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Effect of Defaunation on *In Vitro* Fermentation Characteristics and Methane Emission When Incubated with Forages

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ABSTRACT

An *in vitro* study was conducted to determine the effects of defaunation (removal of protozoa) and forage sources (rice straw, ryegrass and tall fescue) on ruminal fermentation characteristics, methane (CH_4) production and degradation by rumen microbes. Sodium lauryl sulfate, as a defaunation reagent, was added into the mixed culture solution to remove ruminal protozoa at a concentration of 0.375 mg/ml. Pure cellulose (0.64 g, Sigma, C8002) and three forage sources were incubated in the bottle of culture solution of mixed rumen microbes (faunation) or defaunation for up to 24 h. The concentration of ammonia-N was high under condition of defaunation compared to that from faunation in all incubations ($p<0.001$). Total VFA concentration was increased at 3, 6 and 12 h ($p<0.05$~$p<0.01$) but was decreased at 24 h incubation ($p<0.001$) under condition of defaunation. Defaunation decreased acetate ($p<0.001$) and butyrate ($p<0.001$) proportions at 6, 12 and 24 h incubation times, but increased propionate ($p<0.001$) proportion at all incubation times for forages. Effective degradability of dry matter was decreased by defaunation ($p<0.001$). Defaunation not only decreased total gas ($p<0.001$) and CO_2 ($p<0.01$~0.001) production at 12 and 24 h incubations, but reduced CH_4 production ($p<0.001$) at all incubation times for all forages. The CH_4 production, regardless of defaunation, in order of forage sources were rice straw > tall fescue > ryegrass > cellulose ($p<0.001$) up to 24 h incubation.
(**Key words :** Defaunation, Forages, Effective degradability, Total gas, Methane emission)

Ⅰ. INTRODUCTION

The forages make up a large proportion of the diet in ruminant production systems. The feeding of forages as energy sources to ruminants is highly depended on rumen fermentation of the fiber such as cellulose and hemicelluloses by rumen microbes (Wina et al., 2006).

Rumen protozoa are metabolically active and serve a multifunctional role in metabolizing dietary nutrients through several ways (Morgavi et al., 2010). Protozoa are well known to secrete hydrolytic enzymes (Coleman, 1986) and positively contributed to 20% of fiber degradation (Dijkstra and Tamminga, 1995). In addition to their role in fiber degradation, rumen protozoa are positively related to methane (CH_4) production in the rumen. Finlay et al. (1994) described a symbiotic relationship of ruminal ciliate protozoa with methanogens, which has been proved to allow an interspecies H_2 transfer from ciliate protozoa to methanogens for CH_4 synthesis, and the symbiotic methanogens associated with rumen ciliate protozoa may account for 37% of the total CH_4 production. Some studies have shown that defuanation (removal of protozoa in the rumen) decreased CH_4 production up to 10.8% (Kreuzer et al., 1986) or 24.1% (Morgavi et al., 2008) when the cattle were fed forage diets. Furthermore, it is well known that ruminants fed forage-based diets produce more CH_4 than those fed high level of concentrate diets (Johnson et al, 2000).

Since CH_4 emission from ruminants has increasingly caused widespread attention because of energy loss of the ingested diets and its contribution to global greenhouse gas (Johnson and Johnson, 1995) various attempts have been made to suppress CH_4 production. Despite protozoa comprise more than 50% of rumen microbial biomass (Harrison and McAllan, 1980), there is little information about the relationship between various forage feeds and rumen protozoa in CH_4 production from fiber digestion.

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Therefore, the objective of the present study was to investigate the effect of defaunation on fermentation characteristics and *in vitro* CH_4 production by forage feed.

Ⅱ. MATERIALS AND METHODS

1. Preparation of culture solution and *in vitro* incubation with rumen microbes

Rumen contents were obtained 2h after the morning feeding (09:00) from three ruminally-cannulated non-lactating Holstein cows fed 9 kg/d total diets daily (2 kg concentrate and 7 kg ryegrass, as fed basis), twice (09:00 and 18:00 h) per day, in an equal volume. The rumen fluid was strained through 12 layers of cheesecloth to remove the feed particles. Carbon dioxide (CO_2) was flushed into the strained rumen fluid for 30 seconds. Culture solution was prepared by mixing 40 ml strained rumen fluid with 40 ml McDougall's artificial saliva (McDougall, 1948) in 160 ml incubation bottle. Sodium lauryl sulfate (Sigma, L5750) as a defaunation reagent was added into the mixed culture solution to remove ruminal protozoa at concentration of 0.375 mg/ml (Dohme et al., 1999). Pure cellulose (0.64 g, Sigma, C8002) and three forage sources (1.08 g rice straw, 1.0 g ryegrass and 0.94 g tall fescue on an air dried basis) were prepared in a nylon bag (5 × 5 cm; pore size, 50 μm) in order to supply the similar amount of neutral detergent fiber (NDF) between forage sources, and were incubated in the bottle of culture solution of mixed rumen microbes (faunation) or of absence of protozoa (defaunation). The bottles were then sealed with rubber stoppers and were incubated anaerobically in a shaking incubator (VS-8480SR, VISON Science, Bucheon, Korea) at a speed of 135 rpm up to 24 h at 39℃. The *in vitro* incubation was made 3 times in duplicate, each time under the similar conditions. Chemical composition of feed added to the culture solution is shown in Table 1.

2. Measurement and analysis

Incubation was stopped by removing the bottles from the shaking incubator at 3, 6, 12 and 24 h, and pH of culture solution was immediately measured. At the same time an aliquot of culture solution (0.8 ml) was collected from each bottle for ammonia and volatile fatty acid (VFA) analysis. Ammonia concentration was determined by the method of Fawcett and Scott (1960) using a spectrophotometer. The 0.8 ml culture solution was mixed with 0.2 ml 25% phosphoric acid and 0.2 ml pivalic acid solution as the internal standard for the VFA analysis as described by Li et al (2010). Total gas production was also measured at each incubation time through the 3-way stopcock connected to culture bottles. A gas sample was transferred to a 5 ml vacuum tube and analyzed for methane (CH_4) and carbon dioxide (CO_2) by gas chromatography (YL 6100GC, Young Lin Instrument Co., Korea) equipped with flame ionization detector (FID) and thermal conductivity detector (TCD). A 30 m silica capillary column (Agilent HP-PLQT Q, 19095P-Q04, 0.54 mm i.d., USA) was used to identify CH_4 and CO_2 peak analysis. The oven and injector temperatures for gas analysis were 100℃ and 150℃, respectively, and temperatures for FID and TCD detector were kept at 230℃ and 150℃, respectively. The nitrogen (N_2) gas was used as carrier gas at a flow rate of 30 ml/min. The nylon bag containing feed residue was washed with tap water and dried at 60℃ for 48h in the drying oven to measure dry matter (DM) degradation. Crude protein (CP), ether extract (EE), and organic acid (OM) were analyzed according to AOAC (1995). The NDF was analyzed by the methods of Van Soest et al. (1991).

3. Estimation of effective degradability *in vitro*

Percent disappearance of DM at each incubation time was

Table 1. Chemical composition of the feeds sources

Feeds	Chemical composition (%, DM basis)			
	Crude protein	Ether extract	Neutral detergent fiber	Ash
Rice straw	3.62	2.12	70.36	11.34
Tall fescue	3.19	2.95	73.50	5.49
Ryegrass	4.14	2.05	69.48	5.16

calculated from the portion remaining after incubation in the rumen. Disappearance rate was fitted to the equation of Ørskov and McDonald (1979):

$Y(t) = a + b(1-e^{-ct})$

Where $Y_{(t)}$ is the proportion of the incubated material degraded at time *t*; 'a' is the water soluble and instantly degradable fraction; '*b*' is the potentially degradable fraction; '*c*' is the fractional rate of degradation of fraction *b* (h-1). Non-linear parameters a, b and c was estimated by an iterative least square procedure to calculate effective degradability of DM (EDDM) according to the following equation (Ørskov and McDonald, 1979):

Effective degradability = $a + (b \times c) / (c+r)$

Where 'r' is the fractional outflow rate and a hypothetical fractional outflow rate (*k*p) of 0.05/h was used for estimation of effective degradability.

4. Statistical analyses

The present study was conducted as 2 × 4 factorial design that represent two fractions (faunation and defaunation) and four dietary groups (cellulose, rice straw, tall fescue and ryegrass).

Data were analyzed using the general linear models (GLM) procedure of SAS (2002). Eight treatments were replicated twice per time and repeated 3 times. For each variable measured at each time, replicates were averaged, and the total number of observations was 8 (treatments) × 3 (times) = 24 observations. The 24 observations obtained were subjected to least squares analysis of variance according to the following models:

$Y_{ij} = \mu + \tau_i + S_j + (\tau_i \times S_j) + \varepsilon_{ij}$

Where Y_{ij} is observation, μ is the overall mean, τ_i is the effect of forage treatment ($i = 1 \sim 4$), S_j is defaunation effect ($j = 1$ and 2), $(\tau_i \times S_j)$ = interaction effect between forage feed and defaunation and ε_{ij} is the error term. Significances were declared at $p < 0.05$.

Ⅲ. RESULTS

The chemical composition of forage feed sources is shown in Table 1. At each incubation time, microscopic examination was carried out to observe protozoa by using a

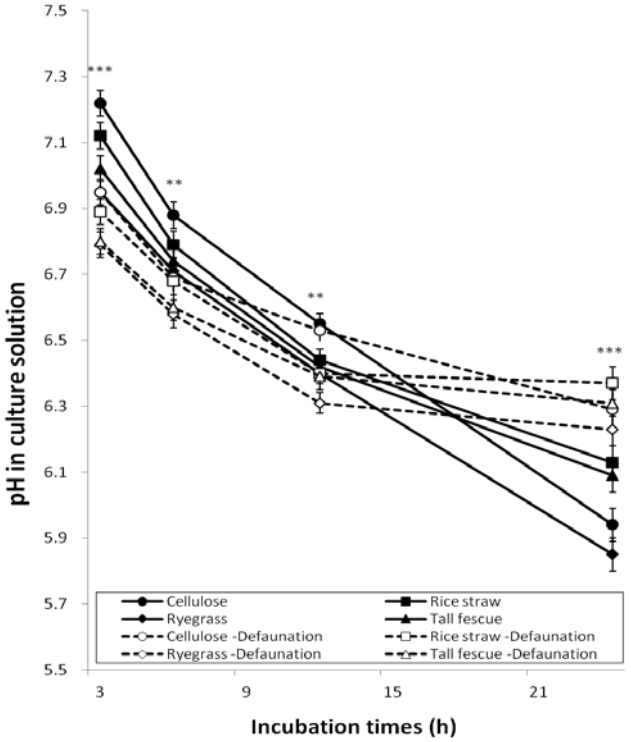

Fig. 1. Effect of forage sources and defaunation on pH in culture solution. **, p<0.01; ***, p<0.001.

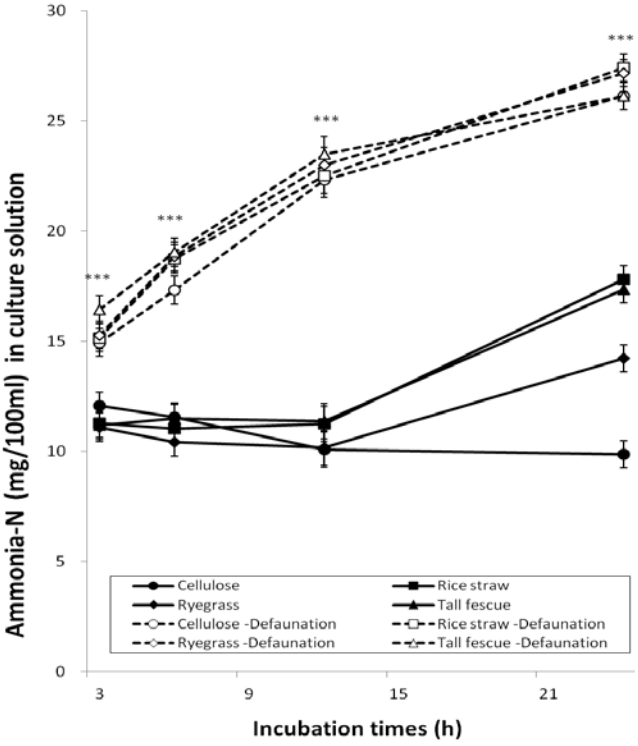

Fig. 2. Effect of forage sources and defaunation on ammonia-N concentration (mg/100ml) in culture solution. ***, p<0.001.

16/0.35 objective, and it showed that sodium lauryl sulfate was effective defaunation agent. After dosing, live protozoa were virtually absent in culture solution. The pH of all treatments in the culture solution tended to decrease with incubation times as shown in Fig. 1. Defaunation decreased pH of culture solution at 3 h ($p<0.001$) and 6 h ($p<0.001$) incubations and then increased it at 24 h ($p<0.001$) when compared with faunation. Among forages, the increased pH was observed in culture solution with cellulose compared with those in culture solutions with other forage sources in 3 h ($p<0.001$), 6 h ($p<0.01$) and 12 h ($p<0.01$) incubation times, and regardless of defaunation the highest pH from rice straw and the lowest pH from ryegrass were found at 24 h incubations ($p<0.001$). Ammonia-N concentration in the culture solution had a trend to increase for all treatments regardless of defaunation except for the cellulose from faunation as the incubation time advanced (Fig. 2). Defaunation significantly increased ammonia-N concentration in all incubations compared with faunation ($p<0.001$). Within the faunation treatments, ammonia-N concentration was not

Table 2. Effects of forage sources and defaunation on total VFA production and molar proportion of VFAs by rumen microbes

Items	Treatments								SEM[1)]	P-values		
	Faunation				Defaunation					Feed (F)	Def.[2)] (D)	F×D[3)]
	Cellulose	Rice straw	Ryegrass	Tall fescue	Cellulose	Rice straw	Rye-grass	Tall fescue				
	3 h											
Total VFAs (mmoles/100ml)	67.09^{b}	70.12^{ab}	72.03^{ab}	71.47^{ab}	72.56^{ab}	72.90^{ab}	77.31^{a}	76.54^{a}	2.467	*	**	NS
Molar proportion (mmoles/100mmoles)												
Acetate (C_2)	66.77^{ab}	68.40^{a}	67.79^{ab}	67.76^{ab}	62.38^{c}	66.07^{b}	66.20^{b}	65.94^{b}	0.891	***	NS	***
Propionate (C_3)	15.43^{d}	17.64^{c}	18.58^{bc}	18.46^{bc}	19.90^{ab}	20.95^{a}	20.97^{a}	20.89^{a}	0.736	***	***	NS
Butyrate (C_4)	10.60^{bc}	11.47^{a}	11.23^{ab}	11.21^{ab}	10.32^{cd}	9.93^{cd}	9.74^{d}	9.98^{cd}	0.592	***	***	*
C_2/C_3	4.33^{a}	3.88^{b}	3.65^{bc}	3.67^{bc}	3.13^{cd}	3.16^{d}	3.16^{d}	3.16^{d}	0.092	***	***	NS
	6 h											
Total VFAs (mmoles/100ml)	73.42^{d}	77.30^{cd}	85.99^{ab}	81.98^{bc}	87.57^{ab}	88.91^{ab}	92.14^{a}	90.74^{ab}	2.180	***	*	NS
Molar proportion (mmoles/100mmoles)												
Acetate (C_2)	68.30^{a}	67.79^{a}	67.63^{a}	67.27^{a}	64.04^{b}	62.27^{c}	62.11^{c}	61.75^{c}	0.739	***	***	NS
Propionate (C_3)	16.81^{c}	17.67^{c}	18.28^{c}	18.03^{c}	24.03^{b}	25.38^{a}	26.29^{a}	24.40^{a}	0.437	***	***	NS
Butyrate (C_4)	12.23^{a}	12.11^{a}	11.88^{a}	12.30^{a}	8.68^{b}	9.13^{b}	8.50^{b}	8.63^{b}	0.379	***	***	NS
C_2/C_3	4.06^{a}	3.84^{b}	3.70^{b}	3.73^{b}	2.67^{c}	2.46^{d}	2.36^{d}	2.34^{d}	0.066	***	***	NS
	12 h											
Total VFAs (mmoles/100ml)	95.78^{c}	105.66^{b}	108.22^{b}	106.75^{b}	96.13^{c}	108.69^{b}	118.41^{a}	110.73^{b}	2.012	***	**	NS
Molar proportion (mmoles/100mmoles)												
Acetate (C_2)	65.11^{a}	65.18^{a}	65.79^{a}	65.91^{a}	59.67^{b}	59.90^{b}	58.97^{b}	59.74^{b}	0.905	***	***	NS
Propionate (C_3)	20.20^{c}	19.20^{c}	19.32^{c}	19.24^{c}	29.18^{ab}	26.67^{b}	29.86^{a}	27.98^{ab}	0.749	***	***	NS
Butyrate (C_4)	12.42^{a}	13.33^{a}	12.69^{a}	12.63^{a}	7.61^{b}	9.65^{b}	7.94^{b}	8.69^{b}	0.857	***	***	NS
C_2/C_3	3.22^{a}	3.40^{a}	3.41^{a}	3.43^{a}	2.04^{b}	2.25^{b}	1.98^{b}	2.14^{b}	0.080	***	***	NS
	24 h											
Total VFAs (mmoles/100ml)	133.70^{a}	126.64^{bc}	134.87^{a}	128.40^{ab}	120.70^{c}	112.88^{d}	124.57^{bc}	119.97^{c}	1.894	***	***	NS
Molar proportion (mmoles/100mmoles)												
Acetate (C_2)	63.31^{a}	64.11^{a}	64.33^{a}	64.21^{a}	58.85^{b}	57.98^{b}	57.07^{b}	58.37^{b}	0.903	***	***	**
Propionate (C_3)	22.79^{b}	19.55^{c}	20.42^{c}	19.85^{c}	29.83^{a}	28.62^{a}	30.91^{a}	28.42^{a}	0.724	***	***	NS
Butyrate (C_4)	11.79^{ab}	13.78^{a}	12.96^{a}	13.45^{a}	7.57^{c}	9.82^{bc}	8.25^{c}	9.55^{bc}	0.711	***	***	NS
C_2/C_3	2.78^{b}	3.28^{a}	3.15^{a}	3.24^{a}	1.97^{c}	2.03^{c}	1.85^{c}	2.07^{c}	0.063	***	***	**

[1)] SEM, Standard error of means; [2)] Def.: defaunation; [3)] F×D, interaction between defaunation and various forages.
[a,b,c] Means in the same row with different superscripts differ regardless of defaunation.
* $p<0.05$; ** $p<0.01$; *** $p<0.001$; NS = Non significant.

Table 3. Effects of forage sources and defaunation on degradation parameters (a, b, and c) and effective degradability of dry matter (EDDM) by rumen microbes

Parameters[1] and ED	Treatments								SEM[2]	Effects		
	Faunation				Defaunation					Feed (F)	Def.[3] (D)	F×D[4]
	Cellulose	Rice straw	Ryegrass	Tall fescue	Cellulose	Rice straw	Ryegrass	Tall fescue				
a	1.545^{d}	1.713^{d}	1.716^{d}	1.726^{d}	1.376^{d}	3.293^{c}	4.429^{a}	3.837^{b}	0.116	***	***	***
b	79.31^{a}	41.77^{e}	46.57^{c}	44.24^{d}	66.78^{b}	33.35^{h}	38.97^{f}	36.64^{g}	0.747	***	***	*
c	0.166^{c}	0.186^{abc}	0.179^{bc}	0.186^{abc}	0.201^{ab}	0.207^{a}	0.192^{abc}	0.174^{c}	0.006	**	**	**
EDDM	62.40^{a}	34.62^{e}	38.13^{c}	36.59^{d}	54.85^{b}	30.16^{g}	35.35^{de}	32.27^{f}	1.161	***	***	***

[1] a, Intercept representing rapidly soluble fraction in the rumen; b, fraction of degradable at time infinity; c, rate constant of disappearance of fraction "b".

[2] SEM, Standard error of means; [3] Def.: defaunation.[4] F×D, interaction between defaunation and various forages.

[a,b,c]Means in the same row with different superscripts differ regardless of defaunation;* $p<0.05$; ** $p<0.01$; *** $p<0.001$.

influenced by forage sources in all incubations except for 24 h incubation. Aammonia-N concentration was highest in the culture solution of faunation incubated with rice straw and tall fescue at 24 h incubation, then followed by ryegrass and cellulose ($p<0.001$, Fig. 2).

Defaunation increased total VFA concentration at 3, 6 and 12 h ($p<0.05$~$p<0.01$) but decreased it at 24 h ($p<0.001$, Table 2). Rice straw produced the lowest total VFA concentration ($p<0.001$) while ryegrass produced its highest concentration ($p<0.001$) after 24 h incubations regardless of defaunation. Defaunation decreased proportions of acetate (C_2, $p<0.001$) and butyrate (C_4, $p<0.001$) at all incubation times except for C_2 at 3 h incubation but increased proportions of propionate (C_3) from 3 h incubation ($p<0.001$). Meanwhile, defaunation decreased C_2 to C_3 ratio for all the forages from 3 h incubation ($p<0.001$).

Degradation parameters (a, b and c) and effective degradability (ED) of major components are presented in Table 3. Defaunation decreased EDDM ($p<0.001$) for all treatments. Furthermore, mean percent EDDM from faunation or defaunation was uniformly ranked as cellulose > ryegrass > tall fescue > rice straw (Table 3).

The effect of defaunation for forages on the gas production is shown in Table 4. Accumulated total gas production was decreased by defaunation at 3, 12 and 24 h incubations ($p<0.001$), and carbon dioxide (CO_2) production was also reduced at 12 h ($p<0.01$) and 24 h ($p<0.001$) incubations. Defaunation clearly decreased CH_4 production at all the incubation times ($p<0.001$) compared with faunation. Meanwhile, emission of total gas ($p<0.001$), CO_2 ($p<0.001$) and CH_4 ($p<0.001$) was lowest from cellulose, but ryegrass showed the highest amount of total gas ($p<0.001$), CO_2 ($p<0.001$) and CH_4 ($p<0.001$) regardless of defaunation at all incubation times. In addition, defaunation was associated with a higher percent of CO_2 ($p<0.001$) and a lower percent of CH_4 ($p<0.001$) in total gas than faunation through all the incubation times. Similarly, defaunation resulted in lower ratio of CH_4 to CO_2 plus CH_4 ($p<0.001$) and CH_4 to CO_2 ($p<0.001$) than faunation during the whole incubation time.

After 24 h incubation, interactions between defaunation and forage feeds were observed in total gas ($p<0.01$), CO_2 ($p<0.01$), CH_4 ($p<0.05$) and EDDM ($p<0.001$, Table 4).

Ⅳ. DISCUSSION

The effective degradability (ED) in the rumen is generally used to measure forage quality, because it can be considered as an assessment of the energy content from forages and typically predicting from forage fiber content. Decrease in fiber digestion has been widely reported after removal of protozoa (Ushida and Jouany, 1990). A general explanation for this is that protozoa are actively involved in fiber digestion by ingesting the feed particles (Akin and Amos, 1979) as well as by secretion of hydrolytic enzymes (Coleman, 1986), thus accounting for 20% of the fiber digestion in the rumen (Dijkstra and Tamminga, 1995). Wolin et al. (1997) reported that fiber degradation is closely associated with methanogenesis and protozoa can activate

Table 4. Effects of forage sources and defaunation on gas production

Items	Treatments								SEM[1]	Effects		
	Faunation				Defaunation							
	Cellulose	Rice straw	Ryegrass	Tall fescue	Cellulose	Rice straw	Ryegrass	Tall fescue		Feed (F)	Def.[2] (D)	F×D[3]
	3 h											
Total gas (ml)	25.33^{e}	36.00^{bc}	42.33^{a}	39.67^{ab}	20.33^{f}	30.67^{d}	36.67^{bc}	33.00^{cd}	1.258	***	***	NS
CO_2 (ml)	17.22^{d}	34.99^{bc}	30.84^{a}	28.66^{ab}	16.18^{d}	23.30^{c}	30.38^{a}	26.90^{abc}	1.149	***	NS	NS
CH_4 (ml)	6.82^{b}	8.81^{a}	10.04^{a}	8.87^{a}	3.52^{d}	5.30^{c}	5.30^{c}	4.48^{cd}	0.365	***	***	NS
CO_2 % in total gas	67.91^{c}	69.40^{c}	72.87^{bc}	72.26^{bc}	79.78^{ab}	76.14^{abc}	82.69^{a}	81.63^{a}	1.942	***	***	NS
CH_4 % in total gas	26.90^{a}	24.47^{ab}	23.74^{ab}	22.35^{b}	17.54^{c}	17.22^{c}	14.48^{c}	13.60^{c}	1.077	***	***	NS
$CH_4/(CH_4+CO_2)$	0.284^{a}	0.261^{ab}	0.245^{b}	0.237^{b}	0.179^{c}	0.185^{c}	0.149^{d}	0.143^{d}	0.009	***	***	NS
CH_4/CO_2	0.397^{a}	0.353^{b}	0.325^{b}	0.311^{b}	0.219^{c}	0.227^{c}	0.176^{cd}	0.167^{d}	0.014	***	***	NS
	6 h											
Total gas (ml)	46.33^{b}	66.33^{a}	73.00^{a}	70.67^{a}	44.33^{b}	65.33^{a}	71.33^{a}	66.33^{a}	1.736	***	NS	NS
CO_2 (ml)	33.21^{c}	48.04^{b}	53.21^{ab}	51.51^{ab}	36.80^{c}	53.43^{ab}	57.69^{a}	53.66^{ab}	1.872	***	*	***
CH_4 (ml)	11.92^{b}	16.80^{a}	16.93^{a}	17.89^{a}	6.817^{c}	10.82^{b}	10.03^{b}	10.80^{b}	0.717	***	***	NS
CO_2 % in total gas	71.71^{b}	72.41^{b}	72.89^{b}	72.89^{b}	83.12^{a}	81.73^{a}	80.73^{a}	80.90^{a}	1.560	***	***	NS
CH_4 % in total gas	25.74^{a}	25.33^{a}	25.19^{a}	25.32^{a}	15.42^{b}	16.47^{b}	14.01^{b}	16.29^{b}	0.918	***	***	NS
$CH_4/(CH_4+CO_2)$	0.264^{a}	0.259^{a}	0.241^{a}	0.258^{a}	0.156^{b}	0.167^{b}	0.148^{b}	0.168^{b}	0.006	***	***	NS
CH_4/CO_2	0.359^{a}	0.350^{ab}	0.318^{b}	0.347^{b}	0.185^{c}	0.201^{c}	0.174^{c}	0.201^{c}	0.009	***	***	NS
	12 h											
Total gas (ml)	92.00^{c}	113.67^{a}	120.33^{a}	118.67^{a}	73.00^{d}	97.00^{bc}	104.00^{b}	98.00^{bc}	2.227	***	***	NS
CO_2 (ml)	66.01^{c}	81.00^{ab}	85.89^{a}	84.55^{a}	60.19^{d}	76.45^{b}	84.91^{a}	80.79^{ab}	1.679	***	**	NS
CH_4 (ml)	23.26^{b}	27.38^{a}	28.56^{a}	28.38^{a}	11.02^{d}	15.47^{c}	14.81^{c}	14.38^{c}	0.711	***	***	NS
CO_2 % in total gas	71.73^{b}	71.25^{b}	71.39^{b}	71.33^{b}	82.44^{a}	78.78^{a}	81.71^{a}	82.44^{a}	1.059	***	***	NS
CH_4 % in total gas	25.32^{a}	24.08^{a}	23.73^{a}	23.90^{a}	15.09^{b}	15.95^{b}	14.24^{b}	14.68^{b}	0.471	***	***	NS
$CH_4/(CH_4+CO_2)$	0.261^{a}	0.253^{a}	0.249^{a}	0.251^{a}	0.155^{c}	0.168^{b}	0.148^{c}	0.151^{c}	0.005	***	***	NS
CH_4/CO_2	0.353^{a}	0.338^{a}	0.332^{a}	0.336^{a}	0.183^{b}	0.203^{b}	0.174^{b}	0.178^{b}	0.008	***	***	NS
	24 h											
Total gas (ml)	157.67^{c}	167.33^{b}	180.67^{a}	170.00^{b}	93.67^{e}	125.33^{d}	131.33^{d}	124.67^{d}	2.444	***	***	**
CO_2 (ml)	111.70^{b}	122.88^{a}	128.14^{a}	123.82^{a}	73.46^{d}	99.90^{c}	107.57^{bc}	101.28^{c}	2.259	***	***	**
CH_4 (ml)	39.62^{a}	40.29^{a}	42.22^{a}	41.31^{a}	14.22^{c}	21.65^{b}	18.72^{b}	20.24^{b}	0.962	***	***	*
CO_2 % in total gas	70.84^{b}	73.44^{b}	70.96^{b}	72.83^{b}	78.43^{a}	79.75^{a}	81.92^{a}	81.23^{a}	1.181	***	***	NS
CH_4 % in total gas	25.13^{a}	24.08^{a}	23.37^{a}	24.30^{a}	15.18^{cd}	17.23^{b}	14.24^{d}	16.24^{bc}	0.531	***	***	NS
$CH_4/(CH_4+CO_2)$	0.262^{a}	0.247^{a}	0.248^{a}	0.250^{a}	0.162^{bc}	0.177^{b}	0.148^{c}	0.167^{bc}	0.005	***	***	*
CH_4/CO_2	0.355^{a}	0.328^{a}	0.330^{a}	0.334^{a}	0.194^{bc}	0.216^{b}	0.174^{c}	0.200^{bc}	0.008	***	***	*

[1] SEM, Standard error of means; [2] Def.: defaunation; [3] F × D, interaction between defaunation and various forages.
[a,b,c,] Means in the same row with different superscripts differ regardless of defaunation; * p<0.05; ** p<0.01; *** p<0.001; NS = Non significant.

fibrolytic bacteria to stimulate fiber digestion by interspecies hydrogen transfer. The synthesis of methane can effectively avoid an accumulation of H_2 in the rumen which in turn can inhibit electron transfer reaction of NADH dehydrogenase, suppressing rumen fermentation and fiber degradation (Morgavi et al., 2010). Thus, it is assumed that defaunation reducing methane production in the present study might be due to indirect shift in an inefficient pathway of H_2 metabolism, leading a higher partial pressure of hydrogen, and this, thereby, inhibiting activities of cellulolytic enzymes and lowering of fiber degradability. In addition, the three forages from defaunation group differed in fiber degradation. As expected, ryegrass and tall fescue had relatively higher fiber degradability than rice straw. It might be related to high lignin content in rice straw compared with ryegrass and tall fescue (Ohet al., 1971). Lignin is a

primary barrier in the ruminal degradation of forage sources by rumen microbes (Baker, 1973). The results of those studies are consistent with ours that pure cellulose used in the present study showed a much high DM degradability than forages irrespective of defaunation. In the present study, defaunation increased pH of the culture solution for both forages and cellulose, and our finding is in line with previous report (Chaudhary et al., 1995).

It is well established that defaunation consistently decreased NH_3-N concentration (Kiran and Mutsvangwa, 2010). However, the result of NH_3-N concentration obtained from this study was completely in contrast to those of the previous reports. An explanation for this might be due to autolysis of protozoa (Ankrah et al., 1990) or the eliminated protozoa as microbial protein source was believed to be digested by the other rumen microbes, leading to high NH_3-N concentration after defaunation.

Microbial fermentation of forage is mainly converted into energy in the form of volatile fatty acids (VFAs) in ruminant, and VFA concentration is positively related to EDDM. In the present study, defaunation significantly decreased total VFA concentration after 24 h incubation. As mentioned earlier, modification of fermentation pattern by defaunation negativity led to the accumulation of H_2 and finally suppressed rumen fermentation, thus the decreased total VFA could mostly be explained by a decreased EDDM up to 24 h of incubation. Simultaneously, defaunation also changed the VFA profile in the culture solution, resulting in a lower molar proportion of C_2 and C_4 but a higher proportion of C_3 and consequently leading to lower C_2 to C_3 ratio than faunation. The current results were in agreement with earlier study (Eugène et al., 2004), where an increase in C_3 was frequently accompanied by a decrease in C_2 and C_4. Molar proportions of C_3 varied inversely with C_4, and C_2 maybe coherent with removal of protozoa which lead to changes in microbial groups and alteration of microbial fermentation, thus led to a shift in the balance of H_2 metabolism between C_3 and methane production in defaunation group. Furthermore, ryegrass showed similar results in comparison with cellulose, and seemed to be more potent in increasing total VFA concentration than both rice straw and tall fescue. This might be associated with the relatively low degree of lignification and high buffer solubility in ryegrass (Jin et al., 2012). It can be indicated that degradation of ryegrass is less limited by lignin and more rapidly available for rumen microbes, contribution to more VFA production.

The *in vitro* gas production technique has been extensively used to estimate nutritive value of forages (Williams, 2000). Defaunation reduced total gas production from cellulose and forages (Table 4). The strong relationship between total gas production and EDDM has been previously reported (Jin et al., 2012). Regardless of defaunation, more gas was produced from forages than from cellulose (Table 4). This can be explained by the presence of non-fibrous ingredient in the forages which was contributed to the additional gas production. High percent of CO_2 but low percent of CH_4 in the total gas was found in defaunation group in the current study (Table 4). One of possible reasons for this may be due to the pathway of methanogenesis, which was selected by methanogens for utilizing H_2 to reduce CO_2 to CH_4 (Finlay et al., 1994). Rumen protozoa are known to have symbiotic relationships involving interspecies hydrogen transfer with methanogens in which methanogen reduces carbon dioxide to methane with transferred hydrogen from protozoa (Hook et al. 2010). Thus, it indicated that decrease in methane production might be accompanied with accumulation of CO_2. For the same reason, lower ratios of $CH_4/(CH_4+CO_2)$ and CH_4/CO_2 from defaunation were observed. Hook et al. (2010) suggested that defaunation may also decrease the protozoa-associated methanogen population, and therefore, decrease the methane production within in the rumen. Also, the removal of protozoa from the rumen not only decreased methane emission but had negative effect on fiber digestion (Morgavi et al., 2010), and defaunation has been shown to reduce methane production by 30%~45% (Hegarty, 1999). In the present study, defaunation markedly reduced CH_4 production from forages and cellulose compared with faunation. Inhibition of interspecies H_2 transfer by defaunation may indirectly result in an altered pattern of hydrogen availability for VFA production, which should also reduce methane production as an alternative pathway for electron sink was available for C_3 production (Morgavi et al., 2008). Li et al. (2009) have confirmed that reduced methane production with increase in C_3 production may be attributed to the competition with methanogens for the

available H_2. These findings are in line with our results that low methane production from defaunation was accompanied by an increased C_3 production. Furthermore, reduced methane production by defauntion is closely related to decreased fiber digestion which was in agreement with the previous report (Morgavi et al., 2010). Hungate et al. (1970) reported that methanogen uses the hydrogen and carbon dioxide produced from carbohydrate fermentation. By removing hydrogen methanogens help the microorganisms involved in fermentation to function optimally and support the complete oxidation of substrate (Sharp et al., 1998). Finally, if the hydrogen which is fermentation end product is not removed, it can inhibit metabolism of rumen microorganisms (Sharp et al., 1998) or stimulate alternative hydrogen sinks such as C_3 production, bio-hydrogenation of unsaturated fatty acids and nitrate reduction (Tandon et al., 2005). Our results demonstrated that reduced methane production might increase the hydrogen in the rumen, then stimulated hydrogen sink toward production of C_3 (Table 3 and Table 4).

Based on the results obtained from the present study defaunation modified fermentation pattern and shifted into an inefficient pathway of hydrogen metabolism which negatively affected metabolic activities of rumen microorganisms, thus resulted in a lower ED, changes in the VFA profile, and simultaneously reduction of methane emission.

Ⅴ. ACKNOWLEDGEMENTS

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