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## Rumen protozoa of different ages of beef cattle raised in tropical pastures during the dry season

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### ABSTRACT

The purpose of this study was to quantify, identify and compare the protozoa populations in the rumen of different ages of beef cattle raised in tropical pastures during the dry season. We sampled the ruminal fluid of 36 steers, 34 cows and 30 calves, all crossbred Nelore. The ruminal fluid was diluted in 10% formaldehyde solution and decimal dilutions were prepared in saline solution to quantify small, medium and large protozoa in Sedgewick Rafter chamber. A total of 135,800 protozoa were evaluated in an optical microscope using Lugol's iodine coloration and the genera were classified according to the morphological characteristics. Total protozoa populations were significantly lower in calf samples. The average of small protozoa population was higher than that of other protozoa groups while the large ciliate population was lower. Protozoa belonging to 17 different genera were identified, showing diversity in this ecosystem. *Charonina* spp. was the most frequent for both bovine groups. *Entodinium* spp. was more prevalent in adult cattle and while *Buetschilia* spp. was more prevalent in calves. In this study, a considerable population of ruminal ciliates presenting high diversity was observed in cattle raised on tropical lignified pastures and their genus profile varied according to the ages of the animals.

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Beef cattle; *protozoa*; ruminal microbiota; tropical pasture; semiarid

## 1. Introduction

Different species of microorganisms belonging to the groups of bacteria, fungi and protozoa are present in the rumen. The interaction between these microorganisms allows the fermentation of structural carbohydrates and, consequently, the use of volatile fatty acids by the ruminant. Rumen ciliates were the first microorganisms to be described in this environment, with a population that varies between  $10^4$  and  $10^6$  protozoa per mL of ruminal content and these eukaryotes can represent up to 50% of the ruminal bi-mass (Ezequiel 2002; Kamra 2005; Newbold et al. 2015).

Forages are the basis of the ruminant diet, and degradation of the cell wall carbohydrates is essential to ruminant digestion and its survival and production (Russel and Rychik 2001). In semiarid tropical regions, the quantity and quality of available pasture are compromised during dry periods when forage digestibility is reduced by the physiological lignification of the vegetative cell wall (Moore and Jung 2001; Carvalho 2008). This reduction of the nutritional value of the pastures promotes substantial transformations in the microbial ecosystem of the rumen (Dirksen 1993).

Enzymes produced by protozoa represent a significant portion of the hydrolytic enzymes in the rumen (Williams and Coleman 1991; Takenaka et al. 2004) and point out the relevance of this microbial group in the degradation of fibrous forages (Santra and Karim 2002). Defaunation decreases the

rumen organic matter (OM) and fibre digestibilities, resulting in the loss of protozoa fibrolytic activities. However, the protozoa elimination can increase microbial protein supply and reduces methane production (Guyader et al. 2014; Newbold et al. 2015).

The protozoa's participation in the digestion and balance of the ruminal ecosystem is well studied for ruminants fed with grains and forages from temperate climate regions (Takenaka et al. 2004; Kamra 2005). However, the role of these ciliates remains unclear (Newbold et al. 2015).

Many protozoan studies have considered only fewer cattle which limits the real comprehension of the dynamic population and the protozoa genus diversity in the ruminal ecosystem. Especially, the influence of the age of cattle raised in tropical lignified pastures is not known. In this research, we aim to quantify, identify and compare the autochthonous protozoan population present in the rumen of different ages of beef cattle raised on tropical pastures during the dry period.

## 2. Material and methods

We collected ruminal fluid samples of 100 crossbred Zebu Nelore cattle: 34 cows, 36 steers and 30 male calves fed on extensive pastures of *Urochloa (Brachiaria)* spp., with low nutritional value, in North Minas Gerais, Brazil. The weaned calves were 6–8 months old, the steers were 24–40 months old,

while the cows were older than 4 years and were multiparous, but not lactating.

The area was located within 16°51'S and 44°55'W and has an average annual temperature of 24.2°C. The climate of the region, tropical humid with dry summer (As), according to the classification of Köppen (Alvares et al. 2014), is marked by a long dry season (from May to September). Ruminal fluid was collected between late May and early September and the rainfall for the sampling period was 136.9 mm (data obtained from the 5th District of the National Institute of Meteorology of Brazil – INMET, 2011).

Thirty days before taking the sampling, the pasture was an only single source of forage and the cattle were supplemented with urea and mineral mix for beef cattle (Table 1). Samples of 20 cm height of the pastures from the farms were collected and frozen to be sent for analysis of nutritional composition according to AOAC (2005). Nutritional composition of the *Urochola* spp. and mineral-nitrogen supplementation is shown in Table 1.

After approximately 8-h fasting, calves were immobilized in a restraint shoot for the collection of ruminal fluid by a puncture. Trichotomy and antiseptics with PVP-iodine solution (1%) were performed in a region of approximately 5 cm<sup>2</sup> on the left ventral abdomen, below the cranial paralumbar fossa and the knee joint (Abrão et al. 2014). Approximately 15 mL of rumen fluid was collected with a catheter (no. 14 G) attached to sterile syringes. This technique could not be used for an adult (cows and steers), because of the greater muscle thickness in the abdomen, and samples of ruminal fluid were obtained in a slaughterhouse with the municipal inspection.

After fasting, the adult cattle were slaughtered by concussion and bleeding. Samples were obtained immediately after slaughter by incision of the rumen ventral sac and approximately 15 mL of fluid was obtained using sterile reverse pipetting with a rubber pipette (Abrão et al. 2014). Samples were transported in insulated boxes and stored for up to 1 h in a sealed sterile test tube kept at 4°C. All procedures were submitted and approved by the Ethics Committee on Animal Experiments of the UFMG (protocol no. 156/05), regulated by the National Council for Control of Animal Experimentation of Brazil.

Tubes were homogenized in a vortex for 1 min, and an aliquot of 1000 µL was transferred to tubes containing 9 mL of 10% formalin. Subsequently, serial dilutions in saline solution were made and protozoa were conducted in Sedgewick Rafter

counting chambers (S52 glass; Pyser-SGI, Edenbridge, Kent, UK). The numbers of small (up to 40 × 60 µm), medium (up to 100 × 150 µm) and large (larger than 100 × 150 µm) ciliates per mL of rumen fluid were determined by light microscopy at 10× magnification (Dirksen 1993).

For protozoa identification, subsamples were placed on slides with cover slips with a drop of Lugol iodine solution (D'agosto and Carneiro 1999). The identification was performed in the optical microscope at 40× objective to characterize a minimum of 200 protozoa per animal (Rufino et al. 2011). In this study, 135.800 protozoa were evaluated and its genus was classified according to the morphologic characteristics described by Dehority (1993) and Ogimoto and Imai (1981).

After exploratory analysis, the data obtained from protozoa counts were transformed to log<sub>10</sub> (x + 10), subjected to the analysis of variance in a split-plot design in agreement to the bovine age groups and size of protozoa and mean values were compared by the Duncan' test. The genus frequencies were analysed by  $\chi^2$ . The data were evaluated using the SAEG 9.1 software with a significance level set at 5%.

### 3. Results and discussion

In this study, significant effects of bovine groups and protozoa sizes, as well as the interaction between them, were verified on the population of rumen ciliates of cattle raised in the tropical pasture during the dry season (Table 2,  $p < 0.05$ ). Calves showed the lowest population of rumen ciliates compared to steers and cows (Table 2,  $p < 0.01$ ). For the three cattle groups, the populations of small protozoa were the highest while the concentration of large protozoa was the lowest (Table 2,  $p < 0.05$ ). The small ciliate protozoa are the most resistant to changes in the ruminal environment and therefore are the most frequent in the ruminal environment, while the large ones are the most sensitive (Leng 1982; Dirksen 1993). Like this, the lower concentration of large ciliates observed in the cattle evaluated in this study could be justified by the nutritional conditions of the mature pastures, which showed low protein and energy contents and high lignin proportions.

In this study, the lower population of rumen ciliates observed in the calves could be justified because during the first months of life, these animals have not fully developed a ruminal microbial ecosystem. After 2 months of life, the concentration of rumen protozoa increases considerably as well as the genus diversity (Valvasori et al. 1992; Li et al. 2012; Yáñez-Ruiz et al. 2015). However, the composition of the diet offered to the young animal influences directly the ruminal development, and the addition of concentrate and grains increases the speed of ruminal development (Suarez et al. 2007). In this study, these ingredients were not offered since the calves were exclusively fed on the pasture without creep feeding, which could explain a late complete establishment of the ciliates on rumen of those animals (>6 months older).

The ruminal protozoa is present in a larger population (>1.10<sup>6</sup> protozoa per mL) when the diet is composed of mixed proportions of forage/concentrated (Ushida et al. 1990). The concentration of protozoa may be relatively low in animals fed exclusively with fodder or may show a high proportion in cattle fed with forage and concentrate (Willians

**Table 1.** Nutritional composition of the pastures and mineral-nitrogen supplementation.

Item (g kg <sup>-1</sup> )	
<i>Urochola</i> spp.	990.0
Mineral-nitrogen supplementation <sup>a</sup>	10.0
Nutrients (g kg <sup>-1</sup> DM)	
DM (g kg <sup>-1</sup> NM)	612.0
MM	71.9
CP	40.6
NDF	773.0
ADF	425.6
EE	14.4
LIG	96.9

DM, dry matter; NM, natural matter; MM, mineral matter; CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre; EE, ether extract; LIG, lignin.

<sup>a</sup>Urea 250.0 g kg<sup>-1</sup>, calcium 54 g kg<sup>-1</sup>, phosphorus 47 g kg<sup>-1</sup>, magnesium 10 g kg<sup>-1</sup>, sodium 145 g kg<sup>-1</sup>, sulphur 24 g kg<sup>-1</sup>, manganese 900 mg kg<sup>-1</sup>, zinc 2600 mg kg<sup>-1</sup>, copper 720 mg kg<sup>-1</sup>, cobalt 80 mg kg<sup>-1</sup>, iodine 120 mg kg<sup>-1</sup>, selenium 18 mg kg<sup>-1</sup>.

**Table 2.** Small, medium and large protozoa averages per mL of ruminal fluid of three bovine categories raised in lignified tropical pasture.

Categories	Small	Medium	Large	Total	CV (%)
Calves	$9.07 \times 10^3$ <sup>Ac</sup>	$6.57 \times 10^3$ <sup>Bc</sup>	$1.60 \times 10^{3Cb}$	$1.72 \times 10^4$ <sup>c</sup>	6.83
Steers	$3.12 \times 10^5$ <sup>Aa</sup>	$1.50 \times 10^5$ <sup>Ba</sup>	$1.91 \times 10^4$ <sup>Ca</sup>	$4.83 \times 10^6$ <sup>a</sup>	5.26
Cows	$7.80 \times 10^4$ <sup>Ab</sup>	$1.34 \times 10^4$ <sup>Bb</sup>	$3.62 \times 10^3$ <sup>Cc</sup>	$9.50 \times 10^4$ <sup>b</sup>	2.36
CV (%)	10.2	9.65	13.67	9.10	

Note: Means followed by different uppercase letters in the rows and lowercase in the columns differ from each other by the Duncan test ( $p < 0.05$ ). CV = Coefficient of variation.

and Coleman 1991). In another study in the North of Minas Gerais, Brazil, during a long dry period was verified decrease of the ruminal ciliates from protozoa Nelore steers raised in *Urochloa* spp. contained high value of lignin. The population of small and medium ruminal protozoa decreased at the end of the dry season. However, the large protozoa population was constant during all the dry season (Silva et al. 2014). The advanced phenological stage of the elephant grass (*Pennisetum purpureum*), which presented low nutritional value, also influenced negatively the concentration of rumen protozoa (Nogueira Filho et al. 1992).

Beef cattle fed *Urochloa* spp. with high maturation level presented higher concentration of the rumen protozoa than those fed high grain and without forage. The acid pH of the rumen reduced large ciliates in cattle fed without roughage (Nigri et al. 2017).

Researchers demonstrated hemicellulolytic and cellulolytic activity in the rumen ciliates, especially for the group of large entodiniomorphs (Frazolin and Franzolin 2000). Principally the large protozoa may colonize fibre fragments and directly ingest plant tissues favouring the action of cellulolytic bacteria (Willians and Coleman 1991).

In this study, a relevant population of large entodinium protozoa ( $>1 \times 10^3$  per mL of rumen fluid) in beef cattle raised in the tropical pasture during the dry period could be detached, since its degradative activity of the vegetal cell wall would contribute to the better utilization of the pastures. Specifically, the large protozoa show an important role in fibre digestion and their enzymes constitute a significant portion of the hydrolytic activity in the rumen (Agarwal et al. 1991; Santra and Karim 2002).

Considering the activity of xylanases and carboxymethyl celluloses among different species of rumen protozoa, a set of carboxymethyl cellulases and xylanases is produced by the large ciliates *Polyplastron multivesiculatum* and *Eudiplodinium maggii* (Béra-Maillet et al. 2006). Additionally, a lower carboxymethyl cellulose activity in the rumen of defaunated lambs was observed, showing lower cellulose digestibility than those faunated (Santra and Karim 2002). Additionally, studies of metagenomic screening of glucosidases and metatranscriptomes have shown that a diverse range of glycoside hydrolases are present in the rumen ciliates (Findley et al. 2011; Qi et al. 2011).

On the three bovine group evaluated, we identified 17 genera. Ciliates of the families Blepharocorythidae (*Charonina* spp.), Isotrichidae (*Isotricha* spp.) and Ophryoscolecidae (*Entodinium* spp., *Eodinium* spp. and *Diplodinium* spp.) showed high occurrence in the rumen of these animals fed on *Urochloa* spp. (Table 3). This diversity, even in the dry season, can indicate a healthy ruminal environment, where different groups of ciliates found conditions to establish themselves (Dirksen 1993; Kamra 2005).

For calves, the most frequent genera were *Buetschilia*, *Charonina* and *Eodinium* ( $p < 0.05$ ). Among steers, *Charonina* spp. and *Entodinium* spp. were the most frequent, followed by *Isotricha* spp. and *Eodinium* spp. For cows, the most frequent genus was *Charonina*, followed by *Entodinium* spp. and *Eodinium* spp. (Table 3,  $p < 0.05$ ).

For the evaluated calves, *Buetschilia* spp. represented the ruminal ciliate most frequent in the ruminal environment, which was not observed for cows and steers (Table 2). Silva et al. (2014) reported similar results to those verified in this study, when shown a high occurrence of *Charonina* spp. and *Entodinium* spp. in steers fed with *U. decumbens* pasture. In young ruminants within two weeks of birth, ciliate protozoa are normally seen in the rumen, with small entodinia established before large endomorphs and holotrich protozoa. However, if animals are isolated from other ruminants shortly after birth, no protozoa establish (Eadie 1962; Dehority 2003). In this study, the calves before weaning were raised together with their mothers and other contiguous calves, favouring the implantation of different genera of ciliates in the rumen of these animals.

In this research, *Entodinium* spp. was identified in higher proportions for steers (16.8%) and cows (14.4%). However, for calves, this genus represented only 4.2% of the identified ciliates, suggesting that this protozoa could establish completely only with the maturity of the animals raised in a tropical lignified pasture. The genus profile for the calves in this study was discrepant of those reported by Manella et al. (2004), who verified the highest prevalence of the genus *Entodinium* in newly weaned Nelore cattle grazing exclusively of *Urochloa brizantha*. Nogueira

**Table 3.** Distribution of ruminal protozoa genera according to bovine categories raised in tropical lignified pastures.

Genera	Calves (n/%)		Steers (n/%)		Cows (n/%)	
<i>Buetschilia</i> spp.	19,620	23.83*	483	6.23	2710	5.92
<i>Isotricha</i> spp.	6390	7.76	733	9.45	4610	10.08
<i>Dasytricha</i> spp.	5000	6.07	443	5.71	1010	2.20
<i>Charonina</i> spp.	13,480	16.37*	1849	23.86*	13630	29.81*
<i>Entodinium</i> spp.	3470	4.21	1303	16.81*	6600	14.43*
<i>Diplodinium</i> spp.	4270	5.18	645	8.32	3910	8.55
<i>Eodinium</i> spp.	12,670	15.38*	743	9.58	6180	13.51*
<i>Eremoplastron</i> spp.	2040	2.47	474	6.11	2050	4.48
<i>Eudiplodinium</i> spp.	3570	4.33	261	3.36	900	1.96
<i>Diploplastron</i> spp.	3480	4.22	232	2.99	680	1.48
<i>Polyplastron</i> spp.	1470	1.78	94	1.21	160	0.34
<i>Ostracodinium</i> spp.	2550	3.09	185	2.37	1220	2.66
<i>Elytroplastron</i> spp.	1460	1.77	35	0.45	180	0.39
<i>Metadinium</i> spp.	840	1.02	30	0.38	1050	2.29
<i>Enoploplastron</i> spp.	200	0.24	45	0.58	150	0.32
<i>Ophryscilex</i> spp.	520	0.63	28	0.36	210	0.45
<i>Epidinium</i> spp.	1300	1.57	167	2.15	470	1.02
Total	82,330	100.0	7750	100.0	45,720	100.0

\*Most frequent genera by category according to the chi-square test with 5% significance.

Filho et al. (1992), when researching Holstein calves fed with *U. decumbens* and concentrate, also recorded a higher frequency of this protozoa genus. We believe that the diet offered by these researchers, including concentrated (corn and soy), showed better nutritional value than those available for the beef cattle in this study, favouring the ciliate.

Other research has also recorded higher occurrences of *Entodinium* spp. in adult cattle. Abrar et al. (2016) observed a large concentration of this eukaryote (80%) in adult animals (Holstein × Wagyu) fed with ryegrass straw and commercial concentrate. This ciliate was also recorded in high concentration diets (79%) for Martinele et al. (2008) in crossbred Dutch-zebu cows receiving different concentrations of elephant grass (60–100%) and Ríspoli et al. (2009) reported the occurrence of 85% of *Entodinium* spp. for Holstein cows fed with 50% corn silage and 50% concentrate (corn in grains and soybean meal). According to Newbold et al. (2015), this genus has been responsible for many of the bacterial protein turnover in the rumen.

The presence of different protozoa genera in the ruminal environment is directly related to the type of diet (Williams and Coleman 1991; Dehority and Odenyo 2003). Studies show that the higher proportion of concentrate in the diet promotes the elevation of the concentration of these eukaryotes (Ushida et al. 1990).

In our study, we use the microscopic identification as the gold standard for analysing rumen protozoa (Williams and Coleman, 1991). Despite a high level of experience by the researcher and laborious and highly demanding (Dehority 2008), the microscopic technique holds several advantages over PCR-based molecular methods. The majority of ruminal and intestinal protozoa have been morphologically characterized; there is a lack of 18S rRNA gene reference sequence for many of the known genera and species. The copy number variation of ribosomal RNA genes across the different genera or under different growth conditions may skew the observed concentration of these genera in a sample (Medinger et al. 2010).

The comparison of diversity and structure of protozoa communities from hay-fed and silage-/grain-fed cattle via T-RFLP analysis yielded similar overall results to microscopy analysis. In both methods, *Entodinium* spp. was more abundant in the silage-/grain-fed cattle. However protozoa diversity was higher for the hay-fed cattle due to greater species evenness (Tymensen et al. 2012).

#### 4. Conclusion

The ruminal protozoa concentration is lower in zebu calves when compared to adult animals. *Entodinium* spp. was more frequent in adult cattle while *Buetschilia* spp. was more frequent in calves. The considerable concentration and genus diversity of ruminal ciliates, detected for cattle of different ages in this study, may indicate the ecologic and nutritional importance of these eukaryotes for the ruminal environment of cattle fed on tropical pastures during the dry season.

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