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# Study of the effect of presence or absence of protozoa on rumen fermentation and microbial protein contribution to the chyme[1]

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**ABSTRACT:** The aim of this study was to investigate the effect of presence or absence of protozoa on rumen fermentation and efficiency of microbial protein synthesis under different diets. Of 20 twin paired lambs, 1 lamb of each pair was isolated from the ewe within 24 h after birth and reared in a protozoa-free environment (n = 10), whereas their respective twin-siblings remained with the ewe (faunated, n = 10). When lambs reached 6 mo of age, 5 animals of each group were randomly allocated to 1 of 2 experimental diets consisting of either alfalfa hay as the sole diet, or 50:50 mixed with ground barley grain according to a 2 × 2 factorial arrangement of treatments. After 15 d of adaptation to the diet, the animals were euthanized and total rumen and abomasal contents were sampled to estimate rumen microbial synthesis using $C_{31}$ alkane as flow marker. Different ($^{15}$N and purine bases) and a novel (recombinant DNA sequences) microbial markers, combined with several microbial reference extracts (rumen protozoa, liquid and solid associated bacteria) were evaluated. Absence of rumen protozoa modified the rumen fermentation pattern and decreased total tract OM and NDF digestibility in 2.0 and 5.1 percentage points, respectively. The effect of defaunation on microbial N flow was weak, however, and was dependent on the microbial marker and microbial reference extract considered. Faunated lambs fed with mixed diet showed the greatest rumen protozoal concentration and the least efficient microbial protein synthesis (29% less than the other treatments), whereas protozoa-free lambs fed with mixed diet presented the smallest ammonia concentration and 34% greater efficiency of N utilization than the other treatments. Although $^{15}$N gave the most precise estimates of microbial synthesis, the use of recombinant DNA sequences represents an alternative that allows separate quantification of the bacteria and protozoa contributions. This marker showed that presence of protozoa decrease the bacterial-N flow through the abomasum by 33%, whereas the protozoa-N contribution to the microbial N flow increased from 1.9 to 14.1% when barley grain was added to the alfalfa hay. Absolute data related to intestinal flow must be treated with caution because the limitations of the sampling and maker system employed.

**Key words:** ciliate, defaunation, microbial synthesis, protozoa, purine base, quantitative polymerase chain reaction



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## INTRODUCTION

Rumen protozoa may represent up to 50% of microbial biomass and play a key role in ruminal N recycling though their intensive bacterial predation (Koenig et al., 2000). Substances such as 2,6-aminoethylphosphonic acid (Abou Akkada et al., 1968) and $^{14}$C (Faichney et al., 1997) have been investigated as potential protozoal markers. However the determination of the protozoal-N flow to the duodenum has been hindered by the lack of appropriate protozoal markers. Therefore, in classical approaches, the contributions of protozoa and bacteria to microbial flow are not distinguished, assuming that

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both populations have a similar microbial marker/N ratio, despite a large body of experimental evidence showing the opposite (Vicente et al., 2004).

The effect of protozoa on rumen fermentation and microbial synthesis has traditionally been studied by their complete elimination (defaunation). In such conditions, defaunation decreases bacterial N recycling and OM digestibility (Eugène et al., 2004b) while increasing the efficiency of microbial protein synthesis (**EMPS**; Ushida et al., 1990). However, the effect of the presence or absence of rumen protozoa on rumen metabolism without the effect of the defaunation process is not completely understood. The use of microbial recombinant DNA (**rDNA**) sequences offers the opportunity to identify and quantify different rumen microbial groups. Sylvester et al. (2005) proposed the use of 18S rDNA as a specific protozoal marker in vivo, whereas Belanche et al. (2010, 2011) evaluated the survival of bacterial 16S and protozoal 18S rDNA throughout the intestinal tract in vitro and in vivo and their potential use as microbial markers.

The assay was planned with 2 aims: 1) to study the effect of the presence or absence of protozoa on rumen fermentation, EMPS, and postruminal protozoal-N flow under different feeding situations using protozoa-free twin lambs; and 2) to compare bacterial and protozoal rDNA sequences as highly specific microbial markers with traditional markers ($^{15}$N and purine bases).

## MATERIALS AND METHODS

Animals were handled by trained personnel and experimental protocols were approved by the Ethics Committee for Animal Research of the University of Zaragoza (protocol reference PI13/05).

### *Animals and Diets*

Twenty twin newborn Rasa Aragonesa lambs (12 males and 8 females) were used. One of each twin was isolated from the ewe within 24 h of lambing, fed with commercial milk replacer, and maintained free of protozoa (**PF**) by avoiding protozoa transmission from adults or from outdoor material. The other twin received natural lactation and remained with the ewe to obtain normal protozoa colonization (faunated). At 45 d old, both groups of lambs were weaned and at 6 mo of age (41 ± 7.3 kg of BW), both groups were subdivided into 2 sub-groups (n = 5: 3 males and 2 females). Animals were randomly allocated to 1 of 2 experimental diets, such that each pair of twins received the same diet. Experimental diets consisted of alfalfa hay (**Alf**; in g/kg of DM: OM, 900; CP, 164; ether extract, 17; NDF, 321; ADF, 259; and ADL, 59) given either as sole diet or mixed (50:50 ratio) with ground barley grain (mixed; in g/kg of DM: OM, 977; CP, 104; ether extract, 20; NDF, 157; ADF, 51; and ADL, 8.6) and supplemented with a vitamin-mineral premix (Norblob, NOREL S.A., Madrid, Spain; declared composition in mg/kg: Mn, 60; Cu, 50; Zn, 40; Fe, 40; I, 30; Co, 20; and Se, 2). Both diets were offered at 1.5 × maintenance (AFRC, 1993) and animals were fed 4 times per day (every 6 h) to minimize diurnal changes in rumen fermentation and digesta composition. At the end of each day, refusals were collected, weighted, and stored for analysis.

### *Experimental Design and Sample Collection*

After diet adaptation (2 wk), animals were placed in metabolic cages for 15 d, allowing 7 d for cage changeover and 8 d for experimental measurements. Feces were collected daily, pooled individually, and dried at 60°C for 48 h for subsequent analysis. From d 10 to 14, 42.7 mg of $^{15}$N/d, as ammonium sulfate, was diluted in 120 mL of water and administered orally in 4 doses per day (every 6 h). One additional lamb from each treatment was infused with nonlabeled ammonium sulfate to establish background isotope abundance.

On d 15, 2 h after feeding, lambs were intramuscularly anesthetized (xylazine 2%, 0.3 mg/kg of BW; Xilagesic Laboratorios Calier S.A., Barcelona, Spain) previous to intravenous euthanization (Thiopental, 10 mg/kg of BW; thiobarbital; Braun Medical S.A., Barcelona, Spain). Finally the digestive tract was dissected and sampled as described by Askar et al. (2005). Total abomasal digesta content was taken and homogenized: 2 samples [0.3 g of fresh matter (**FM**)] were frozen in liquid N and stored at −80°C for DNA analysis and 1 additional sample (100 g of FM) was freeze-dried for general analyses. Ruminal content was strained through 2 cheesecloth layers and 3 samples were taken from the filtrate: 1 (5 mL) was diluted 1:1 (vol/vol) in 18.5% formaldehyde and used for microscopic protozoa counts, whereas the other 2 samples (4 mL) were used for ammonia and VFA analysis, after acidification with hydrochloric [1:1 (vol/vol); 0.1 *M* HCl] and phosphoric acid [4:1 (vol/vol); 0.5 *M* $H_3PO_4$ + 50 m*M* 4-methyl valerate], respectively.

The particulate fraction was diluted 1:1 (vol/vol) with Coleman buffer at 39°C (Williams and Coleman, 1992), mixed gently, and squeezed to maximize the recovery of loosely particulate-associated microorganisms. The filtrate was then divided in 2 fractions to extract liquid-associated bacteria (**LAB**) and protozoa (**LAP**) following Pérez et al. (1996a) and Yáñez-Ruiz et al. (2006) procedures, respectively, whereas the particulate residue was used to isolate the solid-associated bacteria (**SAB**) using carboxymethyl cellulose as detaching agent (Martín-Orue et al., 2000). Two samples (0.3 g of FM) of every microbial extract were sampled for molecular analyses and the remaining pellet was freeze-dried for subsequent analyses.

### *Chemical Analysis*

Dry matter was determined by drying samples to a constant weight at 105°C and OM by combustion at

550°C for 8 h in a muffle furnace. Concentrations of NDF, ADF, and ADL were determined by the sequential procedure described by Van Soest et al. (1991), using the Ankom fiber analyzer (Ankom Technology, Macedon, NY). Concentration of NDF was assayed after treatment with a heat-stable amylase and NDF, ADF and ADL were expressed without residual ash. Total N in feed, refusals, and microbial extracts was determined by the Kjeldahl method following the official method (AOAC, 2005), but using Se as catalyst. Nonammonia N (**NAN**) concentration in abomasal digesta was determined also by the Kjeldahl method but after ammonia evaporation (Firkins et al., 1992); after that, samples were dried and ground to 1 mm. Ammonia-N (Chaney and Marbach, 1962) and VFA (Jouany, 1982) concentrations were determined in the in rumen fluid. Alkanes were extracted (Mayes et al., 1988) and analyzed as described by Valiente et al. (2003). Purine bases (**PB**; Balcells et al., 1992) and $^{15}N$ abundance [VG Prism II mass spectrophotometer, isotope mass spectrophotometry (IRMS) connected in series to Dumas-style N analyzer EA 1108, Carlo Erba, Milan, Italy] were determined in digesta samples and microbial isolates. Isotopic enrichment was calculated from background enrichment determined in nonisotope-infused animals. Protozoa counts and classification were done as described by Dehority (1993).

### *DNA Extraction and Quantitative PCR Conditions*

As PCR amplification is an exponential process, the use of several repetitions during DNA extraction and PCR is crucial to minimize its variability. Genomic DNA was extracted in duplicate from frozen samples using the QIAamp DNA Stool Mini Kit (Qiagen Ltd., Crawley, West Sussex, UK) following the manufacturer's instructions. The only protocol modification was the increment of the temperature of incubation with lysis buffer to 95°C for 10 min to maximize the cell lyses. Similar bacterial population and biodiversity was observed between LAB, SAB, and abomasal samples within the same animal by using denaturing gradient gel electrophoresis (data not shown). Therefore, a clear selection of particular microbial groups during the DNA extraction was ruled out and the DNA extraction efficiency was assumed to be the same for microbial pellets and abomasal samples. This DNA extraction procedure showed acceptable repeatability between extractions (CV = 22%), regardless of sample type. Concentration of total extracted DNA was assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and samples were pooled by animal to maximize their representativeness.

Quantification of total bacteria and protozoa was determined in abomasal samples by quantitative PCR assay (**qPCR**), following the principles described by Maeda et al. (2003) and Sylvester et al. (2004), respectively. The total bacteria 16S rDNA targeted primers used in this study were forward 5′-GTGSTGCAYGGYTGTCGTCA-3′ and reverse 5′-ACGTCRTCCMCACCTTCCTC-3′. Primers designed for detection of protozoal 18S rDNA were forward 5′-GCTTTCGWTGGTAGTGTATT-3′ and reverse 5′-CTTGCCCTCYAATCGTWCT-3′. Quantitative PCR was performed using an ABI PRISM 7000, Sequence Detection System (Applied Biosystems, Carlsbad, CA). A DNA template (2 mL) was added to the amplification reaction (25 μL total volume) containing 25 pmol of each primer, 12.5 μL of platinum SYBR Green qPCR SuperMix-UDG and 0.5 μL of ROX Reference Dye (Invitrogen, Carlsbad, CA). The amplification protocol for total bacterial DNA was 95°C for 10 min and 30 cycles of 95°C for 15 s, 61°C for 30 s, and 72°C for 30 s, and the protozoal protocol was 95°C for 10 min and 40 cycles of 94°C for 10 s, 55°C for 20 s, and 72°C for 30 s.

One aliquot of DNA was taken from the LAP of each faunated animal and pooled; equally, 2 DNA aliquots of all animals (1 from LAB and another from SAB) were pooled to generate the protozoal and bacterial standards, respectively. This decision to generate a single bacterial DNA standard was based on the lack of substantial differences in the bacterial community (>75% similarity) observed between LAB and SAB samples by using denaturing gradient gel electrophoresis (data not shown). True protozoal DNA concentration in the protozoa standard was estimated after have been quantified and subtracted the bacterial DNA. Known standard DNA concentrations were diluted from $10^{-1}$ to $10^{-4}$ to generate the calibration curves and abomasal $C_T$ (threshold cycle) values were always allocated within the calibration curve range. These curves, after data normalization using logarithms (base 10) expression, presented a slope of −3.26 ($R^2 = 0.998$) and −3.58 ($R^2 = 0$ 0.997) for bacterial and protozoal standards, respectively, giving efficiencies of amplification of 103 and 90%, respectively. Similar amplification efficiencies were observed in abomasal samples for bacterial (98%) and protozoal primers (92%).

In the qPCR assay, the average of 2 replicates of each rDNA sample was used to increase the precision (CV = 0.93 and 1.02% between replicates for bacteria and protozoa, respectively). Furthermore, a nontemplate (sterile distilled water) negative control was loaded to screen for possible contamination and dimer formation. Fluorescence readings were taken after each extension step and primer specificity was confirmed after the amplification by a unique sharp peak in the melting analysis. All postrun data analyses were performed using ABI Prism 7000 SDS software.

### *Calculation and Statistical Analysis*

A sacrifice approach based on a single time sampling and total abomasal digesta collection was used. Postruminal flows of DM, OM, and NAN were estimated from

the dietary alkane $C_{31}$, where a complete recovery in abomasal digesta was assumed based on the high recovery rate observed (91 ± 7.7%). Individual daily alkane $C_{31}$ input was estimated according to the DMI and ingredients concentration (363.2 and 13.5 mg/kg of DM, for alfalfa and barley, respectively). Nitrogen concentration in LAP was corrected for individual lambs according to the bacterial N contamination; it was assumed that protozoa-associated bacteria had the same rDNA:N ratio as LAB ($LAP_{LAB}$) or SAB ($LAP_{SAB}$).

The microbial nitrogen (**MN**) contribution to abomasal NAN (**$MN/NAN_{digesta}$**) was estimated from 1) $^{15}N$ enrichment (**E**), 2) PB/N ratio, and 3) rDNA/N ratio in abomasal digesta and microbial extracts as follows: 1) $MN/NAN_{digesta} = E\text{-}NAN_{digesta}/E\text{-}N_{LAB\ or\ SAB}$, 2) $MN/NAN_{digesta} = (PB/NAN)_{digesta}/(PB/N)_{LAB\ or\ SAB}$, and 3) $MN/NAN_{digesta} = [(Bacterial_{rDNA}/NAN)_{digesta}/(rDNA/N)_{LAB\ or\ SAB}] + [(Protozoal_{rDNA}/NAN)_{digesta}/(rDNA/N)_{LAP}]$.

Values of EMPS were calculated as the relation between the MN flow and the true OM digested in the rumen, whereas efficiency of N use (**ENU**) was established as the ratio between MN and the N available to the rumen microorganism (Bach et al., 2005). Data were analyzed by ANOVA with the presence of protozoa ($Pr$), diet ($D$), and their interaction ($Pr \times D$) as fixed effects and twin pair ($T$) and lamb-within-twin pair [$L(T)$] as random effects. Data were analyzed by ANOVA according to the following model:

$$Y_{ijkl} = \mu + Pr_i + D_j + (Pr \times D)_{ij} + T_k + L(T)_{kl} + e_{ijkl},$$

where $Y_{ijkl}$ is the dependent, continuous variable, $\mu$ is the overall population mean, $Pr_i$ is the fixed effect of the presence of protozoa ($i$ = PF or faunated), $D_j$ is the fixed effect of the diet ($j$ = alfalfa or mixed), $(Pr \times D)_{ij}$ is the fixed effect of the interaction between protozoa and diet, $T_k$ is the random effect of the twin pair ($k = 1$ to 10), $L(T)_{kl}$ is the random effect of lamb-within-twin pair ($l = 1$ or 2), and $e_{ijkl}$ is the residual error.

When the interaction was significant, treatment means were compared by post hoc $t$-test. When method ($M$, or microbial extract) formed part of the analyses, protozoa, diet, method (M), and their interactions were treated as fixed effects, whereas random effects were included for twin pair ($T$), lamb-within-twin pair [$L(T)$], method-within-twin pair [$M(T)$], and method-within-lamb-within-twin pair {$M[L(T)]$}:

$$Y_{ijklm} = \mu + Pr_i + D_j + M_m + (Pr \times D)_{ij} + (Pr \times M)_{im} + (D \times M)_{jm} + (Pr \times D \times M)_{ijm} + T_k + L(T)_{kl} + M(T)_{km} + M[L(T)]_{klm} + e_{ijklm},$$

where descriptions of $Y$, $\mu$, $Pr$, $D$, $T$, $L(T)$, $e$, and subscripts $i$, $j$, $k$, and $l$ are defined as above, and subscript $m$ is the index for method ($m = {}^{15}N$, PB, rDNA).

Residual plots were inspected for constant variance and normality, and if these assumptions failed, data were log-transformed (protozoal counts) or analyzed by nonparametric Mann-Whitney test (% Entodiniinae). Findings with $P < 0.05$ were regarded statistically significant, whereas $0.05 < P < 0.10$ was accepted as representing a tendency to differences. Data were analyzed using Genstat (VSN International Ltd., Hemel Hempstead, Hertfordshire, UK).

## RESULTS

### *Intake and Digestibility*

Both groups of lambs showed similar growth rates (273 ± 14 g/d) during the fattening period (from 45 to 90 d). The recovery of alkane $C_{31}$ in feces was high and constant (91 ± 7.7%), independent of the experimental treatments; therefore, a negligible degradation or absorption of this component would be expected. Intake of DM in animals fed alfalfa was 22% greater ($P = 0.039$) than those fed the mixed diet (Table 1), but the alfalfa diet showed a decreased rumen (8.5 percentage points; $P < 0.001$) and whole-tract OM digestibility (13.9 percentage points; $P < 0.001$) compared with the mixed diet; consequently, no differences in digestible OM intake were observed between diets ($P = 0.62$).

The presence of protozoa increased the whole-tract DM ($P = 0.009$) and OM digestibilities ($P = 0.008$) by an average of 3.3 percentage points. This increment was even more pronounced in terms of NDF (11.6 percentage points; $P = 0.001$) and ADF (9.3 percentage points; $P = 0.006$). However, the great variability in digesta flow values caused the presence of ciliates to only tend to increase the true rumen OM digestibility ($P = 0.10$) when lambs were fed a mixed diet. No differences in the apparent or true rumen N digestibility were observed in the present experiment and only values derived from the use of $^{15}N$ and LAB are presented in Table 1.

### *Rumen Fermentation*

The presence of rumen protozoa increased rumen total VFA (Table 2; $P < 0.001$) and ammonia-N concentrations ($P = 0.020$), where the least ammonia-N values corresponded to PF lambs fed the mixed diet (interaction, $P = 0.082$). Barley supply reduced rumen pH, with this effect being more pronounced in faunated animals (interaction, $P = 0.010$). When barley was added to the alfalfa hay, a decrease in the molar proportion of acetate ($P < 0.001$), and an increase in the rest of VFA was observed. Moreover, the largest differences in the molar proportion of acetate, propionate, isobutyrate, valerate, and acetate:propionate ratio were detected between PF groups. Protozoal concentration in faunated lambs increased substantially with barley supplies ($P < 0.001$), even though protozoal distribution was similar for both diets. Rumen protozoal num-

**Table 1.** Effect of diet[1] and absence of rumen ciliates on total tract and rumen digestibility

| | Faunated lamb diet | | Protozoa-free lamb diet | | | Significance[3] | | |
|---|---|---|---|---|---|---|---|---|
| Item | Alfalfa | Mixed | Alfalfa | Mixed | SED[2] | Protozoa | Diet | Pr × D |
| Intake, g/d | | | | | | | | |
| DM | 899 | 759 | 862 | 683 | 72.2 | 0.22 | 0.039 | 0.66 |
| Digestible OM | 499 | 530 | 456 | 471 | 52.0 | 0.078 | 0.62 | 0.74 |
| Total digestibility, % | | | | | | | | |
| DM | 59.3 | 72.0 | 56.2 | 71.1 | 0.94 | 0.009 | <0.001 | 0.10 |
| OM | 61.5 | 74.3 | 58.5 | 73.4 | 1.11 | 0.008 | <0.001 | 0.097 |
| NDF | 50.2 | 48.1 | 43.4 | 44.7 | 2.01 | 0.001 | 0.84 | 0.14 |
| ADF | 50.2 | 43.4 | 44.2 | 41.5 | 1.99 | 0.006 | 0.022 | 0.087 |
| Rumen apparent digestibility, % | | | | | | | | |
| DM | 37.2 | 46.9 | 37.6 | 41.0 | 2.54 | 0.23 | 0.001 | 0.17 |
| OM | 37.9 | 49.3 | 39.4 | 45.1 | 2.22 | 0.51 | <0.001 | 0.17 |
| N | 32.9 | 33.6 | 34.5 | 21.6 | 5.22 | 0.22 | 0.11 | 0.12 |
| Rumen true digestibility,[4] % | | | | | | | | |
| DM | 45.6 | 58.5 | 48.9 | 53.6 | 2.88 | 0.73 | <0.001 | 0.11 |
| OM | 44.8 | 57.9 | 46.6 | 53.6 | 2.21 | 0.42 | <0.001 | 0.11 |
| N | 72.3 | 83.8 | 72.6 | 72.3 | 5.60 | 0.22 | 0.17 | 0.20 |

[1]Twin lambs (1 faunated and 1 protozoa-free) were fed alfalfa hay or 50:50 alfalfa:ground barley (mixed). Animals were euthanized 2 h after feeding and total abomasal content was collected to determine the postruminal flow using $C_{31}$ alkane as the single flow marker.

[2]SED from protozoa × diet interaction (Pr × D; n = 5).

[3]Considering protozoa, diet, and Pr × D as fixed effects and twin pair and lamb-within-twin pair as random effects.

[4]Calculated using $^{15}N$ and liquid-associated bacteria (LAB) as microbial marker and microbial reference, respectively.

bers correlated closely ($R^2$ = 0.971) with the rumen protozoal DNA concentration determined by qPCR.

### *Chemical Composition of Microbial Extracts*

Both the presence of rumen protozoa ($P$ = 0.021) and the alfalfa hay diet ($P$ = 0.015) decreased the $^{15}N$ enrichment of bacterial extracts (Table 3). Moreover LAB showed a greater $^{15}N$ enrichment than SAB ($P <$ 0.001). The rDNA:N ratio in bacterial extracts was not affected by diet, although it peaked in faunated lambs fed the mixed diet (Pr × D, $P$ = 0.040). Both, rDNA:N and PB:N ratios were substantially less in LAB than SAB ($P$ = 0.012 and $P <$ 0.001, respectively). More-

**Table 2.** Effect of diet[1] and absence of rumen ciliates on rumen fermentation

| | Faunated lamb diet | | Protozoa-free lamb diet[2] | | | Significance[4] | | |
|---|---|---|---|---|---|---|---|---|
| Item | Alfalfa | Mixed | Alfalfa | Mixed | SED[3] | Protozoa | Diet | Pr × D |
| pH | 6.79[a] | 5.50[c] | 6.82[a] | 6.03[b] | 0.083 | 0.006 | <0.001 | 0.010 |
| $NH_3$-N, mg/100 mL | 6.15 | 7.97 | 4.82 | 0.84 | 1.959 | 0.020 | 0.43 | 0.082 |
| Total VFA, m*M* | 123 | 122 | 92.5 | 74.2 | 16.33 | 0.004 | 0.41 | 0.47 |
| Molar proportion, % | | | | | | | | |
| Acetate | 77.6[a] | 66.1[b] | 82.7[a] | 56.6[c] | 3.20 | 0.44 | <0.001 | 0.028 |
| Propionate | 15.0[b] | 19.1[ab] | 9.12[c] | 21.5[a] | 2.28 | 0.28 | 0.001 | 0.024 |
| Butyrate | 5.40 | 12.0 | 6.72 | 18.2 | 3.06 | 0.085 | 0.005 | 0.24 |
| Isobutyrate | 0.87[b] | 0.88[b] | 0.63[b] | 1.24[a] | 0.146 | 0.57 | 0.020 | 0.015 |
| Valerate | 0.53[c] | 1.25[b] | 0.35[d] | 1.48[a] | 0.106 | 0.58 | 0.027 | 0.11 |
| Isovalerate | 0.56 | 0.70 | 0.43 | 0.96 | 0.164 | 0.62 | <0.001 | 0.005 |
| A:P ratio[5] | 5.17[b] | 3.47[c] | 9.07[a] | 2.63[d] | 0.554 | 0.009 | <0.001 | <0.001 |
| Protozoal cells,[6] $10^5$/mL | 0.37 | 13.9 | ND | ND | 4.52 | | <0.001 | |
| Distribution,[7] % | | | | | | | | |
| Subfamily Entodiniinae | 99.3 | 99.8 | ND | ND | 0.507 | | 0.68 | |
| Subfamily Diplodiniinae | 0.74 | 0.22 | ND | ND | 0.507 | | 0.68 | |

[a–d]Means within a row without a common superscript letter differ ($P < 0.05$).

[1]Twin lambs (1 faunated and 1 protozoa-free) were fed alfalfa hay or 50:50 alfalfa:ground barley (mixed). Animals were euthanized 2 h after feeding and total rumen content was sampled.

[2]ND = not detected.

[3]SED from protozoa × diet interaction (Pr × D; n = 5).

[4]Considering protozoa, diet, and Pr × D as fixed effects and twin pair and lamb-within-twin pair as random effects.

[5]Acetate:propionate ratio.

[6]Analyzed by 2-sample *t*-test on log-transformed data.

[7]Analyzed by Mann-Whitney nonparametric test.

**Table 3.** Effect of presence or absence of rumen ciliates and type of diet[1] on bacterial and protozoal isolates

| | Faunated lamb diet | | Protozoa-free lamb diet[3] | | | Significance[5] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Item[2] | Alfalfa | Mixed | Alfalfa | Mixed | SED[4] | Protozoa | Diet | Pr × D | Extract | Pr × E | D × E | Pr × D × E |
| $^{15}$N:N, % | | | | | | | | | | | | |
| LAB | 1.78 | 1.90 | 2.10 | 3.02 | 0.284 | 0.021 | 0.015 | 0.21 | <0.001 | 0.347 | <0.001 | 0.016 |
| SAB | 1.67 | 1.74 | 2.08 | 2.70 | | | | | | | | |
| PB:N, mmol/g of OM | | | | | | | | | | | | |
| LAB | 2.07 | 2.45 | 2.23 | 2.27 | 0.124 | 0.23 | <0.001 | 0.39 | <0.001 | 0.027 | 0.014 | <0.001 |
| SAB | 2.65 | 2.84 | 2.54 | 3.38 | | | | | | | | |
| DNA:N, mg/mg | | | | | | | | | | | | |
| LAB | 0.117 | 0.125 | 0.080 | 0.060 | 0.0257 | 0.039 | 0.55 | 0.040 | 0.012 | 0.24 | 0.39 | 0.21 |
| SAB | 0.111 | 0.182 | 0.148 | 0.114 | | | | | | | | |
| LAP | 0.141 | 0.233 | ND | ND | 0.0384[6] | | 0.007 | | 0.17 | | 0.11 | |
| $LAP_{LAB}$ | 0.147 | 0.309 | ND | ND | | | | | | | | |
| $LAP_{SAB}$ | 0.181 | 0.251 | ND | ND | | | | | | | | |

[1]Twin lambs (1 faunated and 1 protozoa-free) were fed alfalfa hay or 50:50 alfalfa:ground barley (mixed). Animals were euthanized 2 h after feeding and rumen content was sampled to isolate microbial extracts.

[2]LAB = liquid-associated bacteria; SAB = solid-associated bacteria; PB = purine bases; LAP = liquid-associated protozoa (LAP, noncorrected): LAP corrected according to LAB ($LAP_{LAB}$) and SAB ($LAP_{SAB}$) cross-contamination.

[3]ND = not detected.

[4]SED from diet × extract interaction (D × E; n = 5); SED from protozoa × D × E interaction (Pr × D × E; n = 5).

[5]Considering protozoa, diet, extract, and their interactions (Pr × D, Pr × E, D × E, Pr × D × E) as fixed effects and twin pair, lamb-within-twin pair, extract-within-twin pair, and extract-within-lamb-within-twin pair as random effects.

[6]Considering diet, extract, and their interaction (D × E) as fixed effects and lamb as random effect.

over, barley addition promoted an increment in the PB:N ratio ($P < 0.001$).

Values of purine bases and $^{15}$N enrichment in LAP extract were not affected by the diet and averaged 139 μmol/g of OM and 1.14 ‰, respectively. The mixed diet prompted a stronger increase in protozoal rDNA concentration ($P < 0.001$) than in N ($P = 0.011$), resulting in an increase in the rDNA:N ratio ($P = 0.007$). Although procedures used for the LAB and SAB isolation removed almost all of the plant matter (checked by microscopic examination) and protozoal DNA contamination (<0.15% based on qPCR analysis), the bacterial contamination in protozoal extracts was considerable. Nevertheless, corrections of LAP values based on the bacterial contamination decreased in a similar way the rDNA (−33 ± 9.9%; $P < 0.001$) and N concentrations (−24 ± 10.3%, $P < 0.001$). Consequently, these corrections did not substantially modify the rDNA:N ratio ($P = 0.171$) and noncorrected (LAP) values were used for subsequent determinations.

### *Microbial N Flow*

Sheep fed alfalfa hay consumed more N ($P = 0.038$) than those fed the mixed diet (Table 4), although these differences were buffered in the rumen. In relation to MN flow, values did not differ between treatments when LAB were used as reference; however, there were variations among microbial markers. The use of PB led to the greatest estimated MN flow followed by rDNA and $^{15}$N ($P = 0.01$). Moreover, the use of rDNA resulted in a greater MN flow in PF than in faunated lambs (9.8 vs. 5.7 g of MN/d), differences that were not evident with the other microbial markers (Pr × M, $P = 0.005$). Using SAB extract as reference, PB and $^{15}$N rendered greater estimates of MN flow than rDNA ($P < 0.001$) and neither diet nor presence of protozoa modified the MN flow; however, these effects were modulated by the microbial marker used (Pr × D × M, $P = 0.003$).

Quantitative PCR allowed to analyze bacterial and protozoa contribution to abomasal flow, bacterial flow through the abomasum increased with protozoal absence (9.8 vs. 5.5 g of N/d; $P = 0.029$) when LAB were used as a reference, whereas data derived from SAB showed that faunated lambs fed the mixed diet (Pr × D, $P < 0.027$) showed the least bacterial N flow. Protozoa-N flow was low (less than 0.5 g of N/d) and not statistically affected by diet ($P = 0.21$). However, when protozoa-N were expressed as proportion of total MN flow, it was observed that barley supplementation resulted in an increment of protozoal contribution from 1.7 (or 2.0) to 12.8% (or 15.3%) using LAB ($P = 0.044$) or SAB ($P = 0.019$) as reference, respectively.

### *Efficiency of Microbial Synthesis*

Variations in EMPS mainly reflected differences between microbial markers and extracts rather than between treatments (Table 5). The presence of protozoa only slightly decreased EMPS when LAB was used as reference ($P = 0.14$), but most of this difference were derived from the use of rDNA data (Pr × M, $P < 0.005$). Considering SAB, the presence of protozoa only decreased EMPS when animals received mixed diet and

**Table 4.** Effect of presence or absence of rumen ciliates and type of diet[1] on postruminal microbial N flow

| Item[2] | Faunated lamb diet | | Protozoa-free lamb diet[3] | | SED[4] | Significance[5] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Alfalfa | Mixed | Alfalfa | Mixed | | Protozoa | Diet | Pr × D | Method | Pr × M | D × M | Pr × D × M |
| Intake, g of N/d | 21.5 | 16.2 | 21.6 | 14.6 | 2.94 | 0.63 | 0.038 | 0.61 | | | | |
| Abomasal flow | | | | | | | | | | | | |
| NAN, g of N/d | 14.6 | 10.6 | 14.2 | 11.5 | 2.41 | 0.85 | 0.14 | 0.61 | | | | |
| Microbial N, g of N/d | | | | | | | | | | | | |
| Considering LAB | | | | | | | | | | | | |
| $^{15}$N | 6.53 | 6.35 | 6.26 | 5.87 | 1.769[6] | 0.40 | 0.25 | 0.38 | 0.010 | 0.005 | 0.77 | 0.47 |
| PB | 10.97 | 8.79 | 8.79 | 8.53 | | | | | | | | |
| rDNA | 7.40 | 4.22 | 9.72 | 9.93 | | | | | | | | |
| Bacterial-N | 7.25 | 3.71 | 9.72 | 9.93 | 2.341 | 0.029 | 0.35 | 0.28 | | | | |
| Protozoal-N | 0.15 | 0.50 | ND | ND | 0.256[7] | | 0.21 | | | | | |
| Considering SAB | | | | | | | | | | | | |
| $^{15}$N | 6.99 | 6.88 | 6.31 | 6.55 | 1.072[6] | 0.44 | 0.15 | 0.40 | <0.001 | 0.14 | 0.011 | 0.003 |
| PB | 8.48 | 7.50 | 7.71 | 5.73 | | | | | | | | |
| rDNA | 6.70 | 2.82 | 4.71 | 5.09 | | | | | | | | |
| Bacterial-N | 6.55[a] | 2.32[c] | 4.71[b] | 5.09[ab] | 1.050 | 0.60 | 0.013 | 0.027 | | | | |
| Protozoal-N | 0.15 | 0.50 | ND | ND | 0.256[7] | | 0.21 | | | | | |

[a–c]Means within a row without a common superscript letter differ ($P < 0.05$).

[1]Twin lambs (1 faunated and 1 protozoa-free) were fed alfalfa hay or 50:50 alfalfa:ground barley (mixed). Animals were euthanized 2 h after feeding and total abomasal content was sampled to determine the postruminal flow using $C_{31}$ alkane as the single flow marker.

[2]NAN = nonammonia N; LAB = liquid-associated bacteria; SAB = solid-associated bacteria; PB = purine bases; rDNA = recombinant DNA.

[3]ND = not detected.

[4]SED from protozoa × diet interaction (Pr × D; n = 5); SED from Pr × D × method interaction (Pr × D × M; n = 5).

[5]Considering protozoa, diet, and their interaction (Pr × D) as fixed effects and twin pair as a random effect. Means without a common superscript differ ($P < 0.05$).

[6]Considering protozoa, diet, method, and their interactions (Pr × D, Pr × M, D × M, Pr × D × M) as fixed effects and twin pair, lamb-within-twin pair, method-within-twin pair, and method-within-lamb-within-twin pair as random effects.

[7]Analyzed by 2-sample *t*-test.

rDNA was used as microbial marker (P × D × M, $P = 0.004$), coinciding with the greatest concentration of rumen protozoa. The EMPS was also affected by diet; barley supply decreased this efficiency using LAB ($P = 0.029$) and SAB ($P < 0.001$); the microbial marker considered also modified the later estimations (D × M, $P = 0.003$).

Protozoa-free lambs fed the mixed diet showed the greatest ENU, independent of the microbial marker or microbial extract considered (Pr × D, $P = 0.05$ for LAB and $P = 0.04$ for SAB). Moreover, the rDNA microbial marker provided the greatest differences between the faunated (0.45 or 0.41) and PF groups (0.65 or 0.51 g of MN/g of available N) using LAB ($P = 0.002$) or SAB ($P = 0.029$), respectively. Barley addition to the diet also increased the ENU using LAB ($P = 0.013$) and SAB ($P = 0.033$); however, the latter was modulated by the microbial marker considered (D × M, $P < 0.001$).

## DISCUSSION

The present protocol, designed to minimize between-individual variation and comparisons between Faunated and PF animals, was performed among twin lambs. Isolation of newborn lambs from natural ciliate sources was considered as the least-aggressive system to obtain PF animals. Single-time sampling procedures based on animal euthanasia have been reported to describe rumen fermentation (Askar et al., 2005; Yáñez-Ruiz et al., 2006) and microbial synthesis in growing lambs (Askar et al., 2007). This procedure allows the collection of the total rumen and abomasum contents, avoiding interference from endogenous duodenal secretions (Belanche et al., 2011). Moreover animals were fed frequently (every 6 h) to minimize diurnal variation in ruminal fermentation or digesta composition (de Veth and Kolver, 2001).

Plant wax *n*-alkanes are commonly used as markers for estimation of intake, digestibility, and diet composition in grazing animals (Dove and Mayes, 1991). Moreover long-chain alkanes have been used as abomasal flow (Askar et al., 2005) or rumen transit markers (Giráldez et al., 2004). However, alkanes may have some concerns about their utilization as internal flow markers because their incomplete fecal recovery. In a recent paper Keli et al., (2008) demonstrated that *n*-alkanes are neither degraded nor synthesized by the rumen microorganisms; thus, the limited $C_{31}$ degradation observed in our experiment (91 ± 7.7%) is likely not happening in the rumen (Ohajuruka and Palmquist, 1991) and was not affected by the experimental treatments. Consequently, digesta flow was calculated based on the $C_{31}$ intake (instead of fecal recovery). Nevertheless, a preferential association of *n*-alkanes with solid digesta, and the sub-

**Table 5.** Effect of presence or absence of rumen ciliates and type of diet[1] on efficiency of microbial protein synthesis and efficiency of N utilization

| | Faunated lamb diet | | Protozoa-free lamb diet | | | Significance[4] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Item[2] | Alfalfa | Mixed | Alfalfa | Mixed | SED[3] | Protozoa | Diet | Pr × D | Method | Pr × M | D × M | Pr × D × M |
| LAB, g of MN/kg of DOMTR | | | | | | | | | | | | |
| $^{15}$N | 18.4 | 15.5 | 17.8 | 17.1 | 3.05 | 0.15 | 0.029 | 0.15 | <0.001 | 0.005 | 0.65 | 0.51 |
| PB | 27.4 | 20.4 | 23.8 | 23.0 | | | | | | | | |
| DNA | 18.4 | 10.2 | 22.7 | 22.2 | | | | | | | | |
| SAB, g of MN/kg of DOMTR | | | | | | | | | | | | |
| $^{15}$N | 19.1 | 16.3 | 17.4 | 18.2 | 1.81 | 0.95 | 0.001 | 0.12 | <0.001 | 0.22 | 0.003 | 0.004 |
| PB | 22.1 | 17.7 | 20.8 | 16.2 | | | | | | | | |
| DNA | 17.4 | 7.0 | 13.1 | 13.4 | | | | | | | | |
| LAB,[5] g of MN/g of available N | | | | | | | | | | | | |
| $^{15}$N | 0.493 | 0.559 | 0.461 | 0.663 | 0.067 | 0.074 | 0.013 | 0.053 | 0.014 | 0.002 | 0.45 | 0.32 |
| PB | 0.609 | 0.624 | 0.549 | 0.735 | | | | | | | | |
| DNA | 0.49 | 0.414 | 0.555 | 0.753 | | | | | | | | |
| SAB,[5] g of MN/g of available N | | | | | | | | | | | | |
| $^{15}$N | 0.509 | 0.577 | 0.463 | 0.685 | 0.06 | 0.31 | 0.033 | 0.045 | <0.001 | 0.029 | <0.001 | <0.001 |
| PB | 0.552 | 0.593 | 0.519 | 0.655 | | | | | | | | |
| DNA | 0.491 | 0.332 | 0.401 | 0.618 | | | | | | | | |

[1]Twin lambs (1 faunated and 1 protozoa-free) were fed alfalfa hay or 50:50 alfalfa: ground barley (mixed). Animals were euthanized 2 h after feeding and total abomasal content was sampled to determine the postruminal flow using $C_{31}$ alkane as the single flow marker.

[2]LAB = liquid-associated bacteria; MN = microbial N; DOMTR = OM truly digested in the rumen; PB = purine bases; SAB = solid-associated bacteria.

[3]SED from protozoa × diet × method interaction (Pr × D × method; n = 5).

[4]Considering protozoa, diet, method, and their interactions (Pr × D, Pr × M, D × M, Pr × D × M) as fixed effects and twin pair, lamb-within-twin pair, method-within-twin pair, and method-within-lamb-within-twin pair as random effects.

[5]N available = N intake – nonammonia N flow + MN.

sequent bias in the digesta flow values, cannot be ruled out; therefore, observations should be carefully interpreted. More sophisticated estimations of the digesta flow (using cannulated animals and double-flow markers) should be considered for further experiments.

### *Digestion and Rumen Variables*

The observed positive response on fiber digestibility and VFA concentration to faunation, especially when structural carbohydrates (alfalfa hay) constitute the main dietary component, confirms the contribution of ciliates to fibrolytic activity (Agarwal et al., 1991). Nevertheless, it remains unclear if this increment in fiber digestibility is attributed specifically to the protozoal activity; to some form of synergy between protozoa, bacteria, and fungi (Chaudhary et al., 1995); or to a reduction in the rumen retention time of defaunated animals (Ushida et al., 1986). Moreover, the presence of ciliates buffered changes in rumen VFA profiles, possibly as a consequence of starch engulfment (Williams and Coleman, 1992) or selective cellulolytic bacteria predation by the protozoa (Dehority, 2003). The decreased rumen ammonia concentration observed in absence of rumen protozoa agrees with decreased bacterial protein recycling and proteolytic activity (Firkins et al., 1992; Koenig et al., 2000). Protozoa-free lambs fed a mixed diet showed an ammonia concentration below the threshold level (5 mg/100 mL) required to maximize microbial growth in vitro (Griswold et al., 2003). However, no significant changes either in rumen fermentation or microbial protein synthesis were detected, suggesting the existence of alternative metabolic pathways to scavenge $NH_3$ under critical ammonia concentrations (Firkins et al., 1992; Tan and Murphy, 2004).

### *Composition of Microbial Extracts*

The decreased $^{15}$N enrichment and PB/N ratio observed in LAP in comparison with bacterial isolates can result in a bias when microbial synthesis is estimated either assuming similar composition of both pellets or ignoring ciliate contribution to the duodenum. Additionally, the greater metabolic activity and preferential $NH_3$ uptake of LAB (Bates et al., 1985) have been invoked to justify its greater PB and N content and $^{15}$N enrichment (Vicente et al., 2004). From our findings rDNA:N ratio was greater in SAB than in LAB, attributable to a differential selection of bacterial species during the detaching treatment (Ranilla and Carro, 2003) or to contamination with salt or plant material. Ciliates modified the chemical composition of both bacterial extracts directly, as a consequence of bacterial predation (Firkins et al., 1998), or indirectly, by modification of rumen conditions, such as increased $NH_3$ concentration (Koenig et al., 2000) or decreased starch availability.

Protozoal pellets isolated from the rumen have been recommended as appropriate references to estimate

protozoal-N flow (Sylvester et al., 2004; Yáñez-Ruiz et al., 2006), but it is difficult to obtain nonbacteria-contaminated protozoal cells. Sylvester et al. (2004) suggested that serial washes of protozoal extract decreases substantially the bacterial contamination. Following the approach of Sylvester et al. (2004), a low bacterial contamination was observed using a mixed diet (8%), but not for alfalfa diets (36%). Even so, these contaminations did not alter significantly the rDNA/N ratio and then, noncorrected LAP values were used assuming that protozoa-associated bacteria are considered as an integral part of the protozoa (Sylvester et al., 2005).

### *Microbial Production in the Rumen: Methodological Aspects*

Average rumen MN outflow was within the range reported using euthanization (Askar et al., 2007) or cannulation protocols (Pérez et al., 1996a). Differences in MN flow among markers ranked as PB > rDNA > $^{15}$N or PB = $^{15}$n > rDNA, considering LAB or SAB as reference, respectively. Endogenous secretions and undegraded dietary PB may account for the overestimation recorded when PB are used as a marker (Pérez et al., 1996b). Stable isotope (i.e., $^{15}$N) originated a consistent set of values that can potentially be considered as a reference to evaluate other microbial markers, as little interference of dietary or endogenous components and little variation between microbial extracts were observed. Microbial N flows derived from rDNA were within the range determined by $^{15}$N and PB when LAB were used as reference extract; however, they were consistently underestimated when SAB were considered. Two major aspects could explain the decreased microbial N flow by using rDNA as microbial marker: the selective retention of protozoa in the rumen and the incomplete recovery of DNA sequences in the abomasum.

In reference to the former, Sylvester et al. (2005) observed comparable protozoal N contribution to the rumen microbial N pool (4.8 and 12.7%) and to the duodenal MN flow (5.9 and 11.9%, using low- or high-fiber diets in dairy cows), suggesting that the protozoal sequestration in the rumen is negligible. On the contrary, under our experimental conditions, protozoal rDNA represented 46 and 8.8% of the total rumen microbial rDNA in sheep fed a mixed or alfalfa diet, respectively, whereas the protozoal contribution to the abomasum decreased to 16.5 and 1.7% of the microbial rDNA (14.1 and 1.9% in terms of protozoal-N, respectively). This protozoal-N contribution to the intestine was similar to that reported by Sylvester et al. (2005), although it was slightly less than the contributions observed in sheep (21.0 to 24.6%) based on similar approaches (Yáñez-Ruiz et al., 2006). Similar figures were also obtained by using $^{15}$N and optical protozoal counts in the omasum [from 4.3 to 10.4% of MN flow (Ahvenjärvi et al., 2002)] or mechanistic models [from 10.1 to 27.6% (Dijkstra et al., 1998)]. Certain fibrolytic protozoa such as Epidinium and Polyplastron feed on plant particles and can be temporally associated with these solid particles (Williams and Coleman, 1988); therefore, it could be hypothesized that they can evade passage with the faster-passing ruminal fluid with subsequent underestimation of the protozoal N flow. In particular, Dijkstra et al. (1998) modeled the passage of protozoa to be one-half that of the solids dilution rate. Our observations seem to support the idea that rumen protozoa could be partially retained in the rumen, even though the protozoal genera cited represented a small proportion in the present experiment.

With respect to incomplete DNA recovery in the abomasum, the DNA extraction protocol used has been reported as one of the most effective and contrasted (McOrist et al., 2002), and the greater correlation observed between rumen concentration of protozoal cells and protozoal DNA seems to confirm this; therefore, only small differences in the efficiency of DNA extraction might be attributed to this method. However, the lack of cell wall seems to make the protozoa more vulnerable to the low abomasal pH than bacteria. To this extent, data from our own laboratory clearly demonstrated that all bacterial rDNA sequences and about 78% of protozoal sequences persisted throughout the in vitro abomasum digestion simulation (Belanche et al., 2010). Similar recovery of protozoal rDNA copies (81%) was observed by Sylvester et al. (2004) after incubating protozoal cells in duodenal liquid for 2 h at room temperature. The assumption of these recovery rates under our experimental conditions would prompt a slight increase of the protozoal N flow to 0.62 and 0.19 g of N/d, which would represent 17.7 and 2.7% of the microbial N flow for animals fed mixed or alfalfa diets, respectively. Nevertheless, it was considered that the in vitro conditions described by the cited authors could be dissimilar to those observed in the abomasum; therefore, no corrections of the protozoal or bacterial DNA recovery in abomasum were conducted (Yáñez-Ruiz et al., 2006). The possible incomplete DNA recovery in postruminal sites was considered a limitation inherent to this particular microbial marker. Nevertheless, an improvement in the DNA extraction protocol (i.e., including bead-beating) and spike and recovery experiments should be considered for further experiments to minimize this potential bias.

### *Efficiency of Microbial Synthesis*

In the present study, the effect of faunation on microbial N flow was weak, probably affected by the inaccuracy in the digesta flow values, and was dependent on the microbial marker and microbial extract considered. Rumen ciliates decreased both the bacterial and MN flow when specific rDNA sequences and LAB were considered. This decrease was only detected in lambs with a great protozoal concentration (faunated lambs fed the mixed diet) when rDNA and SAB were used.

A similar decrease in bacterial N flow was reported by Ushida et al. (1986), who also observed a simultaneous increase of amylolytic bacteria concentrations and a decrease of adherent bacteria concentrations (Kurihara et al., 1978). This protozoal tropism toward cellulolytic bacteria (Koenig et al., 2000) may partially explain the differences observed between LAB and SAB data. Nevertheless, the potential benefits of defaunation on the microbial synthesis were not evident, even when the more contrasted microbial markers were used ($^{15}$N or PB).

Diet was restricted to avoid changes in the feeding level. Such a design allows comparing data under similar energy supply, although it probably hides the maximum microbial synthesis potential derived from defaunation, especially when digesta flow values are limited by a large variability. Moreover, PF lambs were slaughtered 15 d before (and had 14% lighter BW) the faunated animals due to restrictions imposed by farm facilities. The lighter BW of PF lambs (and decreased OM intake) could counteract the potential benefits of defaunation on microbial synthesis in absolute terms (g of MN/d), and would also explain the more evident benefits using relative terms (EMPS or ENU, see below).

Faunated lambs fed the mixed diet showed the greatest rumen protozoal population and the least EMPS (−25%), independent of the microbial marker of bacterial extract considered. In general, it is widely accepted that a decrease (or elimination) of rumen protozoa increases bacterial concentration, MN flow, and EMPS (Eugène et al., 2004b; Ozutsumi et al., 2006), either because the ecological niche is occupied by bacteria, or because of the predatory effect of protozoa. In agreement with the literature (Pérez et al., 1996a; Askar et al., 2005), barley supplementation decreased the EMPS, independent of the method used. This effect has been explained by wasteful recycling and greater maintenance requirements of the amylolytic bacteria (Russell, 1985), but it should only be evident when the concentrate exceeds 70% of the diet (AFRC, 1993).

Although defaunation had a weak effect on EMPS, it substantially improved ENU. Protozoa-free lambs fed mixed diets showed the greatest ENU (34% greater than the other treatments), independent of the method used. This improved dietary N utilization by the rumen microorganisms matched the smallest rumen ammonia concentration (0.84 mg of N-$NH_3$/100 mL) and acetate:propionate ratio (2.63); moreover, ENU figures were close to those described under an optimal rumen microbial synthesis [0.69 g of MN/g available N (Bach et al., 2005)]. This improvement in the ENU has been attributed to the lack of bacteria predation and subsequent improvement in the rumen N recycling in absence of protozoa (Eugène et al., 2004a; Firkins et al., 2007). These benefits were especially evident when rDNA was used as microbial marker; on average, absence of rumen protozoa increased the ENU by 45 and 24% using LAB and SAB, respectively, and these differences were even greater when lambs were fed the mixed diet.

Eugène et al. (2004a) observed a more efficient use of poor diets by faunated animals, considering the increased digestibility as the main advantage. Our findings agree with this observation in terms of digestibility, but suggest that defaunation has a beneficial effect on the ENU in animals fed mixed diets.

### *Conclusions*

This experiment shows that the absence of rumen protozoa can widely modify the rumen fermentation pattern, resulting in a substantial detriment to diet digestibility, VFA production, and rumen ammonia concentration. The effect of defaunation on microbial N flow was weak and affected by the determination method. Faunated lambs fed the mixed diet showed the greatest rumen protozoal concentration and the least EMPS (−29%), whereas PF lambs fed the mixed diet presented the smallest ammonia concentration and a greater ENU than the others (+34%). The rDNA was used successfully as a novel microbial marker and it permitted an independent quantification of the bacterial and protozoal N contributions. This marker showed that the presence of protozoa decreased the bacterial N flow in the abomasum (−33%), whereas the protozoa N contribution increased from 1.9 to 14.1% of the microbial N flow when barley grain was added to the alfalfa hay. However, these observations should be carefully interpreted as a consequence of the high variability observed in digesta flow figures. More accurate studies using cannulated animals and double-flow markers must be conducted to confirm the validity of these findings.

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