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Effects of disodium fumarate on *in vitro* rumen microbial growth, methane production and fermentation of diets differing in their forage:concentrate ratio

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The effects of disodium fumarate on microbial growth, CH_4 production and fermentation of three diets differing in their forage content (800, 500 and 200 g/kg DM) by rumen micro-organisms *in vitro* were studied using batch cultures. Rumen contents were collected from four Merino sheep. Disodium fumarate was added to the incubation bottles to achieve final concentrations of 0, 4 and 8 mM-fumarate, and ^{15}N was used as a microbial marker. Gas production was measured at regular intervals from 0 to 120 h of incubation. Fumarate did not affect ($P>0{\cdot}05$) any of the measured gas production parameters. In 17 h incubations, the final pH and the production of acetate and propionate were increased linearly ($P<0{\cdot}001$) by the addition of fumarate. Fumarate tended to increase ($P=0{\cdot}076$) the organic matter disappearance of the diets and to decrease ($P=0{\cdot}079$) the amount of NH_3-N in the cultures. Adding fumarate to batch cultures tended ($P=0{\cdot}099$) to decrease CH_4 production, the mean values of the decrease being 5·4 %, 2·9 % and 3·8 % for the high-, medium- and low-forage diet, respectively. Fumarate tended to increase ($P=0{\cdot}082$) rumen microbial growth for the high-forage diet, but no differences ($P>0{\cdot}05$) were observed for the other two diets. These results indicate that the effects of fumarate on rumen fermentation depend on the nature of the incubated substrate, the high-forage diet showing the greatest response.

Fumarate: Rumen microbial growth: Methane: Batch cultures

Methane is one of the major end products of anaerobic fermentation of feeds in the rumen. Reducing CH_4 production is an important goal of ruminant nutritionists as it represents a significant loss of energy for the host animal and contributes to global warming (Moss *et al.* 2000). Many attempts have been made to depress rumen methanogenesis through the use of feed additives as ionophores, halogen compounds, unsaturated fatty acids and organic acids. However, some of these substances simultaneously produce adverse effects on rumen fermentation, such as a depression of fibre digestion or a reduction of protozoal growth (Demeyer & Fievez, 2000). Conversely, some organic acids (fumarate and malate) have been shown to produce a decrease in CH_4 production accompanied by an increase in both the production of volatile fatty acids (VFA) and diet degradation (Isobe & Shibata, 1993; Carro *et al.* 1999; López *et al.* 1999*b*; Carro & Ranilla, 2003*a*,*b*).

Reported effects of fumarate on CH_4 production by rumen micro-organisms are variable. In *in vivo* trials, Bayaru *et al.* (2001) reported that fumaric acid supplementation (20 g/kg diet DM) produced a 23 % decrease in CH_4 production in steers fed sorghum silage as the only feed, whereas Newbold *et al.* (2002) only found a 12 % decrease in CH_4 when sheep fed grass hay were supplemented with 80 g fumaric acid/kg hay DM. In semi-continuous fermenters, López *et al.* (1999*b*) observed a 17 % decrease in CH_4 with a mixed diet and 7·35 mM-fumarate. In experiments with batch cultures, the differences between studies are more pronounced. Although small reductions (1·4–7·4 %) have been observed with concentrate feeds (Carro & Ranilla, 2003*a*) and a mixed diet (López *et al.* 1999*b*) for concentrations of fumarate from 4 to 10 mM, greater effects (ranging from 8 % to 17 %) have been reported by other authors (Asanuma *et al.* 1999; Iwamoto *et al.* 1999; López *et al.* 1999*a*) for mixed diets and concentrations of fumarate from 8 to 30 mM.

From these results, it seems that the effect of fumarate on CH_4 production by rumen micro-organisms may depend largely on the nature of the fermented substrate and the level of fumarate, but the different experimental conditions in the earlier cited studies (*in vivo*, fermenters, batch cultures) could also have contributed to the observed differences. The aim of the present study was therefore to evaluate the effects of two doses of fumarate on the CH_4 production and *in vitro* fermentation of three diets differing in their forage:concentrate ratio in batch cultures of mixed rumen micro-organisms.

In addition, several studies have reported a positive effect of fumarate on the growth of different rumen micro-organisms. Nisbet and Martin (1993) reported that 10 mM-fumarate stimulated the growth of *Selenomonas ruminantium* in pure cultures, and Asanuma *et al.* (1999) showed that 30 mM-fumarate increased the growth of *Fibrobacter succinogenes*, *Selenomonas ruminantium*, *Veillonella parvula*, *Selenomonas lactilytica* and *Wolinella succinogenes* in pure cultures. In addition, López *et al.* (1999*b*) found a significant increase in the number of cellulolytic bacteria

Abbreviations: NAN, non-NH_3 N; OM, organic matter; VFA, volatile fatty acids.

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when 7·35 mM-fumarate was added to semi-continuous fermenters. To our knowledge, however, no studies have been conducted to investigate the effects of fumarate on the growth of mixed rumen micro-organisms. We therefore decided to measure the *in vitro* rumen microbial growth in the present experiment using ^{15}N as a microbial marker.

Materials and methods

Diets, animals and experimental procedure

Three diets containing 800 g (high-forage), 500 g (medium-forage) and 200 g (low-forage) forage/kg diet DM were formulated. Forage was composed of alfalfa hay and maize silage (500 and 500 g/kg DM, respectively), and concentrate was based on barley, maize and soyabean meal (500, 350 and 150 g/kg DM, respectively). The chemical composition of the experimental diets is shown in Table 1. Samples of each diet 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 fed alfalfa hay *ad libitum* and 400 g concentrate per d administered in two equal portions at 09.00 and 18.00 hours. Concentrate was based on barley, maize and soyabean meal (390, 400 and 210 g/kg, respectively). The chemical composition of alfalfa hay and concentrate is given in Table 1.

The 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. Feed 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 & Van Soest (1970) in a proportion 1:4 (v/v) at 39°C under continuous flushing with CO_2. A dose of ^{15}N (95 % enriched SO_4 $(^{15}NH_4)_2$; Sigma Chemical, Madrid, Spain) was added to the medium in a proportion of 2·698 mg ^{15}N per litre medium to label the ammonia-N pool. Samples (500 mg) of each diet were accurately weighed into 120 ml serum bottles (Laboratorios Ovejero, SA, León, Spain). Fumarate (disodium salt; Sigma Chemical) was added to achieve final fumarate concentrations of 0, 4 and 8 mM. Fumarate was dissolved in distilled water, and the corresponding solution (1 ml) was added to each bottle immediately before incubation. Control bottles received 1 ml distilled water. Bottles were prewarmed (39°C) prior to the addition of 50 ml buffered rumen contents into each one under CO_2 flushing. Bottles were sealed with rubber stoppers and aluminium caps, and incubated at 39°C.

Fermentation kinetics from gas production curves

Gas production was measured 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. 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. The residue of incubation was washed with 50 ml hot distilled water and dried at 50°C for 48 h; the apparent disappearance of substrate was then calculated. The residue was then analysed for ash to calculate the organic matter (OM) apparent disappearance. Two blanks for each fumarate concentration were included to correct the gas production values for gas release from endogenous substrates and added fumarate. The experiment was repeated on 4 different days so that each treatment was conducted in quadruplicate.

Fermentation parameters and microbial growth

Forty-five bottles with substrate (five bottles for each diet and fumarate concentration) and six bottles without substrate (two bottles per each fumarate concentration; blanks) were incubated. Bottles were withdrawn 17 h after inoculation (corresponding to a passage rate from the rumen of 0·06/h), and total gas production was measured in two bottles per diet and treatment as described earlier. A gas sample (about 15 ml) was removed from each bottle and stored in a Haemoguard Vacutainer (Terumo Europe NV, 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 in ice. One mililitre of the content was added to 1 ml deproteinising solution (metaphosphoric acid (100 g/l) and crotonic acid (0·6 g/l)) for VFA determination, 2 ml were added to 2 ml 0.5 M-HCl for NH_3-N analysis, and 2 ml were stored at −20°C for lactate determination. Finally, the contents of the bottles 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 OM disappearance.

The rest of the bottles were used to obtain samples of total digesta and to isolate microbial pellets in order to determine microbial protein synthesis. The contents of the bottles corresponding to each diet and treatment were mixed and homogenised in a blender at low speed for 1 min. One portion (about 60 g) was stored at −20°C and freeze-dried to determine DM content; this residue, representative of total digesta, was analysed for non-NH_3-N (NAN), and the ^{15}N enrichment in the NAN fraction was determined. The rest of the pooled sample was frozen until

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

	Organic matter	Crude protein*	Neutral-detergent fibre	Acid-detergent fibre	^{15}N enrichment (%)
Sheep diet ingredients					
Alfalfa hay	912	158	472	301	ND
Concentrate	916	198	151	46·8	ND
Incubated diets					
High-forage	947	135	387	236	0·3751
Medium-forage	956	145	297	164	0·3714
Low-forage	966	156	208	91·3	0·3711

ND, not determined.
*N × 6·25.
For details of diets of procedures, see p. 72.

the isolation of microbial pellets by differential centrifugation following the procedure described by Carro & Miller (1999). Homogenising and freezing were used as methods to detach solid-associated micro-organisms (Carro & Miller, 2002), and the isolated microbial pellets were used as a reference to estimate microbial protein synthesis. Microbial pellets were freeze-dried and analysed for N content and ^{15}N enrichment. The experiment was repeated on 4 different days.

Analytical procedures

DM, ash and N were determined according to the Association of Official Analytical Chemists (1999). Neutral-detergent fibre and acid-detergent fibre analyses were carried out according to Van Soest *et al.* (1991) and Goering & Van Soest (1970), respectively. Ammonia-N concentration was analysed by a modified colorimetric method, and VFA concentrations by GC as previously described (Carro *et al.* 1999). Methane was analysed with a gas chromatograph (Shimadzu GC 14B; Shimadzu Corporation, Kyoto, Japan) equipped with a flame ionisation detector and a column packed with Carboxen 1000 (Supelco, Madrid, Spain). He was used as carrier gas, and peaks were identified by comparison with a standard of known composition. Total lactate was determined in centrifuged samples following the method described by Taylor (1996).

Lyophilised samples of digesta (about 1 g) were dampened with distilled water adjusted with 1 M-NaOH to pH > 10 and dried at 90°C for 16 h to remove NH_3-N. The resulting residue was analysed for NAN and for ^{15}N enrichment. The preparation of samples (digesta and microbial pellets) for ^{15}N analysis followed the procedure described by Carro & Miller (1999), and the analysis was performed by isotope ratio MS as described by Barrie & Workman (1984). Diets were also analysed for their natural ^{15}N content, and this value was used for background correction before adding ^{15}N.

Calculations

Gas production values were corrected for gas released from endogenous substrates and added fumarate for each inoculum and fumarate concentration. Corrected values were fitted with time to the exponential model:

$$\text{gas} = A(1 - e^{(-c(t-lag\ \ time))}),$$

where A is the asymptotic gas production, c is the fractional rate of gas production, *lag time* is the initial delay in the onset of gas production and t is the gas-reading time. The parameters A, c and *lag time* were estimated by an iterative least squares procedure using the PROC NLIN of the Statistical Analysis Systems Institute (version 6, 1989; SAS Institute Inc., Cary, NY, USA). The effective degradability of dietary OM was estimated assuming a rumen particulate outflow of 0·06/h, according to the equation proposed by France *et al.* (2000). The average gas production rate (ml gas/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:

$$\text{rate} = Ac/[2\,(\ln 2 + c\ \ lag\ \ time)]$$

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

The proportion of digesta NAN of microbial origin was estimated for each diet and fumarate concentration by dividing the ^{15}N enrichment (atom % excess) of the NAN portion of digesta by the enrichment of the corresponding microbial pellet. Daily microbial N production (mg/d) was estimated in each bottle by multiplying total NAN by the proportion attributed to the microbes (Ranilla *et al.* 2001).

Statistical analyses

Data were analysed as a factorial design with three concentrations of fumarate (0, 4 and 8 mM), three diets (high-, medium- and low-forage), four incubation runs and the interaction fumarate × diet included in the model. Orthogonal polynomial contrasts were performed to determine linear and quadratic effects of fumarate treatment. All the statistical analyses were performed using the general linear models procedures of the Statistical Analysis Systems Institute (version 6, 1989; SAS Institute Inc.). Within each diet, there were four values for each treatment for gas production variables, diet OM disappearance after 17 h of incubation and microbial protein synthesis, and eight values for the rest of the measured variables.

Results

Table 2 shows the effects of fumarate treatment and diet on gas production parameters and OM effective degradability. There were no effects ($P>0{\cdot}05$) of fumarate on any of the measured parameters, but all of them were significantly affected by the incubated diet. The high-forage diet showed the lowest ($P<0{\cdot}05$) values of asymptotic gas production (A) and fractional rate of gas production (c), and the greatest ($P<0{\cdot}05$) values of *lag time*.

The effects of fumarate and diet on final pH, diet OM apparent disappearance and the production of NH_3-N, lactate and microbial N are shown in Table 3. The addition of fumarate increased linearly ($P<0{\cdot}001$) the final pH, and tended to increase the diet OM apparent disappearance ($P=0{\cdot}076$) and decrease the final amount of NH_3-N in the cultures ($P=0{\cdot}079$). There were, however, no effects ($P>0{\cdot}05$) of the addition of fumarate on the amounts of lactate and microbial N, although fumarate tended ($P=0{\cdot}082$) to increase microbial growth with the high-forage diet. No significant ($P>0{\cdot}05$) interactions between fumarate and diet were detected for any of the measured variables.

As shown in Table 4, the addition of fumarate linearly increased ($P<0{\cdot}001$) the production of acetate and propionate, and tended ($P=0{\cdot}099$) to decrease CH_4 production linearly. As a consequence of these changes, the ratio CH_4:VFA decreased linearly ($P<0{\cdot}001$) as the concentration of fumarate increased. Adding fumarate to the cultures did not affect ($P>0{\cdot}05$) the production of butyrate and other VFA. No significant interactions ($P>0{\cdot}05$) between fumarate and diet were detected. The production of both propionate and butyrate was augmented ($P<0{\cdot}001$) as the proportion of concentrate in the diet increased,

Table 2. Influence of disodium fumarate on gas production parameters (*A*, *c* and *lag time*), average gas production (AGPR) and organic matter effective degradability (OMED) of diets with a high (HF), medium (MF) and low (LF) forage content incubated in batch cultures of mixed rumen micro-organisms

(Mean values with their standard errors for four fermentations)*

						Statistical significance of the effects ($P<$)†			
		Fumarate (mM)				Fumarate ‡			
Item	Diet	0	4	8	SED	Linear	Quadratic	Diet	Fumarate × diet
A (ml)	HF	148	151	151	3·0	NS	NS	0·001	NS
	MF	162	166	167					
	LF	176	178	177					
c (h^{-1})	HF	0·0640	0·0645	0·0648	0·00171	NS	NS	0·001	NS
	MF	0·0656	0·0679	0·0678					
	LF	0·0680	0·0688	0·0693					
Lag time (h)	HF	1·75	1·71	1·69	0·254	NS	NS	0·001	NS
	MF	1·20	0·94	1·02					
	LF	0·551	0·531	0·567					
AGPR (ml/h)	HF	5·94	6·12	6·11	0·381	NS	NS	0·001	NS
	MF	6·93	7·47	7·44					
	LF	8·35	8·42	8·42					
OMED (%)	HF	37·7	37·9	37·7	0·98	NS	NS	0·001	NS
	MF	41·9	43·2	42·9					
	LF	46·5	46·3	46·2					

*50 ml diluted buffered rumen contents were incubated for 120 h with 500 mg ground diet; for details of diets and procedures, see p. 72 and Table 1.
† NS; $P>0·10$ (*P* values between 0·05 and 0·10 are considered as trends and reported).
‡ Linear, linear effect of fumarate dose; quadratic, quadratic effect of fumarate dose.

but there were no differences ($P>0·05$) between diets in the production of acetate and other VFA.

Discussion

An increase in gas production due to fumarate supplementation has been consistently observed in batch cultures of mixed rumen micro-organisms (Callaway & Martin, 1996; López *et al.* 1999*b*; Carro & Ranilla, 2003*a*). In the present experiment, when gas production values were corrected only for gas release from endogenous substrates, fumarate increased ($P<0·05$) gas production for the three diets at all sampling times, with the exception of 2 h after incubation (results not shown). As CO_2 is an end product of fumarate fermentation to propionate via the succinate–propionate pathway (Demeyer & Henderickx, 1967), the observed increase in gas production could stem from fumarate

Table 3. Influence of disodium fumarate on final pH, organic matter apparent disappearance (OMAD), production of NH_3 N, lactate and microbial N and efficiency of microbial growth (EMG) during the *in vitro* fermentation of diets with a high (HF), medium (MF) and low (LF) forage content by mixed rumen micro-organisms

(Mean values for eight fermentations for pH, NH_3 N and lactate, and for four fermentations for the rest of parameters)*

						Statistical significance of the effects ($P<$)†			
		Fumarate (mM)				Fumarate‡			
Item	Diet	0	4	8	SED	Linear	Quadratic	Diet	Fumarate × diet
pH	HF	6·35	6·38	6·41	0·021	0·001	0·047	0·001	NS
	MF	6·32	6·38	6·39					
	LF	6·26	6·32	6·32					
OMAD (%)	HF	48·2	49·4	50·1	1·75	0·076	NS	0·001	NS
	MF	57·0	56·9	57·3					
	LF	67·0	66·3	69·0					
Ammonia N (μmol)	HF	15·3	14·6	15·0	0·61	0·079	NS	0·053	NS
	MF	14·9	14·9	14·5					
	LF	15·4	15·2	14·9					
Lactate (μmol)	HF	23·9	21·8	18·1	4·82	NS	NS	0·001	NS
	MF	26·1	24·7	24·1					
	LF	34·6	34·5	32·4					
Microbial N (mg)	HF	15·4	16·9	16·5	0·85	NS	NS	0·011	NS
	MF	16·7	17·4	17·4					
	LF	17·7	18·2	17·9					
EMG (mg microbial N/g OM disappearance)	HF	67·6	72·2	69·7	3·55	NS	NS	0·001	NS
	MF	61·7	64·4	63·5					
	LF	54·7	56·8	53·6					

*50 ml diluted buffered rumen contents were incubated for 17 h with 500 mg ground diet; for details of diets and procedures, see p. 72 and Table 1.
† NS: $P>0·10$ (*P* values between 0·05 and 0·10 are considered as trends and reported).
‡ Linear, linear effect of fumarate dose; quadratic, quadratic effect of fumarate dose.

Table 4. Influence of disodium fumarate on CH_4 and volatile fatty acid (VFA) production during the *in vitro* fermentation of diets with a high (HF), medium (MF) and low (LF) forage content by mixed rumen micro-organisms

(Mean values for eight fermentations)*

						Statistical significance of the effects ($P<$)†			
		Fumarate (mM)				Fumarate‡			
Item	Diet	0	4	8	SED	Linear	Quadratic	Diet	Fumarate × diet
Methane (μmol)	HF	701	661	665	25·2	NS	NS	0·001	NS
	MF	754	737	728					
	LF	812	780	783					
VFA (μmol)									
Acetate	HF	1902	1994	2095	44·2	0·001	NS	NS	NS
	MF	1917	1999	2065					
	LF	1925	2009	2153					
Propionate	HF	699	842	954	20.9	0·001	NS	0·004	NS
	MF	744	874	955					
	LF	759	853	1007					
Butyrate	HF	456	494	495	21·9	NS	NS	0·001	NS
	MF	546	568	539					
	LF	575	584	591					
Others §	HF	125	134	137	7·4	NS	NS	NS	NS
	MF	127	137	126					
	LF	119	125	128					
Total VFA	HF	3182	3463	3682	64·5	0·001	NS	0·001	NS
	MF	3334	3578	3684					
	LF	3378	3572	3879					
Acetate:propionate (μmol:μmol)	HF	2·75	2·38	2·21	0·077	0·001	NS	NS	NS
	MF	2·64	2·31	2·18					
	LF	2·58	2·41	2·15					
Methane:VFA (μmol:μmol)	HF	0·221	0·191	0·181	0·0076	0·001	NS	0·004	NS
	MF	0·228	0·207	0·200					
	LF	0·242	0·219	0·202					

*50 ml diluted buffered rumen contents were incubated for 17 h with 500 mg ground diet; for details of diets and procedures, see p. 72 and Table 1.
† NS: $P>0·10$ (P values between 0·05 and 0·10 are considered as trends and reported).
‡ Linear, linear effect of fumarate dose; quadratic, quadratic effect of fumarate dose.
§ Calculated as the sum of isobutyrate, isovalerate, valerate and caproate.

fermentation itself. In the present experiment, we included blanks containing 4 and 8 mM-fumarate (in addition to the incubation medium) in order also to correct the values for the amount of gas produced by the fermentation of fumarate itself. The lack of effects ($P>0·05$) of fumarate on any of the measured gas production parameters (Table 2) would indicate that the increase in gas production observed in the fumarate-treated cultures stemmed from the fermentation of fumarate.

In agreement with the results previously reported by other authors (Callaway & Martin, 1996; Asanuma *et al.* 1999; López *et al.* 1999*b*; Carro & Ranilla, 2003*a*) for diets of variable composition incubated in batch cultures of mixed rumen micro-organisms, the addition of fumarate increased ($P<0·001$) the final pH linearly in the present experiment. This effect has also been found *in vivo*; thus, Isobe & Shibata (1993) observed a greater pH in the rumen of goats fed two diets (containing 20 % or 50 % crashed maize) when fumaric acid was added to the feed (50 g/kg diet), the authors attributing this effect to a stimulation of saliva secretion caused by added fumarate. In contrast, Bayaru *et al.* (2001) did not find any differences caused by the addition of fumaric acid (20 g/kg diet DM) in rumen pH in steers fed sorghum silage as the only feed. The incubation medium used in batch cultures contains a buffer solution to counteract the drop in pH caused by the production of VFA and lactate. Callaway & Martin (1996) suggested that fumarate might act to buffer rumen contents by a dual mechanism of increased lactate utilisation by *Selenomonas ruminantium* and CO_2 production. In the present experiment, the addition of fumarate did not affect ($P>0·05$) lactate concentrations, although it increased total gas production with all the diets.

H is used to reduce fumarate in the rumen and, as a consequence, there is a decrease in the availability of H_2 for methanogenesis, which could decrease CH_4 production (Callaway & Martin, 1996). A reduction in CH_4 production owing to the addition of fumarate has been found in most of the studies conducted: in steers (Bayaru *et al.* 2001) and sheep (Newbold *et al.* 2002), in semi-continuous fermenters (López *et al.* 1999*b*) and in batch cultures (Asanuma *et al.* 1999; Iwamoto *et al.* 1999; López *et al.* 1999*b*; Carro & Ranilla, 2003*a*). Asanuma *et al.* (1999) stated that the addition of fumarate to ruminant feed possibly increased the number of fumarate-utilising bacteria in the rumen. This could help to explain some of the observed differences in the reduction of CH_4 production reported in the previously cited studies.

Reductions of 12 % (Newbold *et al.* 2002) and 23 % (Bayaru *et al.* 2001) have been observed in sheep and steers, respectively, when animals were fed forage as the only feed, and López *et al.* (1999*b*) found a 17 % decrease by adding fumarate to semi-continuous fermenters fed a mixed diet. In these experiments, fumarate was added to the diet for at least 7 d before measuring CH_4 production. In contrast, CH_4 production in batch cultures has been measured after short periods of time (6–24 h in the

experiments reported), and smaller reductions in CH_4 were found in some of these studies; reductions ranging from 1·4 % to 7·4 % have been reported by López *et al.* (1999*b*) with a mixed diet and by Carro & Ranilla (2003*a*) with concentrate feeds when batch cultures were supplemented with different doses of fumarate (4–10 mM).

Greater reductions have, however, been observed in other *in vitro* studies with batch cultures. Asanuma *et al.* (1999) reported an 11 % decrease with a mixed diet and 30 mM-fumarate, López *et al.* (1999*a*) found an 8 % decrease with a forage-based diet (750 g/kg DM diet) and 8 mM-fumarate, and Iwamoto *et al.* (1999) reported a decrease of 17 % with 10 mM-fumarate and a diet composed of starch and cellulose (666 and 333 g/kg, respectively). Noticeably, López *et al.* (1999*b*) found a 17 % decrease in CH_4 production in semi-continuous fermenters supplemented with 7·35 mM-disodium fumarate, but when the same diet was incubated in batch cultures of mixed rumen micro-organisms for 24 h, the addition of 5 and 10 mM-disodium fumarate decreased the CH_4 production by only 5·3 % and 6·4 %, respectively. In these experiments, rumen fluid from the same sheep was used to inoculate both systems (fermenters and batch cultures), and the differences in pH observed between the two systems (mean values of 6·78 and 6·47 for fermenters and batch cultures, respectively) do not seem to justify the marked differences observed in the reduction of CH_4 production (Van Kessel & Russell, 1996).

The CH_4 decrease found in the present experiment is fairly consistent with the response reported by other authors in batch cultures. Although the decrease in CH_4 was greater for the high-forage diet (5·7 % and 5·1 % for 4 and 8 mM-fumarate, respectively) than for the other two diets (mean values for both fumarate concentrations being 2·9 % and 3·8 % for the medium- and low-forage diets, respectively), the present values indicate that fumarate would be impractical as a means of effectively reducing CH_4 emissions *in vivo*, as has been previously stated (López *et al.* 1999*b*). Further research is needed to clarify whether the observed discrepancies in the effects of fumarate on CH_4 production in the rumen are due to the use of different diets and/or experimental conditions (*in vivo*, rumen fermenters or batch cultures).

Fumarate can be converted into propionate and acetate following different pathways (Demeyer & Henderickx, 1967). In the present experiment, the addition of fumarate linearly increased ($P<0·001$) the production of both acids, but the magnitude of the observed response differed between diets. Calculated recoveries of fumarate as acetate plus propionate were lower than or similar to 100 % for the medium-forage diet (mean values of 88 % and 104 % for 4 and 8-mM fumarate, respectively) and for the low-forage diet when 4 mM-fumarate was added (87 %). In contrast, recoveries higher than 100 % were observed for the high-forage diet (115 % and 110 % for 4 and 8-mM fumarate, respectively) and for the low-forage diet supplemented with 8-mM fumarate (117 %). These results would indicate that the *in vitro* fermentation of these diets was stimulated by the addition of fumarate, but the increase in acetate and propionate production observed for the other incubations could stem from fumarate fermentation itself. When incubations with 4 and 8 mM-fumarate were conducted in absence of incubated substrates (blanks; results not shown), fumarate recovery as acetate plus propionate was 93 % and 86 % for the 4 and 8 mM concentrations, respectively. The fact that these recoveries were lower than 100 % could be explained by an incomplete fermentation of fumarate after 17 h of incubation and/or by the accumulation of other final products (i.e. succinate). However, an incomplete fermentation of fumarate seems improbable, as Asanuma *et al.* (1999) reported that mixed rumen micro-organisms fermenting hay powder and concentrate consumed 100 % of the added fumarate (30 mM) after 9 h of incubation, and no succinate accumulation was detected after 12 h of incubation.

Although several studies (Nisbet & Martin, 1993; Asanuma *et al.* 1999) have reported a positive effect of fumarate on the growth of some rumen micro-organisms in pure cultures, no information on the effects of fumarate on the growth of mixed rumen micro-organisms was available; we therefore used ^{15}N as microbial marker to measure microbial N production in the present study. A trend ($P=0·082$) towards a greater microbial growth produced by fumarate was detected for the high-forage diet, but no significant effects were observed for the other two diets. These results are in agreement with the greater response to fumarate supplementation observed for the high-forage diet in the production of VFA.

As expected, microbial growth increased as the proportion of concentrate in the diet augmented (15·4, 16·7 and 17·7 mg microbial N for the high-, medium- and low-forage diet, respectively). Microbial N production values were in the range of those previously found by our group in batch cultures of mixed rumen micro-organisms fermenting starch and/or cellulose (Tejido *et al.* 2001). In contrast, the values of efficiency of microbial growth were considerably greater than others reported in the literature from *in vitro* experiments (Illg & Stern, 1994). The 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). In addition, microbial growth has several stages (lag, growth, stationary and decline), and in batch cultures these stages relative to incubation time will vary between substrates (Blümmel *et al.* 2003). 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 can partly explain the great efficiency of microbial growth observed. In any case, fumarate treatment did not affect the efficiency of microbial growth with any diet.

The results of the present study suggest that fumarate had a mild beneficial effect on the *in vitro* rumen fermentation of diets of different composition by increasing final pH and the production of acetate and propionate, and by decreasing CH_4 production. The greater response found for the high-forage diet in comparison with the other two diets would indicate that fumarate utilisation *in vitro* could depend on the nature of the fermented substrate. In any case, long-term studies with diets of different composition are required to assess the dietary conditions that influence the effectiveness of fumarate.

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