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# Effect of the dietary supplementation with sunflower and fish oils on the rumen bacterial communities in dairy sheep

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**Abstract.** In ruminants, the dietary addition of lipid sources rich in linoleic acid, such as sunflower oil (SO), and long-chain polyunsaturated fatty acids, such as fish oil (FO), has proved to increase the milk concentration of beneficial conjugated linoleic acid. Milk fatty acid (FA) profile relies on the ruminal FA biohydrogenation, the rumen microbial composition being therefore a key factor to increase the milk contents of beneficial FA. To study the effect of the dietary supplementation with SO and/or FO on the rumen microbiota of dairy sheep, thirty-two lactating ewes were divided in eight lots and offered a high concentrate diet, supplemented or not with lipids (2% SO, SO diet; 1% FO, FO diet; 2% SO plus 1% FO, SOFO diet; and 0% oil, Control diet; 2 lots/diet), for 21 days. Rumen contents were sampled and frozen at –80ºC for DNA analysis using the terminal restriction fragment length polymorphism technique (T-RFLP). Only the SOFO diet altered significantly the rumen bacterial communities, giving a lower proportion of a fragment compatible with bacteria of the order *Clostridiales* and a higher percentage of two fragments that match members of the Clostridial cluster IX (e.g. *Mitsuokella*). Changes in the rumen microbiota due to the supplementation with SO+FO might be partially responsible for variations in the milk FA profile.

**Keywords.** CLA – Lipid supplementation – Rumen microbiota – T-RFLP.

***Effet de la supplémentation de la ration avec de l'huile de tournesol et/ou de l'huile de poisson sur les communautés microbiennes du rumen chez les brebis laitières***

***Résumé.*** *Chez les ruminants, la supplémentation des rations avec des lipides riches en acide linoléique, tel que l'huile de tournesol (SO), et acides gras polyinsaturés à longue chaîne tel que l'huile de poisson (FO), permet d'augmenter la concentration de l'acide linoléique conjugué du lait. Le profil des acides gras (FA) du lait dépend pour partie des bio-hydrogénations des acides gras alimentaires dans le rumen par la communauté microbienne; celle-ci étant en conséquence, un des facteurs clé dans la détermination des teneurs des acides gras bénéfiques du lait. Pour étudier l'effet de la supplémentation de la ration avec SO et/ou FO sur le microbiote du rumen des brebis laitières, trente-deux brebis en lactation ont été divisées en huit lots. Les brebis ont reçu une ration très riche en concentré, supplémentée avec 2% SO (SO), 1% FO, (FO); 2% SO plus 1% FO (SOFO) ou sans l'addition d'huile (témoin) pendant 21 jours, à raison de 2 lots/traitement. Le contenu du rumen a été prélevé et congelé à –80ºC pour l'analyse d'ADN utilisant la technique du polymorphisme de longueurs des fragments de restriction terminaux (T-RFLP). Seulement le traitement de SOFO conduit à un changement significatif des communautés bactériennes du rumen, avec une proportion inférieure d'un fragment compatible avec des bactéries de l'ordre* Clostridiales *et un pourcentage plus élevé de deux fragments correspondant à des membres du cluster* Clostridial *IX (par exemple* Mitsuokella*) comparé aux traitements SO et témoin. En conclusion, les changements du microbiote de rumen dus à une supplémentation avec SO+FO pourraient être partiellement responsables des variations du profil en acides gras du lait.*

***Mots-clés.*** *CLA – Supplémentation lipidique – Microbiote du rumen – T-RFLP.*

# I – Introduction

Ruminant-derived products are known to make a major contribution to beneficial conjugated linoleic acids (CLA) in the human diet (Lawson *et al*., 2001). This is largely due to microbial biohydrogenation (BH) of dietary unsaturated fatty acids (FA) in the rumen, which results in the formation of intermediary metabolites, including health-promoting CLA, mainly rumenic acid (cis-9 trans-11 C18:2), and C18:1 fatty acids, mostly trans-11 (vaccenic acid; VA), a precursor for rumenic acid in the mammary gland and in human tissues (Palmquist *et al*., 2005). It is the microorganisms present in the rumen that carry out the BH, which has important implications for the FA composition of milk and meat, the rumen microbiota structure being therefore a key factor in improving milk FA profile.

Within the rumen microbial ecosystem, bacteria are the most relevant microorganisms in terms of biohydrogenating activity (Jenkins *et al*., 2008). Several researchers have confirmed the active role of the "*Butyrivibrio* group" that includes the genera *Butyrivibrio* and *Pseudobutyrivibrio* and the species *Clostridium proteoclasticum* in the rumen BH (Paillard *et al*., 2007; Jenkins *et al*., 2008). The latter species is one of the few strains known to be able to complete BH to stearic acid (SA, C18:0), together with closely related bacteria (Wallace *et al*., 2006), although recently Boeckaert *et al.* (2008) suggested that other, as yet-uncultivated bacteria, which cluster between the genus *Butyrivibrio* and the genus *Pseudobutyrivibrio*, are involved in C18:0 production. Microbial BH is considered as a detoxification mechanism, since unsaturated FA may prevent bacterial growth. However, differential toxicity of unsaturated FA to ruminal bacteria has been observed (Maia *et al*., 2007) and, among the hydrogenating bacteria, the C18:0 producers appear more vulnerable to inhibition by long chain polyunsaturated fatty acids (PUFA), such as those contained in fish oil. Nonetheless, the effect of different sources of lipids on the rumen microbiota and the relative importance of the known strains and other yet uncultivated bacteria in the ruminal lipid metabolism remain largely unclear. Although traditional culture-based studies have provided fundamental insight into the rumen microbiology, a large proportion of rumen bacteria has not yet been cultivated. Thus, molecular microbial technology based on 16S rRNA genes allows to investigate total eubacterial population changes using modern methodologies, such as the terminal restriction fragment length polymorphism (T-RFLP).

Supplementing ruminant diets with sunflower oil (SO), rich in linoleic acid (LA, a precursor for CLA synthesis), and fish oil (FO), which has high contents of long chain PUFA that inhibit the reduction of VA to SA, has been reported as a good nutritional strategy to improve milk FA profile (Shingfield *et al*., 2006). Therefore, the aim of this study was to investigate the effect of lipid supplementation of dairy ewe diet with sunflower and fish oils on the rumen bacterial communities using a culture-independent molecular technique, the T-RFLP.

# II – Material and methods

## 1. Experimental design and sampling

Thirty-two lactating ewes (86.5 ± 1.21 kg body weight) in mid-lactation were divided in eight lots and offered a high concentrate diet (forage:concentrate ratio, 20:80), supplemented or not with lipids (2% sunflower oil, SO diet; 1% fish oil, FO diet; 2% SO plus 1% FO, SOFO diet; and 0% oil, Control diet). Two lots of four animals were assigned to each diet and after a 3-week adaptation period consuming the control diet, the animals received the experimental ones for 21 days. Then rumen contents were individually sampled 3 hours after the morning feeding through a stomach tube. Samples were strained through two layers of muslin. Equal volumes from each animal were mixed to give approximately 10 ml for each lot and immediately frozen at –80ºC for microbial DNA extraction.

## 2. Analysis of microbial DNA

Stored rumen samples were freeze-dried and thoroughly mixed before DNA extraction, which was performed in duplicate from approximately 80 mg sample by physical disruption using a bead beater (Mini-bead Beater; BioSpec Products, Bartlesville, OK, USA), following Yu and Morrison (2004), with the modification that a higher temperature (95ºC) was used for lysis incubation. Duplicate DNA samples were combined and used as templates for terminal restriction fragment length polymorphism (T-RFLP) analysis, which was performed as follows. A near-full length fragment of the 16S rRNA gene was amplified by PCR using a *Bacteria*-specific primer pair set. The forward primer was labelled with 6-carboxy-fluorescein (FAM) and the fluorescently labelled PCR products were purified on a GE Healthcare PCR purification kit column (GE Healthcare Life Sciences, Buckinghamshire, UK), and eluted in a final volume of 35 µl of sterile milli-Q water. The resultant purified PCR products were digested with *HhaI* (10 IU) (Takara Bio Inc., Otsu, Shiga, Japan) in a total volume of 10 µl at 37ºC for 12 h. 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 550-R (GE Healthcare Life Sciences, Buckinghamshire, UK). Data were analyzed using the GeneMarker Analysis software (SoftGenetics, PA, USA). Sample data consisted of size (base pair, bp) and peak area for each T-RF and were standardized following Kitts (2001). Richness was considered as the number of fragments in each sample once standardized.

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 (http://rdp.cme.msu.edu/).

## 3. Calculations and statistical analysis

From the T-RFLP results, the proportions of each fragment over the total peak area were calculated. These data and the number of fragments were analysed by one-way ANOVA using the SAS software package version 9.1. (SAS Institute Inc., Cary, NC, USA). The peak areas obtained from T-RFLP were analysed using hierarchical clustering with the Ward's method and Euclidean distances to build a dendogram. This analysis was performed with the Community Analysis Package 4 software (Pisces Conservation Ltd., Lymington, Hampshire, UK).

# III – Results and discussion

The microbial DNA extracted from the rumen fluid was analysed using a culture-independent molecular technique, the T-RFLP, which allows to monitor changes in microbial community structures based on variations in the 16S rRNA gene. The areas obtained from each fragment were standardized and used to build the dendogram presented in Fig. 1. Cluster analysis showed a higher similarity between the DNA obtained from the animals offered the SOFO diet than with the rest of the samples (i.e.,derived from the sheep fed the control, SO or FO diets). These results seem to indicate that only supplementing both lipids together (SO at 2% plus FO at 1%) changed substantially the microbial communities in the rumen. The diets with only one lipid source (SO or FO) did not seem to alter considerably the rumen microbiota, which may be due to the lower percentage of lipid addition compared to the SOFO diet. This agrees with a previous study in steers where different dietary FO inclusions (1 and 3% of dry matter intake) were investigated and major changes in the rumen bacterial communities were only observed with the 3% FO supplementation (Kim *et al*., 2008).

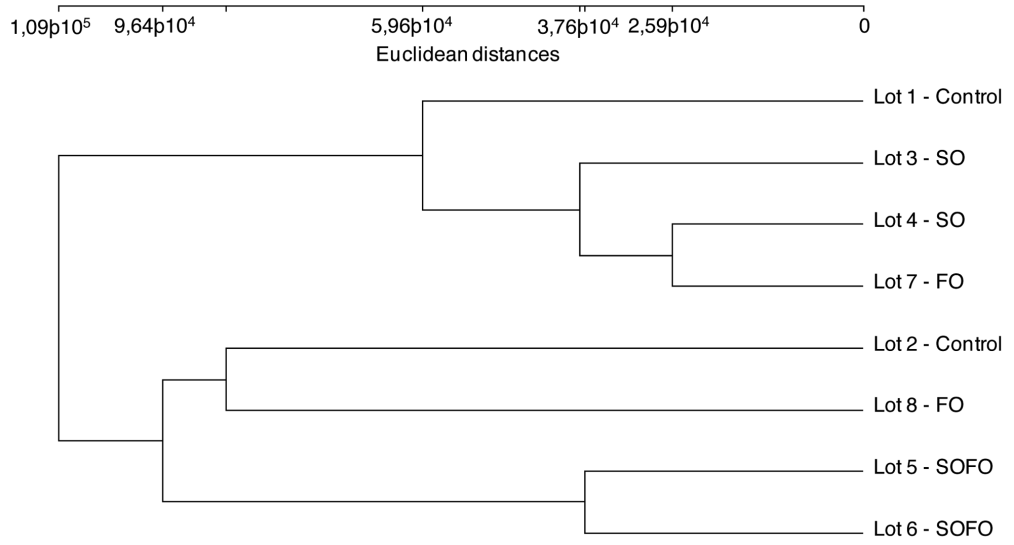


**Fig. 1. Cluster analysis of the T-RFLP profiles of total bacteria present in the rumen fluid of dairy sheep fed a high concentrate diet supplemented with sunflower oil (2%; SO), fish oil (1%; FO), both sunflower and fish oils (3%; SOFO) or 0% oil (Control).**

The number of fragments detected by T-RFLP was not different among diets, the average value being 46.5 ± 2.14. Unlike our results, Kim *et al*. (2008) observed a reduction in the number of bands obtained by another culture-independent molecular technique, the denaturing gradient gel electrophoresis, in steers fed a 3% FO supplemented diet. Fish oil contains a high concentration of long-chain PUFA (basically C20:5 n-3 and C22:6 n-3), which are more toxic to bacteria than the LA (Maia *et al*., 2007), present in the SO. Then, our 3% lipid supplemented diet (SOFO diet) should be less toxic to rumen bacteria than the diet supplemented with 3% FO used by Kim *et al*. (2008), and this might explain our lack of effect on the number of T-RF.

In Table 1 the most relevant fragments detected by T-RFLP are presented with their compatible bacteria, whose potential identity has been inferred using databases of the 16S rRNA gene sequences (Ribosomal Database Project). However, we should point out that different organisms can share similar or identically-sized T-RF and then caution should be taken in the interpretation of the results.

We found that a fragment compatible with bacteria belonging to the phylum *Bacteroidetes* (100 bp), one of the most important microbial groups in the rumen (Edwards *et al*., 2004), was the most abundant (up to 46%), although its proportion was not affected by the experimental treatment (Table 1). The 98 bp fragment showed also a remarkable proportion (up to 26 %) and was affected by the lipid supplementation, being highest with the SOFO diet. The diet supplemented with FO gave a significantly higher proportion than the control diet too, whereas the value obtained with the SO diet was not different from either the FO or the control diets. This 98 bp T-RF might also be compatible with members of *Bacteroidetes*, which have not been reported to date to be involved in the metabolism of unsaturated FA (Maia *et al*., 2007). Alternatively, the 98 bp T-RF may also match some uncultured bacteria belonging to the Clostridial cluster IX, which are related to members of the genus *Mitsuokella*, some of which have been proved to be able to metabolize unsaturated fatty acids (Maia *et al*., 2007). Cluster IX bacteria are also compatible

with the 389 bp T-RF, whose proportion was also significantly higher when the animals received both sources of lipid together (SOFO diet; P<0.05). In animals fed the SO or FO diets this proportion seemed numerically greater than with the control diet too, although again the lower inclusion of lipid might explain the lack of significant effect.

An effect of the experimental treatment was also observed in a different *Bacteroidetes*-compatible T-RF (102 bp) that was strongly reduced with lipid supplementation. Some relevant *Prevotella* spp. from the rumen would match this fragment. Although *in vitro* experiments showed that members of this genus were insensitive to the unsaturated FA (Maia *et al*., 2007), the cultivated strains may represent only a minor proportion of the genus *Prevotella* in the rumen (Stevenson and Weimer, 2007).

The proportion of another interesting fragment, the 65 bp T-RF, tended to be altered by the experimental treatment, being lower with the SOFO diet compared to the control and SO diets. The value obtained with the FO diet did not differ significantly from any of the other diets, although it was numerically lower than with the control and SO diets. Uncultured bacteria belonging to the order *Clostridiales*, which is usually an important rumen bacterial group, may match this fragment. The order *Clostridiales* contains the family *Lachnospiraceae*, which includes bacteria that are inhibited by the long chain PUFA-rich sources, such as fish oil, and have been suggested to be linked to SA production: the *Clostridium proteoclasticum* group (Maia *et al*., 2007) and other related but yet uncultivated microorganisms (Boeckaert *et al*., 2008). Thus, the bacteria compatible with this 65 bp T-RF might be, at least partially, responsible for variations in the ewe milk FA profile, such as the reduction in SA content, when diets were supplemented with FO (Toral *et al*., 2010).

**Table 1. Abundances, expressed in percentages over the total peak area, of several fragments (length in base pairs, bp) obtained by T-RFLP analysis of microbial DNA samples extracted from the rumen fluid of dairy sheep fed a high concentrate diet supplemented with sunflower oil (2%; SO), fish oil (1%; FO), both sunflower oil and fish oils (3%; SOFO) or 0% oil (Control)**

| Length (bp) | Control | SO | FO | SOFO | SEM† | P†† | Compatible bacteria |
|---|---|---|---|---|---|---|---|
| 65 | 2.5 | 2.2 | 1.8 | 1.1 | 0.25 | 0.06 | *Clostridiales* |
| 98 | $10.1^{c}$ | $13.7^{bc}$ | $19.0^{ab}$ | $26.2^{a}$ | 2.23 | 0.03 | *Bacteroidetes* / Cluster IX |
| 100 | 41.9 | 46.5 | 43.6 | 32.1 | 7.99 | NS | *Bacteroidetes* |
| 102 | $5.9^{a}$ | $0.8^{b}$ | $0.5^{b}$ | $0.0^{b}$ | 0.54 | 0.004 | *Bacteroidetes* (*Prevotella*) |
| 191 | 11.7 | 4.0 | 2.6 | 2.2 | 3.07 | NS | *Butyrivibrio*, *Pseudobutyrivibrio* |
| 389 | $0.4^{b}$ | $1.1^{b}$ | $1.8^{b}$ | $7.5^{a}$ | 1.10 | 0.03 | Cluster IX (*Mitsuokella*) |

† Standard error of the means.
†† Statistical significance. NS, non-significant (P>0.1).
Values in a row with different superscript letters are significantly different (P<0.05).

Regarding the known hydrogenating bacteria belonging to the genus *Butyrivibrio* and *Pseudobutyrivibrio* (Jenkins *et al*., 2008), the occurrence of most of them would result in the appearance of a 191 bp T-RF, whose proportion showed a high between-lot variability (CV = 104%). Although variations within this group cannot be discarded, our results did not show any significant changes due to the lipid addition.

## IV – Conclusion

In dairy ewes the dietary supplementation with a combination of SO (2%) and FO (1%) altered the rumen microbiota, as reflected in the microbial T-RFLP profiles. These changes might be responsible at least partially for the variations observed in the milk FA profile, such as the reduction of

the SA content (Toral *et al*., 2010). The decline in a *Clostridiales*-compatible T-RF and the increase in peaks that may potentially match cluster IX bacteria seemed to be also numerically induced by the diets supplemented with only one source of lipid, either SO or FO, although the lower total oil content of these diets might have prevented the variations to be statistically significant.

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## References

**Boeckaert C., Vlaeminck B., Fievez V., Maignien L., Dijkstra J. and Boon N., 2008.** Accumulation of trans C18:1 fatty acids in the rumen after dietary algal supplementation is associated with changes in the Butyrivibrio community. In: *Appl. Environ. Microbiol.*, 74, p. 6923-6930.

**Edwards J.E., McEwan N.R., Travis A.J. and Wallace R.J., 2004.** 16S rDNA library-based analysis of ruminal bacterial diversity. In: *Ant. van Leeuw.*, 86, p. 263-281.

**Jenkins T.C., Wallace R.J., Moate P.J and Mosley E.E., 2008.** Recent advances in biohydrogenation of unsaturated fatty acids within the rumen microbial ecosystem. In: *J. Anim. Sci.*, 86, p. 397-412.

**Kim E.J., Huws S.A., Lee M.R.F., Wood J.D., Muetzel S.M., Wallace R.J. and Scollan N.D., 2008.** Fish oil increases the duodenal flow of long chain polyunsaturated fatty acids and trans-11 18:1 and decreases 18:0 in steers via changes in the rumen bacterial community. In: *J. Nutr.*, 138, p. 889-896.

**Kitts C.L., 2001.** Terminal restriction fragment patterns: a tool for comparing microbial communities and assessing community dynamics. In: *Curr. Issues Intest. Microbiol.*, 2, p. 17-25.

**Lawson R.E., Moss A.R. and Givens D.I., 2001.** The role of dairy products in supplying conjugated linoleic acid to man's diet: a review. In: *Nutr. Res. Rev.*, 14, p. 153-172.

**Maia M.R.G., Chaudhary L.C., Figueres L. and Wallace R.J., 2007.** Metabolism of polyunsaturated fatty acids and their toxicity to the microflora of the rumen. In: *Ant. van Leeuw.*, 91, p. 303-314.

**Paillard D., McKain N., Chaudhary L.C., Walker N.D., Pizette F., Koppova I., McEwan N.R., Kopecny J., Vercoe P.E., Louis P. and Wallace R.J., 2007.** Relation between phylogenetic position, lipid metabolism and butyrate production by different *Butyrivibrio*-like bacteria from the rumen. In: *Ant. van Leeuw.,* 91, p. 417-422.

**Palmquist D.L., Lock A.L., Shingfield K.J. and Bauman D.E., 2005.** Biosynthesis of conjugated linoleic acid in ruminants and humans. In: *Adv. Food Nutr. Res.*, 50, p. 179-216.

**Shingfield K.J., Reynolds C.K., Hervás G., Griinari J.M., Grandison A.S. and Beever D.E., 2006.** Examination of the persistency of milk fatty acid composition responses to fish oil and sunflower oil in the diet of dairy cows. In: *J. Dairy Sci.*, 89, p. 714-732.

**Stevenson D.M. and Weimer P.J., 2007**. Dominance of *Prevotella* and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR. In: *Appl. Microbiol. Biotenchnol.*, 75, p. 185-174.

**Toral P.G., Frutos P., Hervás G., Gómez-Cortés P., Juárez M. and de la Fuente M.A., 2010.** 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. In: *J. Dairy Sci.*, 93, p. 1604-1615.

**Wallace R.J., Chaudhary L.C., McKain N., McEwan N.R., Richardson A.J., Vercoe P.E., Walker N.D., and Paillard D., 2006.** *Clostridium proteoclasticum:* a ruminal bacterium that forms stearic acid from linoleic acid. In: *FEMS Microbiol. Lett.*, 265, p. 195-201.

**Yu Z. and Morrison M., 2004.** Improved extraction of PCR-quality community DNA from digesta and fecal samples. In: *BioTechniques*, 36, p. 808-812.