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Influence of different concentrations of disodium fumarate on methane production and fermentation of concentrate feeds by rumen micro-organisms *in vitro*

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Batch cultures of mixed rumen micro-organisms were used to study the effects of different concentrations of disodium fumarate on the fermentation of five concentrate feeds (maize, barley, wheat, sorghum and cassava meal). Rumen contents were collected from four Merino sheep fed lucerne hay *ad libitum* and supplemented with 300 g concentrate/d. Disodium fumarate was added to the incubation bottles to achieve final concentrations of 0, 4, 7 and 10 mM-fumarate. In 17 h incubations, the final pH and total volatile fatty acid production increased ($P<0·001$) linearly for all substrates as fumarate concentration increased from 0 to 10 mM. Propionate and acetate production increased ($P<0·05$), while the value of the acetate:propionate ratio decreased ($P<0·05$) linearly with increasing doses of fumarate. In contrast, L-lactate and NH_3-N concentrations in the cultures were not affected ($P>0·05$) by the addition of fumarate. For all substrates, fumarate treatment decreased ($P<0·05$) CH_4 production, the mean values of the decrease being 2·3, 3·8 and 4·8 % for concentrations of 4, 7 and 10 mM-fumarate respectively. Addition of fumarate did not affect ($P>0·05$) the total gas production. If the results of the present experiment are confirmed *in vivo*, fumarate could be used as a feed additive for ruminant animals fed high proportions of cereal grains, because it increased pH, acetate and propionate production and it decreased CH_4 production.

Batch cultures: Concentrate feeds: Fumarate: Rumen

High-producing ruminant animals are usually fed high amounts of concentrates. However, negative effects are often observed (Mould, 1988; Carro *et al.* 2000) with diets containing high levels of concentrates (decrease in rumen pH, inhibition of fibre degradation, accumulation of lactic acid, etc.) and therefore antimicrobial compounds are routinely incorporated as feed additives to improve production efficiency. In recent years, there has been increasing concern about the use of antibiotics in animal feeding, and in March 2002 the European Union presented a new proposal that would phase out the authorizations for the four antibiotics being used as growth promoters in animal feed in the European market by January 2006. As a consequence, there is an urgent need for the development of alternatives to the use of these feed additives. Some authors (Callaway & Martin, 1996; Newbold *et al.* 1996) have suggested that organic acids (aspartate, fumarate, malate) may provide an alternative to currently used antibiotic compounds in animals fed high proportions of concentrates.

Nisbet & Martin (1990) showed that the growth of *Selenomonas ruminantium* HD4 in a medium that contained lactate was stimulated approximately twofold by 10 mM-L-aspartate, fumarate or L-malate after 24 h of incubation; moreover, both L-aspartate and fumarate increased L-lactate uptake by *S. ruminantium* more than fourfold, whereas L-malate stimulated uptake more than tenfold. Based on these results, most of the research conducted on the effects of organic acids on rumen fermentation has focused on malate. Both malate and fumarate are key intermediates in the succinate–propionate pathway, which is used by *S. ruminantium* to synthesize succinate and propionate (Martin, 1998). However, compared with the efforts to detail the effects of malate on rumen fermentation, very little research has been conducted to evaluate the effects of fumarate, and most of this work has been conducted with diets containing medium or high proportions of forages (Asanuma *et al.* 1999; López *et al.* 1999). As the effects of fumarate could be influenced by the doses of fumarate and the composition of the diet, the objective of the present study was to evaluate the effects of different concentrations of fumarate on the *in vitro* rumen fermentation of five concentrate feeds (maize, barley, wheat, sorghum and cassava meal).

Abbreviation: VFA, volatile fatty acid.

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Materials and methods

Substrates and experimental procedure

Samples of maize, barley, wheat, sorghum and cassava meal were ground through a 1 mm screen and fermented *in vitro* with buffered rumen contents. Rumen contents were obtained from four rumen-cannulated Merino sheep fed forage (medium-quality lucerne hay) *ad libitum* and 300 g concentrate per d administered in two equal portions at 09.00 and 18.00 hours. Concentrate was based on barley–maize–soyabean meal (39:40:23, by weight on a fresh matter basis). The chemical composition of feeds is given in Table 1. The rumen contents of each sheep were obtained 2 h after the morning feed of concentrate, mixed and strained through four layers of cheesecloth into an Erlenmeyer flask with an O_2-free headspace. Particle-free fluid was mixed with the buffer solution of Goering & Van Soest (1970) in the proportion 1:4 (v/v) at 39°C under continuous flushing with CO_2. Samples of 500 mg of each feed were weighed accurately into 125 ml serum bottles (Laboratorios Ovejero S.A., León, Spain). Fumarate (disodium salt; Sigma, Madrid, Spain) was added to achieve final fumarate concentrations of 0, 4, 7 and 10 mM. Bottles were prewarmed (39°C) prior to the addition of 50 ml buffered rumen contents into each bottle under CO_2 flushing. Bottles were sealed with rubber stoppers and Al caps and incubated at 39°C. The experiment was repeated on four different days, so that each treatment was conducted in quadruplicate.

A total of twenty bottles with substrate (one bottle for each substrate and fumarate concentration) and eight bottles without substrate (two for each fumarate concentration) were incubated each day. Bottles were withdrawn from the incubator 17 h after inoculation (corresponding to a passage rate from the rumen of 0·06 per h) and total gas production was measured using a pressure transducer. A gas sample was removed from each bottle and stored in a Haemoguard vacutainer (Terumo Europe N.V., Leuven, Belgium) before analysis for CH_4 concentration. Bottles were uncapped, the pH was measured immediately with a pH meter and the fermentation was stopped by swirling the bottles on ice. Bottles were emptied into centrifuge tubes and centrifuged (600 ***g***, 4°C, 10 min) to eliminate feed particles. A portion of the supernatant fraction (1 ml) was added to 1 ml deproteinizing solution (metaphosphoric acid (100 ml/l) and crotonic acid (0·6 ml/l)) for volatile fatty acid (VFA) analysis and another 2 ml were added to 2 ml 0·5 M-HCl for NH_3-N analysis. Samples were stored at −20°C. A sample of the supernatant fraction (1·6 ml) was added to 0·1 ml deproteinizing solution (metaphosphoric acid, 200 ml/l), centrifuged (15 000 ***g***, 4°C, 10 min), the pH adjusted to 7·2 with 1 M-NaOH, and concentrations of L-lactate were analysed by an enzymatic–colorimetric method using a diagnostic kit (Sigma). Finally, the contents of the centrifuge tube were transferred to previously weighed filter crucibles. The residue of incubation was washed with 50 ml hot distilled water, dried at 50°C for 48 h and analysed for ash to calculate the apparent disappearance of organic matter.

Analytical procedures

DM, ash and N were determined according to the Association of Official Analytical Chemists (1995). Neutral- and acid-detergent fibre analyses were carried out according to Van Soest *et al.* (1991) and Goering & Van Soest (1970) respectively. NH_3-N concentration was determined by a modified colorimetric method (Weatherburn, 1967). VFA were determined in centrifuged samples (1 ml) by GC as described previously (Carro *et al.* 1999). CH_4 was analysed with a GC (Shimadzu GC 14B; Shimadzu Corporation, Kyoto, Japan) equipped with a flame ionization detector and a column packed with Carboxen 1000 (Supelco, Madrid, Spain). The carrier gas was He and peaks were identified by comparison with a standard of known composition. The volume of gas produced (ml) was corrected for standard conditions (10^5 Pa, 298 K), and the amount of CH_4 produced (μmol) was calculated by multiplying the gas produced (μmol) by the concentration of CH_4 in the analysed sample.

Calculations and statistical analysis

The amounts of VFA produced were obtained by subtracting the amounts present initially in the incubation medium from those determined at the end of the incubation period. Gas production after 17 h of incubation was corrected for gas release from endogenous substrates and added fumarate for each inoculum and fumarate concentration. Data for each substrate were analysed as a one-way ANOVA with four concentrations of fumarate (0, 4, 7 and 10 mM). The sums of squares were further partitioned by orthogonal

Table 1. Chemical composition (g/kg DM) of ingredients of sheep diet and concentrate feeds incubated *in vitro*

	Organic matter	Crude protein*	Neutral-detergent fibre	Acid-detergent fibre
Diet ingredients				
Lucerne hay	912	158	472	301
Concentrate	916	198	151	47
Cereal grains				
Maize	985	90	119	23
Barley	974	116	176	43
Wheat	983	103	142	29
Sorghum	982	114	106	32
Cassava meal	951	21	189	77

* N × 6·25.

polynomial contrast to study linear effects of treatment. Comparisons between treatment means were tested by the least significant difference method. All statistical analyses were performed using the GLM procedure of the Statistical Analysis Systems program (version 6, 1989; SAS Institute Inc., Cary, NC, USA).

Results

The effects of fumarate on *in vitro* rumen fermentation of maize, barley, wheat, sorghum and cassava meal are shown in Tables 2, 3, 4, 5 and 6 respectively. For all substrates, final pH increased linearly ($P<0·001$) as concentrations of fumarate increased, although there were no significant ($P>0·05$) differences between 7 and 10 mM-fumarate. For maize, 10 mM-fumarate increased ($P<0·05$) the apparent disappearance of organic matter, but there were no treatment effects ($P>0·05$) on the apparent disappearance of organic matter for the other substrates.

Whereas the addition of fumarate decreased linearly ($P<0·05$), the production of CH_4 for all substrates, no differences ($P>0·05$) were found in the total amount of gas produced. There were no differences ($P>0·05$) in the production of CH_4 between fumarate at 7 and 10 mM. With all substrates, fumarate treatment increased ($P<0·001$) total VFA production linearly, the greatest values being found at 7 and 10 mM-fumarate. Fumarate treatment increased acetate ($P<0·01$) and propionate ($P<0·001$) production and decreased ($P<0·001$) the acetate:propionate value for all substrates. There was no treatment effect ($P>0·05$) on the production of butyrate and other VFA (calculated as the sum of isobutyrate, isovalerate and valerate) for wheat and sorghum, but the addition of fumarate increased ($P=0·040$) the production of other VFA with maize, and tended ($P=0·057$) to decrease the production of butyrate for cassava meal. With all substrates, adding increasing concentrations of fumarate to batch cultures decreased ($P<0·001$) the CH_4 (μmol):VFA (μmol) value linearly.

There was no significant change ($P>0·05$) in the amount of NH_3-N produced with added fumarate for barley, wheat, sorghum and cassava meal, but fumarate tended to decrease ($P=0·089$) NH_3-N concentration when maize was incubated. There was no effect ($P>0·05$) of fumarate on the concentration of L-lactate in the cultures.

In the absence of exogenous substrates, fumarate addition increased ($P<0·001$) both final pH and gas production, with no differences ($P>0·05$) between 7 and 10 mM (Table 7). Fumarate treatment did not affect ($P>0·05$) the production of CH_4 or NH_3-N concentration. Acetate, propionate and total VFA production were increased ($P<0·001$) by the addition of fumarate, and the values of the acetate:propionate and CH_4:VFA ratios were decreased ($P<0·001$).

Discussion

In the last few years, several papers have investigated the effects of some dicarboxylic acids, including malate, fumarate and aspartate, on rumen fermentation and digestibility of diet in ruminant animals (Callaway & Martin, 1996; López *et al.* 1999; Carro & Ranilla, 2003), although most of the studies have focused on malate. Few studies have been conducted with fumarate, and in most of them animals were fed roughage alone (Bayaru *et al.* 2001) or a mixture of roughage and concentrate was used as

Table 2. Influence of concentration of disodium fumarate on *in vitro* fermentation of maize by mixed rumen micro-organisms in batch cultures†

(Mean values for four fermentations)

	Fumarate (mM)					Statistical significance of the treatment effect ($P=$)	
	0	4	7	10	SED	Control *v.* fumarate‡	Linear§
pH	6·28[a]	6·33[b]	6·36[bc]	6·39[c]	0·019	0·001	0·001
Apparent disappearance of organic matter (%)	69·0[a]	70·4[ab]	70·1[ab]	72·1[b]	1·38	NS	NS*
Gas (μmol)	4076	4063	4022	4018	54·9	NS	NS
CH_4 (μmol)	646[b]	637[b]	615[ab]	598[a]	15·4	0·042	0·007
VFA (μmol)							
Acetate	1600[a]	1706[b]	1731[bc]	1794[c]	29·6	0·001	0·001
Propionate	975[a]	1150[b]	1211[bc]	1259[c]	30·2	0·001	0·001
Butyrate	386	346	381	396	36·6	NS	NS
Others‖	68·3[a]	79·3[b]	71·5[ab]	77·5[b]	0·0047	0·040	NS
Total VFA	3030[a]	3280[b]	3395[bc]	3526[c]	72·7	0·001	0·001
Acetate (μmol):propionate (μmol)	1·65[b]	1·49[a]	1·43[a]	1·43[a]	0·035	0·001	0·001
CH_4 (μmol):VFA (μmol)	0·214[c]	0·194[b]	0·181[ab]	0·170[a]	0·0062	0·001	0·001
NH_3-N (μmol)	663	630	634	654	20·4	NS*	NS
L-Lactate (μmol)	3·43	3·38	3·41	3·34	0·305	NS	NS

VFA, volatile fatty acid.
[a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).
* $P<0·10$.
† 50 ml diluted buffered rumen contents were incubated for 17 h with 500 mg ground maize; for details of diets and procedures, see Table 1 and p. 618.
‡ Orthogonal contrast, control *v.* fumarate: comparison between control and fumarate treatments.
§ Orthogonal polynomials, linear effects of fumarate concentration.
‖ Calculated as the sum of isobutyrate, isovalerate and valerate.

Table 3. Influence of concentration of disodium fumarate on *in vitro* fermentation of barley by mixed rumen micro-organisms in batch cultures* (Mean values for four fermentations)

	Fumarate (mM)					Statistical significance of the treatment effect (P=)	
	0	4	7	10	SED	Control *v.* fumarate†	Linear‡
pH	6·38^{a}	6·41^{ab}	6·45^{bc}	6·49^{c}	0·020	0·002	0·001
Apparent disappearance of organic matter (%)	68·6	70·4	70·7	67·8	2·25	NS	NS
Gas (μmol)	4246	4183	4174	4174	47·3	NS	NS
CH_4 (μmol)	673^{b}	657^{a}	655^{a}	648^{a}	5·86	0·003	0·002
VFA (μmol)							
Acetate	1655^{a}	1690^{ab}	1710^{bc}	1751^{c}	19·7	0·004	0·001
Propionate	941^{a}	1077^{b}	1157^{c}	1282^{d}	26·6	0·001	0·001
Butyrate	406	394	387	392	12·3	NS	NS
Others§	96·8^{b}	94·8^{ab}	90·8^{ab}	86·3^{a}	4·1	NS	0·024
Total VFA	3097^{a}	3255^{b}	3345^{c}	3512^{d}	33·5	0·001	0·001
Acetate (μmol):propionate (μmol)	1·80^{c}	1·59^{b}	1·50^{b}	1·39^{a}	0·048	0·001	0·001
CH_4 (μmol):VFA (μmol)	0·218^{cd}	0·202^{b}	0·196^{b}	0·185^{a}	0·0016	0·001	0·001
NH_3-N (μmol)	831	769	791	831	28·4	NS	NS
L-Lactate (μmol)	3·27	3·00	2·85	2·93	0·232	NS	NS

VFA, volatile fatty acid.
^{a,b,c,d} Mean values within a row with unlike superscript letters were significantly different (P<0·05).
* 50 ml diluted buffered rumen contents were incubated for 17 h with 500 mg ground barley; for details of diets and procedures, see Table 1 and p. 618.
† Orthogonal contrast, control *v.* fumarate: comparison between control and fumarate treatments.
‡ Orthogonal polynomials, linear effects of fumarate concentration.
§ Calculated as the sum of isobutyrate, isovalerate and valerate.

substrate for *in vitro* rumen incubations (Asanuma *et al.* 1999; López *et al.* 1999). As the effects of fumarate could be influenced by the composition of the diet, we decided to investigate the effects of fumarate on the *in vitro* rumen fermentation of several concentrate feeds.

In agreement with the results previously reported by other authors (Callaway & Martin, 1996; Asanuma *et al.* 1999; López *et al.* 1999) with diets of varying composition, the addition of fumarate increased (P<0·001) final pH with all substrates. As suggested by Callaway & Martin (1996), malate may act to buffer rumen contents by a dual mechanism of increased lactate utilization and CO_2 production by *S. ruminantium*. *S. ruminantium* is a common Gram-negative rumen bacterium that can account for up to 51 % of the total viable bacterial counts in the rumen of animals fed on cereal grains (Caldwell & Bryant, 1966). In the present experiment, fumarate addition did not affect (P>0·05) L-lactate concentrations.

Table 4. Influence of concentration of disodium fumarate on *in vitro* fermentation of wheat by mixed rumen micro-organisms in batch cultures* (Mean values for four fermentations)

	Fumarate (mM)					Statistical significance of the treatment effect (P=)	
	0	4	7	10	SED	Control *v.* fumarate†	Linear‡
pH	6·31^{a}	6·37^{ab}	6·41^{b}	6·42^{b}	0·030	0·005	0·003
Apparent disappearance of organic matter (%)	76·7	76·2	74·6	75·7	1·32	NS	NS
Gas (μmol)	4348	4295	4317	4259	64·3	NS	NS
CH_4 (μmol)	701^{b}	682^{b}	678^{a}	675^{a}	6·6	0·002	0·029
VFA (μmol)							
Acetate	1749^{a}	1789^{ab}	1845^{bc}	1902^{c}	29·0	0·001	0·001
Propionate	1017^{a}	1165^{b}	1250^{c}	1362^{d}	20·9	0·001	0·001
Butyrate	404	419	404	406	14·5	NS	NS
Others§	95·0	100	98·0	101	4·5	NS	NS
Total VFA	3264^{a}	3473^{b}	3598^{c}	3771^{d}	49·7	0·001	0·001
Acetate (μmol):propionate (μmol)	1·76^{c}	1·56^{b}	1·50^{b}	1·42^{a}	0·032	0·001	0·001
CH_4 (μmol):VFA (μmol)	0·216^{c}	0·195^{b}	0·189^{ab}	0·181^{a}	0·0037	0·001	0·001
NH_3-N (μmol)	791	769	772	765	23·0	NS	NS
L-Lactate (μmol)	2·49	2·10	2·44	2·29	0·207	NS	NS

VFA, volatile fatty acid.
^{a,b,c,d} Mean values within a row with unlike superscript letters were significantly different (P<0·05).
* 50 ml diluted buffered rumen contents were incubated for 17 h with 500 mg ground wheat; for details of diets and procedures, see Table 1 and p. 618.
† Orthogonal contrast, control *v.* fumarate: comparison between control and fumarate treatments.
‡ Orthogonal polynomials, linear effects of fumarate concentration.
§ Calculated as the sum of isobutyrate, isovalerate and valerate.

Table 5. Influence of concentration of disodium fumarate on *in vitro* fermentation of sorghum by mixed rumen micro-organisms in batch cultures*

(Mean values for four fermentations)

	Fumarate (mM)					Statistical significance of the treatment effect ($P=$)	
	0	4	7	10	SED	Control *v.* fumarate†	Linear‡
pH	6·39[a]	6·46[b]	6·47[b]	6·50[b]	0·021	0·001	0·001
Apparent disappearance of organic matter (%)	59·5	59·0	58·3	59·6	1·36	NS	NS
Gas (μmol)	3813	3719	3768	3688	63·4	NS	NS
CH_4 (μmol)	637[b]	615[b]	607[ab]	605[a]	13·9	0·035	0·050
VFA (μmol)							
Acetate	1469[a]	1489[a]	1563[b]	1594[b]	26·6	0·005	0·001
Propionate	908[a]	1052[b]	1110[c]	1166[d]	18·0	0·001	0·001
Butyrate	347	345	328	331	10·3	NS	NS
Others§	75·0	86·7	72·5	67·0	8·8	NS	NS
Total VFA	2791[a]	2972[b]	3074[c]	3158[c]	38·3	0·001	0·001
Acetate (μmol):propionate (μmol)	1·66[b]	1·43[a]	1·42[a]	1·38[a]	0·031	0·001	0·001
CH_4 (μmol):VFA (μmol)	0·229[c]	0·207[b]	0·197[ab]	0·193[a]	0·0051	0·001	0·001
NH_3-N (μmol)	761	718	725	729	24·8	NS	NS
L-Lactate (μmol)	1·78	1·75	1·60	1·71	0·080	NS	NS

VFA, volatile fatty acid.
[a,b,c,d] Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).
* 50 ml dilute buffered rumen contents were incubated for 17 h with 500 mg ground sorghum; for details of diets and procedures, see Table 1 and p. 618.
† Orthogonal contrast, control *v.* fumarate: comparison between control and fumarate treatments.
‡ Orthogonal polynomials, linear effects of fumarate concentration.
§ Calculated as the sum of isobutyrate, isovalerate and valerate.

Nisbet & Martin (1993) showed that the growth of *S. ruminantium* in a medium that contained DL-lactate was stimulated approximately twofold by 10 mM-fumarate after 24 h of incubation. Fumarate is a key intermediate in the succinate–propionate pathway and is used by *S. ruminantium* to form succinate and propionate (Martin, 1998). In the present study, supplementation with fumarate increased ($P<0·001$) propionate production with all substrates by about 61 μmol/100 μmol fumarate added (mean value for all substrates and concentrations of fumarate). The conversion of fumarate to propionate for maize was 86, 66 and 56 % of added fumarate for 4, 7 and 10 mM treatments respectively (Table 2). Recoveries with barley, wheat and sorghum (Tables 3, 4 and 5) were lower for

Table 6. Influence of concentration of disodium fumarate on *in vitro* fermentation of cassava meal by mixed rumen micro-organisms in batch cultures

(Mean values for four fermentations)

	Fumarate (mM)					Statistical significance of the treatment effect ($P=$)	
	0	4	7	10	SED	Control *v.* fumarate‡	Linear§
pH	6·45[a]	6·50[b]	6·51[bc]	6·53[c]	0·012	0·001	0·001
Apparent disappearance of organic matter (%)	68·4	69·0	69·2	67·5	1·88	NS	NS
Gas (μmol)	4388	4393	4371	4335	46·4	NS	NS
CH_4 (μmol)	614[b]	603[ab]	591[a]	590[a]	9·3	0·029	0·017
VFA (μmol)							
Acetate	1406[a]	1458[ab]	1492[bc]	1542[c]	32·8	0·008	0·002
Propionate	884[a]	988[b]	1028[bc]	1096[c]	31·6	0·001	0·001
Butyrate	379	348	359	351	15·0	NS*	NS
Others‖	20·8	18·0	18·8	18·5	2·24	NS	NS
Total VFA	2690[a]	2793[ab]	2898[bc]	3007[c]	57·6	0·002	0·001
Acetate (μmol):propionate (μmol)	1·65[b]	1·51[a]	1·47[a]	1·46[a]	0·046	0·002	0·003
CH_4 (μmol):VFA (μmol)	0·231[c]	0·217[b]	0·205[a]	0·197[a]	0·045	0·001	0·001
NH_3-N (μmol)	125	128	122	127	8·4	NS	NS
L-Lactate (μmol)	2·93	3·04	2·85	3·05	0·210	NS	NS

VFA, volatile fatty acid.
[a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).
* $P<0·10$.
† 50 ml diluted buffered rumen contents were incubated for 17 h with 500 mg ground cassava meal; for details of diets and procedures, see Table 1 and p. 618.
‡ Orthogonal contrast, control *v.* fumarate: comparison between control and fumarate treatments.
§ Orthogonal polynomials, linear effects of fumarate concentration.
‖ Calculated as the sum of isobutyrate, isovalerate and valerate.

Table 7. Influence of concentration of disodium fumarate on fermentation by mixed ruminal micro-organisms in the absence of added substrates in batch cultures*

(Mean values for eight fermentations)

	Fumarate (mM)					Statistical significance of the treatment effect ($P=$)	
	0	4	7	10	SED	Control *v.* fumarate†	Linear‡
pH	6·81[a]	6·83[b]	6·86[c]	6·86[c]	0·010	0·001	0·001
Gas (μmol)	1125[a]	1205[b]	1228[bc]	1268[c]	31·7	0·001	0·001
CH_4 (μmol)	205	201	201	208	5·8	NS	NS
VFA (μmol)							
Acetate	443[a]	522[b]	567[c]	599[d]	12·6	0·001	0·001
Propionate	98·4[a]	224[b]	304[c]	384[d]	6·4	0·001	0·001
Butyrate	81·5	82·0	77·3	76·9	4·6	NS	NS
Others§	63·1	65·8	63·9	62·8	3·7	NS	NS
Total VFA	686[a]	893[b]	1012[c]	1122[d]	22·2	0·001	0·001
Acetate (μmol):propionate (μmol)	4·86[b]	2·35[a]	1·86[a]	1·55[a]	0·828	0·001	0·001
CH_4 (μmol):VFA (μmol)	0·320[c]	0·230[b]	0·202[a]	0·189[a]	0·0189	0·001	0·001
NH_3-N (μmol)	1071	1118	1093	1056	32·1	NS	NS
L-Lactate (μmol)	1·73	1·69	1·68	1·76	0·119	NS	NS

VFA, volatile fatty acid.
[a,b,c,d] Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).
* 50 ml diluted buffered rumen contents were incubated for 17 h; for details of diets and procedures, see Table 1 and p. 618.
† Orthogonal contrast, control *v.* fumarate: comparison between control and fumarate treatments.
‡ Orthogonal polynomials, linear effects of fumarate concentration.
§ Calculated as the sum of isobutyrate, isovalerate and valerate.

4 mM-fumarate (mean value 70 %), but similar to those for maize at 7 and 10 mM (61 and 62 % respectively). Conversely, cassava meal gave lower values: 51, 40 and 42 % for 4, 7 and 10 mM-fumarate respectively (Table 6). In general, our present values are lower than the 85 and 79 % reported by López *et al.* (1999), when fumarate was added to batch cultures containing a forage diet (500 g/kg) at 5 and 10 mM respectively, but are in reasonable agreement with the values of 60 and 77 % reported by Callaway & Martin (1996), when fumarate was added to batch cultures with cracked maize at 4 and 12 mM respectively. These results indicate that fumarate utilization *in vitro* may depend on the fermented substrate.

Fumarate can be converted into propionate and acetate following different pathways (Demeyer & Henderickx, 1967). In the present experiment, the addition of fumarate increased ($P<0·01$) acetate production with all substrates. Maize gave the greatest conversion of fumarate to acetate (52, 37 and 38 % for 4, 7 and 10 mM treatments respectively), whereas the other four substrates showed considerably lower recoveries (mean values 18, 23 and 25 % for 4, 7 and 10 mM-fumarate respectively). Calculated recoveries of fumarate as acetate plus propionate were always <100 % for barley, wheat, sorghum and cassava meal (mean values 83, 79 and 82 % for 4, 7 and 10 mM treatments respectively); in contrast, recoveries for maize were 138, 103 and 94 % for 4, 7 and 10 mM-fumarate respectively. These results indicate that fumarate at 4 mM, and possibly at 7 mM, stimulated the *in vitro* fermentation of maize. The tendency to lower ($P=0·089$) NH_3-N concentrations in the cultures observed when maize (Table 2) was incubated with fumarate could be due to a greater utilization by rumen microbes. On the other hand, the observed increases in acetate and propionate production with the other four substrates could stem from fumarate fermentation itself. To test this possibility, incubations in the absence of incubated substrates were conducted. In the absence of exogenous substrates, fumarate recoveries as acetate plus propionate were 100, 92 and 87 % for 4, 7 and 10 mM concentrations respectively, which would indicate that 4 mM-fumarate was completely fermented after 17 h. The lower recoveries observed for 7 and 10 mM-fumarate could be due to the accumulation of other final products, such as succinate.

The conversion of glucose to acetate, propionate and butyrate in the rumen results in an overall net release of reducing power. Much of this is used by methanogenic archaea to reduce CO_2 to CH_4, but H can also be used as a substrate in fumarate reduction (Russell & Wallace, 1997). As H is used to reduce fumarate, there is a decrease in the availability of H for methanogenesis in the rumen, which could decrease CH_4 production. Although fumarate significantly decreased ($P<0·05$) CH_4 production with all substrates, the observed decrease was lower than that found by other authors. Bayaru *et al.* (2001) reported that fumaric acid supplementation (20 g/kg diet DM) produced a 23·0 % decrease in CH_4 production in steers fed sorghum silage as the only feed, and López *et al.* (1999) found that adding disodium fumarate (7·35 mM-fumarate) to semi-continuous fermenters fed a mixed diet (500 g grass hay/kg feed) decreased CH_4 production by 17 %. CH_4 production in our present experiment was reduced by 2·3, 3·8 and 4·8 % for 4, 7 and 10 mM-fumarate respectively (mean values for all substrates), which indicates that fumarate would be impractical as a means of reducing CH_4 emissions *in vivo* (López *et al.* 1999). The observed reduction in CH_4 production is consistent with the lower response found by other authors when concentrate feeds were incubated *in vitro* with rumen contents; thus, Asanuma *et al.* (1999) reported that the addition of 20 mM-fumarate to batch cultures containing a concentrate diet (750 g/kg) reduced CH_4 production by 5·3 % after 6 h

of incubation, and Callaway & Martin (1996) found a 3·9 and 2·6 % decrease in CH_4 production when 4 and 12 mM-fumarate were added to *in vitro* fermentations of cracked maize respectively. From these results, it seems that the effect of fumarate on CH_4 production in the rumen may largely depend on the nature of the fermented substrate, as fumarate could be more effective in decreasing CH_4 production with forage-based diets than with high-concentrate diets.

CH_4 production is affected by many factors, such as the type of diet and the rumen pH; thus, fewer methanogens have been detected in the rumen of concentrate-fed animals than in the rumen of forage-fed ones (Demeyer & Fievez, 2000), and pH values in our present incubations were $<6{\cdot}5$, a value below which CH_4 production decreases dramatically (Van Kessel & Russell, 1996). As a consequence of the changes observed in the production of both CH_4 and VFA, the value of CH_4 (μmol):VFA (μmol) decreased linearly as concentrations of fumarate increased for all substrates. If these results are confirmed *in vivo*, the use of fumarate as a feed additive in ruminant animals fed high-concentrate diets could increase the amount of energy obtained in the rumen per unit of fermented substrate.

The results of the present study suggest that fumarate has a beneficial effect on *in vitro* rumen fermentation of concentrate feeds by increasing final pH and the production of acetate and propionate, and by decreasing CH_4 production. Some of these effects are dose-dependent, but in general, no beneficial effects of 10 compared with 7 mM-fumarate were observed. The greater response found for maize in comparison with the other concentrate feeds might indicate that fumarate utilization *in vitro* could depend on the fermented substrate. If the effects observed in the present experiment are confirmed *in vivo* with animals fed high-concentrate diets, fumarate could provide an alternative to currently used feed antibiotic growth promoters. In any case, more studies with diets of different composition are required to assess the dietary conditions that influence the effectiveness of fumarate and its long-term effects.

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References

Asanuma N, Iwamoto M & Hino T (1999) Effect of the addition of fumarate on methane production by ruminal microorganisms in vitro. *J Dairy Sci* **82**, 780–787.

Association of Official Analytical Chemists (1995) *Official Methods of Analysis*, 16th ed., chapter 4, p. 13. Arlington, VA: AOAC.

Bayaru E, Syuhei K & Toshihiko K, *et al.* (2001) Effect of fumaric acid on methane production, rumen fermentation and digestibility of cattle fed roughage alone. *Anim Sci J* **72**, 139–146.

Caldwell DR & Bryant MP (1966) Medium without rumen fluid for non-selective enumeration and isolation of rumen bacteria. *Appl Microbiol* **14**, 794–801.

Callaway TR & Martin SA (1996) Effects of organic acid and monensin treatment on in vitro mixed ruminal microorganism fermentation of cracked corn. *J Anim Sci* **74**, 1982–1989.

Carro MD, López S, Valdés C & Ovejero FJ (1999) Effect of DL-malate on mixed ruminal microorganism fermentation using the rumen simulation technique (RUSITEC). *Anim Feed Sci Technol* **79**, 279–288.

Carro MD & Ranilla MJ (2003) Effect of the addition of malate on *in vitro* rumen fermentation of cereal grains. *Br J Nutr* **89**, 181–188.

Carro MD, Valdés C, Ranilla MJ & González JS (2000) Effect of forage to concentrate ratio in the diet on ruminal fermentation and digesta flow kinetics in sheep. *Anim Sci* **70**, 127–134.

Demeyer DI & Henderickx MK (1967) Competitive inhibition of in vitro methane production by mixed rumen bacteria. *Arch Int Physiol Biochim* **75**, 157–159.

Demeyer DI & Fievez V (2000) Ruminants et environnement: la méthanogenèse (Ruminants and environment: methanogenesis). *Ann Zootech* **49**, 95–112.

Goering MK & Van Soest PJ (1970) *Forage Fiber Analysis (Apparatus, Reagents, Procedures and Some Applications) Agricultural Handbook*, no. 379. Washington, DC: ARS, USDA.

López S, Valdés C, Newbold CJ & Wallace RJ (1999) Influence of sodium fumarate addition on rumen fermentation *in vitro*. *Br J Nutr* **81**, 59–64.

Martin SA (1998) Manipulation of ruminal fermentation with organic acids: a review. *J Anim Sci* **76**, 3123–3132.

Mould FL (1988) Associative effects of feeds. In *Feed Science World. Animal Science, B4*, pp. 279–292 [ER Ørskov, editor]. Amsterdam: Elsevier Science Publishers BV.

Newbold CJ, Wallace RJ & McIntosh FM (1996) Mode of action of the yeast *Saccharomyces cerevisiae* as a feed additive for ruminants. *Br J Nutr* **76**, 249–261.

Nisbet DJ & Martin SA (1990) Effect of dicarboxylic acids and *Aspergillus oryzae* fermentation extract on lactate uptake by the ruminal bacterium *Selenomonas ruminantium*. *Appl Environ Microbiol* **26**, 133–136.

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*. *Curr Microbiol* **26**, 133–136.

Russell JB & Wallace RJ (1997) Energy consuming and yielding mechanisms. In *The Rumen Microbial Ecosystem*, 2nd ed. pp. 246–282 [PN Hobson and CS Stewart, editors]. London: Chapman & Hall.

Van Kessel JAS & Russell JB (1996) The effect of pH on ruminal methanogenesis. *FEMS Microbiol Ecol* **20**, 205–210.

Van Soest PJ, Robertson JB & Lewis BA (1991) Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J Dairy Sci* **74**, 3583–3597.

Weatherburn MW (1967) Phenol-hypochlorite reaction for determination of ammonia. *Anal Chem* **39**, 971–974.