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Preliminary Study on the Effect of Exposure to Low Temperature on the Viability of Both Mixed and Monocultures of Rumen Protozoa

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Summary. The effect of low temperatures on the viability of rumen protozoa was studied. In Trial 1, samples of mixed rumen contents were diluted and cultured at 38, 15 and 5°C for 2, 4 and 6 h, respectively. Viability of *Isotrichidae* was not affected by either the length of incubation or the temperature ($P < 0.10$). Incubation at low temperatures negatively affected the viability of *Entodinium*, *Diplodiniinae* and *Ophryoscolex*. Differences between 15 and 5ºC were not significant except for *Entodinium* spp. ($P = 0.07$). The reduction of viability increased with time, this effect being noticeable ($P < 0.10$) for *Entodinium* (after 4 and 6 h), *Diplodiniinae* (at 6 h) and *Ophryoscolex* (at 2 and 6 h). Preservation of mixed rumen protozoa for at least 4 h at 15 or 5°C did not markedly affect viability, suggesting that cultures can be recovered after short-term refrigeration. In Trial 2, monocultures of *Entodinium caudatum* and *Diploplastron* (*Metadinium*) *affine* were cultured at 5°C for 4, 8 and 24 h with or without milk powder/egg yolk solution as a membrane protector. For both species, viability was not affected after 4 h at 5°C, but decreased from 8 h onwards ($P < 0.01$). The use of a membrane protector improved viability of *D. affine* at all time periods ($P < 0.05$), but only at 24 h for *E. caudatum* ($P = 0.07$), showing that the use of membrane protector may be positive at medium or long exposure to low temperatures, depending on the species.

Key words: *Diploplastron* (*Metadinium*) *affine*, *Entodinium caudatum*, low temperatures, rumen protozoa, viability.

Abbreviations used: c.v. - coefficient of variation; s.e. - standard error.

INTRODUCTION

Survival of rumen protozoa is markedly reduced when they are subjected to low temperatures, making difficult their storage under refrigeration and later recovery. Most rumen ciliate species do not grow below 35°C (Williams and Coleman 1992). Although the cryopreservation of rumen protozoa has been studied (Kisidayova 1996, 1997; Nsabimana *et al*. 2003; de la Fuente *et al*. 2004, 2006), the possibility of maintaining samples of different protozoal species at refrigeration temperatures (below 15°C) for a moderate period of time has not been investigated.

Low temperatures can affect the viability of rumen protozoa either through loss of membrane integrity or lowered metabolic activity. De la Fuente *et al*. (2004) studied the viability of rumen protozoa by the double stain-fluorescence method of Harrison and Vickers (1990). This is based on the ability of carboxyfluorescein

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diacetate to pass across the cell membrane and be converted to carboxyfluorescein by membrane esterases, thus staining the cytoplasm green. At the same time, propidium iodide can pass through the cell membrane only if it is damaged, thus staining nucleic acids in red. Therefore, results of low temperature incubation can be evaluated with respect to both membrane damage and inefficient enzymatic activity.

This experiment studies the effect of maintaining rumen protozoa at low temperatures for a short period of time. Effects of including a membrane protector in the medium on viability, either in mixed rumen samples or in established monocultures of two significant rumen species, was also investigated.

MATERIALS AND METHODS

Trial 1: **Viability of mixed rumen protozoa at refrigeration temperatures**

Rumen contents of a type A fauna (Eadie 1967) were obtained on four different sampling days from a donor rumen cannulated sheep. The animal was fed a 50:50 alfalfa hay: barley straw mixed diet enriched with a vitamin-mineral mixture. Samples were filtered through three layers of gauze and diluted 1:10 in a culture tube with protozoal culture medium M (Dehority 1998) under a stream of CO_2. Four tubes with 10 ml of mixed rumen protozoa at a 10^{-2} dilution were obtained by diluting 1.0 ml samples from the 1:10 dilution into separate culture tubes each containing 9.0 ml of the same medium. Dilution tubes were previously warmed up to 38°C and fed with 0.1 ml of a suspension of 1.5 % ground corn and 1.0 % alfalfa meal gassed with CO_2 for 20 min. One of the tubes was immediately processed to get an initial reference value (INIT) for protozoal concentration and viability, and the other three tubes were incubated at 38, 15 or 5°C. The temperature for refrigerated tubes was reduced to 15°C at 3ºC min^{-1} (for both 15 and 5°C treatments), and from 15 to 5°C at 6ºC min^{-1} for 5°C tubes, and maintained at those temperatures by incubation in a water bath. After 2, 4 and 6 h of inoculation, contents of the tubes were mixed by gentle agitation. Tubes were opened under a CO_2 stream, and 1 ml was sampled for viability studies, resulting four samples for each experimental treatment.

Trial 2: **Viability of pure protozoa cultures at 5°C**

Tubes with monocultures of *Entodinium caudatum* and *Diploplastron* (*Metadinium*) *affine* maintained in our laboratory were used as representative species of the rumen protozoa. Both cultures were previously obtained from strained sheep rumen fluid, by picking up one or two cells and transferring them to tubes with medium M for their anaerobic culture. Establishment and maintenance of cultures were performed as specified by de la Fuente *et al.* (2004).

A 10 ml volume per tube of each culture (38°C) was used in each of three experimental days. Tubes were initially sampled (1 ml) for viability measurement as in Trial 1 (0 h), and then two 4 ml aliquots were used to inoculate two tubes which contained 4 ml of medium M, either alone or with a 0.2 dilution of 11 % milk powder and 5.5 % egg yolk in distilled and de-ionized water as membrane protector. These tubes were previously fed with 0.05 ml of the substrate suspension under CO_2. Tubes were refrigerated at 5°C as in Trial 1 and kept for 4, 8 and 24 h. At each sampling time, 2 ml were removed from each tube and processed as above for determination of viability, resulting in three samples for each experimental treatment. To validate culture recovery, 2 ml of medium M were added to the remaining 2 ml of culture (after 24 h incubation) and a 1.0 ml aliquot was sampled and diluted 1:1 in 18.5 % formaldehyde for cell counts. The remaining 3 ml was incubated for 96 h at 38°C. Tubes were fed anaerobically once daily with 0.05 ml of substrate suspension.

Analyses

The double-stain fluorescence method was performed as described by de la Fuente *et al*. (2004) for viability studies. The 1 ml samples were centrifuged (500 × g, 3 min), 700 µl of the supernatant were discarded and 250 µl from the residue were used. Protozoa cells were fixed in 5 µl of 1.7 mM formaldehyde, and 5 µl of propidium iodide (Sigma; 7.3 µM in distilled water) and 5 µl of carboxyfluorescein diacetate (Sigma; 10 µM in distilled water) were added and the mixture incubated at 37°C for 15 min avoiding light exposure. Two 15 µl subsamples were counted in a fluorescence microscope at 100× magnification with fluorescein and rodamine standard filters. Red-dyed cells were considered as non viable, whereas those only in green were considered as viable. Protozoal concentration was determined by counting in an optical microscope at 100× magnification after staining with brilliant green and diluting in 1 ml 30 % glycerol (Dehority 1984).

Results from both trials for each type of protozoa were statistically analyzed by ANOVA using the Statistix 8.0 statistical package (Analytical Software 2003), considering the experimental day as a block. Non-orthogonal contrasts were planned to compare treatment means. Nine contrasts were established in Trial 1 to study the effect of the incubation along time within protozoal types (INIT *vs.* 2, 4 or 6 h at 38°C), the effect of temperature (INIT *vs*. 15 or 5°C; 15 *vs*. 5°C) at all time intervals and the effect of temperature along time (38 *vs.* 15 and 5°C for 2, 4 or 6 h). The five contrasts planned for each species in Trial 2 were established to study the effect of incubation time (0 h *vs*. 4, 8 or 24 h) and the use of membrane protector, either at all incubation periods (with *vs*. without protector for 4, 8 and 24 h) or only at long-term incubation (with *vs*. without at 24 h).

RESULTS

Trial 1: **Viability of mixed rumen protozoa at refrigeration temperatures**

Protozoal species present in the rumen inocula were *Entodinium* spp. (*E. caudatum, E. dubardi, E. exiguum, E. nanellum, E. vorax), Ophryoscolex caudatus, Diploplastron* (*Metadinium*) *affine, Polyplastron multivesiculatum, Enoploplastron triloricatum, Isotricha prostoma and Dasytricha*

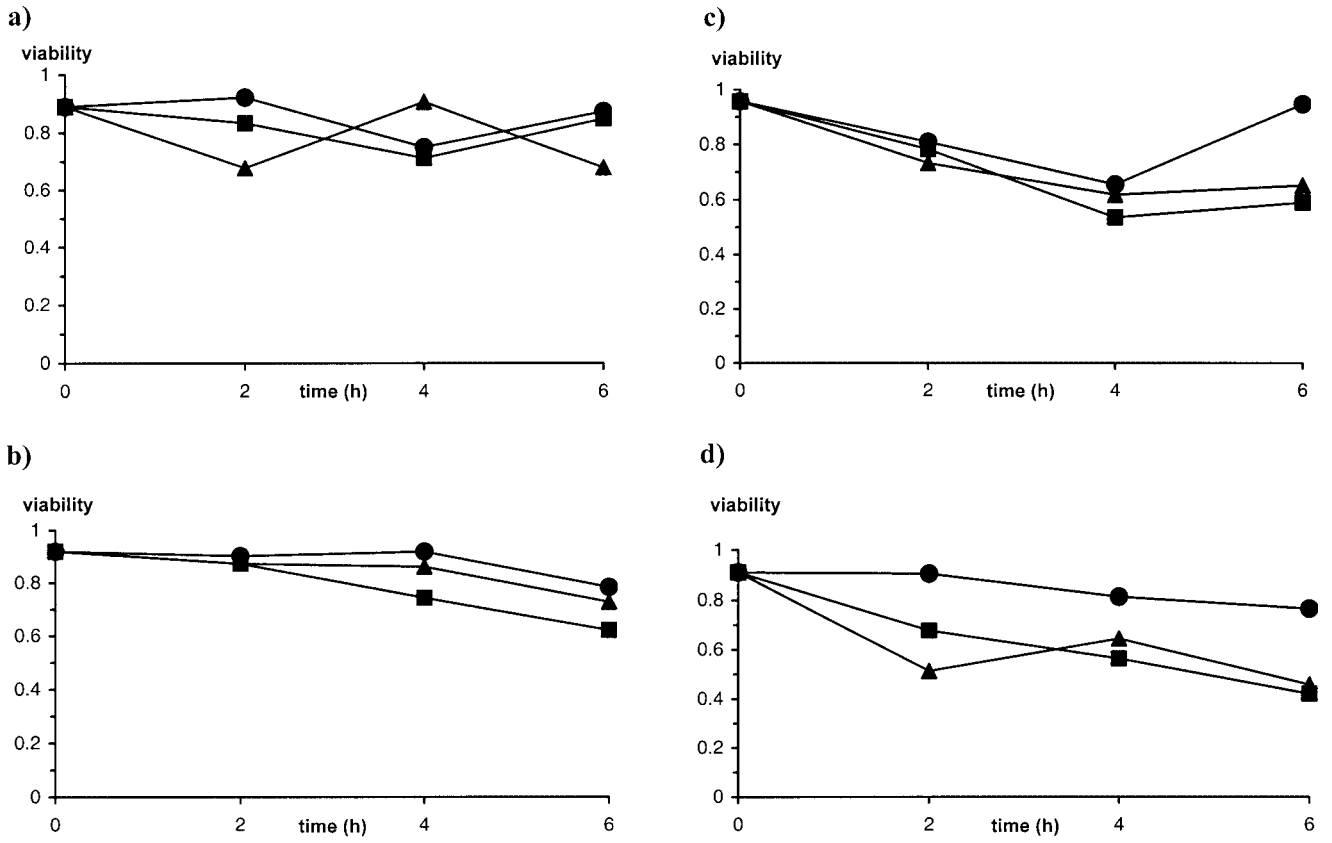

Fig. 1. Viability (proportion of non damaged cells) of *Isotrichidae* (**a**; s.e. = 0.1028), *Entodinium* (**b**; s.e. = 0.0476), *Diplodiniinae* (**c**; s.e. = 0.1145) and *Ophryoscolex* (**d**; s.e. = 0.1162) from mixed rumen samples depending on the temperature (38°C, circles; 15°C, squares; 5°C, triangles) and time of incubation, in Trial 1 (n = 4).

Table 1. Summary (probability, P, and s.e. of the contrast) of the non-orthogonal contrasts established in Trial 1 to study the effects of time (INIT *vs.* 2, 4 or 6 h at 38°C), temperature (INIT *vs.* 15 or 5°C; 15 *vs*. 5°C) and temperature *vs*. time (38 *vs*. 15 and 5°C for 2, 4 and 6 h) on viability of mixed rumen protozoa.

	Isotrichidae		Entodinium		Diplodiniinae		Ophryoscolex	
	P	s.e.	P	s.e.	P	s.e.	P	s.e.
Time								
INIT *vs.* 2 h (38°C)	NS	0.145	NS	0.067	NS	0.162	NS	0.184
vs. 4 h (38°C)	NS	0.163	NS	0.067	0.08	0.162	NS	0.164
vs. 6 h (38°C)	NS	0.145	0.05	0.067	NS	0.162	NS	0.184
Temperature								
INIT *vs.* 15°C	NS	0.356	0.004	0.165	0.03	0.397	0.02	0.403
vs. 5°C	NS	0.364	0.09	0.167	0.04	0.397	0.01	0.403
15 *vs.* 5°C	NS	0.262	0.07	0.120	NS	0.281	NS	0.285
Temp. *vs.* time								
38 *vs.* 15.5°C (2 h)	NS	0.252	NS	0.117	NS	0.281	0.08	0.329
38 *vs.* 15.5°C (4 h)	NS	0.300	0.06	0.117	NS	0.281	NS	0.285
38 *vs.* 15.5°C (6 h)	NS	0.252	0.08	0.120	0.03	0.281	0.07	0.329

NS: non-significant differences (p > 0.10); n = 4 for each experimental treatment.

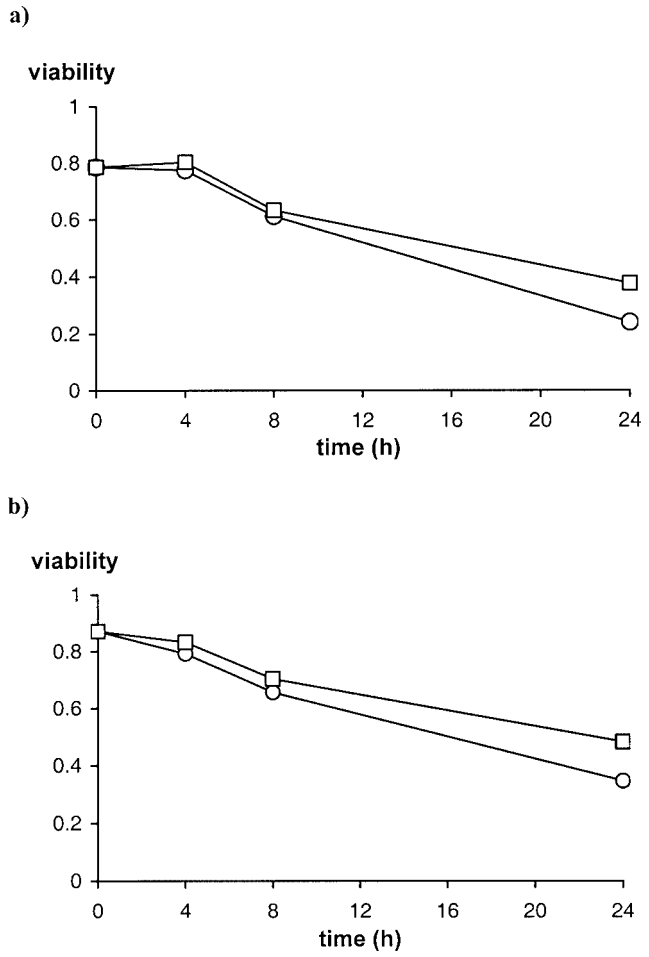

Fig. 2. Viability (proportion of non damaged cells) in monocultures of *Entodinium caudatum* (**a**; s.e. = 0.0476) and *Diploplastron affine* (**b**; s.e. = 0.0326) after 4, 8 or 24 h incubation at 5°C, with (squares) or without (circles) the membrane protector, in Trial 2 (n = 3).

ruminantium. For practical purposes, results of protozoal viability are presented grouped into Family *Isotrichidae*, genus *Entodinium*, Subfamily *Diplodiniinae* and genus *Ophryoscolex*. Protozoal concentration (cells ml^{-1}; n = 4) at the starting of the trial (INIT) were *Isotrichidae*, 223 ± 73.5; *Entodinium*, 5468 ± 693.1; *Diplodiniinae*, 397 ± 74.9; *Ophryoscolex*, 293 ± 151.4. It is worth noting the high variability observed in the initial concentration of cells within the different groups. Average cell viability for INIT ranged from 0.89 to 0.96, with coefficients of variation (c.v.) between 0.08 and 0.13.

The viability (proportion of cells without membrane damage) of cells for each type of protozoa in the mixed samples, as affected by temperature and time of incubation and the significance of the planned contrasts to compare treatments, are shown in Fig. 1 and Table 1, respectively. None of the established contrasts for studying the effects on viability of either the duration of incubation or the temperature during the control period of protozoa from the Family *Isotrichidae* were significant. Incubation of mixed rumen protozoa at 38°C up to 6 h did not affect viability of *Ophryoscolex*, whereas a reduction in this parameter after 6 h was observed in *Entodinium* (0.920 and 0.783 at 0 and 6 h; P = 0.05) as well as a slight (P < 0.10) decrease in the Subfamily *Diplodiniinae* at 4 h incubation (0.957 *vs.* 0.654). The negative effect of incubation at low temperatures on viability compared to the control was observed for *Entodinium*, *Diplodiniinae* and *Ophryoscolex* protozoa, without differences between 15 and 5ºC. However, there was a trend for lower viability of *Entodinium* at 15 than at 5°C (0.746 *vs.* 0.821, respectively), mainly because of differences between both refrigeration temperatures at 6 h.

The reduction of protozoal viability caused by low temperatures increased with time, this effect being noticeable (P < 0.10; Table 1) for *Entodinium* (after 4 and 6 h), *Diplodiniinae* (at 6 h) and *Ophryoscolex* (at 2 and 6 h).

Trial 2: **Viability of pure protozoal cultures at 5°C**

Viability of the cultures at 38°C at the starting of the trial (0 h) was 0.786 ± 0.0324 for *E. caudatum* and 0.872 ± 0.0386 for *D. affine* (n = 3). The average viability of the monocultures of *E. caudatum* and *D. affine* depending on the time of exposure to 5°C and with or without the use of membrane protector is shown in Figure 2 and a summary of the results of the planned contrasts is presented in Table 2. Incubation of *E. caudatum* at 5°C did not affect viability compared with the control (0 h) in the first 4 h of incubation (0.785 and 0.789, respectively), but this effect was significant after 8 and 24 h (0.623 and 0.308). The use of a membrane protector did not improve viability of this species, but a trend for a higher viability was observed after 24 h (0.376 *vs.* 0.242; P = 0.07). In the same way, viability was also significantly reduced for *D. affine* after its incubation at 5°C for 8 and 24 h, though not at 4 h, compared with the control (0.872, 0.812, 0.679 and 0.415 at 0, 4, 8 and 24 h). For this species, the positive effect of the membrane protector was clearly manifested throughout the incubation period, reaching a 0.13 units higher viability after 24 h (0.483 *vs.* 0.347; P = 0.01).

Table 2. Summary (probability, P, and s.e. of the contrast) of the non-orthogonal contrasts established in Trial 2 to study the effect of incubation of monocultures at 5° along time (control *vs.* 4, 8 or 24 h) and the effect of the membrane protector, overall (with *vs.* without) or at long term incubation (with *vs*. without at 24 h).

	E. caudatum		*D. affine*	
	P	s.e.	P	s.e.
Incubation at 5°C				
0 h *vs.* 4 h	NS	0.117	NS	0.080
vs. 8 h	0.02	0.117	0.001	0.080
vs. 24 h	0.001	0.117	0.001	0.080
Membrane protector				
with *vs.* without (all times)	NS	0.117	0.02	0.080
with *vs.* without (24 h)	0.07	0.067	0.01	0.046

NS - non-significant differences ($p > 0.10$); n = 3 for each experimental treatment.

Concentration of *E. caudatum* after 24 h at 5°C was 3223 ± 2308 and 2764 ± 1192 cells per ml with or without membrane protector, respectively. Refrigerated cultures were then maintained at 38°C for 96 h, and these resulted in culture recoveries below 0.20 of the cells counted after 24 h refrigeration. For *D. affine*, the average cell concentration was 582 ± 157.8 and 917 ± 261.6 cells ml^{-1} after 24 h of incubation at 5°C with or without membrane protector, but after 96 h incubation at 38°C cultures were only recovered from tubes from one day of the refrigeration trial (cell concentration 2.97 and 0.54 times the number of cells after 24 h at 5°C, respectively). For both species, it seems that recovery was not improved by the use of the protector.

DISCUSSION

Considering the low concentration of some protozoal species in the rumen (Williams and Coleman 1992, Dehority 2003) mainly in the small sample volume used for viability measurements (15 µl), and because rapid counting is needed in fluorescence microscopy and species identification requires considerable time, we grouped them into genera or families to reach sufficient numbers for more meaningful analyses. This decision may have the drawback of not considering the existing differences in tolerance to low temperatures among species within a genus or subfamily. Except for the *Entodinium* spp., the other types studied in Trial 1 were in low numbers, and results therefore must be considered with caution.

The main goal of this work was to assess the possibility of preserving protozoa for short periods of time at low temperatures (over 0°C) for an easier handling and processing of cells for culture studies. As expected from previous results (de la Fuente *et al*. 2004, 2006), rumen protozoa were affected by incubation at refrigeration temperatures (15 and 5°C). A lower concentration and an adequate medium composition after diluting in protozoa specific culture medium would allow for an increasing resistance to cold stress. Even though, viability was considerably reduced after a short time at either 15 or 5°C for *Entodinium, Diplodiniinae* and *Ophryoscolex*. Despite considerable variation in the response, viability of *Entodinium* was reduced in 0.15 after their culture at 38°C for 6 h compared with the initial numbers (Table 1). A similar response can also be inferred in *Diplodiniinae,* indicating that the response to temperature may be partly confounded by the resistance of some organisms to these *in vitro* culture conditions. However, the fact that results from incubation at 15 and 5°C were in general lower than those at 38°C for the longer incubation times reduces the potential impact of this bias.

Viability proportions after 4 and 6 h incubation at 15°C and 5°C were of a lesser magnitude than those observed in the same periods at 38°C, the response depending on the studied type of protozoa. Values were as low as 0.68 for *Isotrichidae,* 0.62 for *Entodinium*, 0.53 for *Diplodiniinae* and 0.42 for *Ophryoscolex*. Therefore, although in most of the cases the low temperatures affected protozoal viability from 4 h onwards, the effects were of a lower magnitude than those initially

expected considering previous reports (Williams and Coleman 1992). From these results, the preservation of rumen protozoa for at least 4 h at 15 or 5°C without a marked drop of viability could be expected. Even though samples were not cultured at 38°C for a period after being exposed to 5 and 15°C in these incubation trials, the magnitude of the observed viabilities after 4 and even 6 h suggest that the cultures can be successfully recovered after refrigeration and subsequently maintained at 38°C.

In a more specific approach to the effect of low temperature on rumen protozoa for a longer period of refrigeration, *E. caudatum* and *D. affine*, chosen as representative species of a type-A rumen population, were studied in Trial 2. The lack of a negative effect of incubation at 5°C for 4 h suggests that these organisms can be stored for short periods of time at refrigeration temperatures. However, incubation for a longer time (more than 8 h) negatively affected viability of both cultures. In the case of *D. affine*, this depression was more noticeable when membrane protector was not added indicating that some extent of membrane damage primarily occurs with longer refrigeration times. However, this effect was not as clear for *E. caudatum*, suggesting that the response is species dependent. Although this would support the practice of adding membrane protector for preserving some species of rumen protozoa at refrigeration temperatures, results for culture recovery did not show a clear benefit in such practice. In this regard, incubation of both *E. caudatum* and *D. affine* at low temperatures for 24 h does not seem to ensure the culture recovery in a further incubation at 38ºC. It may be speculated that some extent of long term cell damage not detected by the double stain technique would affect cell viability in terms of its metabolic activity, since recovery was even lower than expected from results of incubation at 5°C for 24 h. In all cases, viability at 24 h was considerably lower.

Therefore, refrigeration temperatures affect viability of rumen protozoa, although the effect at incubation times shorter than 4 h is not significant. However, there is no data available on recovery after this incubation time. The variability of this response to refrigeration and the difficulty in culturing some protozoa types *in vitro* limit the possibilities for their manipulation. The use of membrane protectors may be positive for medium to long term refrigeration, the extent of the response depending on the species, but the low viability of cells reduces its potential benefit.

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