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Technical Note

Quantitative and differential analysis of ciliate protozoa in rumen content samples filtered before and after fixation

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ABSTRACT - The objective of this study was to assess whether the straining of rumen content samples influences the estimation of protozoal density. Ninety rumen samples were obtained from 30 cattle (three samples per animal). The samples were subjected to one of three treatments at the moment of collection: 1) fixation in formalin without straining (control treatment), 2) straining before fixation in formalin, or 3) straining after fixation in formalin. To test the hypothesis of the variation in the protozoa composition in the samples, multivariate analyses with non-metric multidimensional scaling (NMDS) were carried out. The diversity and density of rumen protozoa were negatively affected by straining before fixation. In the pre-filtered sample, the number of ciliates from the genus *Entodinium* was reduced, and no individuals from the *Diploplastron*, *Elytroplastron* and *Eudiplodinium* genera were detected; these effects were not observed in the other two treatments. Straining after fixation did not interfere with the diversity of the ruminal community, but the abundance of protozoa was greater than in the control treatment and significantly greater than in the samples filtered before fixation. These factors suggest that post-fixation straining is the recommended technique to analyze rumen protozoa.

Key Words: Ciliophora, *Entodinium*, ruminal protozoa, ruminant

Introduction

The role that ruminal protozoa play in the nutrition of their hosts is still controversial and varies with the nature of their diet. Therefore, the diversity and density of protozoa and their relationship with other constituents of the microbiota are often examined in studies on ruminant nutrition.

The straining of rumen content samples is widely performed in the analysis of various ruminal parameters. A variety of materials have been utilized for this purpose, including gauze (Ríspoli et al., 2009; Trujillo et al., 2010), nylon (Muetzel et al., 2009), sieves (Eriksson et al., 2004), sackcloth (Martinele et al., 2002), fiberglass (Nheta et al., 2005) and mousseline (Rezaeian et al., 2004; Naik et al., 2009).

However, the lack of standardization regarding the filter materials used can impair the quantification and identification of ciliate protozoa because some of these materials have pores that are smaller than the body size of some species; this causes these species to be retained in the filter. According to Martinele et al. (2002), straining allows better visualization of smaller species, such as those of the genera *Entodinium* and *Charonina*, which can be obscured by plant particles. Nevertheless, the authors noted the need to use filters with pores that are large enough to avoid the retention of any genera of ciliates, as they observed when using filters with pore diameter of 500 µm.

In addition to comparing the materials used for straining, this study also considers the timing of the straining because the manipulation of the sample prior to its fixation can be critical to the preservation of ruminal protozoa, as reported by Rossi (2013) after extensive observations of ciliates *in vivo*. Therefore, it has been hypothesized that the manipulation of rumen content samples by filtering before fixation may influence the quantification of ciliated protozoa and consequently alter the interpretation of the role of these protozoa in the physiology of animal digestion.

Thus, the objective of this study was to evaluate the effects of straining rumen content samples on the composition of the protozoa community before and after fixation in formalin using a filter with pores measuring 2000 µm.

Material and Methods

Samples were obtained from the middle of the ruminal mass of 30 crossbred cattle. The cattle used in this study had undefined pedigrees and were allowed to graze on farms located in Juiz de Fora, Minas Gerais, Brazil. Before

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being slaughtered, the animals were deprived of food for 12 hours; during this time they received water *ad libitum*. Three samples of approximately 20 cm^3 each were collected from each eviscerated rumen. These samples were subjected to three treatments, for a total of 30 samples per treatment. For the first (control) treatment, the rumen content samples were immediately fixed in 18.5% (v/v) formalin without straining (Dehority, 1984). For the second treatment (pre-straining), the samples were filtered immediately after collection and then fixed in the same 18.5% formalin solution. Finally, for the third treatment (post-straining), the samples were immediately fixed in the formalin solution and then filtered. Straining was performed using two layers of phyllo cloth with pores measuring 2000 µm.

The density of the rumen protozoa per mL was obtained using a Sedgewick-Rafter counting chamber (Dehority, 1984) with the modifications proposed by D'Agosto & Carneiro (1999), and the protozoa present in each sample were identified based on the criteria described by Ogimoto & Imai (1981).

To test the hypothesis that the composition of the protozoa in the samples would vary due to the different filtration treatments, multivariate analyses were carried out with the PAST program (version 2.17b, 2012). The data were organized in a binary matrix (presence and absence) and plotted on a two-dimensional map with non-metric multidimensional scaling (NMDS); the dissimilarity was calculated using the Bray-Curtis index. This is the most appropriate index for multivariate analysis because it is less affected by the number of rare species in the samples, and the stress index calculated by NMDS with two dimensions is a measure of the fit of the data.

A single-factor similarity analysis (a one-way ANOSIM) was applied with 10,000 permutations using the PAST program (version 2.17b, 2012). This analysis reveals if there are significant differences between the means of the similarities ranked among the samples within the same treatment and between different treatments. This analysis results in an R-statistic, which is a measure of dissimilarity between the treatments. R values near 0 indicate high similarity and those near 1 indicate low similarity. For the ANOSIM calculation, the Bray-Curtis index was also used, in which each R value has its corresponding probability.

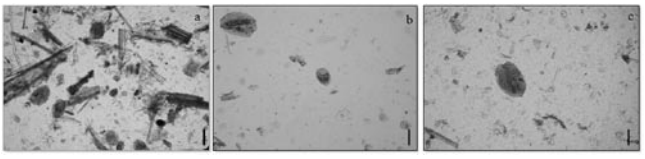
Finally, the data was submitted to similarity percentage analysis (SIMPER) (PAST program version 2.17b, 2012), which allows us to determine which species are primarily responsible for discriminating between different communities, according to the percentage of dissimilarity between pairs of treatments and presenting the percentage of contribution of each genus to that dissimilarity.

Results and Discussion

The straining process removes gross fibrous material from the sample, thus making the visualization of the ciliate protozoa in filtered samples easier than in unfiltered samples (Figure 1); this is true when the filtration occurs either before or after fixation with formalin.

However, the analysis of similarity (ANOSIM) indicated a significant difference ($P<0.05$) between the pre-filtered and post-filtered treatments (Table 1), revealing a different composition of species between the two methods. In addition, the pre- and post-filtered treatments presented a dissimilarity measure (R) that was far from zero relative to the control treatment, indicating high pairwise differences between both of these treatments and the control treatment (Table 1). This difference was also supported by the NMDS, in which the control and post-filtered treatments clustered along axis 1, representing 72% of the data analyzed; the pre-filtered treatment was plotted far from the other groups, indicating that the pre-filtered samples are more distinct with respect to the abundance of ciliates found in them (Figure 2).

The abundance and diversity of ciliates were smaller in the pre-filtered treatment (Table 2). This can be observed by the absence of the genera *Diploplastron, Elytroplastron*



Magnification: 10x.
Scale bar: 100 µm.

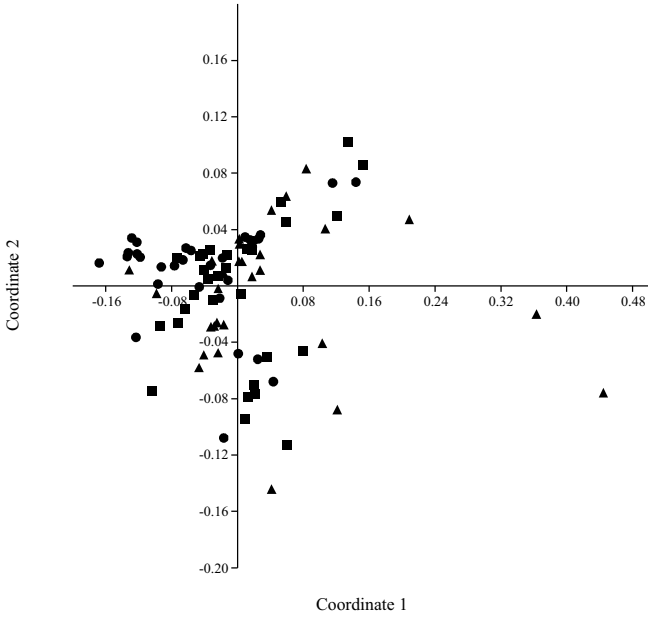
Figure 1 - Photomicrographs of the rumen content of cattle in a randomly chosen field of a Sedgewick-Rafter counting chamber from each of the three treatments: control (a; samples fixed in 18.5% formalin without straining), pre-filtered (b; samples filtered and then fixed) and post-filtered (c; samples fixed and then filtered).

Table 1 - Matrix of similarity between the populations of ciliate protozoa per mL of rumen content of cattle, presented as R values

	Control	Pre-filtered	Post-filtered
Control	0		
Pre-filtered	0.00172	0	
Post-filtered	0.03676[1]	0.04518[2]	0

The control samples were fixed in 18.5% formalin without straining, the pre-filtered samples were filtered and then fixed, and the post-filtered samples were fixed and then filtered.
R values near 1 indicate the treatments have low mutual similarity and R values near 0 indicate high similarity at 5% probability.
[1] P = 0.06.
[2] P = 0.02.

and *Eudiplodinium* in the pre-filtered treatment. However, because these genera were present in the other treatments, their absence may be associated with cell lysis resulting from the manipulation of the sample before fixation and contact with oxygen. Because the ciliate protozoa from the rumen are anaerobic, the exposure of these genera to oxygen can promote the lysis of certain species that are more sensitive to aerobiosis, resulting in the underestimation of their population density.



Analysis using the Bray-Curtis similarity index (stress: 0.1191).

Figure 2 - Multivariate analysis of non-metric multidimensional scaling (NMDS) of the populations of ciliates in the rumen content of cattle in three treatment groups: control (■), pre-filtered (▲) and post-filtered (●).

Table 2 - Scaled contribution percentage and mean abundance of the ciliate genera per mL of rumen content of cattle

Genera	Contribution[1]	Mean abundance		
		Control	Pre-filtered	Post-filtered
Entodinium	20.93	323.200	292.800	433.600
Diplodinium	3.35	30.720	26.240	20.640
Eremoplastron	2.88	25.120	29.440	24.800
Ostracodinium	2.50	29.920	20.320	19.840
Eodinium	2.11	25.920	16.160	17.440
Epidinium	1.53	15.408	10.128	8.960
Isotricha	1.35	11.792	14.240	10.608
Dasytricha	1.15	11.360	9.440	12.752
Eudiplodinium	0.60	8.112	0	907
Metadinium	0.57	4.688	5.120	4.368
Charonina	0.51	5.120	3.088	3.040
Diploplastron	0.02	53	0	212
Elytroplastron	0.01	53	0	1.067

The control samples were fixed in 18.5% formalin without straining, the pre-filtered samples were filtered and then fixed, and the post-filtered samples were fixed and then filtered.
The data were analyzed using the similarity percentage test (SIMPER).
[1]Scaled contribution percentage of each genus for total mean dissimilarity (37.5) between the treatments.

The genera *Isotricha* and *Dasytricha* are normally more represented in the rumen, with percent compositions between 8-11% (D'Agosto & Guedes, 2000; Martinele et al., 2007). In the present study, they had low average abundances (Table 2), with a percent composition of approximately 5%. This may indicate that the isotrichids may have escaped from rumen to the reticle during the fasting period that preceded the slaughter of the animals. The escape of protozoa from the rumen to the reticle during periods of fasting and the subsequent migration of the reticle to the rumen after the animals are fed have been documented in cattle (D'Agosto & Guedes, 2000; Martinele et al., 2007).

Protozoa of the genus *Entodinium* were the most abundant in all treatments, being the best-represented genus among the populations observed. Additionally, this genus explains much of the dissimilarity of the treatments (20.93%, Table 2), indicating that populations of *Entodinium* were more abundant in the post-filtered treatment than in the control and pre-filtered treatments (Table 2); this indicates that the post-filtered treatment is the most recommended treatment. Because the genus *Entodinium* can compose up to 90% of the ciliate protozoa in the rumen of cattle (Martinele et al., 2002), the straining of the rumen content after fixation is justified because most species of the genus *Entodinium* are the smallest species observed in the rumen and are better visualized in filtered samples. However, these species are also more susceptible to the manipulation of rumen samples before fixation (Rossi, 2013). Therefore, the straining after fixation favors the visualization of these smaller species without eliminating them.

Conclusions

The straining of rumen samples allows better visualization and identification of the ciliate protozoa. However, when straining is carried out before fixation in formalin, the ciliate diversity and density are reduced, leading to underestimation of these populations. Therefore, it is recommended to filter the rumen content after fixation in formalin.

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