Fumarate reduces methane production from pasture fermented in continuous culture

E.S. KOLVER, P.W. ASPIN, G.N. JARVIS[1], K.M. ELBOROUGH[1], J.R. ROCHE

Dexcel Ltd., Private Bag 3221, Hamilton, New Zealand

ABSTRACT

This experiment tested the hypothesis that addition of an organic acid (fumarate) would increase the energy captured from a pasture diet during ruminal fermentation. Pasture was fermented with 0, 10, 20, or 30 mM of fumarate constantly infused into four dual-flow continuous culture fermenters. Digestion characteristics responded linearly ($P<0.05$) as fumarate increased from 0 to 30 mM. Increasing fumarate from 0 to 30 mM reduced ($P=0.057$) methane production by 38%, and reduced the ratio of acetate:propionate (2.4 v.s. 1.5). Concentrations of propionate and total volatile fatty acids increased by 74% and 19%, respectively. These results were consistent with fumarate acting as an electron-accepting intermediary in the succinate-propionate pathway. The addition of fumarate increased energy capture from a pasture diet by improving the supply of glucogenic compounds and reducing losses to methane emissions.

Key words: fumarate, organic acids, pasture, rumen, methane

INTRODUCTION

Technologies are required to reduce greenhouse gas emissions, in particular methane emitted by ruminants. Currently there are no methane mitigation technologies that have proven effectiveness and safety, in terms of dairy production (Robertson & Waghorn, 2002). A number of options being investigated in New Zealand include low-methane generating ruminants, alternative forage species, proprietary vaccines, and feed supplements and additives.

Currently available feed additives with methane mitigating properties (e.g. ionophores) may not be compatible with New Zealand's food safety image. However naturally-occurring compounds, such as organic acids, may potentially reduce methane production and increase energy capture. Organic acids are produced by plants, constituting up to 10% of grass dry matter (DM). Of primary interest are the organic acids malate and fumarate, because they are electron-accepting intermediaries in the dicarboxylic acid (succinate-propionate) pathway of ruminal bacteria. In this pathway, additional malate or fumarate would be expected to promote the production of propionate at the expense of methane. Most studies have used malate to modify energy capture, but recent reports suggest that fumarate may be the most effective substance for reducing methane (Asanuma *et al*., 1999).

Limited *in vitro* and *in vivo* studies have been made of the methane mitigating properties of malate and fumarate. Reduced *in vitro* methanogenesis of 40% has been reported by Asanuma *et al*. (1999), Carro *et al*. (1999), Iwamoto *et al.* (1999), Lopez *et al*. (1999), and Martin & Streeter (1995) in response to the addition of malate or fumarate. Bayaru *et al*., (2001) reported a 23% decrease in methane production when fumarate (2% of DM) was added to an all-forage (sorghum silage) diet fed to steers.

Organic acids have also been proposed as a method to prevent ruminal acidosis associated with high grain diets by increasing the conversion of lactate to propionate, and subsequently increasing ruminal pH and digestibility (Asanuma *et al*., 1999; Callaway & Martin 1997; Martin *et al*., 2000). It is unclear whether the addition of malate or fumarate to pasture diets would improve fibre digestion and regulate ruminal pH, as high lactate concentrations are rarely observed during digestion of pasture diets.

This experiment tested the hypothesis that methane emissions would be reduced, and fibre digestibility increased in a dose-dependent manner with addition of fumarate, when pasture was fermented in continuous culture.

MATERIALS AND METHODS

Experimental Design

Four dietary treatments were fermented in continuous culture and compared according to a 4 x 4 Latin square design. A ryegrass pasture substrate was supplemented with 0, 10, 20, or 30 mM fumarate (sodium fumarate dibasic anhydrous $C_4H_2O_4Na_2$; Sigma F1506). Pasture was harvested January 2003 (Table 1). Freeze-dried pasture was ground to 1.6 mm and formed into loose pellets that were readily dispersed in the culture. All cultures received 59.8 ± 0.36 g (mean ± SD) DM/fermenter/day, added in equal portions at 0730 and 1930 hours.

Fumarate was infused continuously at a rate of 1.21 ml/hour using a four-channel syringe pump (Braintree Inc., USA). The 0 mM fumarate treatment received the same volume of distilled water as the fumarate treatments. The 0, 10, 20, and 30 mM fumarate treatments received 0, 2.10 ± 0.043, 4.25 ± 0.11, and 6.29 ± 0.2 g fumarate/fermenter/day, respectively, and resulted in steady state concentrations of 0, 10.9, 21.6, and 31.2 mM

[1] ViaLactia Biosciences (NZ) Ltd, PO Box 109-185, Auckland, New Zealand

fumarate (SED 1.59 mM) within fermenters. These concentrations were equivalent to a dietary DM content of 0, 3.5, 7.1, 10.5% of added sodium fumarate, or 0, 2.5, 5.2, 7.6% of added fumaric acid.

TABLE 1: Chemical composition of the pasture diet (mean ± SD).

Composition	%DM
Metabolizable energy (MJ ME/kg DM)	11.3 ± 0.2
Crude protein	18.4 ± 0.32
Neutral detergent fibre	45.2 ± 1.42
Acid detergent fibre	25.4 ± 0.74
Non-structural carbohydrate[1]	11.9 ± 0.76
Non-fibre carbohydrate[2]	22.6 ± 1.4
Crude fat	4.5 ± 0.12
Ash	9.3 ± 0.25

[1] Water soluble carbohydrate and starch.
[2] NFC = 100-(Crude protein + Neutral detergent fibre + Crude fat + ash).

Continuous Culture Operation

The experiment consisted of 4 periods, with 6 adaption days followed by 3 sample collection days in each period. The dual-flow continuous culture system, inoculation, operation and sampling procedures were as described by de Veth & Kolver (2001), except that the mineral buffer solution of Teather (1990) was used and pH was not regulated.

The culture system comprised of four individual fermenter units maintained at 39 °C. The liquid and solid dilution rates were 12 and 5 %/hour respectively, simulating the ruminal outflow of nutrients. Culture pH was recorded at five-minute intervals by a data logger (XR330 Pocket Logger, Pace Scientific Inc., Charlotte, NC). Oxygen-free nitrogen was purged through each fermenter at a rate of 2.9 ± 0.5 ml/min to maintain anaerobiosis.

Sample Collection and Analysis

Sample collection and analysis of DM constituents, true (corrected for bacterial DM) and apparent DM and organic matter (OM) digestibility, microbial growth, and VFA have been described by de Veth & Kolver (2001). Gas emissions from the fermenters were collected into tedlar gas bladders (2 and 3 L) during the last 3 sampling days of each experimental period. In periods 1, 2, and 3 gas bladders were changed at each feeding (12 h), while in period 4, bladders were changed at morning feeding (24 hours). Volume of empty and inflated bladders were measured by water displacement and 24-hour gas volumes calculated. Gas composition was determined within 5 days of collection using gas chromatography (HP5890 Series 2 chromatograph and FID RGD detector used for CH_4 analysis; HP6890 chromatograph and ECD FID detector used for CO_2 analysis).

Statistical Analysis

Data were analyzed using ANOVA in Genstat 5.4.2 according to a 4 × 4 Latin square design with period, treatment, and period x treatment as main terms. Levels of significance of linear contrasts are presented. No significant quadratic responses were observed.

RESULTS

The gas collected from fermentation included N_2 used to maintain anaerobiosis. A 38% linear (P=0.057) decrease in the concentration of CH_4 was observed from 3.0% of total gas with 0 mM fumarate to 1.9% of total gas with the 30 mM fumarate treatment (Table 2). Relative to the 0 mM fumarate treatment, 10, 20, and 30 mM fumarate reduced total CH_4 production by 41, 35, and 54%, respectively. When the volume of infused N_2 was excluded from calculations, fumarate reduced the CH_4 content of the gases of fermentation from 13.7% (0 mM) to 8.9% (30 mM). The concentration of CO_2 was not influenced by fumarate.

TABLE 2: Concentration of CH_4 and CO_2 in total gas and gas from fermentation in response to four concentrations of fumarate in continuous culture.

	Fumarate (mM)					
	0	10	20	30	SED[1]	*P*[2]
CH_4 (% of total gas collected)[3]	3.0	2.2	2.2	1.9	0.44	0.056
CH_4 concentration (% of control)	100	74	73	62		
CH_4 production (% of control)	100	59	65	46		
CH_4 concentration (% of fermentation gas)[4]	13.7	10.6	9.9	8.9		
CO_2 (%)	18.9	18.6	20.0	19.5	0.91	0.309

[1] Standard error of the difference.
[2] Linear relationship between fumarate treatments.
[3] Expressed as a proportion of total gas collected uncorrected for infusion of N_2.
[4] It is assumed that total gas production from fermentation of pasture DM is represented by the content of CH_4 and CO_2.

TABLE 3: Ruminal pH, concentration of total volatile fatty acids (VFA) and molar proportion of individual acids in response to four concentrations of fumarate in continuous culture.

	Fumarate (mM)					
	0	10	20	30	SED[1]	*P*[2]
Ruminal pH	6.23	6.18	6.27	6.39	0.05	0.017
Total VFA (mM)	55.6	58.1	61.4	66.2	2.76	0.010
Individual (mM)						
Acetate	31.9	30.9	32.6	33.5	2.07	0.369
Propionate	12.8	16.5	18.7	22.3	0.44	<0.001
Butyrate	7.8	7.5	7.1	7.0	0.44	0.117
Lactate	0.10	0.13	0.08	0.15	0.02	0.132
Acetate:propionate	2.44	1.88	1.74	1.51	0.13	0.001

[1] Standard error of the difference.
[2] Linear relationship between fumarate treatments.

TABLE 4: Nutrient digestibility of pasture in response to four concentrations of fumarate in continuous culture.

	Fumarate (mM)					
	0	10	20	30	SED[1]	*P*[2]
True digestibility (%)[3]						
Dry matter	64.6	64.8	62.6	60.7	1.33	0.023
Organic matter	66.0	66.4	64.4	62.8	1.39	0.047
Apparent digestibility (%)						
Dry matter	55.3	55.0	53.2	51.3	1.22	0.017
Organic matter	57.2	57.1	55.5	53.7	1.25	0.029
Neutral detergent fibre	66.4	67.6	66.3	67.6	1.48	0.646
Acid detergent fibre	63.0	65.7	64.8	65.7	1.55	0.199
Non structural carbohydrate	96.1	96.4	97.2	98.6	0.85	0.026

[1] Standard error of the difference.
[2] Linear relationship between fumarate treatments.
[3] Corrected for ruminal bacteria DM.

Culture pH increased linearly (P<0.05) from 6.23 with 0 mM fumarate to 6.39 with 30 mM fumarate (Table 3). A linear (P<0.05) increase was observed in concentrations of total volatile fatty acids (VFA) in fermenter effluent from 55.6 mM with 0 mM fumarate to 66.2 mM with 30 mM fumarate. A 74% linear (P<0.001) increase in the concentration of propionate was observed from 12.8 mM at 0 fumarate to 22.3 mM at 30 mM fumarate. Concentrations of acetate (32.2 ± 1.12 mM), butyrate (7.4 ± 0.35 mM), and lactate (0.12 ± 0.03 mM) were not influenced by fumarate.

Addition of fumarate linearly (P<0.05) reduced the true (corrected for ruminal bacterial DM) digestibility of DM from 64.6% at 0 mM fumarate to 60.7% at 30 mM fumarate (Table 4). True digestibility of OM similarly decreased (P<0.05) from 66% at 0 mM fumarate to 62.8% at 30 mM fumarate.

The apparent digestibility of neutral detergent fibre (67.0 ± 0.73%; mean ± SD) and acid detergent fibre (64.8 ± 1.28%) was unaltered by the addition of fumarate. Apparent digestibility of non-structural carbohydrate linearly increased ($P < 0.05$) from 96.1% at 0 mM fumarate to 98.6% at 30 mM fumarate.

DISCUSSION

The magnitude of the reduction in methane measured at the highest dose of fumarate in the current study is equivalent to reducing the annual methane production of a grazing cow from 112 kg methane (Waghorn *et al*., 2001) to 69 kg methane. This reduction in methane, and the concomitant increase in propionate measured in this study is consistent with fumarate acting as an electron-accepting intermediary in the succinate-propionate pathway. Under fermentation conditions typical of dairy cows grazing pasture, the increased concentration of propionate appeared to be a direct response to the addition of an intermediary substrate (fumarate) in the succinate-propionate pathway. This is in contrast to the mechanism frequently reported for cattle fed high-grain diets. On high-grain diets fumarate increases lactate utilisation, which results in an elevated ruminal pH and subsequent increase in fibre digestibility

(Asanuma *et al*., 1999; Callaway & Martin 1997; Martin *et al*., 2000). These results indicate that the use of naturally-occurring organic acid supplements may be a novel means to reduce methane production and improve energy capture by ruminants grazing pasture. However, the level at which fumarate may be practically included in the diet of dairy cows is yet to be determined.

The role of fumarate in bacterial energy metabolism and methanogenesis has been well established. Some ruminal anaerobic bacteria utilise the reductive or reverse citric acid cycle to produce propionate from pyruvate (Gottschalk, 1986). In this process, fumarate acts as an alternative electron sink for H_2 in the production of succinate, which is decarboxylated to yield propionate. The use of H_2 to reduce fumarate decreases the availability of H_2 for methanogensis in the rumen.

The large increase in ruminal concentrations of propionate, but not acetate or butyrate in the present study suggests that most of the added fumarate was metabolised to propionate. This is consistent with other *in vitro* (Asanuma *et al.,* 1999; Callaway & Martin, 1996; Lopez *et al*., 1999; Iwamoto *et al.,* 1999) and *in vivo* (Isobe *et al*., 1993) studies that used grain-based diets. Every 1 mM of fumarate increased the concentration of propionate by 0.31 mM over the range 0 to 30 mM fumarate, which is similar to the conversion reported by Asanuma *et al*., 1999 (0.24 mM propionate per 1 mM fumarate). *In vitro* studies using lower concentrations (0-12 mM fumarate) report higher conversion efficiencies (0.66 to 0.79 mM propionate per mM fumarate; Callaway & Martin, 1996; Lopez *et al*., 1999).

The 54% reduction in total methane production at 30 mM fumarate in the present study is higher than the 11% reduction (at 30 mM fumarate), but comparable to the 43% reduction (at 20 mM fumarate) in methane production reported *in vitro* by Asanuma *et al*., (1999). *In vitro* studies that have added smaller quantities of fumarate report a smaller reduction in methane. Iwamoto *et al.,* (1999) achieved a 17% reduction with 10 mM fumarate and Lopez *et al*., (1999) reported a 5.5% reduction with 10 mM fumarate and a 19% reduction with 7.4 mM fumarate. The *in vivo* study of Bayaru *et al*. (2001) demonstrated a 23% reduction in methane production when fumarate comprised 2% of a forage diet fed to steers.

Supplementation with malate has also been shown to reduce *in vitro* production of methane (Martin & Streeter, 1995), although this does not always occur, especially when high grain diets result in a low production of methane (Carro *et al*., 1999; Callaway & Martin, 1996; Martin & Streeter, 1995).

This study has also shown that fumarate can increase the supply of glucogenic precursors. However in cattle feeding systems that use high inputs of carbohydrate, malate and fumarate supplements are principally of interest for their ability to stimulate lactate utilisation, thereby raising ruminal pH and decreasing the risk of ruminal acidosis (Martin 1998; Vicini *et al*., 2003). The increased uptake of lactate by *Selenomonas ruminantium* may be as much as 10-fold with addition of malate or fumarate, and has been well documented (Nisbet & Martin 1994), as has the increase in ruminal pH (Asanuma *et al*., 1999; Callaway & Martin, 1996; Lopez *et al*., 1999; Martin, 1998; Martin & Streeter, 1995). In the present study lactate concentrations were low, which is typical of pasture diets (Kolver & de Veth, 2002) and were not changed by fumarate supplementation. This suggests that the increase in propionate production observed when fumarate was added to a pasture substrate was not due to an increased utilisation of lactate. The low concentration of lactate was reflected in the culture pH, with the 0 mM fumarate treatment averaging 6.23 which is close to optimal for forage digestion (de Veth & Kolver, 2001). Additions of fumarate increased culture pH in line with other *in vitro* and *in vivo* studies, probably due to the basic nature of the disodium form of fumarate.

Few studies have measured digestibility in response to fumarate or malate. The 3.9% reduction in true DM digestibility with 30 mM fumarate in the present study was relatively minor and in contrast to the effects of 7.4 mM fumarate *in vitro* with a hay and concentrate substrate where DM digestibility increased by 3.2 percentage units (Lopez *et al*., 1999). The addition of fumarate (2% of DM) did not change DM or NDF digestibility when sorghum silage was fed to steers (Bayaru *et al*., 2001).

In vivo studies of malate or fumarate supplementation have reported mixed production responses. When malate and fumarate have been added to high grain diets that would otherwise result in a low ruminal pH (<6.0), improvements in ruminal pH in dairy cows (Martin *et al*., 1999; Montano *et al*., 1999) and average daily gain in young cattle (Martin *et al*., 1999) have been reported. When diets fed to dairy cows and young cattle contained adequate fibre, additional malate had no effect on ruminal pH, DM intake or milk production (Kung *et al*. 1982; Vicini *et al*., 2003).

In conclusion, the addition of an organic acid (fumarate) to an *in vitro* fermentation increased energy capture by improving the supply of glucogenic compounds from a pasture substrate, and reduced losses to methane emissions. Feeding trials should be undertaken to measure the production responses of animals supplemented with organic acids.

ACKNOWLEDGEMENTS

The authors thank the Dexcel laboratory staff for chemical analysis, B. Dow for statistical analysis, and G. Brailesford (NIWA) for gas analysis. This research was funded by ViaLactia Biosciences (N.Z.) Ltd.

REFERENCES

Asanuma, N.; Iwamoto, M.; Hino, T. 1999: Effect of the addition of fumarate on methane production by ruminal microorganisms *in vitro*. *Journal of dairy science 82:* 780-787.

Bayaru, E.; Kanda, S.; Isobe, Y. 2001. Effect of fumaric acid on methane production, rumen fermentation and digestibility of cattle fed roughage alone. *Animal science journal 72:* 139-146.

Callaway, T.R.; Martin, S.A. 1996. Effects of organic acid and monensin treatment on *in vitro* mixed ruminal microorganism fermentation of cracked corn. *Journal of animal science 74:* 1982-1989.

Callaway, T.R.; Martin, S.A. 1997. Effects of cellobiose and monensin on *in vitro* fermentation of organic acids by mixed ruminal bacteria. *Journal of dairy science 80:* 1126-1135.

Carro, M.D.; Lopez, S.; Valdes, C.; Ovejero, F.J. 1999. Effect of DL-malate on mixed ruminal microorganism fermentation using the rumen simulation technique (RUSITEC). *Animal feed science and technology 79:* 279-288.

De Veth, M.J.; Kolver, E.S. 2001. Digestion of ryegrass pasture in response to change in pH in continuous culture. *Journal of dairy science 84:* 1449-1457.

Gottschalk, G. 1986. Bacterial Metabolism. 2nd edition. Springer-Verlag, NY.

Isobe, Y.; Shibata, F. 1993. Rumen fermentation in goats administered fumaric acid. *Animal science technology 64:* 1024-1030.

Iwamoto, M.; Asanuma, N.; Hino, T. 1999. Effects of nitrate combined with fumarate on methanogenesis, fermentation, and cellulose digestion by mixed ruminal microbes *in vitro*. *Animal science journal 70:* 471-478.

Kolver E.S.; de Veth, M.J. 2002. Prediction of ruminal pH from pasture-based diets. *Journal of dairy science 85:* 1255-1266.

Kung L. Jr.; Huber, J.T.; Krummrey, J.D.; Allison, L.; Cook, R.M. 1982. Influence of adding malic acid to dairy cattle rations on milk production, rumen volatile fatty acids, digestibility, and nitrogen utilization. *Journal of dairy science 65:* 1170-1174.

Lopez S.; Valdes, C.; Newbold, C.J.; Wallace, R.J. 1999. Decreased methane production and altered fermentation in response to the addition of fumaric acid to the rumen simulation technique (Rusitec). *British journal of nutrition 81:* 59-64.

Martin S.A.; Sullivan, H.M.; Evans, J.D. 2000. Effect of sugars and malate on ruminal microorganisms. *Journal of dairy science 83:* 2574-2579.

Martin, S.A. 1998. Manipulation of the ruminal fermentation with organic acids: A review. *Journal of animal science 76:* 3123-3132.

Martin, S.A.; Streeter, M.N. 1995. Effect of malate on *in vitro* mixed ruminal microorganism fermentation. *Journal of animal science 73:* 2141-2145.

Martin, S.A.; Streeter, M.N.; Nisbet, D.J.; Hill, G.M.; Williams, S.E. 1999. Effects of DL-malate on ruminal metabolism and performance of cattle fed a high-concentrate diet. *Journal of animal science 77:* 1008-1015.

Montano M.F.; Chai, W.; Zinn-ware, T.E.; Zinn, R.A. 1999. Influence of malic acid supplementation on ruminal pH, lactic acid utilization, and digestive function in steers fed high-concentrate finishing diets. *Journal of animal science 77:* 780-784.

Nisbet D.J.; Martin, S.A. 1994. Factors affecting L-lactate utilization by Selenomonas ruminantium. *Journal of animal science 72:* 1355-1361.

Robertson, L.J.; Waghorn, G.C. 2002. Dairy industry perspectives on methane emissions and production from cattle fed pasture or total mixed rations in New Zealand. *Proceedings of the New Zealand Society of Animal Production 62:* 213-218.

Teather, R.M. 1990. General protocols for operation and monitoring of the artificial rumen. Pp 14-16. *In* Continuous Culture Fermentors: Frustration or Fermentation. NE ADSA-ASAS Regional Meeting, Chazy, NY.

Vicini J.L.; Bateman, H.G.; Bhat, M.K.; Clark, J.H.; Erdman, R.A.; Phipps, R.H.; Van Amburgh, M.E.; Hartnell, G.F.; Hintz, R.L.; Hard, D.L. 2003. Effect of feeding supplemental fibrolytic enzymes or soluble sugars with malic acid on milk production. *Journal of dairy science 86:* 576-585.

Waghorn, G.C.; Lassey, K.R.; Kolver, E.S.; Molano, G. 2001. Methane emission from dairy cows fed either pasture or balanced rations over one lactation. Report to the New Zealand Dairy Board Environment Global Programme Pp 1-35.