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Research Article

Analysis of Rumen Fermentation and Methanogen Levels in Response to Various Alfalfa Hay, Oat Hay, and Feed Concentrate Ratios

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ABSTRACT

This study aimed to analyze ruminal fermentation, methane emissions, and methanogen levels for different forage feed type and concentrate feed ratios. Alfalfa hay, oat hay, and a feed concentrate were used for *in vitro* fermentation experiments, at ratios of 9:1, 5:5, and 1:9 (forage:concentrate). After 24 h of incubation, rumen fermentation and methanogen level changes were evaluated. In the low forage treatments, the total gas, CH_4, NH_3-N, true dry matter digestibility, and total volatile fatty acid were higher than the other treatments, which were used as the parameters on which to assess rumen fermentation ($P < 0.05$). The feed ratio influenced the copy number for the total archaea and the genus *Methanobrevibacter* ($P = 0.015$, $P = 0.010$). The copy number result trend was like that for CH_4 per digested dry matter (DDM). The PCR results and methanogen copy number analysis indicated that the composition of the methanogens affected the CH_4 levels, not their copy number. The results of this study can be applied to predict rumen fermentation and methane emission patterns for cattle fed a variety of feedstuffs.

(**Key words**: Rumen fermentation, Rumen methanogen, Real time PCR, Alfalfa hay, Oat hay)

Ⅰ. INTRODUCTION

The release of greenhouse gases, namely carbon dioxide and methane (CH_4), is a major cause of global warming. Ruminants are known to release large quantities of methane as well as carbon dioxide during through the microbial digestion of feeds in their stomachs (Woodward et al., 2001). The rumen microbiome involves a complex community of prokaryotes, eukaryotes, and viruses. The diverse microbiome of the intestine drives feed digestion and fermentation in cattle, affecting feed use efficiency and the release of pollutants. Methane generation by ruminants is linked not only to global environmental problems but also to economic losses. This is because methane is generated via the microbial digestion of feed in an animal's digestive tract, and the accompanying energy loss ranges from zero to nearly 12% of the dietary energy (Johnson et al., 2000). To mitigate methane emissions from ruminants, many studies have been conducted with the aim of reducing the partial ruminal fermentation that produces methane. Based on this research, the methane-producing microbiome has been widely investigated using a variety of analysis technologies. Cultivation-independent analysis technologies that use the 16S or 18S rRNA gene as a phylogenetic marker have been developed, as most ruminal microbes cannot be cultured (Kim et al., 2017). Previously, polymerase chain reactions (PCRs) with universal primers had been reported to cause amplification bias (Edwards et al., 2004). For this reason, the identified sequence frequency does not necessarily indicate the relative abundance of the bacterium represented or its importance or weight in rumen function. Specific real-time PCR assays were developed to accurately determine the distributions of the uncultured rumen microbiomes of cattle (Stiverson et al., 2011; Kim et al., 2017). Real-time PCR is known to be a highly sensitive method that is applicable for the detection and quantification of microbial populations without needing to cultivate them in an anaerobic environment (Yu et al., 2005). Previous studies have analyzed rumen methanogens in cattle, but there have been relatively few studies that have focused on the Korean native Hanwoo cattle. Also, there have little study on the effect of feed ratio of roughage to concentrate on the rumen fermentation, though it had been known that the feed proportion has an impact on the rumen work (Suárez et al., 2007). According to the Korean Feeding Standard for Hanwoo (RDA, 2017), the recommended amount of concentrate feed is 1.5-1.6% that of the body weight, at the growing stage (less than 12 months), and it is suggested that roughage makes up at least 90% of the concentrate feed (RDA, 2017). Since the late fattening (22–29 months) period is a period in which the development of intramuscular fat is very

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rapid, feeding management during this period focuses on the improvement of meat quality, especially for marbling. In this case, it is recommended that the roughage be less than 15% of the dry matter intake. The treatments for the feed ratio in this study were determined based on these suggestions. Oat hay and alfalfa hay, which are widely used for Hanwoo in Korea, were selected for this experiment. In this study, the influence of roughage source and the ratio of roughage to concentrate on the ruminal fermentation were analyzed. The objective of this study was thus to analyze methane emissions, rumen methanogens, and microbial changes in response to various forage type and feed concentrate ratios.

Ⅱ. MATERIALS AND METHODS

1. Preparation of Experimental Diets

Alfalfa hay, oat hay, and concentrate feed were used for *in vitro* fermentation experiments. To measure the ruminal fermentation characteristics, the feedstuffs consisted with various combinations as follows: 90% alfalfa and 10% concentrate feed (HA), 50% alfalfa and 50% concentrate feed (MA), 10% alfalfa and 90% concentrate feed (LA), 90% oat hay and 10% concentrate feed (HO), 50% oat hay and 50% concentrate feed (MO), 10% oat hay and 90% concentrate feed (LO). The feed samples were oven-dried at 60 °C for 72 h and then ground through a cyclone mill (cyclotec 1093, Foss, HillerØd, Denmark), with 1 mm and 2 mm screens, for chemical analysis and the *in vitro* trial, respectively.

2. *In vitro* Ruminal Fermentation Experiment

All experimental procedures were approved and performed under the guidelines of the National Institute of Animal Science Institutional Animal Use and Care Committee in Korea. Three cannulated Hanwoo steers were individually housed in a pen and fed a diet composed of 80% concentrate feed and 20% mixed hay (45% tall fescue, 45% orchard grass, and 10% Kentucky bluegrass). Animals were fed twice a day with 4 kg of concentrate and 2.5 kg of roughage in total. Water and mineral blocks were freely accessible. The rumen liquid was collected one hour before the morning feed and squeezed through four layers of cheesecloth, and the pH was then measured. The collected rumen fluid was filtered with 4 layers of cheese cloth and then bubbles with O_2-free CO_2 gas were introduced to maintain anaerobic conditions. The rumen fluid from three donors was pooled and combined with McDougall's buffer in a 4:1 ratio under strict anaerobic conditions (Lima et al., 2010), and 50 mL of inoculum, the mixture of rumen fluid and buffer, was then added (n = 3/treatment). The control setup consisted of three blank flasks containing only inoculum. Each treatment and control flask (125 mL) contained a total of 0.5 g of a fermentation substrate, with the substrates for treatments being combinations of alfalfa, oat hay, and concentrate feed. Then, the flasks were sealed with butyl rubber stoppers and aluminum caps and incubated for 24 h at 39 ℃. Subsequently, the rumen-fluid pH and total gas production were measured; the latter was measured with a glass syringe (Truth, Pvt. Ltd. 100 mL, India). The rumen fluid was centrifuged at 6,000 × *g* at 20 °C for 15 min to remove any remaining feedstuff. Dry matter degradability was calculated by filtering the solid fraction of the rumen fluid after centrifugation and washing the residue in the flask with distilled water. Samples were filtered using filter paper (Whatman, No. 541) and then dried in an oven for 48 h. The remaining undigested samples were used to calculate the digested dry matter (DDM) using a modified version of the methods proposed by Goering and Van Soest (1970). The supernatant was used for the analysis of volatile fatty acids (VFA), ammonia nitrogen, and microbial analysis. For the VFA analysis, a 25% meta-phosphoric acid solution was added to the rumen fluid at approximately 10% volume. The VFA concentration was determined as described by Erwin et al. (1961). The supernatant was injected into a gas chromatograph (6890N, Agilent Tech, USA) equipped with a flame ionization detector (FID) and a capillary column (Nukol ™ Fused silica capillary column, 15 m × 0.53 mm × 0.5 μm, Supelco Inc., PA, USA). The temperatures of the oven, injector, and detector were 110-200 °C, 250 °C, and 250 °C, respectively. Nitrogen was used as the carrier gas, at a flow rate of 25.0 mL/min. The concentration of ammonia was analyzed using the method described by Chaney and Marbach (1962). Samples were stored in a deep freezer (- 80 °C) until analysis. All analyses were performed in triplicate, and the mean values were calculated.

3. DNA extraction and Real-time PCR analysis

Genomic DNA was extracted from the rumen samples using the RBB + C bead-beating method (Yu and Morrison, 2004). Specific primers for the methanogens (16S rRNA) and the genus

Table 1. Chemical compositions of the three feeds used in the *in vitro* experiments (% Dry matter).

Item	Alfalfa	Oat hay	Concentrate
Dry matter	91.4	92.7	91.5
Crude protein	20.1	6.2	19.0
Ether extract	1.5	1.3	3.3
Non-fiber carbohydrate	17.0	27.4	43.8
Neutral detergent fiber[1]	53.1	61.5	25.9
Acid determent fiber	41.9	35.6	9.1
Ash	8.3	3.6	8.0
GE(Kcal/g)	4.2	4.2	4.3

[1]Neutral detergent fiber analyzed using a heat stable amylase and expressed inclusive of residual ash

Methanobrevibacter were used in the real-time PCR (Zhou et al., 2009; Tymensen et al., 2012). The standard plasmid was produced as per the instructions of the manufacturer (Macrogen, Seoul, Korea) and transformed into *Escherichia coli* component cells (pTOP vector). Ampicillin-containing LB agar was used for the growth of the *E. coli* containing a standard plasmid. The recombinant plasmids were extracted using the Qiagen mini-prep plasmid extraction kit (Qiagen, CA). Plasmid DNA containing the total archaea and genus *Methanobrevibacter* gene sequences was used as the standard DNA in the real-time PCR and was obtained by PCR cloning with the specific primer sets (Singh et al., 2012; Kim et al., 2017). The extracted plasmid DNA, standard for real-time PCR, was purified using the Qiagen gel Purification Kit (Qiagen, CA); end-point PCR with specific primers was then conducted, and the results were checked via agarose gel electrophoresis. The concentration of the plasmids was then determined with a Nanodrop spectrophotometer, and the copy number of each standard plasmid was calculated using the following formula: Copy No./μl = concentration of plasmid (gm/ul) × 6.022 × 10^{23}/ length of recombinant plasmid (bp) × 660 (660 = the molecular weight of the nucleotide base; 6.022 × 10^{23} = Avogadro's number). Each standard was serially diluted, and reaction concentrations from 0.25 ng/μl to 0.25×10^{-6} ng/μl were used in the real-time PCR assays. Each real-time PCR assay was conducted in three technical replicates (three PCR reactions from the same DNA template), from which the mean was calculated on a CFX96 thermocycler machine (Bio-Rad, Inc.). The reaction mixture (20 μl) consisted of 10 μL of SYBR green, 2 μl of forward primer, 2 μl of reverse primer, 3 ul of RNase free water, and 3 μl of DNA, for real-time PCR in a 96 well plate. The thermal profile consisted of the following four segments: (i) initial denaturation at 94 °C for 2 min (holding stage); (ii) 35 cycles of 94 °C for 15 s, 60 °C for 30 s, 72 °C for 2 min (PCR stage), and (iii) 95 °C for 10 s, 55 °C for 5 s, and 95 °C for 30 s (melt curve stage). Fluorescence data were collected at the 72 °C stage of the second segment, and, during the last stage of the third segment. Baseline and threshold calculations were performed with the Bio-Rad CFX Manager 3.1 software, using the fluorescence signals acquired, at which the primer dimers were completely denatured and

Table 2. PCR primer sequences, targets, annealing temperatures, and amplicon lengths

Target	Primer Sequence (5′-3′)		Annealing temp (℃)	Product size (bp)	Reference
	Forward	Reverse			
Archaea					
Methanogens (16S rRNA)	CCG GAG ATG GAA CCT GAG AC	CGG TCT TGC CCA GCT CTT ATT C	60	~160	Zhou et al., 2009.
Genus					
Methanobrevibacter	TGG GAA TTG CTG GWG ATA CTR TT	GGA GCR GCT CAA AGC CA	63	231	Tymensen et al. 2012.

would not affect quantification. Following real-time PCR, all products were analyzed using melting curve analysis. The abundance (copies ml^{-1}) was calculated by multiplying the copy number value per real-time PCR with the number of reactions that could be performed with the DNA derived from 1 ml of each sample.

4. Statistical Analysis

The ruminal parameters and diversity index were analyzed using the ANOVA procedure of XLSTAT statistical software (Addinsoft, USA) for the difference between four forage type (rye and oat), between forage-to-concentrate ratio (00:00, 00:00 and 00:00), and their interactions. When the significant difference was found, Duncan's multiple range test was performed at $P < 0.05$ and trends were determined $0.05 \leq P < 0.1$.

Ⅲ. RESULTS

1. *In vitro* Ruminal Fermentation

The *in vitro* fermentation characteristics after 24 h of incubation are presented in Table 3. There was a significant difference in the pH, total gas, CH_4, CH_4 per digested dry matter (DDM), NH_3-N, DDM, total volatile fatty acids (VFA), acetate, propionate, acetate:propionate ratio (A:P ratio), and valerate among the treatments ($P < 0.05$). The pH values of the rumen fluid were significantly different among the forage sources and feed ratios ($P = 0.003$ and $P < 0.001$, respectively) and were significantly lower for the low forage treatments. The total gas and CH_4 production levels were significantly different among the feed concentrate ratios ($P < 0.01$), and the values were significantly higher for the low forage treatments. Conversely, the CH_4 per DDM was significantly lower for the low forage treatments ($P < 0.01$). The DDM showed a significant difference among the forage source and feed concentrate ratios ($P < 0.01$ *and* $P < 0.01$, respectively), and the DDM value increased as the forage ratio decreased. The total VFA values were significantly different among the feed ratios ($P = 0.03$), and the VFA value increased as the forage ratio decreased.

2. Real-time PCR analysis

The results of the real-time PCR analysis for the *in vitro* fermentation are shown in Table 4. The copy numbers of the total archaea in the treatments ranged from 6.5×10^4 to 1.1×10^5 after 24 h of incubation. The copy numbers of the total

Table 3. Effects of the different forage sources and levels after 24 h of *in vitro* rumen fermentation

Item[1]	Treatment[2]						SEM[3]	Significance[4]		
	HA	MA	LA	HO	MO	LO		F	R	F × R
pH	6.67 ^{a}	6.59 ^{b}	6.51 ^{d}	6.62 ^{b}	6.54 ^{c}	6.51 ^{d}	0.010	0.003	<0.001	0.050
Total gas, mL	68.3 ^{e}	80.5 ^{c}	85.3 ^{b}	62.3 ^{f}	75.0 ^{d}	89.0 ^{a}	1.14	0.02	<0.01	<0.01
CH_4, mL	3.9 ^{c}	4.7 ^{b}	5.0 ^{b}	3.3 ^{d}	4.6 ^{b}	5.5 ^{a}	0.14	0.73	<0.01	0.02
CH_4, mL/g DDM	21.8 ^{b}	19.5 ^{bc}	17.5 ^{c}	25.0 ^{a}	21.5 ^{b}	19.6 ^{bc}	0.67	<0.01	<0.01	0.72
NH_3-N, mg/dL	10.4 ^{a}	10.6 ^{a}	9.4 ^{ab}	3.1 ^{d}	6.1 ^{c}	8.3 ^{b}	0.63	<0.01	0.02	<0.01
DDM, %	44.0 ^{d}	57.3 ^{b}	66.0 ^{a}	37.4 ^{e}	51.5 ^{c}	64.9 ^{a}	1.15	<0.01	<0.01	0.07
Total VFA, mM	66.9 ^{bc}	77.8 ^{ab}	79.1 ^{a}	65.9 ^{c}	66.8 ^{bc}	75.0 ^{abc}	3.53	0.09	0.03	0.38
Acetate, mM	46.4 ^{ab}	52.0 ^{a}	50.5 ^{ab}	44.4 ^{ab}	43.6 ^{b}	47.3 ^{ab}	2.33	0.04	0.33	0.37
Propionate, mM	13.6 ^{c}	17.0 ^{ab}	19.4 ^{a}	14.3 ^{c}	15.4 ^{bc}	18.9 ^{a}	0.78	0.50	<0.01	0.37
A:P ratio	3.4 ^{a}	3.1 ^{b}	2.6 ^{d}	3.1 ^{b}	2.8 ^{c}	2.5 ^{e}	0.02	<0.01	<0.01	<0.01
Butyrate, mM	5.0	6.5	7.0	5.8	15.3	6.8	3.87	0.35	0.37	0.47
Valerate, mM	1.61 ^{ab}	1.70 ^{a}	1.69 ^{a}	1.49 ^{b}	1.51 ^{b}	1.62 ^{ab}	0.04	<0.01	0.09	0.39

[1]DDM: digested dry matter; VFA: total volatile fatty acids; A:P ratio: acetate/propionate ratio

[2]HA: 90% alfalfa and 10% concentrate feed; MA: 50% alfalfa and 50% concentrate feed; LA: 10% alfalfa and 90% concentrate; HO: 90% oat hay and 10% concentrate; MO: 50% oat hay and 50% concentrate; LO: 10% oat hay and 90% concentrate.

[3]SEM: standard error of the mean

[4]F: effect of the forage sources; R: effect of the forage to concentrate ratio; F × R: effect of the interactions between the feed sources and the forage to concentrate ratio

Table 4. Copy number for the rumen methanogen after 24 h of *in vitro* fermentation for each treatment

Taxon	Treatments[1]						SEM[2]	Significance[3]		
	HA	MA	LA	HO	MO	LO		F	R	F x R
Total archaea	9.4×10^{5} ^{ab}	1.2×10^{6} ^{a}	5.1×10^{5} ^{bc}	7.9×10^{5} ^{abc}	7.8×10^{5} ^{abc}	2.9×10^{5} ^{c}	1.7×10^{5}	0.096	0.015	0.800
Genus *Methanobrevibacter*	3.5×10^{5} ^{ab}	4.7×10^{5} ^{a}	2.0×10^{5} ^{bc}	2.8×10^{5} ^{abc}	3.0×10^{5} ^{abc}	1.1×10^{5} ^{c}	6.4×10^{4}	0.057	0.010	0.695

[1]HA: 90% alfalfa and 10% concentrate feed; MA: 50% alfalfa and 50% concentrate feed; LA: 10% alfalfa and 90% concentrate; HO: 90% oat hay and 10% concentrate; MO: 50% oat hay and 50% concentrate; LO: 10% oat hay and 90% concentrate.
[2]SEM: standard error of the mean
[3]F: effect of the forage sources; R: effect of the forage to concentrate ratio; F × R: effect of the interactions between the feed sources and the forage to concentrate ratio

archaea differed significantly among the feed concentrate ratios (P = 0.015) and were significantly lower in the low forage treatments. The copy numbers of the genus *Methanobrevibacter* were significantly different among the feed ratios, and there was a trend based on the feed source (P = 0.057, P = 0.010). The copy numbers of the genus *Methanobrevibacter* were lower in the low forage treatments, like those of the archaea.

Ⅳ. DISCUSSION

Overall, the LA and LO treatments resulted in higher total gas, CH_4, NH_3-N, DDM, and total VFA, which are used as indicators of rumen fermentation, than the other treatments (P < 0.05). This result is thought to arise from the relatively high ratio of concentrate feed, accounting for 90% of the total feed in the LA and LO treatments. The general outcomes of the experiment correspond with those from other rumen fermentation investigations. Acetate generation is known to be related to forage, and propionate generation is related to feed concentrate (Sutton et al., 2003; Penner et al., 2009). The A:P ratios for the LA and LO were lower than those of the other treatments. Additionally, feed concentrate has a higher NFC and lower NDF than the forage and thus tends to be easily digested (Hall, 2003). Thus, our overall results regarding the differences in ruminal fermentation among feed composition ratios support those from previous investigations. The copy numbers for the total archaea ranged from 2.9×10^5 to 1.2×10^6, and the lowest were observed for the LA and LO treatments. The copy numbers of genus *Methanobrevibacter* ranged from 1.1×10^5 to 4.7×10^5.

The CH_4 per DDM was significantly lower for the low forage treatments (P < 0.01). Consistent with our results, Kumar et al. (2013) found that the lower forage level leads to low methane per g of substrate. Yanez-Ruiz et al. (2008) also reported that increasing the level of concentrate in diet decrease methane emissions. Generally, starch-rich diets decrease the production of CH_4, enhancing the growth of starch-fermenting bacteria, and thereby resulting in decreased A:P ratios and hydrogen production and less CH_4 generation (Johnson and Johnson, 1995).

The feed ratio influenced the copy number of the total archaea and genus *Methanobrevibacter* (P = 0.015, P = 0.010). The medium level forage treatments had a slightly higher number of methanogens than the high level forage treatments, but the difference was not significant, which corresponds with the results of Danielsson et al. (2012). Additionally, the trend in the PCR results was similar to that for CH_4 per DDM. In both measurements, the low forage treatments had lower values, which corresponded to those obtained by Johnson and Johnson (1995). However, the copy numbers for the methanogens did not reflect the total CH_4 emissions. These results corresponded with those obtained by Zhou et al. (2011), in that the composition of the methanogens affected the CH_4 level but not the total copy number of the methanogens.

This study elucidated the differences in *in vitro* rumen fermentation and the quantity of methanogens associated with different forage types and feed concentrations. The results of this study can be used to predict the rumen fermentation and methane emissions of cattle fed a variety of feedstuffs. For more accurate applications, it is necessary to conduct *in vivo* studies using the same methods with cattle.

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