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Protozoa evolution in Rusitec fermenters fed diets differing in forage to concentrate ratio and forage type

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Abstract. The aim of this study was to investigate the evolution of protozoa numbers over 14 days in 16 Rusitec fermenters fed four different diets. The diets had forage:concentrate (F:C;dry matter (DM) basis) ratios of 70:30 (HF) or 30:70 (HC) with either alfalfa hay (A) or grass hay (G) as forage. Ruminal inoculum from sheep fed the same diets was used to inoculate the fermenters on day 0. Retention time for forage and concentrate was 48 and 24 h, respectively, and dilution rate was preset at 5.14% per h. Total protozoa numbers declined rapidly from the first day after inoculation, but they were present on day 14 in all fermenters at concentrations which ranged from 3.30 to 8.52 x 10^3 / ml. Only Entodiniinae were able to grow in HC-fed fermenters by the end of the trial, but Entodiniinae, Isotrichidae and Diplodiniinae were maintained in fermenters receiving HF diets. The disappearance of Isotrichidae and Diplodiniinae from HC-fed fermenters was attributed to low pH values. Ophryoscolecinae disappeared completely from all fermenters by day 6 of incubation. In general, greater protozoa numbers were observed in the fermenters fed diets containing G compared with those fed A diets. Our results indicate that protozoa could not be maintained at numbers similar to those in the rumen, but responses to changes in F:C ratio in the diet were similar to those observed *in vivo*.

Keywords. Rusitec – Protozoa – Forage: concentrate ratio – Forage.

Évolution des protozoaires dans des fermenteurs Rusitec alimentés avec des régimes ayant différents rapports fourrage : concentré et différents types de fourrages

Résumé. *Le but de cette étude était d'étudier l'évolution des nombres des protozoaires au cours de 14 jours dans 16 fermenteurs Rusitec alimentés avec quatre régimes différents. Les régimes ont un rapport fourrage : concentré (F : C ; rapport calculés sur la base de la matière sèche (DM)) du 70 : 30 (HF) ou du 30 : 70 (HC) avec le foin de luzerne (A) ou le foin d'herbe (G) comme fourrage. L'inoculum ruminal des moutons alimentés avec les mêmes régimes a été utilisé pour inoculer les fermenteurs le jour 0. Le temps de rétention pour le fourrage et le concentré était de 48 et 24 h, respectivement, et le taux de dilution a été préréglé à 5,14 % par h. Les nombres totaux de protozoaires ont rapidement décliné depuis le premier jour après l'inoculation, mais ils étaient présents le jour 14 dans tous les fermenteurs aux concentrations comprises entre 3.30 et 8,52 x 10^3/ml. Seulement les Entodiniinae pouvaient se développer dans les fermenteurs HC vers la fin de l´expérience, mais les Entodiniinae, Isotrichidae et Diplodiniinae ont été maintenus dans les fermenteurs recevant les régimes HF. La disparition des Isotrichidae et des Diplodiniinae des fermenteurs HC a été attribuée aux faibles valeurs de pH. Les Ophryoscolecinae ont complètement disparu de tous les fermenteurs par le jour 6 de l'incubation. On a dénombré plus de protozoaires dans les fermenteurs qui contenaient G comparés avec ceux nourris avec A. Nos résultats indiquent qu'on n'a pas pu maintenir les protozoaires dans des nombres semblables à ceux qui se trouvent dans le rumen, mais les réponses aux changements dans le rapport F : C ont été semblables à ceux observés in vivo.*

Mots-clés. *Rusitec – Protozoaires – Rapport fourrage : concentré – Fourrage.*

I – Introduction

Research on rumen function *in vivo* is mostly carried out with surgically altered (i.e. fistulated animals), which are expensive and difficult to maintain; additionally, in the last years, ethical questions have been raised concerning the use of fistulated animals for experimental purposes. *In vitro* devices simulating rumen fermentation are a good alternative, but in many *in vitro* experiments with continuous-flow fermenters a marked decrease or even a complete disappearance of protozoa with time has been reported (Crawford *et at.*, 1980; Moumen *et al.,* 2007). The semi-continuous flow Rusitec system (Czerkwaski and Breckenridge, 1977) could be more appropriated than continuous-flow fermenters, since solid substrate is enclosed in nylon bags instead of being dispersed by stirring. Dietary factors, such as forage:concentrate (F:C) ratio, forage quality and level of feeding, have been reported to influence protozoa populations in vivo (Jouany, 1989), but few studies have been conducted to determine how these factors affect protozoa populations in fermenters over incubation time. The aim of this work was to investigate the evolution of protozoa populations over time in Rusitec fermenters fed four diets differing in F:C ratio and forage type.

II – Materials and methods

One 14-day incubation trial was carried out with 16 Rusitec fermenters with an effective volume of 600 ml each. Four total mixed diets were formulated according to a 2 x 2 factorial arrangement of treatments. The diets had F:C (dry matter (DM) basis) ratios of 70:30 (HF) or 30:70 (HC) with either alfalfa hay (A) or grass hay (G) as forage, and were designated as HFA, HCA, HFG and HCG. The concentrate was based on barley, gluten feed, wheat middlings, soybean meal, palmkern meal, wheat, corn and mineral-vitamin premix in the proportions of 215, 204, 200, 135, 115, 50, 50 and 31 g/kg, respectively (fresh matter basis). Crude protein content was 186, 177, 121 and 160 g/kg DM for HFA, HCA, HFG and HCG, respectively, and neutral-detergent content was 426, 374, 499 and 401 g/kg DM. Eight rumen-fistulated sheep were used as donors of ruminal inoculum. Two sheep received each of the diet for 21 days before starting the *in vitro* trial. Ruminal contents from each sheep were collected before the morning feeding, pooled by diet, strained through two layers of cheesecloth, and transferred to the corresponding fermenters within 30 minutes after collection. The flow through fermenters was maintained by continuous infusion of artificial saliva at a rate of 740 ml/d (5.14% h^{-1}). Each fermenter received daily 30 g (DM) of the corresponding diet. Forage and concentrate were incubated into separated nylon bags (100 µm of pore size), which remained inside the fermenters for 48 and 24 h, respectively. The general incubation procedure was as described by Carro *et al.* (1992). Fermenters' fluid was sampled every day before the morning feeding and the pH was immediately measured. On days 0, 4, 6, 8, 10, 12 and 14, 2 ml of ruminal fluid were added to 2 ml of 50% formalin solution (18.5% formaldehyde), mixed, and stored at room temperature in dark. Aliquots were counted by duplicate from 10 microscopic fields in a Hausser Nageotte Bright-line counting chamber (0.5 mm depth; Hausser Scientific, Horsham, PA, USA) at a magnification of 40X. Family Isotrichidae and Family Ophryoscolecidae (including Subfamilies Entodiniinae, Diplodiniinae and Ophryoscolecinae) were identified following descriptions by Dehority (1993), and their numbers were separately recorded. When coefficient of variation between replicates was greater than 10%, counting was repeated.

Data were analyzed according to a repeated measures model using the MIXED procedure of the Statistical Analysis Systems statistical package version 8.02 (SAS Institute, Cary, NC, USA). Effects included in the model were time, F:C ratio, forage type (FOR), and the interactions F:C x FOR, F:C x time and FOR x time. Fermenter was considered a random effect. Mean effects were declared significant at $P<0.05$, and when a significant effect of time was detected differences between means were assessed by using the Tukey's multiple comparison test.

III – Results and discussion

As shown in Table 1, total protozoa numbers declined (P<0.001) rapidly from the first day after inoculation, but they were present in all fermenters 14 days after inoculation. Total protozoa numbers were greater for HC than for HF diets (P<0.001), and for G compared with A diets (P<0.001). Concentrations of protozoa at the end of the trial were within the range found in studies with Rusitec fermenters conducted under similar conditions (Czerkawski and Breckenridge, 1977; Carro *et al.*, 1992; Carro *et al.*, 1995).

Table 1. Total protozoa numbers (x 10^3/ml) in Rusitec fermenters fed diets with forage:concentrate (F:C) ratios of 70:30 (HF) or 30:70 (HC) and alfalfa hay (A) or grass hay (G) as forage (FOR) and inoculated on day 0 with buffered rumen fluid from sheep (n = 4)

Day	Diet				SEM†	Statistical effect (P =)					
	HFA	HFG	HCA	HCG		F:C	FOR	Time	F:C x FOR	F:C x Time	FOR x Time
0	463^{a}	392^{a}	826^{a}	598^{a}	3.141	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
2	73.4^{b}	115^{b}	77.7^{b}	103^{b}							
4	35.4^{c}	63.3^{c}	40.4^{c}	45.2^{c}							
6	26.1^{d}	37.1^{d}	19.7^{d}	26.0^{d}							
8	19.3^{de}	26.5^{e}	13.2^{de}	12.0^{e}							
10	15.3^{ef}	15.9^{f}	6.80^{ef}	9.73^{e}							
12	8.00^{fg}	9.10^{f}	2.91^{f}	9.15^{e}							
14	6.14^{g}	8.52^{f}	3.30^{f}	6.76^{e}							

$^{a, b, c, d, e, f, g}$ Mean values within a column with unlike superscripts differ (P<0.05).

† Standard error of the mean.

Dilution rates in the rumen are usually over 1.0 per day, but when similar or greater dilution rates are used in continuous-flow fermenters protozoa numbers decrease markedly, since their generation time becomes greater than the residence one (Abe and Kumeno, 1973; Czerkawski and Breckenridge 1977). In our experiment, liquid dilution rate was 1.20 per day, which would explain the pronounced decrease of protozoa. Low dilution rates have been reported to decrease the washing out of protozoa from the fermenters (Crawford *et al.*, 1980), but they might lead to an accumulation of end-products toxic to microbial populations.

Isotrichidae protozoa numbers declined drastically from the start of the trial, and concentrations at day 8 of incubation were only 2.4 and 0.65 % of those in the inoculum for HF and HC diets, respectively (Table 2). Isotrichidae disappeared completely in the fermenters fed HC diets by day 10, whereas they were maintained in HF-fed fermenters until the end of the trial. Studying the establishment of protozoa in calves and sheep, Eadie (1962) found that Isotrichidae did not develop with pH below 6.5. Thus, the complete disappearance of Isotrichidae in HC-fed fermenters may be pH related. Minimum pH values were observed in all fermenters between 6 and 8 h after feeding (results not shown). Whereas in HF-fed fermenters values did not drop below 6.44 and 6.11 in fermenters fed HFA and HFG, respectively, minimum values were 5.76 and 5.74 in fermenters fed HCA and HCG, respectively. In agreement with our results, Carro *et al.* (1995) found that Isotrichidae represented 1.35 % of total protozoa in Rusitec fermenters fed a HF diet at pH values of 6.36, but they completely disappeared when pH dropped to 6.17, and Carro *et al.* (1992) found that Isotrichidae were 12.2 % of total protozoa in Rusitec fermenters fed the same diet and maintained at pH = 6.86. The greater (P = 0.03) Isotrichidae numbers observed for A diets compared with G diets might also be pH related, as pH mean values over the 12 h after feeding period were 6.43 and 6.17, respectively.

Table 2. Isotrichidae protozoa numbers (x 10^3/ml) in Rusitec fermenters fed diets with forage: concentrate (F:C) ratios of 70:30 (HF) or 30:70 (HC) and alfalfa hay (A) or grass hay (G) as forage (FOR) and inoculated on day 0 with buffered rumen fluid from sheep (n = 4)

Day	Diet				SEM†	Statistical effect (*P* =)					
	HFA	HFG	HCA	HCG		F:C	FOR	Time	F:C x FOR	F:C x Time	FOR x Time
0	47.2[a]	46.3[a]	29.6[a]	31.3[a]	0.276	<0.001	0.03	<0.001	<0.001	<0.001	<0.001
2	7.29[b]	7.48[b]	7.85[b]	9.08[b]							
4	4.54[c]	4.08[c]	4.84[c]	3.79[c]							
6	3.18[d]	1.84[d]	1.14[d]	1.42[d]							
8	1.86[e]	0.42[e]	0.24[e]	0.15[e]							
10	1.62[e]	0.12[e]	0.00[e]	0.00[e]							
12	0.22[f]	0.10[e]	0.00[e]	0.00[e]							
14	0.11[f]	0.03[e]	0.00[e]	0.00[e]							

[a, b, c, d, e, f] Mean values within a column with unlike superscripts differ (P<0.05).

† Standard error of the mean.

Entodiniinae numbers declined (*P*<0.001) rapidly from the inoculation level, but they were present 14 days after inoculation in all fermenters (Table 3). This is in agreement with other studies (Slyter and Putnam, 1967; Carro *et al.*, 1992, 1995) showing that Entodiniinae are the most abundant protozoa in fermenters. Entodiniinae numbers were greater (*P*<0.001) in HC-fed fermenters than in those receiving HF diets. Entodiniinae have been reported to utilize starch and to be the most resistant of the protozoa to low ruminal pH, and therefore they are usually the most abundant protozoa in the rumen of animals given HC diets (Williams and Coleman, 1992; Hristov *et al.*, 2001). In agreement with the results from other studies (Franzolin and Dehority, 1996; Hristov *et al.*, 2001), Entodiniinae represented 83 and 90% of total protozoa in the rumen fluid from sheep fed HF and HC diets, respectively. The proportion of Entodiniinae increased (*P*<0.001) with time, and by the end of the trial it was 94, 99, 100 and 100 in fermenters fed HFA, HFG, HCA and HCG, respectively. Both Entodiniinae numbers and their proportion of total protozoa were greater (*P*=0.03 and <0.001, respectively) in fermenters receiving G diets than in those fed A diets.

Mean retention time of solid digesta in our study was 48 and 24h for forage and concentrate, respectively. This long retention time could help to explain the presence of Entodiniinae in all fermenters for 14 days, because the sequestration of protozoa among particulate digesta has been identified as an important factor in maintaining their concentration (Nakamura and Kurihara, 1978). Crawford *et al.* (1980) reported that continuous-flow fermenters with a solid digesta retention time of 29.7 h maintained relatively stable protozoa numbers after 8 days of incubation, but the reduction of solid digesta retention time to 22.0 and 14.3 h produced a marked decline in protozoa numbers.

As shown in Table 4, Diplodiniinae numbers declined (P<0.001) rapidly, and they disappeared completely from fermenters receiving HC diets by day 10. These observations are in accordance with the results of Franzolin and Dehority (1996), Goad *et al.* (1998) and Hristov *et al.* (2001), who observed that Diplodiniinae disappeared from the rumen when steers were fed HC diets. The disappearance of Diplodiniinae was attributed to their sensitivity to low ruminal pH.

Ophryoscolecinae protozoa numbers (results not shown) were affected by all analysed factors (*P*<0.001) and disappeared completely from all fermenters by day 6 of incubation. Dehority (2004) estimated than the *in vitro* generation time of *Ophryoscolex purkynjei* was 29 h, but generation times of 2-3 days have reported by other authors (Sylvester *et al.*, 2009). Williams *et al.* (1961) reported that dimensions for *Ophryoscolex caudatus* were approximately 200 by 80 µm, depending on the length of time after cell division. Both the long generation time and the large

Table 3. Entodiniinae protozoa numbers (x 10^3/ml) in Rusitec fermenters fed diets with forage: concentrate (F:C) ratios of 70:30 (HF) or 30:70 (HC) and alfalfa hay (A) or grass hay (G) as forage (FOR) and inoculated on day 0 with buffered rumen fluid from sheep (n = 4)

Day	Diet				SEM†	Statistical effect (*P* =)					
	HFA	HFG	HCA	HCG		F:C	FOR	Time	F:C x FOR	F:C x Time	FOR x Time
0	387[a]	326[a]	744[a]	534[a]	2.884	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
2	61.8[b]	102[b]	66.9[b]	86.9[b]							
4	28.5[cd]	56.1[c]	33.8[c]	38.5[c]							
6	20.9[de]	33.2[d]	18.2[d]	23.2[d]							
8	16.4[e]	25.0[e]	12.7[de]	11.1[e]							
10	13.0[e]	15.3[f]	6.73[ef]	9.73[e]							
12	7.47[f]	8.80[f]	2.91[f]	9.15[e]							
14	5.79[f]	8.43[f]	3.30[f]	6.76[e]							

[a, b, c, d, e, f] Mean values within a column with unlike superscripts differ (P<0.05).

† Standard error of the mean.

Table 4. Diplodiniinae protozoa numbers (x 10^3/ml) in Rusitec fermenters fed diets with forage: concentrate (F:C) ratios of 70:30 (HF) or 30:70 (HC) and alfalfa hay (A) or grass hay (G) as forage (FOR) and inoculated on day 0 with buffered rumen fluid from sheep (n = 4)

Day	Diet				SEM†	Statistical effect (*P* =)					
	HFA	HFG	HCA	HCG		F:C	FOR	Time	F:C x FOR	F:C x Time	FOR x Time
0	21.2[a]	18.9[a]	41.7[a]	26.3[a]	0.17	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
2	2.20[b]	2.69[b]	1.20[b]	3.24[b]							
4	1.33[c]	1.77[c]	0.91[b]	1.58[c]							
6	1.20[c]	1.26[d]	0.20[c]	0.80[d]							
8	0.65[d]	0.65[e]	0.14[c]	0.42[de]							
10	0.39[de]	0.27[ef]	0.04[c]	0.00[e]							
12	0.18[de]	0.10[f]	0.00[c]	0.00[e]							
14	0.15[e]	0.04[f]	0.00[c]	0.00[e]							

[a, b, c, d, e] Mean values within a column with unlike superscripts differ (P<0.05).

† Standard error of the mean.

size can help to explain the disappearance of Ophryoscolecinae protozoa from all fermenters in our study. Solid retention time in our study was shorter than the generation time, and the pore size of nylon bags (100 µm) probably limited the entrance of Ophryoscolecinae into the bags and their attachment to digesta. In agreement with this hypothesis, Meyer and Mackie (1986) reported that some of the large protozoa, such as *Polyplastron* spp. and *Ophryoscolex* spp., were unable to enter into bags less than 53 µm of pore size.

IV – Conclusions

The results indicate that under the conditions of the present study, it was not possible to maintain protozoa numbers similar to those in the rumen, but protozoa were present after 14 of incubation in all fermenters. Only Entodiniinae were able to grow in HC-fed fermenters by the end of the trial, but Entodiniinae, Isotrichidae and Diplodiniinae were maintained in fermenters receiving

HF diets. In general, greater protozoa numbers were observed in the fermenters fed diets containing A compared with those fed G diets, and shifts in protozoa populations in response to changes in F:C ratio were similar to those observed *in vivo*.

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