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Changes in the rumen *Butyrivibrio* group in lactating ewes fed a diet supplemented with sunflower oil with or without marine algae

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Abstract. In ruminants, microbial biohydrogenation (BH) of unsaturated fatty acids (FA) can be modulated by diet supplementation with lipids (e.g., sunflower oil -SO- or marine algae -MA-) to improve the fatty acid profile of the milk. The *Butyrivibrio* group contains the most active biohydrogenating bacteria isolated from the rumen and, for this reason, it has been extensively considered the main responsible for the BH process. With the aim of examining the effect of lipid addition on the *Butyrivibrio* population, as well as time-dependent variations, thirty-six lactating ewes were divided in 6 lots (3 lots/treatment) and offered a diet supplemented with either 2.5% SO or 2.5% SO plus 0.8% MA. After 0, 26 and 52 days on treatments, individual samples of rumen fluid were collected through a stomach tube, composited for each lot, and analysed using the terminal restriction fragment length polymorphism (T-RFLP) molecular technique. Results showed no significant variations, due to either diet or time, in the *Butyrivibrio* T-RFLP profiles or in the relative abundances of the major terminal restriction fragments (T-RF) detected. However, some less abundant fragments (i.e., representing less than 4.3% of the total) varied significantly. For example, the frequency of a T-RF compatible with 18:0-producing bacteria increased on day 52 in the diet with only SO (from 0.4 to 4.3%), whereas MA addition precluded this effect. The few changes caused in the *Butyrivibrio* group by a lipid supplementation that is known to alter rumen BH would indicate a low relevance of these bacteria in the ruminal FA metabolism in dairy sheep. Nevertheless, the effect on some small subpopulations would not allow to rule out their involvement in the process.

Keywords. Lipid supplementation – Ruminal bacteria – Biohydrogenation – T-RFLP.

Modifications du groupe* Butyrivibrio *dans le rumen de brebis laitières alimentées avec un régime supplémenté en huile de tournesol en présence ou en absence d'algues marines

Résumé. *Chez le ruminant, la biohydrogénation (BH) microbienne des acides gras (AG) insaturés peut être modulée par des régimes supplémentés en lipides (par exemple en huile de tournesol -HT- ou en algues marines -AM-) pour améliorer le profil en AG du lait. Le groupe* Butyrivibrio*, auquel appartiennent les bactéries les plus actives impliquées dans la BH ruminale, a été considéré comme le principal responsable de ce processus. Afin d'étudier les effets de régimes supplémentés en lipides sur l'évolution au cours du temps de la population* Butyrivibrio*, 36 brebis laitières ont été réparties en 6 lots et ont reçu un régime expérimental supplémenté avec 2,5% de HT ou avec 2,5% de HT et 0,8% de AM (3 lots par traitement). Après 0, 26 et 52 jours de traitement, des échantillons individuels de liquide ruminal ont été prélevés par voie œsophagienne, puis mélangés pour réaliser un échantillon représentatif par lot, et analysés en utilisant la technique moléculaire d'étude du polymorphisme de longueur des fragments terminaux de restriction (T-RFLP). Les résultats montrent qu'il n'y a pas de variations significatives des profils T-RFLP de* Butyrivibrio *ou dans l'abondance relative des principaux fragments terminaux de restriction (T-RF) en fonction de la nature de la ration ou du temps de prélèvement. Cependant, quelques fragments de faible abondance (moins de 4% du total) varient de manière significative. Par exemple, la fréquence d'un T-RF liée aux bactéries produisant le 18:0 a augmenté avec le régime HT après 52 jours de traitement, alors que le régime AM n'a produit aucun effet. Ces premiers résultats semblent donc montrer que les bactéries du groupe* Butyrivibrio *sont peu impliquées dans la BH ruminale chez des brebis laitières nourries avec des régimes supplémentés en lipides. Cependant, les quelques modifications observées sur de petites sous-populations bactériennes ne peuvent pas exclure complètement leurs implications dans le processus de BH.*

Mots-clés. *Supplémentation lipidique – Bactéries du rumen – Biohydrogénation – T-RFLP.*

I – Introduction

In ruminants, dietary lipid supplementation with sunflower oil (SO) in combination with marine lipids, such as marine algae (MA), has been reported to increase the milk concentration of potentially beneficial bioactive lipids (Shingfield *et al*., 2006; Toral *et al*., 2010). This effect has been related to diet-induced alterations in ruminal biohydrogenation (BH) of unsaturated fatty acids (FA), which are mediated by rumen microorganisms. The *Butyrivibrio* group, which includes the genera *Butyrivibrio* and *Pseudobutyrivibrio* as well as phylogenetically related microbes, contains the most active biohydrogenating bacteria isolated from the rumen. For this reason, it has been extensively considered to be the main responsible for the BH process (Lourenço *et al*., 2010). However, studies using culture-independent molecular methods have suggested that identified biohydrogenating bacteria, such as the 18:0-producing *B. proteoclasticus*, may not play a major role in this process, whereas other yet-uncultured bacteria, within the *Butyrivibrio* group, might be more relevant (Boeckaert *et al*., 2008; Belenguer *et al*., 2010; Toral *et al*., 2012).

Results on the milk FA profile in lactating ewes supplemented with SO alone or in combination with marine lipids (Toral *et al*., 2010) suggest long-term variations in the rumen biohydrogenating bacteria. Despite that, information on the *Butyrivibrio* group is limited to a relatively short period (up to 28 days on supplemented diets; Belenguer *et al*., 2010; Toral *et al*., 2012). Therefore, the aim of this study was to investigate the effect of the addition of SO and MA to the diet of dairy ewes on the ruminal *Butyrivibrio* group, as well as time-dependent variations over an extended period, using a culture-independent molecular technique.

II – Materials and methods

Thirty-six Assaf ewes (82.4 ± 5.30 kg body weight; mean ± SD) in mid lactation were randomly distributed in 6 lots (6 ewes/lot) and assigned to one of 2 dietary treatments (3 lots/treatment). Diets consisted of a total mixed ration based on alfalfa hay and a concentrate (forage: concentrate ratio 40:60) and supplemented with either 2.5% SO (Control diet) or 2.5% SO plus 0.8% MA (SOMA diet; DHA Gold Animal Feed Ingredient, Martek Biosciences Corp., Columbia, MD, USA). All ewes were fed the control diet for a 20-day adaptation period before the start of the study. Fresh diets were offered daily *ad libitum* at 09:00 and 19:00 h and clean water was always available. After 0, 26 and 52 days on treatments, samples of ruminal fluid were individually collected 3 h after the morning feeding using a stomach tube. Samples were strained through 2 layers of muslin, composited for each lot, and immediately frozen at –80°C for DNA extraction.

After thorough mixing, DNA was extracted from freeze-dried samples of rumen fluid as described in Belenguer *et al*. (2010). Duplicate DNA samples were used as templates for terminal restriction fragment length polymorphism (T-RFLP) analysis, which was based on *Butyrivibrio* group-specific primers (Boeckaert *et al*., 2008) and one restriction enzyme (*HhaI*; Belenguer *et al*., 2010). The lengths of the fluorescently labelled terminal restriction fragments (T-RF) were determined using the size standard ET-550-R (GE Healthcare Life Sciences, Buckinghamshire, UK) with the GeneMarker Analysis software (SoftGenetics, State College, PA, USA).

To infer the potential phylogenetic bacterial assignment of the fragments, *in silico* restriction for the *Butyrivibrio* group bacteria with the primers and enzyme used in the analysis were obtained from the Ribosomal Database Project II (http://rdp.cme.msu.edu/; Cole *et al*., 2009).

Data from T-RFLP (size in base pairs -bp- and peak area for each T-RF) were analyzed as outlined by Abdo *et al*. (2006), and the number of T-RF and the relative abundances over the total peak area of each T-RF were calculated. Hierarchical clustering analysis with the Ward´s method based on Jaccard distances was performed, using R-project software (R Development Core Team, 2011), to build a dendrogram.

For each T-RF, the relative abundance was analysed by repeated measures, using the MIXED procedure of the SAS software package, version 9.2 (SAS Inst. Inc., Cary, NC, USA). The statistical model included the fixed effects of treatment, day of sampling and their interaction, and the lot as a random effect. Means were separated using the "pdiff" option of the "lsmeans" statement. Significant differences were declared at $P < 0.05$ and tendencies at $P<0.10$.

III – Results and discussion

As plotted in the dendrogram (Fig. 1), results showed no significant variations, due to either diet or time, in the bacterial profiles, which seems to indicate that the addition of MA to a SO-containing diet did not alter noticeably the structure of the rumen *Butyrivibrio* group after 26 or 52 days on treatments. Although similar results were reported for lactating ewes fed fish oil (Belenguer *et al*., 2010), a certain separation of the *Butyrivibrio* group profiles was observed in sheep receiving the same diets as in this study, even though the effect was less clear on this group than on total bacteria (Toral *et al*., 2012).

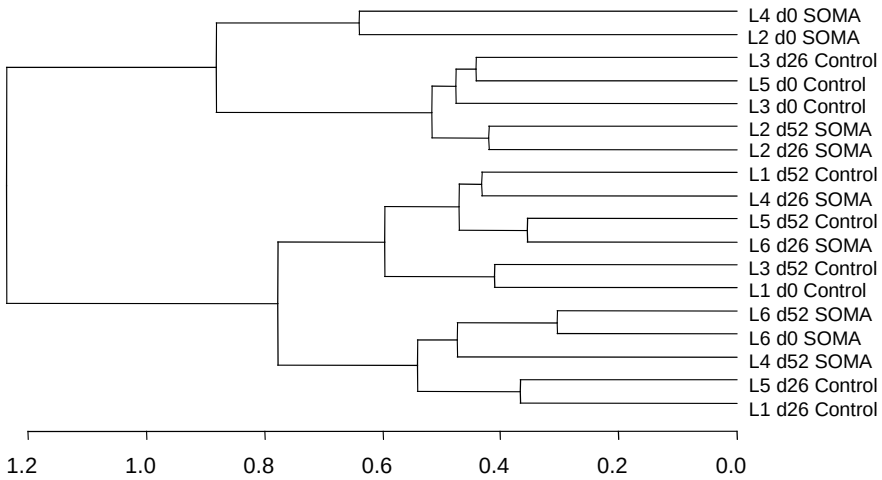

Fig. 1. Terminal restriction fragment length polymorphism (T-RFLP) derived dendrogram showing the relationships among the profiles of the *Butyrivibrio* group. The DNA was extracted from rumen fluid of lactating ewes after 0 (d0), 26 (d26) and 52 (d52) days on a diet supplemented with 2.5% SO (Control) or 2.5% SO plus 0.8% MA (SOMA) (L = lot).

The number of T-RF averaged 29.6 ± 5.75, which is in line with previous studies in sheep fed marine lipids (Belenguer *et al*., 2010; Toral *et al*., 2012), and was only reduced significantly after 52 days of MA supplementation ($P<0.05$). This is consistent with the lack of effects of fish oil (Belenguer *et al*., 2010) or MA (Toral *et al*., 2012) supplementation on this parameter observed for shorter periods of time (up to 28 days).

Samples were composited per lot to reduce the high inter-animal variability described in the rumen bacterial composition (Li *et al*., 2009) and favour the detection of significant effects. Nonetheless, the between-lot variation was still considerable, and there were no modifications, due to diet or time, in the relative abundance of the major T-RF detected (namely, those of 149, 162 and 405 bp). The 162-bp fragment may correspond to bacteria phylogenetically related to 18:0-producers (Boeckaert *et al*., 2009) and the 405-bp T-RF is compatible with uncultured *Lachnospiraceae* bacteria potentially involved in BH (Boeckaert *et al*., 2009). The same lack of

changes in the relative abundance was found for other fragments, such as the 164 and 300 bp T-RF, which are compatible with potentially biohydrogenating *Lachnospiraceae* bacteria (Boeckaert *et al*., 2009). Although this seems to contrast with previous studies showing irregular variations in these fragments (Belenguer *et al*., 2010; Toral *et al*., 2012), it is probably noteworthy that apparently inconsistent changes may be accounted for by different bacteria, with different sensitivity to polyunsaturated FA (Maia *et al*., 2007), resulting in the same fragment size.

Table 1. Number of fragments (T-RF) and relative abundance over the total peak area (%) of several T-RF obtained by T-RFLP in rumen fluid samples of lactating ewes fed a diet supplemented with 2.5% SO (Control) or 2.5% SO plus 0.8% MA (SOMA), on days 0, 26 and 52.

	Control			SOMA			SED[2]	P[1]		
	0	26	52	0	26	52		T	D	T×D
Number of T-RF	28.3	29.0	32.3	33.3[a]	28.0[ab]	26.3[b]	6.49	0.85	0.63	0.02
Length (bp)										
149	31.16	30.16	32.31	30.66	24.88	29.45	7.430	0.32	0.54	0.79
159	0.00[b]	0.15[b]	0.66[a]	0.10	0.28	0.11	0.160	0.45	0.01	<0.01
161	0.44[b]	0.32[b]	4.26[a]	1.40	1.88	2.02	1.024	0.92	<0.01	0.01
162	16.86	10.65	9.53	15.39	11.05	8.54	4.217	0.65	0.13	0.95
164	3.17	3.06	3.90	4.41	1.90	2.43	1.369	0.57	0.43	0.34
300	5.82	5.41	5.42	3.62	5.11	4.45	1.866	0.49	0.79	0.55
405	19.57	22.09	20.16	20.62	16.39	27.51	5.185	0.77	0.44	0.25

[a,b] For each treatment, means within a raw with different superscripts differ significantly ($P < 0.05$). [1] Probability of significant effects due to experimental treatment (T) or day (D). [2] SED = standard error of the difference.

On the other hand, less abundant fragments (representing less than 4.3% of the total) varied significantly over time. For example, the frequency of the T-RF of 161 bp increased on day 52 in the diet with only SO (from 0.4 to 4.3%), whereas MA addition precluded this effect. This T-RF might be compatible with a strain of the 18:0-producing *B. proteoclasticus*, although in previous experiments with sheep (Belenguer *et al*., 2010; Toral *et al*., 2012) and cattle (Huws *et al*., 2011) no changes in this group, due to lipid supplementation, were observed using real time PCR. Nevertheless, as mentioned above, other unclassified bacteria of *Lachnospiraceae* might also match the same T-RF.

IV – Conclusion

Overall, the few changes caused in the *Butyrivibrio* group by a lipid supplementation (sunflower oil + marine algae) that is known to alter rumen BH would indicate a low relevance of these bacteria in the ruminal FA metabolism in dairy sheep. However, some small subpopulations were affected by the addition of MA, which would not allow to rule out their involvement in the process.

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