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SUMMARY

Bentonite (2 g/day) was added to four vessels in the rumen simulation technique (Rusitec) and the effects on the fermentation were compared with four control vessels over a 21-day period. Vessels in both groups were supplied with 20 g/day of a diet containing, per kg, 500 g hay, 299·5 g barley, 100 g molasses, 91 g fishmeal and 9·5 g of mineral/vitamin mix. Numbers of ciliate protozoa were reduced by 69% (4·0 *v.* 12·8 × 10³/ml) in vessels receiving bentonite. The viable count of bacteria was increased by one-third in these vessels (4·8 *v.* 3·6 × 10⁸/ml), and ammonia production was 54·7 and 76·7 mg/day in the bentonite and control vessels, respectively. Other effects on fermentation products and metabolic activities were minor, except that the rate of breakdown of [¹⁴C]leucine-labelled *Selenomonas ruminantium* protein was 47% lower in fluid taken from vessels receiving bentonite. Bentonite did not affect the rate of bacterial breakdown by protozoa immediately when it was added to rumen fluid *in vitro*, although a decline in activity was becoming apparent after 4 h incubation. Microscopic examination of rumen fluid to which bentonite was added indicated that some bentonite was ingested by protozoa, but that its main toxic effect was at the cell surface, causing interference with the motion of cilia and thereby preventing motility of protozoa, particularly the holotrichs.

INTRODUCTION

Bentonite is a colloidal hydrated aluminium silicate (clay) consisting principally of montmorillonite. It has been used in ruminants as an aid to pH control in the rumen, as a source of minerals or merely as a feed binding agent, and some favourable digestion and production responses have been observed with dietary bentonite (Bringe & Schultz 1969; Martin *et al.* 1969; Rindsig *et al.* 1969; Huntingdon *et al.* 1977; Britton *et al.* 1978; May & Barker 1988). Other trials found no benefit of bentonite inclusion (Erwin *et al.* 1957; Jacques *et al.* 1986; Marrero *et al.* 1987; Peiris *et al.* 1988). Its potential for increasing pH in the rumen explains some of the efficacy of bentonite, but there are examples where benefit was obtained without a significant effect on rumen pH (Bringe & Schultz 1969; Rindsig *et al.* 1969; Colling *et al.* 1975). It has also been shown that rumen pH may increase with no productive benefit (Marrero *et al.* 1987).

Recent experiments (Fenn & Leng 1988) have suggested that bentonite may have a beneficial effect on the protein nutrition of ruminants. When bentonite

was supplied in water to faunated sheep on green pasture, it increased wool growth by 17%, implying that the protein supply for tissue growth was increased. Defaunated sheep had a 25% greater rate of wool growth than faunated animals, and bentonite did not further stimulate wool growth to a significant extent (Fenn & Leng 1988). Thus it appeared that bentonite enhanced the flow of protein from the rumen, possibly via an effect on rumen ciliate protozoa.

Bentonite modifies the metabolic activity of rumen micro-organisms (Aitchison *et al.* 1986) and of micro-organisms from habitats other than the rumen (Stotzky 1966; Stotzky & Rem 1966; Marshman & Marshall 1981 *a, b*). These effects were assigned to adsorptive interactions between micro-organisms or their growth substrates and bentonite. The present experiments were undertaken to investigate the effects of bentonite on fermentation by the rumen microbial population, using a well-established long-term rumen simulation technique (Rusitec; Czerkawski & Breckenridge 1977), and to study in particular interactions between bentonite and ciliate protozoa.

MATERIALS AND METHODS

The experiments described here were done at the Rowett Research Institute between November 1989 and April 1990.

Apparatus

The rumen simulation technique Rusitec was used as described by Czerkawski & Breckenridge (1977). The nominal volume in each vessel was 850 ml and the dilution rate was set at 0·88/day, the infused liquid being artificial saliva (McDougall 1948) at pH 8·4. Inocula for the fermentation vessels were obtained from a pooled sample from three rumen-cannulated sheep fed 1·4 kg/day of a forage and concentrate diet of hay, molasses, fishmeal and a minerals and vitamins mixture (500, 299·5, 100, 91 and 9·5 g/kg dry matter, respectively).

The same diet (20 g/day) was supplied to the fermentation vessels after grinding to pass through a 3 mm screen. The food was provided in nylon bags, pore size 50 μm, which were gently agitated in the liquid phase. Two bags were present at any time and one bag was replaced each day to give a 48 h incubation. While the bags were changed, the vessels were flushed with CO_2 to help maintain anaerobiosis.

Analytical methods

Fermentation products were determined on samples taken from the liquid overflow. Analysis of volatile fatty acids, L-lactate and ammonia was carried out by methods described previously (Frumholtz et al. 1989). The digestibility of the diet was estimated from the dry matter remaining in the bags after incubation, obtained by drying at 105 °C for 24 h.

Microbial counts

Counts of ciliate protozoa were carried out microscopically in a counting chamber (Newbold et al. 1987). Counts of total viable bacteria were carried out in a medium containing rumen fluid, medium 2 of Hobson (1969) as described by Frumholtz et al. (1989). Cellulolytic counts were done by a most-probable-number method using filter paper strips (Mann 1968).

Bacterial protein turnover

The activity of micro-organisms in the fermentation liquid in breaking down bacterial protein was determined using [14C]leucine-labelled Selenomonas ruminantium Z108 as described by Wallace & McPherson (1987). Unlabelled L-leucine (5 mM) was added to the fluid to prevent reincorporation of label released as a result of breakdown of S. ruminantium protein, and the rate of breakdown was determined by the release of radioactivity into material soluble in 50 g/l trichloracetic acid.

The influence of bentonite addition on bacterial turnover in rumen fluid was determined using rumen fluid removed 3 h after feeding from four faunated and four defaunated sheep receiving the same diet as added to Rusitec. These sheep were mature animals, fed 1·4 kg/day and fitted with rumen cannulas. The defaunated sheep, prepared by the method of Jouany & Senaud (1979), had been ciliate-free for 9 months. The breakdown of [14C]leucine-labelled S. ruminantium Z108 in strained rumen fluid from these animals was determined as before, in the presence and absence of added bentonite (10 mg/ml).

Enzyme activities

Protease activities were measured by the release of label from [14C]-labelled casein (Wallace 1983). Peptidase activities were measured with alanine peptides as substrates and deaminase activity was determined with casein acid hydrolysate as substrate (Newbold et al. 1990).

Microscopy

Bentonite (1 g) was suspended in 10 ml of 0·5 % malachite green and incubated at room temperature for 1 h. The stained bentonite was sedimented by centrifugation (3000 g, 5 min), washed twice with distilled water, and resuspended in 10 ml of distilled water. One ml of this suspension was incubated at 39 °C with 9 ml of rumen fluid removed from a sheep on the same diet as before, 3 h after feeding. Samples were removed for microscopic analysis 3 h later.

Experimental procedure

Eight vessels were set up as described above and were fed at the same time every day with 20 g of the above diet. In addition, four of the vessels received 2 g of bentonite added to the liquid phase. The duration of the experiment was 21 days. Samples were removed periodically for protozoal counts, but otherwise the measurements were done on samples taken on two days during the last 7 days of the experiment.

RESULTS

Bentonite increased the culture pH in Rusitec vessels by a small but significant extent (Table 1). Production of all three main volatile fatty acids decreased by an average of 17 %, although the relative proportions of acetate, propionate and butyrate remained similar (Table 1). The formation of L-lactic acid was low in control and bentonite-treated vessels, with no significant difference between the groups ($P > 0.05$). Ammonia production decreased by 29 % in the presence of bentonite (Table 1).

Table 1. *Influence of bentonite on digestibility, culture pH, microbial numbers and fermentation products in the rumen simulation technique (Rusitec) in samples taken between days 14 and 21*

	Control	Bentonite	S.E.D. (6 D.F.)
DM digested* (g digested after 48 h incubation)	10·4	10·6	0·29
pH	6·98	7·10	0·03
Total viable bacteria ($\times 10^{-8}$/ml)	3·6	4·8	0·37
Cellulolytic bacteria ($\times 10^{-6}$/ml)	3·0	3·2	1·23
Protozoa ($\times 10^{-3}$/ml)	12·8	4·0	2·46
Acetate (mmol/day)	23·7	19·4	0·62
Propionate (mmol/day)	10·1	8·5	0·41
Butyrate (mmol/day)	12·3	10·4	0·40
L-Lactate (mmol/day)	0·46	0·50	0·038
Ammonia (mg N/day)	76·7	54·7	2·70

* 20 g of a 50 % grass hay:50 % concentrate diet was added daily to four control and four bentonite-treated vessels; 2 g of bentonite was added to the liquid phase at the same time as the diet in the latter group.

Table 2. *Numbers of protozoa in the effluent of the rumen simulation technique (Rusitec) in the presence and absence of bentonite*

	Numbers of protozoa ($\times 10^{-3}$/ml)		
Day	Control	Bentonite	S.E.D. (6 D.F.)
2	154	175	2·80
6	110	65·5	5·20
8	61·8	12·2	5·46
10	15·8	4·4	3·91
12	12·6	3·8	2·29
14	11·5	4·3	2·92
18	14·0	3·8	3·59

Table 3. *Influence of bentonite on microbial protease, peptidase and deaminase activities in the liquid phase of the rumen simulation technique (Rusitec) in samples taken during days 14–21*

	Hydrolytic activity		
	Control	Bentonite	S.E.D. (6 D.F.)
Protease (mg [^{14}C]casein/h per mg protein)	0·597	0·527	0·111
Peptidase (nmol/h per mg protein)			
Ala$_3$	3·45	3·25	0·98
Ala$_4$	3·80	4·16	1·76
Ala$_5$	5·38	4·93	1·43
Deaminase (nmol NH$_3$ produced/h per mg protein)	648	552	222
Protein (mg/ml)	1·8	1·6	0·34

Protozoal numbers fell rapidly during the first 10 days of the experiment, to about one-tenth of their original population density in control vessels and 2–3 % in bentonite vessels (Table 2). The rate of decline during this period was more rapid when bentonite was added. At the end of the experiment, decreased protozoal numbers were accompanied by a 33 % increase ($P < 0.05$) in the total numbers of bacteria (Table 1). The cellulolytic bacterial population, measured by a most-probable-number method, was apparently unaffected, but numbers were highly variable.

No significant effect was observed on the activities of enzymes involved in the breakdown of protein to ammonia. Protease and deaminase activities tended to be higher in control vessels, but again the differences were not significant ($P > 0.05$; Table 3). Peptidase activities with Ala$_2$, Ala$_3$ and Ala$_4$ were unaffected. In

contrast, a 40 % decrease was observed in the rate of breakdown of *S. ruminantium* protein in fluid removed from vessels receiving bentonite (Fig. 1).

Immediate effects of bentonite were investigated by adding bentonite to rumen fluid from sheep fed the same diet as added to Rusitec. The rate of breakdown of labelled *S. ruminantium* was initially similar in the presence of bentonite, but the rate appeared to begin to fall more rapidly after 4 h incubation in the presence of bentonite (Fig. 2).

Bentonite particles stained with malachite green had a characteristic blue colour easily identified in the
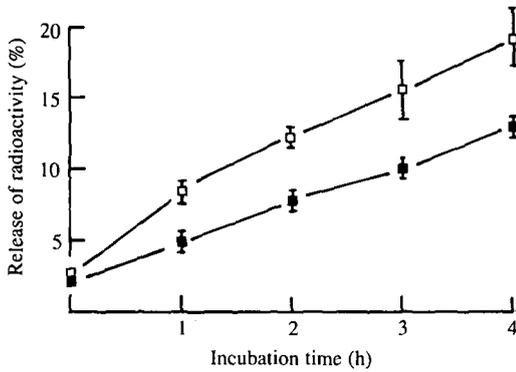
Fig. 1. Release of ^{14}C from [^{14}]leucine-labelled *Selenomonas ruminantium* incubated with mixed micro-organisms from control vessels (□) or vessels receiving bentonite (■) used in the rumen simulation technique (Rusitec). Samples of the liquid phase of Rusitec were added to suspensions of *S. ruminantium* which had been grown in the presence of [^{14}C]leucine. Excess unlabelled leucine (5 mM) was added to the mixture to prevent re-incorporation of [^{14}C]leucine released as the result of bacterial protein breakdown. Breakdown of bacterial protein was determined by adding trichloroacetic acid (50 g/l), centrifuging, and counting the radioactivity of the supernatant fluid. Results are the means±S.E. of samples taken from four vessels in each treatment.
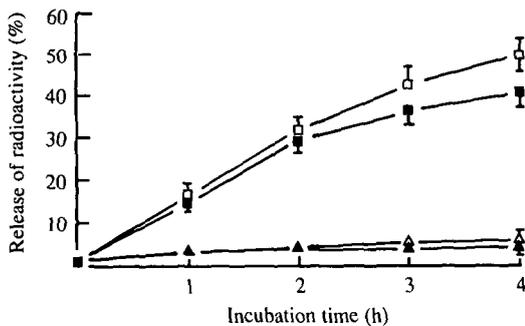

Fig. 2. Release of ^{14}C from [^{14}C]leucine-labelled *Selenomonas ruminantium* incubated with mixed micro-organisms from sheep rumen in the presence (solid symbols) and absence (open symbols) of bentonite (10 mg/ml). Results are the mean±S.E. of incubations with rumen fluid from four faunated (■, □) and four defaunated (▲, △) sheep in each treatment.

light microscope. This material was added to rumen fluid from one of the donor sheep, which did not receive bentonite in its diet, at a concentration of 10 mg/ml. Several types of interaction with protozoa were visible after 3 h incubation. The most obvious effect was on the holotrichs, many of which were static, with a coating of bentonite at the tips of their cilia (Fig. 3). Many holotrichs retained their normal rapid motility, and these had no sign of an ac-
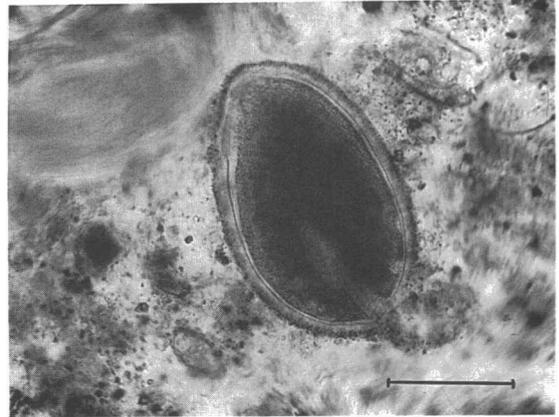

Fig. 3. Influence of bentonite on *Isotricha* sp. Bentonite stained with malachite green was mixed to a final concentration of 10 mg/ml with strained rumen fluid taken from a sheep 3 h after feeding. After 3 h incubation at 39 °C, a blue coating of stained bentonite particles could be seen attached to the outer surface layers of many holotrich protozoa. The protozoon pictured above, coated with bentonite, is immotile, although ciliate movement was seen to occur beneath the bentonite layer. The rapid movement of a similar organism, except not coated with bentonite, is also visible, top left. Bar represents 100 μm.
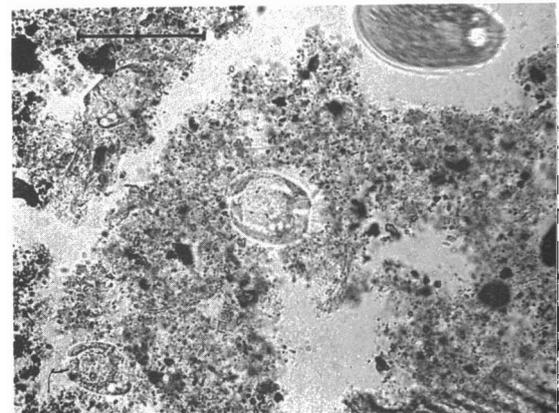
Fig. 4. Influence of bentonite on *Entodinium* sp. In another field of the same sample illustrated in Fig. 3, the attachment of stained bentonite to the cilia of an *Entodinium* sp. (centre) could be seen, although it was much less clear than with the holotrichs. However, it was obvious from the motion of these protozoa that the high viscosity of the suspension caused entodiniomorph rotational movement to be greatly decreased. Bar represents 100 μm.

cumulation of bentonite on their surface. The static organisms had not lost their ability for ciliate motion. The cilia could be seen clearly to be highly active below the layer of bentonite. It appeared that the bentonite shell mainly affected the ability of ciliate motility to generate propulsion.

The effects of bentonite were less clear with entodiniomorphs. Their cilia did not appear to become so coated with bentonite, although their ability to move appeared to be restricted (Fig. 4). The spinning, tumbling type of motility was much slower than in the absence of bentonite.

Neither class of protozoon appeared to engulf bentonite to a significant degree. Bentonite was absent from holotrichs, and the bentonite-clear tracks of healthy holotrichs were clearly visible. Some entodiniomorphs ingested a few particles, which did not appear to pass out of the protozoal cells. Otherwise these protozoa appeared to be normal.

DISCUSSION

The principal effect of bentonite in Rusitec was to depress protozoal numbers. In turn, the rate of bacterial protein breakdown and consequent ammonia production decreased and bacterial numbers increased, as invariably occurs *in vivo* when ciliate protozoa are removed (Williams & Coleman 1988). Bentonite had no influence on proteolytic activity, or on the activities of other enzymes involved in the production of ammonia. The net protein yield resulting from the fermentation might therefore be expected to increase in animals receiving bentonite (Demeyer & Van Nevel 1979).

An improved protein flow in the presence of bentonite was implied in the experiments of Fenn & Leng (1988), but protozoal numbers in the liquid phase of the rumen increased rather than decreased as might be expected. A possible explanation is that bentonite affects protozoa *in vivo* but interferes mainly with migration and sequestration (Abe *et al.* 1981; Dehority & Tirabasso 1989). Thus more protozoa might be subject to washout from the rumen (Fenn & Leng 1988). Alternatively, protozoa may be more susceptible to bentonite in Rusitec than *in vivo*. Rusitec has some limitations in studies of the present type. First, the quantity of bentonite added along with the feed aimed to give a concentration in the liquid phase, rather than the solids phase, of Rusitec that would be similar to that occurring *in vivo*. This is an assumption which has to be made with more-

soluble additives such as ionophores (Wallace *et al.* 1981) because Rusitec is a much more dilute fermentation than occurs *in vivo*. It may not be entirely valid for materials such as bentonite, which attach to solids and also form colloidal suspensions. Furthermore, protozoal numbers in Rusitec initially decline over the first few days (Table 2; Czerkawski & Breckenridge 1977) and conditions are not ideally suited for the migration and survival of protozoa. Nevertheless, it is clear from the present experiments that bentonite is toxic to both entodiniomorphid and holotrich protozoa. Whether this occurred in Rusitec but not *in vivo*, because of the absence of sequestration to the rumen wall, for the reasons already outlined, remains to be resolved by further experimentation.

Many interactions may occur between clays such as bentonite and micro-organisms. Adsorption of microbes to clay and adsorption of protein and carbohydrate growth substrates both occur (Marshman & Marshall 1981 *a*). Bentonite also affects the competition between bacteria and fungi in cultures of non-rumen micro-organisms (Rosenzweig & Stotzky 1979; Marshman & Marshall 1981 *b*). The interactions observed here with rumen ciliate protozoa indicate that suppression of protozoal activity occurred gradually. Even concentrations of bentonite several times larger than would be expected *in vivo* only began to suppress predation of added bacteria after 4 h incubation (Fig. 2). Bentonite was not ingested by the protozoa, but interfered with the effectiveness of ciliate motion in propulsion of the organism, particularly of holotrichs (Fig. 3). Presumably the interaction was between bentonite and ciliary proteins.

Much effort has been expended in finding suitable agents for defaunating ruminants under circumstances where eliminating protozoa would be nutritionally beneficial. Bentonite would appear to offer at least a partial suppression of protozoal activity, potentially leading to a useful improvement in protein flow from the rumen.

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