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Characterization, Metabolites and Gas Formation of Fumarate Reducing Bacteria Isolated from Korean Native Goat (*Capra hircus coreanae*)

Lovelia L. Mamuad[1], Seon Ho Kim[1], Sung Sil Lee[2], Kwang Keun Cho[3], Che Ok Jeon[4], and Sang-Suk Lee[1]*

[1]*Ruminant Nutrition and Anaerobe Laboratory, Department of Animal Science and Technology, College of Bio-industry Science, Sunchon National University, Suncheon 540-742, Republic of Korea*
[2]*Division of Applied Life Science, Graduate School of Gyeongsang National University, IALS, Jinju 660-701, Republic of Korea*
[3]*Department of Animal Resources Technology, Gyeongnam National University, Chinju 660-758, Republic of Korea*
[4]*Research Center for Biomolecules and Biosystems, Department of Life Science, Chung-Ang University, Seoul 156-756, Republic of Korea*

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Fumarate reducing bacteria, able to convert fumarate to succinate, are possible to use for the methane reduction in rumen because they can compete for H_2 with methanogens. In this, we isolated fumarate reducing bacteria from a rumen of Korean native goat and characterized their molecular properties [fumarate reductase A gene (*frdA*)], fumarate reductase activities, and productions of volatile fatty acids and gas. Eight fumarate reducing bacteria belonging to Firmicutes were isolated from rumen fluid samples of slaughtered Korean black goats and characterized their phylogenetic positions based on 16S rRNA gene sequences. PCR based analyses showed that only one strain, closely related to *Mitsuokella jalaludinii*, harbored *frdA*. The growths of *M. jalaludinii* and *Veillonella parvula* strains were tested for different media. Interestingly, *M. jalaludinii* grew very well in the presence of hydrogen alone, while *V. parvula* grew well in response of fumarate and fumarate plus hydrogen. *M. jalaludinii* produced higher levels of lactate ($P \leq 0.05$) than did *V. parvula*. Additionally, *M. jalaludinii* produced acetate, but not butyrate, whereas *V. parvula* produced butyrate, not acetate. The fumarate reductase activities of *M. jalaludinii* and *V. parvula* were 16.8 ± 0.34 and 16.9 ± 1.21 mmol NADH oxidized/min/mg of cellular N, respectively. In conclusion, this showed that *M. jalaludinii* can be used as an efficient methane reducing agent in rumen.

Keywords: fumarate reductase, *Mitsuokella jalaludinii*, Korean native goat, VFA

Introduction

In 2010, global anthropogenic methane emissions estimated 29% came from enteric fermentation and 4% from agriculture manure (Global Methane Initiative, 2012). Approximately two-thirds of these emissions are caused by enteric fermentation, while the remaining one-third is from livestock manure. About 95% of global animal enteric methane is from ruminants because it is one of the major end products of anaerobic fermentation in the rumen (Johnson and Ward, 1996). Due to this phenomena, cattle typically lose 6% of their ingested energy as eructated methane (Johnson and Johnson, 1995).

Fumarate reducing bacteria compete with methanogens by utilizing H_2 through fumarate reductase, which converts fumarate to succinate. Although H_2, formate, acetate, methanol, mono-, di- and tri-methylamine are all potential substrates for methanogens, only H_2/CO_2 and formate are used as methane precursors in the rumen (Miller, 1995). It is more advantageous to reduce methanogenesis by decreasing H_2 production or increasing H_2 consumption in ways other than methanogenesis (Asanuma *et al*., 1999b, 1998). Fumarate reduction plays an important role in maintaining a low partial H_2 pressure in the rumen. A previous study showed that the addition of fumarate and malate to *in vitro* cultures of mixed ruminal microbes resulted in its reduction to succinate via utilization of H_2 or reducing equivalents that were otherwise used to produce H_2 (Asanuma and Hino, 2000). In addition, fumarate reductase serves to provide succinate for biosynthetic use during anaerobic growth when the tricarboxylic acid cycle enzymes are repressed (Spencer and Guest, 1973). The anaerobic route from oxaloacetate to succinate can function in the absence of malate dehydrogenase. Courtright and Henning (1970) proposed a pathway that includes aspartate, which is converted to fumarate by the anaerobically inducible enzyme aspartase. Moreover, Asanuma and Hino (2000) found that fumarate reduction could improve fiber digestion in the rumen. Therefore, this study was conducted to isolate and identify fumarate reducing bacteria, determine the fumarate reductase A (*frdA*) specific gene and its enzyme activity. Furthermore, the effects of electron acceptors and donors on volatile fatty acids, other metabolites, and gas production or consumption were investigated. The results of the present study should lead to improved ruminal fermentation as well as reduced methane production by ruminants.

Materials and Methods

Animals and sampling

Korean native goats (KNG, *Capra hircus coreanae*) from Hwasun, South Korea were used as experimental animal. KNGs of 45 kg body weight were fed timothy and commercial

*For correspondence. E-mail: rumen@sunchon.ac.kr; Tel./Fax: +82-61-750-3237

concentrate at 2% body weight twice a day. Rumen fluid was collected from the rumen of slaughtered KNGs. The samples were strained through four layers of surgical gauze and placed in amber bottles with an oxygen free headspace immediately after collection. The collected rumen fluid was then sealed and maintained at 39°C and immediately transported to the laboratory.

Preparation of media

The isolation techniques employed in this study were similar to those described by Hattori and Matsui (2008). Four types of medium were prepared based on the method described Joblin (1981), with some modification. The basal medium consisted of clarified rumen fluid (Hino *et al*., 1992) and buffer solution (1:3) (Asanuma and Hino, 2000). Sugars were omitted from the basal medium. Hydrogen or formate (40 mM/L) was added as electron donors, while fumarate (40 mM/L) was used as the electron acceptor. The electron donors (hydrogen or formate) were added to the basal medium individually or combined with fumarate. Hydrogen (200 kPa) was added to the headspace of each sample tube after inoculation.

For enrichment incubation, 54 ml of medium were dispensed into a 120 ml serum bottle and sealed with a butyl-rubber septum and aluminum crimp cap. For roll-tubes, 6 ml of medium containing 1.5% Difco Bacto-agar (Becton Dickinson, USA) was dispensed into Balch tubes. For broth medium, 10 ml of medium was dispensed into Balch tubes. All media and anaerobic dilution solutions (Bryant and Burkey, 1953) were prepared under O_2-free N_2 gas and autoclaved for 15 min at 121°C.

Isolation of fumarate reducing bacteria

Enrichment was performed prior to roll-tube isolation. Briefly, 6 ml of strained rumen fluid were inoculated into the enrichment medium and incubated for 24 h with horizontal shaking (80 rpm). Next, 6 ml of this culture fluid were inoculated into the same medium and incubated in the same manner. After enrichment, the culture was serially diluted from 10^{-3} to 10^{-9} with anaerobic dilution solution, inoculated into roll-tubes and then incubated for 24–48 h. Visible colonies in the agar film were subsequently inoculated into broth medium and incubated for 24 h with horizontal shaking at 120 rpm. This isolation step was repeated until the colonies were purified. All incubations were conducted anaerobically at 37°C (Lee *et al*., 2002).

PCR amplification

For amplification of the 16S ribosomal RNA gene (16S rDNA), the 27f and 1492r primers were used, while the *frdA*-Cf and *frdA*-Dr primers were used for amplification of the fumarate reductase gene (Hattori and Matsui, 2008). To amplify the *frdA* fragment from ruminal DNA, a touchdown PCR protocol (Hattori and Matsui, 2008) and general PCR protocol were employed. The general PCR protocol for fumarate reducing bacteria was established in this experiment and consisted of an initial denaturation step at 94°C for 2 min, followed by 30 cycles of 94°C for 54 sec, 72°C for 30 sec and 72°C for 1 min and then a final extension step at 72°C for 10 min.

ARDRA and PCR purification

Amplified Ribosomal DNA Restriction Analysis (ARDRA) was conducted to determine the similarities and differences of isolates. Briefly, the PCR products were double digested with the *Hae*III and *Hha*I restriction endonucleases (TaKaRa, Japan) at 37°C for 5 h. The digested DNA samples were then separated by electrophoresis at 170 V for 80 min using Metaphor agarose gel (BioWhittaker, USA), after which they were visualized using a Kodak Gel Logic 200 imaging system (Eastman Kodak Company, USA). Different bands obtained from ARDRA were then purified, after which the desired PCR products from the 16S rRNA (rDNA), which is 1.2 kb in length, were purified using a QIAquick PCR Purification Kit (Qiagen, USA).

DNA sequencing and phylogenetic tree

Purified PCR products of 16S rDNA were sent to Macrogen for DNA sequencing. Sequenced fragments were assembled using the SeqMan program (DNA Star, Lasergene software, USA). The gene sequences were then compared with 16S RNA gene sequences available in GenBank using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) and EzTaxon (http://147.47.212.35:8080/index.jsp). Approximate phylogenetic affiliations were determined by aligning the gene sequences with those of closely related species using CLUSTAL W version 1.6. A phylogenetic tree was constructed using the neighbor-joining (NJ) method with pair-wise gap removal, while distance matrices were calculated according to Kimura (1980). Finally, the two-parameter NJ method in the PHYLIP package was employed and bootstrap analysis was performed by resampling the data 1,000 times to evaluate the stability of the phylogenetic tree. Only bootstrap values greater than 50% were shown on the internal nodes

Enzyme activity determination

Preparation of the membrane fraction of the microorganisms: *M. jalaludinii* and *Veillonella parvula* KCTC 5019 with cell growth of 0.883 and 0.851 respectively were monitored based on the OD at 600 nm. The cells were harvested during the late log phase by centrifugation (20,000×g, 10 min). The pellets were then resuspended in 5 ml of 50 mM KPi buffer, after which the cells were disrupted by ultrasonication (Ultrasonic Homogenizer 4710 Series, Cole-Parmer Instrument Corp., USA) until 95% of the cells were disrupted. To accomplish this, a needle titanium probe was kept immersed in the samples to a depth of about 5 mm. Samples were kept in a salt ice bath during cell disruption to prevent overheating (30 sec sonication and 30 sec rest per duty cycle and each duty cycle was 0.5 sec with generator acting for 0.5 sec intervals with 0.5 sec of rest), and unbroken cells were counted through microscopy (Gram-Staining). The unbroken cells were then removed by centrifugation (20,000×g, 10 min) (Asanuma and Hino, 2000).

Protein content determination: The protein content of the samples was determined using a Quick Start™ Bradford Protein Assay with bovine serum albumin (BSA) serving as the standard protein. The protein content of the control

(untreated medium used of the sample) was subtracted from the protein content of the samples (media + microorganism). The volume of enzyme in the sample was then determined as follows:

$$M_iV_i = M_fV_f$$

Where:

M_i = sample protein content (μg/ml) – determined by Bradford assay

V_i = x (initial volume needed)

M_f = final protein content needed for the assay

V_f = enzyme assay volume (final volume needed)

Assay for enzyme activity: The fumarate reductase activity was assayed by monitoring the rate of NADH oxidation. The standard assay mixture contained 5 mM fumarate, 0.15 mM NADH, and an enzyme sample (1 mg protein/ml assay mixture) in 50 mM KPi buffer. The reaction was initiated by adding an enzyme sample at room temperature, and the NADH was determined by measuring the absorbance at 340 nm. The activity of NADH oxidase was estimated from the initial rate of NADH oxidation in the absence of fumarate, and this value was subtracted from the initial rate of NADH oxidation in the presence of fumarate to obtain the fumarate reductase activity, which was expressed as mmol NADH oxidized per min per cellular nitrogen (N). This value was taken to represent the amount of enzyme per cell mass because approximately 95% of cells were disrupted by ultrasonication (Asanuma and Hino, 2000).

Cultivation of microorganisms

V. parvula was obtained from the Korean Collection for Type Cultures Biological Resource Center was used as the standard fumarate reducing bacteria. *M. jalaludinii* and *V. parvula* were cultivated using Asanuma and Hino (2000) medium that had been modified with or without addition of one or both of the electron donors as described above. Treatments were as follows: T – treated without addition of fumarate, formate or hydrogen; TH – treated with hydrogen; TF – treated with fumarate; TFH – treated with fumarate + hydrogen; TFo – treated with formate; TFFo – treated with fumarate + formate. Bacterial cultures were grown for 72 h and 5% of each bacterial culture was then added to 10 ml of media. Control bottles, which had been autoclaved, received the same volume and optical density (OD) value of each bacterial culture as treated (15 min at 121°C). Samples at 0, 3, 6, 9, 12, 24, 48, and 72 h of incubation were analyzed for different parameters. These were: total gas [press and sensor machine (Laurel Electronics, Inc., USA)], optical density [OD, UV Spectrophotometer (Biochrom Libra S22, Biochrom Ltd., England)], pH meter [Pinnacle series M530p meter (Schott instruments, Germany)], other gases [gas chromatography (HP 5890, Agilent technology, Germany) using Carboxen 1006 PLOT capillary column 30 m × 0.53 mm, Supelco, USA) according to the method described by (Ørskov and McDonald, 1979) with some minor modifications.], VFA and other metabolites [high performance liquid chromatography (HPLC) (Agilent Technologies 1200 series, USA) using Agilent Technologies Varian MetaCarb 87H (300 × 7.8 mm) and Shodex RSpak DE-613 columns according to the methods described by Tabaru *et al.* (1988), Han *et al.* (2005), and Asanuma and Hino (2000)].

Statistical methods

Data were analyzed by analysis of variance (ANOVA) using the general linear model (GLM) for a randomized complete block design. All treatments were conducted in triplicate

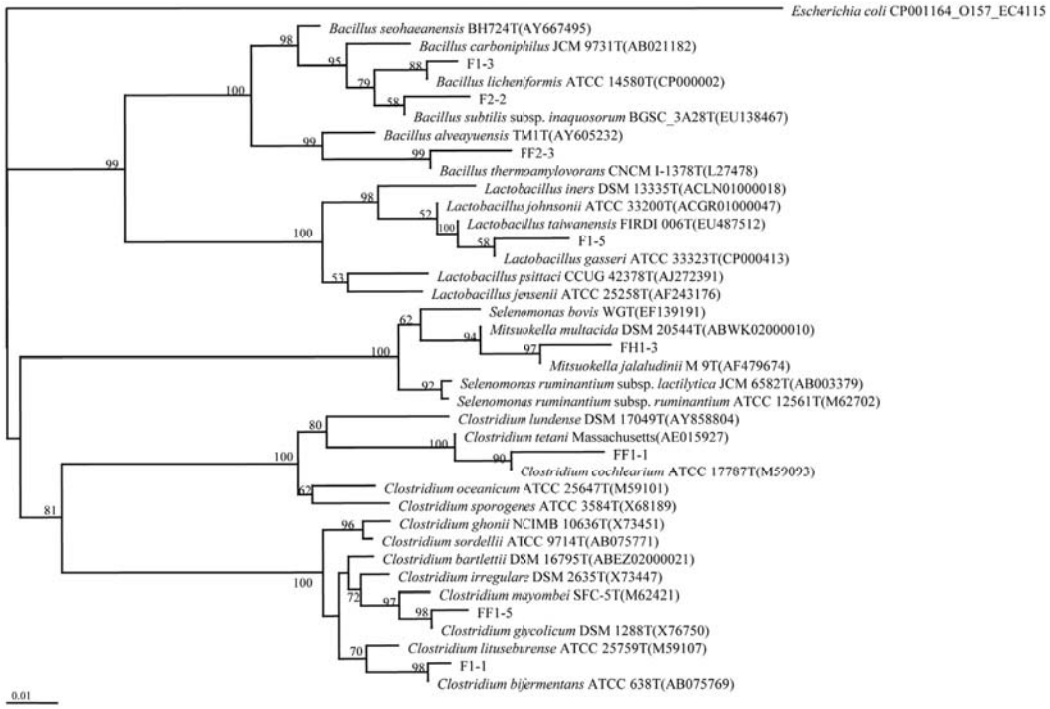

Fig. 1. Phylogenetic tree of fumarate reducing bacteria isolated from Korean native goats constructed using the neighbor joining method based on 16S rRNA gene sequences developed by Kimura (1980) with two parameter correction models. The tree was bootstrap resampled 1,000 times. Only bootstrap values greater than 50% are shown on the internal nodes. *Escherichia coli* was used as an outgroup, 0.01 substitutions per nucleotide position.

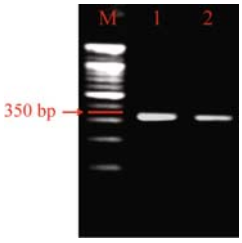

Fig. 2. Agarose gel electrophoresis of polymerase chain reaction products of *frdA* from pure culture. Lanes: M, 100 bp ladder marker; 1, *Mitsuokella jalaludinii*; 2, *Veillonella parvula*.

and Duncan's Multiple Range Test (DMRT) was used to identify differences between specific treatments. A $P \leq 0.05$ was considered to indicate statistical significance. All analyses were carried out using Statistical Analysis Systems (SAS) version 9.1 (2002).

Results

Of thirty seven colonies isolated, eight bacterial species belonging to *Firmicutes* were identified as potential fumarate reducing bacteria (Fig. 1). These bacteria showed high homology (99–100%) to the following bacteria, which show wide diversity, but are primarily isolated from rumens or feces: *Bacillus licheniformis*, *B. subtilis* subsp. *inaquosorum*, *B. thermoamylovorans*, *Clostridium bifermentans*, *C. cochlearium*, *C. glycolicum*, *Lactobacillus johnsonii* and *Mitsuokella jalaludinii*.

The results showed different sized bands from different species of isolated bacteria. Among the species identified, only *M. jalaludinii* had a band with an approximate size of 350 bp (Fig. 2). This band was cut and submitted for sequencing and identification, which revealed that *M. jalaludinii* contained succinate dehydrogenase/fumarate reductase, flavoprotein subunit. In addition, the enzyme activities of *M. jalaludinii* and *V. parvula* were determined to confirm the ability of the microorganisms to reduce fumarate. Table 1 shows the activity per cell mass of *M. jalaludinii* and *V. parvula* at different sonication times. The results revealed that sonication time affects the enzyme activity of microorganisms. *M. jalaludinii* enzyme activity was highest ($P \leq 0.05$) at 15 min sonication time with 6.46±0.13 mmol NADH oxidized/min/mg of cellular N while *V. parvula* at 25 min sonication time with 16.93±1.21 mmol NADH oxidized/min/mg of cellular N. These findings indicate that *M. jalaludinii* has the potential to reduce fumarate or succinate.

Having a positive result molecularly and enzymatically, the growths of *M. jalaludinii* and *V. parvula* strains were tested for different media using different electron donor and acceptor. Figure 3 shows the growth curves of *M. jalaludinii* and *V. parvula* obtained using different media and different incubation times. Hydrogen promoted the rapid growth of *M. jalaludinii*, while fumarate (TF) and fumarate + hydrogen (TFH) in *V. parvula*. The log phase of *M. jalaludinii* started from 0 to 12 h of incubation when no electron acceptor was added (T) and in the TH treatment, then became stationary from 12 to 72 h. Conversely, for treatments TF, TFH, formate (TFo) and fumarate + formate (TFFo) the log phases started at 9 h of incubation and onwards. The *V. parvula* log phases started from 3 to 12 h of incubation, decreased slighly until 24 h and then became stationary. Cell growth was comparatively lower in the T, TH and TFFo treatments.

The gas formation of *M. jalaludinii* and *V. parvula* was also measured using different media. Hydrogen production of *M. jalaludinii* was observed after 48 h in the TFH treatment, while hydrogen consumption was observed in the TFH (24 h) and TH (24 and 48 h) treatments. Conversely, hydrogen production of *V. parvula* was observed in the TF and TFH treatments at 24 and 48 h of incubation, while hydrogen consumption was observed in TH at 24 h of incubation. Carbon dioxide (CO_2) production by *V. parvula* was comparatively higher ($P \leq 0.05$) than that of *M. jalaludinii* in the T (24 and 48 h) and TH (48 h) treatments. *V. parvula* produced CO_2 in the T, TH, TF, and TFH treatments at 24 and 48 h of cultivation, while *M. jalaludinii* produced CO_2 at 24 and 48 h in the T and TH treatments and at 48 h in the TFH treatment (data not shown).

During cultivation of microorganisms, volatile fatty acids (VFA) and other metabolites were measured as fermentation end-products. Cultivation of *M. jalaludinii* with TF, TFH, TFo, and TFFo resulted in higher ($P \leq 0.05$) production of lactate than cultivation of *V. parvula*. In addition, *M. jalaludinii* produced acetate but not butyrate, while *V. parvula* has its opposite for their metabolism. *M. jalaludinii* cultivated with TFo and TFFo and *V. parvula* cultivated with TF and TFFo enhanced the production of propionate ($P \leq 0.05$) than those cultivated in other treatments. Cultivation of *V. parvula* in the TF and TFH treatments resulted in consumption of fumarate as the incubation time became longer. Conversely, cultivation of *M. jalaludinii* in TFH, TFo, and TFFo consumed fumarate but produced this metabolite when cultivated with TF (data not shown).

Correlation analyses of parameters obtained after 24 h of cultivation as affected by *M. jalaludinii* and *V. parvula* are shown in Tables 2 and 3. Gas production from *M. jalaludinii* was negatively correlated ($P \leq 0.05$) to formate. Optical density (OD) was positively correlated to lactate ($P \leq 0.01$), fumarate ($P \leq 0.01$) and acetate ($P \leq 0.05$) but negatively correlated ($P \leq 0.01$) to formate and pH which means as *M. jalaludinii* grew, during its metabolism, it decreased pH and produced lactate, fumarate and acetate but used formate. Also, pH was negatively correlated ($P \leq 0.01$) to lactate and fumarate but positively correlated to ($P \leq 0.05$) formate. In addition, lactate was negatively correlated to formate ($P \leq 0.01$) but

Table 1. Activity per cell mass of *M. jalaludinii* and *V. parvula* at different sonication times

Microorganisms	Sonication time**			
	15 min	20 min	25 min	30 min
Mitsuokella jalaludinii	16.8 ± 0.34[a]	6.5 ± 0.13[b]	1.3 ± 0.03[b]	3.7 ± 1.45[b]
Veillonella parvula	7.7 ± 0.20[b]	4.2 ± 0.30[b]	16.9 ± 1.21[a]	1.3 ± 0.88[b]

Values are Mean ± Standard error of three replications.
Means in the same column with the same superscript letter are not significantly different ($P \leq 0.05$).
**Activity in broken cell suspensions (mmol NADH oxidized/min/mg of cellular N)

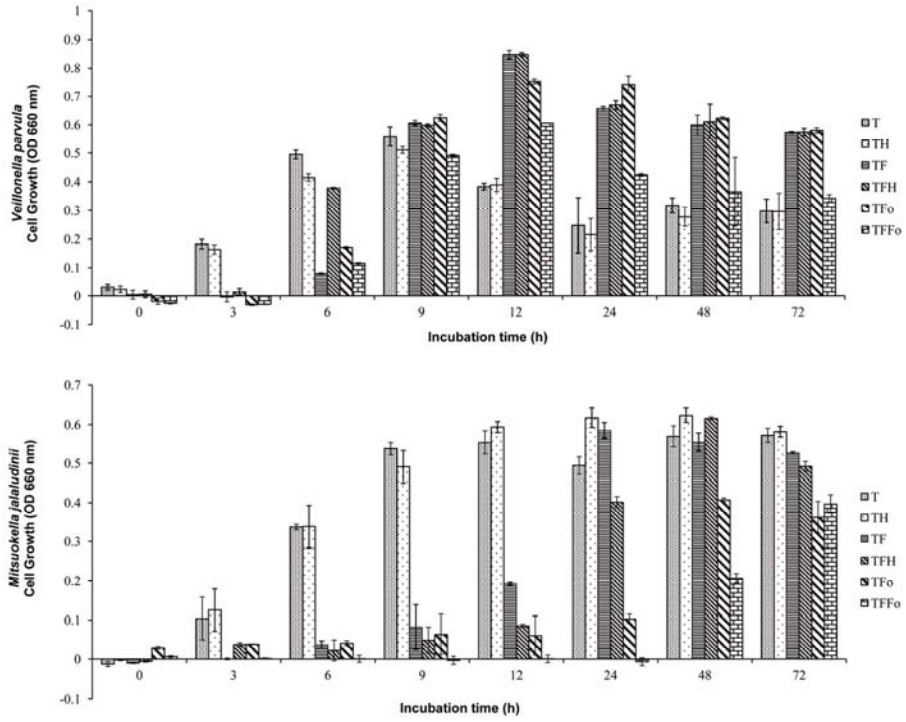

Fig. 3. Growth curve of *M. jalaludinii* and *V. parvula* obtained using different media and incubation times. Values are the means of triplicate analyses and bars indicate the standard error. Treatments: T – treated without addition of fumarate, formate or hydrogen; TH – treated with hydrogen; TF – treated with fumarate; TFH – treated with fumarate + hydrogen; TFo – treated with formate; TFFo – treated with fumarate + formate

positively correlated to fumarate ($P \leq 0.01$) while formate was negatively correlated to acetate ($P \leq 0.05$) and fumarate ($P \leq 0.01$). Moreover, acetate was positively correlated to fumarate ($P \leq 0.05$) but negatively correlated to succinate ($P \leq 0.01$).

Gas production of *V. parvula* was positively correlated ($P \leq 0.01$) to formate while OD was positively correlated ($P \leq 0.05$) to acetate but negatively correlated ($P \leq 0.01$) to formate. During *V. parvula* metabolism, it used formate and fumarate but produced acetate. pH was positively correlated ($P \leq 0.01$) to CO_2, formate and succinate but negatively correlated ($P \leq 0.05$) to acetate while lactate was positively correlated ($P \leq 0.05$) to acetate but negatively correlated ($P \leq 0.01$) to formate and butyrate. Moreover, formate was positively correlated ($P \leq 0.01$) to butyrate but negatively ($P \leq 0.01$) correlated to acetate while acetate was negatively correlated to butyrate ($P \leq 0.01$) and succinate ($P \leq 0.05$). Furthermore, butyrate was negatively correlated ($P \leq 0.05$) to succinate and fumarate.

Discussion

Rumen microbiota harbor many novel microorganisms. Thus, several studies had been conducted regarding rumen microbe's isolation and identification to investigate their role on rumen metabolism and its effect on VFA and methane production. Several pathways had been identified to reduce methane production and one of these is through fumarate reduction. Korean native goat (KNG; *Capra hircus coreanae*) is one of the animal species which was not yet explored. Hence, this study isolated and identified fumarate reducing bacteria which could compete for hydrogen utilization with

Table 2. Correlation analysis of parameters obtained as affected by *M. jalaludinii*

Parameters	GAS	OD	pH	H_2	CO_2	LACTATE	FORMATE	ACETATE	BUTYRATE	PROPIONATE	SUCCINATE	
GAS	1.000	0.380	-0.360	0.417	-0.678	0.514	-0.604[b]	0.332	.	.	-0.519	0.588
OD	0.380	1.000	-0.829[a]	0.168	0.214	0.946[a]	-0.939[a]	0.635[b]	.	.	-0.676	0.934[a]
pH	-0.360	-0.829[a]	1.000	-0.321	-0.783	-0.933[a]	0.761[b]	-0.119	.	.	-0.043	-0.877[a]
H_2	0.417	0.168	-0.321	1.000	.	0.996	0.953	0.933	.	.	0.914	0.615
CO_2	-0.678	0.214	-0.783	.	1.000	.	.	.	.	.	.	.
LACTATE	0.514	0.946[a]	-0.933[a]	0.996	.	1.000	-0.937[a]	0.454	.	.	-0.410	0.984[a]
FORMATE	-0.604[b]	-0.939[a]	0.761[b]	0.953	.	-0.937[a]	1.000	-0.715[b]	.	.	0.699	-0.979[a]
ACETATE	0.332	0.635[b]	-0.119	0.933	.	0.454	-0.715[b]	1.000	.	.	-0.982[a]	0.645
BUTYRATE	.	.	.	.	.	.	.	.	.	.	.	.
PROPIONATE	.	.	.	.	.	.	.	.	.	.	.	.
SUCCINATE	-0.519	-0.676	-0.043	0.914	.	-0.410	0.699	-0.982[a]	.	.	1.000	-0.521
FUMARATE	0.588	0.934[b]	-0.877[a]	0.615	.	0.984[a]	-0.979[a]	0.645[b]	.	.	-0.521	1.000

Values used are the production and consumption obtained after 24 h of incubation using different media.
[a] Values are 1% significantly different ($P \leq 0.01$).
[b] Values are 5% significantly different ($P \leq 0.05$).

Table 3. Correlation analysis of parameters obtained as affected by *V. parvula*

Parameters	GAS	OD	pH	H_2	CO_2	LACTATE	FORMATE	ACETATE	BUTYRATE	PROPIONATE	SUCCINATE	FUMARATE
GAS	1.000	-0.448	-0.341	0.668	-0.232	0.186	0.985[a]	0.089	-0.163	0.945	0.361	-0.299
OD	-0.448	1.000	-0.017	-0.210	0.238	0.386	-0.993[a]	0.636[b]	-0.443	0.371	-0.792[b]	-0.479
pH	-0.341	-0.017	1.000	-0.476	0.956[a]	-0.391	0.998[a]	-0.768[b]	0.554	-0.944	0.927[a]	0.610
H_2	0.668	-0.210	-0.476	1.000	-0.701	-0.746	.	-0.808	0.811	.	-0.117	-0.935
CO_2	-0.232	0.238	0.956[a]	-0.701	1.000	0.410	.	0.453	-0.530	.	0.344	0.230
LACTATE	0.186	0.386	-0.391	-0.746	0.410	1.000	-0.990[a]	0.798[b]	-0.898[a]	-0.404	0.544	0.784
FORMATE	0.985[a]	-0.993[a]	0.998[a]	.	.	-0.990[a]	1.000	-0.997[a]	0.996[a]	-0.980	-0.967	.
ACETATE	0.089	0.636[b]	-0.768[b]	-0.808	0.453	0.798[b]	-0.997[a]	1.000	-0.956[a]	0.560	-0.727[b]	0.905[b]
BUTYRATE	-0.163	-0.443	0.554	0.811	-0.530	-0.898[a]	0.996[a]	-0.956[a]	1.000	0.318	-0.847[b]	-0.889[b]
PROPIONATE	0.945	0.371	-0.944	.	.	-0.404	-0.980	0.560	0.318	1.000	.	.
SUCCINATE	0.361	-0.792	0.927[a]	-0.117	0.344	0.544	-0.967	-0.727[b]	-0.847[b]	.	1.000	0.496
FUMARATE	-0.299	-0.479	0.610	-0.935	0.230	0.784	.	0.905	-0.889[b]	.	0.496	1.000

Values used are the production and consumption obtained after 24 h of incubation using different media.
[a] Values are 1% significantly different ($P \leq 0.01$).
[b] Values are 5% significantly different ($P \leq 0.05$).

methanogens using KBG.

Fumarate reducing bacteria are a diverse and distantly related group of microorganisms that produce fumarate reductase (*frd*). *Frd* is an important respiratory enzyme in the integral membrane protein that catalyzes the interconversion of fumarate to succinate. *Frd* has three subunits, among which subunit A contains the site of fumarate reduction. To verify bacteria isolated, we characterized their molecular properties [fumarate reductase A gene (*frdA*)] and fumarate reductase activities. In this study, we used the *frdA* primer set developed by Hattori and Matsui (2008) for identification of fumarate reducing bacteria. Among the isolated bacteria, only *M. jalaludinii* contained a band at approximately 350 bp. Using *frdA* primers has some limitations (Hattori and Matsui, 2008). They stated that the designed target of the primer set is *Proteobacteria* and that it could not amplify the fumarate reductase gene of known bacteria with a high fumarate reducing activity such as *Fibrobacter succinogenes, Selenomonas ruminantium, Wollinella succinogenes*, and *V. parvula*. Thus, only *M. jalaludinii* has the fumarate reductase gene.

Fumarate is an intermediate compound used in the citric acid cycle to produce energy in the form of adenosine triphosphate (ATP). Lopez *et al.* (1999) stated that fumarate is an intermediate compound in the succinate propionate pathway in the rumen that is reduced to succinate by fumarate reductase. With *V. parvula*'s ability to reduce fumarate (Asanuma and Hino, 2000), it serves as standard microbe. The enzyme activities of *M. jalaludinii* and *V. parvula* were determined to confirm the results obtained using specific primers.

The enzyme activities of *M. jalaludinii* and *V. parvula* were observed after 15 and 25 min of sonication and found to be 16.8 ± 0.34 and 16.9 ± 1.21, respectively. The enzyme activities of both microorganisms were similar, but obtained at different sonication times. In a study conducted by Asanuma and Hino (2000), *V. parvula* utilized a large amount of fumarate containing 15–30 mM. Having almost similar enzyme activities of *M. jalaludinii* and *V. parvula* may also mean large fumarate utilization of *M. jalaludinii.* These findings further indicate that these microorganisms have the potential for fumarate reduction. Furthermore, *M. jalaludinii* isolated and identified in this study contains the succinate dehydrogenase/fumarate reductase flavoprotein subunit, which is responsible for fumarate reductase activity.

M. jalaludinii and *V. parvula* were cultivated using modified *frd* media. During cultivation, response of microbes on the addition of different electron acceptors and donors were observed through its growth, production and consumption of gases, VFA and other metabolites. Results on rapid growth of *M. jalaludinii* with addition of hydrogen was supported by Harris and Reddy (1977) statement that molecular H_2 serves as electron donor for reduction of fumarate to succinate in several organisms. Growth of *V. parvula* in TF led to results comparable to those obtained by Asanuma and Hino (2000), with the production of large amounts of fumarate (15–30 mM). Because both microorganisms grew best in response to addition of hydrogen during their cultivation, they may utilize hydrogen for their metabolic pathway. These findings indicate they have the potential to reduce fumarate (Hattori and Matsui, 2008).

Different gases are produced and consumed during cultivation of *M. jalaludinii* and *V. parvula*. The *V. parvula* profile indicates that the production of hydrogen in TF and TFH treatments and of CO_2 in all treatments were similar to those obtained by Ng and Hamilton (1971) in that hydrogen and carbon dioxide were also produced. Conversely, hydrogen was consumed by TH cultivated *V. parvula* and *M. jalaludinii*. Asanuma *et al.* (1999b) observed that several rumen bacteria including *V. parvula* oxidized H_2 by using fumarate as the final electron acceptor, suggesting that this bacteria competes with methanogens for H_2. However, the affinity of these bacteria to H_2 was lower than their affinity to methanogens. Conversely, *M. jalaludinii* cultivated in TFH consumed hydrogen at 24 h, but produced hydrogen at 48 h. In addition, CO_2 was produced by *M. jalaludinii* cultivated in T, TH and TFH.

Production and consumption of different VFA or metabolites depends on the microorganism's metabolism and pathway. The addition of different electron acceptors and donors affects their metabolism, pathways and VFA and metabolite production and consumption. *M. jalaludinii* produced acetate and lactate in all treatments, while succinate was only produced in the TFo and TFFo treatment and fumarate was produced in the TF treatment. These findings are similar to

those of Lan *et al*. (2002), who found that *M. jalaludinii* major end products from glucose fermentation are lactic, succinic and acetic acid. Conversely, *V. parvula* produced butyrate and lactate in all treatments, while succinate was produced in the TF, TFH, and TFFo treatments and formate was produced in the TFo and TFFo treatments. These findings differ slightly from those reported by Ng and Hamilton (1971). Specifically, they found that acetate and propionate were produced, while in our study acetate was consumed and propionate production was only observed in TF. This difference in results may have been due to differences in the media used. Asanuma *et al*. (1999b) stated that *Fibrobacter succinogenes*, *V. parvula*, *and W. succinogenes* utilized formate, another substrate for methanogenesis, as an electron donor for fumarate reduction. The affinity of these bacteria for formate was greater than the affinity methanogens have for formate. When methanogens were cocultured with an equal amount of each of the fumarate-utilizing bacteria, methane production was markedly decreased, not only from formate, but also from H_2. These results suggest that the addition of fumarate to ruminant feed reduces methanogenesis and enhances propionate production in the rumen.

Correlation analyses on the growth of *M. jalaludinii* after 24 h of cultivation significantly decreased pH and used formate but produced lactate, acetate and fumarate. This result is partly similar with Lan *et al*. (2002) where the end-products from glucose fermentation are lactic, succinic and acetic acid. On the other hand, growth of *V. parvula* significantly used formate but produced acetate which is partly similar to Ng and Hamilton (1971) result where the products are propionate, acetate, carbon dioxide and hydrogen.

Conclusion

Eight bacterial species belonging to Firmicutes were identified in this study and evaluated for the presence of the fumarate reductase gene using *frdA* primers. Only *M. jalaludinii* was found to contain the gene. *M. jalaludinii* and *V. parvula* enzyme activities were highest after 15 and 25 min of sonication, respectively. *M. jalaludinii* grew best in response to the addition of hydrogen alone, while *V. parvula* grew best in response to the addition of fumarate and fumarate + hydrogen. Moreover, *M. jalaludinii* produced comparatively higher levels of lactate than *V. parvula*, and *M. jalaludinii* produced acetate but not butyrate, while *V. parvula* had the opposite metabolism. Furthermore, *V. parvula* produced CO_2 at 24 and 48 h when cultivated in the presence of T, TH, TF, and TFH, while *M. jalaludinii* produced CO_2 when cultivated in the presence of T and TH at 24 and 48 h and in the presence of TFH at 48 h of cultivation. Taken together, the results of this study indicate that *M. jalaludinii* can be used as a fumarate reducing bacteria to compete with methanogens.

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References

Asanuma, N. and Hino, T. 2000. Activity and properties of fumarate reductase in ruminal bacteria. *Gen. Appl. Microbiol.* **46**, 119–125.

Asanuma, N., Iwamoto, M., and Hino, T. 1998. Formate metabolism by ruminal microorganisms in relation to methanogenesis. *Anim. Sci. Technol.* **69**, 9.

Asanuma, N., Iwamoto, M., and Hino, T. 1999. Effect of the addition of fumarate on methane production by ruminal microorganisms *in vitro*. *J. Dairy Sci.* **82**, 780–787.

Bryant, M.P. and Burkey, L.A. 1953. Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. *J. Dairy Sci.* **36**, 205–217.

Courtright, J.B. and Henning, U. 1970. Malate dehydrogenase mutants in *Escherichia coli* k-12. *J. Bacteriol.* **102**, 722–728.

Global Methane Initiative. 2012. Global methane emissions and mitigation opportunities, p. 4. Global Methane Initiative.

Han, S.-K., Kim, S.-H., and Shin, H.-S. 2005. Uasb treatment of wastewater with vfa and alcohol generated during hydrogen fermentation of food waste. *Proc. Biochem.* **40**, 2897–2905.

Harris, M.A. and Reddy, C.A. 1977. Hydrogenase activity and the h2-fumarate electron transport system in bacteroides fragilis. *J. Bacteriol.* **131**, 922–928.

Hattori, K. and Matsui, H. 2008. Diversity of fumarate reducing bacteria in the bovine rumen revealed by culture dependent and independent approaches. *Anaerobe* **14**, 87–93.

Hino, T., Mukunoki, H., Imanishi, K., and Miyazaki, K. 1992. Necessity of ready electron disposal and interspecies hydrogen transfer for the utilization of ethanol by rumen bacteria. *Asian-Australas. J. Anim. Sci.* **5**, 511–517.

Joblin, K.N. 1981. Isolation, enumeration, and maintenance of rumen anaerobic fungi in roll tubes. *Appl. Environ. Microbiol.* **42**, 1119–1122.

Johnson, K.A. and Johnson, D.E. 1995. Methane emissions from cattle. *J. Anim. Sci.* **73**, 2483–2492.

Johnson, D.E. and Ward, G.M. 1996. Estimates of animal methane emissions. *Environ. Monit. Assess.* **42**, 133–141.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16**, 111–120.

Lan, G.Q., Ho, Y.W., and Abdullah, N. 2002. *Mitsuokella jalaludinii* sp. nov., from the rumens of cattle in malaysia. *Int. J. Syst. Evol. Microbiol.* **52**, 713–718.

Lee, P., Lee, S., Hong, S., and Chang, H. 2002. Isolation and characterization of a new succinic acid-producing bacterium, *Mannheimia succiniciproducens* mbel55e, from bovine rumen. *Appl. Microbiol. Biotechnol.* **58**, 663–668.

Lopez, S., Valdes, C., Newbold, C.J., and Wallace, R.J. 1999. Influence of sodium fumarate addition on rumen fermentation *in vitro*. *Br. J. Nutr.* **81**, 59–64.

Miller, T.L. 1995. In ruminant physiology: Digestion, metabolism, growth and reproduction. Stuttgart: Ferdinand Enke-Verlag.

Ng, S.K.C. and Hamilton, I.R. 1971. Lactate metabolism by *Veillonella parvula*. *J. Bacteriol.* **105**, 999–1005.

Ørskov, E.R. and McDonald, I. 1979. The estimation of protein degradability in the rumen from incubation measurements weighted according to rate of passage. *J. Agri. Sci. (Cambridge)* **92**, 499–503.

SAS. 2002. Sas/stat. Statistical analysis systems for windows. Release 9.1. SAS Institute Inc., Cary, N.C., USA.

Spencer, M.E. and Guest, J.R. 1973. Isolation and properties of fumarate reductase mutants of *Escherichia coli*. *Bacteriology* **114**, 563–570.

Tabaru, H., Kadota, E., Yamada, H., Sasaki, N., and Takeuchi, A. 1988. Determination of volatile fatty acids and lactic acid in bovine plasma and ruminal fluid by high performance liquid chromatography. *Nihon Juigaku Zasshi* **50**, 1124–1126.