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Cellulolytic activity in the rumen of lambs fed a high concentrate diet is not affected by the removal of protozoa

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Abstract. Removing protozoa from the rumen may increase animal productivity. However, it is also known that protozoa play an important role in fibre degradation in the rumen. The aim of this work was to study the cellulolytic activity and microbial numbers in the rumen of faunated and protozoa free animals fed a high concentrate diet. Ten crossbred lambs were used. They were taken from the ewes within the first 24 h after birth and kept isolated from adult animals and bottle fed during 6 weeks. Once weaned five of them were given orally 10 ml of rumen fluid collected from adult sheep rumen at the slaughter house (faunated group, F). The other five lambs were given 10 ml of rumen fluid that had been previously centrifuged at 1000 g for 10 min and then frozen and thawed (protozoa free group, PF). Both groups were fed a mix of concentrate supplement and grass hay (60:40) for 5 months. Methane emissions were measured individually over a 3 day period. After slaughter, samples from the rumen were collected to study cellulose degradation using the most-probable number procedure. The numbers of *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, *Ruminoccus albus* and total fungi were quantified by real time PCR using specific primers and DNA isolated from rumen samples (200 mg). PF lambs produced 26% less methane than F lambs. No differences in the cellulolytic numbers were observed between groups. Real time PCR showed no differences in *F. succinogenes* and other cellulolytic bacteria numbers between F and PF animals. Our results show that in animals fed high concentrate diets the removal of protozoa from the rumen does not seem to affect cellulolytic activity.

Keywords. Cellulolytic activity – Protozoa – Real time PCR – Rumen.

L'activité cellulolytique dans le rumen des agneaux recevant une ration riche en concentré n'est pas affectée par la défaunation

Résumé. *La disparition des protozoaires du rumen peut augmenter la productivité animale. Néanmoins, ces micro-organismes peuvent aussi jouer un rôle important dans la dégradation des composés cellulosiques. L'objectif de ce travail était d'étudier l'activité cellulolytique et la population bactérienne présente dans le rumen des animaux avec et sans protozoaires, nourris avec un régime riche en concentré. Dix moutons séparés de leurs mères dans les 24 h qui ont suivi leur naissance, ont été isolés des adultes et nourris par allaitement artificiel pendant 6 semaines. Après leur sevrage, 5 agneaux ont reçu 10 ml du jus de rumen de brebis adultes (groupe F) et les autres 5 agneaux ont reçu 10 ml de surnageant du même jus de rumen centrifugé á 1000 g pendant 10 min, congelé et décongelé (groupe PF). Tous les moutons étaient nourris avec un mélange concentré:foin (60:40) pendant 5 mois. La production de méthane était mesurée sur chaque mouton pendant 3 jours. Après abattage, des échantillons du contenu ruminal ont été utilisés pour étudier la dégradation de la cellulose selon la méthode du nombre le plus probable. La quantité de* Fibrobacter succinogenes *était déterminée par PCR en temps réel en utilisant des amorces spécifiques après extraction de l'ADN des échantillons (200 mg). Il n'y a pas eu de différences entre les deux groupes de moutons concernant le nombre total de bactéries cellulolytiques ainsi que de* F. succinogenes *et des autres bactéries cellulolytiques. Ces résultats montrent que chez les animaux nourris avec un régime riche en concentré l'élimination des protozoaires du rumen ne semble pas affecter l'activité cellulolytique dans le rumen.*

Mots-clés. *Activité cellulolytique – Protozoaires – PCR en temps réel – Rumen.*

I – Introduction

Protozoa represent up to half of the microbial biomass in the rumen. Different authors have shown

that exclusion of protozoa from the rumen has a beneficial effect on growth rate, wool growth and feed conversion efficiency in animals under certain feeding conditions (Jouany *et al.*, 1988). The presence of protozoa in the rumen reduces the overall efficiency of nitrogen metabolism. In addition, they play a key role in the interspecies hydrogen transfer and methane production in the rumen as there is a close association between protozoa and methanogens (Ushida and Jouany, 1996). The production of this gas represents a loss of energy (6 to 10% of the gross energy intake) and it is also detrimental to the environment as ruminants are the main agricultural source of this potent greenhouse gas (Johnson and Johnson, 1995). Elimination of protozoa has been shown to reduce CH_4 production by up to 50% depending on the diet (Hegarty, 1999); although, contradictory results have been reported when short and long term experiments are compared (Morgavi *et al.*, 2005). However, one of the potential disadvantages of eliminating protozoa from the rumen may be a reduction in fibre degradation; although this effect might depend on the type of diet the animal receives (Williams and Coleman, 1992). Investigations of the fibrolytic community in the rumen have been constrained by the limitations of culture techniques, which are time consuming and cumbersome. With the advancement of molecular quantification methods, in particular 16S/18S rRNA gene probing methods, researchers are now able to monitor bacterial and fungal species within the rumen (Denman and McSweeny, 2006). Therefore, as part of an ongoing project with the aim of studying the rumen microbial population involved in methane production, this experiment was undertaken to asses the cellulolytic activity and microbial numbers in the rumen of faunated and protozoa free animals fed a high concentrate diet.

II – Materials and methods

1. Animal, diets and experimental design

Ten crossbred lambs were used. They were taken from the ewes within the first 24 h after birth and kept isolated from adult animals and bottle fed for 6 weeks. Once weaned (19.1 ± 3.23 kg) five animals were given a 10 ml oral dose of rumen fluid collected from adult sheep at the slaughter house (faunated group, F). The other five lambs were given a 10 ml oral dose of rumen fluid that had been centrifuged at 1000 g for 10 min and then the supernatant frozen and thawed to ensure the absence of viable ciliates and the presence of the same bacterial population given to the F group (protozoa free group, PF). Thawed rumen fluid was examined by light microscopy to confirm the absence of viable protozoa due to the formation of crystals during freezing, which affects more protozoa than bacteria (Mazur, 1966). Both groups were kept isolated and fed a mix of concentrate supplement (Wynstay Lamb Finisher) and grass hay (60:40) for three months until they reached 40 kg live weight. At 40 kg they were acclimatised in square polycarbonate chambers (1.8 × 1.8 m) to measure methane emissions over a three day period. Two chambers were used and in each period one lamb from each experimental group was housed in one chamber. Calculation of CH_4 emissions was based on the respective concentration measurements associated with airflows into and out of each chamber, which were obtained every 40 minutes. Animals were fed 340 g of concentrate and 225 g of grass hay in two equal meals at 9:00 and 17:00 hours.

2. Samples collection and analyses

After methane measurements had been completed, animals were slaughtered and samples of rumen contents were taken; a sub sample of 10 g was immediately frozen and kept at -20°C until DNA extraction. The rest of the rumen contents was kept anaerobically at 39°C for bacterial enumeration and gas production measurements: total and cellulolytic bacteria were quantified by using the most-probable number procedure as described by Dehority *et al.* (1989). Gas production was measured over 24 h by incubating the rumen fluid in glass bottles containing 10 ml of rumen fluid, 20 ml of saline buffer and 300 mg of neutral detergent fibre obtained from grass hay.

Total DNA was extracted from freeze-dried rumen samples using a QIAamp® DNA Stool Mini Kit (Qiagen Ltd, Crawley, West Sussex, UK). Real-time PCR was used to quantify rDNA from total

bacteria (Maeda *et al.*, 2003), *Fibrobacter succinogenes*, *Ruminococcus albus*, *Ruminococcus flavefaciens* and total fungi (Denman and McSweeny, 2006). Real-time PCR was performed using a DNA Engine Opticon® System, PTC-200 DNA Engine™ Cycler (MJ research, Braintree, Essex, UK). Total bacteria rDNA was quantified using a standard curve generated from DNA extracted from a mix of 24 h cultures of rumen bacteria pure cultures (Yáñez-Ruiz *et al.*, 2006). The ΔCt method for relative template amplification was used to quantify the rest of the bacterial species relative to the total bacteria (Denman and McSweeny, 2006).

3. Statistical analyses

Data were subjected to ANOVA (Genstat 7; Lawes Agricultural Trust, Rothamsted Experimental Station, Harpenden, Herts, UK) with the absence/presence of protozoa as the sole treatment effect.

III – Results and discussion

The rumen of PF lambs remained free of protozoa during the experiment as neither protozoa nor protozoal DNA was found in their rumen at slaughter. Protozoa free lambs had lower daily methane emissions and methane produced per kg of dry mater intake than faunated lambs ($P < 0.05$). Overall PF lambs produced 26% less methane than F lambs (26.0 and 35.2 l/day by PF and F lambs, respectively).

Using the most-probable number method (Table 1), we observed a higher number of total bacteria in PF lambs compared to F lambs ($P < 0.05$). Most of the experiments carried out on rumen defaunation bacterial numbers increase as a result of the absence of protozoa in the rumen (Hsu *et al.*, 1991). This is in agreement with our findings in protozoa free and faunated lambs fed grass over a 5 months period (Yáñez-Ruiz *et al.*, 2007). In contrast, cellulolytic bacteria numbers did not differ between the experimental groups ($P > 0.05$). This is confirmed by the similar gas production recorded *in vitro* after 24 h incubation on neutral detergent fibre. The results obtained by different authors on the effect of defaunation on fibre degradation show little or no effect in the whole animal and a significant (20%) decrease in the rate of fibre breakdown in the rumen (Williams and Colleman, 1992). However, this effect seems to depend on the type of diet. In the present work, animals fed a diet high in concentrate did not show and difference in fibrolytic activities in the rumen.

Table 1. Bacterial counts (most probable number) and gas volume produced over 24 h of incubation

	PF	F	SEM†	P
Total bacteria, cells × 10^9	46.8	2.97	9.39	0.011
Cellulolytic bacteria, cells × 10^7	66.2	58.2	29.3	0.851
Gas production	9.46	9.74	0.165	0.250

†SEM values for n = 10.

Using real time PCR to quantify individual target species relative to total bacteria we observed no differences in the main fibrolytic bacterial species (Table 2; *F. succinogenes*, *R. flavefaciens* and *R. albus*). However, there was significantly more fungal rDNA in the rumen of lambs lacking of protozoa ($P < 0.01$). Recently, Zhang *et al.* (2007) showed a high synergy between bacteria and fungi in the degradation of cell walls in the rumen. The higher numbers of fungi observed in the rumen of PF lambs in comparison with F lambs could be explained by a compensatory mechanism driven by the microbial community in order to keep fibrolytic activity high.

Table 2. Relative abundance (ΔCt) of *F. succinogenes*, *R. flavefaciens*, *R. albus* and total fungi normalized to total bacteria

	PF	F	SEM†	P
F. succinogenes	0.116	0.123	0.019	0.803
R. flavefaciens	0.0095	0.0008	0.0042	0.178
R. albus	0.00069	0.00072	0.00003	0.61
Total fungi	0.00123	0.00048	0.00014	0.005

†SEM values for n = 10.

IV – Conclusions

Our results show that in lambs fed high concentrate diets, the removal of protozoa from the rumen decreases daily methane emission. However, it does not seem to affect cellulolytic activity. The numbers of the main bacterial species known to be involved in fibre degradation do not change in relation to total bacteria; however there may be a compensatory mechanism by which fungi might partially replace ciliates in the rumen.

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