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Influence of disodium malate on microbial growth and fermentation in rumen-simulation technique fermenters receiving medium- and high-concentrate diets

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Two incubation trials were carried out with the rumen-simulation technique (RUSITEC). In each trial, four vessels received a diet of grass hay and concentrate (600 and 400 g/kg DM, respectively; diet F), and the other four were fed a diet composed of concentrate and barley straw (900 and 100 g/kg DM, respectively; Diet C). Vessels were given 20 g of the corresponding diet daily, and half of them were supplemented with disodium malate to achieve a final concentration of 6·55 mM. There were no effects ($P>0·05$) of malate either on pH or on the daily production of NH_3-N, but malate treatment increased ($P<0·05$) DM, neutral detergent and acid detergent fibre disappearance after 48 h incubation. The daily production of propionate and butyrate increased ($P<0·001$), and the ratio CH_4:volatile fatty acids decreased ($P<0·001$) by supplementing both diets with malate. Whereas adding malate to the F diet produced an increase in acetate production ($P=0·011$) and the growth of solid-associated micro-organisms ($P=0·037$), no effects ($P>0·05$) were observed for diet C. For both diets, there were no differences ($P>0·05$) between treatments in the daily flow of liquid-associated micro-organisms measured using ^{15}N as a microbial marker. These results indicate that malate stimulated the *in vitro* fermentation of both diets by increasing the apparent disappearance of the diet and decreasing the ratio of CH_4:volatile fatty acids, but a greater response was observed with diet F. If these results are confirmed *in vivo*, malate could be used as a feed additive for ruminants fed diets containing medium proportions of forage (i.e. dairy animals) and not only in animals fed high-concentrate diets, as has so far been proposed.

Malate: Rumen fermentation: Microbial protein synthesis: RUSITEC

Organic acids (aspartate, malate, fumarate) have been suggested as an alternative to currently used antibiotic growth promoters (Newbold *et al.* 1996; Martin, 1998). Several papers (Martin & Streeter, 1995; Callaway & Martin, 1996; Carro *et al.* 1999; Carro & Ranilla, 2003*a*; Martin, 2004) have shown that adding malate to *in vitro* fermentations of mixed rumen micro-organisms resulted in changes in the final pH, CH_4 and volatile fatty acids (VFA) that are analogous to ionophore effects. However, the results of some of these studies (Carro & Ranilla, 2003*a*) indicate that the effects of malate are affected by the composition of the incubated diet. Most of the studies reported have been conducted in short-term experiments (incubations of up to 24 h) using batch cultures of mixed rumen micro-organisms, and little is known about the longer-term effects of malate on *in vitro* rumen fermentation. Moreover, in these studies, single feeds (alfalfa hay, barley, wheat, maize, etc.) have been used as incubation substrates, and there are currently no studies available on the effects of malate on the *in vitro* rumen fermentation of diets representative of those fed to animals in practice.

In addition, research has shown that malate can stimulate the growth of *Selenomonas ruminantium* in pure cultures (Nisbet & Martin, 1990, 1993), but no studies have been conducted to investigate the effects of malate on the growth of mixed rumen micro-organisms. The aim of the present study was therefore to investigate the long-term effects of disodium malate on the microbial growth and rumen fermentation of two different diets in rumen-simulation technique (RUSITEC) fermenters. Diets were formulated to be representative of those fed to dairy animals (diet F; medium-concentrate diet) and to growing ruminants under intensive systems of production (diet C; high-concentrate diet).

Materials and methods

Apparatus, diets and experimental procedure

The complete unit of the RUSITEC consisted of eight vessels with an effective volume of 600 ml each, and the general incubation procedure was as described by Czerkawski & Breckenridge (1977). The inoculum was obtained from four ruminally fistulated sheep fed daily 500 g good-quality hay (175 g crude protein and 476 g neutral detergent fibre (NDF) per kg DM) and 500 g commercial concentrate (164 g crude protein and 182 g NDF/kg DM). Sheep were managed according to the protocols approved by the León University Institutional Animal Care and Use Committee. Solid and liquid fermentation inocula were collected from sheep immediately before feeding and transferred to the *in vitro* system within 30 min as previously described (Carro *et al.* 1992). The flow through the vessels was maintained by a

Abbreviations: ADF, acid detergent fibre; LAM, lipid-associated micro-organisms; NAN, non-NH_3-N; NDF, neutral detergent fibre; RUSITEC, rumen-simulation technique; SAM, solid-associated micro-organisms; VFA, volatile fatty acids.

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continuous infusion of McDougall (1948) artificial saliva (pH 8·4) at a rate of 530 ml/d (dilution rate 3·7 %/h). On day 9, a dose of 2·18 mg ^{15}N (95 % enriched $(^{15}NH_4)_2SO_4$; Sigma, Madrid, Spain) was added into each vessel to instantaneously label the NH_3-N pool. A solution of $(^{15}NH_4)_2SO_4$ was then added to the artificial saliva at a daily rate of 4·00 μg ^{15}N/mg dietary N.

Two different diets were formulated, and each vessel received daily 20 g DM of the corresponding diet fed into nylon bags (100 μm pore size). Diet F consisted of grass hay and concentrate (600 and 400 g/kg DM, respectively), and diet C was composed of concentrate and barley straw (900 and 100 g/kg DM, respectively). The composition of both diets is shown in Table 1. Grass hay and barley straw were chopped (to about 0·5 cm size), and concentrate components were ground through a 4 mm sieve and carefully mixed. Sugar beet molasses was added to the concentrates to allow a homogenous mix of the components. From the first day of incubation, four vessels in each incubation run were supplemented with malate (disodium salt; Sigma) to achieve a final concentration of 6·55 mM, the other four vessels receiving no additive. Malate was weighed and carefully mixed with the concentrate before this was placed into the nylon bags.

Two identical incubation runs were carried out independently, and treatments were assigned randomly within each experimental run so that two vessels received each of the treatments; each treatment was, therefore, conducted in quadruplicate. Each incubation run consisted of 14 d. After 8 d of adaptation, on days 9, 10 and 11, samples for gas, VFA and NH_3-N determination were collected, and the apparent disappearance of diet was measured following the procedures described by Carro & Miller (1999). On day 12, the fluid vessel contents were sampled (about 1·5 ml) at 0, 3, 6, 9 and 12 h after feeding, the pH was immediately measured, and samples for VFA and NH_3-N analysis were taken.

On days 12 and 13, 5 ml saturated $HgCl_2$ were added (replacing the H_2SO_4 solution, which can cause bacterial lysis) to the overflow containers, which were held in an ice-water bath to impede microbial growth. For each vessel, the total effluent for days 12 and 13 was mixed and homogenised in a blender at low speed for 1 min. One portion (300 g) was frozen and lyophilised for determination of DM, non-NH_3-N (NAN) and ^{15}N enrichment, the rest of the mix being used to isolate liquid-associated micro-organisms (LAM). The contents of the nylon bags removed on days 12 and 13 were used to determine the growth of solid-associated micro-organisms (SAM). SAM pellets were isolated after treating one portion of the nylon bag contents (about 80 %) with a saline solution (0·85 % NaCl; w/v) of methylcellulose (0·1 %; w/v) at 39°C as described by Ranilla & Carro (2003). The remaining solid content (20 %) was lyophilised to determine DM, NAN and ^{15}N enrichment. Microbial pellets were isolated by differential centrifugation as described by Carro & Miller (1999). Diets were also analysed for their natural ^{15}N content, and this value was used for background correction before ^{15}N infusion.

Adaptive changes in the microbial population of the semi-continuous cultures to each treatment were studied using the vessels' fluid as inoculum for batch cultures and measuring the response in terms of final pH and production of VFA. The fermentative activity of the fluid contained in each vessel was tested against four pure substrates (all manufactured by Sigma): a mixture of starch (40 g wheat, 40 g barley and 200 g potato starch per kg mixture), oat spelt xylan, cellulose and pectin from citrus peel. On the last day of each incubation run, the two nylon bags present in the food container were removed and washed twice with 40 ml the vessel's fluid, the washing liquid being returned to the vessel. Then 440 ml fluid was mixed with 110 ml artificial saliva (enriched with 1·09 g NH_4Cl per litre of saliva), and 50 ml of the final mixture was anaerobically dispensed to 120 ml serum bottles (Laboratorios Ovejero SA, León, Spain) containing 500 mg of one of the substrates described earlier. Eight bottles (two bottles for each substrate) were incubated per each vessel. The bottles were sealed with rubber stoppers and Al caps, and incubated at 39° C. After 6 h of incubation, the bottles were opened, the pH was immediately measured, and samples for VFA determination were taken as described earlier.

Table 1. Composition of basal diets incubated in the rumen-simulation technique system

	Diet F	Diet C
Ingredient composition (g/kg dry matter diet)		
Grass hay	600	–
Barley straw	–	100
Maize grains	150	350
Barley grains	130	350
Soyabean meal	100	180
Molasses, sugar beet	10	10
Mineral–vitamin mixture	10	10
Chemical composition (g/kg dry matter)		
Organic matter	928	957
Nitrogen	25·5	24·7
Neutral detergent fibre	348	220
Acid detergent fibre	150	81·8
Malate	15·1	4·87
^{15}N (% atoms)	0·3678	0·3675

Analytical procedures

DM, ash and N were determined according to the Association of Official Analytical Chemists (1995). NDF and acid detergent fibre (ADF) analyses were carried out according to the method of Van Soest *et al.* (1991). The concentration of NH_3 was determined by a modified colorimetric method (Weatherburn, 1967). VFA were determined in centrifuged samples by GC as previously described (Carro *et al.* 1992). The volume of gas produced was measured with a drum-type gas meter (model TG1; Ritter Apparatebau GmbH, Bochum, Germany), and the concentration of CH_4 was analysed by chromatography as described by Carro & Ranilla (2003b). The volume of gas (l/d) produced was corrected for standard conditions (10^5 Pa, 298 K), and the amount of CH_4 produced (mmol/d) was calculated by multiplying the gas produced by the CH_4 concentration in the analysed sample. Preparation of samples for ^{15}N analysis followed the procedures described by Carro & Miller (1999), and analyses of ^{15}N enrichment were performed by isotope ratio MS as described by Barrie & Workman (1984). The preparation of feed samples for malate analysis and malate analysis by HPLC followed the procedures described by Callaway *et al.* (1997).

Calculations and statistical analyses

The proportion of digesta NAN (liquid or solid) of microbial origin was estimated for each vessel by dividing the ^{15}N enrichment (atom % excess) of the NAN portion of the digesta by the enrichment of the corresponding bacterial pellets (LAM or SAM). Daily microbial N production (mg/d; LAM or SAM) was estimated by multiplying the total NAN production in the

corresponding digesta (liquid or solid) by the proportion attributed to the microbes. Total daily microbial production was calculated as the sum of the flows of LAM and SAM. The amounts of VFA produced in the batch cultures were obtained by subtracting the amounts initially present in the incubation medium from those determined at the end of the incubation period.

Data relative to fermentation parameters were analysed as a split-plot design using the general linear models procedures of the Statistical Analysis Systems program (version 6, 1989; SAS Institute Inc., Cary, NC, USA), with malate treatment as the main plot. The model included malate treatment, vessel nested within treatment, incubation trial, day of sampling, diet, and the treatment × diet interaction. The significance of malate effects was tested using the variance between vessels within treatment as the error term. The effects of other factors were tested against the residual error. In the analysis of data relative to microbial growth in the fermenters and to pH and VFA production in batch cultures, the day of sampling was excluded from the model. Time–sequence data on pH, VFA and NH_3-N concentrations in the liquid phase of the vessels were analysed within each time of sampling.

Results

There were no incidents during the course of the experiments, and the daily amount of effluent was not affected ($P>0·05$) by malate treatment (553 and 555 ml/d for control and malate, respectively) or the incubated diet (555 and 553 ml/d for diets F and C, respectively). The effects of malate treatment on pH, diet disappearance and the daily production of VFA and CH_4 are shown in Table 2. The addition of malate did not affect ($P>0·05$) rumen pH before feeding but increased the apparent disappearance of DM ($P=0·035$), NDF ($P=0·028$) and ADF ($P=0·021$) from the diet.

Compared with the control, the addition of malate increased ($P<0·001$) the daily production of propionate (2·45 and 2·42 mmol/d increase for diets F and C, respectively) and butyrate (0·99 and 2·00 mmol/d for diets F and C, respectively) for both diets. Conversely, acetate production was only increased ($P=0·011$) for diet F (28·8 and 31·8 mmol/d for control and malate treatments, respectively). The daily production of valerate and isovalerate was also increased ($P<0·05$) by adding malate to both diets, but no effects ($P>0·05$) on the production of isobutyrate were observed. As a consequence of these changes, adding malate increased ($P<0·05$) total VFA production by 7·0 and in 5·9 mmol/d for diets F and C, respectively. Supplementation with malate increased ($P<0·05$) the concentration of VFA (mmol/d) at all sampling times for both diets (results not shown) but did not affect ($P>0·05$) rumen pH and NH_3-N concentration (mg/l) at any sampling time.

Adding malate did not affect ($P>0·05$) the production of CH_4 but decreased CH_4:DM apparent disappearance (mmol/g; $P=0·001$) and CH_4:VFA ratio (mmol/mmol; $P=0·012$) for both diets (Table 2). As shown in Table 3, the daily flows of NH_3-N, total NAN and dietary NAN were not affected ($P>0·05$) by the addition of malate, but microbial N flow tended to increase ($P=0·080$) in the vessels supplemented with malate. This effect was due to the increase ($P=0·019$) in the flow of SAM produced by malate (13·3 and 1·9 mg/d for diets F and C, respectively) as there were no differences ($P>0·05$) between treatments in the daily flow of LAM. Efficiency of microbial synthesis, calculated as mg microbial N per g DM apparent disappearance, was not affected ($P>0·05$) by malate supplementation.

As expected, there were marked differences in rumen fermentation between diets. Rumen pH values and NH_3-N concentrations were lower ($P<0·001$) and VFA concentrations higher ($P<0·05$) at all sampling times for vessels fed diet C than for those receiving diet F. The disappearance of DM was greater ($P=0·001$) and the CH_4:VFA ratio (mmol/mmol) lower ($P=0·001$) for diet C than for diet F, although the opposite was observed for SAM flow (41·3 and 60·5 mg SAM-N/d for diets C and F, respectively). There were no ($P>0·05$) malate × diet interactions for any measured parameter.

The results of the *in vitro* incubations with batch cultures designed to detect possible changes in the fermentative activity

Table 2. Effects of malate (6·55 mM) on pH before feeding, apparent disappearance of diet after 48 h incubation and daily production of volatile fatty acid (VFA) and CH_4 in a rumen-simulation technique system fed two different diets

(Values are the mean of three daily observations in each of four vessels; *n* 12)

	Diet F*		Diet C*			Significance level (*P*=)		
Item	Control	Malate	Control	Malate	SED	Malate	Diet	Malate × diet
pH	6·32	6·33	5·90	5·89	0·017	NS	0·001	NS
Apparent disappearance (%) of								
DM	62·3	64·6	64·4	65·7	1·31	0·035	NS†	NS
Neutral detergent fibre	29·7	33·4	24·1	26·0	1·26	0·028	0·001	NS
Acid detergent fibre	24·9	28·4	18·8	20·2	1·00	0·021	0·001	NS
VFA production (mmol/d)								
Acetate	28·8	31·8	40·3	40·8	1·34	NS	0·001	NS
Propionate	6·95	9·40	7·88	10·3	0·32	0·001	0·001	NS
Butyrate	9·21	10·2	13·1	15·1	0·42	0·001	0·001	NS
Isobutyrate	0·828	0·793	0·827	0·852	0·0884	NS	NS	NS
Valerate	1·38	1·53	1·84	2·21	0·102	0·049	0·001	NS
Isovalerate	3·05	3·50	3·17	3·76	0·089	0·007	0·013	NS
Total	50·2	57·2	67·1	73·0	1·78	0·044	0·001	NS
CH_4 (mmol/d)	12·6	12·1	13·1	12·8	0·44	NS	NS	NS
CH_4:DM apparent	1·011	0·936	1·017	0·972	0·0347	0·001	NS	NS
CH_4:VFA (mmol:mmol)	0·251	0·212	0·195	0·175	0·0094	0·012	0·001	NS

* Diet F was composed of grass hay and concentrate (600 and 400 g/kg DM, respectively) and diet C was composed of barley straw and concentrate (100 and 900 g/kg DM, respectively).
NS† $P<0·10$.

Table 3. Effects of malate (6·55 mM) on daily production of NH_3-N and non-NH_3-N (NAN), microbial N flow and efficiency of microbial synthesis (mg microbial N/g DM apparent disappearance) in rumen-simulation technique system fed two different diets

(Values are the mean of three daily observations in each of four vessels (*n* 12) for NH_3-N and the mean of one observation in each of four vessels for the rest of parameters)

	Diet		F*			Significance level (*P*=)		
Item	Control	Malate	Control	Malate	SED	Malate	Diet	Malate × diet
NH_3-N (mg/d)	120·7	121·6	115·6	115·0	2·98	NS	NS†	NS
Total NAN (mg/d)	322	328	318	329	7·42	NS	NS	NS
Dietary NAN (mg/d)	197	190	194	200	11·0	NS	NS	NS
Microbial N flow (mg/d)	125	138	124	129	2·92	NS†	NS	NS
LAM (mg/d)	71·7	72·3	83·7	86·7	2·96	NS	0·015	NS
SAM (mg/d)	54·1	66·8	40·3	42·2	2·18	0·019	0·001	NS
SAM (% of total)	43·3	48·4	32·5	32·7	2·60	NS	0·001	NS
Efficiency of microbial synthesis	10·1	10·8	9·63	9·81	0·250	NS	NS†	NS

LAM, lipid-associated micro-organisms; SAM, solid-associated micro-organisms.

* Diet F was composed of grass hay and concentrate (600 and 400 g/kg DM, respectively) and diet C was composed of barley straw and concentrate (100 and 900 g/kg DM, respectively).

NS† $P<0·10$.

of the semi-continuous cultures in response to the addition of malate are shown in Table 4. For all substrates, there was no difference ($P>0·05$) between the control vessels and those supplemented with malate in terms of the final pH after 6 h incubation. Batch cultures inoculated with rumen fluid from vessels supplemented with malate produced greater ($P<0·001$) amounts of VFA with cellulose and tended ($P=0·071$) to produce greater amounts of VFA with xylan. With all substrates, the final pH was lower ($P<0·001$) in batch cultures inoculated with fluid from vessels fed diet F than in those corresponding to vessels fed diet C. There were, however, no interactions ($P>0·05$) between malate supplementation and diet for any parameter.

Discussion

In the past few years, several papers have investigated the effects of malate on rumen pH in batch cultures (Martin & Streeter, 1995; Callaway & Martin, 1996; Carro & Ranilla, 2003*a*; Martin, 2004) and semi-continuous fermenters (Carro *et al.* 1999), but the results reported are contradictory. Whereas concentrations of malate ranging from 7 to 12 mM consistently increased the final pH in batch cultures containing hay or different concentrate feeds (Martin & Streeter, 1995; Callaway & Martin, 1996; Carro & Ranilla, 2003*a*; Martin, 2004), no effects of 8·0 mM malate were reported in semi-continuous fermenters fed a diet containing 500 g alfalfa hay and 500 g concentrate per kg (Carro *et al.* 1999). The latter results agree with those observed in the present experiment and could be explained by the high buffer capacity of the artificial saliva used in the fermenters. In fact, rumen pH remained fairly stable through the day for both diets.

The increased daily VFA production observed with both diets in this experiment is in agreement with previously reported results. Increases in the production of acetate, propionate and butyrate have been reported when batch cultures containing different concentrate feeds (maize, barley, wheat and sorghum) were supplemented with 7 mM and 10 mM malate (Carro & Ranilla, 2003*a*), although Carro *et al.* (1999), in semi-continuous fermenters fed a mixed diet (500 g alfalfa hay/kg), found that

Table 4. Final pH and production of volatile fatty acid (VFA) in batch cultures containing 500 mg different substrates (starch, cellulose, xylan and pectin) after 6 h incubation with vessels fluid from rumen-simulation technique fermenters fed two different diets and supplemented daily with or without 6·55 mM-malate

(Mean values of eight fermentations)

	Diet F*		Diet			Significance level (*P*=)		
Substrate and item	Control	Malate	Control	Malate	SED	Malate	Diet	Malate × diet
Starch								
pH	5·52	5·59	5·67	5·68	0·022	NS	0·001	NS
VFA (μmol)	3624	3581	3701	3615	107·4	NS	NS	NS
Cellulose								
pH	6·37	6·42	6·63	6·65	0·014	NS	0·001	NS
VFA (μmol)	591	834	490	679	83·9	0·001	NS	NS
Xylan								
pH	6·18	6·19	6·41	6·30	0·046	NS	0·001	NS
VFA (μmol)	1531	1924	1395	1596	118·7	NS†	0·011	NS
Pectin								
pH	5·84	5·88	5·99	6·05	0·026	NS	0·001	NS
VFA (μmol)	3144	3087	2233	2599	145·7	NS	0·001	NS

* Diet F was composed of grass hay and concentrate (600 and 400 g/kg DM, respectively) and diet C was composed of barley straw and concentrate (100 and 900 g/kg DM, respectively).

NS† $P<0·10$.

adding 8 mM malate increased the daily production of propionate, but did not affect ($P>0·05$) the production of acetate or butyrate. These contrasting results could indicate that the effects of malate depend on the incubated diet, and the results of the present study seem to support this hypothesis.

Although malate increased VFA production with both diets, there were some differences in the observed response. Whereas the increase in propionate production was similar for diets F and C (2·45 and 2·42 mmol/d, respectively), the increase in acetate and butyrate production was distinct: 3·04 and 0·53 mmol acetate/d and 0·99 and 2·00 mmol butyrate/d for diets F and C, respectively. As a consequence of the changes in the individual VFA, adding malate at 6·55 mM (which represents 3·93 mmol malate per vessel daily) increased total VFA production by 7·0 mmol/d for diet F and in 5·9 mmol/d for diet C. These results indicate that the observed increase in VFA production could not only stem from malate fermentation itself as it has been reported that malate can be converted into propionate and acetate following different pathways (Demeyer & Henderickx, 1967). The observed increase in the apparent disappearance of the diet (see Table 2) is in agreement with the greater production of VFA and suggests that malate stimulated the *in vitro* fermentation of both diets.

The fact that malate supplementation increased the concentration of VFA at 3, 6, 9 and 12 h after feeding for both diets also indicates a stimulatory effect of malate on fermentation, presumably due to changes in bacterial populations and/or in their activity. Nisbet & Martin (1990, 1993) showed that adding malate to *in vitro* cultures stimulated the growth of *Selenomonas ruminantium* in a medium that contained lactate, but to our knowledge no information about the effects of malate on the *in vitro* growth of mixed-rumen micro-organisms is available. We therefore decided to measure the growth of SAM and LAM in the present experiment. Adding 6·55 mM malate increased ($P=0·037$) the growth of SAM in 12·5 mg SAM-N/d for diet F, but no significant ($P>0·05$) effect was observed for diet C. The greater SAM growth observed for diet F agrees with the reported increase in apparent disappearance of NDF and ADF. These results are in accordance with those from previous research conducted in our laboratory (García-Martínez *et al.* 2004), which showed that 8 mM-malate increased the growth of rumen micro-organisms in batch cultures with a high-forage diet (800 g forage/kg), but no effect was observed with medium- or low-forage diets (500 or 200 g forage/kg, respectively). Other authors have reported increases in the number of cellulolytic bacteria in sheep fed malate (Newbold *et al.* 1996) and in RUSITEC fermenters supplemented with fumarate (López *et al.* 1999). All these results seem to indicate that malate and fumarate can have a stimulatory effect on rumen microbial growth, but this effect can be influenced by diet and experimental conditions.

In the present experiment, the differences observed between diets in the fermentation pattern (pH, production of VFA, disappearance of NDF, etc.) would indicate that a different microbial population was established in the vessels fed the two diets. The pH values in vessels fed diet C ranged from 5·84 to 6·00, and it has been reported that cellulolytic bacteria failed to grow at a pH of less than 6·0 (Stewart, 1977). That could explain the lower apparent disappearance of NDF and ADF ($P=0·001$) observed for diet C compared with diet F (see Table 2), although the differences between both diets in fibre composition should also be taken into account. Nevertheless, the increase in disappearance of NDF and ADF observed by adding malate to both diets would indicate that one of the beneficial effects of malate may be to increase fibre digestion, as has been previously observed by Carro *et al.* (1999) in semi-continuous fermenters and by Newbold *et al.* (1996) in sheep.

As expected, the mean proportion of SAM-N in the particulate fraction of the fermenters (nylon bag residues) was greater ($P=0·001$) for diet F (45·8 %) than for diet C (32·6 %). This proportion was not affected ($P>0·05$) by adding malate to diet C, but malate tended ($P=0·10$) to increase it for diet F, which agrees with the increased disappearance of the diet observed for malate-supplemented vessels fed this diet. For both diets, the growth of LAM was not affected ($P>0·05$) by adding malate. Malate was mixed with the concentrate portion of the diet, and, due to the high compartmentation in the RUSITEC system, it is possible that it had been completely fermented by SAM. Although malate disappearance was not measured in the present experiment, Russel & Van Soest (1984) indicated that 7·5 mM-malate was fermented *in vitro* within 10 h, and Callaway & Martin (1997) found that 7·5 mM-malate disappeared within 24 h in batch cultures. The level of malate used in our experiment (6·55 mM) would justify the assumption that the amount of malate added daily was completely fermented in 24 h. Therefore, the differences observed in the *in vitro* incubations with batch cultures using vessel fluid as the inoculum can only be due to changes in the microbial population of the semi-continuous cultures produced by the addition of malate.

There were, however, only subtle effects of inocula (fluid from malate-supplemented vessels compared with that from unsupplemented vessels) on the *in vitro* fermentation. The greater VFA production ($P=0·001$) observed when cellulose was incubated with fluid from malate-supplemented vessels would indicate a greater fibrolytic activity of the incubation medium, and this observation is in agreement with the trend ($P=0·071$) towards a greater VFA production observed for xylan when it was incubated with fluid from the malate-supplemented vessels. The greater apparent disappearance of NDF and ADF observed in RUSITEC fermenters by adding malate to the diet would support this hypothesis. Nonetheless, it must be taken into account that since only the vessels' fluid was used as inoculum, any effect of malate on the SAM population could not be detected in the batch cultures' trial. Both LAM and the non-adherent micro-organisms washed out of the solid (nylon bags being washed twice with 40 ml of the vessels' fluid, with the washing liquid returned to the vessel before using the fluid as inoculum for the batch cultures' incubations) should have been present in the vessels' fluid. However, SAM and the non-adherent population have been reported to be responsible for most of the feed digestion in the rumen (McAllister *et al.* 1994), and this can explain that only subtle effects of inoculum were observed in batch cultures compared with the greater differences in diet disappearance found in RUSITEC fermenters.

In agreement with the results reported by Carro *et al.* (1999), malate did not affect ($P>0·05$) the daily production of CH_4 but decreased ($P<0·05$) the apparent disappearance of CH_4:DM (mmol/g) and the CH_4:VFA ratio (mmol/mmol) for both diets (see Table 2). The observed decrease in CH_4 production (4·1 % and 2·3 % for diets F and C, respectively) is in the range of previously reported results for similar doses of malate (Callaway & Martin, 1996; Carro & Ranilla, 2003*a*; Tejido *et al.* 2005), and, as has been suggested by other authors for fumarate

(López *et al.* 1999), malate would be impractical as a means of reducing CH_4 emissions *in vivo*. CH_4 production is affected by many factors, such as the type of diet and the rumen pH. The daily CH_4 production values, expressed as mmol/g DM diet apparent disappearance, ranged from 0·936 to 1·017, and were lower than the mean values of 1·4 and 1·6 mmol CH_4/g DM degraded reported by Czerkawski (1986) for concentrate and mixed diets, respectively. Russell (1998) showed that CH_4 production *in vitro* decreased dramatically at pH values below 6·5, and in our experiment rumen pH was always below 6·3 (results not shown), which can explain the low CH_4 production. Our values were in the range of those previously reported in continuous fermenters fed diets differing in their forage:concentrate ratio and maintained at a pH of less than 6·3 (Eun *et al.* 2004).

The results of the present study indicate that malate was effective in increasing diet degradation and VFA production for both diets, although a greater response was observed for diet F in comparison with diet C. If these results are confirmed *in vivo*, malate could be used as additive in ruminants fed medium proportions of forage (i.e. dairy animals) and not only in animals fed high-concentrate diets, as has been proposed up to now.

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