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A meta-analysis of fumarate effects on methane production in ruminal batch cultures

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ABSTRACT: The objective of this study was to understand the effects of fumarate addition on methane (CH_4) and VFA production in the rumen through a meta-analysis of its effects on ruminal batch cultures. Because the reduction of fumarate to succinate can draw electrons away from ruminal methanogenesis, fumarate has been studied as a potential feed additive to decrease CH_4 production in ruminants. The average decrease in CH_4 in batch cultures was of 0.037 μmol/μmol of added fumarate, which is considerably lower than 0.25 μmol/μmol, the decrease predicted from the stoichiometry of the reactions involved. One reason that fumarate was not effective at decreasing CH_4 in batch cultures was that only an average of 48% of added fumarate appeared to be converted to propionate. Secondly, the incorporation of reducing equivalents in the conversion of fumarate to propionate was almost entirely offset by their release from an average of 20% of added fumarate that appeared to be converted to acetate. Thermodynamic calculations indicated that the conversion of added fumarate to both propionate and acetate was feasible. Fumarate appears to be more effective in decreasing CH_4 production and increasing propionate in continuous culture than in batch culture. This suggests that microbial adaptation to fumarate metabolism can be important. Variation in populations of fumarate-reducers, methanogens, and protozoa could all be involved. Fumarate supplementation for an extended period may result in the amplification of otherwise small populations of fumarate-reducers. Addition of some of these organisms may be helpful to improve fumarate conversion to propionate. Strategies based on enhancing the rumen's capacity to convert fumarate to propionate by maintaining a low fumarate concentration have been effective. Thermodynamic considerations should be taken into account when designing strategies for CH_4 abatement through the addition of external electron acceptors.

Key words: fermentation, fumarate, meta-analysis, methane, rumen

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INTRODUCTION

Methane (**CH_4**) is the main electron sink in ruminal fermentation. Its release to the atmosphere represents an energy loss of between 2 and 15% of ingested gross energy (Van Nevel and Demeyer, 1996) and also contributes to global climate change (Moss, 1993). Therefore, there is an interest in decreasing CH_4 production in the rumen for both environmental and economic reasons.

A strategy to decrease ruminal methanogenesis has been the use of organic acids that are intermediates in ruminal fermentation and are alternative electron acceptors to methanogenesis. These compounds can decrease CH_4 production but increase the production of VFA, which are the main source of energy for ruminants (Martin, 1998; Castillo et al., 2004). Fumarate is an intermediate of one of the pathways of propionate formation (Russell and Wallace, 1997) and has been extensively studied as an alternative electron sink to ruminal methanogenesis (Castillo et al., 2004). One mole of fumarate can draw 1 mol of H_2 away from methanogenesis by being reduced to succinate, a propionate precursor (Figure 1). Results obtained with fumarate supplementation in vitro and in vivo have been variable (García-Martínez et al., 2005). The objective of this research was to understand and interpret the effects of fumarate on ruminal methanogenesis and VFA production through a meta-analysis of its effects on batch cultures.

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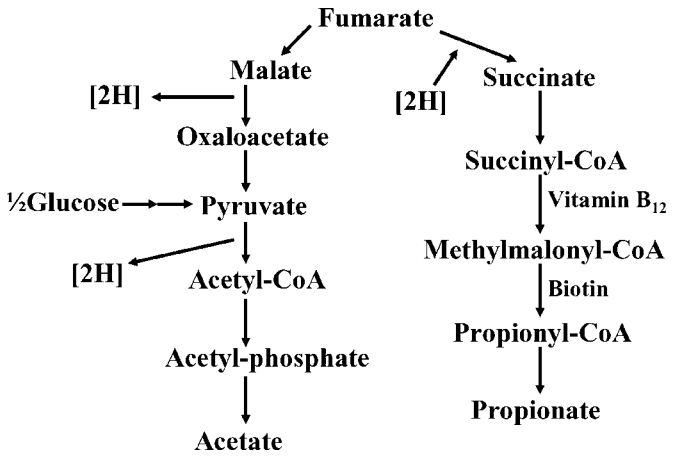

Figure 1. Simplified scheme of fumarate metabolism in the rumen.

MATERIALS AND METHODS

Experiments Not Previously Published

Animal experimentation was carried out under conditions governed by a license issued by the UK Home Office.

Two unpublished experiments where fumarate was added to ruminal batch cultures were used in the meta-analysis, along with 6 published studies. General experimental procedures for these 2 experiments were as described by López et al. (1999). Ruminal fluid was obtained after the morning feeding from 2 fistulated sheep fed a mixed diet (grass hay, barley, molasses, white fish meal, and a vitamin and mineral mixture at 500, 299.5, 100, 91, and 9.5 g/kg of DM, respectively) and strained through 2 layers of cheesecloth. One part of ruminal fluid was mixed with 2 parts of buffer (Menke and Steingass, 1987), and 50 mL of the mixture was delivered into 125-mL serum bottles under anaerobic CO_2 obtained by passing CO_2 through hot copper filings.

In the first experiment, bottles contained either 150 or 300 mg of the mixed diet fed to sheep, previously ground to pass through a 1-mm screen. Ten milliliters of disodium fumarate aqueous solutions were added to the bottles to achieve initial fumarate concentrations of 0, 1.5, 3, 6, 12, or 18 m*M*. In the second experiment, all bottles contained 300 mg of the mixed diet and received either a 1- or 10-mL disodium fumarate solution to achieve initial fumarate concentrations of 0, 1.5, 3, 6, 12, or 18 m*M*. Bottles were sealed under anaerobic CO_2 and placed in a shaking water bath at 39°C.

After 24 h of incubation, total gas volume was measured through the displacement of a syringe plunger. A 1-mL gas sample was taken from each bottle and analyzed for H_2, N_2, CH_4, and CO_2 by gas chromatography (GCV PYE Unicam, Cambridge, UK), with a 3-m-long by 4-mm-i.d. packed column (Porapak Q, Waters Associates Inc., Milford, MA; López et al., 1999). A 4-mL fluid aliquot was sampled from the bottles, added to 1 mL of a 20% (vol/vol) orthophosphoric acid and 20 m*M* 2-ethyl butyrate solution, and centrifuged at 14,000 × *g* for 20 min. The supernatant was then removed and analyzed for VFA by gas chromatography (Hewlett Packard P-5890, Palo Alto, CA) with a 15-m-long × 0.53-mm-i.d. Nukol, fused silica, capillary column (Supelco Canada, Mississauga, Ontario, Canada; López et al., 1999). Buffered ruminal fluid was also sampled before beginning the incubations to determine the initial VFA concentration. Each experiment was conducted on 3 different days with 3 replicates (bottles) per treatment on each day.

Meta-analysis

The effects of fumarate addition on CH_4 and VFA production in ruminal batch cultures were studied through several regression analyses. Experiment effects were included in the models, both as a main random effect and, initially, as their random interaction with independent variables. These terms correct the regression equations for random experiment effects and their possible interactions with the independent variables (St-Pierre, 2001). For all independent variables studied, linear and quadratic models were evaluated. Initial models therefore were: Response = intercept + $x + x^2$ + exp + (exp × x) + (exp × x^2) + residual, where x was the independent variable and exp was the random effect of the experiment. All interactions between experiment and independent variables were not significant (ratio between random interaction variance and residual variance < 1) and were removed from models. Nonsignificant ($P > 0.15$) quadratic effects (x^2) were also removed. Models that included the effects of concentrate percentage in the dietary DM and its interaction with fumarate concentration were also fitted. The REML algorithm of JMP (release 5.0.1.2, SAS Inst. Inc., Cary, NC) was used to fit the models.

The database used for the meta-analysis comprised 74 treatment means for CH_4 production and VFA production or concentration from 7 experiments in 6 published studies (Callaway and Martin, 1996; Asanuma et al., 1999; López et al., 1999; Carro and Ranilla, 2003; García-Martínez et al., 2005; Newbold et al., 2005) and the 2 unpublished experiments described above (Table 1). For some studies (Callaway and Martin, 1996; Asanuma et al., 1999), VFA production could not be calculated because only final VFA concentrations were reported; however, changes in VFA production caused by fumarate addition with respect to controls were calculated from the liquid volumes and final VFA concentrations of the incubations because initial VFA concentrations were assumed to be equal for all treatments.

Treatment means were weighted by the reciprocal of their variance (n/SEM^2) scaled to 1 to account for unequal replication and unequal variances of the means across experiments (St-Pierre, 2001). Normality of residuals was evaluated through the Shapiro-Wilk test

Table 1. Studies included in meta-analysis of fumarate effects on mixed ruminal batch cultures

Study	Substrate	Substrate, g	Incubation, h	Fumarate, m*M*	Fumarate form	Fumarate, % of total substrate[1]
Callaway and Martin (1996)	Corn	0.4	24	0, 4, 8, or 12	Disodium salt	4.4 to 12
Asanuma et al. (1999)	25% hay, 75% concentrate	0.24	6	0, 20, or 30	Not reported	36 to 46
López et al. (1999)	50% grass hay, 50% concentrate	0.2	24	0, 5, or 10	Disodium salt	12 to 22
Carro and Ranilla (2003)	Five concentrates (corn, barley, wheat, sorghum, or cassava)	0.5	17	0, 4, 7, or 10	Disodium salt	4.4 to 10
Newbold et al. (2005) – Experiment 1	75% forage, 25% concentrate	0.4	24	0 or 8	Disodium salt and free acid	10
Newbold et al. (2005) – Experiment 2	Mixed (50% hay) or high-forage (75% hay)	0.4	24	0 or 8	Disodium salt	10
García-Martínez et al. (2005)	High-forage (80% hay), mixed (50% hay) or high-concentrate (20% hay)	0.5	17	0, 4, or 8	Disodium salt	4.4 to 8.5
Original study — Experiment 1	Mixed (50% hay)	0.15 or 0.3	24	0, 1.5, 3, 6, 12, or 18	Disodium salt	3.3 to 45
Original study — Experiment 2	Mixed (50% hay)	0.3	24	0, 1.5, 3, 6, 12, or 18	Disodium salt	2.8 to 29

[1]Total substrate = substrate + fumarate.

(Neter et al., 1996). Plots of residuals against predicted values were examined. Presence of outliers and influential observations were studied by examining studentized residuals, leverage (hat) values, and Cook's distances. Potential influential outliers were identified as cases with a Studentized residual, absolute value larger than 1.96 (2 SD), a leverage value larger than 2k/n (where k was the number of independent variables and n was the number of treatment means used to fit the model; Belsey et al., 1980), or with a Cook's distance greater than 90% of the rest of the cases. Cases identified as potential influential outliers were deleted one at a time, and the models were fitted again in their absence. If most treatment means from one experiment were identified as potential influential outliers, the model was fitted again without all treatment means from that experiment. In all instances, changes in the regression coefficients and their *P*-values after deleting a mean or an experiment were minor, and the mean, or the experiment, were left in.

RESULTS AND DISCUSSION

It had been estimated that under ruminal conditions fumarate reduction should be more exergonic than methanogenesis in terms of Gibbs free energy released per pair of electrons incorporated (Ungerfeld and Kohn, 2006). Figure 2 shows that, over a broad range of fumarate intracellular concentration and at a typical ruminal H_2 pressure of 162 Pa (Kohn and Boston, 2000), Gibbs free energy change per mole of oxidant (fumarate or CO_2) could be comparable for fumarate reduction and methanogenesis. Thus, added fumarate would be expected to compete for H_2 with methanogenesis in the ruminal environment, at least from an energetic viewpoint.

Because fumarate decreases CH_4 production by competing for reducing equivalents, there is an expected stoichiometrical relationship between the decrease in

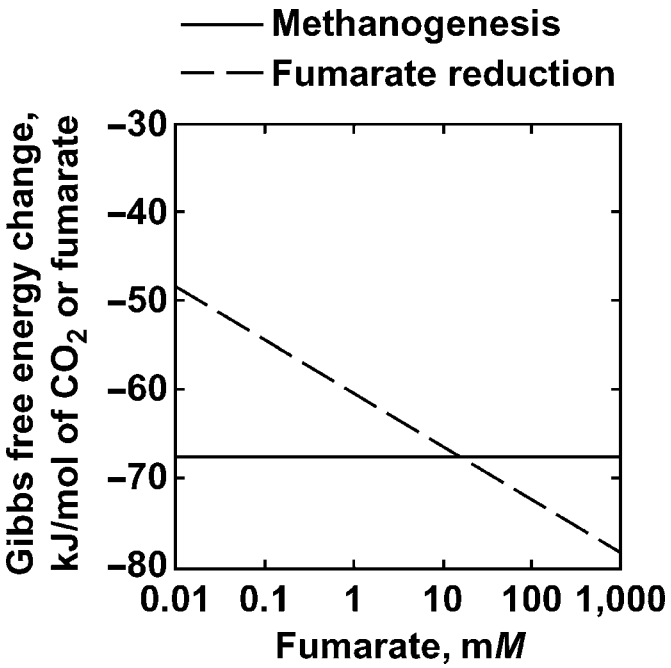

Figure 2. Estimated Gibbs free energy changes of fumarate reduction and methanogenesis in the rumen.

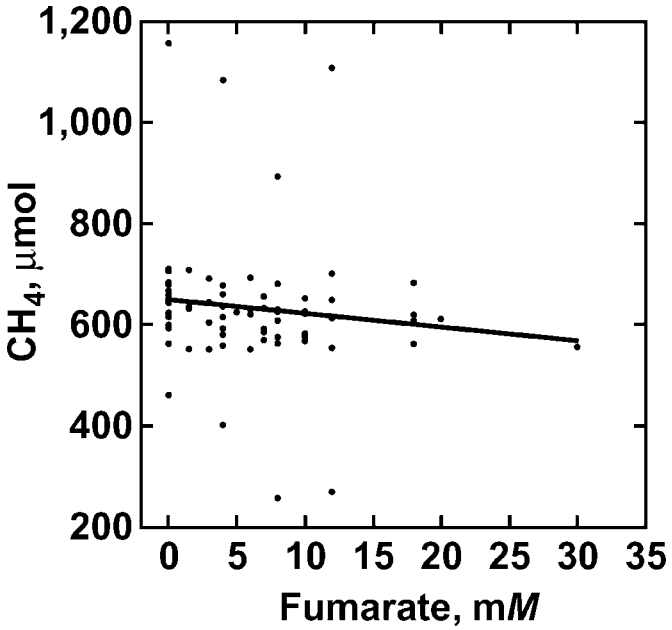

Figure 3. Effect of initial fumarate concentration on methane production in ruminal batch cultures. CH_4, μmol = 642 (± 110, $P < 0.001$) − [2.05 (± 0.91, $P = 0.029$) × initial fumarate, m*M*].

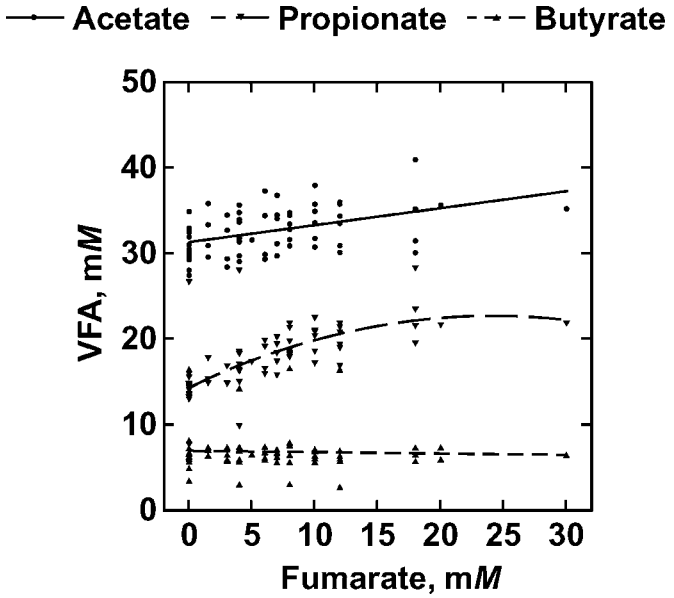

Figure 4. Effects of initial fumarate concentration on final VFA concentration in ruminal batch cultures. Acetate, m*M* = 30.8 (± 3.43, $P < 0.001$) + [0.213 (± 0.0601, $P < 0.001$) × initial fumarate, m*M*]; propionate, m*M* = 14.6 (± 3.09, $P < 0.001$) + [0.591 (± 0.0405, $P < 0.001$) × initial fumarate, m*M*] − [0.0173 (± 0.00420, $P < 0.001$) × initial fumarate, mM^2]; butyrate, m*M* = 6.50 (± 0.84, $P < 0.001$) − [0.00805 (± 0.0257, $P = 0.75$) × initial fumarate, m*M*].

CH_4 potentially achievable and the amount of fumarate added to a system. As 1 mol of fumarate converted to propionate requires the incorporation of 1 mol of reducing equivalents (Russell and Wallace, 1997), it would decrease CH_4 production by 0.25 mol (4 mol of H_2 are needed to produce 1 mol of methane: $CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$). This point was illustrated by Newbold et al. (2005), who showed that 1 mol of fumarate converted to propionate would stoichiometrically decrease CH_4 production by 5.6 L (1 mol of gas ~22.4 L at standard temperature and pressure); for a dairy cow producing 500 L/d of CH_4, a 10% decrease in CH_4 would require the animal to ingest a minimum of 1.4 kg/d of disodium fumarate (8.92 mol).

The present analysis found a linear relationship between CH_4 decrease and fumarate concentration (Figure 3), with a decrease in CH_4 of 0.037 μmol/μmol of added fumarate ($P = 0.03$). The residual plot had greater residuals at higher predicted CH_4 production, but transformations of the independent or dependent variables or removal of influential outliers did not resolve the problem. The observed decrease was more than 6-fold lower than the theoretical stoichiometry of –0.25 mol of CH_4/mol of added fumarate. There was no interaction ($P = 0.63$; data not shown) between the percentage of concentrate in the substrate DM and fumarate addition on CH_4 production. Controlled batch culture experiments that specifically investigated the interaction between fumarate addition and the type of substrate (high-forage, mixed, or high-concentrate) on CH_4 production reached similar conclusions (García-Martínez et al., 2005; Newbold et al., 2005).

The efficacy of fumarate addition to decrease CH_4 production can be analyzed by decomposing it into 2 factors: μmol of CH_4 decrease/μmol of added fumarate = (μmol of extra propionate/μmol of added fumarate) × (μmol of CH_4 decrease/μmol of extra propionate). The average recovery of fumarate as propionate was 0.48 μmol/μmol of added fumarate, less than half of the expected stoichiometry of 1 μmol/μmol. However, the relationship between extra propionate produced and added fumarate was quadratic ($P < 0.001$; Figure 4). Maximum recovery of fumarate as propionate was 0.68 μmol/μmol, and as initial fumarate concentration increased a lower fraction was converted to propionate. There were no interactions ($P = 0.30$; data not shown) between fumarate addition and the percentage of concentrate in the substrate DM regarding fumarate recovered as propionate.

Part of the incomplete conversion of fumarate to propionate could be simply due to incomplete fumarate utilization. Callaway and Martin (1997) found that, of an initial fumarate concentration of 7.5 m*M*, less than 1 m*M* fumarate remained after 12 h incubation in ruminal batch cultures. The presence of cellobiose seemed to accelerate fumarate utilization, compared with the absence of substrates other than fumarate itself. Minimal amounts of fumarate remained after 24 h incuba-

tion, except in the absence of cellulose and monensin, where slightly less than 1 m*M* fumarate remained. Asanuma et al. (1999) found that between 80 and 90% of added fumarate at 20 and 30 m*M* initial concentration disappeared after 6 h of incubation, and all had disappeared after 9 h of incubation. Even though fumarate seems to be metabolized fast, incomplete utilization may partly explain why the conversion of fumarate to propionate was not greater, especially in those studies that used high initial concentrations of fumarate and short incubation periods. In fact, the recovery of fumarate as propionate tended ($P = 0.09$; data not shown) to increase by 0.015 μmol/μmol per hour of incubation. However, incomplete metabolism cannot account for much of the inefficiency in the conversion of added fumarate to propionate.

Succinate turnover in the rumen is very high (Blackburn and Hungate, 1963), and added succinate is metabolized rapidly to propionate in vitro (Samuelov et al., 1999) and in vivo (Sijpesteijn and Elsden, 1952). However, at high fumarate concentration and short incubation times the rate of succinate production may exceed its rate of utilization, resulting in succinate accumulation in batch (Asanuma et al., 1999) and continuous cultures (López et al., 1999). Although succinate conversion to propionate does not involve uptake or release of reducing equivalents, fumarate reduction would become thermodynamically less favorable if succinate accumulated, affecting its capacity to compete for reducing equivalents with methanogenesis. Perhaps the addition of live cultures of succinate utilizers could relieve succinate accumulation and improve the conversion of added fumarate to propionate.

Another strategy could be the addition of an organism capable of converting fumarate to propionate without releasing succinate. *Selenomonas ruminantium* subsp. *lactilytica* metabolized fumarate to propionate with little accumulation of succinate, had a high affinity for H_2, and inhibited CH_4 production more when cocultured with methanogens than most of 5 fumarate-utilizers studied (Asanuma et al., 1999). *Selenomonas ruminantium* subsp. *lactilytica* could therefore be considered as a potential microbial additive to improve the conversion of added fumarate to propionate.

At high succinate concentration its rate of conversion to propionate may become limited by vitamin B_{12} or biotin availability (Figure 1). Supplementation with vitamin B_{12} increased propionate and decreased succinate production by *Prevotella ruminicola* growing on glucose (Strobel, 1992). Perhaps the mixed ruminal microbiota's requirements for vitamin B_{12}, Co, or biotin increase at high rates of succinate production when fumarate is supplemented.

Added fumarate can also be converted to products other than propionate or succinate (López et al., 1999). Concomitant with the increase in propionate, an average increase of 0.20 μmol of acetate/μmol of added fumarate occurred ($P < 0.001$; Figure 4), whereas butyrate production ($P = 0.15$) or concentration ($P = 0.76$; Figure 4) were not affected. There was no interaction ($P = 0.30$; data not shown) between fumarate addition and percentage of concentrate in the substrate DM regarding the increase in acetate per mole of added fumarate. The conversion of 1 mol of fumarate to acetate results in the production of 2 mol reducing equivalents pairs, in malate oxidation to oxaloacetate and in pyruvate oxidative decarboxylation to acetyl-CoA (Demeyer and Henderickx, 1967; Ungerfeld and Kohn, 2006; Figure 1). The production of reducing equivalents in the conversion of fumarate to acetate counterbalanced their incorporation into propionate formation, resulting in a decrease of 0.076 μmol of CH_4/μmol of extra propionate formed (Figure 5) instead of the expected stoichiometry of –0.25 μmol of CH_4/μmol of extra propionate. The 0.076 μmol decrease of CH_4/μmol of extra propionate formed agrees with the average increase in propionate production of 0.48 μmol/μmol of added fumarate and the decrease of 0.037 μmol of CH_4/μmol of added fumarate (Figure 3). The average uptake of 0.48 electron pair μmol/μmol of added fumarate converted to propionate was almost entirely offset by the release of 0.40 electron pair μmol/μmol of added fumarate converted to acetate (2 electron pair μmol/μmol of extra acetate × 0.20 μmol of extra acetate/μmol of added fumarate), resulting in a net uptake of only 0.08 electron pair μmol/μmol of added fumarate. This would theoretically result in a decrease in CH_4 of only 0.02 μmol/μmol of added fumarate (0.08 electron pair μmol/μmol of added fumarate × 0.25 μmol less CH_4/ electron pair μmol), which compares well with the observed value of –0.037 μmol of CH_4/μmol of added fumarate (Figure 3).

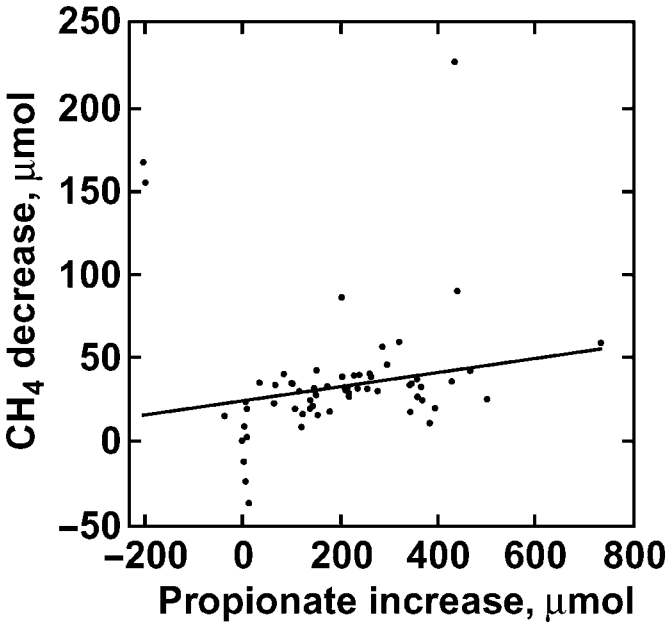

Figure 5. Effect of propionate production on methane production in ruminal batch cultures. Methane decrease, μmol = 14.0 (± 8.24, $P = 0.094$) + [0.0756 (± 0.0119, $P < 0.001$) × initial fumarate, m*M*].

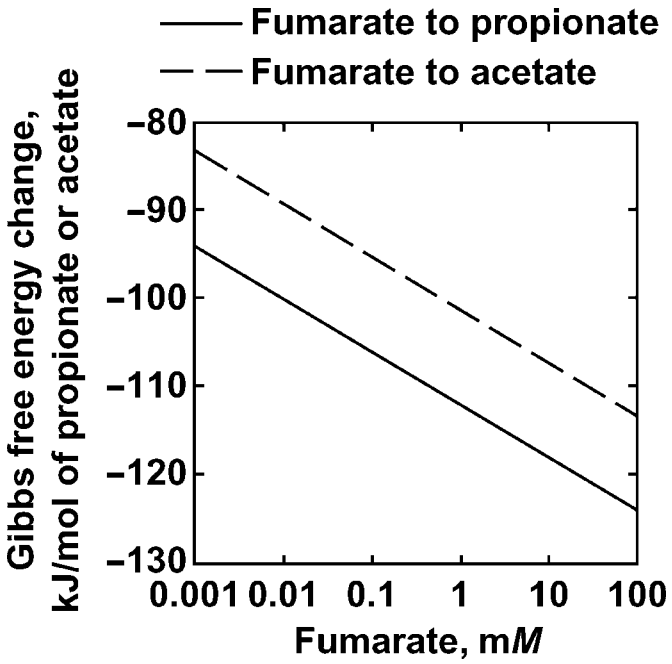

Figure 6. Gibbs free energy changes of fumarate conversion to propionate or acetate.

The increase in acetate with fumarate addition can be understood on the basis of thermodynamics. Gibbs free energy calculations show that acetate production from fumarate should be thermodynamically feasible in ruminal conditions even at very low fumarate concentration (Figure 6; Ungerfeld and Kohn, 2006). Production of acetate from fumarate has been reported for pure cultures of *Ruminococcus albus*, *Prevotella ruminicola* subsp. *brevis*, and *Anaerovibrio lipolytica* (Asanuma et al., 1999; Asanuma and Hino, 2000). Although the production of acetate from fumarate should release reducing equivalents, Schulman and Valentino (1976) observed that acetate production from fumarate in vitro occurred even under a 100% H_2 headspace, which agrees with thermodynamic calculations (not shown).

Reported decreases in CH_4 production in continuous culture studies were 19% (López et al., 1999), 17 to 41% (Moss and Newbold, 2006a), 26 to 38% (Kolver et al., 2004), and 28% (Newbold et al., 2005), compared with an average of 2.3% and a maximum of 18% in batch cultures. In continuous culture studies, propionate production increased on average by 0.85 mmol/mmol of added fumarate, compared with 0.48 μmol of extra propionate/μmol of added fumarate in batch cultures. Acetate production increased on average by 0.16 mmol/mmol of added fumarate in continuous culture, which appears to be similar to batch cultures.

In vivo responses in CH_4 production to feeding fumarate have been variable. Fumarate addition decreased CH_4 production in steers fed sorghum silage (Bayaru et al., 2001) and in sheep fed grass hay (Newbold et al., 2001) or concentrate and straw (Wallace et al., 2006); but had no effect in steers fed barley silage and grain (McGinn et al., 2004; Beauchemin and McGinn, 2006). Fumarate increased CH_4 production in sheep fed a mixed diet (Moss and Newbold, 2006b). The response observed does not seem to be related to animal species or type of diet.

Fumarate added to continuous culture appears in general to be more effective in decreasing CH_4 production than in batch cultures, which suggests that adaptation of ruminal microbiota to metabolize fumarate occurs. Ruminal continuous cultures supplemented with fumarate were not different from controls 5 d after fumarate was withdrawn (López et al., 1999), suggesting that adaptation to utilize added fumarate is lost in its absence. The synthesis of fumarate reductase by 10 fumarate-utilizers studied increased when fumarate was present in the medium, especially in those species with the greatest capacity to reduce fumarate to succinate (Asanuma and Hino, 2000). *Wolinella succinogenes* has a high affinity for H_2 and high fumarate reductase activity (Asanuma et al., 1999; Asanuma and Hino, 2000). *Wolinella succinogenes* is a slow-growing organism present in low numbers in the rumen (Asanuma et al., 1999) and may have little influence on fumarate metabolism in batch cultures, but fumarate supplementation to continuous cultures could result in an amplification of small populations of efficient fumarate-reducers, enhancing the capacity for reducing fumarate to succinate.

Some ruminal methanogens associate with protozoa for greater H_2 availability (Finlay et al., 1994). Protozoa-associated CH_4 production was between 9 and 25% (Newbold et al., 1995) or 37% (Finlay et al., 1994) of total. Associating with protozoa may increase the capacity of methanogens to compete for H_2 with fumarate-reducers. In agreement, fumarate effects on methanogenesis were more pronounced in protozoa-depleted than in protozoa-enriched ruminal fluid (Asanuma et al., 1999). Protozoa tend to die in continuous culture (Sharp et al., 1998), which could give fumarate-reducers an advantage in the competition for H_2 with methanogens compared with batch culture. The importance of protozoa-associated CH_4 production may be a factor affecting fumarate efficacy in vivo.

Formate is also a substrate for ruminal methanogenesis, although most formate is converted to CO_2 and H_2 before being reduced to CH_4 (Russell and Wallace, 1997). Formate donates electrons for fumarate reduction, and fumarate-reducers have a greater affinity for formate than methanogens (Asanuma et al., 1999). Hence, the efficacy of fumarate supplementation to decrease methanogenesis may be also affected by the proportion of pyruvate decarboxylated to acetyl-CoA by pyruvate-formate lyases vs. pyruvate oxido-reductases.

Lambs fed a slow-release form of fumaric acid produced half as much CH_4 as those given the same amount of free fumaric acid (Wallace et al., 2006). Likely, decreased rates of fumarate release resulted in lower and more uniform concentration, and greater recovery of

fumarate as propionate (Figure 4), improving its effectiveness in decreasing CH_4 production (Figure 5).

In summary, responses in CH_4 decrease to fumarate addition to ruminal batch cultures are substantially lower than what is expected from the stoichiometry of electron competition. This low efficacy seems to be explained by incomplete conversion of fumarate to propionate and by the release of electrons by part of fumarate that converts to acetate, the latter being expected based on thermodynamic principles. Because common biochemical and thermodynamic principles are involved, the strategies delineated here are thought to also apply to the use of other organic acids as alternative electron acceptors to ruminal methanogenesis.

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