Influence of detachment procedure and diet on recovery of solid-associated bacteria from sheep ruminal digesta and representativeness of bacterial isolates as assessed by automated ribosomal intergenic spacer analysis-polymerase chain reaction¹

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ABSTRACT

Six ruminally and duodenally cannulated sheep were used in a partially replicated 4 × 4 Latin square experiment designed to evaluate the efficiency of 3 detachment procedures (DP) to recover solid-associated bacteria (SAB) from ruminal digesta. The 4 experimental diets contained forage to concentrate (F:C) ratios of 70:30 or 30:70 with either alfalfa hay or grass hay as the forage. Bacterial biomass was labeled with ¹⁵NH₄Cl. The DP were 1) MET: digesta was incubated at 38°C for 15 min with saline solution (0.9% NaCl) containing 0.1% methylcellulose under continuous shaking; 2) STO: digesta was mixed with cold saline solution and homogenized with a stomacher for 5 min at 230 rpm; 3) FRE: digesta was immediately frozen at –20°C for 72 h, thawed at 4°C, mixed with saline solution and subjected to STO procedure. Common to all treatments was storing at 4°C for 24 h after the treatment, homogenization, filtration, and resuspension of digesta 2 times in the treatment solutions. The automated ribosomal intergenic spacer analysis of the 16S ribosomal DNA was used to analyze the similarity between bacterial communities attached to the digesta and those in the pellet obtained after each DP. There were no significant F:C × DP or forage × DP interactions for any variable. On average, STO treatment detached 65.8% of SAB from ruminal digesta, about 1.2 and 1.5 times more than FRE and MET treatments, respectively. Total recovery of SAB in STO pellets (48.9%) was greater compared with FRE (31.7%) and MET (33.1%), values being greater for high-forage compared with high-concentrate diets. Similarity index between the bacteria attached to diges-

ta and those in the pellets were lower for FRE (48.2%) compared with MET (54.1%) and STO (54.1%), which suggests that FRE could have destroyed cell integrity of some bacterial species, thus reducing the bacterial diversity present in the pellets. The STO method was the most effective removing SAB from digesta, but only a moderate similarity between the bacterial communities attached to digesta and those recovered in the bacterial pellets was obtained. Values of duodenal microbial flow estimated using SAB as reference bacteria were greater with FRE compared with STO and MET, but all DP detected similar differences between diets, and therefore did not influence the interpretation of results.

Key words: solid-associated bacteria, ¹⁵N, bacterial diversity, detachment procedure

INTRODUCTION

The ruminal microbes provide a major proportion of the total AA entering the duodenum of ruminants, and measuring of microbial protein synthesis is essential for optimizing the nutrition of the host animal (Clark et al., 1992; Yang et al., 2001). Methods of estimating ruminal microbial protein synthesis rely on marker techniques in which a particular microbial constituent is related to the microbial N content, but differences between liquid- (LAB) and solid-associated bacteria (SAB) in marker:N ratio have been widely demonstrated (Merry and McAllan, 1983; Carro and Miller, 2002; Molina-Alcaide et al., 2009). Because SAB can account for more than 70% of the total bacteria in the rumen (Yang et al., 2001; Ipharraguerre et al., 2007), their contribution should be taken into account to estimate microbial protein synthesis. Detachment procedures (DP) commonly used to remove SAB have been reported to have an efficiency ranging from 20 (Legay-Carmier and Bauchart, 1989; Olubobokun and Craig, 1990) to 90% (Whitehouse et al., 1994; Ranilla and Carro, 2003; Trbalza-Marinucci et al., 2005), but total recovery of detached microbes is usually lower than 50%, indicating that a pure SAB isolate may not

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Table 1. Ingredients and chemical composition of diets¹

Item	HFA	HCA	HFG	HCG
Ingredient, % of DM				
Alfalfa hay	70	30	—	—
Grass hay	—	—	70	30
Barley	6.4	15.2	6.4	15.2
Gluten feed	6.1	14.5	6.1	14.5
Wheat middlings	5.7	13.7	5.7	13.7
Soybean meal	4.1	9.7	4.1	9.7
Palm kernel meal	3.8	9.0	3.8	9.0
Wheat	1.5	3.5	1.5	3.5
Corn	1.5	3.5	1.5	3.5
Vitamin-mineral premix ²	1.0	1.0	1.0	1.0
Chemical composition				
DM, %	92.7	92.5	92.5	92.4
OM, % of DM	91.3	91.3	92.7	91.9
N, % of DM	2.69	2.83	1.94	2.56
NDF, % of DM	42.6	37.4	49.9	40.1
ADF, % of DM	26.9	18.7	23.8	17.4
ME, ³ Mcal/kg of DM	2.12	2.45	2.00	2.39

¹HFA = 70:30 alfalfa hay:concentrate; HCA = 30:70 alfalfa hay:concentrate; HFG = 70:30 grass hay:concentrate; HCG = 30:70 grass hay:concentrate, all DM basis.

²Vitamin-mineral premix contained per kilogram of DM: 11,250 IU of vitamin A; 2,250 IU of vitamin D₃; 25 mg of vitamin E; and 10 mg of CuSO₄·5H₂O.

³Estimated according to NRC (1985).

be representative of the total SAB. To the best of our knowledge, no studies have been conducted *in vivo* to investigate the efficacy of DP to obtain a representative bacterial pellet. Recently, molecular techniques have been used to profile ruminal and duodenal microbial populations (Sylvester et al., 2005; Karnati et al., 2007) and to assess the richness or diversity metrics of microbial communities in the rumen (Stebulis et al., 2008). The application of these techniques to ruminal samples could constitute a useful tool to evaluate the similarity between bacterial communities attached to digesta and those in the pellet obtained after the detachment process.

Our hypothesis was that the procedure used to detach bacteria from ruminal digesta could affect the characteristics of the bacterial pellet obtained, and therefore the estimation of ruminal microbial protein synthesis. The objectives of this study were 1) to investigate the efficiency of recovery of SAB from sheep ruminal digesta using 3 different DP, 2) to analyze the representativeness of the pellets by comparing their similarity to the bacteria attached to digesta before the detachment process, and 3) to evaluate the estimates of microbial protein synthesis obtained with the bacterial pellets isolated after each DP. Sheep were fed diets differing in the forage to concentrate (**F:C**) ratio and in the type of forage (**FOR**), because these factors can affect both the relative proportion of SAB and LAB in the rumen and the degree of bacterial attachment (Pérez et al., 1998; Ranilla and Carro, 2003; Martínez et al., 2009). The experimental diets were chosen to

be representative of those used in dairy sheep in field conditions in Spain.

MATERIALS AND METHODS

Sheep were cared and handled in accordance with the Spanish Animal Care Regulations, and the experimental protocols were approved by the León University Institutional Animal Care and Use Committee.

Animals and Diets

Six ruminally and duodenally fistulated Merino sheep (58.6 ± 4.46 kg BW) were housed in individual pens and had free access to drinking water throughout the trial. Four total mixed diets were formulated according to a 2×2 factorial arrangement of treatments (Table 1). The diets differed in F:C ratio [70:30 (**HF**) and 30:70 (**HC**); DM basis] and had either alfalfa hay (**HFA** and **HCA**) or grass hay (**HFG** and **HCG**) as forage. Diets were offered to the animals twice daily (0800 and 2000 h) at a daily rate of 52 g of DM/kg BW^{0.75} to minimize feed selection. Refusals and individual feed intakes were recorded daily over the trial.

Experimental Procedure and Sample Collection

The experimental design was a partially replicated 4×4 Latin square, with 4 dietary treatments, 4 periods and 6 sheep. Each experimental period lasted for 19 d, allowing 15 d for adaptation to the diet and 4 d for

Figure 1. Procedures used to detach and isolate solid-associated bacteria (SAB) from ruminal digesta in sheep.

sampling. In each period, the following schedule was employed: continuous infusions of flow and microbial markers from d 10 to 19, testing of DP on d 16, and collection of duodenal digesta from d 17 to 19.

Duodenal Flow. Chromium-mordanted fiber and Co-EDTA were used as solid and fluid phase markers, respectively, to assess the duodenal flow of digesta, and ¹⁵N was used as a microbial marker. Chromium-mordanted fiber was prepared from the diets given to the animals according to Udén et al. (1980). From d 10 to 19, 15 g of Cr-mordanted fiber were administered daily via the ruminal cannula into 4 equal portions at 0800, 1400, 2000, and 0200 h. Co-EDTA (Udén et al., 1980) and ¹⁵NH₄Cl (10% atom excess; Tracer SA, Madrid, Spain) were dissolved in distilled water and infused into the rumen (250 mL/d) at a constant rate of 60 mg of Co/d and 30 mg of ¹⁵N/d by means of a peristaltic pump (Gilson Minipuls Evolution, Gilson Inc., Middleton, WI). On d 17, 18, and 19, duodenal digesta samples were collected at 6-h intervals. The sample collection times were advanced by 2 h each day to obtain a sample that was representative of daily duodenal flow. Samples were pooled by sheep and stored at -20°C. Duodenal samples were thawed at 4°C, homogenized, and half of each sample was centrifuged (1,000 × g for 5 min) to obtain particulate matter (Faichney, 1975). Both samples of whole duodenal digesta and particulate matter were freeze-dried and analyzed for ash, NAN, and ¹⁵N.

Detachment Procedures. On d 16 of each period, about 600 g of rumen contents was withdrawn from each sheep at 4 h after the morning feeding. Rumen contents were squeezed through 4 layers of cheesecloth and the

solid digesta was combined with 300 mL of saline solution (0.9% NaCl) at 38°C, mixed gently, and squeezed again to remove residual LAB. One subsample (about 30 g of fresh matter) was taken, frozen, and freeze-dried to determine DM, NAN, and ¹⁵N enrichment. Three equal portions of digesta (50 g of fresh matter) were sampled and subjected to one of the following treatments (see Figure 1): 1) **MET**: digesta was incubated with saline solution (0.9% NaCl) containing 0.1% methylcellulose at 38°C for 15 min in a continuous-shaking water bath (65 strokes per min), and then 25 mL of cold (4°C) saline solution containing 0.1% methylcellulose were added, and the material was stored at 4°C for 24 h (Ranilla and Carro, 2003); 2) **STO**: digesta were mixed with saline solution (0.9% NaCl), placed in Stomacher standard bags and homogenized with a Stomacher 400 Circulator (Seward Ltd., London, UK) for 5 min at 230 rpm, then 25 mL of cold (4°C) saline solution was added, and the material was stored at 4°C for 24 h; 3) **FRE**: digesta was immediately frozen at -20°C for 72 h, thawed at 4°C for 24 h, mixed with saline solution (0.9% NaCl), and treated as described for STO. All solutions were added at 3 mL/g of solid digesta.

Following treatments, the samples were homogenized for 10 s with a Waring Blender (Waring Products Inc., Torrington, CT), centrifuged at 500 × g for 10 min at 4°C, and the supernatant fraction was removed and retained. The solids were resuspended 2 times in the corresponding treatment solutions, homogenized, and then recentrifuged (500 × g for 10 min at 4°C). The final filtrates were then centrifuged at 29,000 × g for 25 min at 4°C to obtain SAB pellets. These were washed by resuspension in saline solution, and the centrifuga-

tion was repeated. Bacterial pellets were freeze-dried, ground to a fine powder with a mortar and pestle, and analyzed for N and ^{15}N enrichment. The final residue of solid digesta was weighed, frozen, and freeze-dried for determination of DM, NAN, and ^{15}N enrichment.

Analyses of Bacterial Communities by Automated Ribosomal Intergenic Spacer Analysis

To analyze the similarity between bacterial communities attached to the digesta and those in the pellets obtained after the different DP, DNA was isolated from both ruminal digesta (120 mg of DM) and 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 approximately 282 bases of the 16S and 23S sRNA. Primer 23S-125R was fluorescently labeled with the phosphoramidite dye 6-FAM. Each PCR mix (50 μL final volume) contained $1\times$ 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 Biosystems, Foster City, CA), and started with an initial cycle of denaturation (94°C for 3 min), followed by 30 cycles of denaturation (94°C for 60 s), annealing (55°C for 60 s), and elongation (72°C for 120 s), ending with a final extension step at 72°C 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, Wilmington, DE). For each automated ribosomal intergenic spacer analysis (ARISA), about 5 ng of amplicons was mixed with an internal size standard (GS 1200 LIZ, Applied Biosystems) in deionized formamide, then denatured at 94°C 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) with 36-cm by 50- μm capillary and POP-7 polymer (Applied Biosystems). Peak size and area were estimated by comparison with the internal size standard using GeneMarker software (v1.80, Softgenetics, State College, PA). 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 digesta and bacterial pellets. To analyze the representativeness of bacterial pellets, a percent similarity index was calculated from the peak profile patterns in the electropherograms of each digesta and the corresponding isolated pellets, considering only the presence/absence of the different peaks. The Shannon's diversity index was calculated as described by Shannon and Weaver (1949) for each group of samples (each digesta and the 3 corresponding pellets), and was used to evaluate the diversity of bacterial communities. Dendrograms were constructed using the Pearson product-moment correlation coefficient and unweighted pair-group method using arithmetic averages (UPGMA) options in the MVSP software (v3.12d, Kovach Computing Service, Anglesey, Wales, UK).

Analytical Procedures

Dry matter (method 934.01), ash (method 942.05), and N (method 984.13) were determined according to AOAC (1999). Neutral-detergent fiber and ADF analyses were carried out according to Van Soest et al. (1991) using an Ankom²²⁰ Fiber Analyzer unit (Ankom Technology Corporation, Fairport, NY). Sodium sulfite and heat-stable amylase were used in analysis of NDF and ADF, and they were expressed inclusive of residual ash. All samples for ^{15}N analysis were pretreated to remove ammonia by adding 1 N NaOH until pH >10 and drying at 60°C in a forced-air oven for 16 h. Procedures for ^{15}N analysis in feeds, digesta, and bacterial isolates were described by Carro and Miller (1999), and analyses of ^{15}N enrichment were performed by isotope ratio mass spectrometry (VG Prism II, Isogas Ltd., Middlewich, UK) connected in series to a Dumas-style N analyzer (model 1108, Carlo Erba Instruments, Milan, Italy).

Marker (Co, Cr) concentrations in duodenal samples were analyzed by atomic emission spectrophotometry (AES-ICP, Perkin Elmer 2000 DV, Uberlingen, Germany) after wet digestion using the method described in Williams et al. (1962). Wavelengths for Cr and Co were 268 and 228 nm, respectively, and values were corrected for concentrations of Cr and Co determined in each sheep before markers infusion.

Calculations and Statistical Analysis

The proportion of SAB detached (% detachment) for each DP was calculated as $[1 - (\mu\text{g of } ^{15}\text{N in digesta after treatment} / \mu\text{g of } ^{15}\text{N in digesta before treatment}) \times 100]$. The proportion of recovery from detached SAB (% recovery from detached) was calculated as $[\mu\text{g of } ^{15}\text{N in total bacterial pellet} / (\mu\text{g of } ^{15}\text{N in digesta before treatment} - \mu\text{g of } ^{15}\text{N in total after treatment}) \times 100]$. The total recovery of SAB was calculated as $[(\% \text{ detachment} \times \% \text{ recovery}) / 100]$.

Duodenal flow was calculated from the concentrations of Cr and Co in duodenal digesta phases using the dual-phase marker method of Faichney (1975). Samples were mathematically reconstituted to create a representative sample from the concentrations of each analyzed nutrient in each of the duodenal phases (Faichney, 1975). Apparent degradability of OM in the rumen was calculated as (g of OM intake – g of OM duodenal flow) $\times$ (100/g of OM intake).

For each DP, microbial N flow at the duodenum was estimated using SAB as reference bacteria as follows: DM digesta flow (g/d) $\times$ NAN in digesta (g/g DM) $\times$ (^{15}N atom% excess in digesta/ ^{15}N atom% excess in the corresponding SAB isolate). The natural ^{15}N content in diets was used for background correction before ^{15}N infusion.

Data were analyzed according to a partially replicated 4×4 Latin square model using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC) according to the following model:

$$Y_{ijkl} = \mu + c_i + P_j + FC_k + F_l + FCF_{kl} + e_{ijkl},$$

where Y_{ijkl} = the dependent variable; μ = the overall mean; c_i = the random effect of sheep ($i = 1$ to 6); P_j = the fixed effect of period ($j = 1$ to 4); FC_k = the fixed effect of F:C ($k = 1$ to 2); F_l = the fixed effect of FOR ($l = 1$ to 2); FCF_{kl} = the interaction of F:C and FOR; and e_{ijkl} = the residual error. When a significant effect of treatment ($P \leq 0.05$) was detected, differences among means were tested using the Tukey's multiple comparison test.

RESULTS AND DISCUSSION

Based on the results from a previous study comparing several chemical treatments to detach SAB (Ranilla and Carro, 2003), we chose the treatment that showed the greatest SAB recovery (MET) and decided to compare it with 2 physical treatments (STO and FRE). Homogenization and chilling at 4°C were included in all treatments because of their efficacy in removing SAB from ruminal digesta (Minato and Suto, 1978; Martín-Orúe et al., 1998; Trabalza-Marinucci et al., 2005). Because it has been shown that efficiency of DP can be affected by the nature of the treated substrate (Olubobokun et al., 1988; Ranilla and Carro, 2003), the efficiency of the selected DP was evaluated on digesta from sheep fed diets differing in F:C ratio and type of forage. Finally, we decided to use ^{15}N as a microbial marker because it has been reported that ^{15}N reduced the variation in measuring the duodenal flow of microbial protein compared with other markers (Carro and Miller, 2002; Ipharraguerre et al., 2007).

Efficacy of Detachment Procedures and Representativeness of Bacterial Isolates

With the exception of a trend ($P = 0.09$) to a significant F:C $\times$ DP interaction for the percentage of detachment and similarity index, F:C $\times$ DP and FOR $\times$ DP interactions were not significant ($P = 0.11$ to 0.93) for any variables analyzed (Tables 2 and 3); therefore, only results for main effects will be discussed. There were differences ($P < 0.001$) among DP in percentage of detachment, recovery of detached and total recovery (Table 2). Detachment values for STO were on average 1.2 and 1.5 times greater ($P < 0.001$) than those obtained by FRE and MET, respectively. Stomaching has been reported to be an efficient DP in some studies (Merry and McAllan, 1983; Martínez et al., 2009), whereas a lack of effect has been reported in others (Legay-Carmier and Bauchart, 1989; Trabalza-Marinucci et al., 2005). Differences in the type of substrate, bacterial populations, and in the time and intensity of the stomacher treatment could explain these inconsistent results. Freezing the digesta before STO treatment (FRE) reduced ($P < 0.001$) the percentage of detachment compared with STO. In agreement with our results, Trabalza-Marinucci et al. (2005) observed that freezing digesta at -30°C for 24 h before the detaching treatment did not improve the efficacy of any of the tested DP.

The MET treatment produced detachment values lower than the 66.3% mean value reported by Martín-Orúe et al. (1998) in sheep fed diets containing either 80% forage or 80% concentrates. Using MET in digesta from Rusitec fermenters, Ranilla and Carro (2003) reported values greater than 80% for HF and HC diets, and Martínez et al. (2009) found values of 65.3 and 48.6% for forage and concentrate, respectively. These discrepancies in the values observed in different experiments could be because of differences either in the specific DP or in the bacterial species attached to the substrates. Ranilla and Carro (2003) used a 0.1% methylcellulose solution at pH 2.0, whereas the pH of the solution used in the present study and in that of Martínez et al. (2009) was 7.0. It has been shown that decreasing pH from 6.0 to 4.5 reduced the attachment of *Fibrobacter succinogenes* to cellulose, whereas the adhesion of *Ruminococcus flavefaciens* was not affected (Roger et al., 1990). In addition, incubation with a 0.1% methylcellulose solution inhibited the adhesion of *R. flavefaciens* to cellulose, but had no effects on *F. succinogenes* (Rasmussen et al., 1989; Roger et al., 1990). These results suggest that mechanisms of adherence differ among bacterial species, and therefore the efficiency of MET may be influenced by the bacterial species attached to the substrate.

Table 2. Analysis of recovery of solid-associated microbes from ruminal digesta in sheep after applying different detachment procedures

Item ¹	Detachment procedure (DP) ²				Significance level (<i>P</i> -value) ³				
	MET	STO	FRE	SEM	F:C	Forage	DP	F:C × DP	Forage × DP
Detachment, %				2.28	0.002	0.50	<0.001	0.09	0.54
HFA	44.5 ^a	61.9 ^c	52.3 ^b						
HCA	42.9 ^a	68.4 ^c	58.3 ^b						
HFG	44.0 ^a	66.1 ^c	57.2 ^b						
HCG	42.6 ^a	67.4 ^c	58.5 ^b						
Recovery from detached, %				5.12	<0.001	0.80	<0.001	0.40	0.51
HFA	79.8 ^b	81.8 ^b	62.2 ^a						
HCA	67.7 ^b	65.6 ^b	52.9 ^a						
HFG	86.9 ^b	77.6 ^b	56.7 ^a						
HCG	68.7 ^b	70.8 ^b	52.2 ^a						
Total recovery, %				2.83	0.04	0.71	<0.001	0.21	0.88
HFA	35.1 ^a	50.5 ^b	32.4 ^a						
HCA	29.3 ^a	44.9 ^b	30.7 ^a						
HFG	38.2 ^a	50.8 ^b	31.9 ^a						
HCG	29.2 ^a	46.7 ^b	30.8 ^a						
Similarity index, %				2.85	0.28	<0.001	0.006	0.50	0.09
HFA	64.3	63.7	56.9						
HCA	65.6 ^b	63.2 ^b	52.6 ^a						
HFG	40.1	41.4	40.2						
HCG	46.3	48.1	43.2						
Shannon index (H')				0.093	0.03	<0.001	0.02	0.59	0.18
HFA	3.84	3.80	3.62						
HCA	3.90 ^b	3.85 ^b	3.56 ^a						
HFG	3.13	3.17	3.13						
HCG	3.34	3.45	3.28						

^{a-c}Within a row, means without a common superscript letter differ ($P < 0.05$).

¹HFA = 70:30 alfalfa hay:concentrate; HCA = 30:70 alfalfa hay:concentrate; HFG = 70:30 grass hay:concentrate; HCG = 30:70 grass hay:concentrate, all DM basis.

²MET = digesta incubated in 0.9% saline 0.1% methylcellulose (38°C, 15 min); STO = digesta suspended in 0.9% saline solution and treated with stomacher (230 rev/min, 5 min); FRE = digesta frozen (−20°C, 72 h), thawed (4°C, 24 h), and treated as in STO.

³F:C = forage:concentrate ratio; Forage = grass hay vs. alfalfa hay.

In our study, FRE reduced ($P < 0.001$) the recovery of detached bacteria by 26.1 and 24.3% compared with MET and STO, respectively, which might indicate that FRE increased bacterial lysis. The effects of freezing on bacterial integrity are controversial. Merry and McAllan (1983) and Hsu and Fahey (1989) demonstrated significant losses of purine bases by freezing ruminal digesta samples before isolating bacteria, but Ha and Kennelly (1984) and Cecava et al. (1990) observed that the N:purine ratio of bacterial pellets was unaffected by freezing samples before isolation. Cecava et al. (1990) suggested that because of the more fragile nature of gram-negative bacteria compared with gram-positive bacteria, gram-negative bacteria may be more susceptible to cell lysis during freezing and thawing. Bacterial species are strongly affected by the diet fed to the host animals, and differences in bacterial populations would help to explain the conflicting results obtained in these studies. The lack of differences ($P = 0.54$) between MET and STO in the values of recovery of detached bacteria in our study would indicate that the procedures either did not have an adverse effect or they had similar effects on cell integrity. The values of recovery

of detached for MET and STO (65.6 to 86.9%) were greater than those found in other studies using different DP and ¹⁵N as a microbial marker (Craig et al., 1987; Martín-Orúe et al., 1998), which might suggest that the DP used in the present study produced less cell lysis.

Averaged across diets, values of total recovery were 48.6, 31.5, and 33.1% for STO, FRE, and MET, respectively, values being greater ($P < 0.001$) for STO compared with FRE and MET. Total recovery values ranging from 32 to 34% have been previously reported (Craig et al., 1987; Olubobokun et al., 1988), and the comparatively greater total recovery values found in our study for STO would indicate that it constitutes a useful method to recover SAB from ruminal digesta.

The level of detachment was greater ($P = 0.002$) for HC compared with HF diets in the present study, although the F:C × DP interaction tended to be significant ($P = 0.09$). Thus, no differences caused by F:C were found for diets containing grass hay ($P = 0.10$ to 0.99), but STO and FRE treatments produced greater detachment for HC compared with HF when alfalfa hay was the forage in the diet ($P = 0.003$ and 0.005 , respectively). These results would indicate that the ef-

Table 3. Nitrogen content and ^{15}N enrichment of solid-associated bacteria (SAB) from sheep ruminal digesta isolated after applying different detachment procedures

Item ¹	Detachment procedure (DP) ²				Significance level (<i>P</i> -value) ³				
	MET	STO	FRE	SEM	F:C	Forage	DP	F:C × DP	Forage × DP
N, mg/g of DM				2.13	0.01	0.76	0.003	0.35	0.80
HFA	69.8 ^b	67.7 ^{ab}	65.2 ^a						
HCA	64.2	64.8	61.1						
HFG	67.5	65.2	65.4						
HCG	67.9 ^b	65.7 ^b	59.8 ^a						
^{15}N , atom % excess				0.00398	<0.001	<0.001	0.006	0.85	0.82
HFA	0.0574	0.0541	0.0495						
HCA	0.0490	0.0482	0.0410						
HFG	0.0996	0.0995	0.0905						
HCG	0.0695 ^b	0.0609 ^{ab}	0.0556 ^a						
Proportion of bacterial N derived from ammonia-N, %				1.40	0.33	<0.001	<0.001	0.34	0.86
HFA	30.9 ^b	29.4 ^b	26.9 ^a						
HCA	33.3 ^b	31.6 ^{ab}	27.9 ^a						
HFG	40.7	40.7	37.0						
HCG	40.0 ^b	35.1 ^a	32.2 ^a						
Bacterial colonization of solid digesta, % of digesta DM				1.07	<0.001	0.50	0.007	0.53	0.90
HFA	19.1	20.6	23.2						
HCA	26.0 ^a	27.7 ^{ab}	32.8 ^b						
HFG	20.3	21.1	23.0						
HCG	22.7 ^a	26.5 ^{ab}	31.4 ^b						
Duodenal flow of microbial N, flow g of microbial-N/d				1.22	<0.001	<0.001	0.006	0.55	0.93
HFA	16.8	17.6	19.4						
HCA	18.9 ^a	20.2 ^{ab}	22.5 ^b						
HFG	13.0	12.9	14.4						
HCG	17.0 ^a	19.5 ^{ab}	21.3 ^b						
Efficiency of microbial growth, g of microbial-N/kg of OM apparently fermented in the rumen				2.90	<0.001	0.86	0.02	0.51	0.65
HFA	27.8	29.1	32.0						
HCA	35.2 ^a	37.7 ^{ab}	41.7 ^b						
HFG	24.7	24.3	27.1						
HCG	34.8	39.6	43.4						

^{a,b}Within a row, means without a common superscript letter differ ($P < 0.05$).

¹HFA = 70:30 alfalfa hay:concentrate; HCA = 30:70 alfalfa hay:concentrate; HFG = 70:30 grass hay:concentrate; HCG = 30:70 grass hay:concentrate, all DM basis.

²MET = digesta incubated in 0.9% saline 0.1% methylcellulose (38°C, 15 min); STO = digesta suspended in 0.9% saline solution and treated with stomacher (230 rev/min, 5 min); FRE = digesta frozen (−20°C, 72 h), thawed (4°C, 24 h), and treated as in STO.

³F:C: forage:concentrate ratio; Forage: grass hay vs. alfalfa hay.

ficacy of DP might be influenced by the nature of the diet fed to the animals. In agreement with Ranilla and Carro (2003), the recovery of detached bacteria was greater ($P < 0.001$) for HF compared with HC diets (74.1 and 63.0%) which would indicate that microbes attached to forage particles were more resistant to cell lysis than those attached to concentrate particles. Total recovery was also greater ($P = 0.02$) for HF diets than for HC diets, which supports previous studies (Ranilla and Carro, 2003; Martínez et al., 2009). The type of forage did not affect detachment efficiency, recovery of detached bacteria, or total recovery ($P = 0.50$, 0.80, and 0.71, respectively).

Although several studies have investigated the efficiency of different DP, to the best of our knowledge the effects of DP on the profile of the bacterial communities of the pellets obtained have only been tested in a previous study conducted by our group with Rusitec

fermenters and using the PCR-single strand conformation polymorphism approach (Martínez et al., 2009). The PCR-single strand conformation polymorphism analysis involves gel electrophoresis, and therefore only a limited number of samples can be processed at one time. In contrast, ARISA analysis allows rapid analysis of a large number of samples, and it has been recently used to monitor shifts in rumen microbial populations (Stebulis et al., 2008). The number of peaks detected in the ARISA electropherograms of digesta samples was 72, 78, 56, and 61 for HFA, HCA, HFG, and HCG diets, respectively. Lower numbers of peaks were detected in the ARISA electropherograms of the bacterial pellets, averaging 43, 47, 25, and 29 for HFA, HCA, HFG, and HCG diets, respectively. The similarity index between bacterial communities attached to digesta and those in the pellets ranged from 40.1 to 65.6%, and was greater ($P = 0.006$) for STO and MET compared with FRE

Figure 2. Dendrograms of automated ribosomal intergenic spacer analysis profiles of bacterial communities attached to ruminal digesta from sheep fed different diets (HFA = 70:30 alfalfa hay:concentrate; HCA = 30:70 alfalfa hay:concentrate; HFG = 70:30 grass hay:concentrate; HCG = 30:70 grass hay:concentrate, all DM basis.) and those in the pellet obtained after different detachment procedures [MET = digesta incubated in 0.9% saline 0.1% methylcellulose (38°C, 15 min); STO = digesta suspended in 0.9% saline solution and treated with stomacher (230 rpm, 5 min); FRE = digesta frozen (−20°C, 72 h), thawed (4°C, 24 h), and treated as in STO]. All dendrograms correspond to sheep 1.

pellets. However, a trend ($P = 0.09$) to a significant forage $\times$ DP interaction was detected, and differences between bacterial pellets were only detected for diets containing alfalfa hay. The results suggest that freezing could have lysed some bacterial species, thus reduc-

ing the bacterial diversity present in the pellets. This is also indicated by the values of the Shannon index, a measurement of bacterial diversity (Table 1). Averaged across diets, Shannon index was lower for FRE compared with MET ($P = 0.02$) and STO ($P = 0.009$). Both the similarity index and the Shannon index were greater ($P < 0.001$) for diets containing alfalfa hay compared with diets containing grass hay, which would indicate that feeding alfalfa hay promoted a greater diversity of bacterial communities attached to digesta.

Similar results were shown by the dendrograms based on the matrix of similarity. Figure 2 shows the dendrograms of the ARISA profiles of bacterial communities in the ruminal digesta and in the pellets obtained by the 3 DP in sheep 1 as an example. For diets containing alfalfa hay, digesta and STO- and MET-pellets formed a cluster with a 0.56 and 0.54 correlation for diets HFA and HCA, respectively, and FRE-pellets clustered separately. A different clustering pattern was observed for diets containing grass hay. For diet HFG, the 3 pellets formed a group with a 0.62 correlation, but the digesta sample clustered separately. For HCG diet, digesta and STO-pellet formed a cluster with a 0.57 correlation and FRE- and MET-pellets formed a different group with a 0.55 correlation.

Our results indicate a moderate similarity between the bacterial communities attached to digesta and those detached and recovered in the bacterial pellets, with MET and STO pellets more representative than FRE for diets including alfalfa hay. In a previous study conducted by our group with digesta from Rusitec fermenters fed the HCA diet, Martínez et al. (2009) found no differences between MET and STO treatments in the similarity index between bacterial communities attached to digesta and those in the pellets, but values were significantly lower ($P < 0.03$) for FRE. The values of similarity index found in the present study for the HCA diet were lower than the 85, 80, and 70% found by Martínez et al. (2009) for MET, STO, and FRE, respectively, despite the use of the same DP and diet in both studies. This is in accordance with the greater detachment (61%) and recovery from detached bacteria (90%) values found by Martínez et al. (2009). One factor that could help to explain the differences is the time of sampling, because digesta is removed daily after 48 h of incubation in Rusitec fermenters, and microbial attachment could be less tenacious at this time than soon after feeding (Leedle et al., 1982). In agreement with this hypothesis, Craig et al. (1987) and Olubobokun and Craig (1990) observed that the proportion of SAB recovered from ruminal digesta in cattle increased with time after feeding, with the lowest recoveries observed 1 to 3 h after feeding. In addition, modifications in the bacterial populations are produced in Rusitec ferment-

ers over the incubation period (Prevot et al., 1994), and probably bacterial communities in the fermenters were different from those in the rumen of sheep.

Effects of Detachment Procedure and Diet on Composition of Bacterial Isolates and Duodenal SAB Flow

The N content of SAB (Table 3) was in the range of the values previously reported in the literature for SAB detached from ruminal digesta in sheep (Martín-Orúe et al., 1998) and cows (Clark et al., 1992). Pellets detached from HF digesta had greater ($P < 0.01$) N content and ^{15}N enrichment (66.8 mg of N/g of DM and 0.0751 atoms % in excess) compared with those detached from HC digesta (63.9 and 0.0536) which agrees with previous work showing that in ruminal bacteria the N content declined as concentrate in the diet increased (McAllan and Smith, 1977). The greater ^{15}N enrichment observed for pellets detached from HF digesta could be the result of the greater enrichment of the ammonia pool observed in HF fed sheep compared with those found in sheep fed HC diets (0.2154 and 0.1623 atoms% in excess, respectively). Averaged across diets, FRE pellets showed lower N content and ^{15}N enrichment than STO ($P = 0.02$ and 0.04) and MET ($P < 0.001$ and 0.002) pellets (Table 3). The ^{15}N enrichment in microbes depends on their ability to use free ammonia as the N source and on the ^{15}N enrichment of the ammonia-N fraction. Because DP were applied to the same digesta sample, the observed differences cannot be attributed to differences in ammonia- ^{15}N enrichment. The proportion of bacterial N derived from ammonia- ^{15}N was greater ($P < 0.001$) for MET-pellets compared with those obtained using FRE, and tended to be greater ($P = 0.06$) compared with STO-pellets. This is in agreement with previous results from our group (Ranilla and Carro, 2003; Martínez et al., 2009) showing greater ammonia- ^{15}N incorporation by SAB isolated using MET compared with SAB obtained with other DP.

The mean proportion of bacterial DM in the solid ruminal digesta ranged from 19.1 to 32.8% (Table 3), and was in the range of the values reported in the literature (Craig et al., 1987; Legay-Carmier and Bauchart, 1989; Yang et al., 2001). In agreement with others (Yang et al., 2001), the values were lower for HF compared with HC diets ($P < 0.001$; mean values of 21.2 and 27.9%), but they were not affected by the type of forage ($P = 0.50$). Yang et al. (2001) pointed out that the greater bacterial colonization of digesta found when ruminants are fed HF diets probably reflects the increased activity of cellulolytic bacteria caused by greater ingestion of

forage particles. Averaged across diets, the proportion of bacterial DM in the solid digesta was greater for FRE compared with STO ($P = 0.04$) and MET ($P = 0.002$) pellets, with no differences between STO and MET ($P = 0.31$).

As shown in Table 3, the use of the pellets obtained by the different DP affected the estimates of microbial duodenal flow using SAB as reference bacteria ($P = 0.006$) and efficiency of microbial growth ($P = 0.02$). Averaged across diets, the estimates of microbial duodenal flow with FRE treatment were 1.18 and 1.11 greater ($P = 0.002$ and 0.03) than those obtained with MET and STO (Table 3). Similar results were obtained for the efficiency of microbial growth; the use of FRE pellets as reference resulted in greater ($P \leq 0.02$) values of efficiency compared with MET and STO. Although there were differences between DP in the estimates of microbial duodenal flow and its efficiency, all of them detected similar differences among diets and therefore did not influence the interpretation of results. For all DP, the diet HFG promoted the lowest ($P < 0.001$ to 0.03) duodenal microbial flow, with no differences ($P = 0.18$ to 0.96) between HFA, HCA, and HCG diets.

CONCLUSIONS

Results showed that the treatment of ruminal digesta from sheep with STO combined with homogenizing and chilling at 4°C for 24 h removed about 64 and 68% of SAB for HF and HC diets, respectively, but total recovery was only 51 and 46%. As assessed by ARISA, the similarity index between the bacterial communities attached to digesta and those in the pellets obtained after each detachment procedure indicated that STO and MET were the methods that produced a more representative pellet, with no significant differences between them. However, similarity index values for STO and MET were only 64 and 44% for diets containing alfalfa and grass hay, respectively. Further research combining STO and MET or other detachment procedures is needed to develop a method with both high recovery and improved representativeness of the bacterial communities attached to digesta. The DP affected N content and ^{15}N enrichment of SAB, and therefore influenced estimations of duodenal SAB flow. Values of duodenal microbial N flow estimated using SAB as reference bacteria were 1.2 and 1.1 times greater with FRE compared with STO and MET, respectively. In this study, all DP detected similar differences between diets and therefore did not influence the interpretation of results, but the differences observed among DP in microbial N flow would indicate that ranking of treatments might be altered under different conditions.

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