Analysis of microbial communities in Rusitec and single-flow continuous culture fermenters by PCR-SSCP: effects of basal diet

Ranilla M.J., Martín García A.I., Molina Alcaide E., Carro M.D.

in

Papachristou T.G. (ed.), Parissi Z.M. (ed.), Ben Salem H. (ed.), Morand-Fehr P. (ed.). Nutritional and foraging ecology of sheep and goats

Zaragoza : CIHEAM / FAO / NAGREF
Options Méditerranéennes : Série A. Séminaires Méditerranéens; n. 85

2009
pages 239-243

Analysis of microbial communities in Rusitec and single-flow continuous culture fermenters by PCR-SSCP: Effects of basal diet

M.J. Ranilla*, A.I. Martín García, E. Molina Alcaide** and M.D. Carro***

*Departamento de Producción Animal, Facultad de Veterinaria, Universidad de León, Campus de Vegazana s/n, 24071 León (Spain)
**Estación Experimental del Zaidín, CSIC, Profesor Albareda 1, 18008 Granada (Spain)

Abstract. The aim of this work was to analyse microbial communities in two artificial rumen systems, Rusitec and single-flow continuous culture fermenters (SFCCF), fed two diets containing alfalfa hay and concentrate in the proportions of 80:20 and 20:80. Eight fermenters of each type were run for 14 days. On days 13 and 14, total effluent was collected and homogenized in a blender at low speed for 1 min and lyophilized. In order to study microbial diversity, DNA was isolated from samples (120 mg) of lyophilized effluent and the V3-4 region of the 16S rDNA gene was amplified by PCR and analysed by using single-strand-conformation polymorphisms (SSCP). Distinct clusters were observed for Rusitec and SFCCF samples. Microbial diversity, assessed using Shannon's index (H') was higher ($P < 0.01$) in SFCCF than in Rusitec. Within systems, samples were grouped together according to diet, but H' was not affected ($P > 0.05$) by diet or system. Similarity between fermenters in PCR-SSCP banding patterns was higher in the Rusitec than in SFCCF for both diets. Under the conditions of the present experiment, microbial populations were affected by diet in both systems, and microbial diversity was different in both types of fermenters.

Keywords. Rumen microbial communities – PCR-SSCP – *In vitro* – Diet.

Étude des populations microbiennes dans le Rusitec et dans un système de culture à flux simple continu par PCR-SSCP : Effets de la ration de base

Résumé. *Les populations microbiennes ont été étudiées dans deux systèmes de rumen artificiel, le Rusitec et des fermenteurs à flux simple (SFCCF), qui ont reçu deux rations à base de foin de luzerne et de concentré dans des proportions de 80:20 et de 20:80. Huit fermenteurs de chaque type ont été utilisés pendant 14 jours. Aux jours 13 et 14, l'effluent total a été rassemblé et homogénéisé dans un mélangeur à vitesse réduite pendant une minute et lyophilisé. Afin d'étudier la diversité microbienne, l'ADN a été isolé dans les échantillons (120 mg) d'effluent lyophilisé, et la région V3-4 du gène du rDNA 16S a été amplifiée par PCR et analysée en employant les polymorphismes de la conformation des simples brins (SSCP). On a observé deux clusters distincts pour des échantillons du Rusitec et des SFCCF. La diversité microbienne, évaluée en utilisant l'index de Shannon (H') a été plus élevée ($P < 0,01$) dans le SFCCF que dans le Rusitec. Les échantillons ont été groupés par régime dans chaque système, mais l'index H' n'a pas été affecté ($P > 0,05$) par le régime ni par le système* in vitro*. La similitude entre les fermenteurs dans les profils de bandes de PCR-SSCP a été plus élevée dans le Rusitec que dans les SFCCF pour les deux régimes. Dans les conditions de cet essai, les communautés microbiennes ont été affectées par le régime dans les deux systèmes, et la diversité microbienne a été différente dans les deux types de fermenteurs.*

Mots-clés. *Communautés microbiennes ruminales – PCR-SSCP* – In vitro – *Régime.*

I – Introduction

The rumen is a complex ecosystem that is difficult to study. Although most research on rumen fermentation has been carried out with fistulated animals, these studies are expensive and laborious, and conditions are difficult to control. In recent years there has been an increased interest in the development of *in vitro* technologies for simulating rumen fermentation. Two of the

most widely used types of artificial rumen apparatus are the continuous-flow fermenters (Miettinen and Setälä, 1989) and the semi-continuous flow Rusitec system (Czerkawski and Breckenridge, 1977). It is assumed that fermenters are able to maintain a functional microbial community structure similar to the rumen, and that these model rumen systems may serve as a suitable tool for studying aspects of ruminal microbial ecology and may resolve some of the relationships between microbial community structure and function by providing control of experimental conditions. However, little is known about the microbial communities established in these artificial rumen systems, and microbial communities in both types of fermenters have not been compared previously. The aim of this work was to study rumen microbial communities in single-flow continuous culture fermenters (SFCCF) and Rusitec fermenters fed two diets differing in their forage:concentrate ratio, as this is one of the main factors affecting ruminal microbial populations.

II – Materials and methods

The dietary treatments consisted of two complete diets, composed of chopped alfalfa hay and concentrate in the proportions (g/100 g; fresh matter basis) of 80:20 (F80) and 20:80 (F20). Concentrate was based on cracked barley grains, cracked corn grains and soyabean meal. Sugar beet molasses and a mineral-vitamin mixture were added to each diet at rates of 3.5 and 3.0 g per 100 g of DM, respectively. Alfalfa hay and concentrate were weighed separately and mixed before being fed to the fermenters.

One 14-day trial was carried out using a Rusitec unit consisting of eight fermenters, following the general incubation procedure as described by Czerkawski and Breckenridge (1977). Fermenters were inoculated with solid and liquid inocula from four rumen-cannulated sheep. Two sheep were fed diet F80 and the other two received diet F20 for 15 days before commencing the *in vitro* trial immediately before feeding in the morning and transferred to the *in vitro* system within 30 min. Each fermenter (4 per diet) received 15.6 g DM/day of the corresponding diet fed into nylon bags (100-µm pore size) at 09:00 h. A continuous infusion of artificial saliva (McDougall, 1948; pH = 8.4) at a rate of 600 ml/day was maintained throughout the experiment in the fermenters receiving F80 diet. The composition of the artificial saliva was modified (pH = 7.0) for the fermenters fed F20 diet in order to achieve a pH value similar to that found prior to feeding in the rumen of sheep fed this diet. Liquid effluent was collected daily in flasks containing a solution of H_2SO_4 (20%; vol/vol) to maintain pH values below 2. On days 9, 10 and 11, the pH of fluid from the fermenters was determined immediately before feeding, and samples were collected for VFA and ammonia-N determination. In addition, one nylon bag from each fermenter was collected daily to determine disappearance of DM and NDF after 48 h of incubation. On days 13 and 14, liquid effluent was mixed with the solid residue from the nylon bags incubated for 48 h. About 300 ml of the mix were frozen and lyophilized for PCR-SSCP analysis. The rest of the mix was used for estimating daily microbial N synthesis.

Another 14-day incubation trial was carried out using eight SFCCF (Miettinen and Setälä, 1989) with an effective volume of 750 ml each. Fermenters were inoculated with rumen content collected from four rumen-cannulated sheep following the experimental procedure previously described for Rusitec fermenters. Each fermenter (4 per diet) received 27 g DM/day of the corresponding diet in two equal portions at 08:00 and 20:00 h. Fermenters were continuously under CO_2 flux to maintain anaerobic conditions, and the overflow from each fermenter was collected into a flask maintained at 4°C by a cold-water bath to impede microbial growth. Flow through fermenters was maintained by continuous infusion of artificial saliva at a rate of 900 ml/d. Composition of both types of artificial saliva were the same as those described for the Rusitec trial. On days 9, 10 and 11, the pH of the fluid from the fermenters was determined immediately before the morning feeding, and aliquots for VFA and ammonia-N analyses were collected as previously described. On days 13 and 14, the effluent was collected and homogenized in a blender at low speed for 1 min. One sample (about 300 ml) was frozen and lyophilized for PCR-SSCP analysis, and the rest was used for estimating daily microbial N synthesis. Results on ruminal fermentation variables have been published elsewhere (Carro *et al.*, 2007).

In order to study microbial diversity, DNA was isolated from samples (120 mg) of lyophilized effluent following the procedure described by Yu and Morrison (2004). To analyze microbial communities, a fragment of 16S rDNA gene was amplified from DNA extracts by PCR using primers specific to the V3-4 region, B342If and U806Ir (Hori *et al.*, 2006). Primer U806Ir contained a 5'-terminal phosphate group. Each PCR was performed in a volume of 100 µl. Thermocycling, which was conducted in a Applied Biosystem 2720 Thermal Cycler, started with a initial denaturation of 5 min at 94°C; a total of 35 cycles, each including 60 s at 94°C, 60 s at 50°C, and 90 s at 72°C, was followed by a final primer extension step of 4 min at 72°C. The purity of PCR products was analysed after agarose electrophoresis (1.5% agarose gel). PCR products were purified using a Qiaquick PCR Purification Kit (Qiagen, Germany) according to the manufacturer's protocol. Eluted DNA was quantified fluorimetrically using a Nanodrop ND-1000 (NanoDrop Technologies, Delaware, USA). Single-stranded DNA was obtained from the PCR products removing the 5'-phosphorilated strand by λ-exonuclease digestion. Up to 1 µg DNA was digested for 90 min at 37°C in a total volume of 40 µl, with 2.5 U λ-exonuclease in 1X exonuclease buffer (New England Biolabs, Beverly, USA). The digestion was stopped with the first step of the purification with spin columns of the MiniElute DNA cleanup system (Qiagen, Germany). SSCP was performed in a 0.625% MDE gel in 1 × TBE buffer using a DCode system (Biorad, USA). Before loading, samples were denatured in loading buffer (10 mN NaOH, 0.25% xylene cyanol, 0.25% bromophenol blue and 95% formamide) at 95°C for 3 min and subsequently cooled on ice for 3 min. Electrophoresis was performed at 20°C at 500 V for 20 h. The SSCP profiles were visualized by silver staining according to the manufacturer's protocol (Amersham Biosciences, Sweden). Gel images were recorded using a GS-800 Calibrated Imaging Densitometer (Biorad). Comparative analyses of SSCP banding patterns were achieved using Quantity One® software (Biorad). Cluster analysis based on similarity matrices was done by UPGMA (un-weighted pair group method with arithmetic averages). Analysis of PCR-SSCP banding patterns using Shannon's index (*H'*) was performed to measure the richness and evenness of rumen microbial communities.

III – Results and discussion

The SSCP patterns derived from SFCCF and Rusitec for F80 and F20 diets are presented in Fig. 1. The community profiles obtained include up to 50 sharp bands, an indication of community complexity. Microbial diversity, assessed using Shannon's index (*H'*), was higher in SFCCF than in Rusitec ($P < 0.01$) (3.54 *vs* 3.43, respectively).

The dendrogram based on the matrix of similarity for SFCCF and Rusitec fermenters fed both experimental diets is presented in Fig. 2. Two distinct clusters were observed for Rusitec and SFCCF samples, sharing 27% similarity. These results demonstrate a difference in bacterial community composition between the two *in vitro* systems, which could be related both to the differences between original ruminal inocula used in each type of fermenter and to the type of fermenter itself. Some ruminal parameters differ between fermenters, reflecting the different conditions achieved after adaptation in each system.

Within systems, samples grouped together according to diet, indicating that this is one of the major factors affecting rumen environment and, for instance, ruminal microbial communities. In the SFCCF, similarity between F20 and F80 communities was 38%, whereas in the Rusitec the similarity between the diets reached 65%. As suggested by some ruminal parameters (i.e. pH and VFA), the ability of both systems to simulate rumen fermentation is different and it depends on the basal diet. It has been suggested that artificial rumen systems may perform worst with high concentrate diets, and this idea is supported by the higher coefficients of variation seen in the present experiment for diet F20 compared to F80 (see Table 1). Furthermore, the higher pH seen in the Rusitec experiment, even with the C80 diet, would help to explain the smaller variation in microbial populations between diets compared to the SFCCF. It is also noticeable that a longer retention time of the solid phase in the Rusitec system than in SFCCF (48 and 22 h, respectively) can affect the microbial populations that develop in both types of fermenters. Digesta for community

analysis from the Rusitec samples was obtained by mixing liquid effluent with solid residue after 48 h, with many adherent bacteria, whereas in SFCCF total effluent was used; this could help to explain the lower microbial diversity in the Rusitec system and the higher similarity in the microbiota between diets.

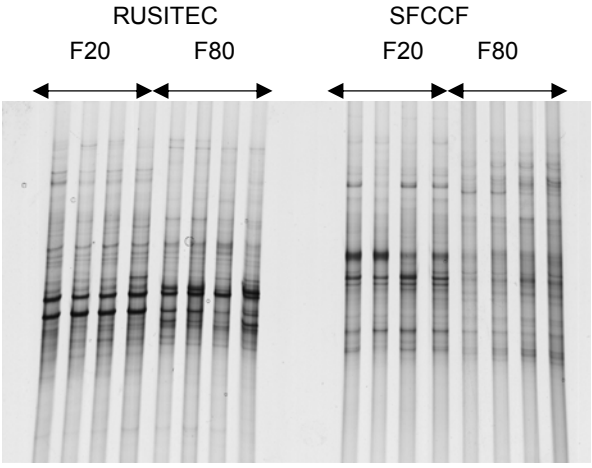

Fig. 1. PCR-SSCP profiles of microbial communities in Rusitec and SFCCF for diets F20 and F80.

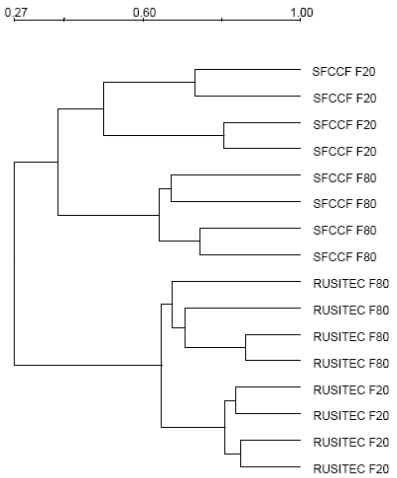

Fig. 2. Similarity index of SSCP profiles obtained from SFCCF and Rusitec fermenters fed diets F20 and F80.

Table 1. Values of coefficient of variation (%) for different fermentation variables measured in Rusitec fermenters and single-flow continuous culture fermenters (SFCCF) fed two diets containing alfalfa hay and concentrate in the proportions of 80:20 (F80) and 20:80 (F20)

Item	Rusitec		SFCCF	
	F80	F20	F80	F20
pH before feeding	0.419	2.62	0.430	3.48
pH at 2 h after feeding	0.245	1.76	0.325	3.30
pH (0-12 h)†	0.277	1.54	0.443	2.74
Total volatile fatty acid (mmol/l)††	1.36	5.48	3.63	5.26
Individual (mol/100 mol)				
Acetate	0.532	1.84	2.17	1.80
Propionate	2.12	4.11	3.81	4.95
Butyrate	1.78	2.86	1.44	1.08
Acetate:Propionate	2.51	4.38	5.87	6.99
Ammonia N (mg/l)	3.91	4.82	2.23	11.1
True dry matter digestibility	1.71	1.64	1.27	3.66
Microbial N (g/d)	2.61	2.72	0.774	2.53

†Values averaged over 12 h sampling period.
††Values averaged over 24 h sampling period for sheep rumen and effluents from fermenters.

In spite of diet clustering, Shannon's index (*H'*) was not affected (P > 0.05) by diet in the SFCCF

(3.25 and 3.30 for F20 and F80 diets, respectively) and in the Rusitec fermenters (3.31 and 3.35 for F20 and F80, respectively). Although different in composition, F20 and F80 bacterial communities seem to have a similar degree of diversity, suggesting that none of the diets had detrimental effects on microbial populations when used in both *in vitro* systems.

Similarity in PCR-SSCP banding patterns between fermenters within the same system was different in the Rusitec than in SFCCF and it depended on the diet. For F20, similarity between the 4 Rusitec fermenters was 81%, in contrast to the 50% found in the SFCCF system. When the F80 diet was incubated, similarity between fermenters was similar in both fermenter systems (68% and 64%, respectively). Our results suggest that, when high concentrate diets are fed to both fermenter systems, the rumen environment is disturbed and parameters fluctuate more than when using high forage diets and this variability is also reflected in microbial communities. In support of these observations, values for coefficient of variation for the main fermentation variables measured in both systems were, in general, higher for F20 than for F80 in both types of fermenters.

IV – Conclusions

Under the conditions of the present experiment, PCR-SSCP analysis of microbial communities in SFCCF and Rusitec fermenter systems show different profiles in both systems, with higher microbial diversity in SFCCF compared to Rusitec. Diet composition (forage:concentrate ratio of 80:20 and 20:80) has a significant effect on the microbial populations that are developed and established in both types of fermenters; however, the inclusion of a high proportion of concentrate did not negatively affect microbial diversity.

Acknowledgements

The authors wish to acknowledge the financial support received from the CICYT (Projects AGL2004-04755-C02-01 and AGL2004-04755-C02-02).

References

Carro M.D., Ranilla M.J., Martín García A.I. and Molina Alcaide E., 2007. Comparison of microbial fermentation of high- and low-forage diets in sheep rumen and two types of fermenters. In: *Anim. Feed Sci. Technol.* (submitted).

Czerkawski J.W. and Breckenridge G., 1977. Design and development of a long-term rumen simulation technique (Rusitec). In: *Br. J. Nutr*., 38. p. 371-384.

Hori T., Haruta S., Ueno Y., Ishii M. and Igarashi Y., 2006. Direct comparison of single-strand conformation polymorphism (SSCP) and denaturing gradient gel electrophoresis (DGGE) to characterize a microbial community on the basis of 16rRNA gene fragments. In: *J. Microbiol. Methods*, 66. p. 165-169.

McDougall E.I., 1948. Studies on ruminant saliva. I. The composition and output of sheep's saliva. In: *Biochem. J.*, 70. p. 99-109.

Miettinen H. and Setälä J., 1989. Design and development of a continuous culture system to study rumen fermentation. In: *J. Agric. Sci. Finland*, 61. p. 463-473.

Yu Z. and Morrison M., 2004. Improved extraction of PCR-quality community DNA from digesta and fecal samples. In: *Bio. Techniques*, 36. p. 808-812.