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***In vitro* predation of pure bacterial species by rumen protozoa from monofaunated sheep, determined by qPCR**

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Abstract. Predation of bacteria has important implications on rumen microbial ecology and metabolism. The predation rate of bacteria by protozoa *in vitro* was studied by bacterial DNA quantification. *Entodinium caudatum* (EC), *Diplodinium dentatum* (DD) and *Metadinium medium* (MM) from monofaunated sheep rumen contents were incubated with two pure cultures of bacteria: the fibrolytic *Ruminococcus albus* (Ra) and the amylolytic *Streptococcus bovis* (Sb). Total bacterial and protozoal numbers were counted in the media by microscopy, and concentration of Ra, Sb and total bacterial DNA was measured in the protozoal fraction as an index of predation. No differences ($P>0.10$) were observed in the proportion of DNA from Ra in the different protozoal fractions, but MM predated a higher amount of total bacteria than EC ($P=0.045$). Proportion of Ra and Sb in the protozoal fraction agrees with that in the medium, precluding selective predation. Protozoal fraction from EC showed a higher ($P=0.018$) proportion of Sb DNA and a lower amount of total bacterial DNA than MM ($P<0.001$). Total DNA in MM cultures either per number of cells or per cell volume was highest, indicating a higher engulfment ability of this protozoal species. No differences were observed among incubation times in any studied parameter ($P>0.10$), suggesting that the predation rate of each protozoa was similar to the intracellular digestion rate.

Keywords. Rumen protozoa – Rumen bacteria – Predation – *In vitro*.

Prédation in vitro de souches bactériennes pures par les protozoaires du rumen chez les ovins monofaunés, déterminée par qPCR

Résumé. La prédation des bactéries par des protozoaires a des implications importantes sur l'écologie et le métabolisme du rumen. Dans ce travail le taux de prédation des bactéries a été étudié *in vitro* par PCR quantitative (qPCR). *Entodinium caudatum* (EC), *Diplodinium dentatum* (DD) et *Metadinium medium* (MM) ont été obtenus des moutons monofaunés et ont été incubés avec deux espèces bactériennes : *Ruminococcus albus* (Ra, fibrolytique) et *Streptococcus bovis* (Sb, amylolytique). Le dénombrement de bactéries totales et de protozoaires a été effectué dans le milieu par microscopie. La concentration de Ra, Sb et des bactéries totales dans la fraction contenant les protozoaires, mesurée par qPCR, a été utilisée comme indicateur de la prédation. La proportion d'ADN provenant de Ra était la même pour toutes les fractions de protozoaires, pourtant MM présentait une activité prédatrice des bactéries totales plus importante que celle de EC ($P=0,045$). Les proportions de Ra et Sb dans la fraction de protozoaires et dans le milieu étaient similaires, excluant ainsi la possibilité d'une prédation sélective. La fraction EC présentait une plus haute proportion d'ADN de Sb que celle de MM ($P=0,018$) avec une moindre quantité d'ADN bactérien total ($P<0,001$). MM avait un nombre supérieur de copies d'ADN de bactéries totales qu'EC et DD, suggérant une plus grande capacité à avaler des bactéries. En revanche, il n'y a pas eu des différences au niveau du temps d'incubation ($P>0,10$), suggérant que le taux de prédation est similaire au taux de digestion intracellulaire.

Mots-clés. Protozoaire – Bactérie – Predation – *In vitro* – Rumen.

I – Introduction

Predation of bacteria by protozoa is a common process in the rumen ecosystem that affects the balance of bacterial communities and that has important implications on the metabolism of the host animal. Predation, by its regulating activity on the bacterial nitrogen turnover in the rumen affects particularly the efficiency of nitrogen utilization by ruminants (Firkins *et al.*, 1992). Rumen protozoa ingest bacteria and use them as the most important single source of nitrogenous nutrients (Coleman, 1979). It has been suggested that all protozoal species tested, with the exception of *E. caudatum*, showed a selective grazing, *i.e.* some bacterial species are more "preferred" than others (Coleman, 1979). Bacterial intake occurs at a fast rate, and intracellular digestion takes place soon after ingestion, being completed in a few hours (Coleman, 1975).

Molecular techniques applied to microbiology have been proved useful for detecting and describing microbial communities (Tajima *et al.*, 2001); we hypothesized that they could be an appropriate tool to quantify predation by single species of protozoa and to check the resistance from bacteria to digestion. The aim of this work was to study the bacterial predation activity of three common protozoal species of the sheep rumen on *Ruminococcus albus* and *Streptococcus bovis*, two bacterial species that play an important role in rumen physiology under different feeding conditions.

II – Materials and methods

Entodinium caudatum (EC), *Diplodinium dentatum* (DD) and *Metadinium medium* (MM) cells were obtained from the rumen of three rumen-cannulated monofaunated sheep. Rumen contents (approximately 1 l) were sampled before feeding and filtered through a 250 μm (400 μm in case of MM rumen fluid) defined aperture nylon mesh. Liquid was decanted with the same volume of Simplex-type solution (StS, Williams & Coleman, 1992) and 30 $\mu\text{g/ml}$ chloramphenicol for one hour at 39°C. Chloramphenicol was added to keep bacterial numbers stable along the experiment without damaging protozoal cells and to facilitate the dislodgement of episymbiotic bacteria. Then, 80 ml were taken, to which 160 ml of StS were added and centrifuged at 500 \times G for 5 min. At the end of the centrifugation, the top 200 ml of the supernatant were discarded and the pellet was washed by centrifugation twice more. After a third centrifugation, 180 ml of supernatant were discarded. Anaerobiosis and temperature (39°C) were maintained during the procedure. Cell concentration in the protozoal suspension was adjusted to reach the highest concentration possible for DD and MM, and at least 10⁵ cells/ml for EC. Cell motility was checked as an index of protozoal viability.

Two rumen bacterial species (*Streptococcus bovis* and *Ruminococcus albus*) cultured in L10 Medium (Caldwell and Bryant, 1966) were subcultured in 75 ml of medium the day of incubation. At the beginning of each trial, 1 ml of culture was fixed in 1 ml formalin (3.7% v/v in PBS) and its optical density (OD) measured spectrophotometrically at 550 nm as an index of bacterial concentration.

A 20-ml volume of bacterial inoculum and 30 $\mu\text{g/ml}$ chloramphenicol were added to 100 ml bottles filled with 20 ml of protozoal suspension in StS and 100 mg of each starch and xylan as substrate. Bottles were incubated at 39°C for either 60 or 210 min before being processed. Bottles without protozoal suspension were also included as a negative control. The experimental procedure was repeated to get three incubation runs per protozoal suspension. At the start of each experimental period (0 h), 1 ml of protozoal suspension was preserved with 1 ml of methyl green formaldehyde saline solution (MFS) and kept in the darkness until analysis for protozoal and bacterial concentration. Each inoculum was also sampled (300 μl) to assess its DNA bacterial concentration. After the established times of incubation, samples of incubation medium were taken for determination of protozoal and bacterial concentration, as above. For bacterial DNA analysis in the protozoal fraction, 30 ml of medium were centrifuged at 500Xg and washed twice with sterile StS, and 300 μl from the residue of the third centrifugation were immediately frozen in liquid

nitrogen. Two 4-ml subsamples, preserved in 0.4 ml of phosphoric acid (5% v/v) or 1 ml of phosphoric acid solution with 2 mg/ml of 4-methyl valerate, were also taken for analysis of ammonia (NH₃) and volatile fatty acid (VFA) concentration, respectively.

The samples for optical microscopic counts were diluted with 30% (v/v) glycerol and protozoa were counted at 100× magnification following Dehority (1993). Bacterial numbers were counted in a Thoma counting chamber in a phase contrast microscope at 400× magnification. Protozoal and bacterial concentrations were expressed on a logarithmic basis (log [no. cells/ml]). Ammonia concentration was determined colorimetrically following Chaney and Marbach (1962). Volatile fatty acids were determined by gas chromatography using a capillary column.

DNA was extracted from frozen samples using QIAamp[®] DNA Stool Mini Kit (Qiagen Ltd, Crawley, West Sussex, UK) following the manufacturer's instructions. DNA concentration and purity (260/280 and 260/230 nm ratios) were determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Moreover, extract DNA (5 µl) was loaded on a 1% agarose gel and visualized by ethidium bromide staining. Total bacterial rDNA concentration in samples was measured by qPCR using the primers designed by Maeda *et al.* (2003) targeting the conserved region on the 16S rDNA: forward 5'-GTGSTGCAYGGYTGTCGTCA-3', reverse 5'-ACGTCRTCCMCACCTTC-CTC-3'. A DNA extract (3 µl) was added to the amplification reactions (25 µl), containing 25 pmol of each primer, 12.5 µl Platinum[®] SYBR[®] Green qPCR SuperMix[®]UDG and 0.5 µl of reference stain ROX (Invitrogen[™]). Primers for *R. albus* and *S. bovis* were used as described by Koike and Kobayashi (2001) and Stevenson and Weimer (2007), respectively. Cycling conditions for total bacteria and *R. albus* were 95°C for 5 min and 45 cycles of: 95°C for 15 seconds, 55°C for 15 seconds and 72°C for 30 seconds. For *S. bovis* samples, the annealing temperature was 60°C. Two replicates of each DNA sample were analysed in the same plate. Standard curves were established with bacterial DNA extracts from mixed cultures, diluted from 10⁻¹ to 10⁻⁴. Individual species were quantified with the Relative Quantification ΔC_T method (Livak and Schmittgen, 2001) and using the total bacteria as reference. Amplification efficiencies were calculated using serial dilutions and only efficiencies above 90% were taken into consideration. Linear relationships were identified between the logarithms of the known DNA quantities of each standard and their respective threshold cycles. All analyses were performed using ABI Prism 7000 SDS software.

Results within each bacterial inoculum were contrasted by ANOVA using the STATISTIX 8.0 statistical package (Analytical Software 2003), considering the protozoal species and incubation time as factors, and these and their interaction were compared by the residual error. Differences among means were compared with the LSD at a P<0.05, and values of P included between 0.05 y 0.10 were considered as significant trends.

III – Results and discussion

Motility of protozoal inocula was checked before the start of incubations, being observed in over 75% of protozoal cells indicating an adequate. Optical microscopy counts and fermentation values are shown in Table 1. The effect of the incubation time and the interaction incubation time x protozoal species were not significant for all studied parameter (P>0.10). Protozoal concentration ranked EC > DD > MM for both bacterial inocula (P<0.05). Total bacterial counts were lower in EC samples (P<0.05), mainly due to the higher dilution needed for this inoculum to reach the desired protozoal concentration.

The NH₃ and VFA concentrations were low, probably due to the washing procedure previous to the incubation and to the inhibition of bacterial metabolism by chloramphenicol. This was especially noticeable for EC as it was reflected in lower total bacterial counts. Samples from DD and MM showed higher NH₃ concentration levels than EC (P=0.009 and 0.012 for Ra and Sb, respec-

Table 1. Effect of incubation time and protozoal species on total bacterial and protozoal numbers (log [no. cells/ml), NH₃ (mg/l) and VFA (mM) concentrations (n = 3)

		60 min			210 min			Probability		
		EC	DD	MM	EC	DD	MM	RSD	Time	Protozoa
Ra	Protozoa	4.97	3.98	3.26	5.19	4.00	3.32	0.153	0.20	<0.001
	Total Bacteria	7.64	8.06	8.30	7.77	8.22	8.29	0.233	0.42	0.002
	NH ₃	4.88	10.82	15.40	5.93	14.61	13.84	4.446	0.61	0.009
	Total VFA	0.70	1.63	4.37	1.60	4.74	4.28	2.420	0.28	0.11
	Acetate	0.70	1.63	3.33	1.60	3.72	3.34	1.893	0.29	0.16
Sb	Protozoa	5.27	4.09	3.36	5.20	3.91	3.19	0.214	0.19	<0.001
	Total Bacteria	7.51	7.92	8.36	7.96	8.03	8.21	0.286	0.25	0.019
	NH ₃	5.03	10.40	12.52	6.27	13.92	13.76	3.923	0.30	0.012
	Total VFA	1.52	2.39	3.61	1.77	3.48	5.09	2.745	0.48	0.27
	Acetate	1.52	1.97	2.75	1.61	2.72	3.88	2.204	0.54	0.41

tively). Valerate, *iso*-valerate and *iso*-butyrate were not detected in any sample, and propionate and butyrate concentrations were only observed in a few samples, mainly from MM suspensions. Consequently, acetate represented 100% of the total VFA molar proportion in many cases. Concentration of VFA in the negative control (without protozoa) was negligible, indicating that either the concentration of VFA in the initial protozoal inocula was high or that protozoal cells were responsible. The latter would be supported by the fact that acetate was the predominant and in many cases the only VFA whereas propionate, which is produced at low amounts by protozoa, was mostly undetected.

Initial (0 h) DNA proportions of pure bacteria in the incubation media with EC, DD and MM were 0.361 ± 0.0940 , 0.115 ± 0.0621 and 0.129 ± 0.0998 for Ra, and 0.354 ± 0.0569 , 0.114 ± 0.0905 and 0.106 ± 0.0630 for Sb, respectively. Total bacterial DNA in the protozoal fraction and the relative DNA proportion of the pure species compared to total bacterial DNA are shown in Table 2. No differences were observed among either incubation times or the interaction time x protozoal species for any studied parameter ($P > 0.10$).

Table 2. Effect of incubation time and protozoal species on the amount of total bacterial DNA in the residue of fermentation, as expressed per volume of residue, per number of protozoal cells and per protozoal cell size, and relative proportion, estimated by ΔC_T (n = 3)

		60 min			210 min			Probability		
		EC	DD	MM	EC	DD	MM	RSD	Time	Protozoa
Ra	tbDNA/ml res.	4.75	3.34	24.85	6.09	2.95	19.63	8.622	0.732	0.045
	tbDNA/1000 prot.	0.045	0.31	12.68	0.03	0.27	9.58	3.392	0.522	<0.001
	tbDNA/mm ³ prot.	2.08	1.78	4.33	1.45	1.54	3.27	1.565	0.400	0.062
	E ^{ΔCt}	0.205	0.341	0.057	0.254	0.094	0.060	0.1462	0.389	0.128
Sb	tbDNA/ml res.	1.07	4.92	28.36	2.47	3.14	21.79	6.292	0.468	<0.001
	tbDNA/1000 prot.	0.007	0.44	12.14	0.017	0.37	16.253	3.499	0.448	<0.001
	tbDNA/mm ³ prot.	0.34	2.51	4.14	0.77	2.11	5.55	1.398	0.499	0.001
	E ^{ΔCt}	0.051	0.004	0.002	0.030	0.005	0.001	0.0222	0.547	0.018

It was observed that total DNA concentration in the incubation residue was higher with MM than EC and DD, either when protozoa were incubated with Ra ($P=0.045$) or with Sb ($P<0.001$), suggesting that a higher predation of total bacteria might occur with the former protozoal species. Differences in the nature of rumen protozoal communities indicate that responses can be caused by the effect of large differences in either the amount of cells or the cell size. When results were expressed as total bacterial DNA per 10^3 protozoa, they were still highest in MM samples ($P<0.001$), as it could be expected because of the larger size of this species compared with DD and EC. However, protozoal concentrations in this *in vitro* work (Table 1) were adapted for the experimental objectives. In *in vivo* conditions, it has to be noted that proportion of *Entodinium* spp. among the rumen protozoa can be over 0.70, whereas that of *Diplodinium* and *Metadinium* spp. would be below 0.01-0.02 (Williams and Coleman, 1992). Coleman (1975) reported a maximum predation rate (bacterial cells per h) for *Entodinium caudatum* of 2700 mixed rumen bacteria engulfed by protozoon, whereas the predation rates for larger protozoa reached 16000 bacterial cells. It is noticeable that the relative size of the three studied species is different enough to be reflected in differences on the total bacteria engulfed.

To allow for the comparison between the three species of protozoa studied at a size level, the volume of protozoal cells was estimated according to the mean length and width proposed by Dehority (1993) and assuming a cylindrical cell shape. Thus, the relative mean volumes of the protozoal species were 2.155×10^{-5} , 17.487×10^{-5} and 293.333×10^{-5} mm^3 for EC, DD and MM. This transformation resulted in an average total cell volume of 3.661 ± 2.760 , 1.863 ± 0.722 and 5.717 ± 1.776 mm^3 by ml of residue for protozoal cultures of EC, DD and MM, respectively. Total bacterial DNA measured per mm^3 of protozoal residue (Table 2) was higher in MM samples than in EC and DD for Ra ($P=0.062$) and Sb ($P=0.001$) indicating the major ability of *Metadinium medium* to engulf bacteria, both per cell and per cell volume. However, some culture conditions might also affect these results. Bacterial density is an important factor that modifies the predation rate (Coleman 1975), and in this sense samples of MM showed higher total bacterial density in the liquid medium ($P<0.05$, data not shown). Nevertheless, there were no differences in bacterial DNA in the residue between incubation times, suggesting an equilibrium in predation rate after the initial 60 minutes or rather, between predation and intraprotozoal digestion rates. Coleman and Sandford (1979) showed that the predation rate of starved *Entodinium* spp. incubated with ^{14}C labeled bacteria decreased after 2 hours. More recently, Wang *et al.* (2008) observed that the engulfment rate of bacteria by protozoa reached a maximum at about 10 min incubation and decreased thereafter as the protozoa digest the engulfed bacteria.

The higher proportion of Ra in EC residues compared with MM (from 4 to 5-fold) could be explained by a higher presence of other bacteria in MM cultures, as it is shown in Table 1. In contrast, differences in the high pure cells proportion between MM and DD samples without marked differences in bacterial numbers could be explained by a selective predation of *D. dentatum* during the first 60 minutes. However, the ratio of Ra in the residue agrees with that in the medium (0.224 ± 0.1658 , 0.152 ± 0.1403 and 0.115 ± 0.0314 for EC, DD and MM samples), precluding selective predation. Protozoal fraction from EC showed the higher proportion of Sb at the starting (0.354 ± 0.0569) but the lowest amount of total bacterial DNA at 60 and 210 minutes ($P<0.001$). Consequently, proportion of Sb was highest in EC residue ($P=0.018$) and liquid medium ($P=0.032$). In all cases, initial proportions of Sb fell acutely after 60 minutes, maybe due to difficulties to growth in the medium. As the media included chloramphenicol, composition of bacterial communities could be affected according to their resistance to this antimicrobial (Fulghum *et al.* 1968). Since total amount of bacterial DNA in the residue of Sb was similar to Ra samples, the biological niche left by Sb would probably be occupied by other chloramphenicol-resistant bacterial species.

IV – Conclusions

Metadinium medium cells exhibited the highest capacity to ingest bacteria compared with *Entodinium caudatum* and *Diplodinium dentatum*, either in absolute terms or when predation was expressed by cell size, suggesting a high rate of engulfment. No differences were observed between *E. caudatum* and *D. dentatum*. No bacterial selectivity was apparent for any protozoal species and no effects of the time of incubation were observed in any studied parameter.

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