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RESEARCH ARTICLE

Effect of progressive inoculation of fauna-free sheep with holotrich protozoa and total-fauna on rumen fermentation, microbial diversity and methane emissions

Alejandro Belanche, Gabriel de la Fuente and Charles J. Newbold*

Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, Aberystwyth, Ceredigion SY23 3DB, UK

*Corresponding author: Charles J. Newbold, IBERS, Aberystwyth University, SY23 3DD, Aberystwyth, Tel.: +44 1970622242; Fax: +44 1970611264; E-mail: cjn@aber.ac.uk

One sentence summary: Methane emissions from ruminants represent a concern of global magnitude. Understanding the interactions of different rumen protozoa with bacteria and methanogens is vital to develop adequate methane mitigation strategies.

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ABSTRACT

Rumen methanogenesis represents an energy waste for the ruminant and an important source of greenhouse gas; thus, integrated studies are needed to fully understand this process. Eight fauna-free sheep were used to investigate the effect of successive inoculation with holotrich protozoa then with total fauna on rumen methanogenesis. Holotrichs inoculation neither altered rumen fermentation rate nor diet digestibility, but increased concentrations of acetate (+15%), butyrate (+57%), anaerobic fungi (+0.82 log), methanogens (+0.41 log) and methanogenesis (+54%). Further inoculation with total fauna increased rumen concentrations of protozoa (+1.0 log), bacteria (+0.29 log), anaerobic fungi (+0.78 log), VFA (+8%), ammonia and fibre digestibility (+17%) without affecting levels of methanogens or methanogenesis. Rumen methanogens population was fairly stable in terms of structure and diversity, while the bacterial community was highly affected by the treatments. Inoculation with holotrich protozoa increased bacterial diversity. Further inoculation with total fauna lowered bacterial diversity but increased concentrations of certain propionate and lactate-producing bacteria, suggesting that alternative H_2 sinks could be relevant. This experiment suggests that holotrich protozoa have a greater impact on rumen methanogenesis than entodiniomorphids. Thus, further research is warranted to understand the effect of holotrich protozoa on methane formation and evaluate their elimination from the rumen as a potential methane mitigation strategy.

Key words: entodiniomorphids; fermentation; holotrichs; methanogenesis; methanogens; rumen protozoa

INTRODUCTION

Enteric methane emissions from ruminants represent both an important source of greenhouse gas emissions and energy losses for the animal, with estimated loses ranging from 2 to 12% of gross energy intake (Johnson and Johnson 1995). Rumen methane is the end product of anaerobic microbial fermentation of feeds, and methanogenesis derives from the need to remove H_2 from the rumen when methanogenic archaea reduce CO_2 to methane. A wide range of approaches to decrease ruminal methane emissions by either (i) minimizing H_2 production by rumen microbes, (ii) stimulation of H_2 utilization

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through non-methanogenic pathways or (iii) by directly inhibiting rumen methanogens have been investigated (McAllister and Newbold 2008). Hristov *et al.* (2013) concluded that most of these approaches have a positive short-term response; however, their long-term effects are still controversial.

The rumen is home to a large population of rumen protozoa composed of two major types which differ in structure and activity: holotrich protozoa, which have flexible pellicles completely covered in cilia and mostly consume soluble substrates; and the entodiniomorphid protozoa, which have a firm pellicle with cilia situated only on the adoral zone and are able to consume particulate material (Dehority 2003). Rumen protozoa are important H_2 producers in the rumen and between 9 and 25% of the rumen methanogens are thought to be associated with them (Newbold, Lassalas and Jouany 1995). As a result, rumen protozoa and their epi- and endosymbiotic methanogens seem to play a key role in interspecies H_2 transfer and ultimately in methane emissions (Tymensen, Beauchemin and McAllister 2012). Protozoa are ubiquitous, but non-essential denizens of the rumen and their elimination have been shown to decrease methane emissions by 9 to 37% (Hook, Wright and McBride 2010; Morgavi *et al.*, 2010) and to increase (34%) the efficiency of N utilization in the rumen (Belanche *et al.*, 2011), which leads to an increase in live-weight gain (11%) and wool yield (14%) (Eugène, Archimède and Sauvant 2004). As a result, the direct elimination of rumen protozoa using defaunating agents, such as lipids and plant extracts, seems to be an attractive strategy to both, decrease methane emissions and enhance animal performance. However, these phytochemicals have generally a broad spectrum activity resulting in a negative impact on fibre degradation and rumen fermentation (Eugène *et al.*, 2004). As a result, not all studies on defaunation have reported positive responses in terms of animal performance and decreased methane emissions (Bird, Hegarty and Woodgate 2008; Morgavi *et al.*, 2011). Therefore, a better understanding of the mechanisms by which rumen protozoa affect rumen methanogenesis, as well as their interactions with methanogens and bacteria, is needed. This understanding would enable the development of more robust methane mitigation strategies based on targeting particular protozoal groups. The objective of this study was to examine the effect of a progressive inoculation of fauna-free (FF) sheep with holotrich protozoa (HOL) and total-fauna (FAU) on rumen fermentation, methane emissions and bacterial and methanogen populations. It was hypothesized that holotrich and entodiniomorphid protozoa might play different roles in rumen methanogenesis (Lloyd *et al.*, 1989).

EXPERIMENTAL METHODS

Animals and diets

All animal procedures were carried out according to the Home Office Scientific Procedures, Act 1986 (PLL40/316; PIL40/9798). Eight Texel-crossbred sheep were separated from their mothers at 24 h after birth and maintained FF by avoiding further contact with other ruminants. These FF sheep were kept grazing pastures which had not been grazed by other sheep for more than 6 months. The experiment began when sheep were 5 years old and had an average body weight of 94 ± 8.6 kg. Experiment consisted of a straight-through design in which all sheep passed through 3 months adaptation phase between each period. For the period 1, sheep remained FF; for the period 2, sheep were inoculated with a mixed holotrich population composed of pure cultures of *Isotricha prostoma*, *I. intestinalis* and *Dasytricha ruminantium*. These cryoprotected pure cultures, which were generously donated by Diego Morgavi (INRA, Clermont-Ferrand, France), were defrosted at 39°C, diluted (1 in 10 mL) in simplex type salts solution (Williams and Coleman 1992) and orally inoculated in all sheep (10 mL per sheep) in order to generate holotrich-fauna sheep (HOL). Finally in period 3, all animals were orally inoculated (10 mL per sheep) with pooled rumen fluid obtained from four control sheep with a natural protozoal population. As a result, the experimental sheep became totally faunated (FAU).

During the first 2 months, after each inoculation all sheep were kept together grazing mixed ryegrass (*Lolium perenne*) and white clover pastures (*Trifolium pratense*) supplemented with ground barley (average of 0.6 kg DM d^{-1} per head). During the last month of each period, sheep were kept in individual pens with free access to fresh water and mineral blocks (Yellow Rockies, Tithebarn Ltd, Winsford, UK) and were fed with the experimental diet. This diet was composed of 67% ryegrass hay and 33% ground barley (composition in Table 1, Supporting Information) and was designed to meet 1.5 times maintenance requirements (AFRC 1993). Diet was distributed in two equal meals per day (09:00 and 19:00 h), and refusals were recovered daily, weighed and analysed in order to determine the true feed intake. Feeds and refusals were analysed for chemical composition using the reference methods (AOAC 2005). Briefly, the dry matter (DM) content was determined by drying in an oven at 105°C for 24 h; organic matter (OM) concentration was determined by heating at 550°C for 6 h in a muffle furnace. Concentration of neutral detergent fibre (NDF) was measured as described by Van Soest, Robertson and Lewis (1991) using heat stable amylase and sodium sulphite, and acid detergent fibre (ADF) concentration was analysed using the Tecator Fibertec System (Tecator Ltd, Thornbury, Bristol, Somerset, UK). All fibre fractions were expressed inclusive of residual ash.

Methane measurement and rumen sampling

After 24 days adaptation to the experimental diet, enteric methane emissions were measured over the last 4 days of each period using respiration chambers (one animal per chamber) (Yáñez-Ruiz *et al.*, 2008). The measurements were based on the mass-balance approach of measuring flows of the air and the concentrations of methane entering and leaving the chamber. Chamber air temperature was maintained between 10 and 15°C, and airflow and methane concentration were continuously measured using anemometers and an ADC MGA3000 Gas Analyser (Spurling Works, Herts, UK), respectively. Methane emission was calculated as air flow multiplied by methane concentration in the effluent air, adjusted for methane concentration of the incoming air and temperature and atmospheric pressure. Moreover, the gas analyser was calibrated daily using pure N_2 gas and 50 ppm methane gas.

At the end of each period, rumen fluid (250 mL per sheep) was withdrawn by orogastric intubation before the morning feeding (09:00 h) in two non-consecutive days; faecal content was also sampled (30 g fresh matter per animal) and dried for subsequent chemical analysis. Rumen fluid was filtrated thought cheesecloth, pH was immediately measured and four subsamples were taken: the first subsample (50 mL) was immediately snap-frozen in liquid N for DNA extraction and microbial characterization; the second subsample (4 mL) was diluted with 1 mL deproteinizing solution (20% orthophosphoric acid containing 10 mM of 2-ethylbutyric acid as an internal standard) for volatile fatty acid (VFA) determination. These rumen VFA

concentrations were also used to estimate the H_2 production based on stoichiometry calculations (Moss, Jouany and Newbold 2000). The third subsample (1 mL) was diluted with 0.6 mL of trichloroacetate (25% wt:vol) for ammonia analysis. The fourth subsample (1 mL) was diluted with 9 mL of formalin (9.25% vol:vol and NaCl 9% wt:vol) for protozoal optical counting and classification into five main groups: *Isotricha* spp., *Dasytricha* sp., subfamily Diplodiniinae, subfamily Ophryoscolecinae and Subfamily Entodiniinae.

For DNA extraction, frozen rumen samples were freeze-dried and physically disrupted using a bead beater (BioSpec Products Inc., Bartlesville, OK). Genomic DNA was extracted using the QIAamp DNA Stool Mini kit (Qiagen Ltd, UK). Duplicate DNA samples were always analysed separately and finally averaged.

Terminal restriction fragment length polymorphism (TRFLP)

Rumen bacteria and methanogenic archaea communities were studied using TRFLP analysis (Yanez-Ruiz *et al.*, 2010). Briefly, specific primer pairs targeting 16S rRNA genes from total bacteria or methanogens were used with forward primers labelled with Cyanine 5 (Table 2, Supporting Information). Each PCR was performed in duplicate and had a final volume of 25 mL containing 500 nmol L^{-1} of each primer, and 1 μL of DNA template and 12.5 μL of master mix (Immomix, Bioline Inc., USA). Amplification conditions were 95°C for 4 min followed by 25 cycles of 55°C for 1 min, 72°C for 1min and 95°C for 1 min with a final step of 10 min at 72°C. Replicates of amplification products were pooled, purified (Millipore MultiScreen PCRm96 plate, Darmstadt, Germany) and the concentration determined by spectrophotometry (Nanodrop ND-100 Spectrophotometer, Thermo Scientific, USA). This purified PCR product (75 ng) was then digested using one of four restriction enzymes (*Hae*III, *Msp*I, *Hha*I or *Rsa*I; for total bacteria and *Hae*III, *Msp*I, *Hha*I or *Taq*I for methanogens; New England Biolabs Ltd, UK) at 37°C for 5 h followed by an inactivation cycle of 20 min at 80°C. The generated DNA fragments were cleaned by ethanol precipitation and size standard was added (600 bp). Finally, the plate was run on the CEQ 8000 Genetic Analysis System (Beckman Coulter, High Wycombe, UK) and the terminal restriction fragments (TRFs) were separated using the Frag4 parameters. Samples were normalized by removing peaks with an area smaller than 0.25% of the sum of all peak areas. To investigate treatment effects on the bacterial or methanogens populations, Bray–Curtis distances were calculated from square-root-transformed data and a dendrogram was generated using the unweighted pair group method with arithmetic mean. Finally, the mean number of TRF (richness) and the Shannon index were determined as indicators of the microbial community diversity and organization (Hill *et al.* 2003).

Quantitative PCR

DNA concentrations from total bacteria, protozoa, anaerobic fungi and methanogens were determined using qPCR and serial dilutions (from 10^{-1} to 10^{-5}) of specific DNA standards. Rumen liquid-associated bacteria were obtained from each animal (Cecava *et al.*, 1990) and pooled to generate a bacterial DNA standard. A protozoal standard was generated by pooling protozoal DNA from individual animals obtained by a sequential filtration and washing of the rumen protozoa to minimize the bacterial contamination (Sylvester *et al.*, 2004). In addition, DNA from three different axenic cultures of anaerobic fungi [*Neocallimastix frontalis* (RE1), *Orpinomyces* spp. SR2 and *Caecomyces* spp. Isol1] and DNA from a plasmid containing the methyl-coenzyme-M reductase (*mcrA*) gene were used as standards for fungi and methanogen quantifications, respectively (Belanche *et al.*, 2012a).

PCR was performed in triplicate using a DNA Engine Opticon system (MJ Research). Amplification reactions (25 μL) contained DNA template (2 μL), 1 mM of each primer and 12.5 mL SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich Ltd, Dorset, UK). Amplification conditions were 95°C for 5 min, then 45 cycles at annealing temperatures described in Table 2 (Supporting Information) for 30 s, 72°C for 30 s and 95°C for 15 s. The C_T value was determined during the exponential phase of amplification, and a final melting analysis was performed to check primer specificity.

To investigate the effect of the protozoal inoculation on the rumen bacterial population, the relative abundance of 13 of the most common cultured bacterial species was measured relative to total bacterial DNA. Species-specific 16S rRNA primers were used following the same cycling conditions described above (Table 2, Supporting Information). Efficiencies of PCR amplification for each primer pair were determined using a regression based on the C_T values obtained from several dilutions of same DNA sample.

Calculations and statistical analysis

The ratio of each bacteria species in respect to total bacteria was determined using the ΔCt method corrected by the efficiencies of amplification (Pfaffl 2001). Microbial data were tested for normality using the Shapiro–Wilk test and in variances which were unequal, data transformations were performed to attain normality. Duplicated samples from the same animal were processed separately and averaged before data were analysed by analysis of variance using of GenStat (15th Edition, VSN International, UK) as follow:

$$Y_{ij} = \mu + F_i + A_j + e_{ij},$$

where YW_{ij} is the dependent, continuous variable ($n = 8$); μ is the overall mean; FW_i is the fixed effect of the protozoal population (i = FF, HOL, FAU); AW_j is the random effect of the animal ($j = 1$ to 8); and eW_{ij} is the residual error.

Treatment effects on TRFLP square-rooted data were analysed by non-parametric permutational analysis of variance using PRIMER-6 software (PRIMER-E Ltd, Plymouth, UK). Moreover, pairwise comparisons were conducted to elucidate differences between treatments. The pseudo F-statistics and *P*-values were calculated after 1000 random permutations of residuals under a reduced model using the Monte Carlo test. Finally, a canonical correspondence analysis was also performed to investigate the relationships between the structure of the microbial community and certain rumen variables, such as ruminal concentration of ammonia, total bacteria, fungi, methanogens, NDF digestibility and methane emissions. The signification of each variable was calculated by using 1000 random permutations.

RESULTS

Intakes, rumen fermentation and methane emissions

Sheep remained in good health and no differences in intakes were detected throughout the experiment (Table 1). A slight decrease in body weight was observed after inoculation with total fauna. No protozoa were detected in FF sheep, whilst a

Table 1. Feed intakes and rumen protozoal numbers in sheep successively inoculated with different protozoal species.

Treatment[1]	FF	HOL	FAU	SED	P-value
Body weight (kg)	93.8[a]	92.9[a]	90.9[b]	0.77	0.003
DM intake (kg d^{-1})	1.58	1.60	1.68	56.2	0.172
Rumen protozoa (log cells mL^{-1})					
Total protozoa	0[c]	4.69[b]	5.85[a]	0.061	<0.001
Isotricha spp.	0[c]	4.01[a]	3.60[b]	0.120	<0.001
Dasytricha sp.	0[c]	4.58[a]	4.17[b]	0.086	<0.001
Subf. Diplodiniinae	0[b]	0[b]	4.55[a]	0.075	<0.001
Subf. Ophryoscolecinae	0[b]	0[b]	4.12[a]	0.096	<0.001
Subf. Entodiniinae	0[b]	0[b]	5.80[a]	0.050	<0.001

[1]Treatments ($n = 8$): FF, fauna-free; HOL, Holotrich monofaunated; FAU, total-faunated sheep. Within a row means without a common superscript differ ($P < 0.05$).

Table 2. Rumen fermentation parameters and methane emissions in sheep successively inoculated with different protozoal species.

Treatments[1]	FF	HOL	FAU	SED	P-value
Rumen fermentation					
pH	6.94[a]	6.89[a]	6.73[b]	0.050	0.002
Ammonia-N (mg dL^{-1})	1.29[b]	1.39[b]	4.85[a]	0.583	<0.001
Total N (mg g^{-1} DM)	36.2[b]	38.8[b]	49.7[a]	2.37	<0.001
Total VFA mM)	78.1[b]	87.3[ab]	94.4[a]	4.64	0.012
Acetate (%)	66.9	68.8	66.9	2.19	0.614
Propionate (%)	21.8[a]	16.3[b]	17.5[b]	1.47	0.005
Butyrate (%)	7.82[b]	11.0[a]	12.0[a]	1.13	0.006
Isobutyrate (%)	1.68	1.44	1.28	0.197	0.163
Valerate (%)	0.79	0.75	0.83	0.152	0.879
Isovalerate (%)	0.85	0.96	1.08	0.134	0.274
Caproate (%)	0.15[b]	0.73[a]	0.36[b]	0.120	<0.001
(Acetate + Butyrate)/Propionate	3.59[b]	4.95[a]	4.52[a]	0.320	0.003
Fecal composition (ratio)					
Ash/OM	0.12	0.12	0.13	0.006	0.057
Ash/NDF	0.16[a]	0.17[a]	0.20[b]	0.010	0.008
Ash/ADF	0.30[a]	0.31[a]	0.36[b]	0.017	0.004
Methane emissions					
L d^{-1}	30.3[b]	49.5[a]	53.5[a]	3.17	<0.001
L kg^{-1} body weight	0.32[b]	0.53[a]	0.59[a]	0.038	<0.001
L kg^{-1} DM intake	19.2[b]	31.2[a]	31.8[a]	1.95	<0.001

[1]Treatments ($n = 8$): FF, fauna-free; HOL, Holotrich monofaunated; FAU, total-faunated sheep. Within a row means without a common superscript differ ($P < 0.05$).

population made up solely of holotrichs was detected in HOL sheep in which *Isotricha* spp. and *Dasytricha* sp. represented 22 and 78% of the population, respectively. FAU sheep had a rumen concentration of total protozoa 14.4 times greater than HOL sheep, being this protozoal population composed of subfamily Entodiniinae (89.3%), subfamily Diplodiniinae (5.1%), subfamily Ophryoscolecinae (2.5%), *Dasytricha* sp. (2.4%) and *Isotricha* spp. (0.7%).

FAU sheep had lower rumen pH and higher concentrations of ammonia and total N than observed in FF or HOL sheep, while no differences were observed between these two later treatments (Table 2). Ruminal concentration of total VFA increased progressively with the successive inoculation of rumen protozoa and differences in molar proportions of individual VFA were also observed. Propionate molar proportions were higher in FF sheep but in butyrate were lower in comparison with HOL and FAU sheep.

Faeces from FF and HOL sheep had lower ratios of ash/OM, ash/NDF and ash/ADF than observed from FAU animals indicating a significant effect of the rumen protozoa on the diet digestibility. Methane emissions were 40% lower in FF compared to HOL or FAU sheep, and no differences were detected between the two latter treatments.

Rumen bacteria community

TRFLP analysis using four restriction enzymes generated a total of 811 different bacterial TRFs and on average each sheep had 192 ± 41 bacterial TRF's. All restriction enzymes generated similar numbers of TRF's (48 ± 13), but slight differences in the rumen bacterial structure and diversity were observed depending on the restriction enzyme used (Table 3, Supporting Information). Therefore, the combination of all enzymes was considered to give the less biased results (Yanez-Ruiz *et al.*, 2010). Dendrogram showed a clear separation between the rumen bacterial structure from FF, HOL and FAU sheep (Fig. 1A and Fig. 1A, Supporting Information). These differences between treatments were confirmed by the permutational analysis of variance (Table 3). Pairwise comparisons of the bacterial community indicated large differences between FF and FAU and also between HOL and FAU sheep, while smaller differences were found between FF and HOL sheep. In order to detect possible correlations

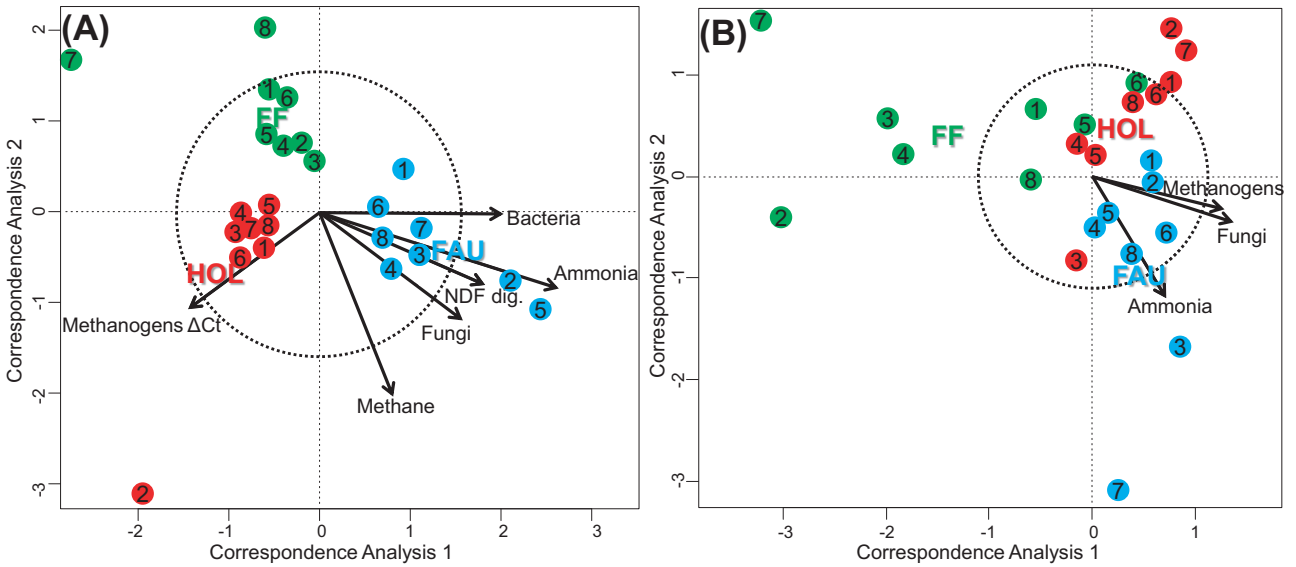

Figure 1. Canonical correspondence analysis illustrating the relationship between the structure of the bacterial (**A**) or methanogen communities (**B**) with the rumen function. Treatment's names indicate the centroid of each treatment. Arrows show the direction of the gradient and their length is proportional to the correlation. Arrows longer than the dotted circle are significant ($P < 0.05$). Treatments: FF, fauna-free; HOL, Holotrich monofaunated; FAU, total-faunated sheep. Animals are indicated with numbers.

Table 3. Changes in the communities of rumen bacteria and methanogens in sheep successively inoculated with different protozoal species.

	Bacteria			Methanogens		
	Similarity	Pseudo-F	*P-value*	Similarity	Pseudo-F	*P-value*
Treatment effect[1]		4.24	0.001		2.86	0.002
Pairwise comparisons						
FF vs HOL	42.3	1.88	0.015	45.4	1.59	0.045
FF vs FAU	39.3	2.12	0.010	46.3	1.85	0.021
HOL vs FAU	44.1	2.18	0.005	53.2	1.63	0.042

[1]Microbial communities were studied using TRFLP with four restriction enzymes. Permutational analysis of variance was performed using Bray–Curtis similarity measurements of square root data: higher Pseudo-F and lower similarities and P-values correspond to greater differences in the microbial community composition. Treatments ($n = 8$): FF, fauna-free; HOL, Holotrich monofaunated; FAU, total-faunated sheep.

Table 4. Changes in the biodiversity indices of rumen bacteria and methanogens in sheep successively inoculated with different protozoal species.

Treatments[1]	FF	HOL	FAU	SED	*P-value*
Bacterial community					
Richness	43.1[b]	59.1[a]	41.6[b]	3.02	<0.001
Shannon index	2.68[b]	3.30[a]	2.52[b]	0.121	<0.001
Methanogens community					
Richness	21.7	20.6	19.3	1.57	0.337
Shannon index	1.97	1.95	1.82	0.071	0.110

[1]Treatments ($n = 8$): FF, fauna-free; HOL, Holotrich monofaunated; FAU, total-faunated sheep. Within a row means without a common superscript differ ($P < 0.05$). TRFLP diversity indices were calculated using the averaged data from four restriction enzymes.

between the samples (treatments) and certain rumen variables, canonical correspondence analysis was performed. Fig. 1A shows a clear separation of the samples on the ordination plot according to the treatments. Moreover, several variables were correlated with this sample distribution: ruminal concentration of ammonia, bacteria, fungi and NDF digestibility were positively correlated to the structure of the bacterial community of FAU samples. Relative abundance of methanogens was positively correlated with the HOL samples. Finally, the structure of the bacterial community in FF samples was negatively correlated with methane emissions (arrow pointing in opposite direction).

Table 5. Ruminal concentration of the different microbial groups and bacterial species in sheep successively inoculated with different protozoal species.

Treatments[1]	FF	HOL	FAU	SED	P-value
DNA concentration (log)					
Protozoa (ng g^{-1} DM)	0^{c}	4.78^{b}	5.78^{a}	0.138	<0.001
Bacteria (ng g^{-1} DM)	6.14^{a}	5.97^{b}	6.26^{a}	0.060	0.001
Methanogens (copies g^{-1} DM)	6.79^{b}	7.20^{a}	7.31^{a}	0.081	<0.001
Anaerobic fungi (ng g^{-1} DM)	1.10^{c}	1.92^{b}	2.70^{a}	0.333	0.001
Relative abundance[2] ($10^3 \times 2^{-\Delta Ct}$)					
R. albus	1.22	1.75	1.36	0.296	0.218
R. flavefaciens	2.85	3.86	2.36	0.896	0.265
F. succinogenes	5.42	5.80	7.25	1.163	0.286
B. fibrisolvens	5.31^{a}	3.84^{ab}	2.66^{b}	0.930	0.041
S. bovis	4.99^{a}	2.43^{ab}	1.11^{b}	1.624	0.086
Prevotella spp.	1.85^{b}	2.02^{b}	7.39^{a}	1.528	0.004
P. bryantii	0.05	0.04	0.05	0.013	0.874
P. albensis	0.28^{b}	0.32^{ab}	0.40^{a}	0.044	0.050
Se. ruminantium	0.05^{ab}	0.03^{b}	0.08^{a}	0.015	0.022
M. elsdenii	0.19^{a}	0.21^{a}	0.10^{b}	0.040	0.040
E. ruminantium	0.07^{b}	0.06^{b}	0.16^{a}	0.023	<0.001
A. lipolytica	0.05^{b}	0.03^{b}	0.20^{a}	0.030	<0.001
Lactobacillus spp.	7.06^{a}	6.17^{b}	7.64^{a}	0.378	0.005
Methanogens	0.24^{c}	0.56^{a}	0.33^{b}	0.040	<0.001

[1]Treatments ($n = 8$): FF, fauna-free; HOL, holotrich monofaunated; FAU, total-faunated sheep. Within a row means without a common superscript differ ($P < 0.05$).
[2]To attain normality analysis of variance was conducted using square-rooted data.

In terms of bacterial biodiversity (Table 4), HOL sheep showed the greatest bacterial richness and Shannon index suggesting an increased bacterial diversity relative to other treatments, and indicating the presence of similar proportions of the different bacterial groups.

Methanogenic archaea community

TRFLP analysis indicated the presence of a complex methanogenic archaea population in the rumen but with a more stable structure than the bacterial community (lower differences between treatments). In particular, TRFLP generated 464 different methanogen signals and on average each sheep had 99 ± 24 TRF's as sum of the four restriction enzymes. Dendrograms showed a modest separation between the three experimental groups (Fig. 1B, Supporting Information). Similarly, canonical correspondence analysis (Fig. 1B) showed that the effect of the treatments on the position of the samples within the ordination plot was less obvious for the methanogen community than for the bacterial one. Moreover, the structure of the methanogen community of these samples was not correlated with most of the rumen variables and only ruminal concentration of ammonia, methanogen DNA and fungal DNA were positively correlated with the structure of the methanogens community of FAU sheep.

Permutational analysis of variance showed that the methanogen population differed between the three experimental treatments (Table 3); pairwise analysis revealed that the greatest differences were between FF and FAU, while HOL sheep had similar differences with FF and with FAU. Methanogen diversity indices were always lower than those observed for bacteria, indicating a less complex and diverse methanogen population. Methanogen diversity indices were unaffected by the experimental treatments suggesting a similar number of methanogen groups. Shannon index remained low and constant across treatments suggesting a methanogen population characterized by the presence of few majority species in high numbers accompanied by many minority species in low numbers.

Microbial numbers

As expected, no protozoal DNA was observed in FF sheep (Table 5), while inoculation of HOL sheep with total fauna (FAU) increased 10 times the protozoal DNA concentration. Rumen concentrations of bacterial DNA were similar between FF and FAU but declined in HOL sheep. FAU sheep showed the greatest concentration of anaerobic fungi, while FF sheep had the lowest concentration of methanogens across treatments.

qPCR analysis (Table 5) revealed that the ruminal abundance of three of the main cellulolytic species (i.e. *Ruminococcus albus*, *R. flavefaciens* and *Fibrobacter succinogenes*) and some amylolytic bacteria (i.e. *Prevotella bryantii*) were unaffected by the experimental treatments. FF sheep had a greater ruminal abundance of *Butyrivibrio fibrisolvens*, *Streptococcus bovis* and *Megasphaera elsdenii* than FAU sheep. Conversely, FAU sheep showed a higher abundance of *Prevotella* spp., *P. albensis*, *Selenomonas ruminantium*, *Eubacterium ruminantium* and *Anaerovibrio lipolytica*. Finally, HOL sheep had the greatest abundance of *M. elsdenii* and the lowest of *Lactobacillus* spp. The expression of methanogen numbers in respect to total bacterial DNA indicated that HOL sheep had the greatest methanogen relative abundance, while FF had the lowest.

DISCUSSION

Rumen colonization

It has been suggested that the effect of defaunation on rumen function may be transitory, particularly when physical or chemical defaunation methods are used (Bird *et al.*, 2008; Morgavi *et al.*, 2011). Other authors suggest that the lower rates of methane observed in FF ruminants could be due to the poor

establishment of certain microbial groups such as methanogens (Hegarty *et al.*, 2008). In our experiment, animals were naturally FF to prevent the alteration of the rumen microbial ecosystem with defaunating agents. More importantly, animals had similar methanogen diversity throughout all experiment indicating that FF sheep had a fully developed rumen microbial ecosystem at the beginning of this experiment. The exposure to microbes during the first 24 h after birth or to contaminated pastures during the subsequent five years seem to be the most likely ways for their rumen microbial colonization (Jami *et al.*, 2013).

Cryopreservation of protozoa cultures allows to recover viable rumen protozoa (de la Fuente, Cebrian and Fondevila 2004) and their symbiotic microbes after thawing. Similarly rumen inoculation with external rumen fluid may result in an initial shift in the bacterial community; however, Weimer *et al.* (2010) demonstrated that ruminants are able to reestablished their original bacterial population within few weeks post-inoculation, even when almost the entire rumen content is exchanged. Thus, the treatment effects observed in this experiment (measured 3 months after inoculation) are likely to be permanent modifications of the rumen rather than transitory effects.

Methane emissions and rumen protozoa

It has been suggested that protozoa might increase methane production in the rumen through a combination of increased feed degradation and/or their symbiotic relation with methanogens (Hegarty 1999a; Morgavi *et al.*, 2011). In the present experiment, FF sheep produced 43% less methane emissions than FAU sheep. In a review, Morgavi *et al.* (2010) observed that rumen defaunation decreased methane emissions by 10.5% on average with a decrease in methane yield of 0.6 g kg^{-1} DM intake per reduction of 10^5 cells mL^{-1}. According to this calculation, half of the increment in methane emissions observed between FF and FAU could have been explained by the increased numbers of rumen protozoa. This observation emphasizes the importance of those dietary interventions focus on lowering the protozoal numbers as methane mitigation strategies (Hegarty 1999a).

However, the substantially higher methane emission in HOL sheep appears to be an 'abnormality' that requires a more detailed examination. It is possible that the effect of holotrichs on rumen methanogenesis could be magnified under our experimental conditions because they were the first protozoa to colonize the rumen, and therefore had no competition from other protozoal species (Williams and Coleman 1992). Further research including animals that undergo an alternate succession of inoculations (entodiniomorphids first and holotrich after) would be needed to evaluate this hypothesis.

It has been suggested that holotrich and entodiniomorphids neither predate each other nor compete for nutrients (Williams 1989). Though, the observed drop in holotrich numbers when a total fauna was established seems to indicate that at least limited competition for the substrate or space between holotrichs and entodiniomorphids did occur.

All rumen protozoa contain hydrogenosomes, organelles with residual respiratory similarities to the mitochondria, which produce large quantities of H_2, CO_2, acetate, butyrate and lactate during anaerobic fermentation (Embley *et al.*, 2003). Hydrogenosomes from holotrichs, in comparison to entodiniomorphids, have a lower isopycnic density (Snyers *et al.*, 1982; Paul, Williams and Butler 1990) as well as a compartmentalization of O_2-sensitive enzyme pyruvate synthase and malate hydrogenase within the hydrogenosomes which may afford some protection to the enzymes enhancing H_2 production even in the presence of O_2 (Lloyd *et al.*, 1989). This may explain why methane production in HOL sheep was an order of magnitude higher than would have been predicted by the equation of Morgavi *et al.* (2010). Indeed HOL sheep produced almost as much methane as FAU sheep since the lower total protozoal numbers observed in HOL sheep (7% of observed in FAU) were somehow compensated by a greater representation of holotrichs. Our calculations, considering a basal methane emission in FF sheep, indicated that holotrich protozoa were associated with the production of 11.8 times more methane per cell than total protozoa observed in FAU sheep and 18.3 times more than entodiniomorphids. This observation agrees with early studies, in which high H_2 production rates were described for pure cultures of *I. prostoma* and *D. ruminantium* (Williams 1986) suggesting that they may play a key role in the rumen methanogenesis (Ushida and Jouany 1996). Considering the great diversity of entodiniomorphids, more research is needed to elucidate possible differences in the methanogenic potential of individual genera.

Fermentation pattern

Diet digestibility was not measured directly in this experiment but can be inferred from fecal analysis of ash (non-digestible) versus other nutrients (Wolk *et al.*, 1998). Based on this ratio, feed digestibility was similar in FF and HOL sheep. Likewise, no differences in the rumen pH or in the concentration of fermentation end-products such as total N, ammonia N and total VFA were observed between both experimental groups. These observations seem to confirm that holotrichs had a limited ability to digest fibre (Ivan 2009) and to predate bacteria in the rumen (Belanche *et al.*, 2012b), and therefore a minor effect on rumen N turnover and microbial protein synthesis (Ivan, Neill and Entz 2000). However, inoculation of FF sheep with holotrichs did shift the molar proportion of the different VFA. Holotrichs exhibit a chemotaxis to simple sugars and are able to engulf, store and utilize large quantities of plant reserve polysaccharides (Dehority and Tirabasso 1989). Moreover, up to 90% of the holotrich cells become attached to solid feed and to the rumen wall preventing them being washed out from the rumen (Orpin and Hall 1983). Holotrichs sequestration within the rumen together with the rapid engulfment of substrates after feeding represents a competitive advantage in respect to bacteria and may explain the decreased bacterial DNA concentration observed in HOL sheep. Thus, holotrichs seem to have an increased fermentation rate and H_2 production after feeding which supports the reported boost on the levels of their endosymbiotic methanogens (Ushida 2010). As a result of that, inoculation of FF sheep with holotrichs increased the ruminal concentrations of acetate (+15%) and butyrate (+57%), which together with lactate, H_2 and CO_2 are the major metabolic end products of holotrich protozoa (Williams 1989). On the contrary, this was accompanied by a decrease in propionate concentration (−17%), an acid which is not synthetized by holotrich protozoa. Considering these proportions of VFA, a positive correlation ($R = 0.71$) was observed between the observed and predicted methane emissions based on stoichiometrical calculations (Moss *et al.*, 2000). This calculation predicted just half of the observed increment in methane emissions indicating that VFA production explains only part of rumen methanogenesis.

The further inoculation of HOL sheep with total fauna promoted a different scenario for the rumen function. Both, FF and HOL sheep had lower total tract digestibility for OM (−12%), NDF (−17%) and ADF (−18%) than observed in FAU sheep. In a

previous experiment, we observed similar reduction (−14% NDF digestibility) in FF sheep fed forage diets compared to faunated sheep in which just entodiniomorphids were present (Belanche *et al.*, 2011). Similarly, the increased ruminal concentration of total VFA, total N and ammonia N observed in FAU sheep indicated that, in contrast to holotrichs, certain entodiniomophids, such as big Diplodiniinae, play a key role in fibre degradation (Ivan 2009) and also in bacterial predation (Belanche *et al.*, 2012b). Interestingly, no significant differences in VFA molar proportions were observed between HOL and FAU sheep indicating that similar metabolic pathways were active in both situations. This observation suggests that either the total protozoa community observed in FAU sheep have very little activity in terms of H_2 production in comparison with holotrichs or it goes into non-methanogenic H_2 sinks.

Bacterial community

TRFLP analysis revealed that rumen inoculation with different protozoal groups promoted a shift in the bacterial community which could indirectly magnify their effect on the rumen function (Koenig *et al.*, 2000; Morgavi *et al.*, 2010). Differences in the rumen bacterial structure between FF and FAU sheep have been reported using DGGE (Yáñez-Ruiz, Williams and Newbold 2007), TRFLP (Belanche *et al.*, 2012a) and 16S rRNA genes clone libraries (Ozutsumi *et al.*, 2005), being generally accompanied with an increased bacterial biodiversity in the presence of total fauna. Our results indicated that the presence of holotrichs alone were also able to modify the structure of the bacterial community and increase its diversity. This increased diversity may be the result of beneficial effects of holotrichs on the bacterial community (i.e. O_2 consumption) but negligible bacterial predatory activity (Williams and Coleman 1992).

Defaunation normally promotes a greater bacterial density in the rumen (Williams and Coleman 1992). This effect was observed when FF and HOL sheep were compared, but not between FF and FAU sheep. Belanche *et al.* (2012a) indicated that the ruminal accumulation of undigested DM in absence of protozoa could minimize these differences when bacterial concentration is expressed per gram of DM.

The structure of the bacterial community was correlated with certain rumen variables: canonical correspondence analysis revealed that the bacterial community of FF sheep was negatively correlated with rumen methanogenesis. Contrarily, the bacterial community of FAU sheep was positively correlated with feed degradation process (i.e. ammonia and NDF digestibility), ruminal concentrations of total bacteria, fungi and methanogens and ultimately with methane emissions. Finally, HOL sheep had a bacterial community positively correlated with relative abundance of methanogen suggesting an efficient interspecies H_2 transfer between bacteria and methanogens (Morgavi *et al.*, 2010).

To further investigate the effect of protozoa on the bacterial community and the interspecies H_2 transfer, the relative abundance of certain bacterial groups was investigated: qPCR analysis detected no differences between treatments in the ruminal abundance of fibrolytic bacteria (*R. albus* and *R. flavefaciens* and *F. succinogenes*). This observation agrees with previous experiments, in which we have shown that these fibrolytic bacteria are sensitive to the N shortage in the rumen but not to the type of carbohydrate (Belanche *et al.*, 2012c) or the presence of rumen protozoa (Belanche *et al.*, 2012a). Similarly, inoculation of FF sheep with holotrichs did not modify the ruminal concentration of most of the microbial groups studied, and only *Lactobacillus* spp. decreased after this inoculation, suggesting that lactate was unlikely to be an important H_2 sink in HOL sheep.

On the contrary, inoculation of HOL sheep with total fauna promoted a symbiotic increase in most fibrolytic microbes, such as protozoa, anaerobic fungi and *B. fibrisolvens*, which may explain the increase in fibre degradation, VFA synthesis and ultimately H_2 production (Morgavi *et al.*, 2010; Belanche *et al.*, 2012c). Since the number of methanogens remained unaffected between HOL and FAU sheep, part of this H_2 could have been diverted to alternative H_2 sinks. To this end, the increased abundance of propionate producers (*Prevotella* spp. and *P. bryantii*), lactic acid producers (*Lactobacillus* spp., *S. ruminatium* and *E. ruminantium*), lipolytic bacteria (*A. lipolytica*) and bacteria involved in fat bio-hydrogenation bacteria (*Prevotella* spp.) in FAU sheep, together with the decreased numbers of lactic acid consumers (*M. elsdenii*), may indicate that propionate, lactate and saturated fats could be relevant H_2 sinks in the rumen of FAU sheep (Morgavi *et al.*, 2010). The direct quantification of these alternative H_2 sinks could help to clarify the latter hypothesis.

Methanogen community

Methanogenic archaea are the sole producers of methane in the rumen (Morgavi *et al.*, 2010), therefore one might expect a clear correlation between methanogen numbers and methanogenesis. However, some authors suggest an uncoupling between methanogenesis and methanogen abundance indicating that a shift in the methanogenic community to a less active or less efficient at producing methane may explain the differences in methane emissions (Hegarty et al., 1999b; Firkins and Yu 2006). The second hypothesis relies on the different methanogenic potential observed among various methanogen groups (Hook *et al.*, 2010; Leahy *et al.*, 2013).

Our data do not give a definitive answer to this dilemma but provides support to the first hypothesis. FF sheep had both lower methanogen abundance and lower methane emissions than observed in HOL and FAU sheep; consequently, a positive correlation was observed between methane production and total methanogen numbers ($R = 0.66$) and methanogens: bacteria ratio ($R = 0.55$). These correlations have been confirmed using a greater number of animals, $R = 0.38$ and 0.49, respectively (Wallace *et al.*, 2014). These increments in the number of methanogens clearly shows a symbiotic relation between rumen protozoa and methanogens that could enhance the interspecies H_2 transfer (Morgavi *et al.*, 2011). However, substantial metabolic differences have been observed between protozoal groups; holotrichs have a lower K_m value for the O_2 than entodiniomorphids which enable holotrichs to scavenge O_2 even when it is at low concentration (Ellis, Williams and Lloyd 1989). The holotrich protozoa's ability to consume O_2, together with the great H_2 production derived from their hydrogenosomes (Paul *et al.*, 1990; Williams and Coleman 1992), seems to provide the perfect environmental conditions for strict anaerobe hydrogenotrophs to grow, and ultimately may explain why HOL sheep had the greatest relative abundance of methanogens.

Nevertheless, the second hypothesis cannot be definitively ruled out because several authors have demonstrated the presence of association patterns between different methanogen groups and specific protozoa (Krumholz, Forsberg and Veira 1983; Ohene-Adjei *et al.*, 2007). In a recent paper, we have also reported that holotrich protozoa have an endosymbiotic methanogen community which differs to that associated with total protozoa (Belanche, de la Fuente and Newbold 2014). This

observation may explain the small change in the structure of the methanogen community after rumen inoculation with protozoa. This shift in the methanogen population was however less pronounced than the changes in the bacterial community and was not accompanied by changes in methanogen diversity indices. Moreover, canonical correspondence analysis demonstrated that the structure of the methanogen community was only correlated with rumen methanogenesis in FAU sheep, and may partially explain the increased methanogenesis observed in FAU sheep, but not in HOL sheep. In a similar study to ours in which FF lambs were inoculated with rumen fluid with our without protozoa, it was also concluded that protozoa was the biggest factor shaping the structure of the bacterial but not the methanogen community (Morgavi *et al.*, 2014).

Our findings agree with previous observations using adult animals, which indicated no substantial changes in methanogens diversity after rumen inoculation with different protozoal groups (Ohene-Adjei *et al.*, 2007), as well as minor differences in the structure of the methanogen community after short- or long-term defaunation (Mosoni *et al.*, 2011). Contrasting results (increased bacterial and methanogen diversity) were however reported when young lambs were inoculated (Morgavi *et al.*, 2014). This discrepancy may be due to the incomplete rumen microbial development of young lambs, since inoculation with rumen fluid with or without protozoa promoted similar responses in the structure of the microbial community in young lambs (Morgavi *et al.*, 2014) but is unlikely to happen in adult animals (Weimer *et al.*, 2010). Further research including control animals with a constant treatment across periods would be needed to confirm this assumption.

IMPLICATIONS

This experiment demonstrated that ruminal methanogenesis is a complex process which require integrated studies of the rumen function and microbiology to be understood. Our findings suggest that holotrich protozoa have a greater impact on rumen methanogenesis than entodiniomorphids. However, further research using control treatments is warranted to fully understand the effect of holotrich protozoa on methane formation and evaluate their elimination from the rumen as a potential methane mitigation strategy. This would help to confirm whether the increase in methanogenesis in HOL sheep was due to the holotrichs activity *per se* or could be magnified by the fact that holotrichs were the first protozoa to colonize the rumen and had no competition from other protozoal groups.

SUPPLEMENTARY DATA

Supplementary data is available at FEMSEC online.

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