Effect of Different Proteins and Carbohydrates Ratio on *In Vitro* Fermentation and Rumen Bacterial Communities

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ABSTRACT

This study compared and analyzed in ruminal *in vitro* fermentation and determined diversity of rumen microbes and identified the dominant microbial species by *in vitro* in the ratio of protein and carbohydrate. Each treatment was mixed with soybean and starch at a ratio of 0:0, 10:0, 7:3, 5:5, 3:7 and 0:10. Parameters of *in vitro* fermentation such as pH, NH_3-N, biogenic amine (BA) concentration were analyzed at 48h while microbial communities were analyzed at 24h. pH value was the lowest in the 0:10 and highest in the 0:0 (control) at 48h. Total gas production was the highest in the 10:0 and lowest 0:0 at 48h of fermentation. The NH_3-N concentration was highest in the 10:0 and lowest in the 0:10 at 48h. Total BA concentrations were detected highest in the 10:0, lowest in the 0:0 and the highest DNA concentration of histamine-forming bacteria was obtained from the 10:0. On the other hand pH, ammonia and BA concentration were comparatively lower in the high carbohydrate ratio. In DGGE (Denaturing Gradient Gel Electrophoresis) profiles of rumen bacterial 16S rDNA amplicons, bands of R2 and R4 strains dominated in high protein, and R7, R8 and R9 strains dominated in high carbohydrate treatments. As a result of analysis of sequence of these strains, R1 to R6 strains were identified as uncultured species isolated from rumen and R7 to R9 strains were identified as *Selenomonas sp*. It is concluded that high carbohydrate ratio is beneficial for the ruminal fermentation on the basis of *in vitro* fermentation and DGGE analysis.

Key words: bacterial community, DGGE, *in vitro*, protein-CHO ratio

INTRODUCTION

Synchronization of protein and energy degradation in rumen is a proposed technique to increase bacterial growth rates and efficiency of nutrient utilization (Huber and Rolando, 1994). DNA fingerprinting techniques such as DGGE (Konstantinov et al., 2003) can be used to describe the microbial diversity of a variety of ecosystems (Felske et al., 1998) based upon electrophoresis of PCR-amplified 16S rDNA fragments, using polyacrylamide gels containing a linear gradient of DNA denaturants (Muyzer et al., 1993). Ruminant animal feeding and management system is consisted of rice straw and concentrated feed, but the bulky roughages are low quality in respect of nutrients. Concentrated and bulky feed in the ruminant supplies energy and protein for the beneficial effect of microbes as well as for the animal. There is a complementary relation between proteolytic and cellulolytic bacteria, but cellulolytic and starch using bacteria are not complementary in ruminant stomach (Hungate et al., 1966). These relation changes condition of ruminant stomach by supplying the special quality of feed that influences the environment of the ruminant stomach for microbial role and finally changes nutrient digestibility. Specially, propionic acid in ruminant stomach increase according to the increased level of concentrated feed and pH decreases (Barnett et al., 1966), consequently coefficient digestibility and fermenta absorption decreases.

Bacterial number and species changes widely than any other biological lives, and they changes easily by environmental condition such as temperature, pH, light etc. For this reason, it is difficult to determine the actual number of bacteria due to their rapid variation (Fuhrman

et al., 1993). Recently, various bacterial community were investigated in molecular biology through 16S rRNA bases sequence (Weller and Ward, 1989), DGGE (denaturing gradient gel electrophoresis) (Muyzer et al., 1993) and T-RFLP (Terminal restriction fragment length polymorphism) (Liu et al., 1997) etc. Cloning of the microorganism was provided using the 16S rDNA analyzing method. DGGE is a molecular fingerprinting method that separates PCR-generated DNA products. The polymerase chain reaction of environmental DNA can generate templates of differing DNA sequence that represent many of the dominant microbial organisms. However, since PCR products from a given reaction are of similar size (p), conventional separation by agarose gel electrophoresis results only in a single DNA band that largely non-descriptive. DGGE can overcome this limitation by separating PCR products based on sequence differences that results in differential denaturing characteristics of the DNA. DGGE using the gels helps to separate the dominance species by the bands.

In this study, analyzed ruminant stomach fermentability and content changes when different ratio of protein (soybean meal) and soluble starch allowed to the artificial rumen. Moreover, microbial community changes in the rumen determined using the DGGE method, when protein and carbohydrate feed allowed by different ratio. Therefore, the purpose to confirm comparison change of ruminant stomach microorganism through influence of the CHO and protein ratio.

MATERIALS AND METHODS

Experimental design

Control (no substrate)-T1, T2=100% SM (2g), T3=70:30 (SM:SS), T4=50:50 (SM:SS), T5=30:70 (SM:SS), T6=100% SS (2g). 80% buffer and 20% rumen fluid added in the serum bottle as 100 ml and incubated for 0, 12, 24 and 48 h. 3 replications was taken for each of the treatment. TG, pH, NH_3-N and BA were analyzed after incubate the samples. The samples were kept in the deep freedge (-50) before used for analyzed.

In vitro method

Rumen fluids were pooled from 3 ruminally-cannulated Hanwoo steers of 24 months of age and body weights of 600±47 kg. The collected fluids were filtered through 4-folded cheese cloth and obtained in glass bottles. The bottles of rumen fluids were placed in a water bath for 1 hour at a temperature of 39˚C and an inoculums was anaerobically transferred (20% v/v) to a medium (pH 6.5) containing different minerals, in accordance with the method described by Russell and Vansoest (1984); Russell and Strobel (1988). Subsequently, 100 ml of the buffered rumen fluid was anaerobically transferred to 160 ml serum bottles containing 1 g of different treatment wise either soybean meal or soluble starch ratio, or containing neither (control) were introduced prior to filling with buffered rumen fluid (pH 6.7 and autoclaved). The filled serum bottles were then sealed and incubated at 39˚C in a shaking incubator at 90 rpm for 0, 12, 24 and 48h with 3 replications for each of the treatments. Samples from *in vitro* fermentation were taken for analysis after incubation was completed.

Analysis for in vitro parameters

Total gas production was measured from each of the serum bottles at different stages with a press and sensor machine (Laurel Electronics, Inc., Costa Mesa, CA). The gas measurement was recorded by PSI and then converted to ml using the equation of $y=0.023x+0.055$ ($r^2=0.996$). The pH value was measured with a Pinnacle series M530p meter (Schott instruments, Mainz, Germany) after uncapping each of the bottles, and fermentation was halted by swirling the bottles on ice at different stages.

The incubated samples were preserved at -20°C until analysis. At the time of testing, the samples were centrifuged with a Micro 17^{TR} (Hanil Science Industrial, Korea) for 15 minutes at 890 ×g at 4°C prior to NH_3-N analysis in the liquid phase. NH_3-N concentration was measured at an optical density (OD) of 630㎚with a Libra S22 spectrophotometer (Biochrom Ltd., CB40FJ, England), according to the methods developed by Chaney and Marbach (1962). The values for NH_3-N were calculated by the equation of y= 0.0004x + 0.0002 and the standard, r^2= 0.9998.

Biogenic amine (BA) concentrations were analyzed from the samples by high performance liquid chromatography (HPLC). Amine separation was conducted using a Waters liquid chromatography (Waters Ltd., Massachusetts, USA) and a Varian column (Pursuit × Rs 5u C-18 250 × 4.6mm) (Varian Inc., CA). Amine compounds were identified and quantified using standard curves constructed from the pure compounds (His, Meth, Ethyl, Tyr, Phenyl, Put and Cad). HPLC samples were centrifuged, filtered, vortex mixed and hydrolyzed. Prior to sample analysis, a standard was developed between 0.98 and 1.0 (r^2).

Histamine froming bacteria community analyzed using the specific PCR-DNA extraction was performed from 24h ruminal fermenta according to the Wizard Genomic DNA Purification Kit, Promega (USA). Specific primer H-PF08: (5`GAT GGT ATW GTT TCK TAT GA) and H-PR08: (5`GAC CAW ACA CCA TAD SCT TS) were used to extracted the DNA (Table 1). The PCR condition was general PCR. Then the concentration was checked by gel electrophoresis (1.8% agarose gel).

Table 1: Information of primers used for specific PCR

Oligonucleotides	Sequence(5'-3')	Specifications
H-PF08	GAT GGT ATW GTT TCK TAT GA	Specific 16S rDNA
H-PR08	GAC CAW ACA CCA TAD SCT TS	Specific 16S rDNA

Microbiological analysis

DNA extraction

The incubated preserved (-20°C) samples (1ml) were used for genomic DNA extraction with Wizard Genomic DNA Purification Kits (Promega, USA). The extracted DNA was then subjected to electrophoresis on a 0.7% agarose gel (W/V) containing gel red to estimate the amount and integrity of the DNA products (23 kb), and visualized using UV light with a Gel Logic 200 Imaging System (Eastman KODAK Company, Rochester, NY).

16S rDNA amplification

16S rDNA amplicons were amplified using the general polymerase chain reaction (PCR). PCR primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGYTACCTTGTTACGACTT-3') were used to amplify the segment of eubacterial 16S rDNA from nucleotide 27 to 1492 (Nübel et al., 1996). PCR was conducted using a Gene Amp C1000^{TM} PCR System (Bio-Rad Laboratories, Inc, Hercules, CA). The thermal cycle was performed using the following conditions: initial denaturation at 94°C for 5 min, followed by 32 cycles of 94°C for 45 s, 65°C for 45 s and 72°C for 1 min, with a final extension at 72°C for 10 min. The products were then analyzed by electrophoresis on (1%) agarose gel to assess the sizes (1.5 kb) and amounts (Zhu et al., 2003).

DGGE- PCR

The following primers with a GC clamp for forward were used to amplify the V3 region of the bacterial 16S rDNA amplicons for nested-touchdown PCR: 341F-GC (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGG CAGCAG-3') and 518R (5'-ATTACCGCGGCKKGCTG-3') (Nübel et al., 1996). PCR was

conducted using a *Taq* DNA Polymerase Kits (Promega, USA) and the same thermal cycler described above to subject the samples to touch-down PCR condition. Agarose gel (2%) electrophoresis was then conducted to estimate the amount and integrity of the products (approximately 230bp).

DGGE

DGGE was conducted using a D-Code Universal Mutation Detection System (Bio-Rad, Hercules, CA). Amplicons of the V3 region of the 16S rDNA were used for sequence-specific separation by DGGE according to the specifications of Muyzer and Smalla (1998).

Similarity analysis and DNA elution of the DGGE gel

The DGGE gel was scanned at 400 dpi and similarity indices were calculated for pairs of DGGE profiles from the densitometric curves of the scanned DGGE profiles using Molecular Analyst 1.12 software (Bio-Rad) based on the Pearson product-moment correlation coefficient (Häne et al., 1993).

Cloning of the gel eluted DNA products & sequence analysis

One microliter of the DGGE gel elutes were used as the template for PCR to amplify the bacterial 16S rDNA using the 341F (without GC clamp): (5'-CCTACGGGAGGCAGCAG-3') and 518R: (5'-ATTACCGCGGCT GCTGG-3') primers (Nübel et al., 1996) using the same method for the touchdown PCR. The purified products were cloned in competent *Escherichia coli* JM109 cells (Takara Bio Inc, Otsu, Japan) using the Promega pGEM-T vector system according to the instructions of the manufacturer (Promega, Madison, WI). The obtained sequences were then compared and analysed.

Statistical analysis

Results obtained from the experiment were analyzed by one-way ANOVA for variance using the GLM (General Linear Model) procedure of the SAS (2004) 9.1 Software Package and authorized synonymy between averages were determined by Duncan's multiple range test method. Significant differences were accepted if $p<0.05$.

RESULTS

Changes of total gas production

Total gas production by different carbohydrate and protein ratio in *in vitro* fermentation at different incubation period was shown in Table 2. The gas production (ml/2g feed) of T1 (16.0) and T6 (34.0) were comparatively lower than other treatment groups at 12h and T1 (23.0) was lowest at 24h of incubation. T2 (102.67), T3 (94.0), T4 (91.0) and T5 (97.67) were produced ($p<0.05$) more total gas than the other treatments at 48h of incubation, and T1 (32.0) and T6 (63.66) produced less gasses than them.

Table 2: Total gas production (ml/2g feed substrate) by rumen microbial *in vitro* fermentation

	Treatments						
hour	**(1)T1**	**(2)T2**	**(3)T3**	**(4)T4**	**(5)T5**	**(6)T6**	**SEM**
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
12	16.00^{d}	53.00^{ab}	63.67^{a}	53.00^{ab}	49.00^{b}	34.00^{c}	3.28
24	23.00^{c}	75.33^{a}	74.67^{a}	70.00^{a}	58.00^{b}	72.33^{a}	2.84
48	32.00^{c}	102.67^{a}	94.00^{a}	91.00^{a}	97.67^{a}	63.66^{b}	5.54

^{abcd}With each row, means with different superscripts differ significantly ($P<0.05$)

$^{(1)}$T1 = Control, $^{(2)}$T2 = Soybean meal 10: Soluble starch 0, $^{(3)}$T3 = Soybean meal 7: Soluble starch 3, $^{(4)}$T4 = Soybean meal 5: Soluble starch 5, $^{(5)}$T5 = Soybean me al 3: Soluble starch 7, $^{(6)}$T6 = Soluble starch 0: Soluble starch 10

pH values

pH values by different carbohydrate and protein ratio in *in vitro* fermentation at different incubation period were shown in Table 3. pH value at 0 hour showed 6.03 to 6.12, and at 48 hour of ruminal fermentation they were between 4.98 to 6.17. The pH value of T6 dropped from 0 h to 48 h by 6.10 and 4.98, respectively, which was lowest among all of the treatments (Table 3).

Table 3: pH values by rumen microbial *in vitro* fermentation

	Treatments						
Hour	**(1)T1**	**(2)T2**	**(3)T3**	**(4)T4**	**(5)T5**	**(6)T6**	**SEM**
0	6.12^{a}	6.07^{ab}	6.03^{b}	6.09^{a}	6.07^{ab}	6.10^{a}	0.01
12	6.03^{a}	5.64^{b}	5.36^{c}	5.43^{c}	5.39^{c}	5.49^{c}	0.04
24	6.10^{a}	5.67^{b}	5.36^{c}	5.21^{d}	5.21^{d}	5.12^{e}	0.02
48	6.17^{a}	5.67^{b}	5.33^{c}	5.15^{d}	5.03^{de}	4.98^{e}	0.04

^{a,b,c,d,e}With each row, means with different superscripts differ significantly($P<0.05$)

Table 4: NH3-N concentration (mg/L) changes by rumen microbial *in vitro* fermentation

	Treatments						
hour	**(1)T1**	**(2)T2**	**(3)T3**	**(4)T4**	**(5)T5**	**(6)T6**	**SEM**
0	440.8^{a}	542.8^{a}	523.7^{a}	523.7^{a}	486.8^{a}	523.6^{a}	0.005
12	447^{ab}	751.2^{a}	613.9^{b}	632.9^{ab}	521.2^{ab}	560.3^{ab}	0.01
24	543.3^{b}	877^{a}	639.5^{b}	575.8^{b}	606.2^{b}	471.2^{b}	0.013
48	395.5^{d}	896.2^{b}	672.8^{ab}	614.5^{bc}	523.7^{dc}	502.8^{d}	0.012

^{a,b,c,d}With each row, means with different superscripts differ significantly($P<0.05$)

Table 5: Biogenic amines concentration (mg/L) at 48 h of rumen microbial in vitro fermentation

	Treatments					
Amines	**(1)T1**	**(2)T2**	**(3)T3**	**(4)T4**	**(5)T5**	**(6)T6**
Histamin	1.455	358.967	237.218	2.221	2.860	1.712
Methylamine	ND	6,136	0,527	ND	ND	ND
Ethylamine	ND	16,952	3,229	ND	ND	ND
Tyramine	ND	ND	ND	ND	ND	ND
Phenylethylamin	ND	ND	ND	ND	ND	ND
Putrescine	ND	76,066	19,601	0.035	0,044	ND
Cadaverine	ND	35,059	7,220	ND	ND	ND
Amine index	1.455	470.092	267.795	2.256	2.904	1,712
Total amine	1.455	493.18	264.039	2.256	2.904	1,712

NH_3-N concentration

NH3-N concentration (mg/L) changes from 0 hour to 48 hours and appeared highest in T2 (896.2) and lowest in T1 (395.5). T6 (502.8) showed lower ammonia concentration than all of the carbohydrate-protein treatment groups at 48 hours (Table 4).

Biogenic amine

Histamine, amine index and total amine concentration (mg/L) was highest in T2 (358.97, 470.09 and 493.18) followed by T3 (237.22, 267.79 and 264.04), T5 (2.86, 2.9 and 2.9), T4 (2.22, 2.26 and 2.26), T6 (1.71, 1.71 and 1.71) and T1 (1.45, 1.45 and 1.45) at 48 hours of incubation (Table 5).

Histamine froming bacteria community

The concentrations of Band's were higher in T2 and T3 than any other treatments due to Histamine froming bacteria, and there was lowest concentration found in T1 due to the absence of protein substrate. Details band results of histamine froming bacteria were shown in figure 1.

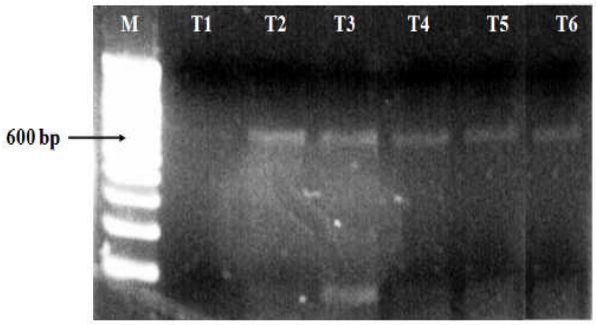

Figure 1: Bacterial amplicons from treatments

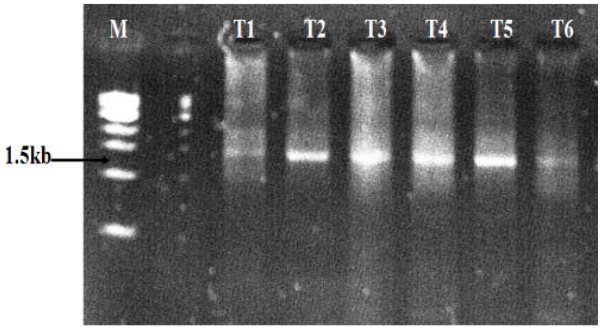

Figure 2: Amplified 16S rDNA PCR products

Microbiological results

Genomic DNA extraction and 16S rDNA- PCR amplicons

DNA extractions from 24h fermented samples were used for electrophoresis in 0.7% agarose gel to confirm by the DNA bands. 27F and 1492R primers used for 16S rDNA PCR that amplify Eubacteria and confirmed 1.5 kb's DNA band (Fig. 2).

DGGE -PCR band

PCR amplification of 16S rDNA at V3 region was amplified using 341F-518R primers. Amplified PCR products were confirmed using ethidium bromide and electrophoresis in 1% agarose gel. PCR products were confirmed along with the band size of 230 bp (Fig. 3).

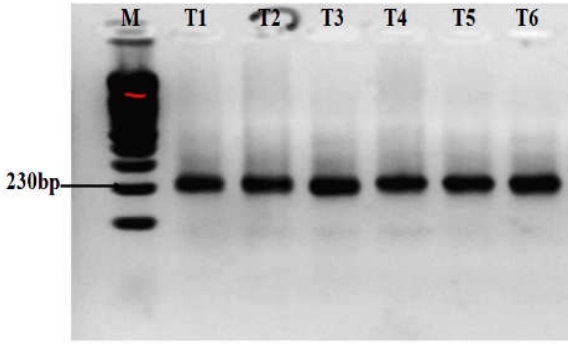

Figure 3: DGGE-PCR amplified from 16S rDNA at V3 region (200 ~ 230 bp)

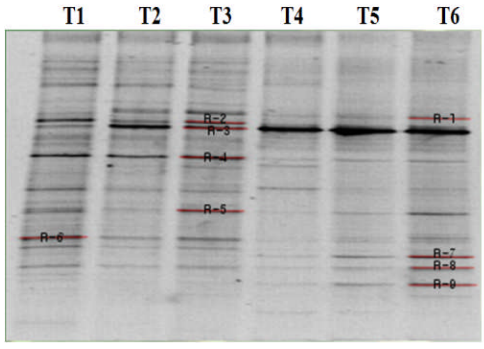

Figure 4: Negative image of DGGE gel bands which were divided from amplified 16S rDNA at V3 region

DGGE

Microbial community analyzed by DGGE method indicated that R2 and R4 band of high protein ratio, and R3, R7, R8 and R9 band of high carbohydrate ratio shows the different specific microorganism (Fig. 4).

Sesequence analysis

Total 9 clones were selected and analyzed for sequences from Macrogen, Korea. Sequence analysis from the DGGE gel cutting band directly appeared as R1, R2, R3, R4, R6, R7, R8 and R9 (Table 6). All of the observed bacteria were available as ruminal microbes except R1 and R3. R7, R8, R9 bands appeared as *Selenomonas sp*.

Table 6: Sequcncing of PCR amplicons generated from clones of individual bands

Sample	Strains (Accession no.)	Identity	Isolation
R-1	Uncultured bacterium clone BB2_h06 (EU458259)	96/110 (87%)	North American black bear feces
R-2	Uncultured bacterium clone RB-5E1 (FJ172825)	130/139 (93%)	rumen
R-3	Uncultured Firmicutes bacterium clone NI_52 (FJ650961)	127/127 (100%)	fecal sample
R-4	Uncultured rumen bacterium clone L7A_C10 (EU381645)	126/128 (98%)	rumen liquid phase of fistulated Holstein Heifer
R-5	No data		
R-6	Uncultured rumen bacterium clone CF376 (EU871409)	134/147 (91%)	rumen
R-7	*Selenomonas sp*. MCB2 (EF195237)	147/149 (98%)	yak rumen in Tibetan plateau
R-8	*Selenomonas ruminantium* gene(AB198437)	148/153 (96%)	isolated from sheep rumen
R-9	*Selenomonas ruminantium* strain LongY6 (EU327402)	146/150 (97%)	rumen

Dendrogram analyses using UPGMA program

Dendrogram analysis results from UPGMA (Unweighted Pair Group Method with Arithmetic mean) was divided into 2 groups as 10:0, 7:3, 0:0 and 5:5, 3:7, 0:10 group. This shows the changes ruminal microorganisms by the effect of feed level especially carbohydrate and protein ratio (Figure 5).

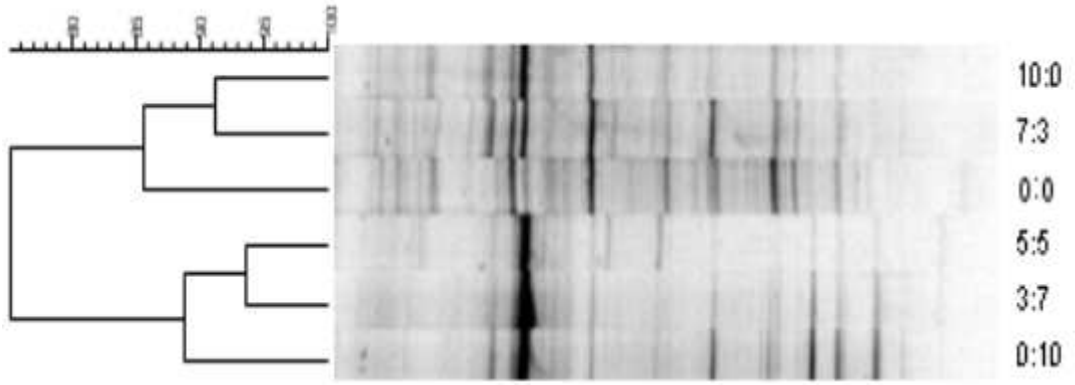

Figure 5: UPGMA cluster analysis of DGGE band pattern of amplified 16S rDNA V3 region from rumen *in vitro* fermentation.

DISCUSSION

When allowed protein feed alone or combined protein and carbohydrate by ratio, total gas production was higher in T2, T3, T4, T5, than others. These results are considered that increase of protein degradation by microbial activity causes high CO_2 production from protein related feed. At 48h of fermentation, pH showed lowest value (4.98) from 6.17 at only soluble starch (0:10) treated fermentation. Walker (1995) reported that starch hydrolysis or fermented by microorganisms causes increased amount of lactic acid and alcohol which are responsible for decrease the pH level in the rumen. Williams, AG (1986) reported that both *Selenomonas ruminantium* and *Streptococcus bovis* enhances their growth by high soluble starch and produce more lactic acid, but usually *S. ruminantium* produces acetate and propionate, and *S. bovis produces* acetate and ethanol. Protozoa usually produces the acetate and butyrate, but when increased the starch level causes they produce more lactic acid and decreased acetate and butyrate. Russell et al. (1979) reported that with the advancement of feed fermentation time (soluble carbohydrate) causes decreased pH value due to the rapid production of lactic acid in ruminant. Low fiber digestion occurs due to the lessening the fiber degrading bacteria, when pH value decreased below 6.2.

Syrjala et al. (1993) reported that NH3-N concentration in ruminant stomach is remained between 1 to 76 mg/100 ml of rumen fluids. The ammonia-N level depends on the presence of microbial protein in the rumen. This change also depends on the level of the carbohydrate level, if the carbohydrate level increase, then both of NH3 and microbial protein will decrease, though microbial protein synthesis efficiency depends on the quantity of carbohydrate fermentation. In the present experiment, NH3-N concentration detected highest in 10:0 of protein and carbohydrate related treatment at 48h of incubation, and measured lowest in 0:10 (proten: carbohydrate). Jae, MW (1997) reported that NH3-N concentration increase with the increased level of microbial protein by the microbial fermentation of higher level of protein ratio in feed.

The BA change amount appeared highest in T2 group (100% protein) and measured lowest in T1 control group during the rumen fermentation among the treatment groups.These result considered that BA is created by decarboxylation of degradable protein by different microbes such as bacillus, coliform, clostridium, lactic acid producing bacteria etc. in the rumen fermentation of cattle. Histamine froming bacteria community band concentration appeared highest in T2 and lowest at T6 at 48h of *in vitro* fermentation. The change of BA concentration determined by HPLC was also showed same results in this reseach.

Microbial community analysis in ruminant stomach through DGGE technique, R2 and R4 strain showed dominant in protein supplemented treatment, and R3, R7, R8 and R9 strain showed dominant in carbohydrate content treatment. Mao et al. (2007) reported that microbial community changes with the kinds of feed and changes of feed level in the rumen. All of the identified microorganisms are available in the ruminant stomach except R1 and R3. Sesequences obtained from cutting band directly in DGGE gel gives information between 120 bp to190 bp. DNA sizes below 200bp has some limitation to give the information regarding the microbes. Wang et al. (2003) reported that the sequence information of 200bp can recognize in Genus level, but other classification accuracy difficult to decide.

If we want more correct systematic classification of microbes, in that case comparative analysis should be considered between 16S rDNA cloning and DGGE clone. Dendrogram analysis results from UPGMA (Unweighted Pair Group Method with Arithmetic mean) was

divided into 2 groups as 10:0, 7:3, 0:0 and 5:5, 3:7, 0:10 group. This result considered that changes of feed level in ruminant stomach diversified microorganism community, and protein feed content effect more than the carbohydrate feed level. Also carbohydrate content appeared some strong microbial community by high concentration of bands. This is considered that sudden decrease of pH by carbohydrate content causes weakend the growing of microorganism except the low pH tolerant dominant microorganisms.

CONCLUSION

In ruminal *in vitro* fermentation and diversity of rumen microbes were compared and analyzed and identified the dominant microbial species by feeding to the Holstein Friesian cattle in the ratio of protein and carbohydrate. pH, ammonia and BA concentration were comparatively lower in the high carbohydrate ratio. As a result of analysis of sequence of these strains, R1 to R6 strains were identified as uncultured species isolated from rumen and R7 to R9 strains were identified as *Selenomonas sp*. It is concluded from the overall result that high carbohydrate ratio is beneficial for the ruminal fermentation on the basis of *in vitro* fermentation and DGGE analysis.

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