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Effects of Defaunation on Fermentation Characteristics and Methane Production by Rumen Microbes *In vitro* When Incubated with Starchy Feed Sources

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ABSTRACT: An *in vitro* experiment was conducted to examine the effects of defaunation (removal of protozoa) on ruminal fermentation characteristics, CH_4 production and degradation by rumen microbes when incubated with cereal grains (corn, wheat and rye). Sodium lauryl sulfate as a defaunation reagent was added into the culture solution at a concentration of 0.000375 g/ml, and incubated anaerobically for up to 12 h at 39°C. Following defaunation, live protozoa in the culture solution were rarely observed by microscopic examination. A difference in pH was found among grains regardless of defaunation at all incubation times ($p<0.01$ to 0.001). Defaunation significantly decreased pH at 12 h ($p<0.05$) when rumen fluid was incubated with grains. Ammonia-N concentration was increased by defaunation for all grains at 6 h ($p<0.05$) and 12 h ($p<0.05$) incubation times. Total VFA concentration was increased by defaunation at 6 h ($p<0.05$) and 12 h ($p<0.01$) for all grains. Meanwhile, defaunation decreased acetate and butyrate proportions at 6 h ($p<0.05$, $p<0.01$) and 12 h ($p<0.01$, $p<0.001$), but increased the propionate proportion at 3 h, 6 h and 12 h incubation ($p<0.01$ to 0.001) for all grains. Defaunation increased *in vitro* effective degradability of DM ($p<0.05$). Production of total gas and CO_2 was decreased by defaunation for all grains at 1 h ($p<0.05$, $p<0.05$) and then increased at 6 h ($p<0.05$, $p<0.05$) and 12 h ($p<0.05$, $p<0.05$). CH_4 production was higher from faunation than from defaunation at all incubation times ($p<0.05$). (**Key Words:** Defaunation, Grains, Fermentation, Degradation, Total Gas, CH_4)

INTRODUCTION

Cereal grains as the principal source of energy in the diets are widely used for intensive production of ruminant livestock all over the world. The primary component in grain is starch, which constitutes approximately 60 to 80% of the total ingredients of cereal grains (Huntington, 1997). Numerous studies (Ø rskov, 1986; Huntington et al., 2006) have reported that the rumen is the main site of starch digestion, where the ruminal microorganisms contribute to 60 to 90% of the starch digestion. The rate and extent of starch digestion is also determined by processing approaches and the properties of starch granule (McAllister and Cheng, 1996). Thus, the potential cereal grain digestion is closely associated with the complex resident microflora involved in the digestion process. Additionally, due to their numerical predominance and metabolic diversity, the ruminal bacteria have a key role in starch digestion in the rumen (Cheng et al., 1991). Ruminal protozoa are also believed to participate in the process by ingesting and digesting starch particles (Huntington, 1997). Some researchers (Coleman, 1992; Nagaraja et al., 1992) suggested that the presence of protozoa in the rumen can help ruminants fed high grain diets stabilize rumen fermentation through slowing starch digestion, thus reducing the risk of acidosis.

However, the fermentation of cereal grains in the rumen is generally accompanied by inevitable losses in heat and methane (Huntgate, 1966). It has been proven that protozoa are indirectly involved in methane production due to their close symbiotic relationship with methanogens, which allowed interspecies hydrogen transfer between them (Finlay et al., 1994). Based on this viewpoint, there is wide interest in investigating the effect of the elimination of protozoa (defaunation) on methane production. Some *in vitro* (Newbold et al., 1995) and *in vivo* (Morgavi et al., 2008) studies have also indicated that methanogens

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associated with protozoa contributed to 9 to 25% of methane production in the rumen. Meanwhile, Johnson and Johnson (1995) indicated that the energy loss through methane emission to the atmosphere represents 2 to 12% of the gross energy ingested by ruminants. Therefore, inhibition of methane production in the rumen can benefit not only ruminant production but also the environment (Becker and Wikselaar, 2011). Consequently, research has been focused on manipulating the number of protozoa to inhibit methane production (Schönhusen et al., 2003; Mohammed et al., 2004).

Many studies (Ø rskov, 1986; Offner et al., 2003) are available describing the characteristics of starch degradation of cereal grains in the rumen. But little information is available on the relationship between cereal grain degradation and methane production as influenced by protozoa. A more efficient use of energy in ruminant diets could result from information regarding cereal grain metabolism and methane production in the rumen. The aim of this *in vitro* study, therefore, was to investigate the effects of defaunation of rumen fluid on fermentation, degradation and methane production when incubated with starch rich - grain feeds.

MATERIALS AND METHODS

Preparation of culture solution and its incubation

Rumen contents were obtained 2 h after the morning feeding (09:00) from three ruminally-cannulated Holstein cows fed 9 kg/d total diet daily (7 kg concentrate and 2 kg ryegrass, as fed basis), feeding was done twice (09:00 and 18:00 h) daily, in an equal quantity. 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 s. Culture solution was prepared by mixing 40 ml strained rumen fluid with 40 ml McDougall's artificial saliva. The preparation of the defaunation of the culture solution was done according to the report of Abel et al. (2006). Sodium lauryl sulfate (Sigma, L5750) was added at a concentration of 0.000375 g/ml as a defaunation reagent into the mixed culture solution to remove ruminal protozoa. The experiment was conducted essentially in a 2 (faunation and defaunation)×3 (three kinds of cereal grains) factorial design. Based on an air dried basis the nitrogen free extract (NFE) contents, as determined by proximal analyses using the AOAC (1995) method, were similar for 0.9 g corn, 1 g wheat and 1.1 g rye. These grains were ground through a 1 mm screen (Wiley mill) and were added to the culture solution with all microbes (faunation) or without protozoa (defaunation) in order to supply a similar amount of NFE contents. Degradability of the ground cereal grains was examined by preparing them in a small nylon bags (5×5 cm, with pore size of 50 μm) and placing them in 160 ml bottles containing the mixed culture solution. 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/min up to 12 h at 39°C. The *in vitro* incubation was done 3 times in duplicate each time under similar conditions. The chemical composition of cereal grains added to the culture solution is shown in Table 1.

Table 1. Chemical composition of cereal grains added to the culture solutions (%, DM basis)

Cereal grains	Chemical composition (%, DM basis)			
	CP	EE	NDF	Ash
Rye	11.73	5.16	21.41	2.23
Wheat	9.57	4.89	18.85	1.48
Corn	7.08	3.04	16.05	1.20

Measurement and analysis

Incubation was stopped by removing the bottles from the shaking incubator at 1, 3, 6 and 12 h, and pH of the 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-N concentration was determined by the method of Fawcett and Scott (1960) using a spectrophotometer. The 0.8 ml of 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 1, 3, 6 and 12 h from the culture bottles through the 3-way stopcock connected to bottle using a 50 ml glass syringe. A gas sample was transferred to a 5 ml vacuum tube and analyzed for methane (CH_4) and carbon dioxide (CO_2) by gas chromatograph (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°C and 150°C, and temperatures for FID and TCD detector were respectively kept at 230°C and 150°C. The Nitrogen (N_2) gas was used as the carrier gas at a flow rate of 30 ml/min. Nylon bags containing feed prior to and post incubation were washed with tap water and dried at 60°C for 48 h in a 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 neutral detergent fiber (NDF) was analyzed by the methods of Van Soest et al. (1991).

Estimation of effective degradability *in vitro*

Percent disappearance of DM at each incubation time

was calculated from the portion remaining in the nylon bags after incubation. Disappearance rate was fitted to the equation of Ø rskov 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 were estimated by an iterative least square procedure to calculate effective degradability of DM (EDDM) according to the following equation (Ø rskov and McDonald, 1979):

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

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.

Statiatical analyses

Data were analyzed using the general linear models (GLM) 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:

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

Where Y_{ijk} is dependent variable, μ is the overall mean, τ_i is the fixed effect of treatment (i = 6), S_j is the random effect of repeated time (j = 3), O_k is the jth incubation time and ε_{ijk} is the error term.

Differences among treatments was considered, and the differences between faunation and defaunation was evaluated by pairwise t-test.

RESULTS

At each incubation time, microscopic examination was carried out to observe live protozoa through a 16/0.35 objective, and protozoa were rarely observed after 1h incubation, indicating that live protozoa in the culture solution were almost eliminated by adding sodium lauryl sulfate. The pH of the culture solution decreased in all treatments regardless of defaunation as the incubation time advanced (Table 2). Defaunation significantly decreased ($p<0.01$ to 0.001) pH of the culture solution for all of the grains except 1 h incubation compared with faunation. Differences in pH of the culture solution was found between the grains regardless of defaunation ($p<0.01$ to <0.001). pH of culture solution from wheat and rye was relatively lower ($p<0.001$) than that from corn in both faunation and defaunation at 6 h and 12 h incubations. While pH from corn by faunation was the highest ($p<0.001$), pH from rye by defaunation was the lowest ($p<0.001$) after 12 h incubation.

Ammonia-N concentration of the culture solution increased for all the grains in both faunation and defaunation with advancing incubation times (Table 2). Defaunation increased ammonia-N concentration at 6 h ($p<0.05$) and 12 h ($p<0.05$) incubation times compared with faunation. Ammonia-N concentration in the rye culture was the highest ($p<0.001$) among the grains and then followed by wheat and corn.

Defaunation increased total VFA concentration at 6 h ($p<0.05$) and 12 h ($p<0.01$) incubation time (Table 2) compared with faunation. The total VFA concentration from both faunation and defaunation also increased when incubated with wheat and rye at 6 h ($p<0.001$) and 12 h ($p<0.001$) incubation compared with corresponding values from corn. Furthermore, rye produced more total VFA ($p<0.001$) than wheat and corn at 6 h and 12 h incubation. Defaunation decreased proportions of acetate (C_2) at 6 h ($p<0.05$) and 12 h ($p<0.01$) and butyrate (C_4) at 3 h ($p<0.05$), 6 h ($p<0.01$) and 12 h ($p<0.01$). Meanwhile, C_2 proportion of corn from faunation and defaunation was higher than wheat and rye at 12 h ($p<0.001$) incubation. Defaunation, however, increased C_3 proportion for all cereal grains in comparison with faunation from 3 h incubation ($p<0.01$ to 0.001). Thus, defaunation decreased C_2 to C_3 ratio for all the grains from 3 h incubation ($p<0.05$ to 0.001).

The effect of defaunation for grains on gas production is shown in Table 4. Defaunation firstly decreased total gas production at 1 h ($p<0.05$) and 3 h ($p<0.05$) and CO_2 production at 1 h ($p<0.05$) incubation, but then increased ($p<0.001$) total gas and CO_2 production at 6 h ($p<0.05$, $p<0.05$) and 12 h ($p<0.05$, $p<0.05$) incubation for all grains. Rye in both faunation and defaunation cultures produced the highest amounts of total gas and CO_2 through all incubation times ($p<0.001$) among grains in the present study. Defaunation clearly decreased ($p<0.05$) CH_4 production at all the incubation times compared with faunation. However, corn resulted in a relatively lower ($p<0.001$) CH_4 generation from both faunation or defaunation than wheat and rye during 12 h incubations. In addition, defaunation was associated with a higher percent of CO_2 ($p<0.05$ to 0.001) and a lower percent of CH_4 ($p<0.01$ to 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 ($p<0.01$ to 0.001) and CH_4 to CO_2 ($p<0.01$ to 0.001) than faunation.

Table 2. pH, ammonia-N concentration and concentration and proportions of major VFAs in the culture solution as influenced by faunation or defaunation when incubated with different cereal grains

Items	Treatment						SEM[1]	Pr>F[2]	Pr>|t|[3] F vs D
	Faunation			Defaunation					
	Corn	Wheat	Rye	Corn	Wheat	Rye			
	---------- 1 h ----------								
pH	7.47^{a}	7.35^{b}	7.26^{c}	7.47^{a}	7.45^{a}	7.39^{ab}	0.022	***	NS
Ammonia (mg/100 ml)	12.41^{c}	12.76^{c}	14.28^{ab}	13.46^{bc}	14.72^{a}	14.82^{a}	0.576	*	NS
Total VFAs(mmoles/100 ml)	55.04^{c}	62.15^{ab}	64.16^{a}	56.28^{bc}	56.78^{bc}	61.41^{ab}	1.450	**	NS
Molar proportion (mmoles/100 mmoles)									
Acetate (C_2)	67.59	68.17	66.75	67.18	67.60	67.90	0.457	NS	NS
Propionate (C_3)	17.81	17.90	18.61	18.55	18.29	18.58	0.476	NS	NS
Butyrate (C_4)	11.17	10.78	11.09	10.60	10.82	10.36	0.347	NS	NS
C_2/C_3	3.80	3.81	3.59	3.63	3.70	3.66	0.111	NS	NS
	---------- 3 h ----------								
pH	7.09^{a}	6.93^{ab}	6.84^{b}	7.00^{ab}	6.96^{ab}	6.84^{b}	0.039	**	NS
Ammonia (mg/100 ml)	11.85^{b}	14.73^{a}	15.39^{a}	16.16^{a}	16.92^{a}	17.52^{a}	0.874	**	NS
Total VFAs (mmoles/100 ml)	67.84	69.86	71.66	64.29	68.48	70.75	3.027	NS	NS
Molar proportion (mmoles/100mmoles)									
Acetate (C_2)	68.46	67.38	66.70	66.8	66.80	67.13	0.593	NS	NS
Propionate (C_3)	17.08^{d}	17.74^{cd}	18.16^{bcd}	19.91^{abc}	20.38^{ab}	20.80^{a}	0.617	**	***
Butyrate (C_4)	11.33^{a}	11.29^{a}	11.68^{a}	9.35^{b}	9.18^{b}	8.37^{b}	0.394	***	*
C_2/C_3	4.01^{a}	3.80^{ab}	3.67^{ab}	3.37^{b}	3.29^{b}	3.24^{b}	0.130	**	*
	---------- 6 h ----------								
pH	6.83^{a}	6.70^{b}	6.49^{c}	6.62^{b}	6.19^{d}	6.09^{e}	0.029	***	NS
Ammonia (mg/100 ml)	12.11^{c}	14.79^{bc}	15.89^{ab}	18.26^{a}	18.88^{a}	19.55^{a}	0.879	***	*
Total VFAs (mmoles/100 ml)	81.07^{d}	85.24^{cd}	96.18^{ab}	90.59^{bc}	99.98^{a}	103.21^{a}	2.287	***	*
Molar proportion (mmoles/100 mmoles)									
Acetate (C_2)	67.54^{a}	66.73^{ab}	66.02^{b}	61.76^{d}	62.52^{cd}	63.54^{c}	0.365	***	*
Propionate (C_3)	17.35^{b}	18.07^{b}	18.58^{b}	27.29^{a}	26.75^{a}	26.95^{a}	0.652	***	**
Butyrate (C_4)	12.34^{a}	12.44^{a}	12.20^{a}	7.52^{b}	7.32^{b}	6.67^{b}	0.480	***	**
C_2/C_3	3.90^{a}	3.71^{a}	3.56^{a}	2.26^{b}	2.34^{b}	2.36^{b}	0.116	***	**
	---------- 12 h ----------								
pH	6.48^{a}	6.33^{ab}	6.38^{b}	5.96^{c}	5.68^{d}	5.63^{d}	0.034	***	*
Ammonia (mg/100 ml)	14.11^{c}	17.47^{b}	19.43^{a}	17.63^{b}	20.22^{ab}	21.35^{ab}	0.759	***	*
Total VFAs (mmoles/100 ml)	104.84^{e}	115.81^{d}	114.14^{d}	140.40^{c}	147.53^{b}	157.06^{a}	2.084	***	**
Molar proportion (mmoles/100 mmoles)									
Acetate (C_2)	64.79^{a}	63.45^{b}	62.87^{b}	59.07^{c}	56.42^{d}	56.69^{d}	0.352	***	**
Propionate (C_3)	18.37^{c}	18.86^{c}	19.14^{c}	33.09^{b}	34.92^{a}	35.51^{a}	0.408	***	***
Butyrate (C_4)	14.11^{a}	14.62^{a}	14.79^{a}	5.38^{b}	6.04^{b}	5.27^{b}	0.441	***	***
C_2/C_3	3.54^{a}	3.36^{ab}	3.29^{b}	1.79^{c}	1.62^{c}	1.60^{c}	0.059	***	***

[1] SEM = Standard error of means. [2] Pr>F = Probability level.

[a,b,c] Means in the same row with different superscripts differ regardless of defaunation.

[3] Pr>|t| = Probability level; Comparison between faunation and defaunation treatment (F = Faunation; D = Defaunation).

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

Defaunation significantly increased EDDM of grains (p<0.05) despite no differences in degradation parameters of a, b and c. Within grains, regardless of defaunation, wheat and rye showed a higher EDDM and parameters c (p<0.001) than corn as shown in Table 3.

DISCUSSION

The rate and extent of starch fermentation are influenced by the grain type, the method of cereal grain processing and rumen microbe species (Hale, 1973; Ø rskov, 1986). Some studies have shown that removal of protozoa generally resulted in a higher cereal grain degradation (Mackie et al., 1978; Mendoza et al., 1993). In the present experiment, our results on degradability were similar to those of previous reports. An explanation for this is that protozoa can manipulate the ruminal cereal grain hydrolysis process by engulfing numbers of bacteria to slow ruminal fermentation rates (Kurihara et al., 1978) or by ingesting starch granules and decreasing the accessibility of these

Table 3. Degradation parameters (a, b, and c) and effective degradability (ED) DM of the experimental diets in the culture solution as influenced by faunation or defaunation when incubated with different cereal grains

Parameters[1] and EDDM	Treatment						SEM[2]	Pr>F[3]	Pr>|t|[4] F vs D
	Faunation			Defaunation					
	Corn	Wheat	Rye	Corn	Wheat	Rye			
a	3.05	2.288	1.636	1.756	3.51	2.83	0.450	NS	NS
b	60.28	64.99	66.66	79.09	74.08	71.84	5.018	NS	NS
c	0.096^{c}	0.596^{b}	0.862^{a}	0.084^{c}	0.365^{b}	0.596^{b}	0.070	***	NS
EDDM	40.52^{d}	62.14^{b}	64.37^{ab}	49.76^{c}	68.66^{a}	69.11^{a}	1.321	***	*

[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] Pr>F = Probability level. [a,b,c] Means in the same row with different superscripts differ regardless of defaunation.
[4] Pr>|t| = Probability level; Comparison between faunation and defaunation treatment (F = Faunation; D = Defaunation).
* p<0.05; ** p<0.01; *** p<0.001; NS = Non significant.

Table 4. Gas production in the culture solution as influenced by faunation or defaunation when incubated with different cereal grains

Items	Treatment						SEM[1]	Pr>F[2]	Pr>|t|[3] F vs D
	Faunation			Defaunation					
	Corn	Wheat	Rye	Corn	Wheat	Rye			
	---------- 1 h ----------								
Total gas (ml)	14.83^{c}	17.33^{b}	21.50^{a}	9.92^{d}	10.75^{d}	11.67^{d}	0.563	***	*
CO_2 (ml)	10.78^{c}	12.45^{b}	15.65^{a}	7.96^{e}	8.75^{de}	9.73^{cd}	0.460	***	*
CH_4 (ml)	3.47^{c}	4.24^{b}	5.19^{a}	1.43^{d}	1.57^{d}	1.69^{d}	0.106	***	*
CO_2 % in total gas	72.65^{b}	71.83^{b}	72.79^{b}	80.45^{a}	81.52^{a}	83.54^{a}	1.601	**	**
CH_4 % in total gas	23.40^{a}	24.50^{a}	24.14^{a}	14.45^{b}	15.79^{b}	13.39^{b}	0.644	***	**
$CH_4/(CH_4+CO_2)$	0.244^{a}	0.254^{a}	0.249^{a}	0.152^{b}	0.162^{b}	0.138^{b}	0.010	***	**
CH_4/CO_2	0.323^{a}	0.341^{a}	0.332^{a}	0.180^{b}	0.194^{b}	0.161^{b}	0.006	***	**
	---------- 3 h ----------								
Total gas (ml)	35.17^{c}	44.63^{b}	53.83^{a}	31.47^{c}	42.37^{b}	49.67^{a}	1.365	***	*
CO_2 (ml)	24.25^{d}	30.03^{c}	36.52^{b}	25.83^{d}	34.29^{b}	40.16^{a}	0.990	***	NS
CH_4 (ml)	8.81^{c}	11.10^{b}	13.54^{a}	4.91^{d}	6.66^{cd}	7.00c^{d}	0.635	***	*
CO_2 % in total gas	69.00^{b}	67.87^{b}	67.79^{b}	82.08^{a}	81.00^{a}	80.93^{a}	1.838	***	***
CH_4 % in total gas	24.97^{a}	25.06^{a}	25.11^{a}	15.60^{b}	15.68^{b}	14.08^{b}	1.298	***	**
$CH_4/(CH_4+CO_2)$	0.265^{a}	0.270^{a}	0.270^{a}	0.160^{b}	0.162^{b}	0.149^{b}	0.022	***	**
CH_4/CO_2	0.365^{a}	0.370^{a}	0.371^{a}	0.190^{b}	0.194^{b}	0.174^{b}	0.012	***	**
	---------- 6 h ----------								
Total gas (ml)	62.50^{f}	80.03^{d}	90.50^{c}	72.14^{e}	101.64^{b}	112.00^{a}	1.324	***	*
CO_2 (ml)	41.42^{f}	53.13^{e}	64.33^{c}	58.24^{d}	78.59^{b}	92.86^{a}	1.603	***	*
CH_4 (ml)	17.10^{c}	21.22^{b}	25.10^{a}	11.39^{d}	15.85^{c}	17.03^{c}	0.638	***	*
CO_2 % in total gas	66.22^{c}	66.40^{c}	70.88^{c}	80.80^{ab}	77.29^{b}	82.90^{a}	1.440	***	**
CH_4 % in total gas	27.39^{a}	26.49^{a}	27.74^{a}	15.77^{b}	15.59^{b}	15.21^{b}	0.585	***	**
$CH_4/(CH_4+CO_2)$	0.293^{a}	0.285^{a}	0.281^{a}	0.163^{b}	0.168^{b}	0.155^{b}	0.011	***	***
CH_4/CO_2	0.414^{a}	0.399^{a}	0.391^{a}	0.195^{b}	0.202^{b}	0.183^{b}	0.006	***	***
	---------- 12 h ----------								
Total gas (ml)	109.50^{d}	128.36^{c}	147.17^{b}	125.81^{c}	151.69^{b}	163.33^{a}	1.959	***	*
CO_2 (ml)	77.75^{e}	87.93^{d}	100.03^{c}	101.95^{c}	123.69^{b}	134.45^{a}	2.344	***	*
CH_4 (ml)	29.53^{c}	34.82^{b}	38.68^{a}	19.88^{e}	23.15^{d}	23.72^{d}	0.922	***	*
CO_2 % in total gas	71.01^{b}	68.47^{b}	67.96^{b}	81.01^{a}	81.54^{a}	82.32^{a}	0.953	***	*
CH_4 % in total gas	26.97^{a}	27.14^{a}	26.29^{a}	15.79^{b}	15.26^{b}	14.52^{b}	0.649	***	***
$CH_4/(CH_4+CO_2)$	0.275^{a}	0.284^{a}	0.279^{a}	0.163^{b}	0.158^{b}	0.149^{b}	0.005	***	**
CH_4/CO_2	0.380^{a}	0.397^{a}	0.387^{a}	0.195^{b}	0.187^{b}	0.175^{b}	0.10	***	**

[1] SEM = Standard error of means. [2] Pr>F = Probability level.
[a,b,c] Means in the same row with different superscripts differ regardless of defaunation.
[3] Pr>|t| = Probability level; Comparison between faunation and defaunation treatment (F = Faunation; D = Defaunation).
* p<0.05; ** p<0.01; *** p<0.001; NS = Non significant.

substrates to prevent its immediate and rapid fermentation by bacteria (Coleman, 1986; Coleman, 1992). Thus both of these factors can decrease degradation of cereal grains and our results indicate that the defaunation agent used in the present study showed strong toxicity to rumen protozoa but without effect on bacteria. This selective toxicity can be explained in that the defaunation agent reacts with cholesterol in eukaryotic membranes but not in prokaryotic cells (Wina et al., 2005), causing protozoal cells to rupture and lyse (Kilta et al., 1996). In addition, the three cereal grains used in the present experiment also differed significantly in EDDM regardless of the presence of protozoa. As expected, wheat and rye had relatively higher EDDM than corn, suggesting that wheat and rye starch was more rapidly fermented by ruminal microbes than corn. Similar results were also observed by others (Ø rskov, 1986; McAllister et al., 1990; Lanzas et al., 2007). Decreased EDDM in corn might be simply due to thickness of the protein matrix which coats starch granules (McAllister and Cheng, 1996), and this matrix is relatively difficult to be hydrolyzed by water and enzymes (McAllister et al., 1990). On the other hand, the distribution of starch granules within the kernel varies with cereal grain type (Swan et al., 2006). The starch granules in rye and wheat endosperms seem to be floury and have a relatively small particle size. Consequently, the smaller starch granules have a larger surface area available for microbial and enzymatic starch hydrolysis which results in rapid degradation.

In the present study, defaunation resulted in lower pH compared with faunation, which is in agreement with other research (Nagaraja et al., 1992; Mendoza et al., 1993). The difference in ruminal pH between the faunated and defaunated cultures was related to total VFA concentration. Thus, the increase in total VFA concentration that was observed from defaunation could be responsible for the lower pH.

Some studies (Abel et al., 2006; Kiran and Mutsvangwa, 2010) reported that defaunation generally led to a decrease in NH_3-H concentration. However, our results in the current study are in contrast to the previous reports. One of the possible reasons for this is that the eliminated protozoa in the culture solution were a source of microbial protein and contributed to the additional NH_3-H production.

The rate and extent of DM digestion may influence the proportion of VFA produced in culture solution. In the present *in vitro* study, defaunation firstly tended to slightly decrease the total VFA concentration at 1 h and 3 h incubations, and this may be related to the defaunating process. But after complete elimination of live protozoa from the culture solution, defaunation led to a significant increase in total VFA concentration up to 12 h incubation. Results of the present experiment are in line with some other reports (Nagaraja et al., 1992; Abel et al., 2006). The higher total VFA concentration might be closely related to the increased EDDM by defaunation. Increased total VFA concentration by defaunation might be due to increased bacterial numbers (Nagaraja et al., 1992). It generally has been observed that bacterial populations are larger after defaunation (Hristov et al., 2001), possibly reflecting both decreased engulfing of bacteria by protozoa and the killed protozoa supplying additional substrate for bacterial reproduction, thus resulting in a higher level of total VFA production (Williams and Withers, 1991; Williams and Coleman, 1992). Additionally, defaunation increased the molar proportion of C3 but decreased the molar proportions of C2 and C4. Numerous studies (Williams and Withers, 1991; Nagaraja et al., 1992; Eugene et al., 2004) have also reported that increased C3 was often accompanied by decreases in C2 and C4 proportions. The shift in proportions of VFA may be associated with the selective engulfment of starch-hydrolyzing bacteria by protozoa (Kurihara et al., 1968). It has been indicated that the high C3 proportion may result from increased bacterial numbers and a shift in the predominant bacterial species by defaunation. Thus, defaunation resulted in a lower C2 to C3 ratio compared with faunation. The individual cereal grains appeared with almost the same trend in VFA characteristics between faunation and defaunation. Meanwhile, rye showed similar results to wheat from the defaunation treatment in comparison with corn, which corresponded with its result of EDDM. That would indicate that NFE content in rye may be more rapidly available for rumen microbes, thus stimulating microbial growth and VFA production.

Our results showed a positive correlation for cereal grains between total gas production and EDDM. Chai et al. (2004) suggested that gas measurement can be considered as a method to estimate potential starch degradation. Thus, the increased total gas production which occurred from these cereal grains from both faunation and defaunation can be attributed to the higher EDDM in the culture solution. Furthermore, our results in the present study also showed a higher percent of CO_2 and a lower percent of CH_4 in the total gas from defaunated cultures. This indicated that the increase in total gas production might be accompanied by increased CO_2 production rather than CH_4. Metabolic H_2 consumed by methanogens is the principal pathway for CH_4 generation in the rumen and the quantity of methane generated is closely related to the end products of carbohydrate fermentation (Yane-Ruiz et al., 2010). Castillo et al. (2004) and Li et al. (2009 a, b) reported that increased C3 production resulted in suppressed methane production due to competition with methanogens for the available H_2. These findings are in line with our results that lower methane production from defaunated cultures was accompanied by increased C3 production (Table 2). Additionally, protozoa also play an important role on

methanogensis, which could be related to their participation in H_2 metabolism or their influences on methanogen populations and the species of the microbiota. Based on the results of *in vivo* and *in vitro* trials Hegarty (1999) and Morgavi et al. (2010) summarized that the complete elimination of protozoa from the rumen would result in a 13% decrease in methane production. Defaunation significantly reduced CH_4 production for all the grain feeds in the current study compared with faunation (Table 4). Finlay et al. (1994) described a symbiotic relationship between ciliate protozoa and methanogens that are found inside or in close association with protozoal cells, which has been established to allow an interspecies H_2 transfer between ciliate protozoa and methanogens. This indicates that protozoa indirectly contributed to methane production. In the present study, defaunation decreased CH_4 production by 11% compared with fauantion (Table 4). Therefore, the number and activity of methanogens are believed to be indirectly restricted by defaunation, and the disturbance of the synergy between protozoa and methanogens could be partly responsible for the reduction of CH_4 production. Meanwhile, other similar defauantion experiments have proved that methanogens were not directly affected by adding a defaunation reagent (Dohme et al., 1999), thus indicating that the present defaunation agent could be applied to eliminate protozoa to study its effects on methanogenesis by methanogens. On the other hand, the difference in gas proportions was not found between cereal grains from both faunation and defaunation cultures. One of the possible reasons for these results might be due to the fact that we used the same amount of NFE content for all the cereal grains, thus resulting in a similar establishment of microbial groups.

Based on the results obtained from the present experiment, it is concluded that defaunation can widely modify the *in vitro* fermentation pattern resulting in a higher EDDM, shifts in molar proportion of VFA production and a reduction of methane emission. Modification of the fermentation pattern can be attributed to the change of rumen micro-flora caused by the elimination of protozoa. Besides, according to the metabolic characteristics of cereal grains in the present study, wheat and rye seem to be more sensitive to this modification when compared with corn. However, more detailed studies are needed to conduct to verify these findings.

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