Effect of Fumaric Acid on Methane Production, Rumen Fermentation and Digestibility of Cattle Fed Roughage Alone

Eruden BAYARU, Syuhei KANDA, Toshihiko KAMADA, Hisao ITABASHI, Sada ANDOH[1], Takehiro NISHIDA[1], Motohiko ISHIDA[1], Toshio ITOH[2], Kunihiko NAGARA[3] and Yoshio ISOBE[4]

Tokyo University of Agriculture and Technology, Fuchu-shi 183-8509, Japan
[1] National Grassland Research Institute, Nishinasuno-machi, 329-2700, Japan
[2] Takeda Chemical Industries Ltd., Chuo-ku, Tokyo 103-0011, Japan
[3] Nippon Shokubai Co.,Ltd., Chiyoda-ku, Tokyo 100-0011, Japan
[4] Isobe Professional Engineer's Office, Suginami-ku, Tokyo 166-0002, Japan

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Abstract Four Holstein steers fed with Sorghum silage were used to examine the effect of fumaric acid supplementation (20 g/kg. diet dry matter (DM)) on methane production, rumen fermentation, blood metabolism and feed digestibility. The protozoal population in the rumen was unaffected by fumaric acid supplementation. The postprandial ruminal concentration of ammonia-N decreased, and that of total volatile fatty acids tended to be higher with fumaric acid supplementation. The proportion of ruminal acetic acid was unaffected, but that of propionic acid increased and that of butyric acid decreased by fumaric acid. The postprandial blood plasma concentration of glucose was increased, whereas that of urea-N was decreased by fumaric acid. The plasma concentration of most of free amino acids was unaffected. Daily methane production decreased by 23.0% and carbon dioxide production decreased by 20.5% with fumaric acid supplementation. Apparent digestibility of dry matter and of neutral detergent fiber were not influenced by fumaric acid. These results indicated that fumaric acid was converted to propionic acid by rumen microorganisms, and that methane production from the rumen was reduced without lowering the ability to digest dietary fiber. However, some dietary conditions that alter the effectiveness of fumaric acid and the long term effect remain to be examined.

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Methane is produced by methanogenic bacteria as one of final products of carbohydrate metabolism in the rumen. Up to 12% of dietary energy consumed by ruminants is lost as methane by eructation in ruminants[4]. Ruminal methane emissions have also received focus due to its contribution to global warming. About 15% of total atmospheric methane emissions originates from ruminants[10]. Therefore, to reduce methane and to inversely increase the amount of useful glucogenic precursors such as propionic acid by diverting metabolic hydrogen in the rumen are important issues.

Many investigators have attempted to reduce ruminal methane production using chemicals

Corresponding : Hisao ITABASHI (Fax : +81 (0) 42-367-5801, e-mail : hita@cc.tuat.ac.jp)

including halogenated methane analogues, ionophore, and unsaturated fatty acids, or by removing ciliate protozoa from the rumen[4,7-9,13,20]. However, these methods have not been practically applied, because fiber digestion may be simultaneously reduced owing to changes in the microbial population of the rumen.

Fumaric acid is an intermediate of propionic acid formation in the rumen that increases propionic acid production in sheep[6] and reduces methane production *in vitro*[1,11].

The objectives of this study were to determine the effect of supplementation with fumaric acid on *in vivo* methane production, rumen fermentation, blood metabolism and digestibility in cattle.

Materials and Methods

Four castrated Holstein steers (mean body weight (BW) ; 572 kg) were housed in stanchion stalls, fed with whole crop Sorghum silage at a maintenance level for energy, and given 196 mg/kg BW of urea to satisfy protein requirement, and 10 g of vitamin-trace mineral mixture (Nippon Zenyaku Kogyo Co., Ltd., Tokyo) daily. The chemical composition of Sorghum silage is as follows : organic matter, 82.8% ; crude protein, 6.8% ; crude fat, 2.1% ; crude fiber, 67.1% ; crude ash, 17.1%. The vitamin-trace mineral mixture contained V.A., 1500 IU/g ; V.D., 500 IU/g ; α-tocopherol, 0.5 mg/g ; water soluble vitamins, 9 mg/g ; Fe, 0.08% ; Co, 0.002% ; I, 0.01% ; Mg, 5.6% ; Mn, 0.2% ; and Zn, 0.4%. Animals were fed twice daily at 0900 h and 1600 h in equal amounts. Water was continuously available.

The experiment consisted of an adaptation period of 7 days, a preliminary period of 7 days and a test period of 7 days for both control and fumaric acid-treated steers. Four steers were fed initially with only the experimental diet described above. Two of them were then fed with the diet supplemented with fumaric acid (Takeda Chemical Industries Ltd., Tokyo and Nippon Syokubai Co. Ltd., Tokyo) at a concentration of 2% of the diet dry matter (DM). Fumaric acid was mixed with the mineral and vitamin mixture and given to the animals.

From days 4 to 7 of the test periods, whole tract digestibility was measured by total collection method. Daily feces samples were composited for each steer, dried at 60℃, ground through a 2 mm screen, then assayed for DM, crude protein (CP), and neutral detergent fiber (NDF). From days 6 to 7 of the test periods, methane and carbon dioxide production were determined. A head cage-type respiration chamber was used to estimate *in vivo* methane and carbon dioxide production.

On day 7 of the test periods, ruminal fluid and jugular venous blood samples were collected at 0, 2, and 5 h after the morning meal. Approximately 300 m*l* of ruminal digesta was collected using a flexible stainless stomach-tube (Fujihira Industries Ltd., Tokyo), and rumen fluid was separated from particulate matter by straining the digesta through two layers of gauze.

Ruminal fluid pH was measured immediately using a glass electrode pH meter. A 1 m*l* of the fluid was diluted with 4 m*l* of methylgreen-formalin-saline to count ruminal ciliate protozoa[7]. Approximately 100 m*l* of the fluid was stored at −30℃ for subsequent analysis. Ruminal fluid was thawed, acidified with 3 N-H_2SO_4 containing 12% metaphosphoric acid, then volatile fatty acid concentrations were determined by gas chromatography (Model GC-8A, Shimadzu Co. Ltd., Kyoto) using a Shimalite TPA column (Shinwa Kako Co. Ltd., Kyoto). Similarly, ammonia-N concentrations were determined by micro-diffusion method as described[7].

Blood samples were collected into heparinized tubes, immediately placed on ice, then were centrifuged at 11,000×g for 15 min. Plasma was removed and stored at −30℃. After thawing, plasma glucose was determined by the o-toluidine boric acid method using a Kit (Wako Pure Chemical Industries Ltd., Tokyo) and urea-N was measured by the diacetyl monooxime method using a Kit (Wako Pure Chemical Industries Ltd., Tokyo). Plasma samples were deproteinized with an equal volume of 10% sulfosalicylic acid, and free amino acids were measured using a Hitachi L-8800 automatic amino acid analyzer (Hitachi Industries, Ltd., Tokyo).

All data were analysed using Student' *t*-test at $P < 0.05$.

Table 1. Effect of fumaric acid on pH, protozoal number, ammonia-N and volatile fatty acid (VFA) in the rumen fluid of steers

Item	Hr. after feeding	Control	Fumaric acid supplemented	SEM
pH	0	6.9	7.1	0.03
	2	6.8	6.9	0.04
	5	6.9	6.8	0.08
Protozoal number ($\times 10^5$/ml)	0	6.4	6.6	1.70
	2	5.9	5.9	0.54
	5	6.3	6.3	1.46
Ammonia-N (mg/100 ml)	0	5.0	4.8	0.35
	2	27.2	22.9*	1.15
	5	10.8	10.9	0.49
Total VFA (mmol/100 ml)	0	5.2	5.7	0.08
	2	6.5	6.9	0.79
	5	6.2	7.0	0.12
VFA composition (molar %)				
Acetic acid (A)	0	69.2	70.0	0.45
	2	60.5	61.5	0.28
	5	62.5	65.5	0.23
Propionic acid (P)	0	18.5	16.9	0.50
	2	23.5	28.4*	1.02
	5	22.9	25.4	0.81
Butyric acid	0	8.5	6.4*	0.64
	2	10.7	6.8*	0.99
	5	9.8	7.0*	0.35
iso-Valeric acid	0	2.7	0.8*	0.39
	2	2.6	1.1*	0.16
	5	2.5	0.9*	0.23
Valeric acid	0	0.9	0.5	0.47
	2	1.7	1.2	0.95
	5	1.6	1.1	0.86
Caproic acid	0	0.2	0.2	0.05
	2	1.0	0.7	0.20
	5	0.7	0.4	0.11

* Significantly different from the control ($P<0.05$).

Results and Discussion

Supplementation with fumaric acid did not influence either the food intake or the health of the steers. The effect of treatment on several rumen fluid parameters is shown in Table 1. The pH value of the fluid was unaffected by fumaric acid supplementation. This disagreed with the results of *in vivo*[6] and *in vitro*[1,3] studies, which showed higher value by the treatment.

Ruminal ciliate protozoa were composed of *Entodinium* spp., *Diplodinium* spp., and *Isotricha* sp.

Table 2. Effect of fumaric acid on concentrations of glucose and urea-N in blood plasma of steers

Item	Hr. after feeding	Control	Fumaric acid supplemented	SEM
Glucose (mg/100 m*l*)	0	61.6	68.8*	1.13
	2	64.3	78.3*	4.33
	5	72.4	81.8*	1.49
Urea-N (mg/100 m*l*)	0	6.7	6.1	0.39
	2	8.4	6.8*	0.58
	5	9.3	8.0*	0.45

* Significantly different from the control ($P<0.05$).

Total number of protozoa was unaffected by fumaric acid supplementation.

The concentration of ruminal ammonia-N increased at 2 h after feeding and decreased thereafter in all steers. Supplementation of fumaric acid significantly reduced ammonia-N at 2 h after feeding, suggesting increased utilization of nitrogen.

The concentration of total ruminal volatile fatty acids (VFA) tended to be higher for steers fed with fumaric acid throughout the sampling period. Similar results have been obtained by Asanuma *et al.*[1] and Lopez *et al.*[12] *in vitro*. The molar proportion of acetic acid was unchanged, but that of propionic acid was increased significantly at 2 h after feeding and that of butyric acid decreased significantly throughout the sampling period by supplementation of fumaric acid. The proportion of *iso*-valeric acid decreased significantly, and those of valeric acid and caproic acid tended to be decrease by fumaric acid. The increased proportion of propionic acid by fumaric acid agreed with the results of other *in vivo*[6] and *in vitro*[1,11] studies. This indicates that metabolic hydrogen was utilized to synthesize propionic acid from fumaric acid.

The blood plasma concentration of glucose was increased significantly by supplementation of fumaric acid at all sampling times (Table 2), most likely because gluconeogenesis from propionic acid increased. On the other hand, the post-feeding plasma concentration of urea-N was decreased significantly by fumaric acid (Table 2). This might be due to a decreased ruminal concentration of ammonia, and suggests increased nitrogen utilization. Most of the plasma free amino acids were unaffected by fumaric acid, but the concentrations of glycine and 3-methylhistidine were decreased significantly (Table 3). Higher concentrations of plasma glycine due to lowered nitrogen utilization have been reported[2]. The concentration of 3 -methilhistidine indicates the degree of muscle protein degradation in animals including ruminants[16]. The present results suggest that fumaric acid has some effect on whole body nitrogen metabolism in ruminants.

The effects of fumaric acid on methane and carbon dioxide production are shown in Table 4. The daily production (*l*/d) and rate of production (*l*/kg DM intake) averaged 100–240 and 17–35, respectively, throughout the experiment, which agreed with the findings of Shibata *et al.*[17] who fed animals with hay alone. The profile of methane production over 24 h is shown in Fig. 1. Methane output increased after feeding in both treated and control steers.

Supplementation with fumaric acid reduced methane production by 23.0%. This is the first report demonstrating that fumaric acid reduced methanogenesis *in vivo*, and which agrees with documented results *in vitro*[1,12]. Lopez *et al.*[11] reported that sodium fumarate addition can decrease methane production *in vitro*, but the diversion of hydrogen to propionic acid formation was incomplete.

Our findings that fumaric acid increased the proportion of propionic acid along with reduced methane production, confirm similar responses in ruminal fermentation patterns where methane production is

Table 3. Effect of fumaric acid on concentrations of free amino acids in blood plasma of steers (Samples taken 5 hr after feeding)

Amino acid	Control	Fumaric acid supplemented	SEM
		μ mol/100 ml	
Aspartic acid	1.1	1.1	0.20
Serine	6.7	5.6	0.38
Glutamic acid	10.6	12.6	0.90
Proline	5.3	5.8	1.32
Glycine	21.7	16.7*	1.03
Alanine	20.2	17.6	2.23
Citrulline	9.1	9.0	0.95
Tyrosine	4.1	4.3	0.08
Ornithine	8.1	8.2	0.33
1-Methylhistidine	0.9	0.8	0.19
3-Methylhistidine	0.9	0.4*	0.08
Total NEAA[a]	86.7	82.1	3.02
Threonine	5.8	5.9	0.87
Valine	23.0	24.7	0.72
Methionine	2.5	1.8	0.27
Isoleucine	9.1	8.3	0.42
Leucine	10.0	10.0	0.39
Phenylalanine	4.2	4.4	0.23
Lysine	8.7	8.4	0.54
Histidine	5.3	4.9	0.23
Arginine	11.1	11.9	0.89
Total EAA[b]	79.7	80.4	1.63
Total amino acids	166.4	162.5	3.65

[a] Non essential amino acids ; [b] Essential amino acids.
* Significantly different from the control ($P<0.05$).

inhibited by various anti-methanogenic chemicals[8,9,13,14].

Stumm *et al.*[18] suggested that up to 20% of the methanogenic bacteria in the rumen may be associated with ciliate protozoa, and Newbold *et al.*[15] reported that 9–25% of the methane production in the rumen is attributable to protozoa-associated methanogenic bacteria. Although fumaric acid unaffected the protozoal population in this study, we observed that the number of protozoa tended to decrease in the experiment using dairy cows (unpublished data). Further experiments are needed to study the effect of fumaric acid on ruminal protozoal population.

Fumaric acid also reduced carbon dioxide production by 20.5%. The reason for this is unclear, but it may be due in part to the changes in ruminal fermentation.

The effect of fumaric acid on energy and nitrogen metabolism, and apparent digestibility is presented in Table 5. More energy and nitrogen were retained by fumaric acid, but the differences between the control and supplemented steers were not significant. The digestibility of dry matter and neutral detergent fiber was not affected, but that of crude protein was significantly increased by fumaric acid. This also indicated that fumaric acid increased feed protein

Table 4. Effect of fumaric acid on methane and carbon dioxide production of steers

Item	Control	Fumaric acid supplemented	SEM
Methane			
(*l*/day)	180.1 (100%)	138.6* (77.0%)	15.60
(*l*/kg/DMI[1])	27.0 (100%)	21.1* (78.2%)	0.55
Carbon dioxide			
(*l*/day)	2355.8 (100%)	1872.1* (79.5%)	153.80
(*l*/kg/DMI)	353.8 (100%)	286.9* (81.1%)	22.75

[1] Dry matter intake.
* Significantly different from the control ($P<0.05$).

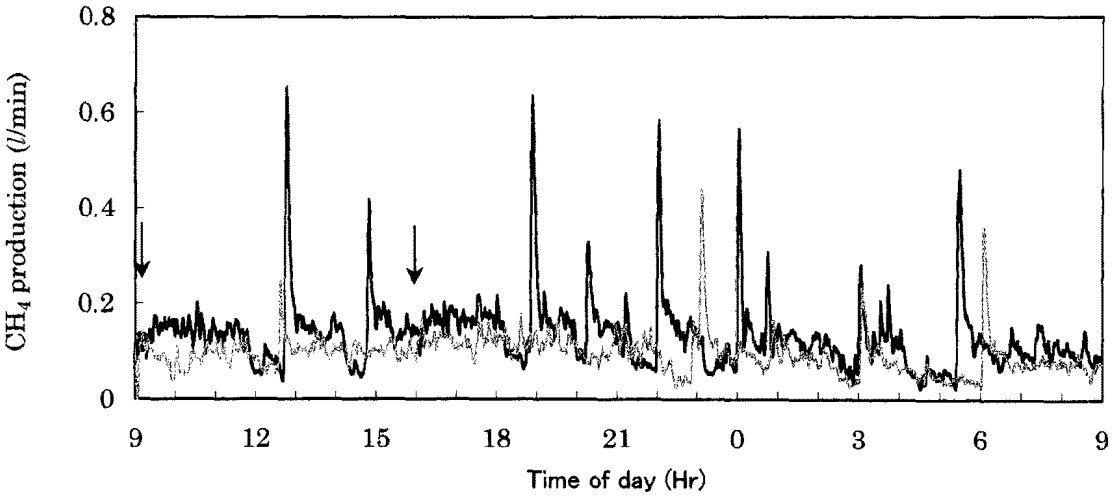

Fig. 1. Profile of methane production over 24 hr in steers fed Sorghum silage with or without fumaric acid.
↓ Time of feeding. — Control. – – Fumaric acid supplemented.

utilization. These results are not in agreement with the results reported by Isobe and Shibata[6] and Lopez *et al.*[12], who found increased digestion of fiber and dry matter, respectively.

We concluded from the present results that supplementing a roughage diet with fumaric acid could reduce methane production from the rumen without decreasing fiber digestion. However, the optimal level of fumaric acid supplementation and the dietary conditions that affect the extent of methane reducton induced by fumaric acid remain to be determined.

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Table 5. Effect of fumaric acid on energy and nitrogen metabolism, and feed digestibility of steers

Item	Control	Fumaric acid supplemented	SEM
Energy metabolism (Mcal/day)			
Intake energy			
GE	23.5	22.9	1.07
DE	14.2	13.9	0.77
ME	11.1	11.5	0.66
Energy loss			
Feces	9.3	8.9	0.32
Urine	1.3	1.1	0.22
Methane	1.7	1.3*	0.28
Heat production	10.7	10.2	1.49
Retention	0.4	1.3	1.00
Nitrogen metabolism (g/day)			
Intake nitrogen	123.5	123.6	6.27
Nitrogen loss			
Feces	56.4	50.7	3.03
Urine	81.8	67.7	14.16
Retention	−14.7	5.2	13.60
Apparent digestibility (%)			
Dry matter	57.4	57.6	0.58
Neutral detergent fiber	71.2	69.8	2.71
Crude protein	54.4	58.9*	0.80

GE ; gross energy, DE ; digestible energy, ME ; metabolizable energy.
* Significantly different from the control ($P<0.05$).

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