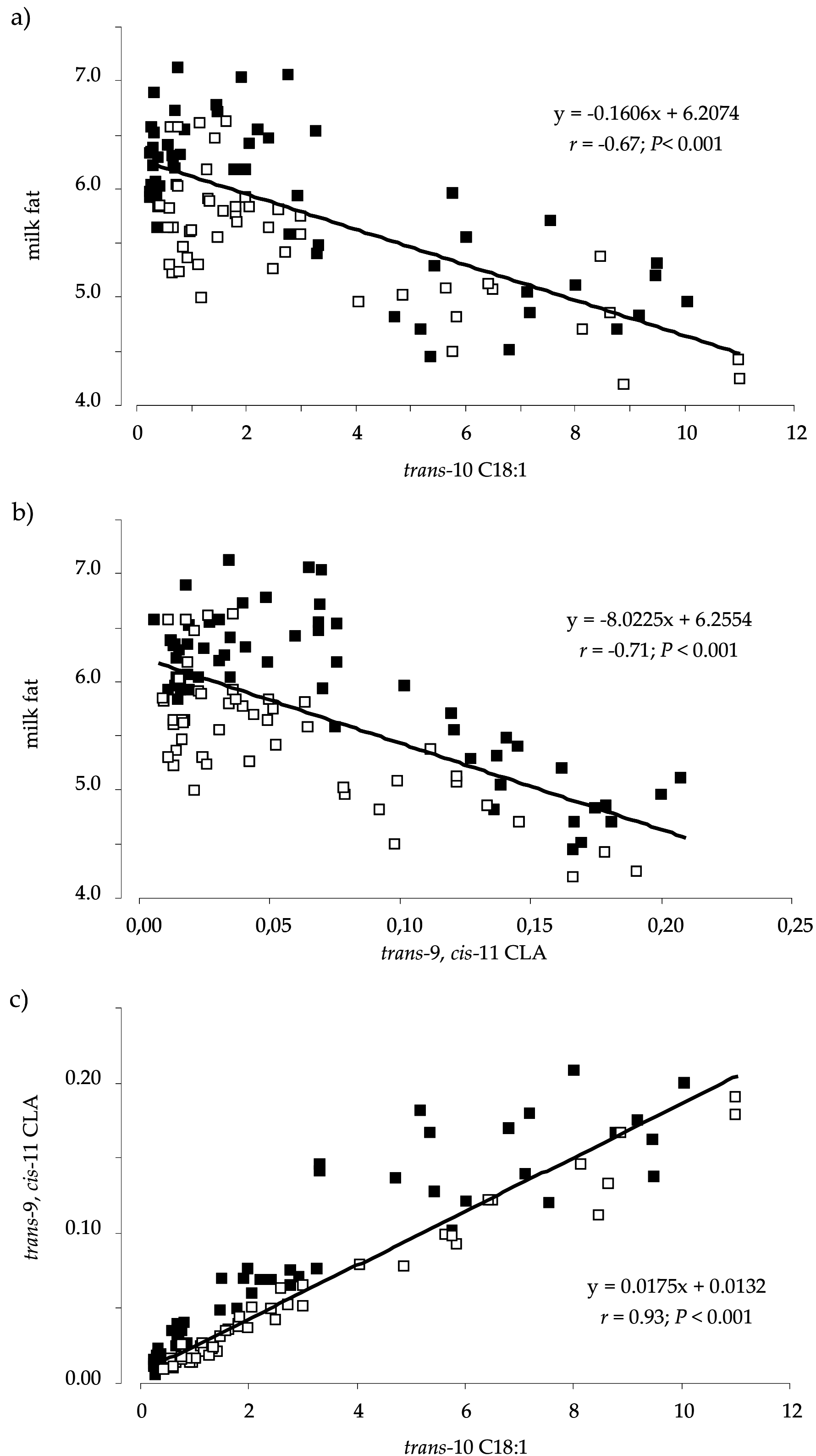

Figure 2. *Relationship between milk fat content (g/100 g raw milk) and corresponding concentrations (g/100 g total fatty acids) of (a)* trans*-10 18:1 or (b)* trans*-9,* cis*-11 CLA, and between (c)* trans*-10 18:1 and* trans*-9,* cis*-11 CLA contents in milk fat. Data are derived from the experiments with lactating ewes described in Chapters I (■; n = 48) and V (□; n = 60).*

2007; Jenkins et al., 2008; see Figure 2 of the General introduction), but the origin of *trans*-9, *cis*-11 CLA has yet to be accurately described. This latter CLA isomer was suggested to be a minor metabolite of 18:2*n*-6 (Wallace et al., 2007), but the incubation of 1-^{13}C-18:2*n*-6 with mixed ruminal microorganisms resulted in the formation of all possible CLA isomers with double bonds in either the 9,11 or 10,12 positions except *trans*-9, *cis*-11 (Jenkins et al., 2008).

Another explanation could be that both *trans*-10 18:1 and *trans*-9, *cis*-11 CLA were produced from a common bacterium or group of bacteria that proliferate in the rumen when SO and marine lipids are fed (Roy et al., 2006; Shingfield et al., 2006; Gama et al., 2008). Remarkably, in the experiment with cannulated ewes (Chapter III), ruminal contents of *trans*-9, *cis*-11 CLA were always very low (< 0.01 g/100 of total FA) and showed no variation after 10 days on the SFO diet, while a 4.5-fold increase in ruminal *trans*-10 18:1 concentration was found at that sampling time. This increment was similar to that observed in milk fat after 7 days on SOFO treatment (+414%, Chapter I), which continued rising up to 950% on day 21. It might therefore have been reasonable to expect a distinct response in the ruminal bacterial community of ewes when samples were collected after 10 or 21 days on the diet containing SO and FO, but the microbiological study (Chapter IV) showed similar changes in the bacteria monitored, despite involving two different experiments (cannulated vs. lactating ewes).

The results from our study indicate, as some other recent papers have suggested (Boeckaert et al., 2008a, 2009; Kim et al., 2008), that the explanation of ruminal lipid metabolism in terms of the bacteria currently supposed to carry out the biohydrogenation is too weak, and new species may be involved. In line with this, a few uncultured strains of the family *Lachnospiraceae*, together with large oval bacteria of the clostridial cluster IX (Figure 3) were stimulated with FO and SO supplementation. These latter bacteria seem to be related to *Quinella ovalis*, which belongs to the same family as *Megasphaera elsdenii* and *Mitsuokella multiacidus*, shown to convert 18:2*n*-6 to *trans*-10, *cis*-12 CLA and to *cis*-9 18:1, respectively (Kim et al., 2002; Maia et al., 2007). Further research is therefore

needed to determine whether these populations have a role in the pathways leading to the accumulation of *trans*-10 18:1. Given the potential impact of this *trans* 18:1 isomer in the regulation of mammary lipogenesis (Kadegowda et al., 2009; Shingfield et al., 2009) and consumers' health (Roy et al., 2007; Shingfield et al., 2008), the identification of the microbial species responsible for its formation would be of great interest.

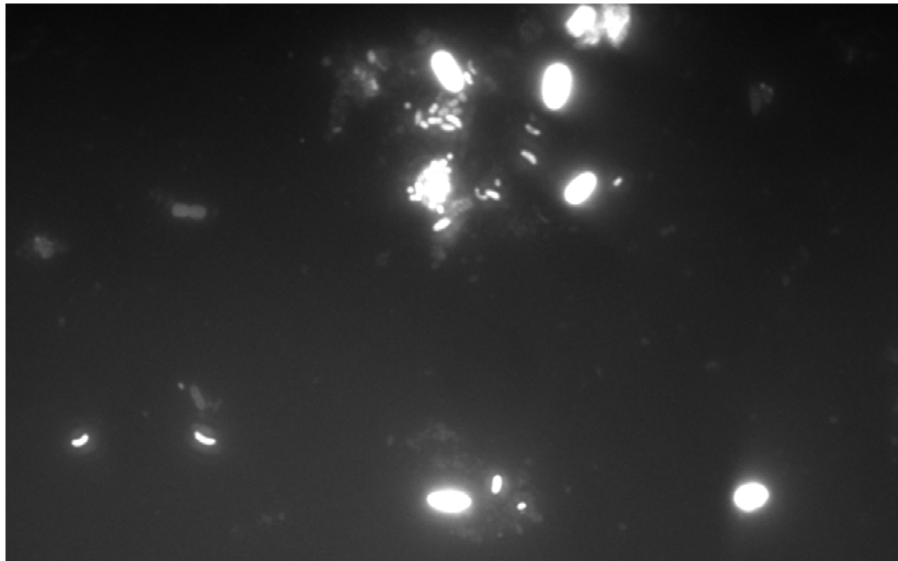

Figure 3. *FISH image of a rumen fluid sample where large oval bacteria, presumably* Quinella*-like, were detected.*

2. MILK FATTY ACID PROFILE

2.1. Saturated and cis*-monounsaturated fatty acids*

The impact of SO and marine lipids on the concentration of saturated FA in milk was consistent in the two experiments with lactating ewes (Chapters I and V) and in agreement with previous studies in lactating cows (AbuGhazaleh et al., 2002; Shingfield et al., 2006; Cruz-Hernandez et al., 2007). All supplemented diets decreased the proportion of even-chain saturated FA (Figure 4) but, while sunflower oil reduced short- and medium-chain FA (synthesized de novo in the

mammary gland), marine lipids affected mainly 18:0 content (derived from the metabolism of unsaturated FA in the rumen).

Regardless of the forage:concentrate ratio of the diet (i.e., comparing the results observed in Chapters I and V), inclusion of SO always resulted in large decreases in the concentrations of 6:0, 8:0 and 10:0, and of 12:0, 14:0 and 16:0. The combination of this plant oil with marine lipids (FO or MA) enhanced this decrease in the case of 6:0, 8:0 and 10:0, but reduced it in that of the potentially atherogenic 12:0, 14:0 and 16:0. Nevertheless, the lower reductions in milk medium-chain FA with FO or MA addition would not be explained by marine oils limiting the inhibitory effect of SO on de novo FA synthesis, but by a dilution effect, as explained in Chapter V (see Figure 3 of that chapter).

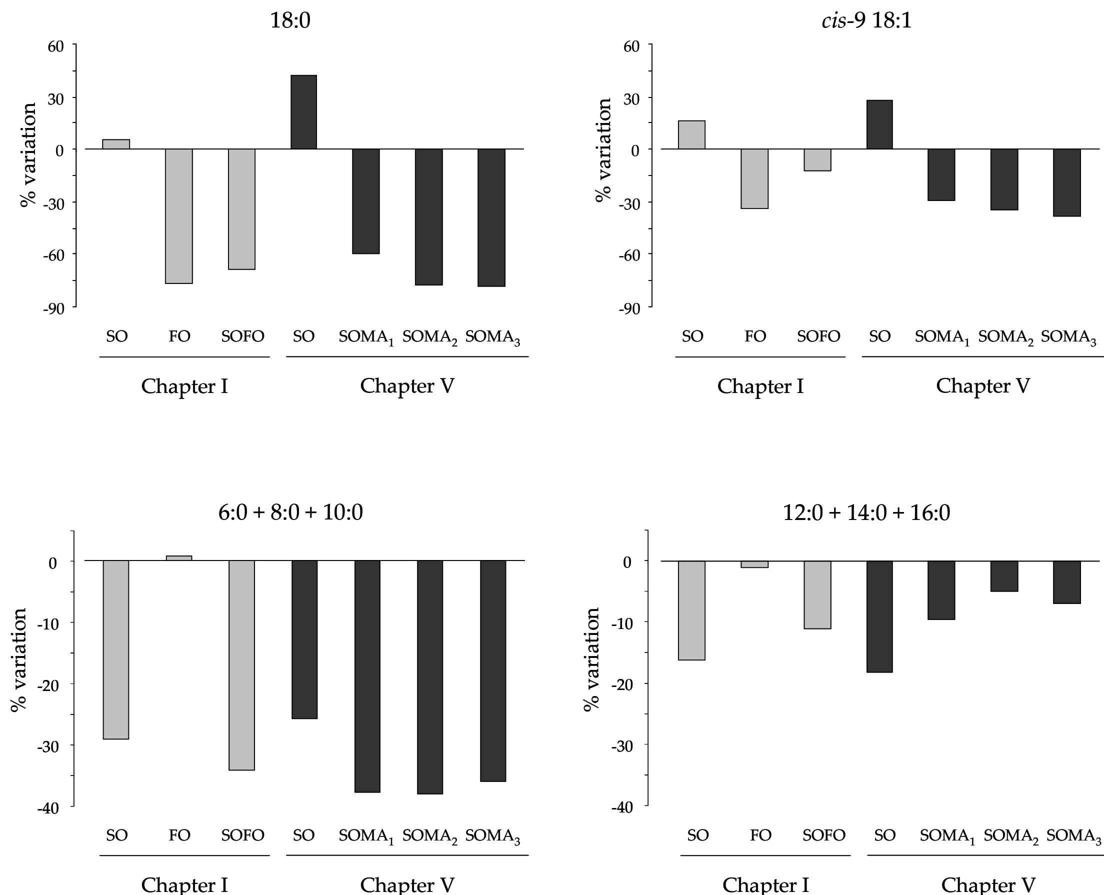

Figure 4. *Percentage of variation in the milk content of 18:0,* cis-9 *18:1, the sum of 6:0, 8:0, and 10:0, and the sum of 12:0, 14:0, and 16:0 in ewes after 28 days on high-concentrate diets supplemented with lipids (Chapters I and V).*

It is well known that ruminal lipid metabolism shares responsibility with mammary gland activity for milk fat composition. Under normal feeding conditions (i.e., for non-supplemented diets), FA leaving the rumen would be expected to be mostly saturated (AbuGhazaleh et al., 2002; Loor et al., 2005; Shingfield et al., 2010), to be consistent with the FA profile of ruminal digesta (Chapter III). Then, in the mammary gland, the Δ^9-desaturase partly reverses the effect of ruminal biohydrogenation by decreasing the saturation level, mainly by 18:0 conversion to *cis*-9 18:1 (Palmquist et al., 2005; Glasser et al., 2008). Mammary desaturation would therefore contribute to explain the lower contribution of 18:0 to total 18 carbon FA in milk than in rumen digesta lipids (62 vs. 22%; Chapters I and III), and the relatively higher content of *cis*-9 18:1 (48 vs. 7%).

Feeding 18:2*n*-6 to ruminants is expected to increase 18:0 and *cis*-9 18:1 in both rumen digesta (Kucuk et al., 2004; Shingfield et al. 2008; Glasser et al., 2008) and milk (Roy et al., 2006; Glasser et al., 2008; Hervás et al., 2008). This is in general agreement with the changes in milk composition when the diets were supplemented only with SO (Figure 4). On the contrary, and despite the different forage:concentrate ratio of the basal diets used in Chapters I and V, when a mixture of SO and marine lipids (either FO or MA) was fed, the effects of these latter prevailed. Marine oils, known to inhibit the final biohydrogenation step where *trans* 18:1 are converted to 18:0 (AbuGhazaleh and Jenkins, 2004), resulted in large decreases in the milk content of 18:0 and consequently, although to a lesser extent, of *cis*-9 18:1 (Figure 4). The experiment with cannulated ewes (Chapter III) confirmed that SFO supplementation reduced the concentration of 18:0 in rumen digesta lipids (reaching a 4.2-fold decrease after 10 days on treatment) but, surprisingly, there were no concomitant reductions in the DNA concentration of *Butyrivibrio* 18:0-producing bacteria in the rumen of those animals (Chapter IV). This contrasts with the significant decrease in this microbial population detected in lactating sheep after 21 days on a similar diet, which, together with the fact that the relative abundance of 18:0-producing *Butyrivibrio* was always low, would support the hypothesis that other as-yet-uncultivated

bacteria must be involved in 18:0 production (Boeckaert et al., 2008b; Kim et al., 2008).

2.2. Trans *fatty acids and conjugated linoleic acid*

The inclusion of SO and marine lipids in the diet led to striking changes in *trans* 18:1 concentration and its profile in milk (Chapters I and V) and rumen digesta lipids (Chapter III). *Trans*-11 and *trans*-10 were the 18:1 isomers whose concentration in milk varied the most in response to lipid supplements (Figure 5). This was particularly clear for MA-containing diets, although it must be noted that the percentage of variation depended on the concentration presented by the corresponding control treatment. In this regard, the milk concentrations of these two isomers in the ewes fed the control diet used in Chapter I (20:80 forage:concentrate) were much higher (70% for *trans*-11 and 160% for *trans*-10 18:1) than those observed in the animals fed the control diet used in Chapter V (50:50 forage:concentrate), which could be attributed to a less extensive ruminal biohydrogenation of unsaturated FA in concentrate-rich diets (Sackmann et al., 2003; Kucuk et al., 2004; AbuGhazaleh and Jacobson, 2007).

The ratio of *trans*-11:*trans*-10 18:1 in milk was also affected by the basal diet composition, with sheep on the 50:50 TMR showing greater values than those on the 20:80 TMR (2.7 vs. 1.8, respectively). It has recently been shown that the shift towards *trans*-10 18:1 accumulation in diets rich in concentrate is not only due to a lower pH (Fuentes et al., 2009), as generally thought, but also to a higher supply of starch (Maia et al., 2009) because this nutrient would presumably favour the growth of ruminal bacteria forming this *trans* FA. Comparing the 2.4-fold increase in milk *trans*-10 18:1 found in ewes fed the 20:80 TMR supplemented with SO (Chapter I) with the non-significant rise when SO was added to the 50:50 diet (Chapter V) could allow to hypothesize that these microbial populations were further stimulated by concentrate-rich diets. According to the results shown in Figure 5, supplementation with FO or MA would notably enhance the milk content of *trans*-10 18:1 in all cases.

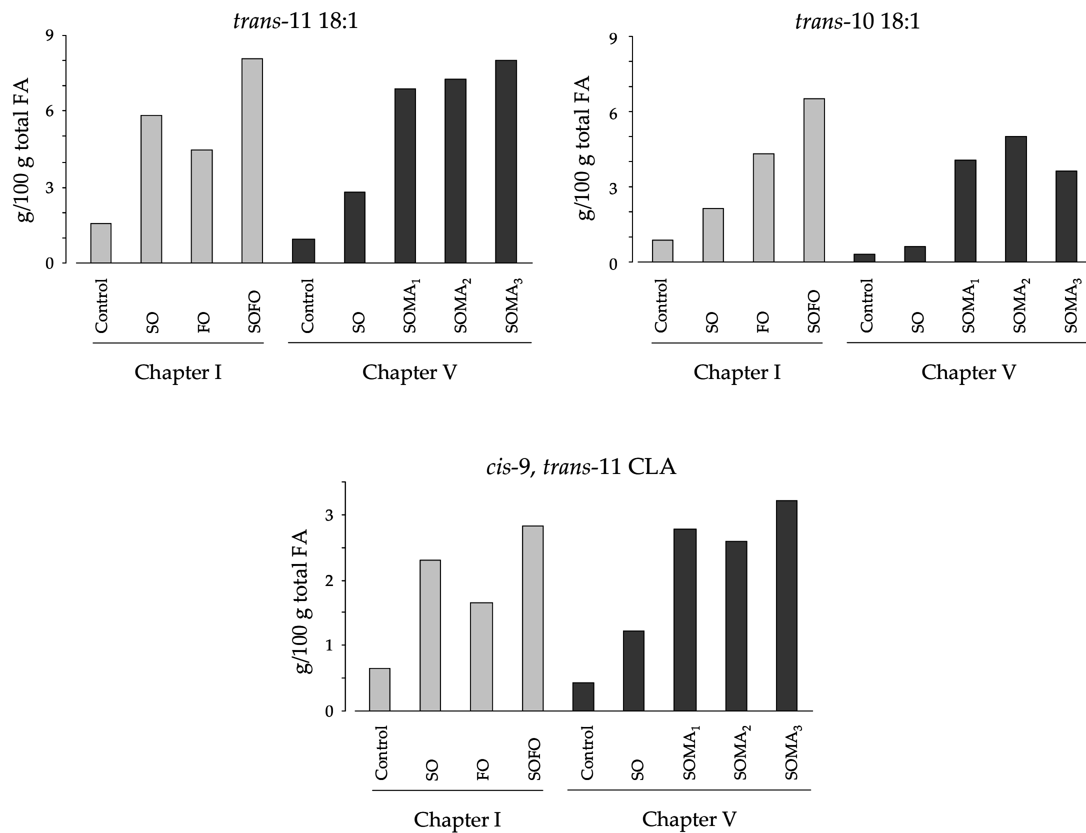

Figure 5. *Mean milk content of* trans-*11 18:1,* trans-*10 18:1, and* cis-*9,* trans-*11 CLA in ewes from experiments described in Chapters I and V.*

Temporary variations in *trans*-10 18:1 in milk (see Figure 1 of Chapter I and Figure 2 of Chapter V) would suggest that the microbiota involved in the biohydrogenation pathways leading to its formation would need at least 2 weeks to suffer the largest variations. However, as previously mentioned, the results of the microbiological study (Chapter IV) showed similar changes in the bacterial community in the rumen samples collected after 10 or 21 days in the diets containing SO plus FO.

Interestingly, when the concentrations of milk *trans*-10 18:1 achieved the maximum values, they only slightly exceeded those of *trans*-11 18:1 in the two experiments conducted with lactating sheep (Chapters I and V), which contrasts with the much greater content of *trans*-10 18:1 often observed in cows fed lipid supplements (Roy et al., 2006; Shingfield et al., 2006; Boeckaert et al., 2008a). These

results are probably related to the fact that, in dairy sheep milk, the large increases in *trans*-11 18:1 concentration achieved with lipid inclusion remained high over time (see Figures 1 of Chapter I, and 2 of Chapter V).

Trans-11 18:1 was the most abundant *trans* 18:1 isomer. In ewes fed the non-supplemented diets, it contributed equally (6%) to total 18 carbon FA in rumen digesta (Chapter III) and milk (Chapter I) lipids, whereas adding SO plus FO resulted in higher proportions in rumen digesta than in milk fat (42 vs. 21% of total 18 carbon FA). This discrepancy could be explained by the mammary desaturation of part of the *trans*-11 18:1 to *cis*-9 *trans*-11 CLA (Palmquist et al., 2005), as well as by the known decrease in uptake and secretion of 18 carbon FA when de novo FA synthesis is limited (Glasser et al., 2008). This latter reason would be consistent with the reduction in milk fat content (i.e., the MFD; Chapter I) occurring concurrently, i.e. from day 14 onwards.

Supplementation with SO alone resulted in stable levels of milk *trans*-11 18:1 over time and, although its percentage of variation (Figure 5) was higher when this vegetable oil was added to the 50:50 diet (Chapter V), greater concentrations were also achieved with the 20:80 (Chapter I).

In relation to CLA isomers, the *cis*-9, *trans*-11 CLA was the most notably affected in all cases, and its changes in milk fat mirrored those of *trans*-11 18:1 (Figure 5), its precursor for endogenous synthesis in the mammary gland (Palmquist et al., 2005). Accordingly, milk fat content of *cis*-9, *trans*-11 CLA was higher (+45%) in animals fed the control diet with the lowest forage:concentrate ratio (20:80, Chapter I) than in those fed the 50:50 diet (Chapter V), and the greatest enhancements occurred during the first days on treatments. However, the subsequent decline in this isomer enrichment was followed, around day 28, by further improvements in MA-containing diets (see Figure 2 of Chapter V).

Other CLA isomers were not substantially modified in response to the supplemented diets, except *trans*-9, *cis*-11 CLA, which, as mentioned above in Section 1.2.2, was increased with marine lipid addition.

2.3. Long-chain n-3 *fatty acids*

The major long-chain *n*-3 FA in the FO and the MA supplements was 22:6*n*-3, which constituted, respectively, 16.4 and 42.3% of total FA. This FA was also the most abundant ≥ 20-carbon FA in the ruminal digesta lipids of the sheep fed SFO, with no variations after 3 or 10 days on treatment (on average 0.71 g/100 g total FA; Chapter III). With regard to milk, the concentrations of this and other long-chain *n*-3 FA were increased sharply after the start of FO (Chapter I) or MA (Chapter V) supplementation and then continued increasing at a modest rate (e.g., 20:5*n*-5) or tended to plateau (e.g., 22:5*n*-3 and 22:6*n*-3). In goats, accumulation of 22:6*n*-3 in milk in response to marine lipids has also been reported to persist over time (Gagliostro et al., 2009), while in the case of cows (Shingfield et al., 2006), the enrichment in long-chain *n*-3 FA appears to be transiently higher during the first days of marine lipid addition, with a relative decline afterwards.

Inconsistent results between studies conducted in cows or small ruminants could be attributed to inter-species differences in ruminal lipid metabolism and to a different adaptation of the microbiota to marine lipid consumption. In this respect, it is well known that PUFA are more toxic to rumen bacteria than monounsaturates, and that biohydrogenation occurs to enable microorganisms to survive the bacteriostatic effects of FA by increasing their saturation level (Maia et al., 2007, 2010). Thus, it is worth noting that most of the *trans*-monounsaturated 20 and 22 metabolites reached transiently higher concentrations in rumen digesta lipids after 3 days on SFO treatment (Chapter III), whereas several PUFA with 20 or more carbons were further enhanced after 10 days or showed no statistical differences for sampling days. These observations, together with the lower ruminal content of 20:0 and 22:0 in SFO_{10} compared with SFO_3, would suggest a relatively slower and less complete biohydrogenation the longer the time on SFO diet. Nevertheless, changes in the cultured bacteria reported to be actively involved in the rumen biohydrogenation (Chapter IV) did not explain the variations observed in the ruminal metabolism of marine lipids, which suggests, once again, that other yet-uncultivated microorganisms may be more relevant in

that process. Moreover, a very recent study (Maia et al., 2010) demonstrated that *Butyrivibrio fibrisolvens*, a species known to play a major role in FA biohydrogenation, is able to metabolize 18:2*n*-6 and 18:3*n*-3 but not 20:5*n*-3 and 22:6*n*-3, the two major PUFA in FO and MA supplements. Considering that extensive ruminal biohydrogenation of long-chain *n*-3 FA is largely responsible for the low transfer efficiencies of these FA from diet into milk, identifying the main bacterial species involved in their metabolism would therefore be of great interest for the production of healthier ruminant-derived foods.

Comparing the two experiments conducted with lactating ewes (Chapters I vs. V), it is notable that the apparent transfer efficiency of 22:6*n*-3 from diet into milk was superior for FO than for MA supplements (on average, 0.095 and 0.046, respectively). This disparity might, at least in part, be attributable to a more extensive biohydrogenation of the unsaturated FA in the diets with a higher forage proportion (i.e., the MA-containing diets; Kucuk et al., 2004; Sackmann et al., 2003).

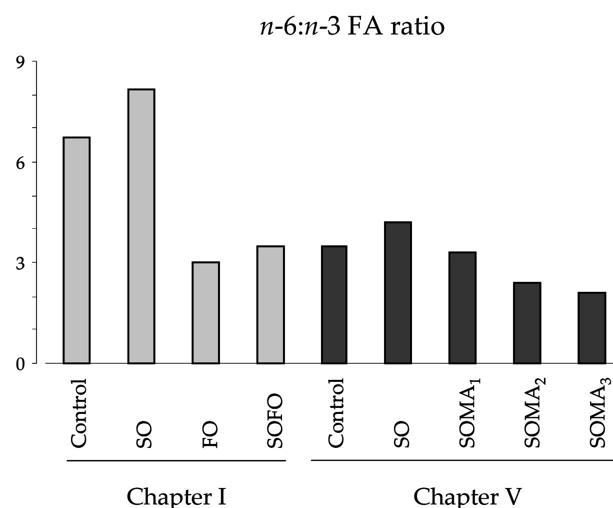

Figure 6. *Mean milk* n-6:n-3 *FA ratio in ewes from the experiments described in Chapters I and V.*

Besides the absolute quantity of *n*-3 FA in foods, the relative balance of *n*-6:*n*-3 FA appears to be of great importance in determining the risk of several chronic diseases (Simopoulos, 2008). In this regard, it is noteworthy that the mean

milk n-6:n-3 FA ratio in ewes fed the control 20:80 TMR (6.7; Chapter I) represents already half of the average value found in some current Western diets (approximately 15; Simopoulos, 2008), and was significantly reduced with FO supplementation to 3.2 (Figure 6). Interestingly, feeding a 50:50 TMR (Chapter V) resulted in a much lower ratio (3.5 for the control diet; Figure 6), and values as low as 2.1 were obtained in response to the supplementation with SO and the highest level of MA ($SOMA_3$).

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CONCLUSIONS

First. Supplementation of high-concentrate diets of dairy ewes with sunflower oil (SO), fish oil (FO), their combination (SOFO), and SO plus three incremental levels of marine algae (MA) proved to be an effective nutritional strategy to modulate milk fat composition, improving the content of some bioactive fatty acids, such as *cis*-9, *trans*-11 conjugated linoleic acid (CLA), *trans*-11 18:1, and, to a lesser extent, long-chain *n*-3 fatty acids. However, the inclusion of either FO or MA also resulted in high levels of *trans*-10 18:1.

Second. All the lipid supplements studied decreased milk protein content, and FO and MA also reduced milk fat concentration, while a lower milk yield was only observed with the inclusion of SOFO. Although the reasons for marine lipid-induced milk fat depression in ewes remain uncertain, the fact that the *trans*-10, *cis*-12 CLA was always detected in very low concentrations and showed no changes over time would rule out this CLA isomer as the principal factor responsible for the reduction observed in milk fat content. On the other hand, the reduction might have been related to the joint action of other putative inhibitors, as well as to the limited ability of the mammary gland to maintain a desirable milk fat fluidity in response to the important increase in *trans* 18:1 together with the decreased availability of 18:0 for *cis*-9 18:1 synthesis.

Third. The addition of SOFO to high-concentrate diets did not detrimentally affect ruminal pH, ammonia and lactate concentrations, in situ disappearance of dry matter, crude protein and neutral detergent fibre, or rates of in vitro gas production, but it caused a shift in the volatile fatty acid profile, with variations in acetate and butyrate. Overall, these results suggest that lipid supplementation did not impair ruminal fermentation in ewes.

Fourth. Supplementing a high-concentrate diet with SOFO caused a time-dependent inhibition of the complete biohydrogenation of 18 carbon unsaturated fatty acids resulting in the accumulation of *trans* 16:1, *trans* 18:1, *trans* 18:2, 20, and 22 carbon metabolites in ruminal digesta, with no evidence of a shift in ruminal biohydrogenation pathways towards *trans*-10 18:1 formation. The detection of several unique 20:1, 20:2, 22:1, 22:3, and 22:4 metabolites provided the first indications that ruminal metabolism of 20:5*n*-3, 22:5*n*-3, and 22:6*n*-3 may proceed via the reduction of the double bond closest to the carboxyl group.

Fifth. The inclusion of SOFO in the diet of lactating ewes promoted important qualitative changes in the total bacteria and *Butyrivibrio* populations that might be partly due to variations in microorganisms belonging to the family *Lachnospiraceae*, and to an increase in the abundance of some members of the clostridial cluster IX, presumably *Quinella*-like bacteria. The results suggested that the microorganisms commonly thought to carry out the ruminal biohydrogenation would not play a dominant role in this process, whereas other yet-uncultured bacteria might be stimulated by lipid supplementation and be more relevant.

CONCLUSIONES

Primera. La suplementación de dietas ricas en alimentos concentrados con aceite de girasol (SO), aceite de pescado (FO), una combinación de ambos (SOFO) y SO más tres niveles crecientes de microalgas marinas (MA) demostró ser una estrategia nutricional efectiva para modificar la composición de la grasa láctea, aumentando el contenido de algunos ácidos grasos bioactivos, como es el caso del isómero *cis*-9, *trans*-11 del ácido linoleico conjugado (CLA), del *trans*-11 18:1 y, en menor medida, de los ácidos grasos *n*-3 de cadena larga. Sin embargo, la inclusión de FO o de MA también dio lugar a niveles elevados de *trans*-10 18:1.

Segunda. Todos los suplementos lipídicos estudiados redujeron el contenido proteico de la leche y el FO y las MA también disminuyeron la concentración de grasa, mientras que solo se observó una reducción de la producción de leche con la inclusión de SOFO. Aunque los mecanismos responsables del síndrome de baja grasa en la leche causado por la adición de lípidos marinos aún no están bien definidos, la concentración de *trans*-10, *cis*-12 CLA en la leche fue siempre baja y no mostró variaciones importantes con el tiempo, lo cual descartaría que este isómero del CLA fuera el principal responsable de la caída de la grasa. Por el contrario, la reducción podría haber estado relacionada con la acción sinérgica entre otros metabolitos supuestamente inhibidores de la síntesis de grasa, así como con la limitada capacidad de la glándula mamaria para mantener una fluidez adecuada de la grasa láctea debido al importante aumento de los *trans* 18:1 y a la menor disponibilidad de 18:0 para la síntesis de *cis*-9 18:1.

Tercera. La adición de SOFO a dietas ricas en alimentos concentrados no afectó negativamente al pH ruminal, las concentraciones de amoniaco y lactato, la desaparición in situ de materia seca, proteína bruta y fibra neutro detergente, ni a los ritmos de producción de gas, pero alteró el perfil de ácidos grasos volátiles, con cambios en las concentraciones de acetato y butirato. En conjunto, estos resultados sugieren que la suplementación con aceites no perjudicó la fermentación ruminal en ovejas.

Cuarta. La suplementación de una dieta rica en alimentos concentrados con SOFO provocó la inhibición de la biohidrogenación completa de los ácidos grasos insaturados con 18 carbonos a lo largo del tiempo, dando lugar a la acumulación en la digesta ruminal de *trans* 16:1, *trans* 18:1, *trans* 18:2 y metabolitos con 20 y 22 carbonos, sin que hubiera evidencia de un cambio en las rutas de biohidrogenación hacia la formación de *trans*-10 18:1. La identificación de varios metabolitos 20:1, 20:2, 22:1, 22:3 y 22:4 únicamente en el contenido digestivo, proporcionó las primeras indicaciones existentes de que el metabolismo ruminal del 20:5*n*-3, 22:5*n*-3 y 22:6*n*-3 podría comenzar con la reducción del doble enlace más cercano al grupo carboxilo.

Quinta. La inclusión de SOFO en la dieta de ovejas lecheras indujo cambios cualitativos de importancia en las bacterias totales y en las poblaciones de *Butyrivibrio*, que podrían ser consecuencia de las variaciones en microorganismos pertenecientes a la familia *Lachnospiraceae*, así como del aumento en la abundancia de bacterias del cluster IX de los Clostridiales, supuestamente microorganismos del género *Quinella* o similares. Los resultados de esta prueba sugirieron que las bacterias que comúnmente se consideraban las principales responsables de la biohidrogenación ruminal, parecen no tener un papel dominante en este proceso, mientras que otras bacterias aún no cultivadas podrían verse estimuladas por la suplementación lipídica y ser más relevantes.