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Diversity of bacterial communities isolated from the solid and liquid phase of the rumen of sheep fed diets of variable composition

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Abstract. The aim of this study was to analyze bacterial diversity in bacterial pellets isolated from the solid (SAB) and liquid (LAB) phase of the rumen in sheep fed different diets. Four rumen-fistulated sheep were fed 4 diets with forage:concentrate ratios (F:C) of 70:30 (HF) or 30:70 (HC) and either alfalfa hay or grass hay as forage (FOR). SAB and LAB were isolated from each sheep immediately before feeding, and the automated ribosomal intergenic spacer analysis (ARISA) of the 16S ribosomal DNA was used to analyze bacterial diversity. A total of 168 peaks were detected in the ARISA electropherograms across the total 32 bacterial pellets, with 6 peaks only found in SAB and 8 found only in LAB. The SAB isolated from sheep fed HF diets had greater number of peaks and Shannon index than those from sheep fed HC diets (P=0.03 and 0.05, respectively), and SAB from sheep fed grass hay diets had greater values of both parameters than those isolated from sheep receiving alfalfa hay diets (P=0.002 and 0.004). In contrast, LAB profile was not affected (P=0.23 to 0.65) by either F:C ratio or FOR. The results indicate that SAB are more markedly affected by dietary characteristics than LAB.

Keywords. Bacterial diversity – ARISA – Forage – Rumen – Sheep.

Diversité des communautés bactériennes isolées dans la phase solide et liquide du rumen de moutons recevant des régimes de composition variable

Résumé. *L'objectif de ce travail a été d'analyser la diversité bactérienne des pellets isolés à partir de la phase solide (SAB) et la phase liquide (LAB) du rumen chez les moutons alimentés avec deux régimes différents. Quatre moutons munis de canules ruminales ont été utilisés selon un schéma en carré latin incomplet. Les quatre régimes expérimentaux avaient des rapports fourrage:concentré 70 : 30 (HF) ou 30 : 70 (HC), étant le fourrage soit le foin de luzerne soit le foin d'herbe. SAB et LAB ont été isolés dans chaque mouton juste avant la prise d'aliment, et la diversité bactérienne a été caractérisée par ARISA (automated ribosomal intergenic spacer analysis). Un total de 168 pics a été détecté dans les électrophérogrammes d'ARISA des 32 pellets bactériens, avec 6 pics seulement trouvés dans les SAB et 8 trouvés seulement dans les LAB. Les pellets SAB isolés chez les moutons recevant les régimes HF ont eu un plus grand nombre de pics et un index de Shannon plus élevé que ceux des régimes HC (P=0,03 et 0,05, respectivement), et les pellets SAB isolés chez les moutons recevant le foin d'herbe ont eu des valeurs plus élevés pour les deux paramètres que ceux isolés chez des moutons recevant du foin de luzerne (P=0,002 et 0,004). En revanche, le profil des pellets LAB n'a pas été affecté (P=0,23 à 0,65) par les rapports fourrage:concentré.ou par le type de fourrage. Les résultats indiquent que les pellets SAB sont plus nettement affectés par les caractéristiques du régime alimentaire que les pellets LAB.*

Mots-clés. *Diversité bactérienne – ARISA – Fourrage – Rumen – Mouton.*

I – Introduction

The rumen is an anaerobic microbial ecosystem which is inhabited by a complex microbial population including bacteria, protozoa, fungi and archaea. Differences in chemical composition and metabolic functions between bacteria isolated from the liquid (LAB) and solid (SAB) phase of the

rumen are widely demonstrated (Merry and McAllan, 1983; Ipharraguerre *et al.*, 2007), but the differences between LAB and SAB in the bacterial communities have received relatively little attention. An additional problem is that the total composition of bacterial communities in the rumen cannot be studied with traditional cultivation techniques. In the last years, different molecular fingerprinting techniques have been used to assess the richness and/or diversity of ruminal microbial communities (Kocherginskaya *et al.*, 2001; Ramos *et al.*, 2009; Welkie *et al.*, 2010). The purpose of this study was to analyze the bacterial diversity in LAB and SAB isolated from sheep fed different diets, through the use of automated ribosomal intergenic spacer analysis (ARISA), a community fingerprinting technique that allows profiling of the culturable and currently unculturable residents of a microbial habitat (Welkie *et al.*, 2010). The ARISA analysis was selected because it is a rapid and effective method for assessing microbial community diversity (Fisher and Triplett, 1999) and it allows a fast analysis of a large number of samples.

II – Materials and methods

Four rumen-cannulated Merino sheep (58.5 ± 3.16 kg body weight) were used. Sheep were housed in individual pens, had continuous access to fresh water and vitamin/mineral block over the experimental period and were cared and handled in accordance with the Spanish Animal Care Regulations. The four experimental diets had forage:concentrate (F:C) ratios (dry matter (DM) basis) of 70:30 (HF) or 30:70 (HC) with either alfalfa hay (HFA and HCA) or grass hay (HFG and HCG) as forage. The concentrate was based on barley, gluten feed, wheat middlings, soybean meal, palmkern meal, wheat, corn and mineral-vitamin premix (215, 204, 200, 135, 115, 50, 50 and 31 g/kg, respectively; fresh matter basis). Crude protein content was 186, 177, 121 and 160 g/kg DM for HFA, HCA, HFG and HCG, respectively, and neutral-detergent fibre content was 426, 374, 499 and 401 g/kg DM. Diets were offered to the animals twice daily (08:00 and 20:00 h) at a daily rate of 56 g DM/kg body weight$^{0.75}$ to minimise feed selection.

The experimental design was a 4 x 4 Latin square, with 21 d of dietary adaptation in each experimental period. On d 22 of each period, about 400 g of ruminal contents were taken through the cannula of each sheep immediately before the morning feeding and strained through 4 layers of cheesecloth. The solid digesta was combined with 100 ml of saline solution (0.9% NaCl) at 38ºC, mixed gently, and squeezed again to remove residual LAB. The procedure was repeated and the solution added to the ruminal fluid before isolation of LAB by differential centrifugation (Ranilla and Carro, 2003). The solid digesta (100 g) was incubated with saline solution (0.9% NaCl; 3 ml per g of digesta) containing 0.1% methylcellulose at 38ºC for 15 min in a continuous-shaking water bath (65 strokes per min); then 50 ml of cold (4ºC) saline solution containing 0.1% methylcellulose were added, and the material was stored at 4ºC for 24 h. The samples were homogenized for 10 s with a Waring Blender, centrifuged at 500 x g for 10 min at 4ºC, and the supernatant fraction was removed and retained. The solids were resuspended two times in saline solution containing 0.1% methylcellulose, and then recentrifuged (500 *x* g for 10 min at 4ºC). The final filtrates were then centrifuged at 20,000 x *g* for 25 min at 4ºC to isolate SAB. The pellets were washed by resuspension in saline solution, and the centrifugation was repeated. Bacterial pellets were freeze-dried and analyzed for bacterial diversity by ARISA.

DNA was isolated from bacterial pellets (30 mg of DM) as described by Yu and Morrison (2004). Extracted DNA was amplified using universal bacterial primers 16S-1392F and 23S-125R (Danovaro *et al.*, 2006; synthesized by Sigma-Aldrich Quimica SA, Madrid, Spain), which amplify the ITS1 region in the rRNA operon plus ca. 282 bases of the 16S and 23S sRNA. Primer 23S-125R was fluorescently labelled with the phosphoramidite dye 6-FAM. Each PCR mix (50 µl final volume) contained 1 x PCR reaction buffer, 1.5 mM $MgCl_2$, 0.25 µM of each primer, each deoxynucleotide triphosphate at a concentration of 0.2 mM, and 2.5 U of Taq Polymerase (Biotools B&M Labs SA, Madrid, Spain). Thermocycling was conducted in a 2720 Thermal Cycler

(Applied Biosystem, Foster City, CA, USA), and started with an initial cycle of denaturation (94ºC for 3 min), followed by 30 cycles of denaturation (94ºC for 60 s), annealing (55ºC for 60 s) and elongation (72ºC for 120 s), ending with a final extension step at 72ºC for 2 min. The PCR products were analyzed for size and quantity by electrophoresis in 1.5% agarose gels, and further purified using a Qiaquick PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Eluted DNA was quantified fluorimetrically using a Nanodrop ND-1000 (NanoDrop Technologies, Delaware, USA). For each ARISA, about 5 ng of amplicons were mixed with an internal size standard (GS 1200 LIZ, Applied Biosystems, Foster City, CA, USA) in deionized formamide, then denatured at 94ºC for 2 min, and immediately chilled on ice. Automated detection of ARISA fragments was carried out using an ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) with 36-cm by 50-µm capillary and POP-7 polymer (Applied Biosystems, Foster City, CA, USA). Peak size and area were estimated by comparison with the internal size standard using the GeneMaker Software v1.80 (SoftGenetics, State College, PA, USA). To include the maximum number of peaks while excluding background fluorescence, a threshold of 100 fluorescence units was used.

It was considered that the peak profile in ARISA electropherograms reflects the predominant bacterial species or populations present in the bacterial pellets, and only the presence/absence of the different peaks was considered for the analysis. The Shannon's diversity index was calculated as described by Shannon and Weaver (1949). To analyze the similarity between LAB and SAB, a percent similarity index was calculated from the peak profile patterns in the electropherograms of both types of bacteria isolated from each sheep within dietary treatment. Dendrograms were constructed using the Pearson product-moment correlation coefficient and unweighted pair-group method using arithmetic averages (UPGMA) options in the MVSP v3.12d software (Kovach Computing Service, Anglesey, Wales, UK), and principal component analysis (PCA) was performed using the MVSP v3.12d software.

Data were analyzed using the MIXED procedure (SAS Inst. Inc., Cary, NC). The effects of F:C ratio, type of forage (FOR), period, and the interaction F:C x FOR were considered fixed, and sheep effect was considered random.

III – Results and discussion

The number of peaks in bacterial pellets isolated from sheep rumen ranged from 42 to 82 for LAB and from 31 to 81 for SAB, with a total of 168 different peaks. There were 8 peaks only found in LAB (4.8% of detected) and 6 appeared only in SAB (3.6% of detected). In a study using ARISA to analyze the ruminal bacterial community in 2 lactating cows fed a 65% forage diet, Welkie *et al.* (2010) observed that 13.5% of the peaks were detected only in the liquid phase, and 1.9% were detected only in the solid phase.

As shown in Table 1, no interaction (P=0.28 to 0.96) F:C x FOR was observed for any variable. Both the number of peaks and the Shannon index were greater in SAB isolated from sheep fed HF diets than from those fed HC diets (P=0.03 and 0.05, respectively), but no effect of F:C ratio was detected on LAB (P=0.39 and 0.23, respectively). The results are in accordance with those reported by Larue *et al.* (2005), who observed that bacterial diversity in SAB tended to be greater in sheep fed only orchardgrass hay compared with those fed a 70:30 orchardgrass:concentrate diet. In contrast, Kocherginskaya *et al.* (2001) found that corn-fed steers displayed more diverse and rich bacterial populations in the ruminal liquid phase than hay-fed steers.

Whereas FOR had no effect (P>0.05) on LAB profile, SAB isolated from sheep receiving grass hay diets had greater number of peaks and Shannon index values than those isolated when alfalfa hay diets were fed (P<0.001 to 0.004). This is in agreement with the results of Koike *et al.* (2003), who

observed that the set of bacteria isolated from orchardgrass seemed to be different from the set isolated from alfalfa when samples of both forages were incubated *in sacco* in the rumen of sheep and bacterial 16S rDNAs were cloned. These authors concluded that the hay source may affect the members of fibre-associated rumen bacterial community, which is in agreement with our results.

Table 1. Values of Shannon index and numbers of peaks detected in the ARISA electropherograms of liquid-associated (LAB) and solid-associated (SAB) bacterial pellets isolated from the rumen of sheep receiving diets differing in forage:concentrate ratio (F:C) and type of forage (FOR), and similarity index (%) of ARISA profiles between LAB and SAB pellets

Item	Diet†				SEM	Statistical effect (*P* =)		
	HFA	HFG	HCA	HCG		F:C	FOR	F:C x FOR
Number of peaks								
LAB	65.0	63.8	62.0	59.5	3.80	0.39	0.65	0.86
SAB	54.5	77.5	48.5	63.0	3.57	0.03	0.002	0.28
Shannon index								
LAB	4.17	4.13	4.09	4.05	0.063	0.23	0.54	0.96
SAB	3.95	4.34	3.88	4.12	0.071	0.05	0.004	0.29
Similarity index	56.5	50.7	51.7	54.1	5.46	0.90	0.76	0.48

† HFA: 70:30 alfalfa hay:concentrate; HFG: 70:30 grass hay:concentrate; HCA: 30:70 alfalfa hay:concentrate; HCG: 30:70 grass hay:concentrate. Dry matter basis.

The similarity index between LAB and SAB ranged from 37.8 to 74.3% (individual values not shown), and was not affected by diet characteristics (*P*=0.90 and 0.76 for F:C and FOR, respectively; Table 1). In addition, there was no relationship (r=0.098; *P*=0.72; n=16) between the number of peaks in LAB and that in SAB. In accordance with our results, Larue *et al.* (2005) and Michalet-Doreau *et al.* (2001) reported differences in the bacterial communities found in the fluid and those in the solid phase of sheep rumen. It must be noticed that whereas LAB are relatively easy to isolate, and a great recovery of the bacteria 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 about 37 and 29% for HF and HC diets (Ramos *et al.*, 2009).

Several studies have pointed out large variations of microbial communities between animals (Mackie *et al.*, 1999; Firkins and Yu, 2006). In order to reduce the inter-animal variability in our study, we decided to use a 4 x 4 Latin square design. Significant effects of sheep were found on the number of peaks and Shannon index of LAB (*P*=0.008 and 0.005, respectively) and SAB (*P*=0.02 and 0.02), confirming previous observations. Bacterial communities in the gastrointestinal tract are influenced by numerous internal host-related factors, such as mastication, rumia, feeding behavior, digesta passage rate, etc.

The dendrograms of the ARISA profiles of LAB showed no clear clustering pattern based on F:C ratio or FOR (dendograms not shown). In contrast, SAB pellets formed two major clusters according to FOR, with all pellets from sheep fed alfalfa hay diets clustered together and the majority of SAB pellets from grass hay-fed sheep grouped in a different cluster. This was confirmed in the PCA analyses (Fig. 1). There was not a clear separation between LAB obtained from sheep fed HF and HC diets, or between diets with alfalfa hay and grass hay (Fig. 1A). In contrast, the PCA analysis of SAB clearly discriminated between alfalfa and grass hay diets, and all SAB from sheep fed alfalfa hay diets grouped together whereas most samples from grass hay-fed sheep formed a distinct group (Fig. 1B).

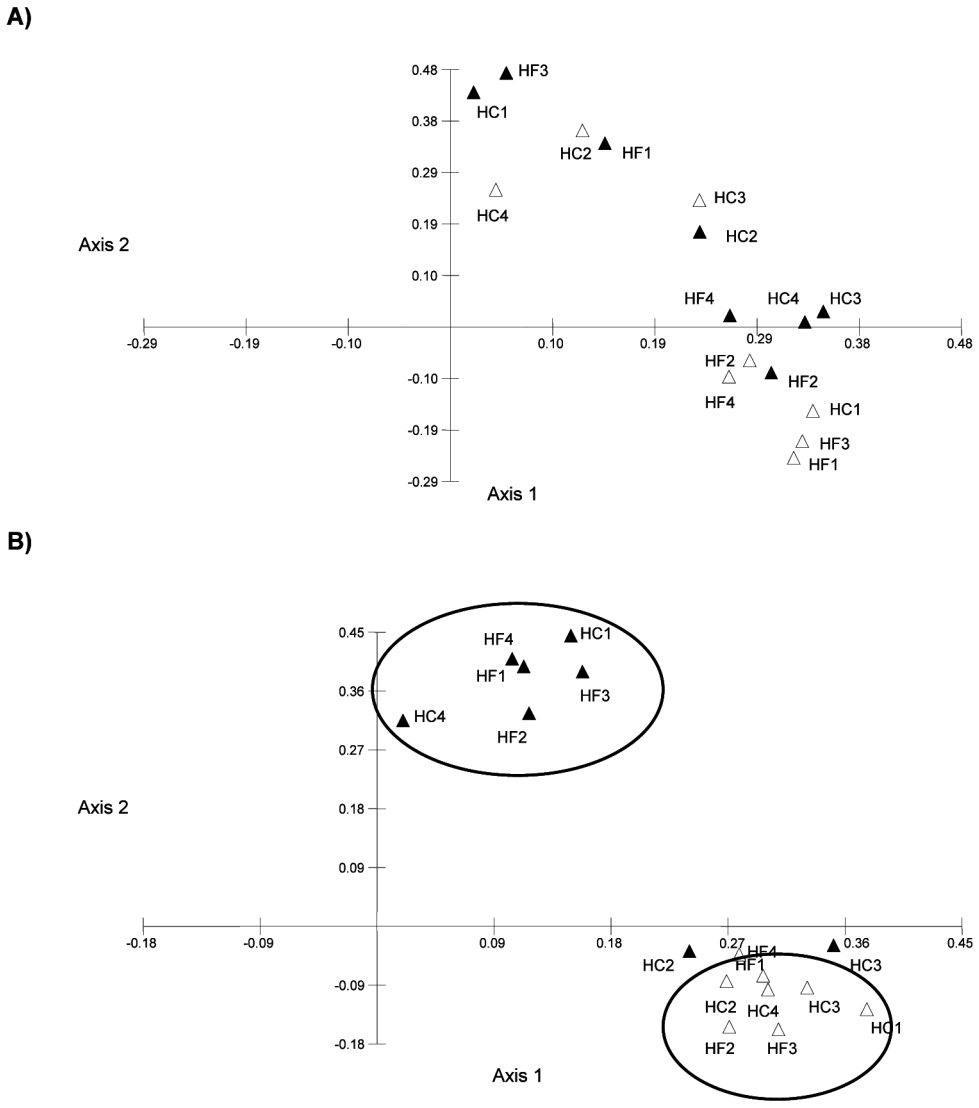

Figure 1. Principal component analysis generated from ARISA profiles of liquid-associated (A) and solid-associated (B) bacteria isolated from sheep fed diets containing alfalfa hay (∆) or grass hay (▲). Sheep received diets with F:C ratios of 70:30 (HF) or 30:70 (HC) and alfalfa hay or grass hay as forage. Numbers 1 to 4 correspond to individual sheep.

IV – Conclusions

Bacterial diversity in SAB pellets was affected by both the type of forage and forage to concentrate ratio in the diet, whereas the structure of LAB communities seemed to be independent of diet characteristics. The similarity index between LAB and SAB ranged from 50.7 to 56.5% and was not affected by the diet. The results indicate that bacteria in the solid phase of the rumen are more markedly affected by dietary characteristics than those in the liquid phase.

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