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Microbial populations and ruminal fermentation of sheep and llamas fed low quality forages

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Highlights

- The forestomach contents of llamas presented higher microbial activity than sheep, which was reflected in a higher NH_4-N and VFA concentrations.
- The llamas had a higher density of total protozoa and of genus *Eudiplodinium*, whereas holotrich protozoa were not detectable.
- No differences were detected between llamas and sheep for total bacterial populations, total methanogens, and fibrolytic bacteria.

Abstract

The objective of this study was to assess the ruminal microbial population and fermentation of sheep and llamas fed low quality forages. Two llamas and three sheep with permanent ruminal cannula were fed *ad libitum* with a low quality fescue hay (*Festuca arundinacea* Schreb). The animals were adapted to forage feeding for 21 days, followed by one day of collection of the ruminal fluid (RF), during 2 experimental periods separated by 7 days. The RF was extracted before morning feeding. The llama forestomach content showed higher degree of fermentative activity than the sheep one, as indicated by NH_3-N and VFA values ($P< 0.001$). Similar densities of total bacteria, methanogens and fibrolytic bacteria were present in both species ($P> 0.050$). However, the forestomach of llamas contained higher density of total protozoa and *Eudiplodinium* ($P< 0.050$), whereas holotrich protozoa were undetectable. In conclusion, the llama forestomach had higher fermentative activity than sheep rumen, different protozoa population, but no differences in bacterial population densities.

Keywords: Camelids; bacteria; protozoa; methanogens; microbiota; forestomach.

Introduction

The domestic South American Camelids (SAC; alpaca and llama) differ from sheep in structure and functioning of their digestive system, as well as in their nutritional

strategies (Vallenas et al., 1971; Engelhardt et al., 1988; San Martin and Bryant, 1989; Jouany et al., 1995). Camelids show an apparently better digestion capacity of low quality food than sheep. It has been speculated that longer retention time of food and more contractions in the forestomach could be involved in this phenomenon (San Martin and Bryant, 1989; Dulphy et al., 1997). In addition, Eckerlin and Stevens (1973) suggested that higher pH stability may be causative, as a result of longer periods of pH within the neutrality range facilitated by an increased secretion of bicarbonate from glandular sacs present on the forestomach walls.

Recent studies have described differences between the biochemical (pH, NH_3-N) and physical (redox potential, osmolarity, pressure and surface tension) characteristics of the forestomach contents of the alpaca with respect to those of the sheep rumen under the same feeding conditions (Liu et al., 2009). Rumen microbiota in sheep and alpacas differ, as a lower population of methanogens and a higher population of fibrolytic fungi and bacteria have been found in alpaca forestomach when compared with sheep as reported by Pei et al. (2013).

Specific characteristics of camelid metabolism may also be involved in the differences of digestion. For instance, camelids have a lower urea excretion rate through urine (probably due to a greater nitrogen recycling), which results in a greater availability of NH_3-N for protein synthesis and metabolic activity of the microbial populations in the forestomach (Hinderer and Engelhardt, 1975).

However, the studies on the ruminal mechanisms underpinning the differences in feeding conditions with hypo-nitrogenous forage diets coupled with microbiota descriptions are scarce. In this work, we studied the mechanisms involved in this apparent superiority of llamas with respect to sheep. We hypothesized that llamas have a more efficient recycling mechanism of nitrogen (N), so that under low N forage diets

they will show higher ruminal concentration of NH_3-N, as well as higher density and microbial activity than sheep. The purpose of this study was to characterize microbial populations and the fermentative parameters of the rumen of sheep and llamas fed low quality forages.

Materials and Methods

Location, experimental conditions and ruminal forestomach sampling.

The study was carried out in the Department of Animal Science of the School of Agriculture (University of Buenos Aires) and at the Laboratory of Rumen Microbiology (National Institute of Agricultural Technology; Argentina). Animal care and husbandry were performed by trained personnel and specialized veterinarians according to the Ethics and Animal Welfare Committee of the Animal Science Magister Program (CEyBA – N° 2014/2).

Two llamas (*Lama glama*) and three sheep (*Ovis aries)*, 76.5 ± 0.7 kg and 68.2 ± 9.2 kg body weight (BW), respectively fitted with rumen cannulas were used. The animals were housed in pens adjacent to each other and fed with low quality tall fescue (*Festuca arundinacea*, Schreb) offered *ad libitum* in two daily rations (08:00 and 16:00 h) and fresh water that was freely available. The animals were adapted to handling and forage feeding for 21-days, followed by one day of collection of ruminal content, during two-experimental periods separated by 7-days.

Samples were collected before morning feeding (100 g; 50: 50 v/v liquid and solid phases) and then filtered through cotton gauze (4-sheets) to obtain the "ruminal fluid" (RF). The samples were aliquoted for DNA analysis, for microscopic protozoa count, and for NH_3-N and VFA analyses.

Evaluation of voluntary feed intake and forage analysis

Dry matter voluntary intake (DMVI) was computed as the difference between the daily offered and rejected forage, throughout the study (38 days). The results were expressed in kilogram of dry matter (DM) or neutral detergent fiber (NDF) per day, percentage of dry matter in basis of body weight per day, and grams of dry matter per kilogram of metabolic weight (g DM/kg $BW^{0.75}$). In addition, measurements of DMVI on day 16 to 20 during the adaptation period were considered for the analysis of the data.

Chemical characterization

The chemical characterization of tall fescue hay was carried out by DM analysis by oven drying (105°C), ash (Ash) by incineration at 550°C for 4 h. (AOAC, 1990, method 942.05). Total nitrogen content was determined by Kjeldahl method and multiplied for 6.25 to obtain crude protein (CP; AOAC, 1990, method 984.13). In addition, we assessed neutral (with alpha amylase) and acid detergent fibers (expressed free of ash, NDF and ADF, respectively) as well as acid detergent lignin (ADL) in a fiber analyzer, ANKOM® 220 (ANKOM Technology, Macedon NY – USA) according to Van Soest et al. (1991).

Determination of volatile fatty acids (VFA), ammoniacal nitrogen (NH_3-N) and pH

Samples of RF were assessed by gas chromatography using Konik 5000B (Konik Group, Miami – USA) with Robokrom GC auto-sampler and a 30 m and 0.32 mm Nukol capillary column. Sample purification was performed according to Friggens et al. (1998). Concentration of NH_3-N was determined in the UV-VIS Spectrophotometer Metrolab® 1500 (Lab Wiener®, USA), at 540 nm, using the uremia kit (Lab Wiener® Rosario - Argentina). All pH values were measured with a pHmeter (Corning Ltd, Halstead, Essex, UK).

Quantification of bacterial and protozoa populations

Genomic DNA was extracted in duplicate from the frozen samples (0.25 g pellet) using the QIAmp DNA Stool Mini Kit (Qiagen, Germany) following the manufacturer's instructions. Total DNA concentration was assessed using NanoDrop (Nanodrop ND-1000, Thermo Fisher Scientific, USA). Total bacteria, *Fibrobacter succinogenes*, *Ruminococcus albus*, *Ruminococcus flavefaciens* and methanogens were determined using qPCR, by comparison with serial dilution (10^1 to 10^8) of specific DNA standards. DNA from a plasmid (PCR®4-TOPOvector®, Invitrogen, USA) containing the *16S rRNA* gen and methyl coenzime-M reductase (*mcrA*) gene were used as standards for bacterial and methanogen quantifications, respectively.

Quantitative qPCR was performed using ABI 7500 v2.3, Sequence Detection System (Applied Biosystems, CA, USA). A total of 2 µl DNA template (10 ng/µl) was added to the amplification reaction (20 µl total volume) containing 20 pmol of each primer, 4 µl 5× HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, Estonia), and DNA/RNA free water adjusted to a total volume. Primer specifications for bacterial and methanogens are described in Table1.

Table 1

The amplification consisted of a cycle of 95°C for 15 min, followed by 40 cycles of 95°C for 30 sec for denaturation; 60°C for 30 sec for annealing, which varied according to the primer used (Table 1), and 72°C for 1 min for extension (Singh et al., 2014). The specificity of the amplicon was verified according to the dissociation curve of the final PCR product, by increasing the temperature 1°C every 30 sec from 60 to 95°C.

The quantification was based on standard curves of already known concentrations of the standard plasmid for the respective target. In each reaction, the linear regression values of the standard curve were within the normal limits (R^2 = 0.99, slope = -3.2 to -3.6 and

efficiency = 100-110%). These values were used to estimate the number of copies of the *16S rRNA* genes from each sample. The following equation was used for this analysis: N° copies/g rumen = (Q × C)/V, where: Q = is the mean of the copies; C = total DNA concentration in each sample (ng/g) and V = is the concentration of DNA used in the reaction (ng).

The density and generic composition of ciliated rumen protozoa were performed according to Dehority (1993). This procedure consisted of mixing the rumen samples with formalin saline solution (formalin 10% in NaCl solution 0.85%). Then the samples were stained with methyl green and observed under an optical microscope (Nikon eclipse E200MV®, Nikon © Tokyo - Japan).

Statistical analysis

Fermentative analysis data (VFA, NH_3-N and pH) and density of total bacteria, methanogens, *F. succinogenes*, *R. albus*, *R. flavefaciens* and protozoa in the rumen of sheep and llamas were collected in two periods and in duplicate (two samples in each Animal × Period experimental unit).

Data were analyzed by general linear models (GLM) procedures of SAS (version 8.0; SAS Institute Inc. Cary, NC, USA) as a randomized complete block design (Block = Period), according to the following model: $Y_{ijk} = \mu + Per_i + Animal_j + \mathcal{E}_{ijk}$, where: Y_{ijk} = response variable; μ = mean; Per_i = period (i = 2); $Animal_j$= animal species (llama *or* sheep; j = 2); $\mathcal{E}_{ijk}$= experimental error (k = 3). Differences among means were considered as significant whenever $P < 0.050$.

Results

Characteristics of forage, voluntary feed intake and ruminal fermentation

The animals received a low protein tall fescue hay throughout the experiment (DM 910 g/kg, CP 63, Ash 87, EE 17, NDF 773, ADF 432 and ADL 40 g/kg DM). The pattern of intake in llama and sheep clearly showed an initial increase, reaching a potential intake after the first six days until stabilized (Figure 1). Then, the hay DMVI (kg/d and % BW) and NDFVI (kg/d) observed for llamas was higher than that of sheep (Table 2).

The pH values of RF in sheep were higher than those of llamas (P= 0.010) although these differences had no biological implications, whereas rumen NH_3-N concentration and total VFA of llamas were twofold those of sheep. Also, the fermentation profile of butyrate and valerate were higher for llamas than sheep (P= 0.008 and 0.024, respectively), with the exception of the iso-valerate, being greater for sheep than for llamas. However, the fermentation profile of acetate, propionate, iso-butyrate and A: P ratio were similar in both species (P= 0.100).

Table 2

Populations of bacteria and protozoa

Methanogen and total bacteria populations present in the rumen of sheep and llamas did not differ (P= 0.360 and 0.630, respectively; Table 3). Moreover, absolute quantification and relative representation of *F. succinogenes*, *R. albus* and *R. flavefaciens*, showed analogous results in both species (P= 0.110, 0.390 and 0.580, respectively). However, the density of total protozoa and entodiniomorphs found in llamas was higher than in sheep (P= 0.010). Furthermore, holotrich protozoa was only detected in the sheep rumen content. The rumen content of sheep had higher proportion

of genus *Entodinium* than the llamas one (92.7 *vs* 67.3%, respectively; $P< 0.001$). By contrast, the genus *Eudiplodinium* along with "other genus" were higher in the forestomach of llamas than sheep (32.8 *vs* 7.3%, respectively; $P< 0.001$).

Table 3

Discussion

Voluntary feed intake and ruminal fermentation

In the present study, a small number of animals were used due to the availability of fistulated llamas and sheep for experimentation. Llamas and sheep reached potential intake at the same time, but llamas had a higher DMVI and NDFVI than sheep. The values of DMVI herein reported, agreed with those reported by López et al. (1998), who fed alpacas with low quality forage (wheat straw and tall fescue; 40 and 57 g/kg $BW^{0.75}$), Also, San Martin *et al.* (1982) observed that the DMVI for llamas was higher than for sheep (1.3 vs 1.2% BW) when fed with native pastures in dry season (< 6.0% CP).

On the other hand, despite that forestomach pH values of sheep overcame those found in llamas ($P= 0.010$), the difference lacked of biological relevance as in both species, it was close to neutrality (6.5 to 7.5; Nilsen et al., 2015). It is worth noting that the lower pH in the forestomach of llamas was associated with higher concentration of total VFA.

Figure 1

The concentration of NH_3-N and total VFA were higher in the forestomach of llamas compared to sheep. These results differ with those of Liu *et al.* (2009), who reported that with 14% protein diets, VFA and NH_3-N concentrations were higher for sheep than for alpaca. Fermentative parameters, digestibility, intake and animal metabolism are

mainly influenced by the type and quality of diet. However, Sponheimer et al. (2003) observed that the digestibility of DM in llamas was greater than in sheep when fed low quality diets (7% CP), but this difference disappeared when the CP diet concentration was improved (14% CP). Therefore, we could mention that in this study, llamas fed with low quality diets retained a higher concentration of NH_3-N, which could lead to greater digestibility of fiber, in coincidence with that reported by López *et al* .(1998) and Sponheimer *et al*. (2003).

The higher concentration of NH_3-N in the RF of llamas could be due to a higher DM intake (major intake of N), and N recycling capacity, which is coherent with our hypothesis. However, more studies are needed to confirm this assertion, as this outcome could also be associated to a higher ruminal protozoa density, as a result of their proteolytic activity on bacteria (Belanche *et al*., 2012), and their role in the degradation of food protein (Ushida *et al.*, 1986). The higher molar proportion of butyrate would be reflecting an increased degradation of dietary protein and turnover of bacterial protein in rumen, due to a higher density of ruminal protozoa in RF of llamas than sheep (Ushida *et al*., 1986). Simultaneously, the increased availability of N in the rumen would favor ruminal microorganism's activity, as evidenced by the higher concentration of total VFA.

Fibrolytic bacteria are highly dependent on NH_3-N availability as a source of N (Russel *et al.*, 1992) and their fermenting capacity is greatly diminished when N is limiting because of their reduced capacity to use peptides and amino acids as N sources. Thus, rumen NH_3-N is critical to ensure optimum rumen functionality. Although the minimum necessary level of NH_3-N in the rumen is dependent on the level of fermentable energy to ensure unrestricted digestion of DM, the values reported (7.2 and 14.2 mg/dL of

NH_3-N for sheep and llamas, respectively) in this experiment were within the limits cited by other authors for ruminants.

In fact, the minimum value of NH_3-N in rumen required to sustain ruminal fermentation activity varies according to different authors (5 mg/dL, Satter and Slyter, 1974; 5 to 23 mg/dL, Mehrez *et al.*, 1977; 11 to 13 mg/dL, Boucher *et al.*, 2007). In addition, this minimum value also varies depending on dietary sources, as shown for barley and maize grains (6 and 13 mg/dL respectively, Odle and Schaefer, 1987), while for wheat straw, Oosting and Waanders, (1993) showed that for goats the minimum necessary value of NH_3-N was from 3.9 to 7.0 mg/dL. In line with this, Van Soest (1994) indicated that protein supplementation would induce an increase in DM digestibility only when forage contains less than 7% CP. Although there are alpaca´s forestomach NH_3-N values reported elsewhere (Liu et al, 2009), in this study, llamas fed with 6% CP in the diet, maintained levels of 14 mg/dL. Hence, these limits are simply orientative, it is difficult to establish an association between rumen microbial activity solely on diet CP concentration, other characteristics as a rumen CP and energy degradability, microbial composition as well as other nutrient required by rumen microbiota influence the general degree of ruminal metabolic activity (Hoover, 1986).

The low CP concentration and high content of indigestible fiber in forages are related to low voluntary feed intake and animal performance (Minson and Milford, 1967). Nevertheless, the maintenance of an optimum and constant NH_3-N forestomach concentration not only would favor DM digestibility, but should also contribute positively to raise intake. This is because higher and constant contribution of N will facilitate microorganism's activity and hence DM disappearance. This would explain the higher values of DMVI observed in llamas with respect to that observed in sheep. This explanation coincides with Oosting and Waanders, (1993) who demonstrated that

DMVI was increased with the higher NH_3-N ruminal concentration (from 3.9 to 9.0 mg/dL) through urea infusion, but this did not significantly modify digestibility. In addition, according to Mehrez *et al*. (1977) the concentration of NH_3-N recorded in our sheep forestomachs would be at the lower limit that could restrict intake.

Analyzing other possible explanations, San Martin *et al.* (1987) observed that despite the lower rumen capacity and longer retention time of solid particles in the forestomach, the intake of DM and NDF was higher for llamas than for sheep when fed with low quality forages. This greater ability to adapt to poor-quality forages is believed to be due mainly to its greater capacity of N recycling to the rumen, and longer retention time of solid particles, which would substantially improve the digestibility of these forages.

Populations of bacteria and protozoa

To date, little information is available on the use of molecular techniques to identify and monitor microbial populations present in forestomach of SAC. Cerón Cucchi *et al.* (2013) identified *F. succinogenes*, *R. albus* and *R. flavefaciens* in the forestomach of llamas by PCR. In addition, Pei *et al.* (2010) quantified and found differences in total bacterial populations in the rumen of sheep and alpacas (7.7 vs 6.9 Log copies/g) fed on alfalfa hay and concentrate. Pei *et al.* (2013) also reported lower percentage of methanogens in alpacas than in sheep. However, our results did not show significant differences in bacteria and total methanogens between llamas and sheep.

In this study, *R. flavefaciens* and *F. succinogenes* were the dominant species in the rumen of sheep and llamas over *R. albus*. This finding is consistent with reports by Koike and Kobayashi, (2001), Michalet-Doreau *et al.*, (2002), Koike *et al.*, (2003) and Singh *et al.*, (2014), who fed sheep and buffaloes with different proportions of concentrate and forage. By contrast, these results differed with those reported by Cobellis *et al.* (2016) and Adeyemi *et al.* (2016), who observed that *R. albus* was the

dominant species in sheep and goat fed with forages and concentrate. On the other hand, Wanapat and Cherthong (2009) observed that *F. succinogenes* were the most abundant fibrolytic bacteria in the rumen of buffaloes fed with forage and concentrate.

The high variability of bacteria populations found could be attributed to different factors, such as diet, age, geographic location, season of the year and animal health (Henderson *et al.*, 2015). Though, this variability could also be due to the technique used (DNA extraction method, primers, real-time PCR *vs* competitive PCR) or to the units used for the analysis (number of copies/g, ml or in µg/ml; Wanapat and Cherthong 2009, Popova *et al.*, 2014; Cobellis *et al.*, 2016).

In addition, the results indicated that llamas had a higher density of total protozoa and entodiniomorphs than sheep, being *Eudiplodinium* the predominant genus. However, Pinares-Patiño *et al.* (2003) and Pei *et al.* (2013) found no difference between alpacas and sheep under the same feeding conditions. Both authors reported the absence of holotrich protozoa in alpacas, which agrees with our results and those reported by Jouany (2000), Del Valle *et al.* (2008) and Cerón Cucchi *et al.* (2016).

The absence of holotrichs protozoa may be due to the diet (Willians and Coleman, 1992, cited by Pinares-Patiño *et al.*, 2003). However, in this study, though both species were fed with the same diet no Hollotrichs protozoa were found in llamas RF, hence it is speculated that other factors such as physical or biochemical forestomach environment should have been involved. This outcome was coherent with recent observation by Bautista *et al.* (2015), who in a total transfaunation experiment (transfer of ruminal content) between sheep and alpacas, showed that in less than 5 days post-transfaunation, the holotrics protozoa disappeared in the forestomach of the alpaca.

The results partially confirmed the hypothesis that llamas were able to maintain higher NH_3-N levels than sheep and contained higher densities of total protozoa populations and entodiniomorphs. However, both species, llamas and sheep, showed similar forestomach densities of total bacterial populations, total methanogens and higher number of fibrolytic bacteria.

Conclusions

When a low protein forage diet was offered, the forestomach contents of llamas presented higher microbial activity than sheep ones, which was reflected in a higher concentration of NH_3-N and VFA. Also, llamas had a higher density of total protozoa and genus *Eudiplodinium*, but holotrich protozoa were not found. Moreover, we did not detect differences between the total bacterial population, total methanogens, and major fibrolytic bacteria.

Conflicts of interest

None

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Figure Captions

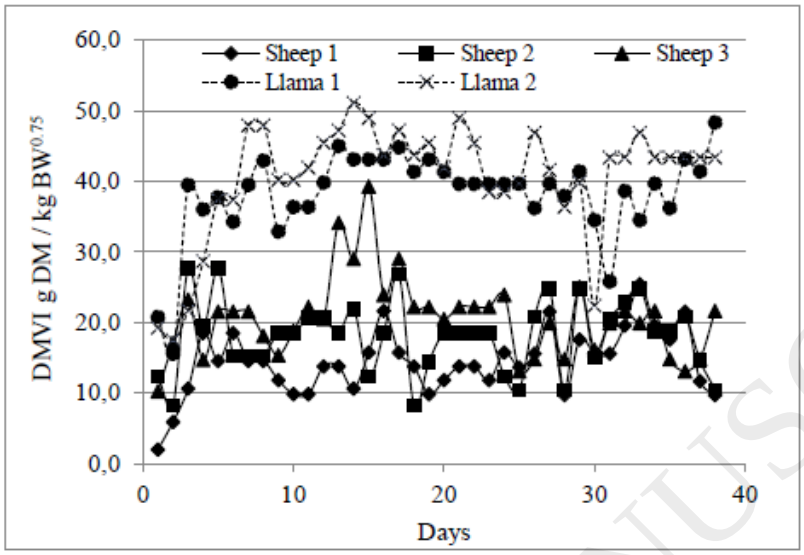

Figure 1. Dynamics of dry matter voluntary intake (g DM/kg $BW_{0.75}$) from throughout the test in llamas and sheep fed tall fescue hay

Table 1. PCR primers used in this study

Target	**Primer sequence (5' – 3')**	**Annealing (°C)**	**Amplicon size (pb)**	**Reference**
Methanogens (*mcrA*)	F-TTC GGT GGA TCD CAR AGR GC R-GBA RGT CGW AWC CGT AGA ATC C	60	140	Denman et al. (2007)
Total bacteria (*16S* rRNA)	F-CGG CAA CGA GCG CGA ACC C R-CCA TTG TAG CAC GTG TGT AGC C	60	130	Denman and McSweeney, (2006)
Fibrobacter succinogenes	F-GGT ATG GGA TGA GCT TGC R-GCC TGC CCC TGA ACT ATC	60	445	Tajima et al. (2001)
Ruminococcus flavefaciens	F-TCT GGA AAC GGA TGG TA R-CCT TTA AGA CAG GAG TTT ACA A	55	295	Koike and Kobayashi, (2001)
Ruminococcus albus	F-CCC TAA AAG CAG TCT TAG TTC G R-CCT CCT TGC GGT TAG AAC A	55	175	Koike and Kobayashi, (2001)

*Table 2.*Body weight, intake and parameters of ruminal *fermentation of sheep and llama* fed on tall fescue hay

Parameter	Species		SEM[a]	*P*-value
	Sheep	Llama		
Body weight (BW, kg)	68.2	76.5	-	-
DMVI (g/d)	467	1146	46.2	0.002
NDFVI (g/d)	361	886	35.7	0.002
DMVI (% BW/d)	0.68	1.50	0.04	<0.001
DMVI (g/kg $BW^{0.75}$)	18.3	39.9	1.90	0.002
pH	6.8	6.7	0.04	0.010
NH_3-N (mg/dL)	7.2	14.2	0.81	<0.001
VFAt (mM)	45.7	88.7	5.74	<0.001
Proportion (mmol/mmol VFAt × 100)				
Acetate	76.7	76.5	0.77	0.812
Propionate	15.9	16.8	0.63	0.194
Butyrate	3.6	4.8	0.35	0.008
Iso-butyrate	1.6	1.3	0.17	0.079
Valerate	0.1	0.6	0.13	0.005
Iso-valerate	1.3	1.0	0.12	0.024
A:P	4.6	4.8	0.21	0.370

DMVI: Dry matter voluntary intake; NDFVI: Neutral detergent fiber voluntary intake; VFAt: Total volatile fatty acids; A: P: acetate and propionate ratio.

[a] SEM: standard error of the mean

Table 3.Density of bacteria and ciliated protozoa in the rumen of sheep and llama fed on tall fescue hay (values expressed as Log_{10})

Item	**Species**		**SEM[A]**	***P*-value**
	Sheep	**Llama**		
Methanogens (copies/g)	7.4	7.3	0.13	0.360
Total bacteria (copies/g)	10.6	10.7	0.10	0.630
F. succinogenes (copies/g)	7.8	8.1	0.17	0.110
R. albus (copies/g)	7.3	7.4	0.17	0.390
R. flavefaciens (copies/g)	8.6	8.7	0.19	0.580
F. succinogenes (%)[B]	0.4	0.7	0.19	0.140
R. albus (%)	0.1	0.1	0.03	0.550
R. flavefaciens (%)	1.3	1.2	0.41	0.860
Total Protozoa (cell 10^4/g)	3.6	5.0	0.35	0.010
Entodiniomorphos (cell 10^4/g)	3.5	5.0	0.36	0.010
Entodinium (%)[C]	92.7	67.3	2.55	<0.001
Eudiplodinium and other (%)	7.3	32.8	2.54	<0.001
Holothics (cell 10^3/g)	1.4	0.0	0.04	0.010

[A] SEM: standard error of the mean.

[B] Values expressed as a percentage of total bacteria.

[C] Values expressed as a percentage of total protozoa