Italian Journal of Animal Science

ISSN: (Print) 1828-051X (Online) Journal homepage: https://www.tandfonline.com/loi/tjas20

Effect of different concentrate diet levels on rumen fluid inoculum used for determination of *in vitro* rumen fermentation, methane concentration, and methanogen abundance and diversity

Seon-Ho Kim, Lovelia L. Mamuad, Eun-Joong Kim, Ha-Guyn Sung, Gui-Seck Bae, Kwang-Keun Cho, Chanhee Lee & Sang-Suk Lee

To cite this article: Seon-Ho Kim, Lovelia L. Mamuad, Eun-Joong Kim, Ha-Guyn Sung, Gui-Seck Bae, Kwang-Keun Cho, Chanhee Lee & Sang-Suk Lee (2018) Effect of different concentrate diet levels on rumen fluid inoculum used for determination of *in vitro* rumen fermentation, methane concentration, and methanogen abundance and diversity, Italian Journal of Animal Science, 17:2, 359-367, DOI: 10.1080/1828051X.2017.1394170

To link to this article: https://doi.org/10.1080/1828051X.2017.1394170

Published online: 25 Oct 2017.

ITALIAN JOURNAL OF ANIMAL SCIENCE, 2018
VOL. 17, NO. 2, 359–367
https://doi.org/10.1080/1828051X.2017.1394170

Taylor & Francis
Taylor & Francis Group

PAPER

Effect of different concentrate diet levels on rumen fluid inoculum used for determination of *in vitro* rumen fermentation, methane concentration, and methanogen abundance and diversity

Seon-Ho Kim[a,b], Lovelia L. Mamuad[a,c], Eun-Joong Kim[d], Ha-Guyn Sung[e], Gui-Seck Bae[f], Kwang-Keun Cho[g], Chanhee Lee[b] and Sang-Suk Lee[a]

[a]Department of Animal Science and Technology, Sunchon National University, Suncheon, South Korea; [b]Department of Animal Sciences Ohio Agricultural Research and Development Center, The Ohio State University, Wooster, OH, USA; [c]Department of Animal Sciences, The Ohio State University, Columbus, OH, USA; [d]Department of Animal Science, Kyungpook National University, Sangju, South Korea; [e]Department of Animal Science and Technology, Sangji University, Gangwon-do, South Korea; [f]Department of Animal Science and Technology, Chung-Ang University, Ansung, South Korea; [g]Gyeongnam National University of Science and Technology, Jinju, South Korea

ABSTRACT

This study investigated the effects of different diet concentrate levels in animal diets as a source of rumen fluid inoculum for *in vitro* ruminal fermentation, CH_4 concentration, and methanogen abundance and diversity. Three non-lactating mature cows assigned to a 3×3 Latin square design were fed with Italian rye grass and concentrate feed at 2 (low), 5 (medium), and 8 (high) kg per animal per day were used as rumen fluid donors. The experiment consisted of three 21-d periods that each consisted of 14 d adaptation followed by 7 d of continuous feeding prior to rumen fluid collection for *in vitro* fermentation evaluation. High concentrate proportion produced the highest total gas in all incubation periods ($p < .05$) while methane (CH_4) concentration was highest in low concentrate proportions and lowest in medium concentrate proportions. Propionate concentration was highest in high concentrate proportions, whereas butyrate concentration was highest in medium concentrate proportions. High concentrate proportion at 0 and 12 h and medium concentrate proportions at 12 h produced archaeal denaturing gradient gel electrophoresis (DGGE) profiles that differed from those of low concentrate proportions. Medium concentrate proportion had the lowest methanogen DNA copies at 12 h of incubation ($p < .05$). Changes in diet influenced the rumen microbiome, CH_4 concentration, and methanogen diversity and abundance in cattle. The rumen conditions of an animal, as along with the microbiome, change as the feed diet changes. As a result, the inoculum for *in vitro* rumen fermentation studies affects Volatile fatty acid (VFA) concentrations, CH_4 production, and methanogen diversity and abundance.

ARTICLE HISTORY
Received 22 February 2017
Revised 16 June 2017
Accepted 25 August 2017

KEYWORDS
Feeding diet ratio; *in vitro* fermentation; methane; methanogen diversity

Introduction

Carbohydrates are an important source of methane (CH_4), and their origin (cellulose, starch, or soluble sugars) influences CH_4 production (Bonhomme 1990). CH_4 production in response to different feed ingredients varies with nutrient composition, grain type and processing, and digestibility (Kim et al. 2013). Moreover, high concentrate diets resulted in lower CH_4 emissions than medium concentrate diets (Wallace et al. 2014), whereas feeding low forage to concentrate ratio diets to finishing beef cattle effectively reduced CH_4 output per unit of product and improved animal productivity (Lovett et al. 2003). CH_4 production in ruminants decreases with increasing forage food quality (Tamminga et al. 2007). Furthermore, lower forage digestibility is generally accompanied by reduced forage intake and an increased ruminal acetate:propionate ratio, which favours the production of CH_4 production per unit of forage consumed (McAllister et al. 1996).

Feeds and feeding diet ratios affect the rumen microflora of animals. Diet had the greatest influence on CH_4 emissions (Wallace et al. 2014), thereby affecting the microbial community, diversity, abundance,

CONTACT Prof. Sang-Suk Lee rumen@sunchon.ac.kr Ruminant Nutrition and Anaerobe Laboratory, Department of Animal Science and Technology, College of Bio-industry Science, Sunchon National University, 413 Jungangno, Suncheon, Jeonnam 57922, South Korea

and methanogen population. Volatile fatty acids (VFAs) and CH_4 production vary with varying ratios of roughage and concentrate fed to the animals. The microbiome composition could be altered by changing the total mixed ration (TMR) forage:concentrate ratio (McCann et al. 2014). Increasing the concentrate ratio increased the Firmicutes, but decreased the Bacteroidetes compositions. Consequently, diet affects the microbiomes, and VFA and CH_4 production in the rumen.

Different feeding diet ratios affect the rumen microbiome, thereby influencing rumen fluids used during *in vitro* fermentation studies. Accordingly, limited information is available regarding the effect of different concentrate ratios. Thus, the present study evaluated the effects of different concentrate levels in animal diets as a source of rumen fluid inoculum for *in vitro* ruminal fermentation, CH_4 concentration, and methanogen abundance and diversity.

Materials and methods

Source of rumen fluid from animals and experimental design

Three trials were conducted to evaluate the effect of different diet concentrate levels using three Holstein–Friesian cows (600 ± 47 kg). The animals were assigned to a 3 × 3 Latin square design and fed Italian ryegrass *ad libitum* and commercial concentrates at 2 (low), 5 (medium), and 8 (high) kg twice daily (8:30 a.m. and 4:00 p.m.) on a DM basis. The chemical composition of Italian ryegrass and concentrate feed provided to the animals is shown in Table 1. All animals were given free access to fresh drinking water and trace mineral salts (NongHyup, Inc. Ansung, South Korea) throughout the experiment. The experiment consisted of three 21-d periods and at the end of the feeding period ruminal fluid was collected 2 h after feeding. Ruminal contents were collected from the animals through stomach tubing for the evaluation of *in vitro* fermentation profiles. Samples were mixed, squeezed, and strained through a 4-layered cheesecloth. The ruminal fluids samples were immediately placed in amber bottles, sealed, maintained at 39 °C, and immediately transported to the laboratory.

Table 1. Feed ingredients and chemical composition of the experimental diet.

Ingredients, % of DM	Concentrate feed	Italian Rye grass
Corn grain	27.47	–
Wheat grain	17.00	–
Cane molasses	5.00	–
Tapioca	6.00	–
Wheat flour	3.00	–
Corn gluten feed	20.00	–
Rapeseed meal	4.00	–
Palm kernel meal	8.82	–
Cottonseed hull	1.00	–
Tallow	0.62	–
Salt dehydrated	0.50	–
Limestone (1 mm)	1.96	–
Vitamin premix[a]	0.10	–
Mineral premix[b]	0.10	–
Chemical composition, %		
Dry matter	88.68	88.74
Crude protein	12.90	5.91
Ether extract	3.76	2.16
Crude fibre	6.10	36.07
Ca	0.91	0.17
P	0.41	0.12
Crude ash	6.62	6.25
NDF	35.50	36.20
ADF	22.50	15.80
TDN	73.00	46.18

[a]Vitamin premix contained the following ingredients diluted in cellulose (g/kg premix): L-ascorbic acid, 121.2; DL-α-tocopherol acetate, 18.8; thiamine hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin, 36.4; Ca-D-pantothenate, 12.7; myo-inositol, 181.8; D-biotin, 0.27; folic acid, 0.68; p-aminobenzoic acid, 18.2; menadione, 1.8; retinal acetate, 0.73; cholecalciferol, 0.003; cyanocobalamin, 0.003.
[b]Mineral premix contained the following ingredients (g/kg premix): Mg $SO_4 \cdot 7H_2O$, 80.0; NaH_2PO_4 $2H_2O$, 370.0; KCL, 130.0; ferric citrate, 40.0; $ZnSO_4 \cdot 7H_2O$, 20.0; Ca-lactate, 356.5; CuCl, 0.2; $AlCl_3 \cdot 6H_2O$. 0.15; KI, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4 \cdot H_2O$, 2.0; $CoCl_2 \cdot 6H_2O$, 1.0.
*Concentrate feed was provided by NongHyup Company.
DM: Dry matter; NDF: Neutral detergent fibre; ADF: Acid detergent fibre; TDN: Total digestible nutrients.

In vitro *fermentation*

A prepared buffer was composed of 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 per litre with a final pH of 6.9 as described by Asanuma et al. (1999). Buffer and rumen fluid were mixed at a 3:1 ratio and then bubbled with O_2-free N_2. Next, 100 mL buffered rumen fluid was anaerobically transferred to 160 mL serum bottles containing Italian rye grass as a substrate at 1.0% (w/v) on a DM basis and sealed with butyl-rubber stoppers and aluminium caps. Each sample was replicated three times and maintained in a shaking incubator (80 rpm) at 39 °C (Hanbaek Scientific Co., Siheung, South Korea). During incubation, *in vitro* fermentation parameters were monitored at 3, 6, 12, and 24 h. Changes in diversity were analysed using bacterial and archaeal 16S rDNA denaturing gradient gel electrophoresis (DGGE), whereas methanogen abundance 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., Costa

Mesa, CA, USA). pH was measured with a Pinnacle series M530p metre (Schott Instruments, Mainz, 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 millilitre of the TG produced during the *in vitro* fermentation process was used to determine the CH_4 and carbon dioxide (CO_2) emitted in the incubation period. Gas chromatography (Agilent Technologies HP 5890, CA, USA) was conducted using a TCD detector with a Column Carboxen 1006PLOT 30 m × 0.53 mm capillary column (Supelco, Sigma-Aldrich, USA).

Analyses of VFAs and other metabolites were conducted using high performance liquid chromatography (HPLC; Agilent Technologies 1200 series, CL, USA) with a UV detector set at 210 and 220 nm. A MetaCarb 87H (Varian, Germany) column was used in the determination of fermentation products, and a 0.0085 N H_2SO_4 buffer applied to the column at a rate of 0.6 mL/min as described by Han et al. (2005).

Evaluation of methanogen diversity and abundance using denaturing gradient gel electrophoresis and quantitative real time polymerase chain reaction

Genomic DNA (gDNA) from fermented samples (preserved) taken at 0, 12, and 24 h were extracted using Wizard Genomic DNA Purification Kits (Promega, WI, USA). Methanogen gDNA amplification was conducted using Met 86F (5′-GCT CAG TAA CAC GTG G-3′) and Met 1340R (5′-CGG TGT GTG CAA GGA G-3′) (Wright and Pimm 2003). The ARC344f (5′-ACG GGG YGC AGC AGG CGC GA-3′) with 40-bp GC clamp (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3′) and 519R primers (5′-GWA TTA CCG CGG CKG CTG-3′) (Nübel et al. 1996) were used to amplify the V3 region of the 16S rDNA amplicons (Bano et al. 2004). DGGE was performed using a D-Code Universal Mutation Detection System (BioRad, Hercules, CA, 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 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 densitometric curves of the scanned DGGE profiles using Molecular Analyst 1.12 software (Bio-Rad, CL, USA) and the Pearson product-moment correlation coefficient (Häne et al. 1993) 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-archaea primers. The PCR product was purified with a QiaQuick PCR purification kit following the manufacturer's instructions, after which PCR products were sent to Macrogen (Seoul, Korea) for sequencing. The results were compared with those in the GenBank database using the BLAST tool of the National Center for Biotechnology Information (NCBI) and EzTaxon.

For qRT-PCR, external standards were prepared using a mixture of pure cultures of *Methanobrevibacter ruminantium*, *M. smithii, M. millerae,* 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 by using the protocol developed by Christophersen (2007). Amplification was performed using an Opticon Monitor 3.1.32 (MJ Geneworks, Inc., MA, USA; BioRad Laboratories, Inc. CL, USA) with a volume of 25 μL containing the following reagents: 12.5 μL 2 × SensiMix Plus SYBR & Fluorescein (Bioline, London, UK), 1.0 μL (each) PCR primers, 1.0 μL 50 mM $MgCl_2$ (Quantance), 4.5 μL dH_2O, and 5.0 μL template DNA. Real-time amplification was carried out at 95 °C for 15 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s. Melting-curve analysis occurred 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.

Statistical analysis

Means of triplicate samples from treatments and the control were analysed by analysis of variance (ANOVA) using a general linear model (GLM) for complete randomised design. Duncan's multiple range test (DMRT) was used to compare specific treatment differences. To describe the functional relationships among treatment levels, the linear and quadratic effects of feed concentrate among treatments were analysed. In addition, the relationship between total VFA and CH_4 concentration was determined using the Pearson product-moment correlation coefficient. All tests of significance were compared at the 5% probability level and all analyses were carried out using Statistical Analysis Systems (SAS Institute Inc., NC, USA) version 9.1 (2002).

Results

The animals consumed all the commercial concentrates at 2 (low), 5 (medium), and 8 (high) kg given

during the periods. At the start of *in vitro* fermentation, pH was lower from low to medium concentrate and then high concentrate proportion, with values of 6.36, 6.30, and 6.28, respectively. pH was significantly higher when rumen fluid inocula were taken from low and medium concentrate than that of high concentrate proportion at 3 h and was the lowest in medium proportion of concentrate after 6 h ($p < .006$) and 12 h ($p < .039$) of incubation. pH was linearly significant after 3 h ($p < .010$) and quadratically significant after 6 ($p < .004$) and 12 h ($p < .021$) of incubation. Total gas was significant linearly and quadratically produced at all incubation times except for 24 h, when it was not significant linearly ($p < .131$). High proportion of concentrate produced the highest ($p < .05$) total gas in all periods of incubation, with 28.87 mL being the highest total gas produced after 24 h of incubation (Table 2). Overall, CH_4 concentration was not significant linearly, but was quadratically significant at 3 ($p < .004$) and 6 h ($p < .009$) of incubation. CH_4 concentration was the highest ($p < .05$) in low concentrate and lowest ($p < .05$) in medium concentrate proportion at 3, 6, and 12 h of incubation with 0.201, 0.212, and 0.296 mM/mL, respectively. High concentrate had the lowest ($p < .05$) CO_2 production of 0.192 and 0.217 mM/mL at 12 and 24 h of incubation, respectively.

Table 3 shows the VFA concentration of *in vitro* fermentation using rumen fluid inocula from cattle fed low, medium, and high proportions of concentrate. Acetate concentrations increased linearly as concentrate given to the animals increased at all incubation times. High proportion of concentrate had the highest ($p < .05$) acetate concentrations of 39.29, 45.48, 52.05, and 57.71 mM/mL after 3, 6, 12, and 24 h of incubation, respectively. It was notable that butyrate was linearly (3, 6, 12, and 24 h) and quadratically (6, 12, and 24 h) significant ($p < .05$), with the highest level ($p < .001$) observed in medium concentrate proportion after 24 h of incubation with 15.77 mM/mL. Propionate concentrations were highest in medium concentrate at 3 and 6 h of incubation, and became highest in high followed by medium, and then low concentrate proportion after 12 and 24 h of incubation. Total VFA concentrations were highest ($p < .05$) in high concentrate proportion after 6, 12, and 24 h of incubation, with values of 65.88, 76.36, and 86.07 mM/mL, respectively. Low and high concentrate proportions had comparable acetate to propionate ratio after 24 h of incubation and both significantly higher than medium concentrate proportion. However, higher acetate to propionate ratios were observed in 3, 6, and 12 h of incubation. Propionate and total VFA were linearly significant ($p < .05$) at all incubation times and quadratically significant ($p < .01$) at 3 and 6 h. As shown in Figure 1, higher correlation coefficients between VFA and CH_4 concentrations were observed in high ($R^2 = 0.6143$) than medium ($R^2 = 0.3157$) and low ($R^2 = 0.363$) concentrate proportions.

The similarity index of amplified methanogen DGGE bands obtained using total genomic DNA extracted

Table 2. Gas, pH, methane, and carbon dioxide of *in vitro* fermentation from cattle fed different feed concentrates.

	Treatment				*p* Value		
Parameters/incubation time	Low	Medium	High	SEM	Treatment	Linear	Quadratic
pH							
3	6.14[a]	6.07[a]	5.82[b]	0.053	.022	.010	.255
6	5.76[a]	5.44[b]	5.62[a]	0.038	.006	.074	.004
12	5.34[a]	5.17[b]	5.27[ab]	0.030	.039	.194	.021
24	5.15	5.17	5.25	0.035	.270	.141	.545
Total gas production, mL							
3	11.20[b]	11.30[b]	14.67[a]	0.267	<.001	<.001	.003
6	14.97[b]	15.53[b]	20.27[a]	0.562	.002	.001	.040
12	19.87[b]	19.60[b]	25.47[a]	0.987	.017	.013	.068
24	25.33[ab]	22.00[b]	28.87[a]	1.167	.040	.131	.027
Methane, mM/mL							
3	0.317[a]	0.201[b]	0.276[a]	0.015	.009	.160	.004
6	0.376[a]	0.212[b]	0.326[a]	0.028	.020	.290	.009
12	0.418[a]	0.296[b]	0.331[ab]	0.028	.058	.084	.062
24	0.445	0.542	0.471	0.053	.765	.518	.787
Carbon dioxide, mM/mL							
3	0.319[a]	0.209[b]	0.205[b]	0.383	.004	.003	.038
6	0.401[a]	0.212[b]	0.210[b]	0.341	.013	.009	.074
12	0.382[a]	0.246[b]	0.192[c]	0.271	<.001	<.001	.042
24	0.383[a]	0.341[ab]	0.271[b]	0.027	.068	.027	.607

*Values are the means of triplicates ± standard error; means with different superscripts ([a, b, c]) among treatments are significantly different (a > b > c > d, $p < .05$).
Commercial concentrates were given low (2 kg), medium (5 kg), and high (8 kg).

from *in vitro* fermentation of rumen fluid inocula from cattle fed low, medium, and high concentrate proportions is shown in Figure 2. The treatments were grouped into three clades that represented 0 h of low, medium, and high concentrate proportions. In addition, high proportion of concentrate at 0 and 12 h and medium concentrate proportion at 12 h produced archaeal DGGE profiles that differed from that

Table 3. Volatile fatty acid concentration (mM) of *in vitro* fermentation from cattle fed different feed concentrates.

	Treatment				*p* Value		
Parameters/incubation time (h)	Low	Medium	High	SEM	Treatment	Linear	Quadratic
Acetate							
3	32.02^{c}	36.39^{b}	39.29^{a}	0.231	<.001	<.001	.0431
6	36.76^{c}	41.06^{b}	45.48^{a}	0.330	<.001	<.001	.8937
12	42.22^{c}	45.75^{b}	52.05^{a}	0.436	<.001	<.001	.0691
24	47.72^{b}	46.22^{b}	57.71^{a}	0.785	.001	.001	.003
Propionate							
3	8.73^{c}	11.11^{a}	9.71^{b}	0.194	.001	.022	.001
6	9.82^{b}	11.17^{a}	10.98^{a}	0.142	.001	.001	.005
12	11.11^{c}	12.27^{b}	12.92^{a}	0.171	.001	.001	.308
24	12.43^{b}	12.91^{b}	14.78^{a}	0.376	.024	.011	.254
Butyrate							
3	4.85^{b}	6.96^{a}	6.94^{a}	0.440	.047	.031	.149
6	5.54^{b}	9.56^{a}	9.42^{a}	0.400	.002	.002	.015
12	7.77^{b}	12.08^{a}	11.40^{a}	0.348	.003	.003	.009
24	11.19^{c}	15.77^{a}	13.57^{b}	0.158	<.001	<.001	<.001
Total VFA							
3	45.59^{b}	54.46^{a}	55.94^{a}	0.738	<.001	<.001	.014
6	52.11^{c}	61.80^{b}	65.88^{a}	0.680	<.001	<.001	.017
12	61.11^{c}	70.10^{b}	76.36^{a}	0.806	<.001	<.001	.287
24	71.34^{b}	74.90^{b}	86.07^{a}	1.104	.001	<.001	.076
A:P Ratio							
3	3.67^{b}	3.28^{c}	4.05^{a}	0.045	<.001	.002	<.001
6	3.74^{b}	3.67^{b}	4.14^{a}	0.035	<.001	.001	.003
12	3.80^{b}	3.73^{b}	4.03^{a}	0.025	.001	.002	.002
24	3.84^{a}	3.58^{b}	3.90^{a}	0.047	.011	.442	.004

*Values are the means of triplicates ± standard error; means with different superscripts (^{a,b,c}) among treatments are significantly different (a > b > c > d, $p < .05$).
Commercial concentrates were given low (2 kg), medium (5 kg), and high (8 kg).
A:P ratio: acetate to propionate ratio; VFA: volatile fatty acid.

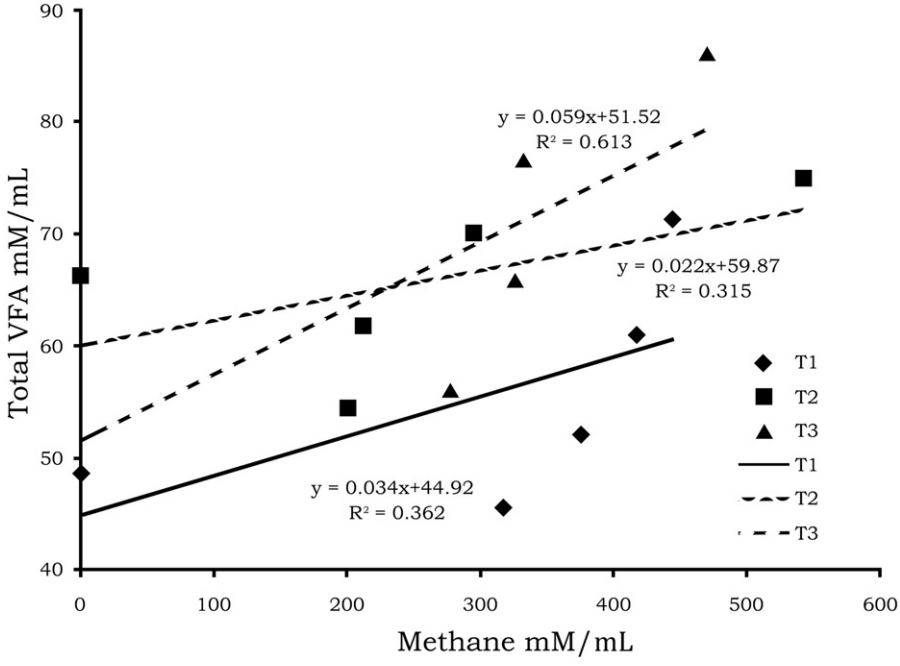

Figure 1. Correlation coefficients of methane and total volatile fatty acid (VFA) concentrations of *in vitro* fermentation using rumen fluid inocula from cattle fed with low (T1), medium (T2), and high (T3) feed concentrate. Total VFA is the sum of acetate, propionate, and butyrate. The methane and total VFA concentration are indicated in millimoles per millilitres.

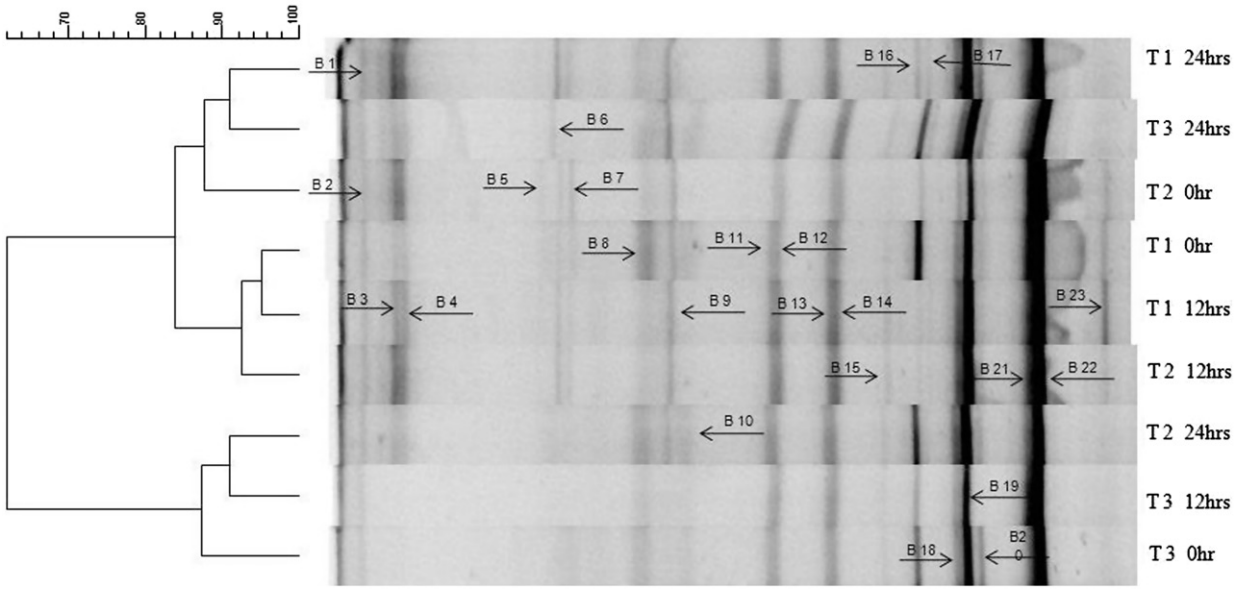

Figure 2. Similarity analysis and negative image of amplified methanogen 16S rDNA denaturing gradient gel electrophoresis band profiles using the total genomic DNA extracted from *in vitro* fermentation using rumen fluid inocula from cattle fed with low (T1), medium (T2), and high (T3) feed concentrate incubated at 0, 12, and 24 h. Arrows indicate the identified bands.

Table 4. Identified dominant bands from methanogen amplified denaturing gradient gel electrophoresis.

PCR-DGGE bands	Most related taxon (GenBank accession no.)	Similarity (%)
1,2,8,9,11,12,18,19,20	*Methanobrevibacter smithii* ATCC 35061T (CP000678)	99.33
3,16,17	*Methanobrevibacter olleyae* KM1H5-1PT (AY615201)	98.67
4,6	*Methanobrevibacter ruminantium* M1T (CP001719)	98.67
5,7,13,14,15,21,22,23	*Methanobrevibacter millerae* ZA-10T (AY196673)	99.33
10	*Methanosarcina vacuolata* DSM 1232T (FR733661)	96.67

PCR–DGGE: Polymerase chain reaction– denaturing gradient gel electrophoresis.

of low concentrate proportion. A total of 23 distinct bands were detected from the PCR–DGGE profiles of groups low, medium, and high concentrate proportions after 0, 24, and 48 h of incubation (Table 4). The 23 gel cut bands identified had 90–100% similarity with sequences available in the GenBank database of validly described methanogens including *M. smithii* ATCC35061 (CP000678) (nine bands), *M. millerae* ZA-10 (AY196673) (eight bands), *M. olleyae* KM1H5-1PT (AY615201) (three bands), *M. ruminantium* M1T (CP001719) (two bands), and *Methanosarcina vacuolata* DSM 1232T (FR733661) (one band). *Ms. vacuolata* was present in all incubation time of low concentrate proportion while *M. olleyae* and *M. millerae* in medium and high proportions of concentrate, respectively. *M. smithii, M. olleyae,* and *M. ruminantium* were not present in low and high concentrate proportions while *Ms. vacuolata* was not present in medium and high concentrate proportions after 24 h of incubation. In the present study, variations in the number of methanogens were observed. Specifically, medium concentrate proportion, which also showed the lowest pH and CH_4 concentration at 12 h, had the lowest number of methanogen DNA copies ($p < .05$) (Figure 3).

Discussion

In vitro rumen fermentation can be used to evaluate CH_4 production of different substrates, supplements, probiotics, etc. However, the results differ among regions because of the variation in the feed provided to the animals as their source of rumen fluid inoculum for *in vitro* fermentation studies. For example, Mamuad et al. (2014) and Lamba et al. (2014) used the same substrate (rice straw), but the CH_4 production differed. These findings imply that the source of rumen fluid inoculum for *in vitro* fermentation differs depending on the diet provided to the animals. Feed intake, digestibility, species, physiological state, concentrates, and roughage ratio all influence CH_4 production in the rumen (Moe and Tyrrell 1979).

During rumen fermentation, pH is considered a major factor affecting fermentation, rumen microbiome, CH_4 production, and VFA concentration.

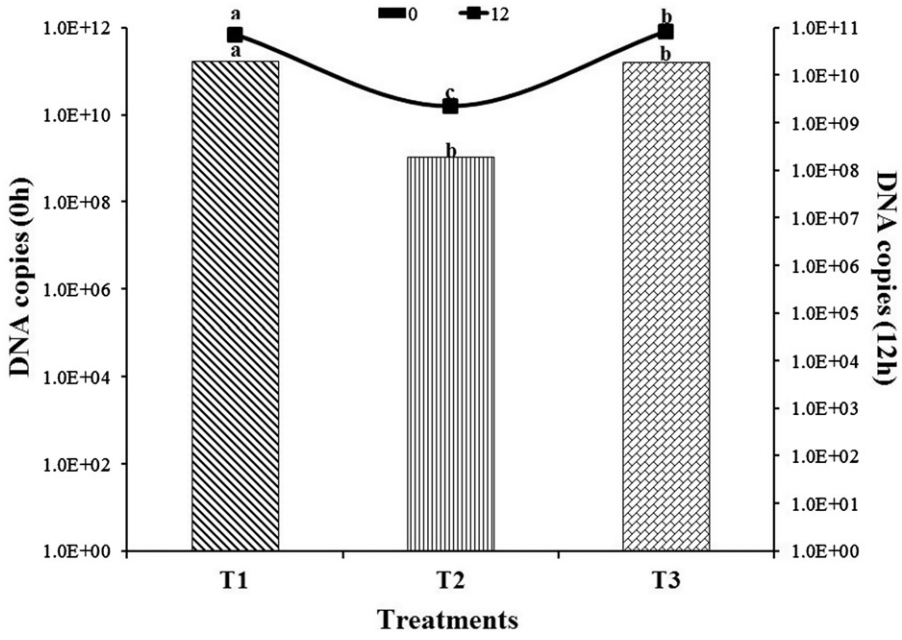

Figure 3. Methanogen DNA copies of *in vitro* fermentation using the total genomic DNA extracted from *in vitro* fermentation using rumen fluid inocula from cattle fed with low (T1), medium (T2), and high (T3) feed concentrate incubated at 0 and 12 h. Values are means of triplicate analyses. Means with the same letter are not significantly different.

Ruminant animals depend on cellulolytic ruminal bacteria to digest cellulose; hence, the higher pH observed in low concentrate proportion might be due to the low concentrate diet provided to the cattle. Specifically, this possibly led to increased forage consumption, resulting in slower digestibility and increasing rumination. Russell and Wilson (1996) explained that the major consequence of ruminal pH <6 is that fibre digestion declines dramatically. This can occur for two reasons; the enzymes necessary for fibre breakdown do not function effectively at pH <6.0, and the growth rate of fibrolytic activity declines markedly at low pH.

Concentrate digestibility is faster than forage digestibility, which explains the higher total gas production observed in high proportion of concentrate. The linear significance of total gas production indicates a directly proportional relationship between total gas production and the amount of concentrate provided. Concentrate feed has lower cell wall components than forage; therefore, increasing concentrate diets has been proposed for CH_4 mitigation. However, commercially produced concentrates vary in nutrient compositions and therefore differ in CH_4 production (Kim et al. 2013). One of the feed ingredients that decreases CH_4 in the rumen is cereal grain (Blaxter 1962) through propionate production of starch-fermenting bacteria. Also, CH_4 production in the rumen is directly associated with H_2 formation (Janssen 2010); therefore, CH_4 production can be reduced by depriving methanogens of H_2. The high dietary content of fibre increases the production of acetate (an H_2-liberating reaction), whereas diets with higher starch content favour propionate formation (Valadares et al. 1999) and therefore decrease H_2 formation. In addition, specific activities of methanogenesis were positively correlated with acetate:propionate ratio (Lana et al. 1998). Thus, the lower CH_4 production observed in medium proportion of concentrate, which correlates the lower acetate:propionate ratio as well as high propionate concentration in the same treatment. However, the CH_4 production differed as the incubation time became longer. This might be due to the substrate, rice straw, used during *in vitro* fermentation.

It should be noted that in previous studies, the ruminal fluid used as fermentation inocula was collected from one animal, whereas in the present study rumen fluid from animals fed with different levels of concentrate corresponding to different experimental treatments was used. Linearly increased of butyrate concentrations indicates that acetate was a precursor of butyrate (Gray and Pilgrim 1952), which was validated by Sheppard et al. (1959). The high VFA production in high concentrate and low VFA production in low proportion of concentrate in this study also corroborate Lana et al. (1998) observation when they fed steers increasing amounts of concentrates, the VFA concentration increased linearly. These findings provide evidence that the diet changed *in vitro* fermentation VFA production significantly. Tamminga et al.

(2007) reported that the presence of non-structural carbohydrates (starch and sugars) such as concentrates normally fermented faster than forage, resulting in elevated propionate levels. These findings explain the higher VFA production, especially propionate, observed in high proportion of concentrate. However, ruminal pH is negatively correlated with the VFA concentration. Hence, if VFA production is greater than absorption, the pH in the rumen will drop, leading to disruption of the rumen microbiota (Plaizier et al. 2008). Meanwhile, high acetate to propionate ratios observed in high concentrate were due to high concentration of acetate on the same treatment. The higher and comparable acetate to propionate ratios of low and high concentrate than control in this study were similar with the results reported by Chen et al. (2015) in the rumen fermentation of Yaks. The difference in the VFA concentrations at other incubation time might be due to rumen microbiota present of the animal and the forage used.

Analysis of the methanogenic community structure revealed that rumen fluids (0 and 12 h) from different diets had different methanogen diversity. The dominant methanogens in the rumen fluid were related to the genus *Methanobrevibacter*, which concurred with the results of other studies (Hook et al. 2009; Zhou et al. 2009). The population of pH sensitive methanogens could have decreased from the fermenta as pH was reduced in medium proportion of concentrate. In addition, Hook et al. (2010) showed that high concentrate feed did not significantly affect the methanogen density of the rumen, but significantly affected methanogen diversity. Another factor that affects survival of methanogens is their close association with protozoa, as they are able to live extra- and intracellularly in these eukaryotic organisms, thereby receiving some protection from lower pH (Krumholz et al. 1983).

Conclusions

The rumen conditions of an animal, as along with the microbiome, change as the feed diet changes. Medium concentrate proportion had the lowest methanogen DNA copies at 12 h of incubation. Changes in diet influenced the rumen microbiome, CH_4 concentration, and methanogen diversity and abundance in cattle. As a result, the inoculum for *in vitro* rumen fermentation studies affects VFA concentrations, CH_4 production, and methanogen diversity and abundance. Future work on the analysis of microbial communities using pyrosequencing is necessary to further understand the effect of diet on rumen fluid used for *in vitro* studies.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This research was supported by the Cooperative Research Program for Agriculture Science and Technology Development, (Project No. PJ012697), Rural Development Administration, Republic of Korea.

ORCID

Lovelia L. Mamuad http://orcid.org/0000-0002-1866-0897
Sang-Suk Lee http://orcid.org/0000-0001-8804-3416

References

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

Bano N, Ruffin S, Ransom B, Hollibaugh JT. 2004. Phylogenetic composition of arctic ocean archaeal assemblages and comparison with antarctic assemblages. Appl Environ Microbiol. 70:781–789.

Blaxter KL. 1962. The energy metabolism of ruminants. Thomas: The University of Michigan.

Bonhomme A. 1990. Rumen ciliates: their metabolism and relationships with bacteria and their hosts. Anim Feed Sci Tech. 30:203–266.

Chaney AL, Marbach EP. 1962. Modified reagents for determination of urea and ammonia. Clin Chem. 8:130–132.

Chen GJ, Song SD, Wang BX, Zhang ZF, Peng ZL, Guo CH, Zhong JC, Wang Y. 2015. Effects of forage:concentrate ratio on growth performance, ruminal fermentation and blood metabolites in housing-feeding yaks. Asian-Australas J Anim Sci. 28:1736–1741.

Christophersen CT. 2007. Grain and artificial stimulation of the rumen change the abundance and diversity of methanogens and their association with ciliates [dissertation]. Perth: School of Animal Biology, University of Western Australia.

Gray FV, Pilgrim AF. 1952. Origins of the volatile fatty acids in the rumen. Nature. 170:375–376.

Han SK, Kim SH, Shin HS. 2005. UASB treatment of wastewater with VFA and alcohol generated during hydrogen fermentation of food waste. Proc Biochem. 40:2897–2905.

Häne BG, Jager K, Drexler HG. 1993. The pearson product-moment correlation coefficient is better suited for identification of DNA fingerprint profiles than band matching algorithms. Electrophoresis. 14:967–972.

Hook SE, Northwood KS, Wright ADG, McBride BW. 2009. Long-term monensin supplementation does not significantly affect the quantity or diversity of methanogens in the rumen of the lactating dairy cow. Appl Environ Microbiol. 75:374–380.

Hook SE, Wright AD, McBride BW. 2010. Methanogens: methane producers of the rumen and mitigation strategies. Archaea. 2010:11.

Janssen PH. 2010. Influence of hydrogen on rumen methane formation and fermentation balances through microbial

growth kinetics and fermentation thermodynamics. Anim Feed Sci Technol. 160:1–22.

Kim SH, Mamuad LL, Jeong CD, Choi YJ, Lee SS, Ko JY, Lee SS. 2013. *In vitro* evaluation of different feeds for their potential to generate methane and change methanogen diversity. Asian-Aust J Anim Sci. 26: 1698–1707.

Krumholz LR, Forsberg CW, Veira DM. 1983. Association of methanogenic bacteria with rumen protozoa. Can J Microbiol. 29:676–680.

Lamba JS, Wadhwa M, Bakshi MPS. 2014. In vitro methane production and in sacco degradability of processed wheat and rice straws. Indian J Anim Nutr. 31:345–350.

Lana RP, Russell JB, Van Amburgh ME. 1998. The role of pH in regulating ruminal methane and ammonia production. J Anim Sci. 76:2190–2196.

Lovett D, Lovell S, Stack L, Callan J, Finlay M, Conolly J, O'Mara FP. 2003. Effect of forage/concentrate ratio and dietary coconut oil level on methane output and performance of finishing beef heifers. Livest Prod Sci. 84:135–146.

Mamuad L, Kim SH, Jeong CD, Choi YJ, Jeon CO, Lee SS. 2014. Effect of fumarate reducing bacteria on *in vitro* rumen fermentation, methane mitigation and microbial diversity. J Microbiol. 52:120–128.

McAllister TA, Cheng KJ, Okine EK, Mathison GW. 1996. Dietary, environmental and microbiological aspects of methane production in ruminants. Can J Anim Sci. 76:231–243.

McCann JC, Wickersham TA, Loor JJ. 2014. High-throughput methods redefine the rumen microbiome and its relationship with nutrition and metabolism. Bioinform Biol Insights. 8:109–125.

Moe PW, Tyrrell HF. 1979. Methane production in dairy cows. J Dairy Sci. 62:1583–1586.

Muyzer G, Smalla K. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. A Van Leeuw. 73:127–141.

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

Plaizier JC, Krause DO, Gozho GN, McBride BW. 2008. Subacute ruminal acidosis in dairy cows: The physiological causes, incidence and consequences. Vet J. 176:21–31.

Russell JB, Wilson DB. 1996. Why are ruminal cellulolytic bacteria unable to digest cellulose at low pH? J Dairy Sci. 79:1503–1509.

Sheppard AJ, Forbes RM, Johnson BC. 1959. Rate of acetic acid production in the rumen determined by isotope dilution. Proc Soc Expl Biol Med. 101:715–717.

Tamminga S, Bannick A, Dijkstra J, Zom R. 2007. Feeding strategies to reduce methane loss in cattle. Report 34, Animal Science Group. Available from: http://edepot.wur.nl/28209.

Valadares RFD, Broderick GA, Filho SCV, Clayton MK. 1999. Effect of replacing alfalfa silage with high moisture corn on ruminal protein synthesis estimated from excretion of total purine derivatives. J Dairy Sci. 82:2686–2696.

Wallace RJ, Rooke JA, Duthie CA, Hyslop JJ, Ross DW, McKain N, de Souza SM, Snelling TJ, Waterhouse A, Roehe R. 2014. Archaeal abundance in post-mortem ruminal digesta may help predict methane emissions from beef cattle. Sci Rep. 4:5892.

Wright ADG, Pimm C. 2003. Improved strategy for presumptive identification of methanogens using 16S riboprinting. J Microbiol Methods. 55:337–349.

Zhou M, Hernandez-Sanabria E, Guan LL. 2009. Assessment of the microbial ecology of ruminal methanogens in cattle with different feed efficiencies. Appl Environ Microbiol. 75:6524–6533.