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Effects of Defaunation on Fermentation Characteristics, Degradation of Ryegrass Hay and Methane Production by Rumen Microbes *In Vitro* When Incubated with Plant Oils

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ABSTRACT

This study was conducted to examine the effects of defaunation (removal of live protozoa) on fermentation characteristics, degradation of ryegrass hay and CH_4 (methane) production by rumen microbes when incubated with plant oils (SO, sunflower oil and LO, linseed oil) *in vitro*. Sodium lauryl sulfate (0.000375 g/ml) as a defaunation reagent was added into the culture solution and incubated anaerobically up to 24 h at 39℃. pH from defaunation was increased for all treatments from 6 h incubation times ($p<0.01$-0.001) compared with those from fauantion. Concentration of ammonia-N from defaunation is higher than that from faunation at 3 h ($p<0.001$), 12 h ($p<0.05$) and 24 h ($p<0.001$) incubation times. Defaunation decreased ($p<0.01$-0.001) total volatile fatty acid concentration at all incubation times. Molar proportions of C_2 (acetate, $p<0.05$-0.001) and butyrate ($p<0.01$-0.001) were also decreased by defaunation at all incubation times. Molar proportion of C_3 (propionate), however, was increased by defaunation at all incubation times ($p<0.001$). Thus the rate of C_2 to C_3 was decreased by defaunation at all incubation times ($p<0.001$). Defaunation decreased ED (effective degradability) of dry matter ($p<0.001$) and ED of neutral detergent fiber ($p<0.001$) of ryegrass hay. Defaunation decreased total gas, CH_4 production, CH_4 % in total gas and CH_4/CO_2 at all incubation times ($p<0.001$). Oil supplementation decreased total gas ($p<0.05$-0.001), CH_4 production ($p<0.001$) and CH_4 % in total gas ($p<0.001$) compared with control at all incubation times. The result of this study showed that defaunation combined with oil supplementation may cause an alteration of microbial communities and further medicate the fermentation pattern, resulting in both reduction of degradation of ryegrass hay and CH_4 production. No difference, however, was observed in all the examinations between SO and LO.
(**Key words :** Defaunation, Ryegrass hay, Soybean oil, Linseed oil, Methane)

Ⅰ. INTRODUCTION

Rumen protozoa are believed to actively participate in the fermentation processes of the diet in the rumen (Hungate, 1966), and they contribute to 20% of fiber degradation (Dijkstra and Tamminga, 1995). The ruminal protozoa are also strongly related to CH_4 (methane) production during the ruminal fermentation. Removal of protozoa (defaunation) clearly reduced CH_4 production when incubated with starchy feeds (Qin et al., 2012) and with forages (Qin et al., 2013). A symbiotic relationship between ruminal ciliate protozoa and methanogens was uncovered by Finlay et al. (1994), and they indicated that the symbiotic methanogens associated with the protozoa may account for 37 % of the total CH_4 production.

Meanwhile, lipid supplementation to the diet has negatively affected digestibility both in the rumen and hind gut of cattle (Song et al., 1994), and even feed intake (Song et al., 1992). The ruminal fiber degradation was decreased when more than 4% of lipid was supplemented (Jenkins et al., 1989). Supplementation of crushed flaxseed (Song et al., 1994) and oil infusion to the rumen of sheep (Song et al.,

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1999) clearly decreased ruminal NDF (neutral detergent fiber) degradation. Ruminal degradation rate of the diet was decreased with increased degree of unsaturation (Huang et al., 1999). Unlikely to the bacteria, ruminal protozoa may have a minor contribution in bio-hydrogenation of poly unsaturated fatty acid as well as in lipid degradation (Dawson and Kemp, 1969).

This study was conducted to examine the effect of defaunation on fermentation characteristics, degradation of ryegrass (*Lolium multiflorum*) hay and CH_4 production by rumen microbes when incubated with plant oils which are different in fatty acid composition.

Ⅱ. MATERIALS AND METHODS

1. *In vitro* incubation

Rumen contents were obtained 2 h after the morning feeding (09:00) from three cannulated Holstein cows fed 9 kg/d total diets daily (7 kg concentrate and 2 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 buffer (McDougall, 1948). Sodium lauryl sulfate (0.000375 g/ml, Sigma, L5750) as a defaunation reagent was added into the mixed culture solution to remove ruminal protozoa (Dohme et al., 1999). One gram of ($C_{18:2}$) linoleic acid -rich SO (sunflower oil) or ($C_{18:3}$) (linolenic acid) -rich LO (linseed oil) with 0.4 g albumin emulsifier each dissolved in 1 ml buffer solution was added to 80 ml culture solution in 160 ml bottle. Culture solution without any supplements was also prepared for incubation (control). One gram of ground ryegrass (*Lolium multiflorum*) hay through 1 mm screen on a DM (dry matter) basis as substrate was prepared in a nylon bag (5 × 5 cm, a pore size 50 μm) and was added to the mixed culture solution of presence of protozoa (faunation) or absence of protozoa (defaunation) in the bottle. The bottles were sealed with rubber stoppers, and then were incubated anaerobically in a shaking incubator (VS-8480SR, VISON Science, Bucheon, Korea) at a speed of 135 rpm/min up to 24 h at 39℃. The *in vitro* incubation was made 3 times in duplicate under similar conditions. Chemical composition of ryegrass hay is shown in Table 1 and composition of major fatty acids of plant oils was shown in Table 2.

Table 1. Chemical composition of ryegrass hay added into the culture solution (%, DM basis)

Substrate	Chemical composition (%, DM basis)			
	CP	EE	NDF	Ash
Ryegrass hay	4.14	2.05	69.48	5.16

Table 2. Fatty acid composition of plant oils added into the culture solution (%, total fatty acid)

	Sunflower oil	Linseed oil
$C_{12:0}$	0.00	0.00
$C_{16:0}$	6.49	5.09
$C_{18:0}$	3.59	3.82
$C_{18:1}$	26.13	20.61
$C_{18:2}$	62.38	15.70
$C_{18:3}$	0.10	54.30
$C_{20:0}$	0.26	0.24
$C_{22:0}$	0.70	0.15
Other fatty acids	1.10	0.81

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 VFA (volatile fatty acid) analyses. Ammonia concentration was determined by the method of Fawcett and Scott (1960) using a spectrophotometer (Optizen 3220UV, Mecasys Co. Ltd., Daejeon, Korea). 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. The VFA concentration was determined by a gas chromatograph (GC, HP5890 series II, Hewlett Packard Co. USA) equipped with a FID (flame ionization detector). The oven temperature for VFA analysis was 120°C, and the temperatures of the injector and detector were maintained at 170°C and 200°C, respectively. A 30-m fused silica capillary column (HP-FFAP, 19091F-

112, 0.32 mm i.d. USA) was used. Ultra high purity He was used at a flow rate of 50 ml/min. Identification and quantification were carried out by the external standard method following injection of a VFA standard (Supelco 47058, WSFA-4, USA) of known concentration. Total gas production was 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 CH_4 and CO_2 by gas chromatography (YL 6100 GC, Young Lin Instrument Co., Korea) equipped with FID and TCD (thermal conductivity detector). 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 N_2 (nitrogen) gas was used as carrier gas at a flow rate of 30 ml/min. The nylon bag containing feed residue after incubation was washed with tap water and dried at 60℃ for 48 h in the drying oven to measure DM degradation. The CP (crude protein), EE (ether extract), and ash of feeds (ryegrass hay) prior to incubation and feed residue after incubation were analyzed according to AOAC methods (1995). The NDF (neutral detergent fiber) was analyzed by the methods of Van Soest et al. (1991).

3. Estimation of effective degradability *in vitro*

Percent disappearance of DM and NDF at each incubation time was calculated from the remained portion 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*. Non-linear parameters a, b and c were estimated by an iterative least square procedure to calculate effective degradability of DM (EDDM) and NDF (EDNDF) according to the following equation (Ørskov and McDonald, 1979): Effective degradability = a + (b×c)/(c+r)

Where 'r' is the fractional outflow rate and a hypothetical fractional outflow rate of 0.05 h was used for estimation of effective degradability.

4. Statistical analyses

The present study was shown as 2×3 factorial design that represent two fractions (faunation and defaunation) and three treatments (control, SO or LO supplementation).

Data were analyzed using the GLM (general linear models) procedure of SAS (V 9.1, 2002). Six 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 6 (treatments) × 3 (times) = 18 observations. The 18 observations obtained were subjected to least squares analysis of variance according to the following models:

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

Where Y_{ijk} is observation, μ is the overall mean, τ_i is the effect of oil treatment (i=1-3), S_j is microbial effect (j=1-2), O_k is the jth incubation time, $(\tau_i \times S_j)$ = interaction effect between oil and microbe and ε_{ijk} is the error term. Data between treatments were compared by S-N-K (Steel and Torrie, 1980) and significant differences were declared at $p<0.05$.

Ⅲ. RESULTS

Prior to each incubation, it was found that live protozoa were virtually absent when the culture solution was examined under the microscope, thus it was confirmed that sodium lauryl sulfate could be an effective agent against protozoa. The effect of oils or defaunation on pH, NH_3-N and VFA are presented in Table 3.

pH from defaunation was increased for all treatments from 6 h incubation times ($p<0.01$-0.001) compared with those from fauantion. Concentration of ammonia-N from defaunation is higher than that from faunation at 3 h ($p<0.001$), 12 h ($p<0.05$) and 24 h ($p<0.001$) incubation times. Defaunation decreased ($p<0.01$-0.001) total VFA concentration at all incubation times. Molar proportions of C_2 (acetate, $p<0.05$-0.001) and butyrate ($p<0.01$-0.001) were also decreased by defaunation at all incubation times. Molar proportion of C_3 (propionate), however, was increased by

Table 3. pH, ammonia-N concentration, and concentration and proportions of major VFAs in the culture solution as influenced by presence (faunation) or absence (defaunation) of protozoa as associated with plant oils

	Faunation			SEM[1]	Pr>F[2]	Defaunation			SEM	Pr>F	Effects[3]		
	Control	SO	LO			Control	SO	LO			F vs D	C vs O	O×D
3h													
pH	6.65	6.66	6.67	0.010	0.378	6.68	6.69	6.69	0.021	0.903	NS	NS	NS
NH_3-N	12.16	11.96	11.80	0.229	0.575	12.60	12.78	13.09	0.181	0.234	***	NS	NS
Total VFA (mmoles/100ml)	61.12	60.53	60.49	0.416	0.520	60.08	58.83	59.31	0.398	0.164	**	NS	NS
Molar proportion (mmoles/100mmoles)													
Acetate (C_2)	64.09	63.91	64.13	0.549	0.955	63.50^a	63.37^a	62.32^b	0.243	0.026	*	NS	NS
Propionate (C_3)	18.37	19.01	18.99	0.299	0.296	21.64^b	21.60^b	22.19^a	0.085	0.005	***	NS	NS
Butyrate (C_4)	11.70	13.23	13.37	0.494	0.097	11.53	11.60	12.06	0.183	0.164	**	*	NS
C_2/C_3	3.49	3.36	3.38	0.058	0.291	2.94^a	2.93^a	2.81^b	0.011	0.0003	***	*	NS
6h													
pH	6.45	6.49	6.46	0.021	0.509	6.51	6.51	6.52	0.017	0.948	**	NS	NS
NH_3-N	16.35	16.30	16.33	0.407	0.997	15.65	16.08	17.53	0.595	0.143	NS	NS	NS
Total VFA (mmoles/100ml)	73.89	72.12	72.41	0.533	0.114	70.30	70.77	69.31	0.528	0.218	***	NS	NS
Molar proportion (mmoles/100mmoles)													
Acetate (C_2)	63.77	63.07	62.86	0.325	0.200	60.57	59.61	60.18	0.342	0.214	***	NS	NS
Propionate (C_3)	19.07	19.13	20.38	0.389	0.094	24.02	25.31	25.25	0.422	0.126	***	*	NS
Butyrate (C_4)	13.34	13.44	13.50	0.441	0.967	10.97	10.84	10.48	0.292	0.494	***	NS	NS
C_2/C_3	3.34	3.30	3.09	0.053	0.031	2.52	2.36	2.39	0.051	0.114	***	**	NS
12h													
pH	6.23	6.24	6.25	0.026	0.893	6.40	6.37	6.36	0.021	0.460	***	NS	NS
NH_3-N	26.50	24.14	24.88	0.769	0.1654	26.50	26.75	27.24	0.587	0.585	*	NS	NS
Total VFA (mmoles/100ml)	91.13^a	89.57^a	87.24^b	0.552	0.007	83.70	85.39	83.94	0.458	0.079	***	**	**
Molar proportion (mmoles/100mmoles)													
Acetate (C_2)	62.53^a	60.48^b	60.40^b	0.267	0.002	58.11	58.35	57.03	0.450	0.290	***	**	NS
Propionate (C_3)	18.99^b	21.24^a	21.28^a	0.356	0.006	23.33^b	24.95^a	24.16^{ab}	0.354	0.048	***	***	NS
Butyrate (C_4)	13.10	12.56	13.33	0.197	0.080	11.99	10.80	11.61	0.409	0.191	***	*	NS
C_2/C_3	3.29^a	2.85^b	2.84^b	0.055	0.002	2.49	2.30	2.36	0.048	0.069	***	***	*
24h													
pH	5.90	6.05	6.08	0.052	0.106	6.37	6.31	6.31	0.017	0.116	***	NS	*
NH_3-N	34.02	31.81	32.48	0.986	0.335	36.37	37.09	36.76	1.117	0.901	***	NS	NS
Total VFA (mmoles/100ml)	109.52^a	101.51^b	101.39^b	0.898	0.001	90.74^b	94.32^a	93.63^a	1.665	0.007	***	**	***
Molar proportion (mmoles/100mmoles)													
Acetate (C_2)	62.50^a	59.85^b	58.98^b	0.562	0.011	58.84^a	56.42^b	56.55^b	0.833	0.005	***	***	NS
Propionate (C_3)	19.74^b	21.43^a	21.60^a	0.538	0.028	21.38^b	24.39^a	24.29^a	0.855	0.010	***	***	NS
Butyrate (C_4)	13.17	12.81	13.23	0.470	0.801	11.67	11.21	11.67	0.389	0.642	***	NS	NS
C_2/C_3	3.17^a	2.78^b	2.75^b	0.069	0.009	2.72^a	2.44^b	2.59^b	0.128	0.008	***	***	NS

[1] SEM, standard error of means.
[2] Pr>F, probability level; [a,b,c] Means in the same row with different superscripts differ.
[3] F vs D, faunation vs defaunation; C vs O, control vs oil regardless of defaunation; O × D, interaction between oil supplementation and defaunation.
* $p<0.05$; ** $p<0.01$; *** $p<0.0001$; NS = Non significant.

Table 4. Degradation parameters (a, b, and c) and effective degradability (ED) of ryegrass hay in the culture solution as influenced by presence (faunation) or absence (defaunation) of protozoa as associated with plant oils

	Faunation			SEM[1]	Pr>F[2]	Defaunation			SEM	Pr>F	Effects[3]		
	Control	SO	LO			Control	SO	LO			F vs D	C vs O	O×D
a	7.34	7.14	7.24	0.130	0.594	6.67	6.01	6.08	0.271	0.242	***	NS	NS
b	42.7	39.55	40.6	0.839	0.093	30.6^{b}	34.3^{a}	33.9^{a}	0.256	0.0001	***	NS	***
c	0.18^{b}	0.20^{a}	0.18^{b}	0.003	0.016	0.20	0.20	0.19	0.005	0.390	*	*	NS
EDDM	40.8	38.7	39.1	0.704	0.017	31.6^{b}	33.4^{a}	32.7^{a}	0.402	0.015	***	NS	***
a	5.05	5.76	4.62	0.335	0.129	3.34	4.07	3.65	0.265	0.232	***	*	NS
b	48.42^{a}	45.39^{b}	46.40^{ab}	0.634	0.038	27.82^{b}	30.65^{a}	31.86^{a}	0.704	0.017	***	NS	***
c	0.08	0.09	0.09	0.011	0.970	0.20	0.28	0.18	0.046	0.364	***	NS	NS
EDNDF	35.90	34.29	34.24	0.785	0.114	25.54^{b}	29.56^{a}	28.66^{a}	0.556	0.005	***	NS	***

[1] SEM, standard error of means.
[2] Pr>F, probability level; [a,b,c] Means in the same row with different superscripts differ.
[3] F vs D, faunation vs defaunation; C vs O, control vs oil regardless of defaunation; O × D, interaction between oil supplementation and defaunation.
* p<0.05; *** p<0.0001; NS = Non significant.

defaunation at all incubation times (p<0.001). Thus the rate of C_2 to C_3 was decreased by defaunation at all incubation times (p<0.001). Frequency of oil effect in difference of VFA composition was not consistent by incubation times while interaction between oil supplementation and defaunation was seldom appeared in most observation items at most incubation times.

Defaunation decreased values of degradation parameters a and b (p<0.001), and *in vitro* EDDM (p<0.001) of ryegrass hay compared with relevant values of faunation (Table 4). Similar trends to EDDM were observed in ED of NDF degradation. Oil supplementation only increased degradation parameter c of DM and degradation parameter a of NDF. Interaction between oil supplementation and defaunation was found from degradation parameter b (p<0.001), and EDs of DM and NDF (p<0.001).

Defaunation decreased total gas, CH_4 production, CH_4 % in total gas and CH_4/CO_2 at all incubation times (p<0.001), and CO_2 production from 12 h incubation times (p<0.001) while clearly increased CO_2 % in total gas at all incubation times (p<0.001, Table 5). Oil supplementation decreased total gas (p<0.05-0.001), CH_4 production (p<0.001) and CH_4 % in total gas (p<0.001) at all incubation times, and CH_4/CO_2 (p<0.001) from 6 h incubation times while increased CO_2 production (p<0.05-0.001) and CO_2 % in total gas (p<0.001) at all incubation times compared with corresponding values of control. Interactions between oil supplementation and defaunation were observed mainly from production of total gas (p<0.001), CO_2 (p<0.001) and CH_4 (p<0.01-0.001) at 12 h and 24 h incubation times.

Ⅳ. DISCUSSION

Kiran and Mutsvangwa (2010) showed that elimination of protozoa from the rumen lowered dietary CP degradation and resulted in a decreased concentration of NH_3-N. This indicates the active proteolytic activity of protozoa in the rumen. But in the present study, increased NH_3-N concentration by defaunation may be due to the fact that defaunating agent (sodium lauryl sulfate) killed protozoa cells, and the dead protozoa might be lysed by bacteria, thus led to a higher NH_3-N concentration rather than bacterial CP analyses of ryegrass hay. The present result in concentration of NH_3-N is in agreement with Qin et al. (2012).

The concentration of total VFA is, generally, positively correlated with feed digestibility. In the present study, decreased EDDM of ryegrass hay by defaunation could be closely related with the reduced concentration of total VFA, confirming that rumen protozoa actively participate in the fermentation processes of the diet in the rumen (Hungate, 1966). Oil supplementation in association with faunation in the current study further decreased total VFA concentration.

Table 5. Gas production from culture solution incubated for 24 h and it's major gas composition as influenced as influenced by presence (faunation) or absence (defaunation) of protozoa when associated with plant oils based on ryegrass hay

	Faunation			SEM[1]	Pr>F[2]	Defaunation			SEM	Pr>F	Effects[3]		
	Control	SO	LO			Control	SO	LO			F vs D	C vs O	O×D
						3h							
Total gas (ml)	36.33	36.00	36.33	0.272	0.630	32.33^{b}	32.00^{b}	34.00^{a}	0.192	0.0007	***	***	**
CO_2 (ml)	23.09	23.86	23.71	0.316	0.264	23.65^{b}	23.70^{b}	25.14^{a}	0.319	0.027	*	*	NS
CH_4 (ml)	10.98	10.12	10.24	0.400	0.325	6.41^{a}	5.46^{b}	5.92^{ab}	0.215	0.054	***	*	NS
CO_2% in total gas	63.45	66.16	65.25	0.652	0.064	73.19	74.05	73.93	0.574	0.554	***	*	NS
CH_4% in total gas	30.09	28.15	28.18	0.503	0.056	19.83^{a}	17.06^{b}	17.41^{b}	0.502	0.016	***	***	NS
CH_4/CO_2	0.47	0.43	0.43	0.027	0.445	0.27	0.23	0.24	0.010	0.053	***	NS	NS
						6h							
Total gas (ml)	65.00	64.67	65.00	0.509	0.870	60.67^{b}	60.00^{b}	62.33^{a}	0.430	0.021	***	*	NS
CO_2 (ml)	40.19^{b}	43.86^{a}	43.94^{a}	0.507	0.003	43.32	42.92	45.37	0.741	0.116	*	**	*
CH_4 (ml)	20.60	18.63	18.48	0.677	0.122	12.30	10.83	10.22	0.583	0.106	***	*	NS
CO_2% in total gas	61.90^{b}	67.84^{a}	67.58^{a}	0.456	0.0001	71.51	71.49	72.77	0.547	0.245	***	***	***
CH_4% in total gas	31.68^{a}	28.83^{b}	28.41^{b}	0.351	0.001	20.31^{a}	18.04^{b}	16.94^{c}	0.351	0.0007	***	***	NS
CH_4/CO_2	0.52	0.43	0.42	0.025	0.063	0.28^{a}	0.25^{b}	0.23^{c}	0.007	0.004	***	**	NS
						12h							
Total gas (ml)	114.15^{a}	104.08^{b}	105.31^{b}	0.577	<.0001	85.34^{c}	88.71^{b}	90.07^{a}	0.544	0.0009	***	***	***
CO_2 (ml)	75.53^{a}	72.38^{b}	72.95^{b}	0.464	0.007	63.02^{b}	68.56^{a}	68.89^{a}	0.499	0.0003	***	*	***
CH_4 (ml)	35.36^{a}	29.35^{b}	28.39^{b}	0.539	0.0002	21.01^{a}	18.07^{b}	16.71^{c}	0.318	0.0002	***	***	**
CO_2% in total gas	66.40^{b}	69.58^{a}	69.51^{a}	0.511	0.006	74.20^{b}	77.36^{a}	76.05^{ab}	0.604	0.028	***	***	NS
CH_4% in total gas	31.04^{a}	28.21^{b}	27.00^{b}	0.431	0.002	24.75^{a}	20.40^{b}	18.48^{b}	0.580	0.0007	***	***	NS
CH_4/CO_2	0.47^{a}	0.41^{b}	0.39^{b}	0.016	0.029	0.34^{a}	0.26^{b}	0.24^{b}	0.018	0.027	***	***	NS
						24h							
Total gas (ml)	170.67^{a}	163.33^{b}	159.42^{c}	0.544	<.0001	105.33^{b}	116.83^{a}	117.56^{a}	0.509	<.0001	***	*	***
CO_2 (ml)	114.56	114.54	110.98	1.276	0.152	77.33^{b}	86.97^{a}	87.16^{a}	0.593	<.0001	***	***	***
CH_4 (ml)	51.73^{a}	45.48^{b}	42.60^{c}	0.527	<.0001	25.33^{a}	22.83^{b}	22.78^{b}	0.379	0.005	***	***	***
CO_2% in total gas	67.13^{b}	70.14^{a}	69.82^{a}	0.627	0.028	73.41	74.96	74.48	0.603	0.255	***	**	NS
CH_4% in total gas	30.30^{a}	27.84^{b}	26.79^{b}	0.381	0.002	24.05^{a}	19.69^{b}	19.48^{b}	0.453	0.0006	***	***	NS
CH_4/CO_2	0.45^{a}	0.40^{b}	0.38^{b}	0.010	0.007	0.33^{a}	0.26^{b}	0.26^{b}	0.012	0.013	***	***	NS

[1] SEM, ptandard error of means.

[2] Pr>F, probability level; [a,b,c] Means in the same row with different superscripts differ.

[3] F vs D, faunation vs defaunation; C vs O, control vs oil regardless of defaunation; O × D, interaction between oil supplementation and defaunation.

* p<0.05; ** p<0.01; *** p<0.001; NS = Non significant.

The decreased total VFA concentration as one possible reason may be attributed to the toxicity of plant oils to the protozoa (Ivan et al., 2001), resulting in reduced EDDM and EDNDF. Qin et al. (2012) observed that oil supplementation or defaunation influenced the VFA profiles by shifting in a decreased molar proportion of C_2 and C_4 but an increased molar proportion of C_3, thus resulted in a decreased ratio of C_2 to C_3. It may indicate that these treatments led to alteration of microbial communities and further medication of fermentation pattern. It could also be explained that defaunation and oil supplementation cause a higher partial pressure of hydrogen due to inhibition of CH_4 production

and thus stimulated another hydrogen consuming pathway (such as C_3 production) to maintain optimal hydrogen levels.

Defaunation is known to decrease fiber digestion (Ushida and Jouany, 1990) and protozoa were generally responsible for 20% of fiber digestion (Dijkstra and Tamminga, 1995). The results of the present study are consistent with the finding of above reports. This could be simply attributed to the contribution of fibrolytic enzymes by the rumen protozoa (Takenaka et al., 2004). Meanwhile, EDDM and EDNDF of ryegrass hay in the present study were not affected by oil supplementation in the faunated culture solution. Result of another research (Pavan et al., 2007), however, has shown that oil supplementation reduced NDF digestion as PUFA (polyunsaturated fatty acid) have the direct toxicity to fibrolytic bacteria (Maczulak et al., 1981) through coating feed particle and inhibition of attachment of fibrolytic bacteria on feed particle (Dong et al., 1997). Supplementation of oil in the present study seemed to be less adverse effect on those bacteria. This could be due to the fact that plant oil was added into the culture solution in form of emulsion, which failed to result in prevention of bacterial colonization on the surface of fiber. In addition, oil in combination with defaunation resulted in an increased EDNDF compared with control. This could be closely related to the fact that elimination of protozoa and their associated methanogens led to an inefficient inter-species hydrogen transfer between fibrolytic bacteria and methanogens. Cheng et al. (2009) has proved that the presence of methanogens could maintain a lower hydrogen level and be beneficial for the fiber degradation. Thus, addition of oils rich in PUFA to defaunation culture solution may supply an alternative pathway for hydrogen sink via microbial bio-hydrogenation which indirectly promoted fiber digestion. Ruminal degradation rate of the diet was decreased with increased degree of unsaturation (Huang et al., 1999) but in the current study, no difference was found in degradation of DM and NDF of ryegrass hay between $C_{18:2}$ rich sunflower oil and $C_{18:3}$ rich linseed oil.

Measurement of gas production is considered as indirect indicator for fermentation kinetics. Jin et al. (2012) demonstrated that gas production was closely correlated with the disappearance rate of dietary DM in the rumen. In the present study, defaunation significantly reduced total gas production. This could be attributed to the relative lower EDDM caused by defaunation. It has been recognized for many years that elimination of rumen protozoa can effectually reduce CH_4 production by interruption of interspecies hydrogen transfer between protozoa and their ecto- and endo-symbiotically associated with methanogens (Finlay et al. 1994; Hegarty, 1999; Qin et al., 2012). Meanwhile, oil supplementation was considered to be a promising dietary strategy for reduction in CH_4 production and has been widely reported by *in vitro* (Li et al., 2011) and *in vivo* (Jordan et al., 2006; Li et al., 2009) studies. In the present study, SO or LO supplementation to faunated- or defaunated culture solution reduced CH_4 production. Previous studies have also observed the suppression of CH_4 production by SO (McGinn et al., 2004) and LO (Martin et al., 2008) added to the diet. The toxicity of oil to protozoa could partly be responsible for the reduction of CH_4 production as Nagaraja et al. (1997) observed decrease in protozoal numbers with addition of oil lipid. However, decreased CH_4 production was also found from oil supplementation in combination with defaunation. It may be due to a direct toxic effect of PUFA rich in plant oil on methanogens via consumption of metabolic hydrogen for bio-hydrogenation (Machmüller et al., 2003). Thus, it implied that shift in metabolic hydrogen toward bio-hydrogenation of PUFA may inhibit the normal metabolic activities of all the species of methanogens since the protozoa associated and free-living methanogens showed the different physiological characteristics (Tokura et al., 1997). It also indicated that the effects of oil supplementation are mediated by the simultaneous elimination of protozoa. One of possible reasons might be that defaunation increased the number of *B. fibrisolvens* and thus enhanced the utilization of metabolic hydrogen for bio-hydrogenation, especially on forage-based diet which resulted in fibrolytic bacteria as the predominant rumen microbes.

The result of this study showed that defaunation combined with oil supplementation might cause an alteration of microbial communities and further medicate the fermentation pattern, resulting in both reduction of degradation of ryegrass hay and CH_4 production. No difference, however, was observed in all the examinations between SO and LO.

Ⅴ. ACKNOWLEDGEMENT

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