Influence of sodium fumarate addition on rumen fermentation *in vitro*

S. López*†, C. Valdés†, C. J. Newbold and R. J. Wallace
Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, UK

(Received 6 August 1997 – Revised 15 July 1998 – Accepted 28 September 1998)

The influence of sodium fumarate on rumen fermentation was investigated *in vitro* using batch and semi-continuous cultures of mixed rumen micro-organisms taken from three sheep receiving a basal diet of hay, barley, molasses, fish meal and a mineral–vitamin supplement (500, 299·5, 100, 91 and 9·5 g/kg DM respectively). Batch cultures consisted of 10 ml strained rumen fluid in 40 ml anaerobic buffer containing 200 mg of the same feed given to the sheep. Sodium fumarate was added to achieve a final concentration of 0, 5 or 10 mmol/l, as a result of the addition of 0, 250 or 500 μmol, equivalent to 0, 200 and 400 g/kg feed. CH_4 production at 24 h (360 μmol in the control cultures) fell ($P < 0·05$) by 18 and 22 μmol respectively (SED 7·5). Total gas production was increased by the addition of fumarate without significant accumulation of H_2. Substantial increases in acetate production (92 and 194 μmol; SED 26·7, $P < 0·01$) were accompanied by increases in propionate formation (212 and 396 μmol; SED 13·0, $P < 0·001$). Longer-term effects of fumarate supplementation on ruminal fermentation and CH_4 production were investigated using the rumen simulation technique (Rusitec). Eight vessels were given 20 g basal diet/d, and half of them received a supplement of fumarate (disodium salt) over a period of 19 d. The response to the daily addition of 6·25 mmol sodium fumarate was a decrease in CH_4 production of 1·2 mmol (SED 0·39, $P < 0·05$), equivalent to the consumption of 4·8 mmol H_2, and an increase in propionate production of 4·9 mmol (from 10·4 to 15·3 (SED 1·05) mmol/d, $P < 0·01$). The inhibition of CH_4 production did not decline during the period of time that fumarate was added to the vessels. Thus, the decrease in CH_4 corresponded well to the fraction of the fumarate that was converted to propionate. Fumarate had no significant ($P > 0·05$) effect on total bacterial numbers or on the number of methanogenic archaea, but numbers of cellulolytic bacteria were increased (8·8 *v.* 23·9 (SED 2·49) $\times 10^5$ per ml, $P < 0·01$). Fumarate also increased DM digestibility of the basal diet after 48 h incubation (0·476 *v.* 0·508 (SED 0·0123), $P < 0·05$). Thus, it was concluded that sodium fumarate may be a useful dietary additive for ruminants, because it diverts some H_2 from CH_4 production and because it is able to stimulate proliferation of cellulolytic bacteria and digestion of fibre.

Rumen: Fumarate: Methane: Rusitec

CH_4 formation represents a substantial loss of energy to the ruminant animal, and it is also a significant source of greenhouse gas emissions from agriculture (Moss, 1993). Inhibition of CH_4 production by ruminants would therefore have significant economical and environmental benefits (Van Nevel & Demeyer, 1996). Many chemical compounds (mainly antimicrobial compounds) have been tested as potential feed additives for ruminants on the basis of their direct or indirect effects on CH_4 production in the rumen (Moss, 1993; Van Nevel & Demeyer, 1996). These include ionophores, halogenated CH_4 analogues and unsaturated fatty acids. One possible way to decrease CH_4 formation in the rumen is to promote alternative metabolic pathways to dispose of the reducing power, competing with methanogenesis for the H_2 uptake. Fumaric acid is a four-C dicarboxylic acid that is an intermediate in the propionate pathway, in which it is reduced to succinate by fumarate reductase (*EC* 1.3.99.1). Reducing equivalents are needed in this reaction and therefore fumarate may provide an alternative electron sink for hydrogen. As hydrogen is used to reduce fumarate, there is a decline in the availability of H_2 for methanogenesis in the rumen. Fumarate and other dicarboxylic acids also seem to stimulate the growth and activity of the lactic acid-utilizing rumen bacterium *Selenomonas ruminantium* (Nisbet & Martin, 1990), providing an electron sink for this organism (Martin & Park, 1996). CH_4 production by

Abbreviations: Rusitec, rumen simulation technique.

* **Corresponding author:** Dr Secundino López at his present address, fax +34 987 291 311, email DP1SLP@ISIDORO.UNILEON.ES
† **Present address:** Departamento de Producción Animal, Universidad de León, E-24071 León, Spain.

mixed rumen bacteria was decreased for short periods *in vitro* when fumarate was added to the medium (Demeyer & Henderickx, 1967). The objectives of the present study were to evaluate the effect of fumarate on CH_4 production in short-term *in vitro* batch incubations and to determine the long-term effects of fumarate on ruminal fermentation in the semi-continuous rumen simulation technique (Rusitec).

Materials and methods

In vitro *batch fermentations*

Short-term *in vitro* incubations were carried out with rumen fluid withdrawn from three rumen-cannulated sheep. The sheep received 1·4 kg/d of a mixed diet consisting of grass hay, barley, molasses, white fishmeal and a vitamin and mineral mixture (500, 299·5, 100, 91 and 9·5 g/kg DM respectively) in two equal meals. Rumen fluid was withdrawn, via the cannula, 2 h after the morning feed and, after mixing the samples from the three sheep, it was strained through two layers of muslin and maintained at 39° under O_2-free CO_2. Rumen fluid was anaerobically transferred (200 ml/l) to a Simplex-type buffer containing (/l): 5 g K_2HPO_4, 4 g KH_2PO_4, 0·52 g NaCl, 70 mg $MgSO_4.7H_2O$, 35 mg $CaCl_2$, 5·9 g $NaHCO_3$ and 174 mg cysteine hydrochloride, which had been prepared as described by Coleman (1987). After mixing, 50 ml buffered rumen fluid was anaerobically dispensed into each of twelve 120 ml serum bottles containing 200 mg of the diet described earlier, previously ground to pass through a 1 mm mesh screen. Weighed amounts of fumarate (disodium salt; Sigma Chemical Co., Poole, Dorset, UK) were added to achieve final concentrations of 0, 5 and 10 mmol/l. The bottles were sealed (CO_2 atmosphere) with rubber stoppers and aluminium caps and were placed in a shaking water bath at 39°. After 24 h incubation, total gas production was measured using a 100 ml lubricated syringe connected to a needle, which was inserted through the stoppers into the headspace. A gas sample (1 ml) was removed from each bottle and analysed for CH_4 and H_2 by GLC on a 4 mm × 3 m glass column packed with Porapak Q mesh 60-80 (Waters Associates Inc., Milford, MA, USA). The oven temperature was 250° and the carrier gas (He) flow rate was 30 ml/min; a katherometer detector was used. Peaks were identified by comparison with gas standards of known composition. The bottles were then uncapped and the pH was measured using a pH electrode connected to a Russell 660 pH meter (Russell pH, Auchtermuchty, Fife, UK). Fermentation fluid (4 ml) was added to 1 ml of an acid solution containing 200 ml orthophosphoric acid/l and 20 mM-2-ethyl-butyric acid. Acidified samples were centrifuged (27 800 ***g***, 5°, 15 min) and volatile fatty acids were determined by GLC using ethylbutyric acid as the internal standard as described by Stewart & Duncan (1985).

Fermentation in the Rusitec

The Rusitec was used as described by Czerkawski & Breckenridge (1977). The nominal volume in each reaction vessel was 850 ml and the dilution rate was set at 0·88 per d, the infused liquid being artificial saliva (McDougall, 1948) at pH 8·4. Inocula for the fermentation vessels were obtained from a pooled sample (liquid and particulate rumen contents) from the three rumen-cannulated sheep used in the *in vitro* trial. On the first day of the experiment 300 ml strained rumen fluid and 300 ml artificial saliva were placed in each reaction vessel. Solid rumen contents (80 g) were weighed into a nylon bag and one of these was placed inside the food container in each vessel together with a bag of food. The food was the same forage–concentrate diet fed to the donor animals, and 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. The bags that were removed from the vessels were placed in plastic bags, and their contents washed and squeezed with 40 ml artificial saliva. This was done twice for each bag, and the combined washings were poured back into the reaction vessels. Fermentation vessels were flushed with anaerobic-grade CO_2 before filling, after filling, and then every day during feeding (when the nylon bags with the food were changed).

Eight vessels were set up as described earlier (Czerkawski & Breckenridge, 1977) and were supplied at the same time every day with 20 g of the basal diet fed to the donor animals. The duration of the experiment was 26 d, which consisted of period 1 (days 1–19) in which four vessels received 1 g fumarate (disodium salt) daily, which was added to the basal diet, the remaining vessels being controls, and period 2 (days 20–26) in which no fumarate was added to any vessel. Gas samples were taken over days 8–19 and on the last 2 d in period 2, and analysed by GLC as described earlier, to determine H_2 and CH_4 concentrations.

During the last 2 d of each period, fermentation products were determined on samples taken from the liquid overflow, as described by Newbold *et al.* (1997). Fermentation acids were detected by capillary GC, using the derivatization method described by Richardson *et al.* (1989). NH_3 was measured by the phenol–hypochlorite method of Weatherburn (1967). In liquid samples from period 2, volatile fatty acids were analysed, with no derivatization, by GLC (Stewart & Duncan, 1985). The volume of the liquid overflow, together with the concentration of fermentation products, was used to calculate the daily output of fermentation products. pH was measured as described earlier in samples of fermentation fluid withdrawn from around the nylon bags at the time of feeding. The digestibility of the diet was estimated from the DM remaining in the bags after 48 h incubation. DM in feed samples and in incubation residues was determined by drying at 105° for 48 h.

Samples for microbial counting were taken on the last 2 d of the first period (days 18 and 19) of the Rusitec experiment. Protozoa in the liquid phase were enumerated microscopically in a counting chamber (Newbold *et al.* 1987). Hungate's methods (Hungate, 1969) were used to prepare media and to cultivate bacteria. Media were dispensed into Hungate tubes sealed with butyl rubber stoppers (Belco Glass Inc., Vineland, NJ, USA). A sample of fermentation fluid (20 ml) taken directly from the reaction vessel and a sample of the digesta (1 g) remaining in the nylon bags incubated for the last 48 h were homogenized together, under O_2-free CO_2, for 1 min using an MSE top-bladed

homogenizer (MSE, Crawley, Sussex, UK) at full speed. Serial 10-fold dilutions were prepared under O_2-free CO_2 for each sample by the anaerobic method of Bryant (1972) using an anaerobic diluent (Mann, 1968). Total viable bacteria were enumerated in roll tubes with a complex rumen fluid–sugars medium to which 20 g agar/l was added (Medium M2, Hobson, 1969), and these tubes were incubated for 72 h at 39°. Cellulolytic counts were done by a most-probable-number method based on the degradation of filter paper strips (Mann, 1968). Numbers of methanogenic archaea were determined by a most-probable-number technique, based on the production of CH_4 in a medium containing formate, acetate and H_2 (Morvan *et al.* 1994).

Statistical analyses

In vitro incubations were performed on 2 d with two replicates per d (n 4). Data were analysed as a one-way ANOVA with three concentrations of fumarate (0, 5, 10 mmol/l). Orthogonal polynomial contrasts were performed to study linear effects (SAS Institute Inc., 1989). Comparisons between treatment means were tested by the least significant difference method. For the trial carried out in the Rusitec, reaction vessels were randomly assigned to control (untreated) and fumarate groups. Variables measured over 12 d (gas and CH_4 production, DM digestibility) were analysed as a repeated measures ANOVA with the Greenhouse-Geisser adjustment as given by PROC GLM in the Statistical Analysis Systems program (SAS Institute Inc., 1989). Effect of fumarate addition was tested using vessel within treatment (df 6) as the error term, whereas sampling day was the within-subject effect (SAS Institute Inc., 1989) as repeated measures taken on the same experimental unit. For all the other variables, treatment effects were established by a t test (Steel & Torrie, 1980), and significance was declared at $P < 0{\cdot}05$ unless otherwise stated. Computations were performed using the general linear models procedure of the Statistical Analysis Systems program (SAS Institute Inc., 1989).

Results

In vitro *batch incubations*

The addition of fumarate significantly increased the final pH ($P < 0{\cdot}05$) and total gas production ($P < 0{\cdot}01$) during the 24 h incubation (Table 1). The CH_4 concentration in the gas produced was significantly ($P < 0{\cdot}05$) decreased by the addition of fumarate (256, 229 and 219 mmol CH_4/mol gas for 0, 5 and 10 mM-fumarate respectively), thus resulting in a significant ($P < 0{\cdot}05$) fall in CH_4 production as the dose of fumarate added to the medium increased. However, 250 and 500 μmol fumarate caused decreases of only 18 and 22 μmol in CH_4 production (5·3 and 6·4 % of the CH_4 produced in the control cultures).

Acetate production was increased ($P < 0{\cdot}01$) by about 100 μmol for each 250 μmol fumarate addition, whereas fumarate addition had no significant ($P > 0{\cdot}10$) effect on butyrate production. Propionate production increased ($P < 0{\cdot}001$) by about 200 μmol for each 250 μmol fumarate added.

Effects of fumarate on fermentation in the Rusitec

Total gas production was unaffected ($P > 0{\cdot}05$) by fumarate during period 1, whereas CH_4 production measured over days 8–19 of the experiment was significantly decreased ($P < 0{\cdot}05$) by fumarate addition (Table 2), but only by 1·2 mmol (17 %) for an addition of 6·25 mmol sodium fumarate. This effect was consistent over the time that fumarate was added to the vessels, with no significant ($P = 0{\cdot}361$) differences between sampling days and no significant interaction ($P = 0{\cdot}669$) between treatment and sampling day (both P values are those adjusted for a Greenhouse-Geisser epsilon of 0·2942). The inhibition in CH_4 production by adding fumarate did not result ($P > 0{\cdot}05$) in the accumulation of H_2 gas. Adding fumarate also increased the digestion of the basal diet in the fermenter, since the DM digestibility was 6·3 % higher when fumarate was added to the diet compared with the control cultures. The only significant effect of fumarate on microbial numbers was that cellulolytic bacteria were increased ($P < 0{\cdot}01$). Methanogenic archaea were unaffected. Fumarate had no significant ($P > 0{\cdot}05$) effect on the pH in the fermentation vessels (Table 3). Fumarate addition resulted in a significant ($P < 0{\cdot}01$) increase in propionate production, but did not affect the production of formate, lactate, butyrate and other volatile fatty acids (Table 3). An increase of 4·9 mmol propionic acid occurred in response to the

Table 1. Influence of sodium fumarate on 24 h fermentation by mixed rumen micro-organisms in *in vitro* batch cultures*
(Mean values for four fermentations with the standard error of difference between means)

	Sodium fumarate added (μmol)			SED	Statistical significance of the treatment effect: $P=$	
	0	250	500	(df 8)	C *v.* F†	L‡
pH	6·42[a]	6·49[b]	6·50[b]	0·023	0·0145	0·0241
Total gas production (μmol)	1412[a]	1496[b]	1544[b]	31·8	0·0045	0·0033
Methane (μmol)	360[a]	342[b]	338[b]	7·5	0·0456	0·0473
Volatile fatty acids (μmol)	1228[a]	1541[b]	1820[c]	45·2	0·0002	0·0002
Acetate (μmol)	789[a]	881[b]	983[c]	26·7	0·0027	0·0021
Propionate (μmol)	259[a]	471[b]	655[c]	13·0	0·0001	0·0001
Butyrate (μmol)	138	151	145	6·8	0·6578	0·9836

[a,b,c] Mean values within a row not sharing a common superscript letter were significantly different, $P < 0{\cdot}05$.
* For details of procedures, see pp. 60–61.
† Orthogonal contrasts, C *v.* F: comparison between control and fumarate treatment.
‡ Orthogonal polynomials, linear (L) effects of fumarate dose.

Table 2. Influence of sodium fumarate addition (6·25 mmol/d)* on the output of methane and hydrogen from, and the digestion of, dry matter and on microbial numbers in the rumen simulation technique (Rusitec)†

(Mean values for four vessels, with the standard error of difference between means)

	Control	Fumarate	SED (6 df)	Statistical significance of the difference, $P=$
Gas production (mmol/d)‡	58·0	54·2	3·51	0·3196
Hydrogen (mmol/d)‡	0·31	0·22	0·116	0·4675
Methane (mmol/d)‡	7·0	5·7	0·39	0·0167
Digestion of DM (g/kg) after 48 h incubation‡	476	508	12·3	0·0410
Total bacteria ($\times 10^8$/ml)§	5·4	4·7	0·69	0·3495
Methanogenic archaea ($\times 10^6$/ml)§	14·2	14·9	4·21	0·8734
Cellulolytic bacteria ($\times 10^5$/ml)§	8·8	23·9	2·49	0·0010
Protozoa ($\times 10^3$/ml)§	4·4	3·8	0·68	0·4115

* Sodium fumarate was added to Rusitec vessels for 19 d.
† For details of procedures, see pp. 60–61 .
‡ Each value is the mean of the observations taken during the last 12 d of fumarate addition.
§ Each value is the mean of the observations taken during the last 2 d of fumarate addition.

addition of 6·25 mmol sodium fumarate. Fumarate also caused the accumulation of succinate in the culture, whereas no succinate was detected in the effluent of control vessels. NH_3 production was unaffected by the addition of fumarate. When fumarate treatment was stopped the differences in DM digestibility, CH_4 and volatile fatty acid production disappeared (Table 4).

Discussion

Recently, some dicarboxylic acids, such as aspartate, malate and fumarate, have been tested as feed additives for ruminants (Callaway & Martin, 1996; Martin & Park, 1996). The results presented in the present paper describe the short- and long-term effects of one of these dicarboxylic acids, sodium fumarate, on rumen fermentation.

The 6 % decrease in CH_4 formation found when fumarate was added to batch cultures in the present study is fairly consistent with the low response found by Callaway & Martin (1996), but very different to the value of 60 % found by Demeyer & Henderickx (1967). Since many factors can influence CH_4 production, responses may

Table 3. Influence of sodium fumarate (6·25 mmol/d)* on 24 h fermentation (output of end-products, mmol/d) in the rumen simulation technique (Rusitec)†

(Mean values for four vessels, with the standard error of difference between means)

	Control	Fumarate	SED (6 df)	Statistical significance of the difference, $P=$
pH	6·74	6·77	0·048	0·5768
Formate	0·49	0·43	0·056	0·3586
Acetate	26·2	29·7	2·30	0·1818
Propionate	10·4	15·3	1·05	0·0033
Butyrate	8·6	8·7	0·86	0·8776
Valerate	3·3	4·1	0·33	0·0572
Isobutyrate	0·29	0·31	0·033	0·5587
Isovalerate	0·16	0·20	0·038	0·3216
Succinate	0·00	0·47	0·053	0·0001
Lactate	0·21	0·08	0·130	0·3626
Ammonia	2·77	2·75	0·246	0·9378

* Sodium fumarate was added to Rusitec vessels for 19 d, and each value is the mean of the observations taken during the last 2 d of that period.
† For details of procedures, see pp. 60–61.

Table 4. Fermentation characteristics of rumen simulation technique (Rusitec) vessels following removal of the fumarate treatment*†

(Mean values for four vessels, with the standard error of difference between means)

	Previous treatment			
	Control	Fumarate	SED (6 df)	Statistical significance of the difference, $P=$
Methane (mmol/d)	6·6	6·5	1·16	0·9341
Digestion of DM (g/kg) after 48 h incubation	487	499	13·5	0·4083
Acetate (mmol/d)	24·4	22·1	1·48	0·1712
Propionate (mmol/d)	12·8	13·3	0·97	0·6247
Butyrate (mmol/d)	10·6	9·5	0·84	0·2383

* For details of procedures, see pp. 60–61.
† Sodium fumarate had been added to Rusitec vessels for 19 d, and each value is the mean of the observations taken over days 6–7 after withdrawal of the fumarate treatment.

well be different with different diets (García-López *et al.* 1996), and indeed the pH of rumen fluid can have a major influence on CH_4 production (Van Kessel & Russell, 1996). Here, the pH was much higher than the critical value of 6·0 enabling maximal CH_4 formation (Van Kessel & Russell, 1996).

The decrease in CH_4 production observed in batch cultures was very low considering the amounts of fumarate added, and appeared to increase only slightly when the dose was increased from 250 to 500 μmol per incubation. Fumarate was a more effective alternative hydrogen sink to CH_4 in the Rusitec compared with batch cultures. Based on the equation $4H_2 + CO_2 = CH_4 + 2H_2O$ and the reduction of fumarate to succinate by 2H, in the Rusitec 6·25 mmol fumarate caused a 1·2 mmol fall in CH_4, equivalent to an efficiency of hydrogen trapping of $1{\cdot}2 \times 4/6{\cdot}25 = 77\,\%$, indicating that this organic acid may act as an effective hydrogen sink, competing with methanogenesis. But the inhibition achieved was still only 17 %, and thus fumarate would be impractical as a means of eliminating CH_4 emissions *in vivo*. The fumarate concentrations were indeed high, selected to be similar to the anticipated CH_4 production, so that the maximum potential of fumarate to divert H_2 away from CH_4 could be explored. It emerged that the limit was quite low, presumably because of the much lower affinity of fumarate reductase for H_2 than hydrogenase of methanogenic archaea.

There may be other beneficial effects of fumarate, however. During fermentation in the Rusitec, fumarate stimulated the numbers of cellulolytic bacteria threefold. The reason for this increase is not clear. Although it has been demonstrated that cellulolytic organisms benefit from the presence of methanogenic or other H_2-utilizing bacteria because of interspecies H_2 transfer (Wolin & Miller, 1988), H_2 accumulation was not affected by fumarate in the Rusitec. Nevertheless, an increased DM digestibility was observed in the Rusitec trial, consistent with the increased numbers of cellulolytic bacteria, indicating that one of the beneficial effects of fumarate may be to increase fibre digestion.

Fumarate and other dicarboxylic acids also promote lactate utilization by the predominant ruminal anaerobe *S. ruminantium* (Nisbet & Martin, 1990), stimulating its growth *in vitro* (Nisbet & Martin, 1993). Improved removal of lactate may help to prevent acidosis, and would indirectly stimulate fibre digestion. However, lactate concentrations in the Rusitec were very low, and pH was stable, so this effect would be of minimal importance in the present experiment. The addition of fumarate also resulted in a significant increase in total gas production in batch cultures, which has to be attributed to increased CO_2 production, as there was no effect on H_2 production and CH_4 was decreased. The increase in CO_2 production may act together with the removal of lactate to buffer ruminal fermentation.

Redirecting the hydrogen produced during rumen fermentation caused changes in the outputs of other metabolic intermediates and end-products. The response to the daily addition of 6·25 mmol fumaric acid to the Rusitec vessels was a decrease in CH_4 production of 1·2 mmol (equivalent to the consumption of 4·8 mmol H_2) and an increase in propionate production of 4·9 mmol. Thus, the decrease in CH_4 corresponded well to the fraction of the fumarate that was converted to propionate. The conversion via succinate, and thereafter to propionate, was the way by which most (89 %) of the added fumarate was fermented in the Rusitec. Most of the fumarate was recovered as propionate and a small part as succinate, which accumulated before being converted into propionate. The recoveries of fumarate as propionate in the batch cultures were 85 % and 79 % for the 5 and 10 mmol/l additions respectively, which is in reasonable agreement with results observed in the Rusitec. However, H recoveries in the Rusitec (calculated from 2H produced $= 2A + P + 4B + 3V$, 2H recovered $= 2P + 2B + 4V + 4CH_4 + H_2$, where A is acetate, P is propionate, B is butyrate and V is valerate; Marty & Demeyer, 1973) were lower (74 and 72 % for the control and fumarate treatments respectively) than those recorded in the batch cultures (92–98 %). This inconsistency may indicate that some reduced end-products were missing from the analysis or that alternative pathways may have been operative, possibly direct utilization of O_2 (Demeyer *et al.* 1972), resulting from the higher rate of O_2 leakage in the Rusitec (Hillman *et al.* 1985). It will be important to confirm the effects of fumarate on ruminal stoichiometry *in vivo*.

Organic acids, and fumarate in particular, can be converted into propionate and acetate following different pathways (Demeyer & Henderickx, 1967). Increases in acetate were observed in batch and semi-continuous cultures, although the increase in acetate production was not significant in the Rusitec. Other additives give rise to increases in propionate at the expense of acetate.

Little information is available on the effects of fumarate on ruminal digestion and animal performance *in vivo*. Isobe & Shibata (1993) observed that ruminal fermentation in goats was enhanced by the addition of fumarate, increasing cellulose digestion, which is in agreement with our results in the Rusitec. There is also evidence that other dicarboxylic acids such as malate may improve animal performance in dairy (Kung *et al.* 1982) and beef cattle (Sanson & Stallcup, 1984; Streeter *et al.* 1994). The results of the present study suggest that dietary fumarate would have a beneficial effect via decreased methanogenesis, increased propionate production and stimulation of fibre breakdown by rumen micro-organisms.

Acknowledgements

S. López was supported by a fellowship under the OECD Project on Biological Resources Management. C. Valdés was supported by a fellowship of the DGICYT of the Spanish Ministry of Education and Science. Financial support by the collaborative research project 'Acción Integrada HB1996-0150' is also gratefully appreciated. The Rowett Research Institute is funded by The Scottish Office Agriculture Environment and Fisheries Department. We thank Freda McIntosh for skilled technical assistance.

References

Bryant MP (1972) Commentary on the Hungate technique for culture of anaerobic bacteria. *American Journal of Clinical Nutrition* **25**, 1324–1328.

Callaway TR & Martin SA (1996) Effects of organic acid and monensin treatment on *in vitro* mixed ruminal micro-organisms

fermentation of cracked corn. *Journal of Animal Science* **74**, 1982–1989.
Coleman GS (1987) Rumen entodiniomorphid protozoa. In In Vitro *Methods for Parasite Cultivation*, pp. 29–51 [AER Taylor and JR Baker, editors]. London: Academic Press.
Czerkawski JW & Breckenridge G (1977) Design and development of a long term rumen simulation technique (Rusitec). *British Journal of Nutrition* **38**, 371–384.
Demeyer DI & Henderickx HK (1967) Competitive inhibition of *in vitro* methane production by mixed rumen bacteria. *Archives Internationals de Physiologie et de Biochimie* **75**, 157–159.
Demeyer DI, Van Nevel CJ & Henderson C (1972) Stoichiometry of oxygen utilization by rumen contents. In *Proceedings of the 2nd World Congress on Animal Feeding*, vol. 5, part 2, pp. 33–37 [International Veterinary Association for Animal Production, editors]. Madrid: Industrias Gráficas España.
García-López PM, Kung L & Odom JM (1996) *In vitro* inhibition of microbial methane production by 9,10-anthraquinone. *Journal of Animal Science* **74,** 2276–2284.
Hillman K, Lloyd D & Williams AG (1985) Continuous monitoring of fermentation gases in an artificial rumen system (Rusitec) using a membrane-inlet probe on a portable quadrupole mass spectrometer. In *Gas Enzymology*, pp. 201–206 [D Degn, editor]. Dordrecht: Reidel Publishing Company.
Hobson PN (1969) Rumen bacteria. *Methods in Microbiology* **3**B, 133–159.
Hungate RE (1969) A roll tube method for cultivation of strict anaerobes. *Methods in Microbiology* **3**B, 117–132.
Isobe Y & Shibata F (1993) Rumen fermentation in goats administered fumaric acid. *Animal Science and Technology* **64**, 1024–1030.
Kung L, Huber JT, Krummrey JD, Allison L & Cook RM (1982) Influence of adding malic acid to dairy cattle rations on milk production, rumen volatile acids, digestibility, and nitrogen utilization. *Journal of Dairy Science* **65**, 1170–1174.
McDougall EI (1948) Studies on ruminal saliva. 1. The composition and output of sheep's saliva. *Biochemical Journal* **43**, 99–109.
Mann SO (1968) An improved method for determining cellulolytic activity in anaerobic bacteria. *Journal of Applied Bacteriology* **31**, 241–244.
Martin SA & Park CM (1996) Effect of extracellular hydrogen on organic acid utilization by the ruminal bacterium *Selenomonas ruminantium*. *Current Microbiology* **32**, 327–331.
Marty RJ & Demeyer DI (1973) The effect of inhibitors of methane production on fermentation pattern and stoichiometry in vitro using rumen contents from sheep given molasses. *British Journal of Nutrition* **30**, 369–376.
Morvan B, Doré J, Rieu-Lesme F, Foucat L, Fonty G & Gouet P (1994) Establishment of hydrogen-utilizing bacteria in the rumen of the newborn lamb. *FEMS Microbiology Letters* **117**, 249–256.
Moss AR (1993) *Methane Global Warming and Production by Animals*. Canterbury: Chalcombe Publications.
Newbold CJ, Wallace RJ & McIntosh FM (1997) Mode of action of the yeast *Saccharomyces cerevisiae* as a feed additive for ruminants. *British Journal of Nutrition* **76**, 249–261.
Newbold CJ, Williams AG & Chamberlain DG (1987) The *in vitro* metabolism of D,L-lactic acid by rumen microorganisms. *Journal of the Science of Food and Agriculture* **38**, 9–19.
Nisbet DJ & Martin SA (1990) Effect of dicarboxylic acids and *Aspergillus oryzae* fermentation extract on lactate uptake by the ruminal bacterium *Selenomonas ruminantium*. *Applied and Environmental Microbiology* **56**, 3515–3518.
Nisbet DJ & Martin SA (1993) Effects of fumarate, L-malate, and an *Aspergillus oryzae* fermentation extract on D-lactate utilization by the ruminal bacterium *Selenomonas ruminantium*. *Current Microbiology* **26**, 133–136.
Richardson AJ, Calder AG, Stewart CS & Smith A (1989) Simultaneous determination of volatile and non-volatile fatty acidic fermentation products of anaerobes by capillary gas chromatography. *Letters in Applied Microbiology* **9**, 5–8.
Sanson DW & Stallcup OT (1984) Growth response and serum constituents of Holstein bulls fed malic acid. *Nutrition Reports International* **30**, 1261–1267.
SAS Institute Inc. (1989) *SAS/STAT® User's Guide*, version 6, 4th ed. Cary, NC: SAS Institute Inc.
Steel RGD & Torrie JH (1980) *Principles and Procedures of Statistics*, 2nd ed. London: McGraw-Hill.
Stewart CS & Duncan SH (1985) The effect of avoparcin on cellulolytic bacteria of the ovine rumen. *Journal of General Microbiology* **131**, 427–435.
Streeter MN, Nisbet DJ, Martin SA & Williams SE (1994) Effect of malate on ruminal metabolism and performance of steers fed a high concentrate diet. *Journal of Animal Science* **72**, Suppl. 1, 384 Abstr.
Van Kessel JAS & Russell JB (1996) The effect of pH on ruminal methanogenesis. *FEMS Microbiology Ecology* **20**, 205–210.
Van Nevel CJ & Demeyer DI (1996) Control of rumen methanogenesis. *Environmental Monitoring and Assessment* **42**, 73–97.
Weatherburn MW (1967) Phenol–hypochlorite reaction for determination of ammonia. *Analytical Chemistry* **39**, 971–974.
Wolin MJ & Miller TL (1988) Microbe–microbe interactions. In *The Rumen Microbial Ecosystem*, pp. 343–359 [PN Hobson, editor]. London: Elsevier Applied Science.