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Ciliate protozoa of the forestomach of llamas (*Lama glama*) from locations at different altitude in Argentina

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Abstract

This study describes the diversity and concentration of the protozoal population from the forestomach of llamas in Argentina at three altitudinal locations. Protozoal diversity was studied in samples from eight llamas from Hurlingham (Buenos Aires, 43 m altitude), four from Tilcara (Jujuy, 2465 m altitude) and six llamas from Cieneguillas (Jujuy, 3800 m altitude). The total concentrations of protozoa in the forestomach contents were 7.9, 9.1 and 4.1 cells x 10⁴ ml⁻¹ in Hurlingham, Tilcara and Cieneguillas, respectively (P>0.05). *Entodinium* spp. represented 97.9, 92.3 and 71.4% of the protozoal community in Hurlingham, Tilcara and Cieneguillas, respectively, and the remaining protozoa belonged to the *Eudiplodinium* genus. *Entodinium* spp. were identified as *E. caudatum* (mostly morphotype *dubardi*), *E. longinucleatum*, *E. parvum*, *E. bovis*, *E. exiguum*, *E. dubardi*, and a minor presence of *E. bimastus* (in three animals) and *E. ovibos* (in one animal). In regards to the rest of protozoal species, *Eudiplodinium maggii* is the first reported host record for the genus in llamas. This species was present in the forestomach of 14 out of 18 llamas tested, and in one case it was the unique protozoal species. The vestibuliferids, *Dasytricha* and *Isotricha* were absent from the forestomach of llamas. Similarly, other species such as those from the *Caloscolex* genus, *Diplodinium cameli* and *Entodinium ovumrajae*, commonly found in Old World Camelids, were also absent from llamas.

Key words: Forestomach protozoa, South American Camelids, entodiniomorphids

Introduction

The llama (*Lama glama*) is one of the two domestic species of South American Camelids (SACs) together with the alpaca (*Vicugna pacos*), whereas the guanaco (*Lama guanicoe*) and the vicuña (*Vicugna vicugna*) are wild species. The llama preferably feeds on tall and coarse bunchgrasses from the drier areas of the Andean Altiplano (3500 to 4500 m altitude), and in Argentina is naturally present mainly in the North–Western provinces of Jujuy, Salta, Catamarca and La Rioja. Llamas have been used since pre–Hispanic times as multipurpose animals, providing fiber, meat and leather, and as beast of burden.

The microbial ecosystem of the forestomach of SACs in general, and of the llama in particular, is poorly described. Only few authors have recently tried to characterize the different communities (Ceron Cucchi *et al.*, 2013; Del Valle *et al.*, 2008; Pei *et al.*, 2010). As in all wild and domesticated ruminants, camelids harbor ciliate protozoa, and rumen protozoal counts of dromedaries and SACs are similar to those of ruminants (Dehority, 1986; Jouany, 2000). These authors also reported that the protozoal population in camelids is only type B (Eadie, 1962) and the family Isotrichidae had never been observed. A previous report on protozoal of SACs from La Paz, Bolivia, have demonstrated that protozoal communities differ between SAC hosts, total concentration being 3.6 times

higher in the stomach contents of alpacas than in llamas (Del Valle *et al.*, 2008). In such report, the authors observed 4 to 11 species, all from the genus *Entodinium*, in llamas, whereas in alpacas, they detected 8 to 9 species of *Entodinium* and minor proportions of *Diplodinium* (*D. anisacanthum*, *D. dogieli*, *D. rangiferi*), *Eudiplodinium* (*E. bovis*, *E. maggii*, *E. neglectum*) and *Epidinium* (*E. ecaudatum*).

Protozoa are ubiquitous, but not essential denizens of the rumen. The large population of protozoa that inhabit the rumen and their ability to attack the major components of feeds suggest that, though not essential, they play an important role in ruminal fermentation (Coleman, 1985; Dehority, 2003; Veira, 1986). The presence of ciliates has been observed to affect ruminal factors such as pH, volatile fatty acid, ammonia concentration, volume and dilution rate, and bacterial biomass, all of which can affect the rate and extent of digestion (Veira, 1986). The mentioned differences in protozoal communities between SACs can be attributed to their different feeding habits, even when graze in shared locations (Castellaro *et al.*, 2004; Tichit & Genin, 1997). In a similar way, if intake of a high quality forage may justify a larger and more diverse protozoal community in the rumen (Williams & Coleman, 1992), differences among forages grazed at different altitudes above sea level may also influence the forestomach ecosystem of llamas reared at those locations.

This work was planned to assess whether the rearing environments of llamas at different altitudinal locations lead to differences in the protozoal populations and diversity in their forestomach. To our knowledge, this is the first report on the diversity and concentration of forestomach protozoal population of llamas from different environments of Argentina.

Material and methods

The forestomach contents of llamas were sampled by trained personnel and specialized veterinarians by following animal use and care guidelines of Institutional Committee for the Care and Use of Experimental Animals CICUAE-National Institute of Agricultural Technology INTA CICVyA (N°5 2013). Eighteen adult males (2 – 4 year old, from 90 – 140 kg live weight) were used in the study, which took place between October and November 2012. It involved eight llamas from Hurlingham–Buenos Aires (34°36'S, 58°40'W; 43 m altitude), four llamas from Tilcara–Jujuy (23°34'S, 65°22'W; 2465 m altitude) and six llamas from Cieneguillas–Jujuy (22°08'S, 65°08'W; 3800 m altitude).

Animals in Hurlingham (pampa, humid conditions) were fed alfalfa hay *ad libitum* once daily (09:00–14:00 h), those in Tilcara (valley conditions in Los Andes) had freely available alfalfa hay once daily (09:00–13:00 h), with a minimum intake of native grassland, available from 17:00–19:00 h (routinely management), and those in Cieneguillas (traditional extensive breeding system in dry Puna) grazed mostly a native grassland dominated by vegas (*Festuca argentinensis*) and tola (*Parasthephia quadrangularis*). Samples of offered forages and dominant species on grasslands were obtained for chemical composition analyses (Table 1).

TABLE 1. Dry matter content (g/kg fresh matter), chemical composition (g/kg dry matter) and estimated dry matter digestibility coefficient of forages consumed by the llamas at the different locations.

	<i>Alfalfa hay</i> (Hurlingham)	<i>Alfalfa hay</i> (Tilcara)	<i>Festuca argentinensis</i> (Cieneguillas)	<i>Parasthephia quadrangularis</i> (Cieneguillas)
Dry matter	904	904	872	940
Crude protein	127	192	34	68
Neutral detergent fiber	571	504	712	501
Acid detergent fiber	468	337	470	349
Lignin	111	74	56	126
*Dry matter digestibility	524	626	523	617

*Dry matter digestibility was estimated from the acid detergent fiber.

Contents from the first stomach chamber (approximately 40 ml) were collected by esophageal tube, during the first hours of the morning and before feeding time. The samples were taken using a clear vinyl tube (approximately, outside diameter 1.2 cm, inside diameter 1 cm, length 115 cm) attached to a 60 ml syringe. The tube was lubricated

with Lidocaine 2% and was introduced in the oral cavity. Once the tip of the tube was in the oral pharynx, gentle pressure stimulated the animal to swallow it. The samples obtained by aspiration were filtered through a double layer of gauze and immediately mixed 1:1 with 18 % formaldehyde saline solution and preserved in a dark place for later study. To reduce performance error among the animal sampling, all samples were collected by one person and tubes individually sterilized were used for each animal to avoid contamination between samples. Total and generic ciliate concentrations were determined by previously described procedures (Dehority, 1984), by using a Sedgewick-Rafter counting chamber. Previous reports (Dehority, 1986, 1993; Dogiel, 1927; Göçmen, 1999; Lubinsky, 1957; Sládeček, 1946; Wertheim, 1935) were used for the species identification and it was mainly based on morphological descriptions (size, body shape, skeletal plates and shape of the macronucleus). *Entodinium* has one ciliary zone, one contractile vacuole and a macronucleus that lies between the micronucleus and nearest body side. By contrast *Eudiplodinium* is larger and has two ciliary zones, two or more contractile vacuoles, skeletal plates and micronucleus that lie between a macronucleus and nearest body side. The species distribution and cellular morphology were determined from 20 cells for each species/morphotype, with methyl-green as a nuclear stain and Lugol's iodine as a stain for skeletal plates. Samples of forages from the three locations (alfalfa hay from Hurlingham and Tilcara, and *F. argentinensis* and *P. quadrangularis* from Cieneguillas) were analysed for their content in dry matter by oven drying (65°C, 48h). Crude protein was determined according to AOAC (1995). Contents of neutral detergent fiber, acid detergent fiber and lignin were determined according to Goering and Van Soest (1970). Dry matter digestibility (DMD) was estimated from the acid detergent fiber (ADF) as follows: %DMD=88.9-(0.779 x %ADF) (Rohweder *et al.*, 1978). All the determinations were made by the Animal Food Evaluation Laboratory–Catholic University of Argentina.

The data for total protozoal concentration were analyzed by one-way ANOVA using the Statistix 10 package (AnalyticalSoftware, 2013) and the differences between the means groups were compared by the Tukey t test at a $P < 0.05$. Moreover, a canonical correspondence analysis (CCA) was performed, including the location of the samples as constraining effect in the model.

Results

Total concentrations, numbers of species and species proportions of protozoal communities are shown in Table 2. No differences ($P > 0.05$) of protozoa among the three locations (7.9, 9.1 and 4.1 cells $\times 10^4$ ml⁻¹ for Hurlingham, Tilcara and Cieneguillas, respectively). A higher number of protozoal species were observed in individuals from Tilcara compared with those from Cieneguillas ($P = 0.003$), but it is worth mentioning that one llama from the latter location was monofaunated with *Eudiplodinium maggii*. This animal also presented the lowest concentration of protozoal cells (0.7 cells $\times 10^4$ ml⁻¹). On average, *Entodinium* spp. represented 97.9, 92.3 and 71.4% of protozoa in llamas from Hurlingham, Tilcara and Cieneguillas, respectively and the remaining protozoa belonged to the species *Eudiplodinium maggii*. No other protozoal species or genera were detected. The most common *Entodinium* species were *E. caudatum* m. *dubardi*, *E. longinucleatum*, and *E. parvum*. In contrast, *E. ovibos* was detectable in one single llama in Hurlingham and *E. bimastus* was only detected in three out four animals from Tilcara, whereas *E. dubardi* was not detected in animals from Cieneguillas. Because of this, the occurrence of these less common species was not compared statistically. The species *Eud. maggii* was present in all animals but four llamas from Hurlingham, and it was the only protozoa detected in one animal of Cieneguillas, as mentioned earlier. Concentration of *E. caudatum* was highest in Hurlingham ($P < 0.001$), and that of *E. parvum* was higher in Hurlingham than in Cieneguillas ($P = 0.018$), whereas the opposite occurred with the presence of *E. longinucleatum* ($P = 0.026$) and *Eud. maggii* ($P = 0.048$).

CCA analysis showed also that samples clustered by location (see Figure 1), in which *E. bimastus* was associated with samples coming from Tilcara, *E. ovibos* with samples coming from Hurlingham and *Eud. maggii* with samples coming from Cieneguillas. CCA is known to be a useful tool to explain the structure of a multivariate data table by using environmental variables, assuming a unimodal distribution of species. Thus, the ordination diagram represents not only a pattern of community distribution, but also the main features of the distribution of species along the environmental variables, in this case, location.

Length and width of protozoa observed in this study are shown in Table 3. No major morphological differences among locations were observed among cells of the same species, except for cells of *E. exiguum* in Hurlingham and

those of *E. longinucleatum* in Cieneguillas, that had higher and smaller sizes, respectively, than those of the same species from other locations.

TABLE 2. Average total concentration (number of cells $\times 10^4 \text{ ml}^{-1}$) and species distribution (%) of protozoa in the forestomach contents from llamas at Hurlingham (n=8), Tilcara (n=4) and Cieneguillas (n=6). Standard errors of means are given in brackets.

	Hurlingham	Tilcara	Cieneguillas	P
Total concentration	7.9 (0.120)	9.1 (0.169)	4.1 (0.138)	0.22
Species observed	6.3 (0.50) ab	8.3 (0.70) a	4.5 (0.58) b	0.003
<i>Entodinium caudatum</i>	59.7 (4.30) a	36.1 (6.07) b	26.0 (4.96) b ¹	<0.001
<i>E. longinucleatum</i>	15.8 (5.22) b	20.3 (7.38) ab	39.7 (6.03) a ¹	0.026
<i>E. exiguum</i>	4.1 (3.18)	13.5 (4.49) ²	1.4 (3.67) ³	0.14
<i>E. parvum</i>	11.8 (1.87) a	5.3 (2.65) ab	2.9 (2.16) b ¹	0.018
<i>E. bovis</i>	2.7 (0.84) ⁴	4.3 (1.19) ²	1.3 (0.97) ³	0.19
<i>E. dubardi</i>	4.2 (4.10) ⁵	7.5 (7.38)	N.O.	---
<i>E. ovibos</i>	15.0 ⁶	N.O.	N.O.	---
<i>E. bimastrus</i>	N.O.	7.1 (1.40) ²	N.O.	---
<i>Eudiplodinium maggi</i>	2.1 (7.18) b ⁵	7.7 (10.2) ab	33.3 (9.09) a	0.048

Within rows, different letters indicate significant differences ($P < 0.05$); N.O.: not observed.

¹: present in 5 out of 6 llamas; ²: present in 3 out of 4 llamas; ³: present in 3 out of 6 llamas; ⁴: present in 7 out of 8 llamas;

⁵: present in 4 out of 8 llamas; ⁶: present in 1 out of 8 llamas

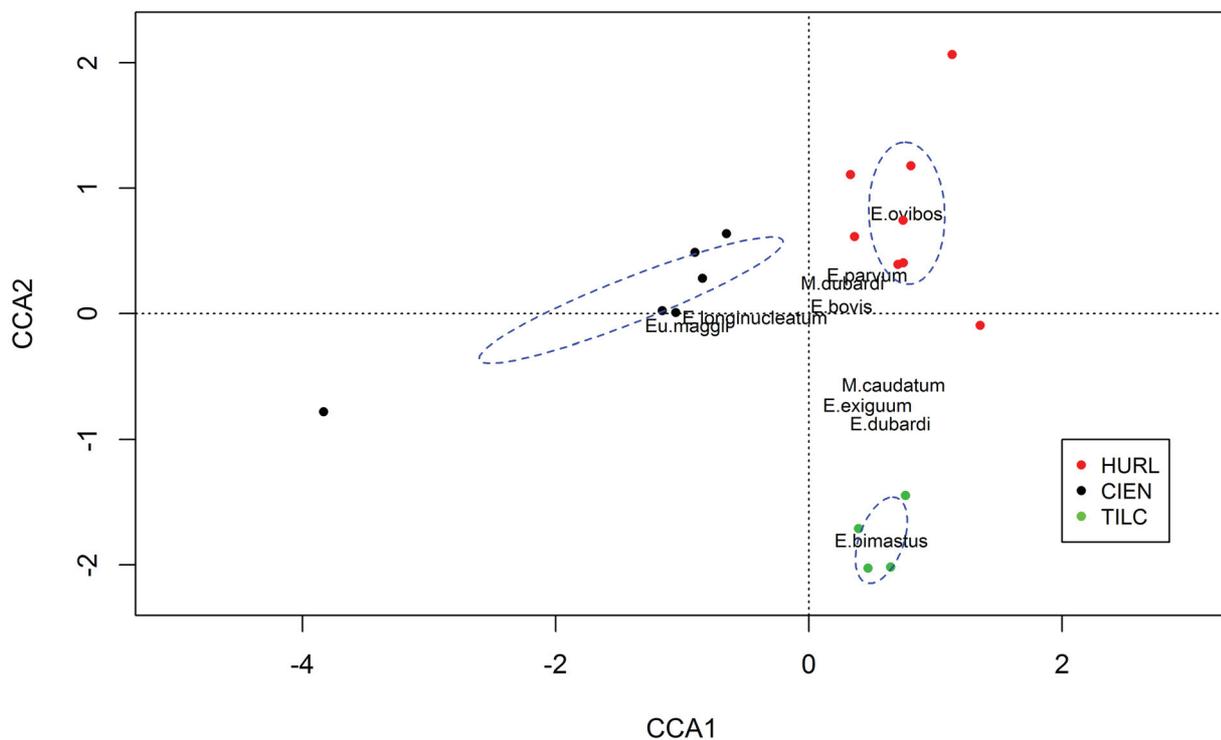


FIGURE 1. Canonical correspondence analysis illustrating a pattern of community distribution and the main features of the distribution of forestomach protozoa species in llamas from Hurlingham, Tilcara and Cieneguillas. Hurlingham (HURL), Tilcara (TILC) and Cieneguillas (CIEN).

TABLE 3. Average dimensions (μm) of cells of the protozoal cells of *Entodinium* and *Eudiplodinium* species/morphotypes found in the contents of llamas at Hurlingham (n=8), Tilcara (n=4) and Cieneguillas (n=6). Values in brackets show the dimension range (minimum–maximum).

Species observed	Hurlingham		Tilcara		Cieneguillas	
	length	width	length	width	length	width
<i>Entodinium caudatum</i>						
morphotype <i>caudatum</i>	67 (58–76)	31 (30–32)	74 (62–80)	38 (36–43)		
morphotype <i>dubardi</i>	43 (38–50)	28 (20–35)	43 (30–50)	31 (23–38)	42 (35–48)	26 (25–26)
<i>E. longinucleatum</i>	55 (42–75)	39 (25–47)	62 (45–95)	38 (28–48)	42 (30–55)	27 (23–30)
<i>E. exiguum</i>	37 (35–40)	25 (23–28)	33 (30–35)	17 (13–23)	30 (28–33)	21 (18–23)
<i>E. parvum</i>	40 (35–45)	27 (22–30)	41 (35–45)	26 (23–28)	40 (38–43)	24 (23–30)
<i>E. bovis</i>	38 (33–45)	26 (25–27)	40 (35–45)	29 (23–30)	38 (33–45)	26 (25–26)
<i>E. dubardi</i>	38 (35–43)	25 (23–28)	42 (35–45)	30 (25–33)		
<i>E. ovibos</i>	41 (35–50)	25 (22–27)				
<i>E. bimastus</i>			46 (45–47)	31 (28–35)		
<i>Eudiplodinium maggii</i>	164 (110–173)	106 (77–125)	170 (125–195)	100 (83–117)	165 (125–190)	105 (80–125)

Discussion

Sampling forestomach contents by esophageal tube allows for repeated sampling of the same animal when maintenance of cannulated animals is not possible. Despite differences exist in environmental parameters among forestomach sites, Shen *et al.* (2012) did not observe differences in rumen parameters between this method and cannula sampling, and support that oral tubes can be inserted in different animals reaching the same site. Sampling of all animals was carried out in a short period of time, so possible bias in this regard can be discarded.

The protozoal concentration of dromedaries (Dehority, 1986; Jouany, 2000) and SACs such as alpacas (Del Valle *et al.*, 2008; Pinares-Patino *et al.*, 2003) was similar to reported values in ruminants. However, values observed here were lower, even at the lower range (from 9.1 to 104.6 x cells 10^4 x ml^{-1}) observed for this host species by Del Valle *et al.* (2008). There is no apparent explanation for this, and only rumen environmental characteristics (Lemosquet *et al.*, 1996) can be argued, since there were no differences in protozoa concentrations among locations despite considerable differences in altitude and feeding conditions. In previous studies in Bactrian camels in Mongolia, Imai and Rung (1990) observed a mean protozoal concentration of 211 cells x 10^4 ml^{-1} (74 to 437 cells x 10^4 ml^{-1}) with seven genera containing 14 species and five formae. In dromedaries concentrations of 13.9 cells x 10^4 ml^{-1} (4.9 to 109.4 cells x 10^4 ml^{-1}) with 10 genera containing 31 species and 16 morphotypes and 5.8 cells x 10^4 ml^{-1} (2.8 to 7.5 cells x 10^4 ml^{-1}) with six genera containing 13 species and 7 morphotypes have been reported by Kubesy and Dehority (2002) and Selim *et al.* (1999), respectively. The results in forestomach contents in dromedary indicate that this camelid contains more genera and species of protozoa than llamas. As in previous reports (Baker & Day, 1993; Del Valle *et al.*, 2008; Pinares-Patino *et al.*, 2003) no holotrich protozoa (*Isotricha* and *Dasytricha* spp.) were observed in this study. Similarly, species such as *Caloscolex* spp., *Buetschlia* spp. and *Diplodinium cameli*, commonly found in Old World Camelids (Dehority, 1986; Kubesy & Dehority, 2002). Dromedaries (Kubesy & Dehority, 2002) have shown a wider protozoal diversity than llamas, but even alpacas have shown the presence of other genera, such as *Diplodinium*, *Eudiplodinium* and *Epidinium* (Baker & Day, 1993; Del Valle *et al.*, 2008).

Del Valle *et al.* (2008) reported the presence of four to eleven protozoal species of protozoa (all from the genus *Entodinium*) in the forestomach contents of llamas from bolivian Altiplano. The present study did not observe up to six species of those cited by Del Valle *et al.* (2008), but the presence of *E. bovis* and *E. parvum* was more generalised among the experimental animals from the three locations.

To our knowledge, the present study is the first report of *Entodinium bimastus* (in three out of 18 animals) and mainly *Eudiplodinium maggii* (in 14 out of 18 animals) as new protozoal species found in llamas as host. The

discrepancies of the diversity of protozoal of host animals might be attributed to difference in the geographical locations, type and amount of feed consumed and physiological conditions (Dehority, 2003; Hungate, 1966; Warner, 1962). Ruminant and SACs differ not only in dietary features but most importantly in their digestive anatomy and physiology, hence it can be expected that they differ in their forestomach microbial diversity and populations and overall ecosystem environmental. Specific forestomach conditions in llamas may favor the presence of certain protozoal populations (*Entodinium* spp and *Eudiplodinium maggii*), and can be detrimental to other species (holotrichs). The genus *Entodinium* can be considered ubiquitous for most host species and, in general, dominates the rumen faunae (Imai, 1998). Large Entodiniomorphid protozoa, such as *Eudiplodinium* spp. play an important role in fiber digestion in ruminants (Coleman, 1985), and probably could be applied also to llamas, especially *Eudiplodinium* that has high cellulolytic activity (Ivan, 2009). The presence of *Eudiplodinium* in llamas would improve the fiber degradation in low quality forage based diet, which explains their presence in higher proportions in llamas from Cieneguillas, where llamas live in extensive conditions with no direct contact with other animal species and fed native species (*Festuca argentinensis* and *Parastephia quadrangularis*). On the other hand, llamas from Tilcara are fed on alfalfa hay and are in contact with guanaco and sheep individuals during few hours a day, and those from the lowlands in Hurlingham live indoors, fed on alfalfa hay and without direct contact with other animals, even though there are cows and sheep in the same location.

In general, the range of length and width for *Entodinium* and *Eudiplodinium* cells observed in this study were similar to those reported by Dehority (1993), Ogimoto and Imai (1981) and Williams and Coleman (1992), except for minor deviations of some species, such as *E. exiguum*, *E. longinucleatum* and, to same extent, *Eud. maggii*. Therefore, it is difficult to associate such differences with any environmental aspect. In order to corroborate the protozoal diversity identification data, DNA from forestomach contents of three llamas from Hurlingham was amplified and it showed that only 18S rRNA genes from *Entodinium* and *Eudiplodinium* were detected (unpublished data), hence supporting the hypothesis that the llamas in this study contains only these two genera.

Conclusions

Entodinium was the dominant genus found in the forestomach contents in llamas from Argentina. The identified members of this genus were *E. caudatum*, *E. longinucleatum*, *E. bovis*, *E. exiguum*, and *E. parvum* and, in some host individuals, *E. dubardi*, *E. ovibos* and *E. bimastus*. The presence of the genus *Eudiplodinium* is a new host record in llamas, being present in 14 out of 18 animals tested, although, they occurred mostly in low concentrations. No major effects of environment were apparent, except for frequency variations, probably attributable to feeding differences.

The vestibuliferids *Dasytricha* and *Isotricha* were absent from the forestomach of llamas. Further studies are needed to confirm the differences shown between the microbial communities from the SACs with their relatives in Eurasia.

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