Journal of Microbiology (2014) Vol. 52, No. 2, pp. 120–128
DOI 10.1007/s12275-014-3518-1

Effect of Fumarate Reducing Bacteria on *In Vitro* Rumen Fermentation, Methane Mitigation and Microbial Diversity

Lovelia Mamuad[1], Seon Ho Kim[1], Chang Dae Jeong[1], Yeon Jae Choi[1], Che Ok Jeon[2], 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]*Research Center for Biomolecules and Biosystems, Department of Life Science, Chung-Ang University, Seoul 156-756, Republic of Korea*

(Received Oct 8, 2013 / Revised Jan 9, 2014 / Accepted Jan 15, 2014)

The metabolic pathways involved in hydrogen (H_2) production, utilization and the activity of methanogens are the important factors that should be considered in controlling methane (CH_4) emissions by ruminants. H_2 as one of the major substrate for CH_4 production is therefore should be controlled. One of the strategies on reducing CH_4 is through the use of hydrogenotrophic microorganisms such as fumarate reducing bacteria. This study determined the effect of fumarate reducing bacteria, *Mitsuokella jalaludinii*, supplementation on *in vitro* rumen fermentation, CH_4 production, diversity and quantity. *M. jalaludinii* significantly reduced CH_4 at 48 and 72 h of incubation and significantly increased succinate at 24 h. Although not significantly different, propionate was found to be highest in treatment containing *M. jalaludinii* at 12 and 48 h of incubation. These results suggest that supplementation of fumarate reducing bacteria to ruminal fermentation reduces CH_4 production and quantity, increases succinate and changes the rumen microbial diversity.

Keywords: DGGE, fumarate reducing bacteria, *in vitro*, *Mitsuokella jalaludinii*, qRT-PCR

Introduction

Scientists are currently exploiting biochemical pathways in competing with methanogenesis. One of the methods that can be used to accomplish this is through alternative hydrogen sink via fumarate reduction. Fumarate is an intermediate compound used in the citric acid cycle to produce energy in the form of adenosine triphosphate (ATP). In the rumen, fumarate is reduced to succinate by fumarate reductase through the succinate propionate pathway (Lopez *et al.*, 1999). The molecular H_2 serves as an electron donor for reduction of fumarate to succinate (Harris and Reddy, 1977) and hence, competes with methanogens in H_2 utilization.

Fumarate reduction plays an important role in maintaining a low partial H_2 pressure in the rumen as well as improving its fiber digestion (Asanuma and Hino, 2000). Thus, supplementation of fumarate is a possible method for CH_4 reduction. Hence, several studies have been conducted regarding addition of the organic acid fumarate and its effects on CH_4 production (Nisbet and Martin, 1993; Asanuma *et al.*, 1999b; Lopez *et al.*, 1999; Garcia-Martinez *et al.*, 2005; Ungerfeld *et al.*, 2007; Mao *et al.*, 2008; Zhou *et al.*, 2011; Lin *et al.*, 2013). Most of these have shown positive results regarding CH_4 reduction during *in vitro* fermentation, but the *in vivo* results were inconsistent. In addition, the current cost of organic acid fumarate is uneconomical.

Biotic agents such as fumarate reducing bacteria can be used an alternative to organic acid fumarate as well as in H_2 utilization. Fumarate reducing bacteria are hydrogenotrophs that are diverse and distantly related group of microorganisms. They produce fumarate reductase that catalyzes the interconversion of fumarate to succinate. In our previous research, we isolated a strain from Korean black goat, which is fumarate reducing bacteria that was closely related to *Mitsuokella jalaludinii* (Mamuad *et al.*, 2012). This microorganism was found to harbor the *frdA* gene, which contains the succinate dehydrogenase/fumarate reductase flavoprotein subunit. Moreover, *M. jalaludinii* has a fumarate reductase activity of 16.8±0.34 mmol NADH oxidized/min/mg of cellular N.

Accordingly, *M. jalaludinii* can be used to compete with methanogens in H_2 utilization (Mamuad *et al.*, 2012). However, there are no reports on the effect of *M. jalaludinii* addition on rumen fermentation. *In vitro* fermentation method is usually used by the researchers to simulate the rumen fermentation processes (Johnson, 1966). This method has been shown to be a valuable tool in various studies such as evaluation of the nutritive value of feedstuffs (Liu *et al.*, 2002; Akinfemi *et al.*, 2009; Murillo *et al.*, 2011) and mitigation of methane production in ruminants (Do *et al.*, 2011; Hassanat and Benchaar, 2012). Therefore, the objective of this study was to investigate the effects of *M. jalaludinii* supplementation *in vitro* rumen fermentation and microbial diversity and quantity.

Materials and Methods

Cultivation of microorganisms

The standard fumarate reducing bacteria, *Veillonella parvula* KCTC 5019, was obtained from the Korean Collection for Type Cultures Biological Resource Center while the treated

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

microbe, *Mitsuokella jalaludinii*, was previously isolated in our laboratory. For cultivation of microbes, basal media was mixed with clarified rumen fluid (3:1) (Asanuma and Hino, 2000) and amended with 2 g/L of glucose. The prepared medium was then dispensed anaerobically under an O_2-free N_2 atmosphere, after which it was autoclaved at 121°C for 15 min (Hattori and Matsui, 2008). H_2 was then added after inoculation of the microbes and bacterial cultures were grown anaerobically for 24–48 h under 120 rpm horizontal shaking at 37°C until the optical density was approximately 1.0 (Asanuma *et al.*, 1999b).

Rumen fluid collection and buffer preparation

The animals used in this experiment were cared for in accordance with the guidelines established by National Institute of Animal Science (NIAS), South Korea. Ruminal contents were obtained from ruminally cannulated Holstein cow with body weight of 600±47 kg that was fed twice a day with feed concentrate and rice straw at a 2:8 ratio. The sample was then 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 subsequently sealed and maintained at 39°C and immediately transported to the laboratory.

The buffer used in this study was composed of (per L) 0.45 g K_2HPO_4, 0.45 g KH_2PO_4, 0.9 g $(NH_4)_2SO_4$, 0.12 g $CaCl_2{\cdot}2H_2O$, 0.19 g $MgSO_4{\cdot}7H_2O$, 1.0 g trypticase peptone, 1.0 g yeast extract, and 0.6 g cysteine·HCl (Asanuma *et al.*, 1999b). The medium was adjusted to pH 6.9 sodium hydroxide (NaOH), after which it was dispensed anaerobically under a 100% N_2 atmosphere and autoclaved at 121°C for 15 min (Hattori and Matsui, 2008). The chemical composition of rice straw in dry matter (DM) used in this experiment contained 3.90% crude protein (CP), 1.22% ether extract (EE), 51.58% nitrogen free extract (NFE), 30.14% crude fiber (CF), 13.16% ash, and 47.26% total digestible nutrients (TDN). Next, 2% DM (2 mm) of rice straw was prepared by adding straw to 160 ml serum bottles and then mixing it with 50 ml rumen fluid and basal medium that had been prepared with a 1:3 ratio and transferred into serum bottles under a stream of O_2-free N_2. The bottles were then sealed with rubber stoppers and aluminum caps, after which *M. jalaludinii* or *V. parvula* were inoculated into the serum bottles anaerobically 1% (approximately 1.0 OD value) of total volume. The samples were then incubated at 37°C while shaking horizontally at 120 rpm (Hattori and Matsui, 2008). *In vitro* fermentation parameters were measured at 0, 12, 24, 48, and 72 h in triplicate. Changes in diversity were analyzed using bacterial and archaeal 16S rDNA denaturing gradient gel electrophoresis (DGGE), while methanogen quantity was determined using quantitative real time polymerase chain reaction (qRT-PCR).

Analyses for *in vitro* fermentation parameters

Total gas (TG) production at different stages was measured in each of the serum bottles using a press and sensor machine (Laurel Electronics, Inc., USA). pH was measured with a Pinnacle series M530p meter (Schott Instruments, Germany) after uncapping each of the bottles. The ammonia nitrogen (NH_3-N) concentration was measured according to the methods developed by (Chaney and Marbach, 1962).

One ml of the TG produced during the *in vitro* fermentation process was used to determine the CH_4 and carbon dioxide (CO_2) emitted during the incubation period. Gas chromatography (Agilent Technologies HP 5890, USA) was conducted using a TCD detector with a Column Carboxen 1006PLOT 30 m × 0.53 mm capillary column (Supelco, USA). CH_4 produced was estimated using the formula described by Ørskov and McDonald (1979).

Volatile fatty acid (VFA) and other metabolite analyses were conducted using HPLC (Agilent Technologies 1200 series) with a UV detector set at 210 nm and 220 nm. Two columns were used for determination of fermentation products, a MetaCarb 87H (Varian, Germany) column using 0.0085 N H_2SO_4 buffer applied at a rate of 0.6 ml/min as described by Tabaru *et al.* (1988) and Han *et al.* (2005), and an RSpak DE-613 (Shodex, Japan) column using 3 mM perchloric acid buffer applied at a rate of 0.8 ml/min.

Denaturing Gradient Gel Electrophoresis (DGGE)

Fermented samples (preserved) taken at 0, 24, 48, and 72 h were extracted using Wizard Genomic DNA Purification Kits (Promega, USA). Genomic DNA (gDNA) PCR amplification was performed using the 27F and 1492R primers for bacteria, while Met 86f and Met 1340r were used for methanogens. To amplify the V3 region of 16S rDNA amplicons, the 341F-GC and 518R primers (Nübel *et al.*, 1996) were used for bacteria, while ARC344f-GC and 519r were used for archaea. DGGE was performed using a D-Code Universal Mutation Detection System (Bio-Rad, USA).

Amplicons of the V3 region of 16S rDNA were used for sequence-specific separation by DGGE according to the specifications of Muyzer and Smalla (1998). The DGGE gel was then scanned at 400 dpi and similarity indices were calculated for pairs of DGGE profiles. The number of DGGE bands and similarity indices were calculated from the densitometric curves of the scanned DGGE profiles using the Molecular Analyst 1.12 software (Bio-Rad) and the Pearson product-moment correlation coefficient (Häne *et al.*, 1993; Simpson *et al.*, 1999) by the Central Microbiology Laboratory of SCNU in Korea. Bands of interest were excised from the gel, eluted in 50 µl sterile distilled water and incubated overnight at 4°C. Eluted gels were then amplified using non-GC bacteria and archaea primers. The PCR products were purified with a QiaQuick PCR purification kit in accordance with the manufacturer's instructions, after which PCR products were sent to Macrogen, Seoul, Korea for sequencing. The results were then compared to those in the GenBank database using the BLAST tool of the National Center for Biotechnology Information (NCBI) and EzTaxon.

Quantitative real-time PCR (qRT-PCR)

External standards were prepared using a mixture of pure cultures of *Methanobrevibacter ruminantium*, *M. smithii*, *M. millarae*, and *Methanosarcina barkeri*. Methanogen real-time PCR primers Met630F (5′-GGA TTA GAT ACC CSG GTA GT-3′) and Met803R (5′-GTT GAR TCC AAT TAA ACC GCA-3′) were used to enumerate the methanogens represented in the extracted DNA from rumen samples using the

Table 1. Effect of fumarate reducing bacteria on *in vitro* rumen fermentation parameters incubated at 0, 24, 48, and 72 h

Parameters/Time/Treatments	Control	*Mitsuokella jalaludinii*	*Veillonella parvula*
Total gas (ml)			
12	13.7±0.36	15.50±1.66	15.13±0.84
24	17.93±0.07[b]	18.50±0.29[b]	21.07±0.64[a]
48	23.00±0.58	22.03±0.55	23.33±1.20
72	22.33±2.17[ab]	21.40±1.03[b]	26.50±0.55[a]
Carbon dioxide (mM/ml)			
12	4.46±0.07	4.70±0.34	4.63±0.26
24	5.50±0.19	6.16±0.59	6.78±0.10
48	8.25±0.19	7.60±0.56	8.42±0.05
72	8.57±0.25[b]	7.76±0.31[b]	10.49±0.51[a]
pH			
0	5.65±0.05[b]	5.91±0.02[ab]	6.19±0.14[a]
12	5.35±0.02[b]	5.51±0.03[b]	5.72±0.10[a]
24	5.08±0.06[b]	5.23±0.01[ab]	5.25±0.04[a]
48	5.19±0.01	5.25±0.05	5.41±0.14
72	5.16±0.03[b]	5.36±0.08[a]	5.38±0.03[a]
Ammonia-nitrogen (mg/L)			
0	430.39±14.45	407.36±35.59	405.55±17.78
12	609.48±7.45	642.52±47.32	696.15±24.77
24	681.00±66.15	660.70±4.85	743.12±10.79
48	746.76±31.62	750.39±18.98	747.06±13.91
72	767.97±39.63	764.64±4.30	858.58±23.10

[a] Values are mean ± standard error of three replications
[b] Means in the same row with the same superscript letter are not significantly different (P<0.05) by DMRT.

protocol described by Christophersen (2007). Amplification was performed using Opticon Monitor 3.1.32 (MJ Geneworks, Inc., Bio-Rad Laboratories, Inc.) with a volume of 25 µl containing the following reagents: 12.5 µl of 2× SensiMix Plus SYBR & Fluorescein (Quantance, USA), 1.0 µl (10 pmol) PCR primers, 1.0 µl 50 mM $MgCl_2$ (Quantance), 4.5 µl dH_2O, and 5.0 µl of template DNA (50 ng final concentration). Real-time amplification commenced with a hot start at 95°C for 15 min, followed by 40 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 60 sec. Melting-curve analysis was conducted by monitoring fluorescence continuously between 60°C and 95°C, with 10-s increments of 0.5°C (Hook *et al.*, 2010). DNA amplification was performed in triplicate.

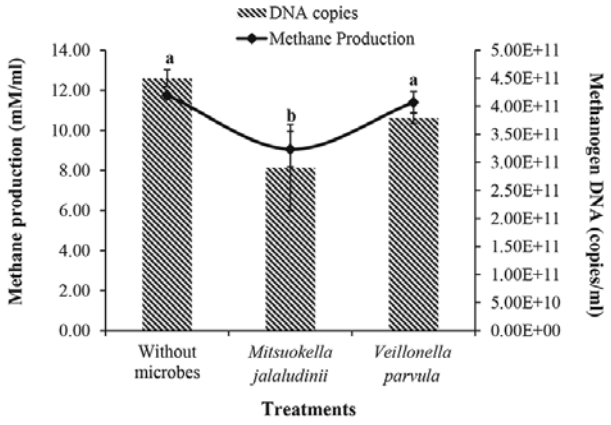

Fig. 1. Methane production (mM/ml) and methanogen DNA (copies/ml) of *in vitro* rumen fermentation amended with or without fumarate reducing bacteria after 48 h of incubation. Values are the means of triplicate analyses and bars indicate the standard error. Means with the same letter are not significantly different (P<0.05) by DMRT.

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

Results

Effect of adding fumarate reducing bacteria on *in vitro* parameters

Total gas (TG) production, CO_2, pH and NH_3-N concentration of *in vitro* rumen fermentation amended with *M. jalaludinii* and *V. parvula* at different incubation times using rice straw as a substrate are shown in Table 1. TG production and CO_2 and NH_3-N concentration were directly proportional and increased with increased incubation time. Additionally, TG, CO_2, and pH were found to be highest (P<0.05) in treatments in which *V. parvula* was added at different incubation times. Moreover, NH_3-N was found to be highest in the treatment amended with *V. parvula*, followed by *M. jalaludinii* and then the control; however, it did not differ significantly among incubation times. Conversely, CH_4 production as well as methanogen DNA copies were found to be lowest (P<0.05) for the treatment amended with *M. jalaludinii* at 48 h (9.06 mM/ml) (Fig. 1).

Table 2. Effect of fumarate reducing bacteria on *in vitro* rumen fermentation volatile fatty acid (VFA) and other metabolites concentration incubated at 0, 24, 48, and 72 h (mM)

VFA/Metabolites	Time	Control	*Mitsuokella jalaludinii*	*Veillonella parvula*
Acetate	0	28.09±0.10	28.10±0.78	28.26±0.62
	12	36.14±0.35	36.70±1.34	37.49±0.63
	24	40.00±1.27	38.14±0.14	41.44±1.53
	48	42.12±0.46[a]	39.08±0.92[b]	42.28±0.95[a]
	72	42.35±1.61[a]	38.31±0.44[b]	48.08±1.19[a]
Propionate	0	7.15±0.19	7.36±0.24	7.57±0.13
	12	10.29±0.13	10.44±0.20	10.22±0.55
	24	11.38±0.43	11.91±0.11	12.36±0.43
	48	11.32±0.28	12.86±0.62	12.41±0.80
	72	12.46±1.12	11.93±0.51	12.97±0.19
Butyrate	0	3.84±0.39[a]	2.94±0.01[b]	2.87±0.02[b]
	12	6.73±0.23	6.54±0.14	6.36±0.55
	24	8.52±0.59	8.04±0.19	9.06±0.52
	48	10.41±1.57	10.82±1.29	9.74±1.51
	72	12.18±0.97[a]	8.59±0.40[b]	8.62±1.09[b]
Succinate	0	ND	ND	ND
	12	19.53±5.70	24.99±5.59	13.60±0.49
	24	13.57±1.20[b]	18.97±1.84[a]	16.32±1.05[ab]
	48	9.20±0.65	11.10±1.07	11.14±1.14
	72	10.06±2.62	8.33±0.87	ND
Fumarate	0	ND	0.18±0.02	0.21±0.03
	12	0.25±0.01	ND	ND
	24	ND	ND	ND
	48	ND	ND	ND
	72	ND	ND	ND
Total VFA	0	39.08±0.38	38.40±0.89	38.70±0.60
	12	53.17±0.51	53.68±1.55	54.07±0.86
	24	59.90±1.71[ab]	58.08±0.16[b]	62.86±0.93[a]
	48	63.85±2.03	62.76±2.63	64.43±2.45
	72	66.99±1.46[a]	58.83±1.25[b]	69.67±2.33[a]
A/P*	0	3.93±0.10	3.82±0.13	3.73±0.11
	12	3.51±0.06	3.52±0.12	3.69±0.20
	24	3.53±0.20	3.20±0.04	3.37±0.22
	48	3.73±0.11[a]	3.05±0.12[b]	3.44±0.26[ab]
	72	3.47±0.40	3.22±0.12	3.71±0.05

[a] Values are mean ± standard error of three replications.
[b] Means in the same row with the same superscript letter are not significantly different ($P<0.05$) by DMRT.
* A/P – Acetate to propionate ratio.

Table 2 shows the volatile fatty acid (VFA) and other metabolite production of *in vitro* rumen fermentation. Although the values did not differ significantly, propionate was found to be highest in treatments amended with *M. jalaludinii* after 12 and 48 h of incubation and with *V. parvula* at 24 and 72 h. Moreover, succinate was highest ($P<0.05$) in treatments amended with *M. jalaludinii* (18.97 mM) followed by *V. parvula* (16.32 mM) and then the control (13.57 mM) at 24 h

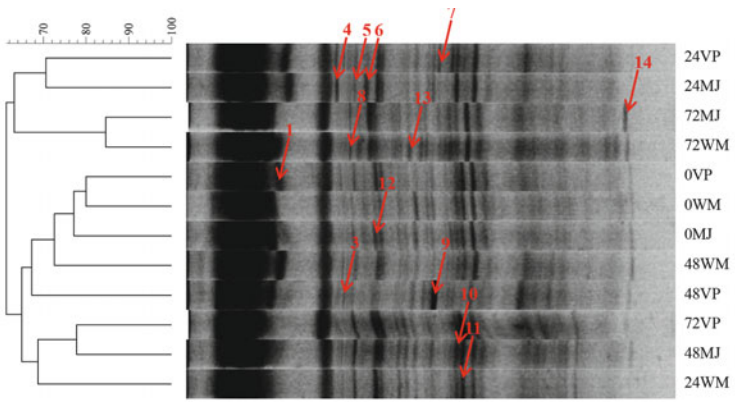

Fig. 2. Similarity analysis and negative image of bacterial 16S rDNA amplified DGGE band profiles using total genomic DNA extracted from *in vitro* fermentation amended with or without fumarate reducing bacteria incubated at 0, 24, 48, and 72 h. Arrows indicate the identified bands. Treatments: WM, without microbes added (control); MJ, *Mitsuokella jalaludinii*; VP, *Veillonella parvula*.

Table 3. Identified bands from bacterial 16S rDNA amplified DGGE band profiles using total genomic DNA extracted from *in vitro* fermentation amended with or without fumarate reducing bacteria incubated at 0, 24, 48, and 72 h

Band no.	Closest relative	Identity %	Isolation
1	*Clostridium aminophilum*	100 (123/123)	Dairy cow
3	Uncultured bacterium clone GB7N87003GMEOM small subunit ribosomal RNA gene, partial sequence	83 (100/120)	Unvegetated soil environments
4	Uncultured bacterium gene for 16S rRNA, partial sequence, clone:2-A06	90 (89/99)	Fecal sample from cattle
5	Uncultured bacterium clone Camel_AAR_187 16S ribosomal RNA gene	96 (132/138)	Rumen
6	*Clostridium thermopalmarium* strain KU-M1 16S ribosomal RNA gene	98 (120/122)	Anaerobic sludge
7	Uncultured bacterium clone PF7252 16S ribosomal RNA gene, partial	100 (121/121)	Feces
8	Uncultured rumen bacterium clone S11 16S ribosomal RNA gene, partial sequence	91 (116/128)	Goat rumen
9	*Succinivibrio dextrinosolvens* DSM 3072(T)	99 (123/124)	Rumen cattle
10	Uncultured bacterium clone Camel_AAR_002 16S ribosomal RNA gene, partial sequence	100 (130/130)	Rumen
11	Uncultured rumen bacterium clone Lq-H-8C 16S ribosomal RNA gene, partial sequence	99 (120/121)	Liquid fraction of cattle rumen content
12	Uncultured rumen bacterium gene for 16S rRNA, partial sequence, clone	95 (122/128)	Rumen contents from Holstein heifer
13	Uncultured rumen bacterium clone RuLiq_191 16S ribosomal RNA gene, partial sequence	98 (127/129)	Rumen liquid of goat from semi-arid region
14	*Olsenella umbonata* lac31(T) (FN178463)	99 (134/135)	Ruminal fluid of a sheep

incubation. Finally, total VFA was highest in the treatment amended with *V. parvula* at 24 and 72 h of incubation.

Molecular analyses of *in vitro* fermenta

The similarity analysis and negative image of bacterial 16S rDNA amplified DGGE band profiles obtained using the total genomic DNA extracted during *in vitro* fermentation at 0, 24, 48, and 72 h was shown in Fig. 2. At 0 h, the treatments had almost similar bacterial diversity. However, the bacterial diversity differed after 24, 48, and 72 h of incubation. It was noticeable that after 24 h of incubation, treat-

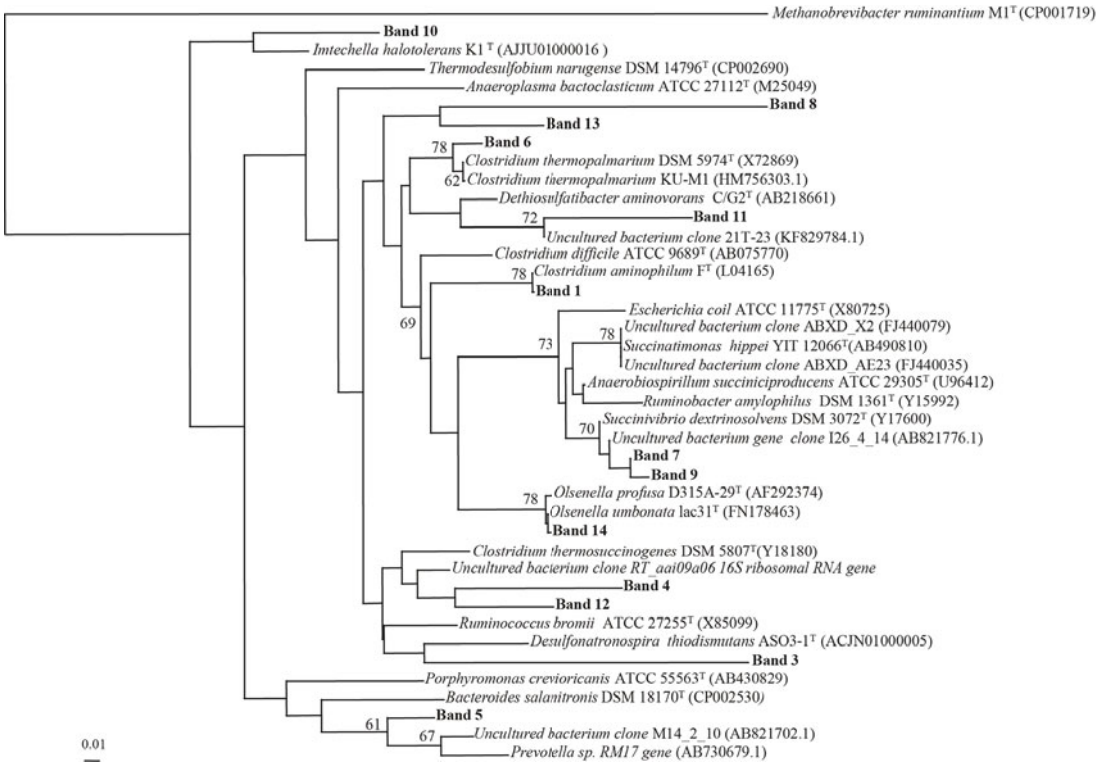

Fig. 3. Phylogenetic tree of identified bands from bacterial 16S rDNA amplified DGGE constructed using the neighbor joining method based on 16S rRNA gene sequences developed by Kimura (1980) with two parameter correction models. The tree was bootstrap resampled 1,000 times. Only bootstrap values greater than 60% are shown on the internal nodes. *Methanobrevibacter ruminantium* was used as an outgroup, 0.01 substitutions per nucleotide position.

Table 4. Identified bands from archaeal 16S rDNA amplified DGGE band profiles using total genomic DNA extracted from *in vitro* fermentation incubated at 0, 24, 48, and 72 h

Band No.	Strains	Identity %	Isolation
1,3,7,9,10,24,25	*Methanobrevibacter ruminantium*	99 (147/149)	Bovine rumen contents
2,4,6,12,13,15,16,17, 18,20,22,23,27,29,30	*Methanobrevibacter smithii*	99 (148/149)	Human feces, sewage
5,14,19	*Methanobrevibacter millerae*	100 (148/148)	Ovine and bovine rumen
8	*Methanobrevibacter olleyae*	99 (147/148)	Ovine and bovine rumen
11,21,26,28	*Methanobrevibacter thaueri*	99 (147/148)	Bovine feces

ments amended with *M. jalaludinii* and *V. parvula* were on the same clade and was 71% similar to each other. A total of 13 dominant bands were selected, cut, sequenced and identified (Table 3). They have identity of 83 to 100%, of which, most of the bacteria were isolated from rumen or feces except band 3 and 6. Band 9, *Succinivibrio dextrinosolvens*, was found in treatment added with *V. parvula* as well as with *M. jalaludinii* at 24 h. In addition, the phylogenetic tree of the identified bands from bacterial 16S rDNA amplified DGGE band was shown in Fig. 3. It 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 60% are shown on the internal nodes. *Methanobrevibacter ruminantium* was used as an outgroup, 0.01 substitutions per nucleotide position.

As shown in Fig. 4, the similarity analysis and negative image of archaeal 16S rDNA amplified DGGE band profiles obtained using the total genomic DNA extracted from *in vitro* fermentation at 0, 24, 48, and 72 h. At 0 h, the treatments were grouped into one cluster with 99% similarity. However, the methanogen diversity of the samples was grouped according to treatments after 24, 48, and 72 h of incubation. A total of 30 bands were selected, cut, sequenced and identified (Table 4). Among these, 15 were *Methanobrevibacter smithii*, seven *M. ruminantium*, four *M. thaueri*, three *M. millerae*, and one *M. olleyae*. They have identity of 99 to 100%, of which, all of them were isolated from rumen or feces. Figure 5 shows the phylogenetic tree of the identified bands from archaeal 16S rDNA amplified DGGE. It 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 60% are shown on the internal nodes. *Aquifex pyrophilus* was used as an outgroup, 0.01 substitutions per nucleotide position.

Discussion

Effect of adding fumarate reducing bacteria on *in vitro* parameters

The total gas production observed in this study agreed with the findings reported by Robinson *et al.* (1989), who found that total gas production increased with incubation time due to increased microbial fermentation. Higher gas production in the treatment containing *V. parvula* might have been due to the addition of microbes, which increased the rumen microbial fermentation production during metabolism and conversion of the substrates. However, it could also be due to the high contents of easily fermentable starches, sugars, or hemicelluloses as substrate to rumen microbes for gas production. Getachew *et al.* (1998) reported that the digestibility of the measured organic matter is closely related to that predicted from gas production and the crude protein and ash contents of feeds.

In the present study, the pH of all of the treatments including the control decreased from 12 to 72 h. NFE, TDN, and ruminal fermentation have a directly proportional relationship; therefore, increases in ruminal fermentation result in reduced pH. Stabilization of pH has been observed in treatments amended with microorganisms. The addition of *M. jalaludinii* and *V. parvula* could elicit its mode of action as

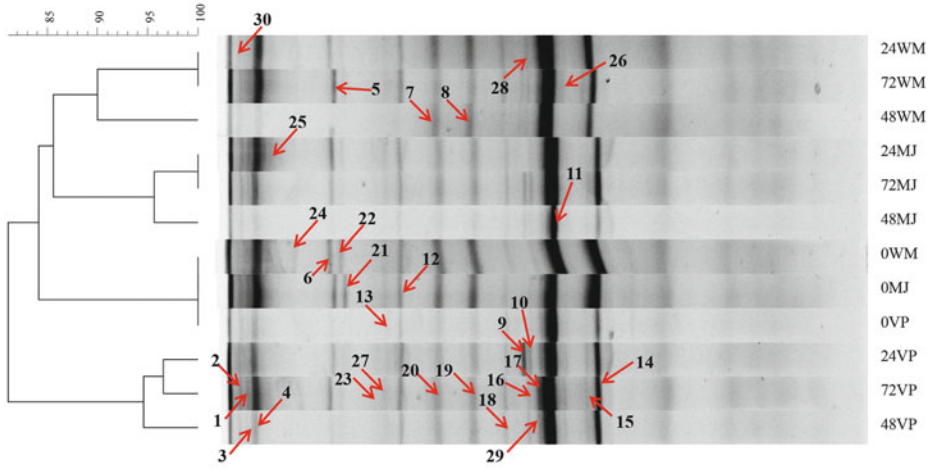

Fig. 4. Similarity analysis and negative image of archaeal 16S rDNA amplified DGGE band profiles using total genomic DNA extracted from *in vitro* fermentation amended with or without fumarate reducing bacteria incubated at 0, 24, 48, and 72 h. Arrows indicate the identified bands. Treatments: WM, without microbes added (control); MJ, *Mitsuokella jalaludinii*; VP, *Veillonella parvula*.

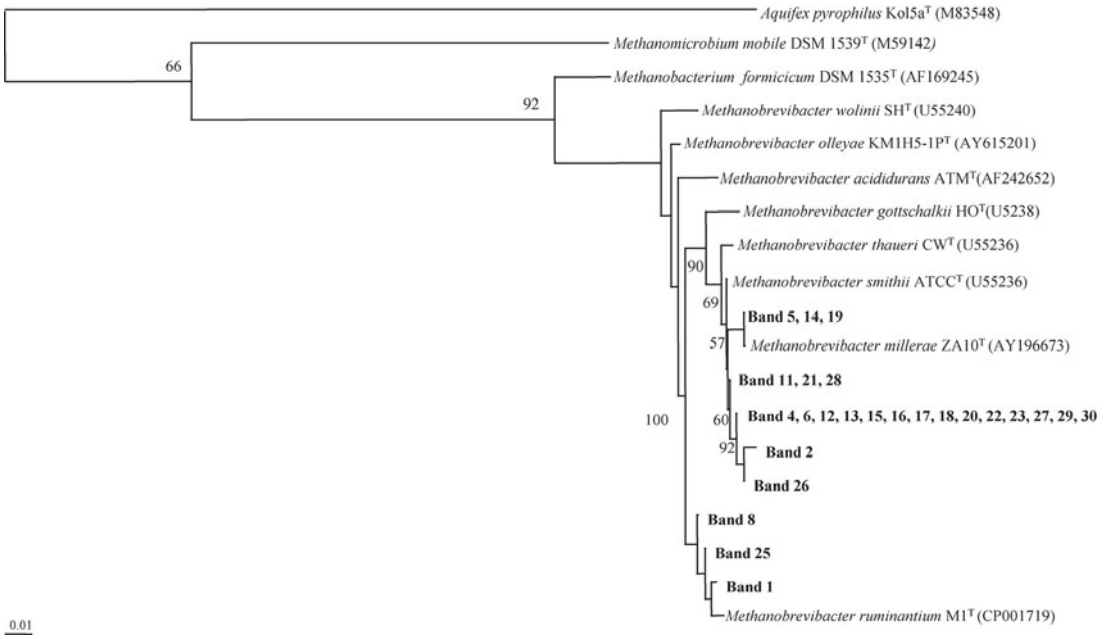

Fig. 5. Phylogenetic tree of identified bands from archaeal 16S rDNA amplified DGGE constructed using the neighbor joining method based on 16S rRNA gene sequences developed by Kimura (1980) with two parameter correction models. The tree was bootstrap resampled 1,000 times. Only bootstrap values greater than 60% are shown on the internal nodes. *Aquifex pyrophilus* was used as an outgroup, 0.01 substitutions per nucleotide position.

a direct fed microbe (DFM) through stabilization of ruminal pH (Seo *et al.*, 2010) and therefore lessen the occurrence of ruminal acidosis. Moreover, McNamara *et al.* (2000) stated that the pH of ruminal fluid is dependent on the concentration of HCO_3^- and CO_2 pressure. Thus, the higher CO_2 concentration observed in *V. parvula* resulted in a higher pH. CO_2 is used by intestinal microbes, as well as the animal itself to maintain bicarbonate levels in the saliva. Normally, CO_2 in the rumen is about 2–3 times greater than that of CH_4, but it is usually reduced by methanogens for CH_4 production.

NH_3-N is regarded as the most important nitrogen source for microbial protein synthesis in the rumen (Bryant, 1974), and the levels in the rumen are generally high when feeds are more digestible (Kim *et al.*, 2012). The results observed in the present study showed the same trend of increasing NH_3-N concentrations with increased incubation time. These results were supported by Erdman *et al.* (1986), who found that NH_3-N contents increased with increased digestion period in the ruminant. Moreover, the NH_3-N level depends on the presence of microbial protein in the rumen. Although the values did not differ significantly, higher NH_3-N was observed in treatments amended with either *M. jalaludinii* or *V. parvula*, indicating that there was more N available for microbial utilization and protein synthesis.

H_2 is one of the key elements associated with CH_4 reduction because it is a major substrate for CH_4 production. H_2 was not detected in treatments containing *M. jalaludinii* and *V. parvula* (data not shown), indicating that *M. jalaludinii* and *V. parvula* might use H_2 in the conversion of fumarate to succinate. Conversely, feed intake and nutrient content are factors affecting CH_4 production via carbohydrate degradation by rumen bacteria (Moe and Tyrrell, 1979). CH_4 production has also been reported to be affected by the type of carbohydrate fed to the animals. Cattle fed forage typically produce more CH_4 and have a higher ruminal pH than those fed grain (Russell and Rychlik, 2001). Thus, high CH_4 production was observed in this study relative to other studies due to the use of rice straw as a substrate. Conversely, numerous microorganisms in the rumen use carbohydrates as an energy source, and 40% of the total gas produced in the fermentation process is CO_2 (McDonald *et al.*, 1995). Substrates such as acetate, methanol, and methylamine are utilized by most methanogens to generate CH_4 from CO_2 and H_2 (Miller *et al.*, 1986). Hydrogenotrophic microorganisms such as fumarate reducing bacteria are methanogen competitors; therefore, the addition of fumarate reducing bacteria significantly lowers the CH_4 concentration in the rumen, as observed in the treatment amended with *M. jalaludinii*. This observation was also supported by the results of qRT-PCR and DGGE, which showed that the number of methanogen DNA copies and occurrence of methanogens were lower in the treatment amended with *M. jalaludinii* (Figs. 3 and 4).

There are many factors affecting the rumen fermentation. Concentrations of microbial metabolites and pH in digesta have been used as indicators of intestinal health and microbial activity (Nyachoti, *et al.*, 2006). Additionally, van Beers-Schreurs *et al.* (1998) explained that the quantity of VFA depends on the amount and composition of the substrate, as well as the types of microbes present. Lower VFA concentrations reflect lower amounts of fermented substrate, lower quantities of microbial activity and lower energy intake. The fumarate reducing bacteria utilize H_2 through fumarate reductase, which converts fumarate to succinate. Thus, increased in succinate was observed after 12, 24, and 48 h of

incubation in *M. jalaludinii*-treatment.

Propionate production is primarily via the dicarboxylic acid pathways (Lehloenya, 2007). Although there was an increase in propionate production with addition of *M. jalaludinii*, it was not markedly increased as chemically added fumarate (Nisbet and Martin, 1993; Lopez *et al.*, 1999; Asanuma *et al.*, 1999b; Garcia-Martinez *et al.*, 2005; Ungerfeld *et al.*, 2007; Mao *et al.*, 2008; Zhou *et al.*, 2011; Lin *et al.*, 2013). This might have been due to the amount of *M. jalaludinii* added and the presence of microbes (van Beers-Schreurs *et al.*, 1998) that are capable of decarboxylating succinate to propionate such as *Selenomonas ruminantium* (Scheifinger and Wolin, 1973; Scheifinger *et al.*, 1975) and *Propionibacterium* (Blackburn and Hungate, 1963). On the other hand, the present results showed higher favorable VFA (propionate and succinate) in treatments amended with *M. jalaludinii* or *V. parvula*, which is a good indication of the energy and health of an animal.

Molecular analyses of *in vitro* fermenta

Addition of microorganisms alters the microbial community in the rumen. Mao *et al.* (2008) stated that the microbial community changes in response to changes in feed and feed level in the rumen. Several good bacteria for rumen fermentation were identified in the present study; namely, Band 9, *S. dextrinosolvens*, which is a succinate producing bacteria; band 14, *Olsenella umbonata*, which is a lactic acid bacterium; band 1, *Clostridium aminophilum*, which is an amino-acid fermenting bacterium; and band 6, *C. thermopalmarium*, which is a butyrate-producing bacterium. One important ruminal microorganism identified in this study was *S. dextrinosolvens*, which is the predominant isolate from the rumen when the diet of the animal is high in starch. The end products of *S. dextrinosolvens* are acetate, formate, lactate and succinate (O'Herrin and Kenealy, 1993). Succinate is an important intermediate for propionate formation, with the latter being readily absorbed from the rumen for glucogenesis. These findings explain the increased succinate and propionate concentration in the treatments amended with either *M. jalaludinii* or *V. parvula.*

Whitford *et al.* (2001) stated that the rumen has a highly diverse population of methanogens. Janssen and Kirs (2008) recently reviewed the diversity of archaea in the rumen and found that *Methanobrevibacter* accounts for nearly two-thirds of rumen archaea. Moreover, Zhou *et al.* (2011) stated that most of the sequences obtained from the DGGE bands observed from previous PCR-DGGE profiling analyses of the rumen ecosystem belonged to the general *Methanobrevibacter* or *Methanosphaera.* Grant *et al.* (1985) indicated that most ruminal methanogens could be classified as *M. ruminantium*, which explains the seven bands identified as such in the present study. Moreover, the occurrence of identified methanogens was lower in treatments amended with *M. jalaludinii*. These results are correlated with the lower CH_4 concentration and lower methanogen DNA copies observed.

Conclusion

Addition of *M. jalaludinii* increases favorable VFA (propionate and succinate), which is a good indication of animal energy and health. Moreover, *M. jalaludinii* significantly reduces the CH_4 concentration, which was supported by the reduced number of methanogen DNA copies and occurrence of methanogens observed following their addition in the present study. Overall, these results indicate that *M. jalaludinii* has the potential for use as a direct fed microbe. *In vivo* studies should be conducted to determine the actual effects of *M. jalaludinii* on ruminants.

Acknowledgements

This research was supported by Bio-industry Technology Development Program, Ministry of Agriculture, Food and Rural Affairs, and Cooperative Research Program for Agriculture Science and Technology Development, Rural Development Administration and with support of "Cooperative Research Program for Agriculture Science and Technology Development (Project No. PJ0091562014)", Rural Development Administration, Republic of Korea.

References

Akinfemi, A., Adesanya, A.O., and Aya, V.E. 2009. Use of an *in vitro* gas production technique to evaluate some Nigerian feedstuffs. *Am. Eur. J. Sci. Res.* **4**, 240–245.

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

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.

Blackburn, T.H. and Hungate, R.E. 1963. Succinic acid turnover and propionate production in the bovine rumen. *Appl. Microbiol.* **11**, 132–135.

Bryant, M.P. 1974. Nutritional features and ecology of predominant anaerobic bacteria of the intestinal tract. *Am. J. Clin. Nutr.* **27**, 1313–1319.

Chaney, A.L. and Marbach, E.P. 1962. Modified reagents for determination of urea and ammonia. *Clin. Chem.* **8**, 130–132.

Christophersen, C.T. 2007. Grain and artificial stimulation of the rumen change the abundance and diversity of methanogens and their association with ciliates. *School of Animal Biology*. University of Western Australia, Perth, Ph. D. thesis.

Do, H.Q., Thi Thuy, T., Phuc Hao, T., Preston, T.R., and Leng, R.A. 2011. Effects of nitrate and sulphur on *in vitro* methane production and dry matter degradation. *Livestock Res. Rural Develop.* **23**, http://www.lrrd.org/lrrd23/10/hqdo23211.htm.

Erdman, R.A., Proctor, G.H., and Vandersall, J.H. 1986. Effect of rumen ammonia concentration on *in situ* rate and extent of digestion of feedstuffs. *J. Dairy Sci.* **69**, 2312–2320.

Garcia-Martinez, R., Ranilla, M.J., Tejido, M.L., and Carro, M.D. 2005. Effects of disodium fumarate on *in vitro* rumen microbial growth, methane production and fermentation of diets differing in their forage:concentrate ratio. *Br. J. Nutr.* **94**, 71–77.

Getachew, G., Blümmel, M., Makkar, H.P.S., and Becker, K. 1998. *In vitro* gas measuring techniques for assessment of nutritional quality of feeds: a review. *Anim. Feed Sci. Technol.* **72**, 261–281.

Grant, W.D., Pinch, G., Harris, J.E., De Rosa, M., and Gambacorta, A. 1985. Polar lipids in methanogen taxonomy. *J. Gen. Microbiol.* **131**, 3277–3286.

Häne, B.G., Jäger, K., and Drexler, H.G. 1993. The Pearson product-

moment correlation coefficient is better suited for identification of DNA fingerprint profiles than band matching algorithms. *Electrophoresis* **14**, 967–972.

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. *Process Biochem.* **40**, 2897–2905.

Harris, M.A. and Reddy, C.A. 1977. Hydrogenase activity and the H_2-fumarate electron transport system in *Bacteroides fragilis*. *J. Bacteriol.* **131**, 922–928.

Hassanat, F. and Benchaar, C. 2012. Assessment of the effect of condensed (acacia and quebracho) and hydrolysable (chestnut and valonea) tannins on rumen fermentation and methane production *in vitro*. *J. Sci. Food Agric.* **93**, 332–339.

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.

Hook, S.E., Wright, A.D.G., and McBride, B.W. 2010. Methanogens: methane producers of the rumen and mitigation strategies. *Archaea* **2010**.

Janssen, P.H. and Kirs, M. 2008. Structure of the archaeal community of the rumen. *Appl. Environ. Microbiol.* **74**, 3619–3625.

Johnson, R.R. 1966. Techniques and procedures for *in vitro* and *in vivo* rumen studies. *J. Anim. Sci.* **25**, 855–875.

Kim, S.H., Alam, M.J., Gu, M.J., Park, K.W., Jeon, C.O., Ha, J.K., Cho, K.K., and Lee, S.S. 2012. Effect of total mixed ration with fermented feed on ruminal *in vitro* fermentation, growth performance and blood characteristics of Hanwoo steers. *Asian-Aust. J. Anim. Sci.* **25**, 213–223.

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.

Lehloenya, K.V. 2007. Direct fed microbial supplementation to cattle: effects on milk yield and components, reproduction and digestion. ProQuest Information and Learning Company, Ann Arbor.

Lin, B., Lu, Y., Salem, A.Z.M., Wang, J.H., Liang, Q., and Liu, J.X. 2013. Effects of essential oil combinations on sheep ruminal fermentation and digestibility of a diet with fumarate included. *Anim. Feed Sci. Technol.* **184**, 24–32.

Liu, J.X., Susenbeth, A., and dekum, K.H.S. 2002. *In vitro* gas production measurements to evaluate interactions between untreated and chemically treated rice straws, grass hay, and mulberry leaves. *J. Anim. Sci.* **80**, 517–524.

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.

Mamuad, L.L., Kim, S., Lee, S., Cho, K., Jeon, C., and Lee, S.S. 2012. Characterization, metabolites and gas formation of fumarate reducing bacteria isolated from Korean native goat (*Capra hircus coreanae*). *J. Microbiol.* **50**, 925–931.

Mao, S.Y., Zhang, G., and Zhu, W.Y. 2008. Effect of disodium fumarate on ruminal metabolism and rumen bacterial communities as revealed by denaturing gradient gel electrophoresis analysis of 16S ribosomal DNA. *Anim. Feed Sci. Technol.* **140**, 293–306.

McDonald, P., Edwards, R.A., and Greenhalgh, J.F.D. 1995. Animal Nutrition 5th. Longman, London, UK.

McNamara, J.P., France, J., and Beever, D.E. 2000. Modelling nutrient utilization in farm animals. CAB International, New York, USA.

Miller, T.L., Wolin, M.J., Zhao, H.X., and Bryant, M.P. 1986. Characteristics of methanogens isolated from bovine rumen. *Appl. Environ. Microbiol.* **51**, 201–202.

Moe, P.W. and Tyrrell, H.F. 1979. Methane production in dairy cows. *J. Dairy Sci.* **62**, 1583–1586.

Murillo, M., Herrera, E., Reyes, O., Gurrola, J.N., and Gutierrez, E. 2011. Use *in vitro* gas production technique for assessment of nutritional quality of diets by range steers. *Afr. J. Agric. Res.* **6**, 2522–2526.

Muyzer, G. and Smalla, K. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek* **73**, 127–141.

Nübel, U., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A., Amann, R.I., Ludwig, W., and Backhaus, H. 1996. Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. *J. Bacteriol.* **178**, 5636–5643.

Nisbet, D.J. and Martin, S.A. 1993. Effects of fumarate,L-malate, and an *Aspergillus oryzae f*ermentation extract on D-lactate utilization by the ruminal bacterium *Selenomonas ruminantium*. *Curr. Microbiol.* **26**, 133–136.

Nyachoti, C.M., Omogbenigun, F.O., Rademacher, M., and Blank, G. 2006. Performance responses and indicators of gastrointestinal health in early-weaned pigs fed low-protein amino acid-supplemented diets. *J. Anim. Sci.* **84**, 125–134.

O'Herrin, S.M. and Kenealy, W.R. 1993. Glucose and carbon dioxide metabolism by *Succinivibrio dextrinosolvens*. *Appl. Environ. Microbiol.* **59**, 748–755.

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. Agric. Sci.* **92**, 499–503.

Robinson, J.A., Smolenski, W.J., Ogilvie, M.L., and Peters, J.P. 1989. *In vitro* total-gas, CH_4, H_2, volatile fatty acid, and lactate kinetics studies on luminal contents from the small intestine, cecum, and colon of the pig. *Appl. Environ. Microbiol.* **55**, 2460–2467.

Russell, J.B. and Rychlik, J.L. 2001. Factors that alter rumen microbial ecology. *Science* **292**, 1119–1122.

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

Scheifinger, C.C., Linehan, B., and Wolin, M.J. 1975. H_2 production by *Selenomonas ruminantium* in the absence and presence of methanogenic bacteria. *Appl. Microbiol.* **29**, 480–483.

Scheifinger, C.C. and Wolin, M.J. 1973. Propionate formation from cellulose and soluble sugars by combined cultures of *Bacteroides succinogenes* and *Selenomonas ruminantium*. *Appl. Microbiol.* **26**, 789–795.

Seo, J.K., Kim, S.W., Kim, M.H., Upadhaya, S.D., Kam, D.K., and Ha, J.K. 2010. Direct-fed microbials for ruminant animals. *Asian-Aust. J. Anim. Sci.* **23**, 1657–1667.

Simpson, J.M., McCracken, V.J., White, B.A., Gaskins, H.R., and Mackie, R.I. 1999. Application of denaturant gradient gel electrophoresis for the analysis of the porcine gastrointestinal microbiota. *J. Microbiol. Methods* **36**, 167–179.

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.

Ungerfeld, E.M., Kohn, R.A., Wallace, R.J., and Newbold, C.J. 2007. A meta-analysis of fumarate effects on methane production in ruminal batch cultures. *J. Anim. Sci.* **85**, 2556–2563.

van Beers-Schreurs, H.M.G., Nabuurs, M.J.A., Vellenga, L., Valk, H.J.K.-v.d., Wensing, T., and Breukink, H.J. 1998. Weaning and the weanling diet influence the villous height and crypt depth in the small intestine of pigs and alter the concentrations of short-chain fatty acids in the large intestine and blood. *J. Nutr.* **128**, 947–953.

Whitford, M., Teather, R., and Forster, R. 2001. Phylogenetic analysis of methanogens from the bovine rumen. *BMC Microbiol.* **1**, 5.

Zhou, Y.W., McSweeney, C.S., Wang, J.K., and Liu, J.X. 2011. Effects of disodium fumarate on ruminal fermentation and microbial communities in sheep fed on high-forage diets. *Animal* **6**, 1–9.