

Propionate precursors and other metabolic intermediates as possible alternative electron acceptors to methanogenesis in ruminal fermentation *in vitro*

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Fifteen potential precursors of propionate were tested for their ability to decrease CH₄ production by ruminal fluid *in vitro*. Sodium acrylate and sodium fumarate produced the most consistent effects in batch cultures, with 50% of the added precursors being fermented to propionate and CH₄ production decreasing by between 8 and 17%, respectively. Additives were more effective when added as free acids, but this also decreased the pH and may have inhibited fibre digestion. Changing the dietary substrate from predominantly grass hay to predominantly concentrate had no influence on the effectiveness of acrylate and fumarate. In an *in vitro* fermentor (the rumen simulating technique, Rusitec) with a grass hay—concentrate (50:50, w/w) diet as substrate, both compounds were again fermented to propionate (33 and 44% conversion to propionate, respectively). However, fumarate appeared more effective as a H₂ sink compound. It was calculated to capture 44% of the H₂ previously used for CH₄ formation compared with a 22% capture of H₂ with acrylate. Fumarate also caused a stimulation in fibre digestion. Thus, sodium fumarate was the preferred propionate precursor for use as a feed ingredient to decrease CH₄ emissions from ruminants.

Methane: Fumarate: Rumen: Organic acids

Reducing equivalents generated during ruminal fermentation are disposed of partly by the formation of CH₄, which is energetically wasteful and environmentally damaging. Many chemical compounds have been tested as potential feed additives for ruminants on the basis of their direct or indirect effects on CH₄ production in the rumen (Moss, 1993; Van Nevel & Demeyer, 1996; Demeyer & Fievez, 2000). These compounds include ionophores, halogenated CH₄ analogues and unsaturated fatty acids (Van Nevel & Demeyer, 1996; Demeyer & Fievez, 2000). Different biotechnological approaches have been investigated that might induce changes in the ruminal microbial population, such as defaunation (Newbold *et al.* 1995), which decreases the number of protozoa-associated methanogenic archaea, or the enhancement of acetogenesis in the rumen (López *et al.* 1999a).

Another means of diverting H₂ away from CH₄ formation would be to promote alternative electron-sink metabolic pathways to dispose of the reducing power (López *et al.* 1999b; Ungerfeld *et al.* 2003a). Both fumarate and malate are key intermediates in the succinate–propionate pathway, in which malate is dehydrated to fumarate and fumarate reduced to succinate, which is then decarboxylated to propionate (Fig. 1). Reducing equivalents are consumed in the reduction of fumarate to succinate, and hence both fumarate and malate have been shown to compete

successfully for H₂ in the rumen, with subsequent decreases in methanogenesis both *in vitro* and *in vivo* (Asanuma *et al.* 1999; López *et al.* 1999b; Bayaru *et al.* 2001; Jalc & Ceresnakova, 2002; Carro & Ranilla, 2003a,b). However, the diversion of H₂ to propionate was incomplete and may have been affected by the propionate precursor used, the quantity added or the basal diet (Callaway & Martin, 1996; Carro & Ranilla, 2003a,b).

The present study was undertaken to explore a range of precursors of propionate, either as free acids or as salts, as possible feed additives that would divert metabolic H₂ away from CH₄ to more useful products. Short-term *in vitro* batch incubations were used to evaluate whether the efficacy of the additives was affected by the forage:concentrate ratio of the diet. The long-term effects of promising candidates were then further evaluated in the rumen-simulating fermentor (Rusitec).

Materials and methods

Experiment 1: Influence of adding propionate precursors as sodium salts or free acids

Fourteen compounds were tested, all of which were propionate precursors or metabolic intermediates in the different pathways of

Abbreviation: VFA, volatile fatty acids; Rusitec, rumen simulating fermentor.

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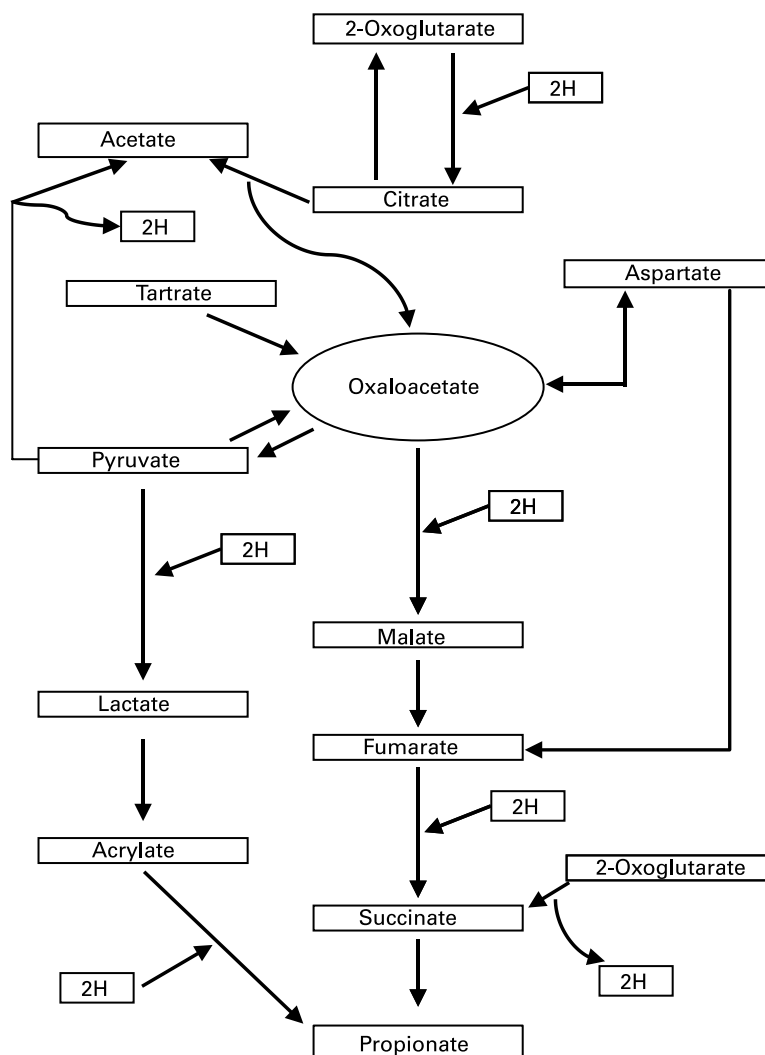


Fig. 1. Possible fermentation pathways for selected propionate precursors in the rumen.

volatile fatty acid (VFA) synthesis during ruminal fermentation (Fig. 1). Propionate itself was included as a control. The compounds used as additives were: pyruvate (sodium salt; Sigma Chemical Co., St Louis, MO, USA), L-lactate (sodium salt; Fluka Chemie, Buchs ZH, Switzerland), acrylate (sodium salt; Aldrich Chemical Co., St Louis, MO, USA), DL-aspartate (potassium salt; Fluka Chemie), fumarate (disodium salt; Sigma Chemical Co.), DL-malate (disodium salt; Aldrich Chemical Co.), citrate (trisodium salt; Sigma Chemical Co.), 2-oxoglutarate (α -ketoglutaric acid disodium salt; Sigma Chemical Co.), L(+)-tartrate (disodium salt dihydrate; Sigma Chemical Co.) and propionate (sodium salt; Sigma Chemical Co.). To test whether the form (salt or acid) of the compound added had any influence on the effect of the additive, the following acids were also used: DL-aspartic acid (Fluka Chemie), fumaric acid (Sigma Chemical Co.), DL-malic acid (Fluka Chemie) and citric acid (Sigma Chemical Co.).

In vitro incubations were carried out with ruminal fluid obtained 2 h after the morning feeding, from three rumen-cannulated sheep fed 1 kg/d of a good-quality grass hay. Ruminal contents were mixed and strained through two layers of muslin and maintained at 39°C under O_2 -free CO_2 . Ruminal fluid was diluted anaerobically into the medium described by Goering & Van Soest

(1970) containing (per litre): 475 ml distilled water, 1.25 ml trace elements solution (13.2 g $CaCl_2 \cdot 2H_2O$, 10 g $MnCl_2 \cdot 4H_2O$, 1 g $CoCl_2 \cdot 6H_2O$ and 0.8 g $FeCl_2 \cdot 6H_2O$ per litre), 237.5 ml buffer solution (35 g $NaHCO_3$ and 4 g $(NH_4)HCO_3$ per litre), 237.5 ml macromineral solution (5.7 g Na_2HPO_4 , 6.2 g KH_2PO_4 and 0.6 g $MgSO_4 \cdot 7H_2O$ per litre), 1.25 ml resazurin solution (1 g/L) and 47.5 ml freshly prepared reducing solution (6.25 g cysteine hydrochloride, 40 ml 1M-NaOH and 6.25 g $Na_2S \cdot 9H_2O$ per litre). After mixing, 50 ml buffered ruminal fluid were dispensed anaerobically to 120 ml serum bottles containing 400 mg diet F (Table 1), containing 750 g/kg forage and 250 g/kg concentrate, previously ground to pass through a 1 mm mesh screen. Solutions (0.4 M) of the different additives used in each experiment were prepared in distilled water, and 1 ml of each solution (400 μ mol additive) was added to each of two incubation bottles, to achieve final concentrations of 8 mM. Another two bottles received 1 ml distilled water (control cultures).

The bottles were sealed under a CO_2 atmosphere with rubber stoppers and aluminium caps, and were incubated at 39°C. Incubations were performed on 3 d with two replicates per day, and a total of thirty bottles (fourteen additives plus control, with two replicates each) was incubated in each run. After 24 h

Table 1. Ingredients and chemical composition of diets used as substrates in *in vitro* incubations

	General-purpose diet	High-forage diet	High-concentrate diet
Ingredients (g/kg DM)			
Grass hay	500		
Alfalfa hay		750	210
Maize		80	320
Barley	299.4	70	280
Soyabean meal		30	120
Fish meal	91		
Molasses	100	30	30
Vitamin–mineral mixture	9.5	40	40
Chemical composition (g/kg DM)			
Organic matter	832	856	908
Crude protein	148	175	162
Neutral detergent fibre	515	326	187
Acid detergent fibre	244	204	81
Acid detergent lignin	ND	31	4

ND, not determined.

incubation, fermentation was arrested by chilling to 4°C, and the quantities of fermentation products (total gas, CH₄ and VFA) formed were determined in each bottle following the methods of analysis described below.

Total gas was measured using a 100 ml lubricated syringe connected to a needle, which was injected through the stoppers into the headspace. A gas sample (1 ml) was removed from each bottle and analysed for CH₄ and H₂ by GLC as described by López *et al.* (1999b), using PYE 4500 Unicam GC containing a 4 mm × 3 m glass column packed with Porapak Q mesh 60–80 (Waters Associates Inc., Milford, MA, USA). The oven temperature was 65°C and the carrier gas (He) flow rate was 30 ml/min; a katherometer detector was used. Peaks were identified by comparison with gas standards of known composition.

The bottles were then uncapped and (only in Expt 1) the pH measured using a pH meter. Fermentation fluid (1 ml) was added to 0.2 ml of an acid solution containing 200 g/l orthophosphoric acid and 20 mM 2-ethyl-butyric acid. Acidified samples were centrifuged (27 800 g, 5°C, 15 min), and VFA were determined by GLC using ethyl-butyric acid as the internal standard, as described by Stewart and Duncan (1985). In Expt 1 only, DM disappearance (apparent DM degradation) was measured by filtering all the bottle contents using previously tared 50 ml, 40 mm plate, coarse-porosity, fritted-glass crucibles under a moderate vacuum.

The number of replicates for each experimental treatment was six (three experimental days with duplicated observations each day). To assess the differences between additives, one-way ANOVA was performed on data from the investigation into adding propionate precursors as sodium salts on *in vitro* fermentation, with additive as the treatment factor (ten additives plus control) and experimental day as a blocking factor and *Quercus Suber* L., considered. The statistical model used was:

$$y_{ijkl} = \mu + A_i + D_j + \varepsilon_{ijk}$$

where y_{ijk} is the value for each observation, μ is the overall mean, A_i represents the i th treatment effect (additive), D_j is the effect of the j th block (day) and ε_{ijk} is the random error.

Propionate addition was considered as a 'positive' control, representing the case in which an equimolar amount of the end-product is directly added to the medium. Dunnett's test was performed to determine whether any treatments were significantly different

from either the control (no acid) or the propionate treatment. To evaluate differences between the addition of the acid or its corresponding salt, ANOVA was performed using a nested design with additive and form of presentation (acid or salt) within each additive as the treatment factors. In this case, the statistical model used was:

$$y_{ijkl} = \mu + A_i + S_{j(i)} + D_k + \varepsilon_{kl(ij)}$$

where y_{ijk} is the value for each observation, μ is the overall mean, A_i represents the i th treatment effect (additive), $S_{j(i)}$ is the effect of the j th level of factor S (additive form, salt or acid) within each i th level of factor A , D_k is the effect of the k th block (day) and $\varepsilon_{kl(ij)}$ is the random error.

Experiment 2: Influence of diet on effectiveness of additives

The effects of adding propionate precursors on *in vitro* fermentation were compared with forage or concentrate diets as substrate. The compounds used as additives in this case were acrylate (sodium salt), DL-aspartate (potassium salt), fumarate (disodium salt), DL-malate (disodium salt), 2-oxoglutarate (α -ketoglutaric acid disodium salt), oxaloacetate (free acid) and propionate (sodium salt).

The donor sheep in this experiment received a mixed diet (1.4 kg/d) consisting of grass hay, barley, molasses, white fish-meal and a vitamin and mineral mixture (general-purpose diet, Table 1) in two equal meals. The two diets (F and C) described in Table 1 were used as substrates for *in vitro* incubation, so the total number of bottles incubated in each run was thirty-two (seven additives plus control in each of two diets, with two replicates each), and incubations were again performed on three non-consecutive days. The fermentation parameters measured in each bottle after 24 h incubation were total gas and CH₄ production, and VFA concentrations.

Data from the study investigating the effect of adding propionate precursors on the *in vitro* fermentation of forage or concentrate diets were analysed by two-way ANOVA according to a factorial design with diet and additive (seven additives plus control) as main factors of variation, and with experimental day as a blocking factor. In this case, the statistical model used was:

$$y_{ijkl} = \mu + A_i + F_j + AF_{(ij)} + D_k + \varepsilon_{ijkl}$$

where y_{ijk} is the value for each observation, μ is the overall mean, A_i represents the effect of i th level of factor A (additive), F_j is the effect of the j th level of factor F (feed), $AF_{(ij)}$ is the effect of the interaction $A \times F$, D_k is the effect of the k th block (day) and $\epsilon_{kl(ij)}$ is the random error. A one-way ANOVA to study the effects of the additive used was then performed for each diet separately. The model was then $y_{ijk} = \mu + A_i + D_j + \epsilon_{ijk}$, as explained above.

Experiment 3: Influence of fumarate and acrylate on fermentation in *Rusitec*

The rumen simulation technique (Rusitec) was used as described by Czerkawski & Breckenridge (1977). Twelve vessels were supplied at the same time every day with 20 g basal diet fed to the donor animals. The duration of the experiment was 21 d, during which four vessels received 6.25 mmol/d fumarate (disodium salt)/d, four vessels received 6.25 mmol/d acrylate (sodium salt) added to the basal diet, and the remaining vessels were controls. The nominal volume in each reaction vessel was 850 ml, and the dilution rate was set at 0.88/d, the infused liquid being artificial saliva (McDougall, 1948) at pH 8.4. Inocula for the fermentation vessels were obtained from a pooled sample (liquid and particulate rumen contents) from three ruminally cannulated sheep fed 1.4 kg/d general-purpose diet (Table 1) in two equal meals.

On the first day of the experiment, 300 ml strained ruminal fluid and 300 ml artificial saliva were transferred to each reaction vessel. Solid ruminal contents (80 g) were weighed into a nylon bag that was placed inside the food container in each vessel together with a bag of food. The food was the same diet (general-purpose diet; Table 1) fed to the donor animals and was provided in nylon bags, pore size 50 μ m, which were gently agitated in the liquid phase. Two bags were present at any time, and one bag was replaced each day to give a 48 h incubation. The bags that were removed from the vessels were placed in plastic bags, and their contents were washed and squeezed with 40 ml artificial saliva. This was done twice for each bag, the combined washings being poured back into the reaction vessels. Fermentation vessels were flushed with anaerobic grade CO₂ before filling, after filling and then every day during feeding (when the nylon bags with the food were changed).

Gas samples were taken over days 10 to 19 and analysed for CH₄ and H₂ by GLC as described above. During the last 2 d of the experiment, fermentation products were determined on samples taken from the liquid overflow, as described by Newbold *et al.* (1997). Fermentation acids were detected by GLC as described above. Ammonia was measured by the phenol–hypochlorite method of Weatherburn (1967). The volume of the liquid overflow, together with the concentration of fermentation products, was used to calculate the daily output of individual VFA and ammonia. pH was measured as described above in samples of fermentation fluid withdrawn from around the nylon bags at the time of feeding. The apparent degradation of the diet was estimated from the DM remaining in the bags after 48 h incubation. DM in feed samples and in incubation residues was determined by drying at 105°C for 48 h.

Samples for microbial counts were also taken on the last 2 d of the period. Protozoa in the liquid phase were enumerated microscopically in a counting chamber (Newbold *et al.* 1987). Hungate's methods (Hungate, 1969) were used to prepare media and to cultivate bacteria. Media were dispensed into Hungate

tubes sealed with butyl rubber stoppers (Bellco Glass Inc., Vineland, NJ, USA). A sample of fermentation fluid (20 ml) taken directly from the reaction vessel together with a sample of the digesta (1 g) remaining in the nylon bags incubated for the last 48 h were homogenized, under O₂-free CO₂, for 1 min using an MSE top-bladed homogenizer Machine Shop Equipment Ltd, Crawley, Surrey at full speed. Serial ten-fold dilutions were prepared under O₂-free CO₂ for each sample by the anaerobic method of Bryant (1972) using an anaerobic diluent (Mann, 1968). Total viable bacteria were enumerated in roll tubes with a complex rumen fluid plus sugars medium to which 20 g/l agar was added (medium M2; Hobson, 1969), and these tubes were incubated for 72 h at 39°C. Cellulolytic bacterial counts were made by a most-probable-number method based on the degradation of filter paper strips (Mann, 1968).

Results were analysed by one-way ANOVA. Each vessel was considered as an experimental unit, with the acid added (control, fumarate or acrylate) as the treatment factor. Again, the model used was $y_{ijk} = \mu + A_i + D_j + \epsilon_{ijk}$, as explained above. Following a significant F test ($P < 0.05$), differences among means were examined by the least significant difference test (Steel & Torrie, 1980).

Results

Effects of adding propionate precursors as sodium salts or free acids on in vitro fermentation (Experiment 1)

Ten sodium salts (Table 2) and five free acids (Table 3) were tested for their influence on fermentation in Expt 1. Propionate concentrations increased most with sodium acrylate (+52%), sodium fumarate (+59%) and sodium malate (+51%). Sodium citrate produced abundant acetate, but propionate and butyrate production were small, and there was no significant inhibition of CH₄ formation. The greatest inhibition of CH₄ formation with the sodium salts occurred with acrylate (−14%) and fumarate (−9%). 2-Oxoglutarate also caused a significant decrease in CH₄ (−13%), in spite of a relatively small increase in propionate concentration. Acetate and butyrate were the main products formed from 2-oxoglutarate. Butyrate, together with acetate and propionate, was also formed from sodium lactate, but no decrease in CH₄ formation was observed. Aspartic, fumaric and malic acids produced propionate, fumaric acid giving the greatest decrease in CH₄ formation (Table 3). CH₄ production was inhibited more when fumarate was added as a free acid (−17%) rather than a sodium salt (−8%). Supplementation with the sodium salts of fumarate, 2-oxoglutarate, lactate, malate, pyruvate and tartrate stimulated apparent DM degradation by between 10 and 16%, and no stimulation was observed when the free acids were added.

Only sodium pyruvate, malic and tartaric acids significantly decreased pH. Even when statistically significant, however, the effect was very small, all changes being a difference of 0.11 units or less, and the lowest pH recorded was 6.49. The calculated H₂ recovery was usually 80% or above, with the exceptions of sodium citrate, citric acid and 2-oxoglutarate, which gave about 70% recovery.

Effects of adding propionate precursors on in vitro fermentation of high-forage or high-concentrate diets (Experiment 2)

Sodium fumarate and sodium acrylate caused significant decreases in CH₄ production and increases in propionate

Table 2. The effects of adding propionate precursors as sodium salts on DM degradation and ruminal fermentation *in vitro*

	DM degradation (g/kg)	pH	Gas (ml/d)	CH ₄ (μmol/d)	Acetate (μmol/d)	Propionate (μmol/d)	Butyrate (μmol/d)	VFA (μmol/d)	H ₂ recovery* (%)
No addition	571	6.60	72.8	636	1420	458	187	2131	95.0
Acrylate	592	6.56	70.9	546 ^{a,b}	1519	696 ^{a,b}	113 ^b	2280 ^b	88.2
Aspartate	607	6.56	76.5 ^{a,b}	596	1556 ^a	666 ^{a,b}	205	2496 ^a	89.9
Citrate	627	6.58	80.7 ^{a,b}	614	2162 ^{a,b}	495 ^b	206	2933 ^{a,b}	68.4
Fumarate	640 ^a	6.59	75.9 ^{a,b}	582 ^b	1515	730 ^{a,b}	198	2513 ^a	91.9
2-Oxoglutarate	662 ^a	6.56	75.0	552 ^{a,b}	1841 ^{a,b}	505 ^b	242 ^{a,b}	2656 ^a	72.0
Lactate	657 ^a	6.57	76.0 ^{a,b}	641	1579 ^{a,b}	609 ^{a,b}	270 ^{a,b}	2530 ^a	89.3
Malate	638 ^a	6.61	77.8 ^{a,b}	613	1600 ^{a,b}	693 ^{a,b}	227 ^{a,b}	2591 ^a	89.5
Pyruvate	657 ^a	6.54 ^a	79.0 ^{a,b}	586 ^b	1643 ^{a,b}	506 ^b	202	2417 ^{a,b}	81.9
Tartrate	629 ^a	6.57	80.2 ^{a,b}	654	1769 ^{a,b}	436 ^b	222 ^{a,b}	2493 ^a	81.0
Propionate	612	6.58	72.4	663	1450	904 ^a	194	2620 ^a	106.3
SED	22.9	0.018	1.18	23.6	41.2	28.4	7.8	67.0	

VFA, volatile fatty acids.

^{a,b}Treatment was significantly ($P < 0.05$) different from control and propionate treatments, respectively.*H₂ recovery was calculated as described by Demeyer and Van Nevel (1975), in which 2H released = 2A + P + 4B and 2H accepted = 4M + 2P + 2B.**Table 3.** The effects of adding propionate precursors as free acids on DM degradation and ruminal fermentation *in vitro*

	DM degradation (g/kg)	pH	Gas (ml/d)	CH ₄ (μmol/d)	Acetate (μmol/d)	Propionate (μmol/d)	Butyrate (μmol/d)	VFA (μmol/d)	H ₂ recovery* (%)
No addition	571	6.60	72.8	636	1420	458	187	2131	95.0
Aspartic acid	594	6.57	76.9 ^a	576 ^a	1525 ^a	603 ^{a,c}	203	2397 ^a	88.0
Citric acid	603	6.53 ^c	86.1 ^{a,c}	618	2005 ^{a,c}	457	194	2714 ^{a,c}	72.0
Fumaric acid	576 ^c	6.55 ^c	75.5	526 ^{a,c}	1452	656 ^{a,c}	196	2368 ^{a,c}	87.8
Malic acid	575 ^c	6.49 ^{a,c}	77.1 ^a	552 ^{a,c}	1564 ^a	662 ^a	222 ^a	2509 ^a	85.3
Tartaric acid	608	6.51 ^{a,c}	84.4 ^{a,c}	630	1695 ^a	435	218 ^a	2409 ^a	81.4
SED (acid v. salt)	27.0	0.024	1.46	24.6	42.3	25.8	8.9	64.7	
SED (acid v. control)	30.0	0.023	1.65	25.2	44.1	25.9	10.5	68.2	

VFA, volatile fatty acids.

^aTreatment was significantly ($P < 0.05$) different from control.^cSignificant difference ($P < 0.05$) between the response to sodium salts (see Table 2) and the corresponding free acids (Table 3).*H₂ recovery was calculated as described by Demeyer and Van Nevel (1975), in which 2H released = 2A + P + 4B and 2H accepted = 4M + 2P + 2B.

production in incubations with high-forage (+42 and 38%; Table 4) and high-concentrate (+42 and 26%; Table 5) diets. The inhibition of CH₄ production was similar in the two diets for sodium fumarate (-6 v. -9% high-forage v. high-concentrate diet) and sodium acrylate (-8 v. -7%, respectively). As before, sodium malate formed a considerable amount of propionate but had less of an effect on CH₄ production than fumarate or acrylate. The accumulation of H₂ was minimal with all treatments, and calculated H₂ recoveries were high.

Effects of fumarate and acrylate on fermentation in *Rusitec* (Experiment 3)

The addition of acrylate and fumarate increased the production of propionate in *Rusitec* by 28 and 37% respectively (Table 6). Total VFA produced tended to be higher in the fumarate-supplemented vessels ($P = 0.087$). CH₄ production decreased by 28% in fumarate-supplemented vessels ($P < 0.05$), and acrylate supplementation had no significant ($P > 0.05$) effect on CH₄

Table 4. The effects of adding propionate precursors on *in vitro* fermentation with a high-forage diet

	Gas (ml/d)	CH ₄ (μmol/d)	H ₂ (μmol/d)	Acetate (μmol/d)	Propionate (μmol/d)	Butyrate (μmol/d)	VFA (μmol/d)	H ₂ recovery (%)
No addition	82.9	620	0.78	1430	552	263	2349	94.2
Acrylate	80.8	569 ^{a,b}	1.04	1534	760 ^{a,b}	193 ^{a,b}	2573 ^{a,b}	92.8
Aspartate	86.2	606	0.25	1458	665 ^{a,b}	244	2475 ^b	95.1
Fumarate	82.3	581 ^{a,b}	0.85	1512	783 ^{a,b}	261	2659 ^a	93.1
2-Oxoglutarate	83.4	593	0.00	1777 ^{a,b}	596 ^b	276	2775 ^a	80.4
Malate	90.9 ^a	594	0.21	1456	750 ^{a,b}	244	2553 ^b	98.8
Oxaloacetic acid	97.0 ^a	608	1.18	1582 ^a	589 ^b	282	2535 ^b	87.4
Propionate	88.2	634	0.87	1485	950 ^a	278	2818 ^a	101.0
SED	3.18	16.5	0.442	56.8	24.2	14.1	87.2	

VFA, volatile fatty acids.

^{a,b}Treatment was significantly ($P < 0.05$) different from control and propionate treatments, respectively.

Table 5. The effects of adding propionate precursors on *in vitro* fermentation with a high-concentrate diet

	Gas (ml/d)	CH ₄ (μ mol/d)	H ₂ (μ mol/d)	Acetate (μ mol/d)	Propionate (μ mol/d)	Butyrate (μ mol/d)	VFA (μ mol/d)	H ₂ recovery (%)
No addition	98.7	641	0.61	1597	660	392	2772	88.0
Acrylate	96.9 ^a	596 ^{a,b}	0.83	1735 ^a	833 ^{a,b}	362	3037 ^a	84.7
Aspartate	92.1 ^{a,b}	604	2.13	1530	783 ^{a,b}	319 ^{a,b}	2746 ^b	92.7
Fumarate	98.1 ^b	582 ^{a,b}	0.87	1696	938 ^{a,b}	376	3133 ^a	86.6
2-Oxoglutarate	96.8 ^b	609	0.19	1850 ^{a,b}	735 ^b	358	3083 ^a	81.2
Malate	101.4	621	1.55	1654	861 ^{a,b}	378	3022	89.3
Oxaloacetic acid	118.3 ^{a,b}	622	0.67	1781 ^a	733 ^b	390	3017	82.7
Propionate	105.8 ^a	650	1.32	1607	1118 ^a	385	3232 ^a	96.9
SED	2.60	17.5	0.793	64.1	37.2	20.9	111.1	

VFA, volatile fatty acids.

^{a,b}Treatment was significantly ($P < 0.05$) different from control and propionate treatments, respectively.**Table 6.** Influence of acrylate and fumarate addition (6.25 mmol/d) on pH and the production of volatile fatty acids (VFA), ammonia and gases (mmol/d) in the rumen simulating fermentor (Rusitec)

	Control	Acrylate	Fumarate	SED	<i>P</i> value
pH	6.71 ^b	6.71 ^b	6.78 ^a	0.026	0.0314
H ₂	0.34	0.26	0.27	0.103	0.6900
CH ₄	2.46 ^a	2.12 ^{a,b}	1.78 ^b	0.208	0.0287
Acetate	16.3	17.3	17.9	0.952	0.2673
Propionate	7.6 ^b	9.7 ^a	10.4 ^a	0.560	0.0020
Iso-butyrate	0.23	0.21	0.26	0.021	0.1190
Butyrate	8.1	8.6	9.6	1.091	0.4473
Iso-valerate	1.71 ^a	0.41 ^b	1.90 ^a	0.232	0.0002
Valerate	5.8	6.1	6.9	0.537	0.1105
Total VFA	39.7 ^b	42.4 ^{a,b}	47.1 ^a	2.914	0.0867
Acetate:propionate	2.14 ^a	1.79 ^b	1.73 ^b	0.067	0.0004
Ammonia	58.0	60.0	62.2	5.290	0.7350

^{a,b}Values in a row not sharing a common superscript letters were significantly different, $P < 0.05$.

production. The only other substantial change was that the production of iso-valerate was decreased compared with the control when acrylate was added. Fumarate, but not acrylate, increased pH within the Rusitec, but in all cases the pH was relatively stable at near neutral (6.71–6.78).

The addition of acrylate or fumarate did not affect microbial numbers (Table 7). There was a non-significant ($P = 0.18$) increase in DM digestion after 24 h fermentation in vessels supplemented with fumarate (Table 7).

Discussion

Many chemical compounds, such as ionophores, halogenated CH₄ analogues and unsaturated fatty acids (Van Nevel & Demeyer,

1996; Demeyer & Fievez, 2000), anthraquinones (Kung *et al.* 2003), sulphur-containing amino acids (Takahashi, 2001), inhibitors of methanogenic archaea (Miller & Wolin, 2001) and inhibitors of oxidative pyruvate decarboxylation (Ungerfeld *et al.* 2003b) have been studied as potential feed additives to suppress CH₄ production in the rumen (Moss *et al.* 2000). Some of these compounds inhibit the growth of CH₄-producing archaea, whereas others have an effect on the metabolic pathways involved in producing H₂ as a substrate for methanogenesis.

Although some chemicals may be capable of decreasing ruminal CH₄ production, there is growing concern over the use of chemicals not normally associated with human foods in farm animals. As methanogenesis is principally a sink for metabolic H₂ in the rumen, a possible alternative is the use of compounds,

Table 7. Influence of acrylate and fumarate addition (6.25 mmol/d) on microbial numbers and DM digestion in the rumen simulating fermentor (Rusitec)

	Control	Acrylate	Fumarate	SED	<i>P</i> value
Total viable bacteria ($\times 10^8$ /ml)	5.53	5.06	4.90	1.388	0.8977
Cellulolytic bacteria ($\times 10^6$ /ml)	4.26	5.50	8.51	2.641	0.3020
Protozoa ($\times 10^3$ /ml)	0.73	0.90	0.67	0.316	0.7621
DM (g) digested after 24 h	2.80	3.00	3.75	0.485	0.1754
DM digestibility after 24 h (g/kg)	166	176	217	28.0	0.2150
DM (g) digested after 48 h	6.34	6.22	6.98	0.709	0.5419
DM digestibility after 48 h (g/kg)	383	365	402	41.7	0.6803

such as propionate or butyrate precursors (Asanuma *et al.* 1999; López *et al.* 1999b; Ungerfeld *et al.* 2003a), that may act as electron sinks competing with methanogens for the available H₂. Dicarboxylic acids, including aspartate, malate and fumarate, which may act as H₂ acceptors in the rumen, have been tested (Callaway & Martin, 1996; Martin & Park, 1996). These organic acids or their metabolites are reduced by ruminal bacterial species that use either H₂ or formate as electron donors (Asanuma *et al.* 1999). The rumen microflora has a high capacity to reduce fumarate (Asanuma & Hino, 2000), and although the fumarate-utilizing bacteria have a lower affinity for H₂ or formate than methanogens (Asanuma *et al.* 1999), the reduction of fumarate may to some extent compete with methanogenesis for the metabolic H₂, such that in the presence of this acid, CH₄ production can be decreased (Asanuma & Hino, 2000). The results presented here extend these observations to describe the short- and longer-term effects of a number of propionate precursors on CH₄ production. Possible fermentation pathways in the rumen of the compounds used are shown in Fig. 1. All are potential precursors of propionate.

The molar conversion of organic acids to propionate in batch cultures varied between zero for tartrate and 68% for fumarate. This compares well with previous studies in which the percentage transfer of fumarate to propionate varied between 40 and 85% (Callaway & Martin, 1996; López *et al.* 1999b; Carro & Ranilla, 2003b). Carro & Ranilla (2003a), studying the fermentation of cereal grains, suggested that diet might influence the efficiency with which organic acids are converted to propionate; however, no consistent effect of diet was found in the present study, with 52, 58 and 50% of acrylate, fumarate and malate being converted to propionate in fermentations with a forage diet, and 43, 69 and 50%, respectively, being converted with a concentrate diet.

Although a number of compounds were found to decrease CH₄ production, the most consistent decreases in CH₄ production and increases in propionate production were found with fumarate and acrylate. Demeyer & Henderickx (1967) observed that the addition of 500 µM fumarate inhibited *in vitro* CH₄ production by 60%. The largest decrease noted in the present study was a 17% decrease in CH₄ production in response to the addition of 400 µM fumaric acid. This decrease is consistent with the decreases in CH₄ observed *in vitro* when fumarate was included in the incubations of Callaway & Martin (1996) and Carro & Ranilla (2003b) (15 and 5%, respectively). Ungerfeld *et al.* (2003a) previously reported a variable decrease in CH₄ production when some propionate and butyrate enhancers and analogues were evaluated *in vitro*.

Based on the equation $4\text{H}_2 + \text{CO}_2 = \text{CH}_4 + 2\text{H}_2\text{O}$, and the reduction of fumarate to succinate or acrylate to propionate by 2H (Fig. 1), the efficiency of H₂ capture by fumarate was on average 70% in batch cultures, whereas the figures for acrylate were nearer 100%. Thus, despite the more efficient conversion of fumarate to propionate in comparison with the conversion of acrylate to propionate, both caused on average a 10% decrease in CH₄ production over all the *in vitro* batch incubations. A greater response occurred with free fumarate and malate compared with sodium salts. Although the addition of free acids was associated with a decrease in the pH of the cultures, the change was very small and the resultant pH remained in the range optimal for CH₄ formation (Russell, 1998). Unless solubility or rate of dissolution was affected, it is unclear why the free acids were more effective.

Additives used to decrease CH₄ production in ruminal fermentations have had a variable effect depending on the substrate used as feed for the fermentation (García-López *et al.* 1996). Thus, a subset of the most promising compounds was examined with two diets of different forage:concentrate ratio added to the *in vitro* incubations. Again, the greatest response, in terms of a reduction in CH₄, was observed with fumarate and acrylate, with little difference between both diets in terms of the effect of the acids on CH₄ production (decreases of 8 and 6% with a forage diet, and of 7 and 9% with a concentrate diet when adding either acrylate or fumarate, respectively).

Stoichiometrically, the decrease in CH₄ production accounted for 68 and 85% of the H₂ used to convert fumarate into propionate when forage or concentrate diets were fermented, respectively, although no differences were detected between diets in total H₂ recovery (93 and 87% for the forage and concentrate diet, respectively).

Acrylate and fumarate were further evaluated in Rusitec. Of the added acrylate and fumarate, 33 and 44% respectively was recovered in propionate. However, fumarate appeared more efficient in decreasing CH₄ production (28 v. 14% decrease), and this effect was consistent over the period of time the Rusitec experiment lasted, in agreement with the results of López *et al.* (1999b), who also found that the effect of fumarate on CH₄ production in semi-continuous cultures persisted. The decrease of 0.68 mmol/d in CH₄ production with sodium fumarate corresponds to a utilization of $4 \times 0.68 = 2.72$ mmol H₂/d, close to the 2.8 mmol/d increase in propionate production, suggesting an efficient capture of H₂ with fumarate. With sodium acrylate, the decrease in CH₄ production was 0.34 mmol/d, equivalent to the diversion of 1.36 mmol H₂/d. Propionate production increased by 2.1 mmol/d, indicating that H₂ capture by acrylate was less efficient than by fumarate.

Other beneficial effects of adding propionate precursors may occur, for reasons that may not be related to methanogenesis. Apparent DM degradation was increased in batch cultures by between 10 and 16% by the addition of fumarate, 2-oxoglutarate, malate and tartrate. Increases in DM degradation were not observed when free acids were used. Ungerfeld *et al.* (2003a) observed that some propionate and butyrate precursors or analogues increased apparent substrate fermentation *in vitro*. Carro and Ranilla (2003b) observed a small increase (approximately 4%) in the apparent *in vitro* digestibility of maize supplemented with fumarate. Fumarate also increased numerically the apparent DM degradation in Rusitec (Table 7).

The ability of dicarboxylic acids to stimulate ruminal fermentation may be associated with differences in pH, although these may be small, and to the removal of H₂. In the current study, no consistent effect of the additives used on pH in batch cultures were observed, possibly owing to the inherent buffering capacity of the incubation medium used. In Rusitec, fumarate but not acrylate caused an increase in pH; again, the response was small, probably reflecting the buffering capacity of the medium used. The addition of fumarate might be associated with higher pH values, in part because these dicarboxylic acids stimulate lactate uptake and utilization by some bacterial species (Martin, 1998). It is noteworthy that CH₄ production is decreased with the addition of fumarate, even though an increased CH₄ production may be expected in response to a higher ruminal pH (Russell, 1998).

In addition, it has been shown that cellulolytic organisms benefit from the presence of methanogenic or other H₂-utilizing bacteria as a result of interspecies H₂ transfer (Wolin *et al.* 1997). Thus, if H₂ removal were increased by the presence of propionate precursors, this might also stimulate the growth of fibrolytic bacteria (López *et al.* 1999b) and enhance cellulose digestion (Asanuma & Hino, 2000).

It is thus apparent that organic acids might be used to decrease ruminal methanogenesis in farm livestock. In steers, CH₄ production from the rumen was reduced by 23% by fumaric acid supplementation (20 g/kg) without affecting the digestibility of the dietary fibre (Bayaru *et al.* 2001). The results of the present study suggest that we might expect to see approximately 50% of the fumarate being used to divert H₂ from CH₄ formation, mainly owing to the conversion of fumarate into products other than propionate. Thus, in order to decrease CH₄ production by 1 litre (1/22.4 = 0.045 mol, assuming that 1 mol gas = 22.4 litre), one would require 4 × 0.045 mol fumarate at 100% efficiency, or 2 × 4 × 0.045 = 0.36 mol sodium fumarate at 50% efficiency. The M_r of disodium fumarate is 160, so 0.36 × 160 = 58 g disodium fumarate would be required for a 1 litre decrease in CH₄ formation. In a dairy cow producing 500 litres CH₄/d, a decrease in CH₄ of just 10% would require 2.9 kg sodium fumarate (or 2.1 kg fumaric acid) to be fed. The implications of feeding these quantities of fumarate need to be considered.

The fumarate necessary for a 10%, or 50 litre, decrease in CH₄ formation would produce 50 × 0.18 = 9 mol propionate. One might argue that such an increase in propionate production would lead to an increase in glucose synthesis and hence in milk lactose: assuming a 70% efficiency in the conversion of propionate to glucose and a 40% conversion of glucose to lactose (Baldwin & Kim, 1993), the fumarate could lead to benefits in terms of 200 g additional lactose or 4.5 litres liquid milk at a lactose concentration of 4.8%. Furthermore, as already noted, there may be other benefits from fumarate addition. Martin *et al.* (1999) found that malate increased average daily gain in cattle fed a high-concentrate diet, apparently by improving ruminal function.

There is also the practicality of feeding such substantial amounts of fumarate. Feeding the free acid may be problematic because of concerns over rumen pH. However, as a salt, 2 kg disodium fumarate would provide 550 g Na, which is well in excess of all current recommendations and may indeed induce toxicity (National Research Council, 2001). Similarly excess propionate in the rumen may lead to a decrease in feed intake and problems such as reduced milk fat content in dairy cows and soft fat in lambs (Ørskov & Ryle, 1990; Oba & Allen, 2003). Thus, although organic acids such as fumarate may be of interest as a means of decreasing CH₄, thought needs to be given to the economics of the approach and the mechanism of supply.

A further consideration is that the effects of certain propionate and butyrate enhancers would be expected to be additive. 2-Oxoglutarate was almost as effective as sodium acrylate in decreasing CH₄ formation. In all incubations except those with the high-concentrate diet, 2-oxoglutarate did not form as much propionate as some other salts, but it formed butyrate as well as propionate. This result may indicate that combining certain compounds, forming more than one H₂ sink, may have a cumulative effect. For example, adding fumarate alone diverts H₂ into the dicarboxylic acid pathway for propionate formation. Adding acrylate as well could increase propionate production even more because it feeds into a different mechanism for propionate formation, the acrylate

pathway, which occurs in organisms different from those using the dicarboxylic acid pathway. Adding a butyrate enhancer could utilize further reducing equivalents. Thus, the apparent limit of a 10% decrease in CH₄ formation for single H₂-sink compounds may be modest in comparison with what might be achieved by combining electron-sink feed additives.

In conclusion, although it might be possible to use organic acids, and fumarate in particular, to decrease ruminal CH₄ production, the quantities required to make a major impact on daily CH₄ production might well be impractical.

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