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Abstract. The aim of this study was to investigate the evolution of protozoa numbers in two types of rumen-simulating fermenters [single-flow continuous culture fermenters (SFCCF) and semi-continuous flow fermenters (Rusitec)] receiving high-forage diets. Ruminal inoculum from sheep and goat were used in SFCCF trials, whereas only sheep inoculum was used for Rusitec fermenters. In both systems incubation trials were conducted for 14 days by following the methodologies usually utilized in each of the participant laboratories. The flow through all fermenters was maintained by continuous infusion of artificial saliva at a dilution rate of 5.3%/h (1.3 per day). Retention time of solid digesta was 19 and 48 h in SFCCF and Rusitec fermenters, respectively. Protozoa numbers decreased ($P < 0.001$) rapidly with time from the first day after inoculation in all fermenters. The decrease was more pronounced in SFCCF than in Rusitec over the first 2 days post-inoculation, with values dropping to 17.8 and 33.0% of those on day 0 in SFCCF and Rusitec, respectively. In contrast, on day 4 SFCCF maintained greater protozoa numbers than Rusitec fermenters (8.75×10^4 and 4.1×10^4 per ml, respectively). Protozoa completely disappeared after 7 days in SFCCF, whereas they were present in Rusitec fermenters even 14 days after inoculation (1.0×10^4 per ml). Holotrich protozoa disappeared completely in SFCCF by the second day after inoculation, but they were maintained in Rusitec fermenters for 12 days. The better ability of Rusitec fermenters than that of SFCCF to maintain protozoa might be related to the greater retention time of solid digesta in the Rusitec system.

Keywords. Single-flow continuous culture fermenters – Rusitec – Protozoa.

Evolution du nombre de protozoaires dans les fermenteurs à simple flux continu ou à flux semi-continu (Rusitec)

Résumé. *L'évolution du nombre de protozoaires dans deux types de fermenteurs de simulation du rumen [fermenteurs à simple flux continu (SFCCF) et à flux semi-continu (Rusitec)], nourris avec des régimes riches en fourrage, a été étudiée. Du jus de rumen de moutons et de chèvres a été utilisé dans SFCCF, et seulement celui des moutons dans les fermenteurs Rusitec. Dans les deux systèmes les essais se sont déroulés pendant 14 jours. Le flux dans les fermenteurs a été maintenu par infusion continue de salive artificielle à un taux de dilution de 5,3%/h (1,3 par jour). Le temps de rétention du solide était de 19 et 48 h dans les SFCCF et Rusitec, respectivement. Le nombre de protozoaires a diminué ($P < 0,001$) rapidement avec le temps depuis le premier jour d'incubation dans les deux types de fermenteurs. La diminution a été plus prononcée dans les SFCCF que dans les Rusitec après les 2 premiers jours d'incubation en chutant jusqu'à 17,8 et 33,0% des valeurs du jour 0 dans SFCCF et Rusitec, respectivement. Au contraire, au quatrième jour, dans SFCCF a été maintenu un plus grand nombre de protozoaires que dans les Rusitec ($8,75 \times 10^4$ et $4,1 \times 10^4$ par ml, respectivement). Les protozoaires ont complètement disparu après 7 jours dans SFCCF, tandis qu'ils étaient présents dans les Rusitec même pendant le jour 14 après l'inoculation ($1,0 \times 10^4$ par ml). Les Holotriches ont disparu complètement dans le SFCCF après 2 jours d'incubation, mais ils ont été maintenus dans le Rusitec même pendant 12 jours. La meilleure capacité des fermenteurs Rusitec à maintenir les protozoaires par rapport aux SFCCF pourrait être liée à une rétention de la phase solide plus longue dans le Rusitec.*

Mots-clés. *Fermenteurs à simple flux continu – Rusitec – Protozoaires.*

I – Introduction

The rumen is a complex ecosystem comprising a wide diversity of bacterial, protozoal and fungal species that are able to breakdown plant material. Most research on ruminal fermentation has been carried out with fistulated animals, although these studies are laborious and expensive. In addition, and given the rumen complexity, it is difficult to conduct *in vivo* studies under controlled and well-defined conditions. These problems, together with the increased public awareness of the animal rights and the need for decreasing the number of fistulated animals used for experimental purposes, have contributed to the development of *in vitro* devices to simulate ruminal fermentation. Several types of rumen-simulating apparatus have been described in the literature; two of the most widely used are the continuous (Hoover *et al.*, 1976; Miettinen and Setälä, 1989) and the semi-continuous (Czerkawski and Breckenridge, 1977) flow fermenters. Conditions in fermenters have been shown to affect negatively some microbial populations. One of the most frequently reported difference to the rumen environment is the drastic decline in protozoa numbers from fermenters over the incubation period (Hoover *et al.*, 1976; Mansfield *et al.*, 1995). This has been ascribed to the exposition of fermenters contents to atmospheric oxygen (Hillman *et al*., 1991), and also to wash out of slow-growing protozoa from the fermenters (Czerkawski and Breckenridge, 1977; Mansfield *et al.*, 1995). The purpose of this work was to determine the evolution of rumen protozoa with time in single-flow continuous culture (SFCCF) and Rusitec fermenters when both were fed high-forage diets.

II – Materials and methods

1. Apparatus, diet and experimental procedure

A. Single-flow continuous culture fermenters (SFCCF) trial

A 14-day incubation trial was conducted using six SFCCF (Miettinen and Setälä, 1989) with an effective volume of 750 ml each. Three fermenters were inoculated with ruminal liquor from three rumen-fistulated Segureña sheep, and the other three with ruminal liquor from three rumen-fistulated Granadina goats. All animals were fed a diet composed of alfalfa hay (93%) and a commercial mineral-vitamin mixture (7%). Diet was offered at maintenance level in two equal portions at 09:00 and 16:00 h. Neutral-detergent fibre (NDF), acid-detergent fibre (ADF) and crude protein (CP) content of diet was 390, 246 and 183 g/kg dry matter (DM), respectively. Ruminal contents from each animal were collected two hours after the morning feeding, pooled by animal species, and strained through two layers of cheesecloth. The fluid was transferred to the fermenters (700 ml per fermenter) within 30 minutes after ruminal contents collection. The flow through fermenters was maintained by continuous infusion of McDougall (1948) artificial saliva (pH = 8.3) at a rate of 960 ml/d (dilution rate of 5.3%/h). Anaerobic conditions were maintained by continuous infusion of CO_2 and fermenters were kept in a bath at 39ºC. Each fermenter received daily 50 g DM of the same diet fed to donor animals, administered in two equal portions at 09:00 and 16:00 h. The general incubation procedure was as described by Martín García *et al.* (2006).

B. Rusitec fermenters trial

A 14-day incubation trial was carried out with three Rusitec fermenters (Czerkawski and Breckenridge, 1977) with an effective volume of 550 ml each. Each fermenter was inoculated with 300 ml of rumen liquor, 200 ml of artificial saliva (McDougall, 1948) and 80 g of solid rumen contents. Ruminal contents were obtained from three rumen-fistulated Merino sheep fed daily 1 kg DM of a diet consisting of 41% grass silage, 34% corn silage and 25% concentrate (DM basis). NDF, ADF and CP content of diet was 401, 220 and 155 g/kg DM, respectively. Ruminal contents from each sheep were collected two hours after the morning feeding, mixed, strained through two layers of cheesecloth, and transferred to the fermenters within 30 minutes after collection. The flow through fermenters was maintained by continuous infusion of a modified McDougall (1948) artificial

saliva (pH = 7.0) at a rate of 700 ml/d (dilution rate of 5.3%/h). Fermenters were kept in a bath at 39°C. Each fermenter received daily 10 g DM of the same diet fed to donor animals contained into nylon bags of 100 µm of pore size. Once every day (09:00 h) a bag that had spent 2 days in the fermenters was removed and a new bag was introduced. The general incubation procedure was as described by Carro *et al.* (1992).

2. Sampling, analytical procedures and statistical analyses

In both trials, fermenters' fluid was sampled every day before the morning feeding and the pH was immediately measured. For protozoa counting, 5 ml of fluid were added to 5 ml of 50% formalin solution (18.5% formaldehyde), mixed, and stored at 4°C (Dehority, 1984). Protozoa were counted from 20 microscopic fields in a Neubauer counting-chamber. Entodiniomorphida and Holotrichia were separately recorded. DM, NDF, ADF and CP content of feeds were determined as described by Yáñez Ruiz *et al.* (2004). Data from each trial were analysed independently according to a one-way ANOVA using the GLM procedure of SAS (SAS Inst., Inc., Cary, NC). Effects included in the model were fermenter and time. When the effect of time was significant ($P < 0.05$), differences between means were assessed by LSD test.

III – Results and discussion

Protozoa numbers in the ruminal fluid used to inoculate the fermenters were in the range of those reported previously in the rumen of sheep and goat fed high-forage diets (Santra *et al.*, 1998; Yáñez Ruiz *et al.*, 2004). As shown in Table 1, protozoa numbers declined ($P < 0.001$) rapidly from the first day after inoculation in all fermenters. The decrease was more pronounced in SFCCF than in Rusitec over the first 2 days post-inoculation, with values dropping to 17.8 and 33.0% of those on day 0 in SFCCF and Rusitec, respectively. In contrast, on day 4 SFCCF maintained greater protozoa numbers than Rusitec (8.75×10^4 and 4.1×10^4 per ml, respectively). Whereas protozoa completely disappeared after 7 days in SFCCF, they were present in Rusitec 14 days after inoculation (1.0×10^4 per ml). A drastic decrease of protozoa numbers in fermenters has been identified as the most obvious difference in microbial ecology compared to *in vivo* rumen (Crawford *et al.*, 1980; Mansfield *et al.*, 1995), which has been attributed to factors such as the washing out of slow-growing protozoa from fermenters and the exposure of fermenters contents to atmospheric oxygen (Abe and Iriki, 1978; Hillman *et al.*, 1991; Mansfield *et al.*, 1995).

Dilution rates in the rumen are usually over 1.0 per day, but when similar or greater dilution rates are used in continuous culture fermenters protozoa numbers decrease markedly, since their generation time becomes greater than the residence one (Williams, 1986). In the present trials, liquid dilution rates were 1.3 per day in both types of fermenters, which would explain the pronounced decrease of protozoa during the first days after incubation. Abe and Kumeno (1973) were unable to maintain protozoa in fermenters with dilution rates of 1.2 to 1.5 per day. In a later study, Abe and Iriki (1978) found that protozoa numbers could be maintained in continuous-flow fermenters equipped with a dialysis system and at a low dilution rate of 0.48 per day. In agreement with these results, Czerkawski and Breckenridge (1977) also reported a decrease in protozoa numbers from 12.6×10^4 to 3.4×10^4 per ml by increasing dilution rate from 0.33 to 0.96 per day. These findings suggest that high dilution rate would exceed the growth rate of the protozoa, resulting in washing out of protozoa from the fermenters. However, low dilution rates in fermenters might lead to an accumulation of end-products toxic to microbial populations.

Although in the present study liquid dilution rates were similar in both types of fermenters, mean retention times of solid digesta were 2.5 times greater in Rusitec than in SFCCF (19 and 48 h, respectively), which would help to explain the presence of protozoa in Rusitec fermenters for 14 days and their disappearance after 7 days in SFCCF. In agreement with this hypothesis, Crawford *et al.* (1980) reported that dual-flow continuous culture fermenters with a solid digesta retention time of 29.7 h maintained relatively stable protozoa numbers (1.0×10^4 per ml) after 8 days of

incubation, but the reduction of solid digesta retention time to 22.0 and 14.3 h produced a decline of protozoa numbers to 1.0×10^3 and 1.2×10^2 per ml, respectively. In the present study, protozoa numbers in Rusitec fermenters were similar to those found in previous studies conducted under similar conditions (Czerkawski and Breckenridge, 1977; Carro *et al.*, 1992). A complete disappearance of protozoa after 7 days of incubation has been reported previously (Hoover *et al.*, 1976; Bas *et al.*, 1990) in continuous culture fermenters using a similar dilution rate to that in the present trial with SFCCF.

Table 1. Effects of incubation day on protozoa numbers and proportion of holotrichs in single-flow continuous (SFCCF) and semicontinuous (Rusitec) culture fermenters fed high-forage diets (n = 3)

Day after inoculation	SFCCF sheep†		SFCCF goats††		Rusitec†††	
	Total ($\times 10^4$/ml)	Holotrichs (% of total)	Total ($\times 10^4$/ml)	Holotrichs (% of total)	Total ($\times 10^4$/ml)	Holotrichs (% of total)
0	82.0^{a}	4.88^{a}	93.0^{a}	4.30^{a}	70.0^{a}	6.60^{a}
1	35.7^{b}	0.00^{b}	27.0^{b}	1.33^{b}	59.0^{b}	2.66^{bc}
2	15.5^{c}	0.00^{b}	15.5^{c}	0.00^{b}	23.1^{c}	2.99^{abc}
3	15.5^{c}	0.00^{b}	14.0^{c}	0.00^{b}	7.21^{d}	3.99^{ab}
4	7.50^{d}	0.00^{b}	10.0^{d}	0.00^{b}	4.09^{e}	4.25^{ab}
5	2.50^{de}	0.00^{b}	3.50^{e}	0.00^{b}	3.71^{ef}	4.17^{ab}
6	2.00^{e}	0.00^{b}	1.50^{f}	0.00^{b}	2.37^{efg}	4.21^{ab}
7	0.00^{e}	0.00^{b}	0.00^{g}	0.00^{b}	2.31^{efg}	3.57^{abc}
8	0.00^{e}	0.00^{b}	0.00^{g}	0.00^{b}	2.01^{fg}	3.54^{abc}
9	0.00^{e}	0.00^{b}	0.00^{g}	0.00^{b}	2.09^{fg}	3.59^{abc}
10	0.00^{e}	0.00^{b}	0.00^{g}	0.00^{b}	1.51^{g}	3.49^{abc}
11	0.00^{e}	0.00^{b}	0.00^{g}	0.00^{b}	1.42^{g}	2.77^{bc}
12	0.00^{e}	0.00^{b}	0.00^{g}	0.00^{b}	1.37^{g}	1.58^{bc}
13	0.00^{e}	0.00^{b}	0.00^{g}	0.00^{b}	1.15^{g}	0.00^{c}
14	0.00^{e}	0.00^{b}	0.00^{g}	0.00^{b}	1.00^{g}	0.00^{c}
SEM††††	1.734	0.014	0.517	0.576	0.679	1.285
P value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.108

†SFCCF were inoculated with rumen fluid from sheep, and fed daily 50 g DM of a 93:7 alfalfa hay:mineral-vitamine mixture diet.
††SFCCF were inoculated with rumen fluid from goats, and fed daily 50 g DM of a 93:7 alfalfa hay:mineral-vitamine mixture diet.
†††Rusitec fermenters were inoculated with rumen fluid from sheep, and fed daily 10 g DM of a 75:25 silage:concentrate diet.
††††Standard error of the mean.
^{a,b,c,d,e,f,g}Mean values within a column with different superscripts differ ($P < 0.05$).

Differences between the two types of fermenters were also observed in the percentage of Holotricha, which disappeared completely in SFCCF by the second day after inoculation but were maintained in Rusitec fermenters for 12 days. Holotricha are especially vulnerable because of their relatively long generation time (Williams, 1986), which exceed the retention time of solid digesta in SFCCF; in addition, the homogeneous conditions of these fermenters did not facilitate their sequestration within the solid phase (Mansfield *et al.*, 1995). It is known that ruminal protozoa are able to maintain themselves in the rumen because they are attached to particulate matter and the rumen wall, which prevented their passage at the more rapid fluid turnover rates (Dehority, 1998).

Eadie (1962) found that *Entodinum* became established in the rumen of lambs and calves at pH above 6.0, but Holotricha did not develop with pH below 6.5. Values of pH in Rusitec fermenters (measured before feeding) ranged from 6.10 to 6.54 (mean value 6.29 ± 0.019), and could have contributed to the disappearance of Holotrichia protozoa by the end of the trial. In agreement with

this hypothesis, Carro *et al.* (1995) found that Holotrichia represented 1.35% of total protozoa in Rusitec fermenters fed a high-forage diet at pH values of 6.36, but they completely disappeared when pH dropped to 6.17. Carro *et al.* (1992) found that Holotrichia were 12.2% of total protozoa in Rusitec fermenters fed the same diet and maintained at pH = 6.86. Mean values of pH in SFCCF were 6.33 ± 0.088 and 6.35 ± 0.080 for trials with sheep and goat inoculum, respectively.

IV – Conclusions

The results obtained indicate that under the conditions of the present trials, it was not possible to maintain protozoa numbers similar to those in the rumen in any type of fermenter. However, protozoa were present in Rusitec fermenters on day 14 of incubation, whereas they completely disappeared by day 7 in SFCCF. Holotrichia protozoa disappeared in SFCCF early after incubation whereas in Rusitec they disappeared only by the end of the incubation period. A greater stratification and a lower dilution rate might improve protozoa maintenance in SFCCF, and higher values of pH in Rusitec fermenters might help to maintain Holotrichia protozoa.

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