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Effects of forage type on diversity in bacterial pellets isolated from liquid and solid phases of the rumen content in sheep and goats

L. Abecia*, E. Molina-Alcaide*, G. Cantalapiedra-Hijar*, E. C. Soto* and M.D. Carro**

*Estación Experimental delZaidín (CSIC), ProfesorAlbareda 1, 18008 Granada (Spain)
**Departamento de Producción Animal, E.T.S.I. Agrónomos, Universidad Politécnica de Madrid, Ciudad Universitaria, 28040 Madrid (Spain)

Abstract. Two sheep and two goats, fitted with a ruminal cannula, received two diets composed of 30% concentrate and 70% of either alfalfa hay (AL) or grass hay (GR) as forage in a two-period crossover design. Solid and liquid phases of the rumen were sampled from each animal immediately before feeding and 4 h post-feeding. Pellets containing solid associated bacteria (SAB) and liquid associated bacteria (LAB) were isolated from the corresponding ruminal phase and composited by time to obtain 2 pellets per animal (one SAB and one LAB) before DNA extraction. Denaturing gradient gel electrophoresis (DGGE) analysis of *16S* ribosomal DNA was used to analyze bacterial diversity. A total of 78 and 77 bands were detected in the DGGE gel from sheep and goats samples, respectively. There were 18 bands only found in the pellets from sheep fed AL-fed sheep and 7 found exclusively in samples from sheep fed the GR diet. In goats, 21 bands were found only in animals fed the AL diet and 17 were found exclusively in GR-fed ones. In all animals, feeding AL diet tended ($P < 0.10$) to promote greater NB and SI in LAB and SAB pellets compared with the GR diet. The dendrogram generated by the cluster analysis showed that in both animal species all samples can be included in two major clusters. The four SAB pellets within each animal species clustered together and the four LAB pellets grouped in a different cluster. Moreover, SAB and LAB clusters contained two clear subclusters according to forage type. Results show that in all animals bacterial diversity was more markedly affected by the ruminal phase (solid *vs.* liquid) than by the type of forage in the diet.

Keywords. DGGE – Forage – Goats – Ruminal bacteria – Sheep.

Effets du type de fourrage sur la diversité bactérienne mesurée sur des pellets obtenus à partir des phases liquide et solide du contenu de rumen chez des ovins et des caprins

Resumé. *L'objectif de cette étude était d'analyser la diversité des communautés bactériennes à partir d'extraits isolés dans les phases liquide (LAB) et solide (SAB) du contenu de rumen d'ovins et de caprins nourris avec des régimes différant par le type de fourrage. Les deux régimes expérimentaux consistaient (base matière sèche) en 30% de concentré et 70% de foin de luzerne (AL) ou de foin de graminées (GR). Deux brebis et deux chèvres, porteuses de canule ruminale, ont reçu le régime alimentaire selon un schéma en cross-over avec deux périodes. Les bactéries SAB et LAB ont été isolées à partir de chaque animal immédiatement avant la distribution de l'alimentation (0 h) ou 4 h après cette distribution. A chaque période, les extraits bactériens ont été échantillonnés afin d'avoir 2 extraits bactériens par animal (un SAB et LAB) avant extraction de l'ADN. L'analyse de l'ADN ribosomal 16S par la technique d'electrophorèse sur gel en gradient dénaturant (DGGE) a été utilisée pour établir la diversité bactérienne. Deux gels DGGE différents ont été réalisés, l'un pour les échantillons des brebis et l'autre pour ceux des chèvres. Au total, 78 et 77 bandes ont été détectées dans le gel des échantillons, respectivement, de brebis et de chèvres. Parmi elles, 18 bandes ne se sont retrouvées que chez les brebis recevant le régime AL, et 7, que dans des échantillons provenant de brebis nourries avec le régime GR. Pour les caprins, 21 bandes ne se trouvaient que chez les animaux nourris avec le régime AL et 17 ont été trouvées exclusivement chez les animaux recevant le régime GR. Il n'y a eu aucune interaction espèce animale x régime alimentaire ($p > 0,05$), que ce soit dans le nombre de bandes (NB) ou pour l'indice de Shannon (IS), ce qui indique que les populations bactériennes des deux espèces animales ont répondu de la même façon aux changements liés au type de fourrage. Il n'y a eu aucune différence ($P > 0,05$) entre les deux espèces animales pour le nombre ou l'indice de Shannon pour les extraits SAB, mais les extraits LAB des brebis avaient un nombre plus élevé ($P < 0,05$) de bandes et un index SI supérieur aux chèvres. Chez tous les animaux, la distribution du régime AL tend ($P < 0,10$) à augmenter NB et SI pour les extraits LAB et SAB par rapport à celle du régime GR. Le dendrogramme généré par l'analyse typologique a montré que, chez les deux espèces animales, tous les*

échantillons peuvent être inclus dans deux grands clusters. Les quatre extraits SAB se sont regroupés dans un cluster différant de celui regroupant les quatre extraits LAB. Par ailleurs, les clusters SAB et LAB contenaient deux sous-groupes bien différenciés correspondant au type de fourrage. Les résultats montrent que, chez tous les animaux, la diversité bactérienne a été plus influencée par l'extrait bactérien analysé (LAB ou SAB) que par le type de fourrage dans le régime.

Mots-clés. *DGGE – Fourrage – Caprin – Bactéries du rumen – Ovin.*

I – Introduction

Differences in chemical composition and metabolic functions between bacteria isolated from the liquid (LAB) and solid (SAB) phases of the rumen are widely demonstrated (Carro and Miller, 2002; Ipharraguerre *et al.*, 2007; Molina-Alcaide *et al.*, 2009), but differences in the bacterial communities isolated in LAB and SAB pellets have received relatively little attention. Additionally, composition of bacterial communities in the rumen cannot be studied with traditional cultivation techniques. In the last years, molecular fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), single strand conformation polymorphism (SSCP) or automated ribosomal intergenic spacer analysis (ARISA) have been used to assess the diversity of ruminal bacterial communities. Some studies have analyzed the changes in bacterial diversity promoted by diet in cattle (Tajima *et al.*, 2001; Welkie *et al.*, 2010; Weimer *et al.*, 2010) and sheep (Kocherginskaya *et al.*, 2001; Martinez *et al.*, 2010), but studies in goats are very scarce (Shi *et al.*, 2007; Cunha *et al.*, 2011). The purpose of this study was to analyze the changes in bacterial diversity in liquid and solid phases of the rumen of sheep and goats fed diets differing in forage type. The DGGE technique was selected for this study because it has been shown to be a powerful tool for profiling diversity of microbial communities in the gastrointestinal tract of different animal species (Simpson *et al.*, 2002). To our knowledge this is the first comparative study to examine bacterial diversity in sheep and goats fed the same diets, although Belenguer *et al.* (2011) have previously analyzed the structure of rumen bacterial community in sheep, goats and cows grazing in the same plot.

II – Materials and methods

1. Diets, animals and experimental design

This study was conducted with an experiment investigating the nutrient utilization and N balance in sheep and goats fed different diets (Carro *et al.,* 2012). The two experimental diets contained 30% concentrate (dry matter (DM) basis) and 70% of either alfalfa hay (AL) or grass hay (GR) as forage. The concentrate was based on barley, gluten feed, wheat middlings, soybean meal, palmkern meal, wheat, corn and mineral-vitamin premix in the proportions of 215, 204, 200, 135, 115, 50, 50 and 31 g/kg, respectively (fresh matter basis). Crude protein content was 186 and 121 g/kg DM for AL and GR diet, respectively, and neutral-detergent fibre content was 426 and 499 g/kg DM, respectively.

Two Murciano-Granadina goats (44.5 ± 1.00 kg body weight (BW)) and 2 Merino sheep (55.1 ± 2.90 kg BW) were chosen to investigate changes in rumen bacterial diversity promoted by the experimental diets. Animals fitted with ruminal cannulas were used in a trial with 2 periods of 25 days each. In each period, one sheep and one goat were fed AL diet and the other 2 animals were fed the GR diet. Animals were housed in individual pens and had continuous access to fresh water and vitamin/mineral blocks over the experimental period. Animals were cared and handled in accordance with the Spanish Animal Care Regulations (Royal Decree 1201/2005 of October 10th on the protection of animals used for experimentation or other scientific purposes). Diets were offered to the animals twice daily (08:00 and 14:00 h) at a daily rate of 56 g DM/kg

$BW^{0.75}$ to minimize feed selection. On days 23 and 25 of each period, rumen contents (600 g) were withdrawn from each animal at 0 and 4 h after the morning feeding. Rumen contents were squeezed through 4 layers of cheesecloth and the solid digesta was combined with an equal volume of saline solution (0.9% NaCl) at 39ºC, mixed gently, and squeezed again to remove residual non-attached bacteria. The filtrate obtained at each sampling time was kept at 4ºC, pooled per animal(150 mL from each sampling time), and used to isolate liquid-associated bacteria (LAB) by differential centrifugation (Ranilla and Carro, 2003). The solid digestawastreated with saline solution (0.9% NaCl) containing 0.1% methylcellulose as described by Ranilla and Carro (2003) before isolation of solid-associated bacteria (SAB). Bacterial pellets (LAB and SAB) were lyophilized and ground to a fine powder with a mortar and pestle. Bacterial pellets were composited to have 2 pellets (one SAB and one LAB) per animal and period before DNA extraction. In addition, pH of the fluid was immediately measured, and 5 mL of fluid were added to 5 mL of deproteinizing solution (100 g of metaphosphoric acid and 0.6 g of crotonic acid per L) for volatile fatty acid analysis. Samples were analyzed as described by Carro *et al.* (2012).

2. DNA extraction and DGGE analyses

Samples (40 mg DM) of freeze-dried LAB and SAB were homogenized with steel beads in a Mini-Bead-beater (BioSpecInc, Bartlesville, OK, USA). DNA was extracted from homogenized samples with the QIAmp® DNA Stool Mini Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer´s instructions. Purity and yield of the extracted DNA were assessed using a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Denaturing gradient gel electrophoresis (DGGE) was used to investigate the effects of experimental diets on bacterial diversity. The V3 region of the *16S rRNA* gene was amplified from the extracted DNA by PCR using the bacterial primers 338f forward 5´-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3´ and 534r reverse 5´- ATTACC GCG GCT GCT GG-30 (Muyzer *et al.*, 1993). The PCR amplification was performed using the following steps: one cycle (94 ºC for 4 min), 30 cycles (94 ºC for 1 min, 55 ºC for 1 min, 72 ºC for 1 min), and one cycle (72 ºC for 7 min). The PCR reaction solution contained 50 ng DNA in a 50 µL mix containing 1 mM buffer, 1.25 mM of each primer, 0.8mM of dNTPs, 2.5 mM $MgCl_2$ and 2.5U of Taq DNA polymerase in 10 mMTrisHCl (pH 9.0). The resulting amplicons were visualized on a 2% (w/v) TBE (89mM Tris, 89mM Boric acid, 2mM Na2EDTA; pH 8.3) agarose gel stained with GelRed.

The DGGE was performed using a BDH system from VWR International Ltd (UK), following the manufacturer's guidelines. The PCR products (10 µl) were loaded onto 8% (w/v) TAE polyacrylamide gels (40 mmol /L of Tris base, 20 mmol/L of acetic acid and 1 mmol/L of EDTA, pH 8.3), which contained a 40–60% denaturant gradient (100% denaturant, 7 mol/L of urea and 40% (v/v) of deionized formamide). Electrophoresis was performed at a constant voltage of 100 V and temperature of 60ºC for 16 h. The DNA was visualised by silver staining with a Bio-Rad Silver stain kit, and scanned DGGE images were analysed with the Quantity One Software (BioRad, Madrid, Spain). Each band position present in the gel was binary coded for its presence or absence within a lane and each lane was compared by using a similarity matrix. Two different DGGE gels were run, one including samples from sheep and another with samples from goats. DGGE profiles within the same gel were compared by using Dice coefficient and the unweighted pair group method with arithmetic averages (UPGMA) clustering algorithm, and shown graphically as a dendrogram. The richness of the bacterial community was determined from the number of bands (NB) in each lane. The Shannon index (SI), a measure of diversity, was calculated by the following equation $H = -\sum (pi \cdot \ln pi)$, where pi is the abundance of every species.

3. Statistical analyses

Data were analyzed using the MIXED procedure (SAS Inst. Inc., Cary, NC). The effects of animal species (AS), type of forage in the diet, period, and the interaction AS x diet were considered fixed, and animal within species effect was considered random. Within each animal, differences between LAB and SAB in their NB and SI were analysed by a paired Student's *t* test.

III – Results and discussion

A total of 78 and 77 bands were detected in the DGGE gel from sheep and goats samples, respectively. In sheep, 18 bands were only found in samples from AL-fed animals, and 7 were found only in samples from animals fed the GR diet. In goats, 21 bands were found only in AL-fed animals and 17 were found exclusively in GR-fed goats. The mean values of NB and SI in LAB and SAB pellets from sheep and goats fed the two experimental diets are shown in Table 1. There were no AS x diet interactions (P = 0.165 to 0.259) in any of these parameters, indicating that the individuals from the two AS responded similarly to changes in the type of forage. In sheep, the NB ranged from 43 to 50 and from 27 to 35 for AL and GR diets, respectively. In goats, the NB ranged from 30 to 45 and from 28 to 34 for AL and GR diets, respectively. There were no interspecies differences in the NB (P = 0.655) and SI (P = 0.728) in SAB pellets. In contrasts, LAB pellets from sheep had greater NB (P = 0.016) and SI (P = 0.016) compared with goats, which would indicate a greater diversity of LAB communities in the rumen of sheep. In both animal species, AL diet tended to promote a greater NB and SI in LAB (P = 0.072 and 0.071, respectively) and SAB (P = 0.089 and 0.099) pellets compared to the GR diet.

Table 1. Band numbers and Shannon index calculated from the total bacterial DGGE profiles of LAB and SAB samples obtained from sheep and goats fed diets containing 30% of concentrate and 70% of alfalfa hay (AL) or grass hay (GR) as forage

Item	Bacterial pellet	Animal species	Diet		SEM	Significance levels (*P* =)		
			AL	GR		AS[1]	Diet	AS x Diet
Band number	LAB	Sheep	48.0	33.0	1.07	0.016	0.072	0.165
		Goat	32.5	28.0				
	SAB	Sheep	43.5	30.0	2.88	0.655	0.089	0.259
		Goat	37.5	33.0				
Shannon index	LAB	Sheep	3.87	3.50	0.028	0.016	0.071	0.225
		Goat	3.50	3.33				
	SAB	Sheep	3.77	3.40	0.081	0.728	0.099	0.245
		Goat	3.61	3.50				

[1]AS: animal species.

The similarity index between LAB and SAB ranged from 31.6 to 66.7% (data not shown), and they did not differ (P = 0.481) between the two animal species (51.6 and 45.9% for sheep and goats, respectively). Whereas in sheep both NB and SI were greater in LAB compared to SAB (P= 0.050 and 0.036, respectively), no differences between LAB and SAB were found either in NB (P = 0.269) or SI (P = 0.257) in goats. Moreover, in sheep there was a positive correlation between the NB in LAB and that in SAB (r = 0.966; P = 0.034), but no correlation (r= 0.039; P = 0.961) was observed in goats. In accordance with our results, others have reported differences in the bacterial communities found in the fluid and those in the solid phase of the rumen in sheep (Michalet-Doreau *et al*., 2001; Larue *et al.*, 2005; Stiverson *et al.,* 2011) and goats

(Cunha *et al.,* 2011). However, it must be noticed that whereas LAB are relatively easy to isolate, and a great recovery of the bacterial populations should be expected, recovery of SAB from ruminal digesta is usually low, indicating that a pure SAB isolate may not be representative of the total SAB population. The treatment of sheep ruminal digesta with the detachment method used in the present study has been reported to recover less than 40% of the total SAB in sheep fed high-forage diets (Ramos *et al.*, 2009), but no other method has been shown to be more effective in detaching SAB from solid digesta.

Several studies have pointed out large variations of microbial communities between animals (Firkins and Yu, 2006; Weimer *et al*., 2010). In order to illustrate the inter-animal variability in our study, Fig. 1 shows the NB in LAB and SAB pellets in each animal. The NB in LAB pellets varied from 31 to 50, but it was numerically lower for GR compared with AL diet in all animals. The NB in SAB pellets was numerically lower for GR compared with AL diet in the two sheep and goat 1, but goat 2 showed the opposite results. Bacterial communities in the gastrointestinal tract are influenced by numerous host-related factors, such as mastication, rumination, feeding behavior, digesta passage rate, genetics, etc. Interestingly, the two sheep and goat 1 showed numerically lower values in pH andacetate:propionate ratio in ruminal fluid for GR than for AL diet (Fig. 2), but goat 2 showed the opposite response, and this may be related to the higher SAB diversity observed when this animal was fed GR (Fig. 1).

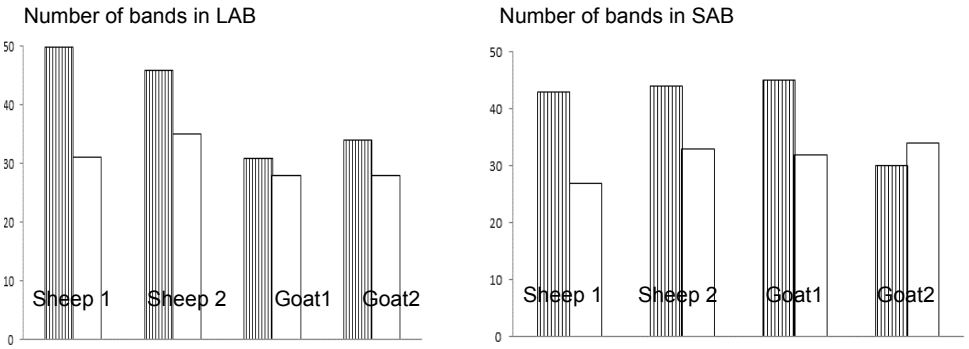

Fig. 1. Total number of bands in bacterial DGGE profiles of liquid-associated (LAB) and solid-associated (SAB) bacterial pellets obtained from sheep and goats fed diets containing 30% concentrate and 70% of either alfalfa hay (striped bars) or grass hay (unfilled bars) as forage. Numbers 1 and 2 correspond to individuals within each animal species.

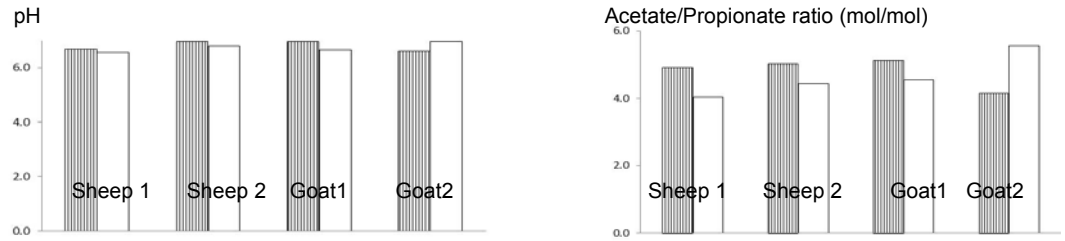

Fig. 2. Ruminal pH and acetate/propionate ratio in ruminal fluid (mean values of samples taken at 0 and 4 h morning post-feeding) from sheep and goats fed diets containing 30% of concentrate and 70% of either alfalfa hay (striped bars) or grass hay (unfilled bars) as forage. Numbers 1 and 2 correspond to individuals within each animal species.

As shown in Fig. 3A, the UPGMA dendrogram generated by the cluster analysis shows that all samples from sheep can be included in two major clusters. The four SAB pellets clustered together and the four LAB pellets grouped in a different cluster. In goats, the four SAB pellets clustered together, but clustering of LAB pellets was not so clear (Fig. 3B). In both animal species, SAB and LAB clusters contained two clear subclusters according to forage type.

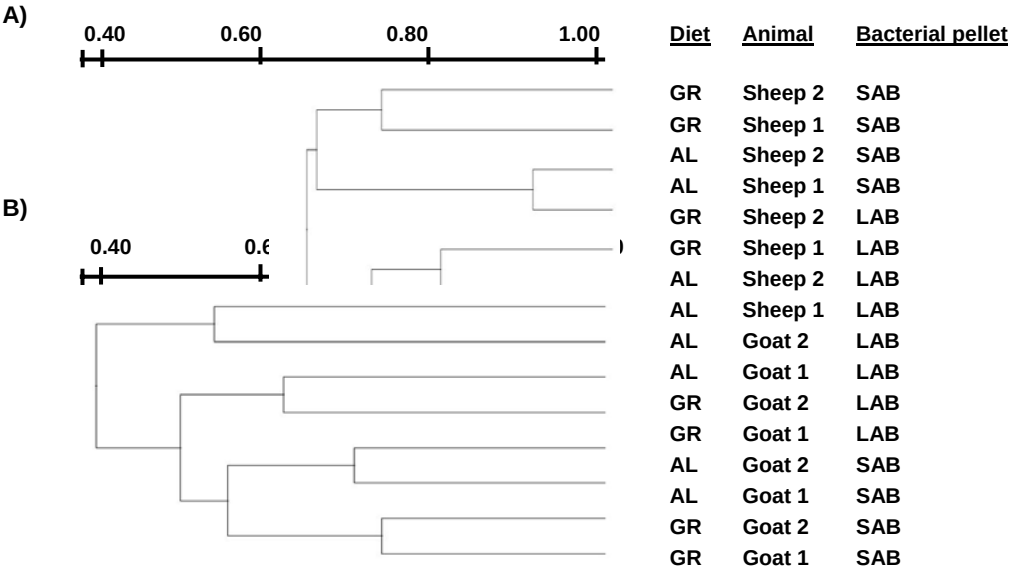

Fig. 3. Dendrograms from DGGE analysis of liquid-associated (LAB) and solid-associated (SAB) bacterial pellets in sheep (A) and goats (B) fed diets containing 30% of concentrate and 70% of alfalfa hay (AL) or grass hay (GR) as forage. Numbers 1 and 2 correspond to individuals within each animal species.

IV – Conclusions

Despite the reduced number of animals used in this study, in all of themLAB and SAB diversity was affected by the type of forage in the diet, and bacterial pellets from AL-fed animals tended to have greater diversity than those from animals fed the GR diet. These results indicate differences in the composition of the bacterial communities in the liquid and solid phases of the rumen. Inter-animal variability was observed, with 3 animals responding similarly to changes in forage type and one animal showing the opposite response in ruminal pH, acetate/propionate ratio and diversity in SAB pellets.

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