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Effect of diet and absence of protozoa on the rumen microbial community and on the representativeness of bacterial fractions used in the determination of microbial protein synthesis¹

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ABSTRACT: Accurate estimates of microbial synthesis in the rumen are vital to optimize ruminant nutrition. Liquid- (LAB) and solid-associated bacterial fractions (SAB) harvested from the rumen are generally considered as microbial references when microbial yield is calculated; however, factors that determine their composition are not completely understood. The aim of this study was to evaluate the effect of diet and absence or presence of rumen protozoa on the rumen microbial community. It was hypothesized that these treatments could modify the composition and representativeness of LAB and SAB. Twenty twin lambs (*Ovis aries*) were used; one-half of the twins were kept protozoa-free, and each respective twin sibling was faunated. At 6 mo of age, 5 animals from each group were randomly allocated to the experimental diets consisting of either alfalfa hay as the sole diet, or 50:50 mixed with ground barley grain. After 15 d of adaptation to the diet, animals were euthanized, rumen and abomasum contents were

sampled, and LAB and SAB isolated. The presence of protozoa buffered the effect of diet on the rumen bacterial population. Faunated animals fed alfalfa hay had a greater abundance of *F. succinogenes*, anaerobic fungi and methanogens, as well as an enhanced rumen bacterial diversity. Cellulolytic bacteria were more abundant in SAB, whereas the abomasal abundance of most of the microorganisms studied was closer to those values observed in LAB. Rumen and abomasal samples showed similar bacterial DNA concentrations, but the fungal and protozoal DNA concentration in the abomasum was only 69% and 13% of that observed in the rumen, respectively, suggesting fungal and protozoal sequestration in the rumen or possible preferential degradation of fungal and protozoal DNA in the abomasum, or both. In conclusion, absence of protozoa and type of diet extensively modified the chemical composition of LAB and SAB as a consequence of changes in the microbial composition of these fractions.

Key words: bacterial structure, denaturing gradient gel electrophoresis, microbial synthesis, quantitative polymerase chain reaction, rumen protozoa, terminal restriction fragment length polymorphism

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INTRODUCTION

Microbial protein synthesized in the rumen often represents the main source of AA for the ruminant (AFRC, 1993). However, estimates of microbial

yield rely on marker techniques in which the microbial marker flow must be determined at postruminal sites. Moreover, a microbial reference, sampled from the rumen, must be isolated to establish the marker/N ratio. Conventionally, liquid- (**LAB**) and solid-associated bacteria (**SAB**) harvested from the rumen have been used as microbial references (Pérez et al., 1998), but factors that determine the chemical composition of these fractions are not completely understood. The extent to which these microbial fractions represent microorganisms that flow out from the rumen is therefore

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crucial to the precision of the whole estimation procedure.

Rumen protozoa interact with other microbes in the rumen (i.e., bacteria, anaerobic fungi, and methanogens) and can modify the structure of the rumen microbial ecosystem (Yáñez-Ruiz et al., 2006; Mosoni et al., 2011). However, there is little evidence as to whether these changes are due to protozoal predatory activity (Newbold et al., 1989) or protozoa-substrate interactions, in which case, it would be modulated by the diet.

In a companion paper (Belanche et al., 2011a), we demonstrated that rumen protozoa and diet exert an important effect on rumen fermentation, digestibility, and microbial protein synthesis. However, figures of microbial synthesis were variable depending on the bacterial isolate and microbial marker considered (i.e., ^{15}N , purine bases or DNA sequences). Thus, the aim of this study was to investigate the effect of protozoa and diet on the rumen microbial populations. It was hypothesized that i) fermentation pattern and animal performance are related to the rumen microbial structure, and ii) the estimations of the microbial synthesis would be affected by the microbiological composition of the fractions used as reference.

MATERIALS AND METHODS

Experimental animals were handled by trained personnel, and experimental protocols were approved by the Ethical Committee for Animal Research of the University of Zaragoza (Ref. PI13/05).

Animals and Diets

Twenty Rasa Aragonesa, twin lambs were used. One of each twin was isolated from its mother within the first 24 h after lambing, fed with commercial milk replacer, and maintained protozoa-free (PF) by avoiding protozoa transmission from adult animals. Their siblings (control) remained with their mothers and in contact with the flock to allow the rumen protozoal colonization. At 45 d, all lambs were weaned and at 6 mo of age, both groups were subdivided into 2 subgroups ($n = 5$; 3 males and 2 females) and randomly allocated to 1 of 2 experimental diets, such that each pair of twins received the same diet. Experimental diets consisted of alfalfa (*Medicago sativa* L.) hay (in g/kg DM: OM 900, CP 164, ether extract 17, NDF 321, ADF 259 and ADL 59) given either as sole diet (ALF) or mixed (MIX) at 50:50 ratio with ground barley (*Hordeum vulgare* L.) grain (in g/kg DM; OM 977, CP 104, ether extract 20, NDF 157, ADF 51 and ADL 8.6). Diets were offered at 1.5 maintenance (AFRC, 1993), animals had free access to fresh water and vitamin-mineral premix (Norblob, NOREL S.A., Madrid, Spain)

and were fed every 6 h to minimize diurnal changes in rumen fermentation and digesta composition.

Experimental Design and Sample Collection

After diet adaptation (2 wk), individual intakes were recorded over 1 wk, and ^{15}N -ammonium sulphate (42.7 mg $^{15}\text{N}/\text{d}$, Cambridge Isotope Laboratories, Inc., Andover, MA) was orally administered every 6 h to label microbial rumen populations. Lambs were euthanized 2 h after feeding (BW = 41 ± 7.0 kg) by intravenous Thiopental administration (10 mg/kg BW Tiobarbital, BRAUN Medical S.A., Barcelona, Spain), and the digestive tract was removed and sampled as described by Askar et al. (2007). Total rumen or abomasal contents were homogenized and 2 samples (0.3 g fresh matter) were frozen in liquid N for DNA analysis. Ruminal contents were strained through 2 cheesecloth layers and sampled: 1 sample (5 mL) was diluted with 5 mL of 18.5% (vol/vol) formaldehyde and used for microscopic protozoa counts, and the remaining filtrate was divided into 2 fractions for LAB and protozoa isolation following the protocols described by Pérez et al. (1998) and Sylvester et al. (2004), respectively. Isolation of SAB from solid digesta was conducted using carboxymethyl cellulose to detach attached microbes (Martin-Orue et al., 2000), because previous work has shown that this gives a representative SAB population (Ramos et al., 2009). Microbial fractions were sampled (0.3 g fresh matter) for DNA analysis, and the remaining sample was frozen and freeze-dried for subsequent analysis.

Chemical Analysis and DNA Extraction

The N content of microbial fractions was determined by the Kjeldahl method using Se as the catalyst (AOAC, 2005). Purine base (PB) concentration was determined following the methods proposed by Balcells et al. (1992), and the isotope abundance of ^{15}N was determined using mass spectrophotometry (VG PRISM II, IRMS connected in series to DUMAS-style N analyser EA 1108, Carlo Erba, Milan, Italy). Protozoa counts were done as described by Dehority (1993). Genomic DNA was extracted from frozen samples using the QIAamp DNA Stool Mini Kit (Qiagen Ltd., Crawley, West Sussex, UK) and following the manufacturer's instructions. The only protocol modification was the increase of the temperature of incubation with lysis buffer to 95°C for 10 min to maximize the cell lyses. Nucleic acids were extracted in duplicate from rumen and microbial fractions and in triplicate from abomasal samples, then they were pooled to maximize the microbial diversity within each fraction. The concentration of DNA was assessed by spectrophotometer (NanoDrop, Thermo Scientific, Wilmington, DE), then corrected according to sample weight and dilution during extraction.

Table 1. Authors and sequences of primers used in the present experiment

Target	Author	Primers		Size, bp
		Forward ¹	Reverse	
TRFLP ² bacteria	Amann et al. (1990)	AGAGTTTGATCCTGGCTCAG	ACGGGCGGTGTGTRC	1350
DGGE ³ bacteria	Nubel et al. (1996)	ACGGGGGCTACGGGAGGCAGCAG	ATTACCGGCTGCTGG	200
DGGE protozoa	Regensbogenova et al. (2004)	GGTGGTGCATGGCCG	AATTGCAAAGATCTATCCC	300
Quantitative PCR				
Total bacteria	Maeda et al. (2003)	GTGSTGCAYGGYTGTCTGCA	ACGTCRTCCMCACCTTCTC	150
<i>R. albus</i>	Koike and Kobayashi (2001)	CCCTAAAAGCAGTCTTAGTTCG	CCTCCTTGGGTTAGAACA	175
<i>R. flavefaciens</i>	Tajima et al. (2001)	GGACGATAATGACGGTACTT	GCAATCYGAACTGGGACAAT	835
<i>F. succinogenes</i>	Koike and Kobayashi (2001)	GGTATGGGATGAGCTTGC	GCCTGCCCTGAACTATC	446
<i>P. ruminicola</i>	Tajima et al. (2001)	GGTTATCTTGAGTGAGTT	CTGATGGCAACTAAAGAA	485
<i>S. ruminantium</i>	Tajima et al. (2001)	TGCTAATACCGAATGTTG	TCCTGCACTCAAGAAAGA	513
<i>S. bovis</i>	Stevenson and Weimer (2007)	TTCCTAGAGATAGGAAGTTCTTCGG	ATGATGGCAACTAACAAATAGGGGT	127
Methanogens	Denman et al. (2007)	TTCGGTGGATCDCARAGRGC	GBARGTCGWAWCCGTAGAATCC	140
Anaerobic fungi	Denman and McSweeney (2006)	GAGGAAGTAAAAGTCGTAACAAGTTTC	CAAATTCACAAAGGTTAGGATGATT	120
Total protozoa	Sylvester et al. (2005)	GCTTCGWTGGTAGTGATT	CTTGCCCTCYAATCGTWCT	223
<i>Entodiniinae</i>	Skillman et al. (2006)	GAGCTAATACATGCTAAGGC	CCCTCACTACAATCGAGATTAAGG	283

¹T (terminal)-RFLP forward primers labeled with Cyanine 5 at the 5' end.

²TRFLP = terminal restriction fragment length polymorphism.

³DGGE = denaturing gradient gel electrophoresis.

T-RFLP and DGGE

Rumen bacterial biodiversity was evaluated by terminal restriction fragment length polymorphism (T-RFLP) as described by Liu et al. (1997). Briefly, PCR was performed in a final volume of 25 μ L, with 5 μ L of 5 \times buffer, 0.5 μ L of deoxynucleotide triphosphate (10 mM each), 1.75 μ L of MgCl₂ (25 mM), 1.25 U of Go Taq Flexi DNA polymerase (Promega, Southampton, UK), 1 μ L of template (75 ng DNA), and 12.5 pmol of each of the universal bacterial primers described in Table 1. The conditions were set as follows: 95°C for 4 min followed by 25 cycles of 55°C for 1 min, 72°C for 1 min, and 95°C for 1 min; and a final step of 30 min at 72°C to complete the elongation and avoid false positive peaks (Popa et al., 2009). The PCR product was visualized on a 1% agarose gel, purified (Millipore MultiScreen PCR_{μ96} plate PCR plate with -6.8×10^4 Pa of Hg vacuum) and concentration determined within each sample. Purified PCR products (75 ng) were digested by 1 of 4 restriction enzymes (*Hae*III, *Msp*I, *Hha*I and *Rsa*I; New England Biolabs, Hitchin, UK) at 37°C for 5 h followed by an inactivation cycle of 20 min at 80°C. The restricted DNA was cleaned by ethanol precipitation, resuspended in Sample Loading Solution (Beckman Coulter, High Wycombe, UK) containing a 600 bp size standard. Finally the plate was run on the CEQ 8000 Genetic Analysis System (Beckman Coulter) and the terminal restriction fragments (TRF) were separated using the Frag4 parameters. To remove the smaller peaks detected/noise and to increase repeatability, peaks with an area smaller than 0.25% of the sum of all peak areas were not considered. The mean number of electropherogram signals presented per restriction enzyme was considered as an indicator of the bacterial species richness.

Cluster analysis was performed using the unweighted pair group method with arithmetic mean grouping method (UPGMA) and the Bray-Curtis distance using R statistics software (version 2.10.0). Shannon's diversity index (the proportional abundance of species in a community) was determined as follow $H' = -\sum p_i \times \ln(p_i)$, where p_i is the proportion of signal in respect to the total.

Changes in the bacterial and protozoal community between the different sample origins (i.e., rumen, abomasum, LAB and SAB) were evaluated by denaturing gradient gel electrophoresis (DGGE). Three DGGE gels were run following the protocol described by Belanche et al. (2010a); 1 gel compared the rumen and abomasal protozoal diversity in Control lambs, and 2 gels compared the bacterial diversity in rumen, abomasum, LAB and SAB (6 animals per gel). Each band position present in the gel was binary coded (presence or absence within a lane) and the DGGE profile was analyzed by UPGMA cluster analysis and Hamming distance using R statistics software.

Quantitative PCR

Quantitative PCR was performed using the procedure and equipment described by Belanche et al. (2010a). Briefly, the absolute concentration of total bacteria, total protozoa, methanogens, and anaerobic fungi in the ruminal and abomasal content was determined by amplifying serially diluted DNA standards (from 10⁻¹ to 10⁻⁵). A total bacterial DNA standard was generated from a mix of equal volumes of genomic DNA from LAB and SAB from all experimental animals. A protozoal DNA standard was generated pooling protozoal extracts, and its true protozoal DNA concentration was estimated after having been quan

tified and subtracted from the bacterial DNA (Belanche et al., 2011b). An anaerobic fungal DNA standard was prepared with equal amounts of genomic DNA from 3 different axenic cultures of anaerobic fungi [*Neocallimastix frontalis* (R_{E1}), *Orpinomyces* sp. SR2, and *Caecomycetes* sp. Isol1]. Finally, a methanogens DNA standard was based on the *mcrA* gene (140 bp) inserted into a plasmid pCR-TOPO (Invitrogen, Carlsbad, CA). Gene copies present in each plasmid extract were calculated using the plasmid DNA concentration and the molecular weight of the vector with the insert. Cycling conditions for bacterial quantification were 95°C for 10 min and 30 cycles of: 61°C for 30 s, 72°C for 30 s and 95°C for 15 s, and for protozoal quantification were 95°C for 10 min and 40 cycles of: 55°C for 20 s, 72°C for 30 s, and 94°C for 10 s. For the rest of the microorganisms studied, common cycling conditions of 95°C for 10 min and 45 cycles of: 55°C for 30 s, 72°C for 30 s, and 95°C for 15 s were used. A negative control was loaded to screen for possible contamination and primer dimer formation; moreover, the efficiency of real-time PCR amplification was checked in every plate, and only reactions with an efficiency of amplification ($E = 10^{-1/\text{slope}} - 1$) close to 100% were considered valid and comparable.

Calculations and Statistical Analysis

Relative abundance of *Ruminococcus albus*, *Ruminococcus flavefaciens*, *Fibrobacter succinogenes*, *Prevotella ruminicola*, *Selenomonas ruminantium* and *Streptococcus bovis* in respect to total bacteria was estimated using the ΔC_T method proposed by Pfaffl (2001) where the conserved 16S ribosomal DNA was considered as the reference gene (or protozoal 18S rDNA for *Entodiniinae*). Anaerobic fungi and methanogens are not included in the eubacteria domain, but their numbers in the abomasum, LAB, and SAB were expressed in respect to the total bacteria to make data comparable between different sample origins.

The relative abundance of every population was calculated after data normalization according to the efficiency of amplification (E):

$$\% \text{ species} = 100 \times \frac{E^{\text{Ct total bacteria}}}{E^{\text{Ct target}}}$$

Rumen derived data were analyzed by ANOVA according to the following model:

$$Y_{ijkl} = \mu + \text{Pr}_i + \text{D}_j + (\text{Pr} \times \text{D})_{ij} + \text{T}_k + \text{L}(\text{T})_{kl} + e_{ijkl}$$

where Y_{ijkl} is the dependent, continuous variable; μ is the overall population mean; Pr_i is the fixed effect of the presence of protozoa ($i = \text{PF}$ or control), D_j is the fixed effect of the diet ($j = \text{ALF}$ or MIX), $(\text{Pr} \times \text{D})_{ij}$ is the fixed ef-

fect of the interaction between protozoa and diet, T_k is the random effect of the twin pair ($k = 1$ to 10), $\text{L}(\text{T})_{kl}$ is the random effect of lamb-within-twin pair ($l = 1$ or 2), and e_{ijkl} is the residual error. When different types of samples were compared, data were analyzed using the same model, but included sample type (S_m , where $m = \text{abomasum}$, LAB, or SAB) and its interactions as fixed effects, whilst sample-within-twin pair [$\text{S}(\text{T})$], and sample-within-lamb-within-twin pair [$\text{S}[\text{L}(\text{T})]$] were random:

$$Y_{ijklm} = \mu + \text{Pr}_i + \text{D}_j + \text{S}_m + (\text{Pr} \times \text{D})_{ij} + (\text{Pr} \times \text{S})_{im} + (\text{D} \times \text{S})_{jm} + (\text{Pr} \times \text{D} \times \text{S})_{ijm} + \text{T}_k + \text{L}(\text{T})_{kl} + \text{S}(\text{T})_{km} + \text{S}[\text{L}(\text{T})]_{klm} + e_{ijklm}$$

When the interaction was significant, treatment means were compared by a post-hoc *t*-test. Residual plots were inspected for constant variance and normality, and if these assumptions failed, data were log-transformed or analyzed by the nonparametric Mann-Whitney test. Findings with $P < 0.05$ were regarded as statistically significant, and $0.05 < P < 0.10$ was accepted as a tendency to differences. Data were analyzed using Genstat (VSN International Ltd., Hemel Hempstead, Herts, UK). Rumen bacterial distribution derived from T-RFLP data were analyzed by nonparametric permutational multivariate analysis of variance (PERMANOVA) using the Adonis vegan option in R statistics software (v. 2.10.0). A PERMANOVA R-value was determined for each treatment effect by evaluation against 1,000 random permutations of the Bray-Curtis similarity values (Anderson, 2001). Probability values represent the proportion of R-values that exceeded the values from nonrandomized comparisons.

RESULTS

Rumen Microbial Ecosystem

All primer sets generated a single PCR product, and efficiencies of amplification were always between 90 and 110%, except for *R. flavefaciens* and anaerobic fungi, for which efficiencies ranged between 75 and 90%. No protozoa cells were detected in PF lambs, but barley supplementation increased the concentration of protozoal cells ($P < 0.001$) and protozoal DNA ($P = 0.005$) in control lambs. *Entodiniinae* subfamily accounted for practically the entire protozoal population, independent of the diet and the quantification method ($99.5 \pm 0.4\%$ or $98.0 \pm 6.3\%$ by optical counting or quantitative PCR, respectively), whereas the *Dipodiniinae* subfamily was present at a low concentration ($0.5 \pm 0.4\%$). The presence of rumen protozoa increased the numbers of anaerobic fungi ($P = 0.028$) and methanogens ($P = 0.043$). In contrast, the MIX diet had a negative effect on anaerobic fungi ($P = 0.005$) and methanogen numbers ($P = 0.020$), this effect being especially obvious in control animals for the

later microbial group ($\text{Pr} \times \text{D}$, $P < 0.001$). Total bacterial DNA concentrations in the rumen were not affected by the experimental treatments; however, the proportions of the different species studied were affected. Absence of rumen protozoa increased the proportion of *R. albus* ($P = 0.004$), *P. ruminicola* ($P = 0.004$), and *S. bovis* ($P = 0.027$), but decreased the proportion of *S. ruminantium* ($P = 0.072$). Barley supplementation increased the abundance of *S. ruminantium* ($P = 0.016$), and control lambs fed ALF showed the greatest abundance of *F. succinogenes* ($\text{Pr} \times \text{D}$, $P = 0.013$).

The T-RFLP derived dendrogram (Figure 1) showed that rumen content from control and PF animals had a different bacterial population (protozoa effect PERMANOVA, $R^2 = 0.243$, $P = 0.001$), and they clustered as 2 independent groups. Within groups, PF lambs presented lower between-animal dissimilarities than control lambs. Type of diet also had a significant effect on the rumen bacterial population (diet effect PERMANOVA, $R^2 = 0.101$, $P = 0.002$); however, this effect was more pronounced in the PF group (PERMANOVA for $\text{Pr} \times \text{D}$, $R^2 = 0.082$, $P = 0.012$). A differential effect of diet was observed in the bacterial diversity indexes dependent on the faunation status; ALF increased the TRF numbers ($\text{Pr} \times \text{D}$, $P = 0.058$) and Shannon index ($\text{Pr} \times \text{D}$, $P = 0.090$) in the control group, whereas MIX increased this figures in the PF group.

Rumen Microbial Contribution to Abomasal Flow

Concentration of total bacterial DNA in rumen and abomasal fluid (Figure 2) was not modified by the diet, the absence of rumen protozoa, or the sampling site. Abomasal concentrations of protozoal and anaerobic fungal DNA were less than those in the rumen ($P = 0.001$ and $P = 0.022$, respectively), whereas the opposite happened with methanogens ($P = 0.012$). Protozoal DNA concentrations increased when animals were fed the MIX diet ($P = 0.005$), independent of the sampling site considered. Concentrations of anaerobic fungal DNA were enhanced by the presence of rumen protozoa ($P = 0.003$) or ALF consumption ($P = 0.003$). Similarly, the greatest methanogen concentration was found in control animals fed ALF ($\text{Pr} \times \text{D}$, $P < 0.001$).

The general structure of the protozoal population in the rumen and abomasum from control animals was studied by DGGE (Figure 3a). The set of primers used produced an average of 9.1 bands per animal. Most of the protozoal bands detected in the rumen content ($91 \pm 6.6\%$) were also found in the abomasal sample from the same animals; therefore, both sites showed a high degree of similarity ($>75\%$). However, the protozoal Shannon index was less in abomasum than in rumen (2.00 vs. 2.34, $P = 0.014$). The protozoal banding profile was not clearly affected by the diet, but animals fed MIX

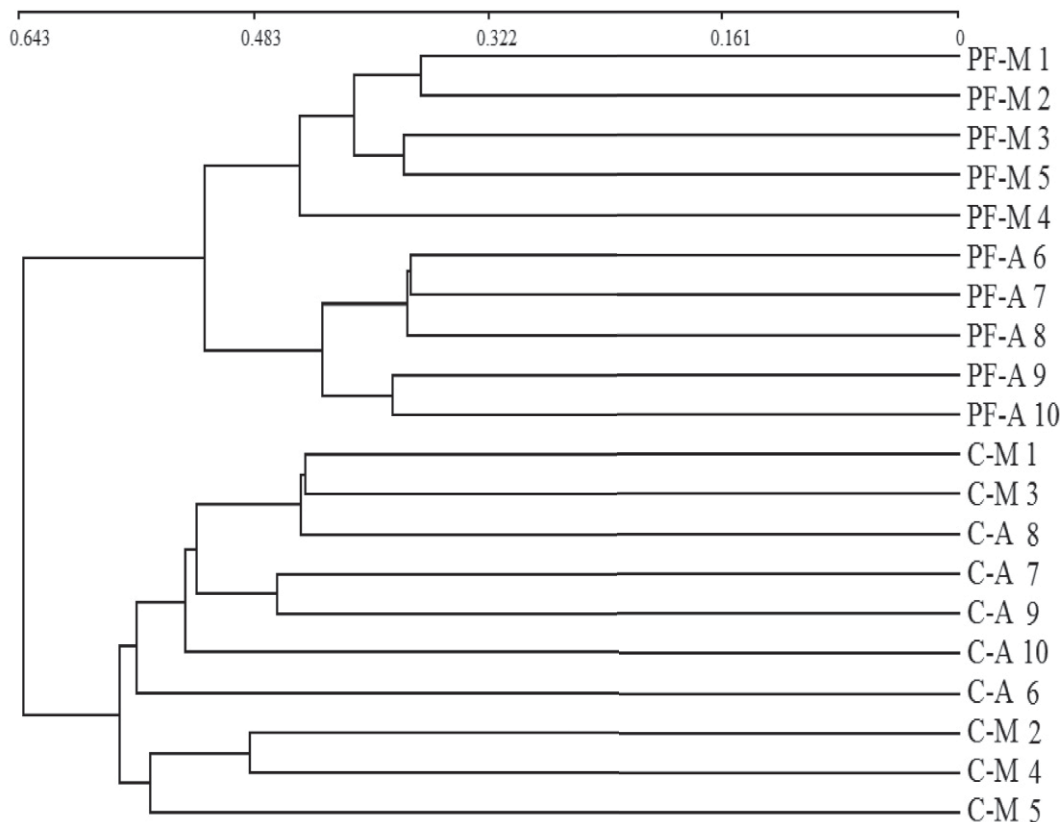


Figure 1. T-RFLP derived dendrogram illustrating the effect of diet (A = alfalfa hay; M = mix of alfalfa and barley) and absence of rumen protozoa (C = control; PF = protozoa-free) on the rumen bacterial population (16S rDNA gene). The scale bar shows Bray-Curtis dissimilarity distances and twin lambs are depicted with the same number.

showed a greater number of DGGE bands than those fed ALF (10.5 vs. 7.6 bands, $P = 0.029$). Quantitative PCR showed that the relative abundance of *Entodiniinae* in abomasal content was similar to that observed in the rumen ($94.4 \pm 11.6\%$) and was not affected by the diet.

Microbial Fractions Composition and Representativeness

A detailed description of the effect of diet and absence of rumen protozoa on LAB and SAB composition was provided in the companion paper (Belanche et al., 2011a). Briefly, LAB had a greater ^{15}N enrichment than SAB (2.20 mg/g vs. 2.05 mg/g, $P < 0.001$) but decreased PB/N (2.25 mmol/g vs. 2.85 mmol/g, $P < 0.001$) and DNA/N ratios (0.10 g/g vs. 0.14 g/g, $P = 0.012$). The presence of rumen protozoa decreased the ^{15}N enrichment (2.47 mg/g vs. 1.77 mg/g, $P = 0.021$) and increased the DNA/N ratio (0.10 g/g vs. 0.13 g/g, $P = 0.039$) in both bacterial fractions. This dietary effect on DNA/N ratio was particularly obvious in animals fed MIX diets (0.15 g/g vs. 0.09 g/g, $P = 0.04$). These bacterial fractions had also greater ^{15}N enrichment (2.34 mg/g vs. 1.91 mg/g, $P < 0.015$) and PB/N ratio (2.73 mmol/g vs. 2.37 mmol/g, $P < 0.001$) in animals fed MIX than ALF diets.

Regarding to the protozoal extracts composition, the PB content and ^{15}N enrichment averaged 139 $\mu\text{mol PB/g OM}$ and 1.14 mg/g and was not affected by the diet; however, the DNA/N ratio was greater in lambs fed MIX than ALF diet (from 0.14 to 0.23 mg/mg, $P = 0.007$).

The set of DGGE primers used to study the general bacterial structure in different sample sites generated 54 major different bands and an average of 20 ± 2.3 bands were observed per animal. Treatment effects on rumen bacterial structure visualized by DGGE (Figure 3b and 3c) showed a general agreement with the T-RFLP observations; but most importantly, DGGE showed that the bacterial banding profiles (and bacterial diversity index) did not differ between rumen, abomasum, and bacterial fractions within the same animal ($>75\%$ similarity).

Quantitative PCR was used to evaluate the representativeness of the LAB and SAB in respect to the microorganisms flowing out of the rumen to the abomasum. Eubacterial DNA was used as reference genes for comparing the different microbial groups. Abomasal numbers of microorganisms studied were comparable with those observed in rumen (Table 2) and also with those in the bacterial fractions (Table 3). Abomasum content and LAB had a similar abundance of the 3 cellulolytic bacteria studied (*R. albus*, *R. flavefaciens*, and *F. succinogenes*), but their abundance was greater in SAB ($P < 0.007$). In contrast, the abomasal abundance of *S. ruminantium* was similar to that observed in SAB and less than recorded in LAB ($P < 0.001$). Abomasal proportions of methanogens and anaerobic fungi were greater than those observed in bacterial fractions ($P < 0.001$), and the opposite was true with *P. ruminicola* and *S. bovis* ($P < 0.006$).

The effect of protozoa and diet on the concentrations of the different microorganisms studied in the bacterial fractions and abomasal samples (Table 3) were similar to effects described in rumen samples (Table 2). Absence of protozoa increased the concentration of *R. albus* ($P = 0.031$), especially in the SAB ($\text{Pr} \times \text{S}$, $P < 0.001$). Control and PF lambs fed ALF showed the greatest and least abundance of *F. succinogenes*, respectively ($\text{Pr} \times \text{D}$, $P = 0.010$), whereas control lambs fed MIX had the greatest proportion of *S. ruminantium* ($\text{Pr} \times \text{D}$, $P = 0.034$), especially in the LAB isolate ($\text{Pr} \times \text{D} \times \text{S}$, $P = 0.006$). Both the absence of rumen protozoa ($P = 0.026$) and barley supplementation ($P = 0.007$) increased the abundance of *S. bovis*, particularly in bacterial fractions ($\text{Pr} \times \text{D} \times \text{S}$, $P < 0.001$). Control animals fed ALF had the greatest abundance of methanogens ($\text{Pr} \times \text{D}$, $P < 0.001$), particularly in abomasal samples ($\text{D} \times \text{S}$, $P < 0.001$). Finally, the presence of protozoa ($P = 0.024$) and ALF ($P = 0.009$) increased the anaerobic fungi abundance, this effect being particularly obvious in abomasal samples ($\text{Pr} \times \text{S}$, $P = 0.034$ and $\text{D} \times \text{S}$, $P = 0.016$).

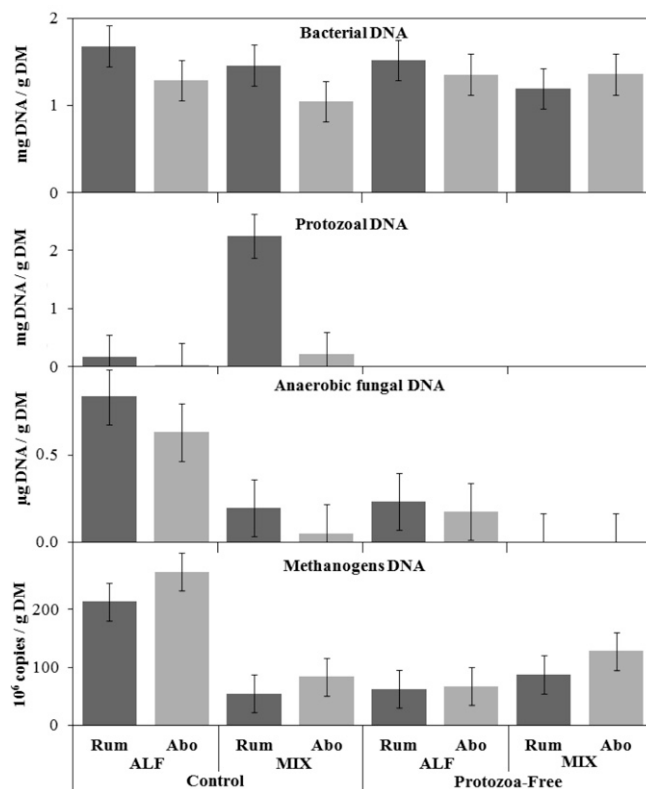


Figure 2. Effect of diet (ALF = alfalfa hay; MIX = alfalfa and barley), absence of rumen protozoa (control; protozoa-free) and sampling site (Rum = rumen; Abo = abomasum) on the rDNA concentration of bacteria, protozoa, anaerobic fungi, and methanogens. Animals were euthanized 2 h after feeding. Each value is the average of 5 observations; bars indicate the SED.

DISCUSSION

Methodological Approach

The protocol used was designed to minimize between-individual variation; therefore, the effect of the presence or absence of rumen protozoa was investigated using twin lambs. Isolation of newborn lambs from natural ciliate sources was used as a nonaggressive system to obtain PF animals compared with chemical defaunation methods. Circadian changes in digesta composition were minimized by increasing the number of meals (de Veth and Kolver, 2001) and single-time sampling procedures based on animal euthanasia allowed the collection of the total digesta content, making it feasible to describe rumen fermentation (Yáñez-Ruiz et al., 2007; Belanche et al., 2010a) and microbial synthesis (Askar et al., 2007; Belanche et al., 2011a).

Early studies, showed that the cultured bacteria population in the rumen ranges from 5 to 15% of the total direct cell count determined microscopically (Krause and Russell, 1996). Our experiment based on molecular analysis agrees with this, because the sum of the 6 bacterial species studied (currently considered as some of the most common in the rumen) represented <11% of the total bacterial DNA in the rumen; this suggests the existence of a vast group of microorganisms in the rumen that cannot be cultured. The success of molecular techniques depends on primer specificity and the coverage of different isolates within each target microorganism. The primer sets used in this study responded to both criteria. Some discrepancies, such as the incomplete amplification of some *F. succinogenes* groups (Mosoni et al., 2007) or the simultaneous

amplification of *S. ruminantium* and *Mitsuokella multacida* (Stevenson and Weimer, 2007), may bias our estimations in absolute terms, but do not prevent us from studying the dynamic responses to environmental changes.

Effect of Rumen Protozoa and Diet

The partial isolation of the control lambs at 3 mo of age may have resulted in an incomplete faunation; therefore *Entodinium*, which is one of the first protozoa to colonize the rumen (Belanche et al., 2010a), constituted almost the entire protozoal population. In a companion paper (Belanche et al., 2011a), the treatment effects on the rumen fermentation pattern and microbial synthesis were described; briefly, absence of protozoa reduced fiber digestibility, rumen ammonia, and VFA concentration, while increasing the efficiency of N utilization by rumen microorganisms. These effects have traditionally been attributed to protozoal fibrolytic activity (Agarwal et al., 1991) and a negative effect of ciliates on the rumen N metabolism (Koenig et al., 2000). Nevertheless, it remains unclear if these effects are attributable specifically to protozoal activity or represent some form of synergy between protozoa, bacteria, anaerobic fungi, and methanogens (Chaudhary et al., 1995). To this end, T-RFLP analysis showed that PF lambs contained a different rumen bacterial population to control lambs. Similar findings have been observed using DGGE (Yáñez-Ruiz et al., 2007) and 16S rDNA clone libraries (Ozutsumi et al., 2005). This latter author also observed an increase in bacterial richness in the presence of rumen protozoa. Under our experimental conditions,

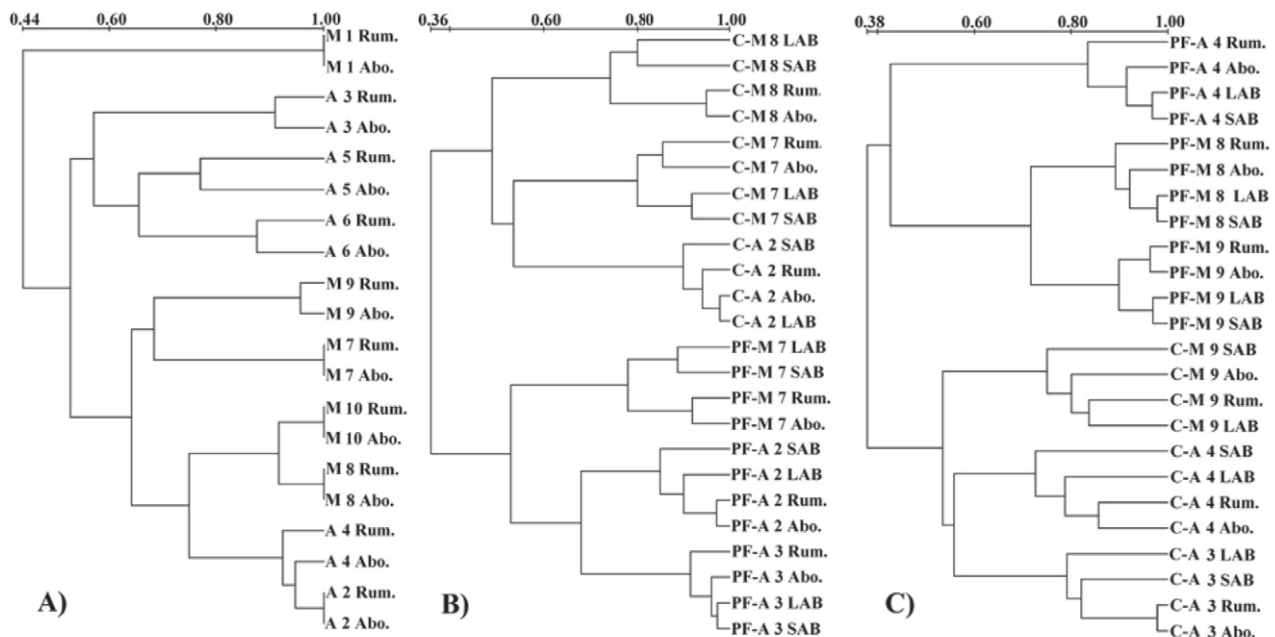


Figure 3. Denaturing gradient gel electrophoresis (DGGE) derived dendrograms illustrating the effect of diet (A = alfalfa hay; M = mix of alfalfa and barley), absence of rumen protozoa (C = control; PF = protozoa-free) and sampling site (Rum = rumen; Abo = abomasum; LAB = liquid-associated bacterial fractions; SAB = solid-associated bacterial fractions) on the protozoal (Panel A) and bacterial population (Panels B and C). The scale bars show Hamming distances and twin lambs are depicted with the same number.

however, this increased diversity was only observed when animals were fed ALF, because a more specialized and diverse bacterial community seems to be required to degrade fibrous diets in the absence of protozoa.

Defaunated animals usually have a greater concentration of rumen bacteria (per milliliter) determined by classical viable bacteria counts than faunated animals (Yáñez-Ruiz et al., 2009); nevertheless, these differences were not detected in terms of bacterial DNA concentration under our experimental conditions. The lower diet digestibility observed in PF than in control lambs (Belanche et al., 2011a) and the subsequent accumulation of undigested DM in the rumen could have diluted the bacterial DNA, maintaining similar bacterial numbers per gram of DM. *Ruminococcus albus*, *R. flavefaciens*, and *F. succinogenes* represent the 3 major cultivable cellulolytic bacteria in the rumen (Koike and Kobayashi, 2001). However, their joint abundance only represented 2 to 10% of the total bacteria in our animals; thus, a significant proportion of cellulose degradation in the rumen may be caused by other cellulolytic microorganisms, such as anaerobic fungi, protozoa, and novel bacterial species. In agreement with the

literature (Eugène et al., 2004; Yáñez-Ruiz et al., 2009), our findings showed no effect of defaunation on the abundance of the cellulolytic bacteria was detected. However, the defaunation effect seems to be modulated by diet (see below). *Prevotella* constitutes up to one-half of the bacterial 16S rDNA sequences in the rumen of dairy cows (*Bos taurus*), but the most common species appear to make only a minor contribution [i.e., *P. ruminicola* 1.7%, *P. bryantii* 1.4%, and *P. brevis* 0.14% (Stevenson and Weimer, 2007)]. This experiment confirmed such findings in sheep, and also demonstrated a negative relationship between rumen protozoa and ruminal numbers of *P. ruminicola*, *S. bovis*, and *R. albus*. A symbiotic relationship between *R. albus* and *P. ruminicola* has been described in co-cultures (Debroas and Blanchart, 1993), whereas competition for substrate (starch, protein, or both) and the accumulation of fermentation end-products (VFA and NH₃) may explain the negative effect of protozoa on amylolytic or proteolytic or both bacterial species (Ozutsumi et al., 2005). The presence of rumen ciliates tended to increase the abundance of other starch-fermenting bacteria such as *S. ruminantium*. This shift in bacterial species with a similar activity may reflect

Table 2. Effect of diet and absence of rumen protozoa on the abundance of some microorganisms and on bacterial diversity in the rumen

Item ¹	Control lamb diet		Protozoa-free lamb diet ²		SED ³	Significance ³		
	ALF	MIX	ALF	MIX		Pr	D	Pr × D
Optical counts								
Total protozoa, ⁴ 10 ⁵ cells/mL	0.37	13.89	ND	ND	4.520		<0.001	
Subfamily Entodiniinae, ⁵ %	99.3	99.8	ND	ND	0.507		0.68	
Subfamily Diplodiniinae, ⁵ %	0.74	0.22	ND	ND	0.507		0.68	
Absolute quantification								
1Bacterial DNA, mg/g DM	1.68	1.46	1.52	1.20	0.216	0.22	0.090	0.75
1Protozoal DNA, ⁴ mg/g DM	0.17	2.25	ND	ND	0.524		0.005	
Fungal DNA, µg/g DM	0.841	0.199	0.235	0.003	0.1882	0.028	0.005	0.21
Methanogens, ⁴ 10 ⁶ copies/gDM	213.4 ^a	54.7 ^b	63.0 ^b	87.8 ^b	35.05	0.043	0.020	<0.001
Relative abundance, 100 × 2 ^{-ΔCt}								
<i>R. albus</i> ⁴	0.024	0.041	0.182	0.132	0.0871	0.004	0.56	0.68
<i>R. flavefaciens</i> ⁴	2.304	0.905	1.496	2.041	0.8840	0.96	0.70	0.13
<i>F. succinogenes</i>	7.765 ^a	2.167 ^b	0.020 ^b	5.047 ^{ab}	2.4672	0.18	0.88	0.013
<i>P. ruminicola</i> ⁴	0.936	0.461	5.547	3.028	1.8541	0.004	0.104	0.80
<i>S. ruminantium</i>	0.320	3.981	0.001	1.397	1.0842	0.072	0.016	0.14
<i>S. bovis</i> ⁴	0.013	0.011	0.016	0.022	0.0037	0.027	0.57	0.21
Subfamily Entodiniinae ⁴	98.6	97.4	ND	ND	8.430		0.88	
Bacterial diversity								
TRF ⁶ per enzyme	56.6	53.5	50.0	54.0	2.552	0.095	0.83	0.058
Shannon index	3.33	3.18	3.16	3.30	0.1001	0.74	0.97	0.090

^{a-c}Means within a row without a common superscript letter differ ($P < 0.05$).

¹Twin lambs (1 faunated and 1 protozoa-free) were fed alfalfa hay (ALF) or 50:50 alfalfa:ground barley (MIX). Animals were euthanized 2 h after feeding and total rumen content was sampled. Each value is the average of 5 observations.

²ND = not detected.

³Considering presence of protozoa (Pr), diet (D), and Pr × D as fixed effects and twin pair and lamb-within-twin pair as random effects. SED is the error term for the Pr × D interaction ($n = 5$).

⁴Analyzed on log₁₀ transformed data to attain normality.

⁵Analyzed by Mann-Whitney nonparametric test.

⁶TRF = terminal restriction fragments.

a preferential uptake of specific bacterial groups by the protozoa, depending on the bacterial size and attachment to substrate (Orpin and Letcher, 1984).

Massive supplementation of the diet with concentrate feed has a detrimental effect on rumen protozoal concentrations as a consequence of ruminal acidification, but constrained use of cereals (as in the present experiment) which does not exceed the buffering capacity of the rumen (pH > 5.5) increased the rumen protozoal concentration and promoted a shift in the rumen bacterial population structure. In particular, this supplementation increased bacterial diversity in PF lambs, leading to substantial changes in the fermentation pattern (Belanche et al., 2011a), but the effect on control lambs

was less obvious. The capacity of protozoa to buffer dietary disruptions in the rumen, such as lactate accumulation, has been described (Hristov et al., 2001; Brossard et al., 2004) and may explain the greater microbial stability in the rumen of control lambs. In agreement with the T-RFLP data, quantitative PCR showed that the effect of diet on microbial numbers was less evident than the protozoal effect. Several studies have shown that, during the transition from forage to concentrate diets, fibrolytic bacteria generally become less prevalent and amylolytic bacteria increase (Weimer et al., 1999; Tajima et al., 2001); however, when transition is completed, the size of the various carbohydrate-utilizing microbial populations is remarkably constant in rumi-

Table 3. Effect of diet and absence of rumen protozoa on the relative abundance of certain rumen microorganisms in the abomasum, LAB and SAB. Data expressed relative to total bacteria as $100 \times 2^{-\Delta C_t}$

Item ¹	Control lamb diet		Protozoa-free lamb diet		SED ²	Significance ²						
	ALF	MIX	ALF	MIX		Pr	D	Pr × D	Sam	Pr × Sam	D × Sam	Pr × D × S
<i>R. albus</i> ³												
Abomasum	0.027	0.224	0.066	0.056	0.4034	0.031	0.25	0.28	<0.001	<0.001	0.53	0.16
LAB	0.011	0.024	0.206	0.121								
SAB	0.140	0.501	0.990	1.185								
<i>R. flavefaciens</i> ³												
Abomasum	0.521	0.595	0.303	0.225	1.5210	0.36	0.35	0.22	<0.001	0.22	0.63	0.11
LAB	0.748	0.768	0.293	1.309								
SAB	6.118	4.566	3.152	6.590								
<i>F. succinogenes</i>												
Abomasum	4.677	1.383	0.005	1.621	3.1670	0.28	0.99	0.019	0.007	0.95	0.60	0.029
LAB	5.218	1.475	0.003	2.542								
SAB	10.620	2.962	0.011	10.598								
<i>P. ruminicola</i> ³												
Abomasum	1.198	0.475	3.860	1.050	1.2650	0.14	0.13	0.61	0.006	0.32	0.12	0.22
LAB	2.780	1.280	2.938	1.346								
SAB	1.643	1.218	2.595	2.962								
<i>S. ruminantium</i>												
Abomasum	0.075	0.576	3 × 10 ⁻⁴	0.096	1.6540	0.017	0.011	0.034	<0.001	0.002	0.002	0.006
LAB	0.763	12.428	0.001	1.448								
SAB	0.161	1.976	0.001	0.688								
<i>S. bovis</i> ³												
Abomasum	0.012	0.017	0.014	0.009	0.0045	0.026	0.007	0.82	0.006	<0.001	0.014	<0.001
LAB	0.011	0.018	0.022	0.036								
SAB	0.011	0.017	0.013	0.041								
Methanogens ³												
Abomasum	0.647	0.164	0.053	0.124	0.1149	<0.001	0.015	<0.001	<0.001	0.13	<0.001	0.41
LAB	0.617	0.038	0.052	0.026								
SAB	0.302	0.111	0.053	0.092								
Anaerobic fungi												
Abomasum	0.371	0.080	0.078	0.001	0.0612	0.024	0.009	0.11	<0.001	0.034	0.016	0.34
LAB	0.039	3 × 10 ⁻⁴	0.010	1 × 10 ⁻⁴								
SAB	0.086	0.007	0.001	1 × 10 ⁻⁴								

¹Twin lambs (1 faunated and 1 protozoa-free) were fed alfalfa hay (ALF) or 50:50 alfalfa:ground barley (MIX). Animals were euthanized 2 h after feeding, abomasal content was sampled and liquid- (LAB) and solid-associated bacteria (SAB) isolated. Each value is the average of 5 observations.

²Considering presence of protozoa (Pr), diet (D), sample type (Sam), and their interactions (Pr × D, Pr × Sam, D × Sam, Pr × D × Sam) as fixed effects and twin pair, lamb-within-twin pair, sample type-within-twin pair, and sample type within-lamb-within-twin pair as random effects. SED is the error term for the Pr × D × S interaction (n = 5).

³Analyzed on log₁₀ transformed data to attain normality.

nants fed forage or concentrate diets at moderate levels of intake (Leedle et al., 1982). In agreement with this, our results showed that cellulolytic flora remains stable when the rumen ecosystem was not intensely disturbed by the diet (Koike and Kobayashi, 2001; Mosoni et al., 2007). Nevertheless, barley supplementation increased the abundance of lactate producers (i.e., *S. bovis*) and consumers (i.e., *S. ruminantium*), an observation which fits with previous findings (Tajima et al., 2000; Fernando et al., 2010). As expected, the abundance of anaerobic fungi was positively related with forage intake and indirectly accompanied by changes in methanogen numbers. To this end, control lambs fed ALF had the greatest concentration of anaerobic fungi and *F. succinogenes*, a bacterium with high cellulolytic activity (Kobayashi et al., 2008). This particular microbiota resulted in a substantial increase in fiber digestibility (+6.8%) and the acetate/propionate ratio (from 4.7 to 7.3) in the rumen (Belanche et al., 2011a), which should result in a considerable increase in H₂ production and may explain the greater concentration of methanogens recorded and the increased methane emissions (about 10%) usually observed in presence of protozoa (Morgavi et al., 2010).

Rumen Out-flow and Representativeness of the Bacterial Fractions

Two major aspects determine the success of a microbial marker in accurately estimating microbial synthesis in the rumen: i) the survival of the marker at the postruminal sampling site, and ii) the microbial reference (harvested from the rumen) considered to establish the marker/N ratio. Bacterial and protozoal rDNA sequences have been proposed as high specific microbial markers (Sylvester et al., 2005), although potential degradation of some gene sequences during gastric digestion can limit their application (Belanche et al., 2010b). The present study showed that bacterial DNA was not degraded through abomasal digestion, and no differences in DNA concentration, clustering pattern, or bacterial biodiversity were detected between rumen and abomasal samples. This validates the use of 16S ribosomal gene as a microbial marker or as a reference gene, or both when a relative quantification is conducted postminimally. In contrast, the low protozoal and fungal DNA concentration and diversity observed in the abomasum compared with the rumen seems to support the theory of protozoal and fungal sequestration in the rumen (Abe et al., 1981; Ankrah et al., 1990). This sequestration may be a consequence of their long generation time [between 8 and 36 h in protozoa and about 24 h in fungi (Van Soest, 1994)], and tropism towards fibrous components with long rumen retention times (Edwards et al., 2008). However, the lack of bacterial wall can facilitate, to some extent, fungal and protozoal lysis under acidic conditions leading to

DNA degradation (Sylvester et al., 2005; Belanche et al., 2010b) and subsequent underestimation of their N contribution to the intestine (Belanche et al., 2011a). In contrast, methanogens had a slightly greater abundance in abomasal compared with rumen contents (+40 ± 23%), possibly as a result of their association with the liquid digesta (or small particles). Liquid digesta is able to pass through the reticulum-omasal orifice and has a low retention time in the abomasum, aspects that could minimize the lysis of methanogens. To this end, the type of diet, compartment retention time, acidity, and amplicon size seems to modulate DNA survival (Belanche et al., 2010b); therefore, spike and recovery approaches should be considered for further experiments (Sylvester et al., 2005).

Bacterial populations studied using DGGE, did not reveal qualitative differences between bacterial fractions and digesta samples from the rumen or abomasum, and all samples showed a high degree of similarity within each individual and detectable amounts of all microorganism studied. However, the quantitative abundance of these microorganisms differed between the 4 types of samples considered. Cellulolytic adherent bacteria were associated with SAB, and were 7.6, 12.4, and 3.2 times more abundant than the concentrations observed in abomasal samples for *R. albus*, *R. flavefaciens*, and *F. succinogenes*, respectively (7.4, 3.0, and 1.6 greater in SAB than rumen samples). Our results agree with Michalet-Doreau et al. (2001), who demonstrated that cellulolytic bacterial concentrations were nearly double in SAB than in rumen contents because they rapidly colonize and remain attached to fibrous material (Krause et al., 2003; Edwards et al., 2007). Cellulolytic bacteria tropism towards solid digesta may explain the underrepresentation of these microbes in many clone libraries prepared from rumen liquor (Pei et al., 2010). In contrast, *S. ruminantium* is a motile microorganism; hence its preferential association with LAB. The abundance of methanogens and anaerobic fungi in both microbial fractions was less than observed in digesta samples. Anaerobic fungi are strongly associated with fibrous material (Edwards et al., 2008), and a substantial part of methanogens is associated with rumen protozoa [up to 25% (Newbold et al., 1995)] and varies between digesta fractions (Mosoni et al., 2011). These and other physiological peculiarities (e.g., a lower sedimentation coefficient than bacteria) lead to a poor recovery using protocols developed to isolate rumen bacteria. As expected, the ruminal abundance of most of the microorganisms studied was within the range delimited by LAB and SAB. Moreover, their abundance in the abomasum was generally comparable to those observed in rumen and at least 1 of the 2 bacterial fractions considered. The closer match of microbial composition between abomasum and LAB (compared with abomasum and SAB) may help explain our more consistent values of the microbial synthesis when the former fraction was used as reference (Belanche et al., 2011a).

The decreased ^{15}N enrichment and PB/N ratio observed in protozoal compared with bacterial fractions could result in a bias when microbial synthesis is estimated, either assuming a similar composition of both microbial groups or by ignoring the ciliate contribution at the duodenum. Moreover, the origin of the chemical differences among LAB and SAB is still not clear (Martinez et al., 2009; Ramos et al., 2009). Belanche et al. (2011a) provided evidence that the microbial fractions used to estimate microbial synthesis can generate greater differences (up to 24%) than observed between diets (16%) or between control and PF lambs (2%). The absence of rumen protozoa modified substantially the abundance of cellulolytic species and the PB/N ratio in SAB but not in LAB; whereas the opposite happened with *S. ruminantium*. Selective bacterial predation might explain a differential effect of protozoa on the LAB and SAB composition. The type of diet consumed also modified the chemical (^{15}N enrichment and PB/N ratio) and microbial composition of bacterial fractions (abundance of *P. ruminicola*, *S. ruminantium*, *S. bovis*, methanogens, and anaerobic fungi), with LAB and SAB affected differently. Traditionally, the dissimilar metabolic activity between LAB and SAB and the differential contamination with salt and/or plant material have been used to justify such chemical differences (Pérez et al., 1998). However, our findings suggest that these chemical differences are intimately related with differences in their microbiological structure. More sophisticated and refined procedures (i.e., isolation of microorganism from post-ruminal sites) should be considered to increase the representativeness of the microbial fractions to those microorganisms that leave the rumen.

Conclusions

This study showed that the presence of protozoa modified the rumen bacterial structure to a greater extent than the type of diet consumed by the ruminant. In particular, use of forage diets in the presence of rumen protozoa increased the abundance of *F. succinogenes*, anaerobic fungi and methanogens, as well as rumen bacterial diversity. Such a shift in the microbial ecosystem seems to make the rumen better adapted to fiber use. The chemical composition of LAB and SAB was also affected by the diet and the presence/absence of rumen protozoa, as a consequence of substantial changes in their microbial structure. In particular, cellulolytic bacteria were especially abundant in SAB, whereas the abomasal abundance of most of the microorganisms studied was closer to those values observed in LAB. As consequence, our results suggest that both microbial fractions must be considered to minimize potential bias in the estimation of microbial protein synthesis. Finally, a similar bacterial DNA concentration was observed in the rumen and abomasum, but the protozoal

and fungal DNA concentration in the later site accounted for 69 and 13% of that recorded in the former, suggesting a protozoal and fungal sequestration into the rumen or a possible degradation of their DNA sequences. More accurate studies using cannulated animals and double-flow markers must be conducted to confirm these findings.

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