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Original article

Preliminary study on the requirements of *Entodinium exiguum* and *E. caudatum* for live or dead bacteria when cultured in vitro

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Abstract — Whether live bacteria are required to culture the rumen protozoa *Entodinium exiguum* and *E. caudatum* in vitro was studied. Treatments were protozoa plus antibiotics (PA), PA plus autoclaved bacteria (PAB) or protozoa plus live bacteria (PLB). Generation times at 24 h were 22.8 and 31.0 h for *E. exiguum* and *E. caudatum*. Protozoal concentrations were unaffected by the absence of bacteria up to 48 h. After 72 h, *E. exiguum*, concentrations were higher in PLB than PA or PAB. With *E. caudatum* differences between PLB and PA were only observed at 96 h. Thus, a requirement for live bacteria appears to be manifested in culture periods longer than 48 (*E. exiguum*) and 72 (*E. caudatum*) h. Although differences between PLB and PAB indicate a metabolic dependence for bacteria or a long-term antibiotic effect, non-significant differences between PAB and PA suggest that the effect is also related to a nutritive bacterial contribution.

rumen protozoa / *Entodinium* / generation time / in vitro culture

Résumé — Étude préliminaire sur les besoins en bactéries des protozoaires *Entodinium exiguum* et *E. caudatum* cultivés in vitro. Pour étudier le besoin en bactéries des protozoaires *Entodinium exiguum* et *E. caudatum* cultivés in vitro on a comparé trois traitements qui consistaient en des protozoaires décontaminés par des antibiotiques (PA), PA plus des bactéries autoclavés (PBA) ou des protozoaires plus des bactéries vivantes (PBV). Les temps de génération à 24 h ont été 22,8 et 31,0 h pour *E. exiguum* et *E. caudatum* respectivement. La concentration des protozoaires n'a pas été affectée par l'absence de bactéries avant 48 h, mais la concentration de *E. exiguum* a été supérieure dans le milieu PBV en comparaison aux milieux PA et PBA après 72 h, et celle de *E. caudatum* a été supérieure dans le milieu PBV par rapport à PA aprés 96 h. Les résultats montrent que *E. exiguum* et *E. caudatum* ont besoin de bactéries vivantes après 48 et 72 h de culture. Ils traduisent une dépendance

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métabolique des protozoaires envers des bactéries vivantes. Bien que non significatives, les différences entre PBA et PA, suggèrent également une dépendance nutritive des protozoaires à des constituants de la biomasse bactérienne.

protozoaires du rumen / *Entodinium* / temps de génération / culture in vitro

1. INTRODUCTION

Protozoa contribute up to half of the microbial mass in the rumen. Among them, *Entodinium* spp. are the most abundant, reaching up to 90% of total population in high concentrate diets [9, 13]. However, knowledge about protozoal metabolism is limited, mostly because of their dependence of bacteria [11], which confounds their in vitro culture, mainly affecting generation time and survival in long-term incubations. It has been suggested that this dependence is not nutritive [11], but the causative factor(s) remain (s) unknown. In vitro, observed generation time for *E. caudatum* ranged from 13 to 25 h [3, 6]. However, in other studies generation times as short as 9 h have been estimated in vivo [13] and as long as 40 h in vitro [11]. No reports exist about growth and generation time of *E. exiguum*, nor of the effects on these parameters when these two species are cultured in the absence of live bacteria.

In this study, *E. exiguum* and *E. caudatum* were cultured in the presence or absence of live or dead bacteria, to evaluate the extent of their dependence on viable bacteria for survival and growth especially in short term incubations, and the nature of such dependence.

2. MATERIALS AND METHODS

Protozoa were isolated from rumen contents from a fistulated sheep fed on alfalfa hay 6 h after the morning feeding. Sample was diluted in anaerobic dilution solution [2], and individual cells were obtained from a droplet by using a capillary pipette under the microscope. The organisms were cultured anaerobically at 39 °C in medium M [6] in 16 × 150 test tubes. After checking for purity, cultures were allowed to establish for one month before the experimental period. Tubes were daily opened under a stream of CO_2 and fed with 0.1 mL of a suspension of 1.5% (w/v) ground wheat and 1.0% (w/v) orchardgrass hay (both ground to pass a 40-mesh screen). Cultures were maintained routinely by transferring 5 mL every 4 days to another tube with 5 mL f fresh medium. Morphology of *E. exiguum* and *E. caudatum* (two forms, ecaudatum without spines and caudatum with spines) are shown in Figure 1.

In the control period, experimental treatments were protozoa alone (PA), protozoa plus dead bacteria (PAB) and protozoa plus live bacteria (PLB). Both species were cultured in medium M. All tubes (two per treatment) contained 6 mL of medium plus 0.1 mL of feed suspension. For PA, 1 mL of antibiotic solution (12 000 U penicillin plus 780 U streptomycin per mL) was added; for PAB, 1 mL of an antibiotic solution with dead rumen bacteria (obtained by centrifuging 50 mL filtered rumen fluid at 21 000 × *g* for 7 min, drying the pellet, resuspending it in CO_2 gassed distilled water, autoclaving and adding antibiotics up to the same concentration as in PA) was added; or for PLB, 1 mL of sterile distilled water was added. Tubes were then inoculated with 2.5 mL of the protozoal stock cultures. In order to avoid any effect of live bacteria even at the beginning of the experimental period, initial bacterial concentration was minimized by incubating the inoculum for

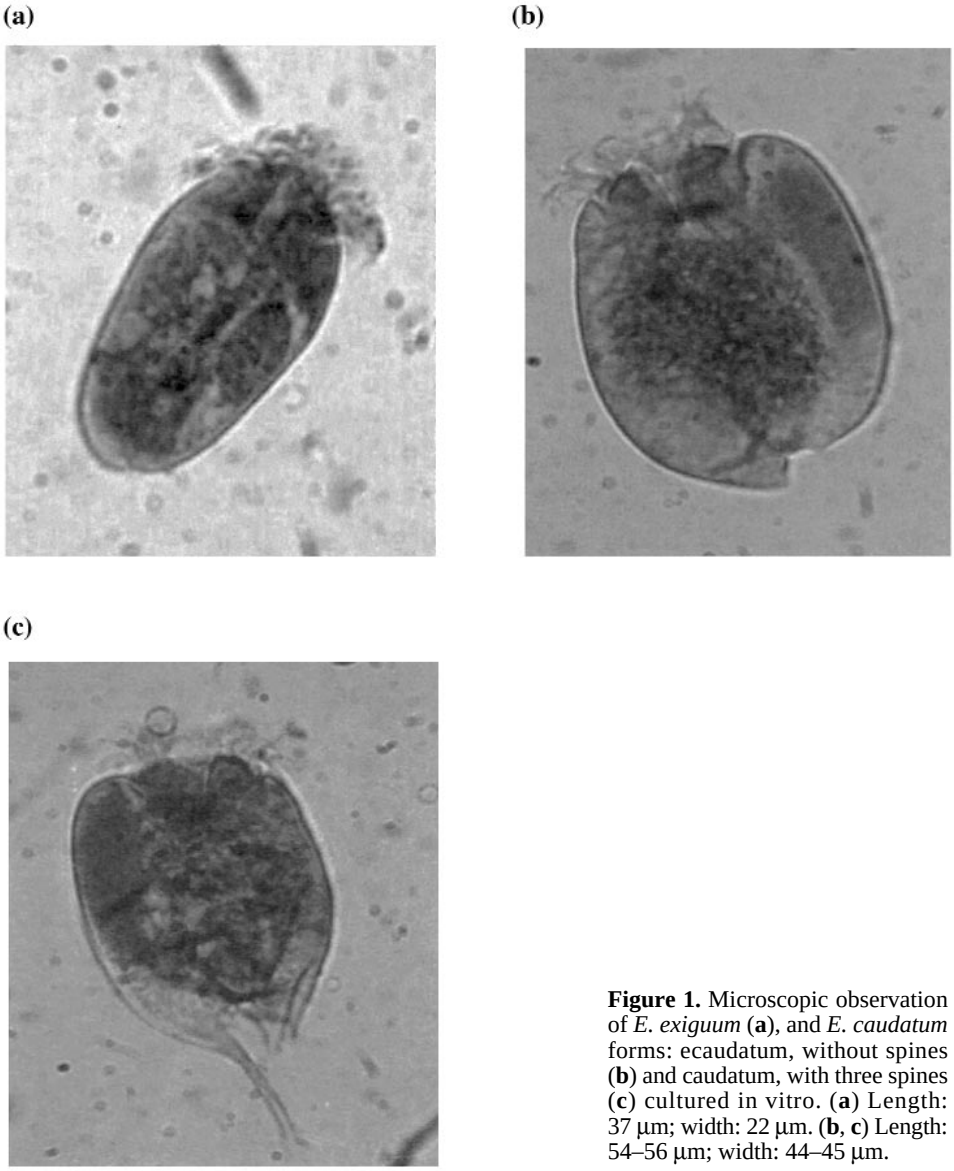

Figure 1. Microscopic observation of *E. exiguum* (**a**), and *E. caudatum* forms: ecaudatum, without spines (**b**) and caudatum, with three spines (**c**) cultured in vitro. (**a**) Length: 37 µm; width: 22 µm. (**b**, **c**) Length: 54–56 µm; width: 44–45 µm.

treatments PA and PAB with 0.20 mL antibiotic solution per ml for 4 h before inoculation. The tubes were daily opened anaerobically, and 0.1 mL of substrate suspension was added. Samples (1 mL) for protozoal counts were obtained after 12 h and then at 24, 48, 72 and 96 h (just before the daily feeding) and mixed immediately with 1 mL of 18% formaldehyde; 8–10 h later fixed samples were diluted with 2 mL of 30% glycerol and counted [5]. Protozoa concentrations are reported as a percentage of initial concentration. Generation time for each incubation period was calculated using

the exponential growth equation [4], related to the initial protozoal concentration.

Treatments were analyzed by time with ANOVA, using STATISTIX [1], considering species, treatment and their interaction as sources of variation. Means were contrasted by the least significant difference at $P < 0.05$.

3. RESULTS AND DISCUSSION

Preliminary results [7] of bacterial counts using MPN had shown that the antibiotic solution effectively killed more than 99% of the bacteria in 4 h, and average bacteria:protozoa ratio drop from 1408:1 in the inoculum to 11.4:1 and 0.03:1 at 4 and 8 h incubation. The same antibiotic mixture had previously shown no bacterial concentration after either 7 or 14 days [10]. No apparent negative effect on protozoal concentrations after 24 h incubation was observed. Numerous studies have reported results with protozoal cultures containing various antibiotics, i.e., penicillin, ampicillin, neomycin, chloramphenicol, streptomycin and several others [12]. In general, bacteria were markedly reduced in concentration with minimal effects being observed on the protozoa, although in several studies with high antibiotic concentrations, protozoal survival was limited.

Growth patterns of *E. exiguum* and *E. caudatum* are presented in Figures 2 and 3, respectively. The overall mean of *E. exiguum* concentrations (as a% of initial concentration) were greater than *E. caudatum* concentrations at 12 and 24 h ($P < 0.05$) Generation times (Tab. I) were shorter for *E. exiguum* than *E. caudatum* at 24 and 96 h ($P < 0.05$); however, no differences were found at the other sampling times ($P > 0.10$). Overall generation times when in coculture with live bacteria (treatment PLB) throughout all the incubation periods were 24 ± 7.48 h for *E. exiguum* and 32.7 ± 4.00 h for *E. caudatum*. Results of growth and generation time of *E. exiguum* are in agreement with other observations from similar experiments by the authors [7, 8]. However, the

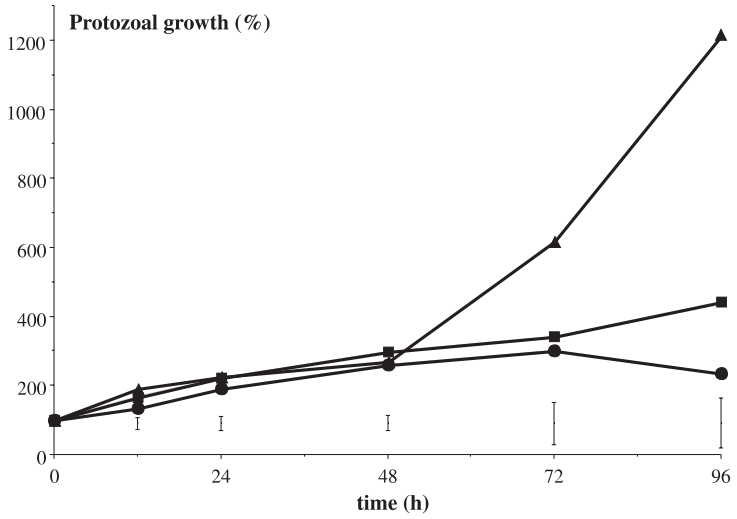

Figure 2. Growth (as % of initial concentration) of *E. exiguum*, when cultured alone (PA, ●), with dead bacteria (PAB, ■) or with live bacteria (PLB, ▲). Lower bars show standard error of means for each sampling time.

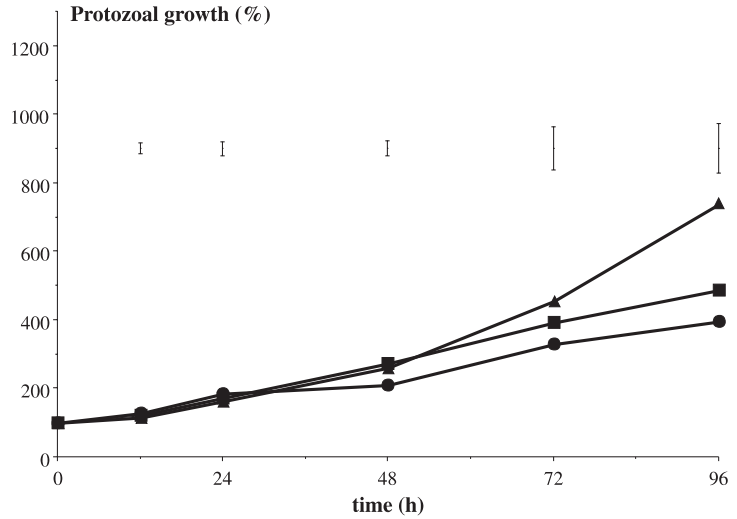

Figure 3. Growth (as % of initial concentration) of *E. caudatum*, when cultured alone (PA, ●), with dead bacteria (PAB, ■) or with live bacteria (PLB, ▲). Upper bars show standard error of means for each sampling time.

Table I. Generation time (h) of *E. exiguum* and *E. caudatum* when cultured alone (PA), with dead bacteria (PAB) or with living bacteria (PLB).

Sampling time	*E. exiguum*			*E. caudatum*			
	PA	PAB	PLB	PA	PAB	PLB	r.s.d.
12 h	39.3	16.6	13.4	32.6	45.3	25.6	14.77
24 h	25.7	20.8	21.9	28.1	30.3	34.6	5.44
48 h	36.0	30.6	33.4	45.5[a]	33.2[b]	35.2[a,b]	5.32
72 h	45.5[a]	41.8[a]	27.6[b]	42.8	36.4	34.4	5.68
96 h	77.6[a]	45.0[b]	26.7[c]	48.4[a]	42.7[a]	33.6[b]	3.59

[a, b, c] Within a row, treatment differences within the same species are significant ($P < 0.05$).

generation time for *E. caudatum* was longer than that cited by [3] and [6], partly because it has been shown that transfer periods of 24 h increase the generation time [6].

Concentrations of both species were unaffected by the absence of bacteria up to 48 h and survival was apparently not affected up to 96 h. However, in *E. exiguum* cultures at 72 h, the concentration in PLB was higher than either PA or PAB (620, 300 and 342%, respectively; $P < 0.05$). For *E. caudatum* at 72 h, differences were only significant between PLB and PA (331, 395 and 455% for PA, PAB and PLB), because of the magnitude of the error term (coefficient of variation 0.21). After 96 h, differences in concentrations of *E. exiguum* between PLB and PA and PAB were greater (1214, 236 and 442%; $P < 0.01$). At this time, differences between PLB and PAB in

E. caudatum were not significant. In agreement with growth results, generation time (Tab. I) was only affected by treatment after 72 h ($P < 0.05$) and 96 h ($P < 0.001$), being shorter in PLB than in PA or PAB, except for *E. caudatum* at 72 h, where differences between PLB and PAB were non-significant ($P > 0.10$).

These responses of both *Entodinium* species to the experimental treatments are in close agreement with those observed by the authors with *E. exiguum* when cultured in vitro for 48 h [7, 8]. The need for live bacteria was manifested at incubation times longer than 48 h for *E. exiguum*, and 72 h for *E. caudatum*, which in both cases is about two-fold the generation time of each organism. Differences between PA and PAB, though only significant after 96 h, indicate that in longer incubations the effect of the presence of dead bacteria might be related to a certain extent to a nutritive bacterial contribution, since no extra source of nutrients (i.e. protein), except for the feeding suspension, was included in the media. Other factors related to a metabolic dependence of protozoa for live bacteria or to a long-term effect of antibiotics over protozoal replication, may be the main factors causing this response. Our inability to culture protozoa axenically at this time does not permit us to carry out the experiments needed for a definitive answer to the effect of antibiotics on protozoa; however, this possibility cannot be overlooked.

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