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In vitro microbial growth and rumen fermentation of different substrates as affected by the addition of disodium malate

M. L. Tejido, M. J. Ranilla, R. García-Martínez and M. D. Carro†

Departamento de Producción Animal I, Universidad de León, 24071 León, Spain

†Corresponding author. E-mail: DP1MCT@UNILEON.ES

Abstract

The effects of two concentrations of disodium malate on the in vitro *fermentation of three substrates differing in their forage : concentrate ratio (0·8 : 0·2, 0·5 : 0·5 and 0·2 : 0·8; g/g dry matter; low-, medium- and high-concentrate substrates, respectively) by rumen micro-organisms were studied using batch cultures. Rumen contents were collected from four Merino sheep offered lucerne hay* ad libitum *and supplemented daily with 400 g concentrate. Disodium malate was added to the incubation bottles to achieve final concentrations of 0, 4 and 8 mmol/l malate and ^{15}N was used as a microbial marker. Gas production was measured at regular intervals from 0 to 120 h of incubation to study fermentation kinetics. When gas production values were corrected for gas released from added malate, no effects (*P*>0·05) of malate were detected for any of the estimated gas production parameters. In 17-h incubations, the final pH and total volatile fatty acid (VFA) production were increased (*P*<0·001) by the addition of malate, but no changes (*P*>0·05) were detected in the final amounts of ammonia-N and lactate. When net VFA productions were corrected for the amount of VFA produced from malate fermentation itself, adding malate did not affect (*P*>0·05) the production of acetate, propionate and total VFA. Malate reduced methane (CH_4) production by proportionately 0·058, 0·013 and 0·054 for the low-, medium- and high-concentrate substrates, respectively. Adding malate to batch cultures increased (*P*<0·01) rumen microbial growth (mean values of 16·6, 18·3 and 18·4 mg of microbial N for malate at 0, 4 and 8 mmol/l, respectively), but did not affect (*P*>0·05) its efficiency of growth (55·5, 56·7 and 54·3 mg of microbial N per g of organic matter apparently fermented for malate at 0, 4 and 8 mmol/l, respectively). There were no interactions (*P*>0·05) malate × substrate for any of the measured variables, and no differences (*P*>0·05) in pH, CH_4 production and microbial growth were found between malate at 4 and 8 mmol/l. The results indicate that malate had a beneficial effect on* in vitro *rumen fermentation of substrates by increasing VFA production and microbial growth, and that only subtle differences in the effects of malate were observed between substrates. Most of the observed effects, however, seem to be due to fermentation of malate itself.*

Keywords: in vitro *culture, malic acid, rumen fermentation.*

Introduction

In the last few years there has been an increasing concern regarding the use of antibiotics in animal feeding and in September 2003 the European Union approved a new Directive that will phase out by January 2006 the authorizations of the four antibiotics being used as growth promoters in the European market. As a consequence, there is a demand for the development of alternatives to the use of these food additives (European Commission, 2003). Organic acids (aspartate, fumarate, malate) have been proposed as an alternative to currently used antibiotic compounds in ruminant animals (Martin, 1998; Castillo *et al*., 2004) but most of the research conducted on this topic has been focused on malate. Several papers (Callaway and Martin, 1996; Carro *et al*., 1999; Carro and Ranilla, 2003; Gómez *et al*., 2005) have shown that adding malate to *in vitro* fermentations of mixed rumen micro-organisms resulted in changes in final pH and production of methane (CH_4) and volatile fatty acids (VFA) that are analogous to ionophore effects. Moreover, malate reduced the drop in rumen pH usually observed 2 to 4 h after feeding in animals given high-concentrate diets (Martin *et al*., 1999; Montaño *et al*., 1999).

Research has shown that malate can stimulate the growth of *Selenomonas ruminantium* in pure cultures (Nisbet and Martin, 1990 and 1993). As *S. ruminantium* is a common Gram-negative ruminal bacterium that can account for up to 51% of the total viable bacterial counts in the rumen of animals given cereal grains (Caldwell and Bryant, 1966), most of the research conducted to investigate the effects of malate on rumen fermentation has been carried out using concentrate foods as substrate for *in vitro* experiments (Martin and Streeter, 1995; Callaway and Martin, 1996; Carro and Ranilla, 2003) or high-concentrate diets for *in vivo* experiments (Martin *et al*., 1999; Montaño *et al*., 1999; Carro *et al*., 2003). However, results from recent studies (Carro and Ranilla, 2003; Martin, 2004; Gómez *et al*., 2005) indicate that

malate utilization *in vitro* could depend on the nature of the fermented substrate. In addition, there is little information about the effects of malate on the growth of mixed rumen micro-organisms. Therefore, the aim of this study was to investigate the effects of disodium malate on microbial growth and rumen fermentation of three different substrates in batch cultures. Because in most of the conducted studies single foods (hay, barley, wheat, maize, etc.) have been used as substrates, we decided to incubate three substrates composed of forage and concentrate in different proportions. Ingredients of substrates were selected to be representative of those given to ruminants in practice.

Material and methods

Substrates, animals and experimental procedure

Three substrates containing 200 (low-concentrate), 500 (medium-concentrate) and 800 (high-concentrate) g of concentrate per kg dry matter (DM) were formulated. Forage was composed of lucerne hay and maize silage (500 and 500 g/kg DM, respectively) and concentrate was based on barley, maize and soya-bean meal (500, 350 and 150 g/kg DM, respectively). The chemical composition of experimental substrates is shown in Table 1. Samples of each substrate were ground through a 1-mm screen and fermented *in vitro* with buffered rumen fluid.

Rumen fluid was obtained from four rumen-cannulated Merino sheep given lucerne hay *ad libitum* and 400 g of concentrate per day administered in two equal portions at 09:00 and 18:00 h. Concentrate was based on barley, maize and soya-bean meal (390, 400 and 210 g per kg fresh matter, respectively). The chemical composition of the lucerne hay and concentrate is given in Table 1. Rumen contents of each sheep were obtained immediately before the morning feeding, mixed and strained through four layers of cheesecloth into an Erlenmeyer flask with an O_2-free headspace. Food particles were allowed to settle to the bottom (5 min) and finally the fluid was strained through two layers of nylon cloth (40-μm pore size). Particle-free fluid was mixed with the buffer solution of Goering and Van Soest (1970; no trypticase added) in a proportion 1 : 4 (v/v) at 39ºC under continuous flushing with CO_2. A dose of ^{15}N (95% enriched $(^{15}NH_4)_2SO_4$; molecular weight 134·1; Sigma Chemical, Madrid, Spain) was added to the medium in a proportion of 2·698 mg ^{15}N per l medium to label the ammonia-N pool. Samples of 500 mg of each substrate were accurately weighed into 120-ml serum bottles. Malate (disodium salt; Sigma Chemical, Madrid, Spain) was added to achieve final malate concentrations of 0, 4 and 8 mmol/l. Bottles were pre-warmed (39ºC) prior to the addition of 50 ml of buffered rumen contents into each bottle under CO_2 flushing. Bottles were sealed with rubber stoppers and aluminium caps and incubated at 39ºC.

Fermentation kinetics from gas production curves

Gas production was measured in one bottle per substrate and per malate treatment at 3, 6, 9, 12, 16, 21, 26, 31, 36, 48, 60, 72, 96 and 120 h using a pressure transducer and a calibrated syringe, and the gas produced was released after each measurement. After 120 h of incubation, the fermentation was stopped by swirling the bottles in ice, the bottles were opened and their content was transferred to previously weighed filter crucibles (40 to 60-μm pore size). The undegraded substrate was washed with 50 ml of hot distilled water (50ºC), dried at 50ºC for 48 h, and analysed for ash to calculate the substrate organic matter (OM) apparent disappearance after 120 h of incubation (OMD_{120}; Carro and Ranilla, 2003).

Because in previous experiments we noticed that fermentation of malate itself can influence the amount of gas produced in batch cultures of rumen micro-organisms, two bottles without substrate for each malate concentration (0, 4 and 8 mmol/l) were included to correct the gas production values for gas released from buffered rumen contents and added malate. The experiment was repeated on four different days so that each treatment was conducted in quadruplicate.

Fermentation parameters

A total of 45 bottles with substrate (five bottles for each substrate and malate concentration) and six bottles without substrate (two per each malate concentration) were incubated. Bottles were withdrawn 17 h after inoculation (corresponding to a passage rate from the rumen of 0·06 per h) and total gas production was measured in two bottles per substrate and treatment as described above for fermentation kinetics. A gas sample (about 15 ml) was removed from each bottle and stored in a haemoguard vacutainer 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 in ice. One ml of content was added to 1 ml of deproteinizing solution (metaphosphoric acid (100 g/l) and crotonic acid (0·6 g/l)) for volatile fatty acid (VFA) determination, 2 ml were added to 2 ml 0·5 mol/l HCl for ammonia-N analysis, and 2 ml of content were stored

Table 1 *Chemical composition (g/kg dry matter) of ingredients of sheep diet and substrates incubated* in vitro

	Organic matter	Crude protein	Neutral-detergent fibre	Acid-detergent fibre	Malate	^{15}N:N (g/100 g)
Sheep diet ingredients						
Lucerne hay	912	158	472	301	ND†	ND†
Concentrate	916	198	151	46·8	ND†	ND†
Incubated substrates						
Low-concentrate	947	135	387	236	10·3	0·3751
Medium-concentrate	956	145	297	164	6·45	0·3714
High-concentrate	966	156	208	91·3	2·58	0·3711

† ND: not determined.

at –20ºC for lactate determination. Finally, the contents of the bottles were mixed with those from the remaining three bottles, and the resulting mixture was used to obtain samples of total digesta and to isolate mixed microbial pellets in order to determine microbial protein synthesis.

The mixture corresponding to each substrate and treatment was homogenized in a blender at low speed for 1 min. One portion (about 60 g) was stored at –20ºC and freeze-dried to determine DM content; this residue, representative of total digesta, was analysed for non-ammonia N (NAN) and the ^{15}N enrichment in the NAN fraction was determined. The rest of the pooled sample (about 140 g) was frozen until the isolation of mixed microbial pellets by differential centrifugation following the procedure described by Carro and Miller (1999). Briefly, samples were thawed at 4ºC, homogenized as described above, and strained through four layers of cheesecloth to remove particulate material. The strained material was centrifuged at 500 **g** for 10 min at 4ºC, and the supernatant fraction was then centrifuged at 18000 **g** for 25 min at 4ºC to obtain a mixed microbial pellet. This was washed by resuspension in distilled water and the centrifugation was repeated. Homogenizing and freezing were used as methods to detach solid-associated micro-organisms (Carro and Miller, 2002), and the mixed microbial pellets were used as reference to estimate microbial protein synthesis. Microscopic examination of final microbial pellets showed that they were essentially free from food particles, and the pellets were freeze-dried and analysed for N content and ^{15}N enrichment. The experiment was repeated on four different days.

Analytical procedures

DM, ash and N were determined according to the Association of Official Analytical Chemists (1999). Neutral- and acid-detergent fibre analyses were carried out according to Van Soest *et al*. (1991) and Goering and Van Soest (1970), respectively. NH_3-N concentration was determined by a colorimetric method (Weatherburn, 1967), and VFA and CH_4 were determined by gas chromatography as previously described (Carro *et al*., 1999). Total lactate was determined in centrifuged samples following the method described by Taylor (1996). Preparation of substrate samples for malate analysis and the subsequent malate analysis by high-performance liquid chromatography followed the procedures described by Callaway *et al*. (1997).

Lyophilized samples of total digesta were dampened with distilled water adjusted with 1 mol/l NaOH to pH > 10 and dried at 90ºC for 16 h to remove ammonia-N. The resulting NAN was collected by the Kjedahl method, steam distilled, and the distillate was collected in 5 ml of a solution of boric acid (30 ml/l). The distillate was acidified with excess H_2SO_4 and evaporated down to about 1·5 ml volume on a hot plate at 55ºC. The dried residue was redissolved in distilled water to give a solution containing an appropriate amount of N for ^{15}N analyses. The same procedure, with the exception of removing ammonia-N, was followed for the analysis of microbial pellets. Analyses of ^{15}N enrichment were performed by isotope ratio mass spectrometry. Substrates were also analysed for their natural ^{15}N content, and this value was used for background correction before ^{15}N addition.

Calculations and statistical analyses

In the 120-h incubations, gas production values for each inoculum and malate concentration were corrected for gas released from buffered rumen fluid and added malate. Corrected values were fitted with time to the exponential model : gas = A (1-e $^{(-c\ (t-lag))}$), where A is the asymptotic gas production, c is the fractional degradation rate and *lag* is the initial delay in the onset of gas production. The parameters A, c and *lag* were estimated by an iterative least-squares procedure using the PROC NLIN of the Statistical Analysis Systems Institute (1988). The effective degradability of substrate OM (OMED) was estimated assuming a rumen particulate outflow (p) of 0·06 per h according to the following equation:

$$OMED = [(OMD_{120}\ c)/(c + Kp)]\ e^{(-\ c\ lag)}.$$

The average fermentation rate (ml gas per h) was defined as the average gas production rate between the start of the incubation and the time at which the cumulative gas production was half of its asymptotic value, and was calculated as rate = $A\ c/[2\ (\ln 2 + c\ lag)]$.

In the 17-h incubations, the volume of gas (ml) produced was corrected for the gas produced from buffered rumen contents and added malate, and for temperature (273 K) and pressure ($1{\cdot}013 \times 10^5$ Pa) conditions to calculate the µmol of gas produced. The amount (µmol) of CH_4 produced was calculated by multiplying the gas produced by the concentration of CH_4 in the analysed sample. The amounts of VFA produced were obtained by subtracting the amount present initially in the incubation medium from that determined at the end of the incubation period.

The proportion of digesta NAN of microbial origin was estimated for each substrate and malate concentration by dividing the ^{15}N enrichment (atom% excess) by the enrichment of the corresponding NAN portion of digesta of the corresponding microbial pellet. Microbial production (mg/day) was estimated in each bottle by multiplying total NAN by the proportion attributed to the microbes (Ranilla *et al*., 2001). The amount of OM apparently fermented (OMAF) was estimated from net productions of acetate, propionate and butyrate, following the calculations described by Demeyer (1991). This value was used to estimate the efficiency of microbial growth (mg microbial N per g OMAF).

Data were analysed as a factorial design with three concentrations of malate (0, 4 and 8 mmol/l), three substrates (low-, medium- and high-concentrate), four incubation runs and the interaction malate × substrate included in the model. The sums of squares were further partitioned by orthogonal polynomial contrast to analyse differences among malate treatments, and the contrasts were distributed as follows : (1) control *v.* malate, and (2) 4 *v.* 8 mmol/l malate. All the statistical analyses were performed using the general linear models procedures of the Statistical Analysis Systems Institute (1988). For each treatment and substrate, there were four values for gas production parameters, substrate OM disappearance after 120 h of incubation, microbial protein synthesis and CH_4 production, and eight values for the rest of the measured variables.

Table 2 *Influence of disodium malate on gas production parameters (*A, c *and lag time), average gas production (AGPR), and organic matter effective degradability (OMED) during the* in vitro *fermentation of substrates with low (LC), medium (MC) and high (HC) concentrate content incubated in batch cultures of mixed rumen micro-organisms as estimated after correction for gas released from added malate (no. = 4)*†

						Significance of effects			
		Malate (mmol/l)				Malate (M)‡		Substrate (S)	M × S
	Substrate	0 (control)	4	8	s.e.d.	Control *v.* malate	4 *v.* 8 mmol/l		
A (ml)	LC	148	151	151	3·1			***	
	MC	162	165	164					
	HC	176	174	176					
c (per h)	LC	0·0640	0·0640	0·0657	0·00182			***	
	MC	0·0656	0·0658	0·0662					
	HC	0·0680	0·0701	0·0702					
lag time (h)	LC	1·75	1·75	1·79	0·242			***	
	MC	1·20	1·17	1·17					
	HC	0·55	0·53	0·52					
AGPR (ml/h)	LC	5·94	6·04	6·00	0·383			***	
	MC	6·93	7·10	7·08					
	HC	8·35	8·48	8·55					
OMED (g/g)	LC	0·377	0·377	0·375	0·0105			***	
	MC	0·419	0·413	0·416					
	HC	0·464	0·470	0·473					

† 50 ml diluted buffered rumen contents were incubated for 120 h with 500 mg ground substrate; for composition of substrates see Table 1.
‡ Orthogonal contrasts: control *v.* malate = comparison between control and the two malate treatments; 4 *v.* 8 mmol/l = comparison between malate at 4 and 8 mmol/l.

Results

The effects of malate treatment and substrate on gas production parameters and OMED are shown in Table 2. There were no effects of malate ($P>0·05$) on any of the estimated parameters, but all of them were significantly affected by the incubated substrate. The values of asymptotic gas production (*A*), average gas production rate and OMED increased ($P<0·05$) whereas those of lag time (*lag*) decreased ($P<0·05$) as the proportion of concentrate in the substrate increased. There were no significant ($P>0·05$) interactions between malate and substrate for any of the measured variables.

Table 3 *Influence of disodium malate on final pH, production of ammonia-N, lactate and microbial N and efficiency of microbial growth (EMG) during the* in vitro *fermentation of substrates with low (LC), medium (MC) and high (HC) concentrate content incubated in batch cultures of mixed rumen micro-organisms (no. = 8 for pH, ammonia-N and lactate, and no. = 4 for the rest of parameters)*†

						Significance of effects			
		Malate (mmol/l)				Malate (M)‡		Substrate (S)	M × S
	Substrate	0 (control)	4	8	s.e.d.	Control *v.* malate	4 *v.* 8 mmol/l		
pH	LC	6·35	6·39	6·40	0·013	***	*	***	
	MC	6·32	6·37	6·38					
	HC	6·26	6·31	6·35					
Ammonia-N (μmol)	LC	15·3	14·6	15·2	0·38				
	MC	14·9	14·8	14·7					
	HC	15·4	15·8	15·7					
Lactate (μmol)	LC	23·9	23·9	21·8	3·76			***	
	MC	26·1	24·8	25·9					
	HC	34·6	32·8	31·4					
Microbial N (mg)	LC	15·4	17·0	18·0	0·92	**		*	
	MC	16·7	19·1	19·1					
	HC	17·7	18·4	18·1					
EMG (mg microbial N per g OM apparently fermented)§	LC	54·2	55·4	54·1	2·82				
	MC	55·0	59·3	57·3					
	HC	57·2	55·5	51·5					

† 50 ml diluted buffered rumen contents were incubated for 17 h with 500 mg ground substrate; for composition of substrates see Table 1.
‡ Orthogonal contrasts: control *v.* malate = comparison between control and the two malate treatments; 4 *v.* 8 mmol/l = comparison between malate at 4 and 8 mmol/l.
§ Organic matter (OM) apparently fermented was estimated from net production of acetate, propionate and butyrate as described by Demeyer (1991).

Table 4 *Influence of disodium malate on methane (CH_4) and volatile fatty acid (VFA) production during the* in vitro *fermentation of substrates with low (LC), medium (MC) and high (HC) concentrate content incubated in batch cultures of mixed rumen micro-organisms (no. = 4 for CH_4 and no. = 8 for VFA)*†

		Malate (mmol/l)				Significance of effects			
						Malate (M)‡		Substrate (S)	M × S
	Substrate	0 (control)	4	8	s.e.d.	Control *v.* malate	4 *v.* 8 mmol/l		
CH_4 (μmol)	LC	701	662	657	27·4	*		***	
	MC	754	746	742					
	HC	812	762	773					
VFA (μmol)									
Acetate	LC	1902	2002	2129	40·5	***	***		
	MC	1917	2001	2108					
	HC	1925	2032	2154					
Propionate	LC	699	823	941	28·7	***	***	**	
	MC	744	838	920					
	HC	759	871	1006					
Butyrate	LC	456	484	518	22·8	**		**	
	MC	546	568	555					
	HC	575	615	595					
Others §	LC	125	129	140	7·37	**			
	MC	127	134	130					
	HC	119	164	139					
Total VFA	LC	3182	3438	3728	71·7	***	***	***	
	MC	3334	3541	3713					
	HC	3378	3682	3894					
Acetate : propionate (μmol : μmol)	LC	2·75	2·43	2·25	0·072	***	***	**	
	MC	2·64	2·39	2·29					
	HC	2·58	2·33	2·14					
CH_4 : VFA (μmol : μmol)	LC	0·221	0·193	0·177	0·0107	***	*	**	
	MC	0·226	0·211	0·201					
	HC	0·240	0·209	0·199					

† 50 ml diluted buffered rumen contents were incubated for 17 h with 500 mg ground substrate; for composition of substrates see Table 1.
‡ Orthogonal contrasts: control *v.* malate = comparison between control and the two malate treatments; 4 *v.* 8 mmol/l = comparison between malate at 4 and 8 mmol/l.
§ calculated as the sum of isobutyrate, isovalerate, valerate and caproate.

The effects of malate and substrate on final pH and the production of ammonia-N, lactate and microbial N are shown in Table 3. The addition of malate increased final pH ($P<0·001$) and microbial N ($P<0·01$; 16·6, 18·3 and 18·4 mg microbial N for malate at 0, 4 and 8 mmol/l, respectively). There were, however, no effects ($P>0·05$) of malate addition on the final amounts of ammonia-N and lactate and on the efficiency of microbial growth (55·5, 56·7 and 54·3 mg of microbial N per g of OMAF for malate at 0, 4 and 8 mmol/l, respectively). No significant ($P>0·05$) interactions between malate and substrate were detected. Level of malate did not affect ($P<0·05$) any of these parameters, with the exception of final pH of the cultures, which was higher ($P<0·05$) for 8 mmol/l than for 4 mmol/l malate. As expected, microbial growth increased as the proportion of concentrate in the substrate was augmented (15·4, 16·7 and 17·7 mg microbial N for the high, medium and low-forage substrates with no malate, respectively).

As shown in Table 4, the addition of malate increased ($P<0·01$) the production of acetate, propionate, butyrate and other VFA (calculated as the sum of isobutyrate, isovalerate, valerate and caproate), and reduced ($P<0·05$) CH_4 production (mean values of 756, 723 and 724 μmol for malate at 0, 4 and 8 mmol/l, respectively). As a consequence of these changes, the ratio CH_4 : VFA decreased ($P<0·001$) on adding malate to the cultures. No significant interactions ($P>0·05$) between malate and substrate were detected for VFA and CH_4 production. The production of both propionate and butyrate was augmented ($P<0·01$) as the proportion of concentrate in the substrate increased, but there were no differences ($P>0·05$) between substrates in the production of acetate and other VFA. The production of CH_4 was higher ($P<0·05$) for the high-concentrate substrate than for the low-concentrate one (782 and 673 μmol, respectively), with the medium-concentrate substrate having intermediate values (747 μmol).

Discussion

In most of the studies conducted to investigate the effects of malate on *in vitro* rumen fermentation (Martin, 1998; Carro and Ranilla, 2003), an increase in total gas production has been consistently reported. In the present experiment, gas production values were higher ($P<0·05$) for malate-supplemented cultures than for control ones at all sampling times (results not shown), but when gas production values were corrected for the amount of gas released from malate fermentation, no differences between treatments were detected in any of the estimated gas parameters. These

results would indicate that the observed increase in gas production was produced by fermentation of malate itself, as CO_2 is an end product of malate fermentation to propionate via the succinate-propionate pathway (Demeyer and Henderickx, 1967).

Concentrations of malate ranging from 4 to 12 mmol/l consistently increased final pH in batch cultures containing different concentrate foods (Callaway and Martin, 1996; Carro and Ranilla, 2003), but Martin (2004) did not find this effect when hay was used as substrate. In the present experiment, malate significantly increased final pH for all substrates, although the observed increase was low (mean values for all substrates of 6·31, 6·34 and 6·38 for malate at 0, 4 and 8 mmol/l, respectively). Callaway and Martin (1996) suggested that malate may act to buffer rumen contents by a dual mechanism of increased lactate utilization and CO_2 production by *S. ruminantium*. Although CO_2 production was not measured in the present experiment, adding malate increased gas production for all substrates; since CH_4 production was reduced by malate addition, the observed increase in gas production can only be due to an increased CO_2 production. However, no changes in the amount of lactate were found for any substrate.

The increased VFA production observed with all substrates is in accordance with previously reported results (Russell and Van Soest, 1984). Increases in the production of both acetate and propionate have been reported when batch cultures containing different cereal grains were supplemented with 7 and 10 mmol/l malate (Carro and Ranilla, 2003), although other authors found increases in propionate production without significant changes in the production of acetate when malate (4 to 12 mmol/l) was added to batch cultures containing cracked maize or hay (Martin and Streeter, 1995; Martin, 2004) and to Rusitec fermenters given a high-concentrate substrate (Gómez *et al*., 2005). These contrasting results might indicate that effects of malate could depend on the incubated substrate and/or the rumen inoculum. Although in the present experiment, adding malate increased acetate, propionate and butyrate production with all substrates, there were some differences in the observed response. Whereas the increases in total VFA production were similar for the low- and high-concentrate substrates (256 and 304 μmol for 4 mmol/l malate, and 546 and 516 μmol for 8 mmol/l malate, respectively), lower values were observed for the medium-concentrate substrate (207 and 379 μmol for 4 and 8 mmol/l, respectively). The reason for the lower increases in VFA production with this substrate is unknown, but it was observed in each of the four incubations conducted on different days; since the same rumen inoculum was used each day to inoculate all batch cultures, the differences observed between substrates in their response to malate addition can not be attributed to inoculum characteristics.

Russell and Van Soest (1984) showed that 7·5 mmol/l malate was completely fermented by mixed rumen micro-organisms within 10 h and the primary fermentation products were acetate and propionate. In our experiment, incubations with 0, 4 and 8 mmol/l malate were conducted in the absence of incubated substrate and the production of VFA was determined. The amount of VFA produced from fermentation of malate itself was calculated as the difference between the production in the cultures without substrate but with added malate (4 and 8 mmol/l) and that in the cultures containing only buffered rumen fluid. When VFA production in the cultures with substrate (values reported in Table 4) was corrected for the amount of VFA produced from malate fermentation itself, adding malate did not affect ($P>0·05$) the production of acetate (mean values for all substrates of 1915, 1918 and 1943 μmol for malate at 0, 4 and 8 mmol/l, respectively) and propionate (734, 755 and 757 μmol), but increased ($P<0·05$) the production of butyrate (526, 553 and 547 μmol). These results would indicate that most of the observed increase in VFA production was because of fermentation of malate itself.

Nisbet and Martin (1991) hypothesized that malate might act as an electron sink for hydrogen, after dehydration of malate to fumarate. As hydrogen is used to reduce fumarate, there can be a decrease in the availability of hydrogen for methanogenesis in the rumen, which could explain the observed decrease in methane production when substrates were incubated with malate. The proportionately 0·05 to 0·06 decrease in CH_4 production found in this study for the low- and high-concentrate substrates is fairly consistent with the response observed previously for similar doses of malate in experiments with batch cultures of mixed rumen micro-organisms (Callaway and Martin, 1996; Carro and Ranilla, 2003) and indicates that malate would be impractical as a means of effectively reducing CH_4 emissions *in vivo*. The reason for the lower reduction in CH_4 formation (proportionately 0·013) observed for the medium-concentrate diet is unknown.

Although previous studies (Nisbet and Martin, 1990 and 1993) have reported a positive effect of malate on the growth of *Selenomonas ruminantium* in pure cultures, to our knowledge no studies have investigated the effects of malate on the growth of mixed rumen micro-organisms; therefore, we determined microbial N production in the present study. Adding malate increased microbial N by proportionately 0·13, 0·14 and 0·03 for the low-, medium- and high-concentrate substrates, respectively. These results are in accordance with those reported by Gómez *et al*. (2005), who observed that 6·55 mmol/l malate increased microbial growth in Rusitec fermenters given a substrate containing 600 g/kg forage, but had no significant effect with one containing 900 g/kg concentrate. These results might indicate that malate can stimulate the growth of bacteria fermenting structural carbohydrates and not only the growth of *S. ruminantium*, as has been reported by Nisbet and Martin (1990 and 1993); therefore, studies investigating the effects of malate on the growth of individual rumen micro-organisms are needed. Adding malate, however, did not affect microbial growth efficiency (expressed as mg of microbial N per g of OM apparently fermented), which might indicate that the greater microbial N observed in malate-supplemented cultures was due to a greater availability of fermentable OM in these cultures.

Microbial N production values were in the range of those found previously by our group in batch cultures of mixed rumen micro-organisms fermenting starch and/or cellulose

and using ^{15}N as a microbial marker (Tejido *et al*. 2001), but values of efficiency of microbial growth were considerably greater than others reported in the literature from *in vitro* experiments (Illg and Stern, 1994; Ranilla *et al*., 2001). Efficiency of microbial growth is affected by a great number of factors, but the values obtained can also be strongly influenced by the techniques and microbial markers used to measure it (Dewhurst *et al*., 2000). It has been reported (Harmeyer *et al*., 1976) that estimates of *in vitro* microbial growth obtained indirectly from incorporation of isotopic markers are usually higher than those based on other methods, and it is possible that the use of ^{15}N as a microbial marker overestimated microbial growth in our experiment. In addition, microbial growth has several stages (lag, growth, stationary and decline), and in batch cultures the amount of microbial N varies relative to incubation time (Harmeyer *et al*., 1976). In this experiment, microbial N production was measured after 17 h of incubation, which could be considered as a stage of great microbial growth, and this might partly explain the great efficiencies of microbial growth observed.

In conclusion, malate had a beneficial effect on rumen fermentation by increasing final pH, VFA production and microbial growth, and only subtle differences were observed between substrates in their response to malate addition. The results seem to indicate, however, that most of the observed effects might be due to malate fermentation itself. Long-term studies with substrates of different composition would be required to asses if malate supplementation can increase microbial growth.

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