Journal of Animal and Veterinary Advances 12 (10): 1000-1006, 2013
ISSN: 1680-5593
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Effects of Exogenous Fibrolytic Enzyme on *in vitro* Ruminal Fermentation and Microbial Populations of Substrates with Different Forage to Concentrate Ratios

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Abstract: This research was conducted to investigate the effects of exogenous fibrolytic enzyme on *in vitro* ruminal fermentation and microbial populations with substrates in different Forage to Concentrate ratios (F:C). Four levels (0, 40, 80 and 120 U g^{-1} dry matter substrate) of fibrolytic enzyme were supplemented to buffered rumen fluid and incubated with three different F:C ratios (80:20, 50:50 and 20:80) substrates at 39°C. After 24 h incubation, the results showed that in three different F:C ratios, fibrolytic enzyme significantly increased ($p<0.001$) total gas production, *in vitro* dry matter disappearance, concentration of total volatile fatty acid and molar proportion of propionate and decreased ($p<0.001$) pH but it did not significantly ($p>0.05$) affect molar proportion of butyrate. And in the high (F:C 80:20) and medium (F:C 50:50) forage substrates, fibrolytic enzyme increased ($p<0.05$) the molar proportion of propionate and decreased ($p<0.05$) the ratio of acetate to propionate but the effects did not observed in high concentrate (F:C 20:80) substrates. It was also found that fibrolytic enzyme increased ($p<0.05$) the number of *Fibrobacter succinogenes* and methanogens in the F:C 80:20 substrate but there was no significant ($p>0.05$) effects on microbial populations in the F:C 50:50 and 20:80 substrates. The results indicated that the fibrolytic enzyme improved the degradation of substrates and had different effects on the ruminal fermentation pattern and microbial populations under three F:C ratio substrates. The efficiency of fibrolytic enzyme on high and medium forage substrates was greater than low forage substrates.

Key words: Exogenous fibrolytic enzyme, ruminal fermentation, forage to concentrate, *in vitro*, microbial populations

INTRODUCTION

Forage is the most important carbohydrate source in ruminant diet, although rumen microorganisms can produce enzymes to hydrolyze cellulose and hemicellulose in the forage, excessively complex structure of carbohydrates may decrease the efficiency of feed utilization due to insufficient fibrolytic enzymes. Thus, exogenous fibrolytic enzymes as candidate additives for ruminant diets have been studied for a long time. Previous studies reported that fibrolytic enzymes could enhance ruminal degradation and nutrient utilization (Beauchemin *et al*., 2003; Torrentera *et al*., 2005; Giraldo *et al*., 2008b; Arriola *et al*., 2011; Chung *et al*., 2012). However, the effects of fibrolytic enzyme on molar proportions of individual Volatile Fatty Acid (VFA) seemed to be inconsistent in published literatures. Some studies demonstrated that supplementation of exogenous fibrolytic enzymes significantly increased the VFA production and decreased Acetate to Propionate ratios (A:P) (Giraldo *et al*., 2007, 2008a; Gonzalez-Garcia *et al*., 2010). But it was also found that exogenous fibrolytic enzyme did not affect the concentration of total VFA and molar proportions of individual VFA (Chung *et al*., 2012) and increased the molar proportion of acetate in rumen fluid (Beauchemin *et al*., 2000; Gado *et al*., 2009). The effect of fibrolytic enzyme may be influenced by the nature (forage to concentrate ratio) of substrate and conditions of the fermented substrate such as *in vivo*, Rusitec fermentation and batch cultures. In addition to the knowledge, few studies have focused on the effects of exogenous fibrolytic enzyme on rumen microbial populations. Therefore, in the present study, researchers evaluated the effects of fibrolytic enzyme on ruminal fermentation and microbial populations with substrates in different Forage to Concentrate ratios (F:C).

MATERIALS AND METHODS

This study was approved by the Animal Care and Use Committee of the College of Animal Science and Technology of the Northwest A&F University.

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Animals and design: Exogenous fibrolytic enzyme (cellulase) was from *Aspergillus niger* (1.07 unit mg^{-1}, product of Japan, 22178-25G, Sigma Inc.). *In vitro* experiment was conducted as described by Menke and Steingass (1988) with some modifications. Substrate (1,200 mg) was accurately weighed into 500 mL bottle. The ingredient and chemical composition of substrate treatments are shown in Table 1. Fibrolytic enzyme was added at 0, 40, 80, 120 U g^{-1} of DM substrate in three different F:C (80:20, 50:50 and 20:80) with four replicates per treatment. Four fistulated adult goats (mean body weight 55±2 kg) were fed a diet of alfalfa hay and concentrate mixture in 50:50 ratio at 08:00 and 18:00 daily. The rumen liquor was collected from the goats before the morning feeding and transferred into three thermos flasks immediately under anaerobic conditions. The rumen fluid was filtered through four layers of cheesecloth and the filtered rumen fluid was mixed with two volume of buffer under CO_2.

Then, total 120 mL mixture of rumen fluid and buffer were added into each bottle. Each bottle was filled with CO_2 gas, sealed with rubber stoppers and placed in a constant temperature incubation shaker at 39°C for 24 h.

Measurements and analytical methods: After 24 h incubation, all the bottles were cooled in the ice-water to terminate fermentation. The total gas of each bottle was measured using a calibrated syringe with a needle through the rubber stopper. Gas sample in each bottle was collected and stored in the vacuum tube for analysis of methane concentration. The final pH was measured with a pH meter (Sartorius PB-10). To determine total and individual VFA proportions, 1 mL deproteinzing solution of metaphosphoric acid (100 g L^{-1}) and crotonic acid (0.4 g L^{-1}) as an internal standard was added to 1 mL of the fermented fluid and stored at -40°C until analysis.

Samples for VFA were centrifuged at 12,000×g for 15 min to remove protein thoroughly. The supernatant was filtered through a 0.45 μm filter membrane. The VFA was determined by a gas chromatography equipped with FFAP column (30 m×0.25 mm×0.33 μm, Lanzhou Atech, China) and flame ionization detector. The oven temperature was 45°C which was then increased by 10°C min^{-1} to 150°C and held at this temperature for 7 min. The injector temperature was 250°C, the detector temperature was 250°C and the carrier gas was nitrogen. The 1 mL of the fermented fluid was added to 1 mL hydrochloric acid (0.5 M) and stored at -40°C for ammonia-N analysis.

Samples for ammonia-N concentration were analyzed according to Weatherburn (1967). The volume of total gas production was corrected at the same temperature (0°C) and pressure (1.013×10^5 Pa) conditions to calculate the mol of total gas according to Giraldo *et al*. (2008a). The methane concentration of the total gas sample was analyzed by gas chromatograph (Agilent Technologies 7820A, USA), methane concentration was determined using methods as described by Hu *et al*. (2005). The methods of the AOAC (1995) were used for measurements of DM (ID 930.5), Crude Protein (CP, ID 984.13) and ash (ID 942.05). Acid detergent fibre and neutral detergent fibre were analyzed as described by Van Soest *et al*. (1991). Alpha amylase (Sigma A3306; Sigma-Aldrich, Shanghai, China) and sodium sulphite were added to each sample separately for NDF determination. *In vitro* Dry Matter Disappearance (IVDMD) was calculated according to Elwakeel *et al*. (2007).

The total DNA was extracted with the modified method according to Murray and Thompson (1980) and Zhou *et al*. (1996). The relative quantification of different microorganism was analyzed with real-time PCR (BIO-Rad iCycler iQ 5, Inc. Hercules, CA, USA). The primer sets of total bacteria, methanogens, *Fibrobacter succinogenes*, anaerobic fungi and protozoa are listed in Table 2. The 25 μL reaction system was composed as follows: 12.5 μL SYBRGreen dye (SYBR® Premix Ex Taq™ II, TaKaRa Biotechnology (Dalian) Co., Ltd.), 1.0 μL forward primer (10 μM concentration), 1.0 μL reverse primer (10 μM

Table 1: Ingredient and chemical composition of substrate treatments

Items	Forage:Concentrate ratio		
	80:20	50:50	20:80
Ingredient composition (g kg^{-1} DM)			
Alfalfa hay	800	500	200
Corn grain	88	338	587
Soybean	112	162	213
Chemical composition (g kg^{-1} DM)			
Dry matter	937	933	931
Organic matter	915	931	946
Crude protein	154	154	155
Neutral detergent fibre	456	393	347
Acid detergent fibre	243	170	98

Table 2: The primers for real-time PCR assay

Target group	Forward primer (5'-3')	Reverse primer (5'-3')	Size (bp)
Total bacteria*	CGGCAACGAGCGCAACCC	CCATTGTAGCACGTGTGTAGCC	130
Methanogens†	TTCGGTGGATCDCARAGRGC	GBARGTCGWAWCCGTAGAATCC	140
Protozoa‡	GCTTTCGWTGGTAGTGTATT	CTTGCCCTCYAATCGTWCT	223
*Fibrobacter succinogenes**	GTTCGGAATTACTGGGCGTAAA	CGCCTGCCCCTGAACTATC	121
Anaerobic fungi*	GAGGAAGTAAAAGTCGTAACAAGGTTTC	CAAATTCACAAAGGGTAGGATGATT	120

*Cited by Denman and McSweeney (2006); †Cited by Denman *et al*. (2007); ‡Cited by Sylvester *et al*. (2004); bp = Base pairs

concentration), 2.0 μL template DNA (30 ng μL^{-1}) and 8.5 μL sterile distilled water. Real-time PCR amplification according to the manufacturer's protocol: two step standard cycling program: the first step: predenature, 95°C, 30 sec, 1 cycle, the second step: PCR reaction, 95°C, 5 sec, 60°C, 30 sec, 40 cycles. Threshold cycles were calculated automatically by the iCycler. Program melting curve as shown : 95°C, 2 min; 72°C, 1 min; 95°C, 30 sec, step 0.5°C sec^{-1}; 30°C, 1 min. Quantification for *Fibrobacter succinogenes*, methanogens, fungi and protozoa were expressed as a proportion of total rumen bacterial 16S rRNA according to the equation as described by Zhang *et al*. (2008):

$$\text{Relative quantification} = 2^{-(\text{Ct target-Ct total bacteria})}$$

where, Ct represents threshold cycle.

Statistical analysis: All data were analyzed using the General Linear Model (GLM) of SPSS 18.0. When a significant effect of treatment ($p<0.05$) was detected, differences between means were assessed by the Least Significant Differences (LSD).

RESULTS AND DISCUSSION

As shown in Table 3 with the increase of fibrolytic enzyme and decrease of F:C substrates, total gas production, methane production, *In vitro* Dry Matter Disappearance (IVDMD) and methane: VFA were significantly increased ($p<0.001$) while pH and ammonia-N were decreased ($p<0.001$). There were significant interactions ($p<0.05$) between F:C and fibrolytic enzyme for total gas production, pH, methane production, methane:VFA and ammonia-N.

The effects of fibrolytic enzyme and diet on VFA are shown in Table 4. Fibrolytic enzyme increased ($p<0.001$) the concentration of total VFA and molar proportion of propionate and decreased ($p<0.001$) A:P and molar proportion of butyrate. There were however no effects ($p>0.05$) of the fibrolytic enzyme on molar proportion of acetate and propionate and A:P in F:C 20:80 substrate. All the VFA parameters were significantly affected by the F:C ratios substrates. A significant ($p<0.001$) interaction between F:C and fibrolytic enzyme for the concentration of total VFA were found.

Table 5 showed the effects of fibrolytic enzyme and diet on microbial population. Fibrolytic enzyme increased ($p<0.05$) the number of *Fibrobacter succinogens* and methanogens. With the decrease of F:C substrates, the number of *Fibrobacter succinogenes*, methanogens, total bacteria and anaerobic fungi decreased ($p<0.01$). The interactions between F:C and fibrolytic enzyme were observed for *Fibrobacter succinogenes* and methanogens ($p<0.05$). In the F:C 80:20 substrate, the fibrolytic enzyme increased ($p<0.05$) the number of *Fibrobacter succinogenes* and methanogens and total bacteria. There was however no difference on microbial populations by adding fibrolytic enzyme in the F:C 50:50 and 20:80 substrates.

Effects of fibrolytic enzyme and diet on total gas production and IVDMD: Total gas production reflects the degree of microbial fermentation, it had a high correlation with digestibility of dry matter (Menke *et al*., 1979). Giraldo *et al*. (2007, 2008a) reported that exogenous fibrolytic enzyme significantly increased total gas production and IVDMD. Some researchers also reported that exogenous fibrolytic enzymes enhanced digestibility of DM in *in vitro* (Feng *et al*., 1996; Hristov *et al*.,

Table 3: Effects of fibrolytic enzyme on total gas, rumen pH, IVDMD, methane production and ammonia-N *in vitro* of substrates with different forage to concentrate ratios

Fibrolytic enzyme ($U\ g^{-1}$)	Forage to concentrate	Total gas (mmol)	pH	IVDMD (%)	Methane (mmol)	Methane:VFA	Ammonia-N (mg 100 mL^{-1})
0	80:20	10.280[d]	6.230[a]	58.420[d]	1.760[d]	0.206[c]	25.960[a]
40	-	11.920[c]	6.160[b]	62.630[c]	2.020[c]	0.232[b]	25.050[ab]
80	-	12.280[b]	6.120[c]	65.100[b]	2.170[b]	0.240[a]	24.090[b]
120	-	12.560[a]	6.080[d]	69.730[a]	2.250[a]	0.243[a]	25.040[ab]
0	50:50	12.910[b]	6.060[a]	63.280[d]	2.330[b]	0.260[bc]	21.840[a]
40	-	13.090[b]	6.020[b]	67.240[c]	2.370[b]	0.255[c]	22.360[a]
80	-	15.010[a]	6.010[b]	71.980[b]	2.750[a]	0.282[b]	22.620[a]
120	-	15.500[a]	5.970[c]	75.490[a]	2.880[a]	0.311[a]	20.300[b]
0	20:80	17.290	5.940[a]	74.200[d]	3.170[b]	0.329	20.670[a]
40	-	17.460	5.900[b]	76.200[c]	3.230[b]	0.330	20.450[a]
80	-	17.550	5.860[c]	81.290[b]	3.370[a]	0.338	20.170[ab]
120	-	17.780	5.800[d]	84.510[a]	3.420[a]	0.336	19.460[b]
SEM	-	0.373	0.018	1.119	0.080	0.007	0.320
p-value	F:C	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	Enzyme	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	F:C x Enzyme	<0.001	<0.001	0.510	0.020	0.001	<0.001

F:C = Forage to Concentration ratios; IVDMD = *In vitro* Dry Matter Disappearance; VFA = Volatile Fatty Acid; SEM: Standard Error of Mean. [a-d]Means in the same column within F:C 80:20, 50:50 and 20:80 subgroups with different superscripts differ based on single degree of freedom contrasts ($p<0.05$)

Table 4: Effects of fibrolytic enzyme on VFA *in vitro* of substrates with different forage to concentrate ratios

Items				Individual VFA (mol/100 mol)			
Fibrolytic enzyme (U g^{-1})	Forage to concentrate	Total VFA (mmol L^{-1})	A:P	Acetate	Propionate	Butyrate	BCVFA
0	80:20	71.210^{c}	3.480^{a}	59.310^{a}	17.030^{d}	14.420^{ab}	9.240^{a}
40	-	72.400^{c}	3.310^{b}	58.450^{b}	17.670^{c}	14.640^{a}	9.250^{a}
80	-	75.230^{b}	3.290^{b}	59.310^{a}	18.030^{b}	14.180^{b}	8.470^{b}
120	-	77.300^{a}	3.190^{c}	58.470^{b}	18.350^{a}	14.590^{a}	8.590^{b}
0	50:50	74.650^{c}	3.390^{a}	57.610	17.010^{b}	17.190	8.190
40	-	77.440^{b}	3.300^{a}	57.640	17.490^{b}	16.970	7.890
80	-	81.100^{a}	3.350^{a}	57.790	17.250^{b}	17.060	7.900
120	-	77.120^{b}	3.150^{b}	57.190	18.180^{a}	17.000	7.630
0	20:80	80.330^{c}	3.310	57.640	17.430	17.920	7.000
40	-	81.790^{bc}	3.210	57.390	17.910	17.930	6.770
80	-	83.120^{ab}	3.150	57.050	18.120	18.140	6.700
120	-	84.980^{a}	3.110	57.000	18.310	18.160	6.530
SEM	-	0.620	0.019	0.139	0.082	0.225	0.084
p-value	F:C	<0.001	0.002	<0.001	0.004	<0.001	<0.001
	Enzyme	<0.001	<0.001	0.103	<0.001	0.851	<0.001
	F:C x Enzyme	<0.001	0.524	0.568	0.314	0.406	0.144

A:P = Acetate to Propionate, F:C = Forage to Concentration ratios, SEM = Standard Error of Mean; VFA = Volatile Fatty Acid, BCVFA = Branched-Chain Volatile Fatty Acid; $^{a-d}$Means in the same column within F:C 80:20, 50:50 and 20:80 subgroups with different superscripts differ based on single degree of freedom contrasts ($p<0.05$)

Table 5: Effects of fibrolytic enzyme on microbial populations *in vitro* of substrates with different forage to concentrate ratios

Fibrolytic enzyme (U g^{-1})	Forage to concentrate	*Fibrobacter succinogenes* ($\times10^{-2}$)	Methanogens ($\times10^{-3}$)	Total bacteria	Anaerobic fungi ($\times10^{-3}$)	Protozoa
0	80:20	0.893^{b}	3.192^{b}	1.000^{c}	1.655	0.118
40	-	1.248^{ab}	4.939^{ab}	1.366^{ab}	1.954	0.105
80	-	0.938^{b}	3.331^{b}	1.047^{bc}	1.745	0.084
120	-	1.969^{a}	6.911^{a}	1.546^{a}	1.906	0.833
0	50:50	0.863	1.961	1.186	1.167	0.570
40	-	0.837	3.143	1.100	1.064	1.198
80	-	0.866	2.622	1.367	0.873	0.207
120	-	0.758	1.872	0.904	0.877	0.190
0	20:80	0.265	0.944	0.767	0.259	0.263
40	-	0.479	1.337	0.972	0.319	0.246
80	-	0.232	0.716	0.626	0.219	0.203
120	-	0.314	0.579	0.662	0.209	0.184
SEM	-	0.086	0.320	0.060	0.126	0.098
p-value	F:C	<0.001	<0.001	<0.001	<0.001	0.386
	Enzyme	0.035	0.007	0.595	0.886	0.637
	F:C x Enzyme	0.008	<0.001	0.054	0.971	0.306

F:C = Forage to Concentration ratios; SEM = Standard Error of Mean; $^{a-d}$Means in the same column within F:C 80:20, 50:50 and 20:80 subgroups with different superscripts differ based on single degree of freedom contrasts ($p<0.05$)

1996) and *in vivo* (Yang *et al*., 1999; Elwakeel *et al*., 2007). In the present study, researchers also found that the fibrolytic enzyme significantly increased the total gas production and IVDMD. Beauchemin *et al.* (2003) suggested that the addition of exogenous fibrolytic enzymes improved digestion and the colonization of ruminal microorganism of cell wall which promoted the utilization of cellulose by microorganism and increased the IVDMD. The effects of fibrolytic enzyme increased IVDMD for High Forage (HF), Medium Forage (MF) and Low Forage (LF) substrates with 12.67, 13.10 and 8.72% increased, respectively. It is indicated that the low forge diet (F:C 20:80) showed the lowest efficiency by fibrolytic enzyme than F:C 80:20 and 50:50 substrates.

Arriola *et al.* (2011) reported that the dairy cows with lower F:C ratio diets showed higher digestibility of DM. In the present study with the decrease of F:C ratio, total gas production and IVDMD increased, indicating that the great mass of non-structural carbohydrates in concentrate could be degraded and utilized rapidly by microorganisms.

Effects of fibrolytic enzyme and diet on VFA and pH: The acetate, propionate and butyrate form an important part of the ruminant's energy. Previous research reported that adding fibrolytic enzyme resulted in higher total VFA and lower A:P ratio (Giraldo *et al.*, 2007, 2008b; Gonzalez-Garcia *et al.*, 2010; Arriola *et al.*, 2011). In the current study, fibrolytic enzyme significantly increased the concentration of VFA, propionate molar proportion and decreased A:P. The change of ruminal fermentation pattern may reflect a shift in the species of colonizing bacteria in feed (Wang *et al.*, 2001) and improve efficiency of energy utilization in ruminant (Srinivas and Gupta,

1997). Forage diets are mainly composed of cellulose and hemicellulose, fibrolytic enzymes act in concert to hydrolyze fiber of plant cell wall to glucose, cellobiose or cellooligosaccharides (Murad and Azzaz, 2010). In this experiment, higher forage and medium forage substrates showed the greater beneficial effects after 24 h incubation, fibrolytic enzyme increased concentration of VFA by 5.29, 5.23 and 3.69% (mean values for three doses) for HF, MF and LF substrates, respectively. Researchers suggested that higher F:C ratio diet contain more forage which more match with by the fibrolytic enzyme and also that could be related to the greater fermentation rates of high-concentrate substrates compared to those of high-forage ones (Garcia-Martinez *et al*., 2005) and in the F:C 80:20 and 50:50 substrates, fibrolytic enzyme increased the propionate molar proportion and decreased A:P. However, in F:C 20:80 substrate, the fibrolytic enzyme did not affect fermentation pattern. Indicated that the effect of fibrolytic enzyme on ruminal fermentation may dependent on the different F:C substrates.

Getachew *et al*. (2004) reported that *in vitro* gas production at 24 h positively correlated with total VFA production. Hodgson and Thomas (1975) found that as F:C of ruminant diets decreased, the A:P ratio reduced along with the ratios of production of methane and carbon dioxide. Homan and Wattiaux (1996) reported that non-structural carbohydrates produced lower acetate proportion and higher propionate molar proportion. Yang *et al*. (2001) reported that cows fed with higher forage diets showed higher acetate and lower propionate molar proportions which resulted in a higher A:P ratio. In the present experiment, total VFA concentration was increased and A:P was decreased with F:C decreased. Giraldo *et al*. (2008b) reported that fibrolytic enzyme decreased rumen pH. Similar results were observed in the present study. Fibrolytic enzyme decreased the rumen pH, the higher concentration of total VFA may contribute to the lower pH.

Effects of fibrolytic enzyme and diet on methane and ammonia-N: The forage in diet was fermented by the microorganism in rumen, released H^+ and CO_2 provided the source to produce methane by methanogens (Stewart *et al*., 1997). Previous studies reported that fibrolytic enzyme increased methane production (Dong *et al*., 1999; Giraldo *et al*., 2007, 2008a; Chung *et al*., 2012). In the present study, similar results were observed.

The concentration of ammonia-N was usually used to reflect the balance status between the degradation and utilization of nitrogen (Aufrere *et al*., 2003). Carbohydrates and proteins are the major nutrients required for rumen microbes, synchronization of both the nutrients availability is necessary for optimizing microbial growth and fermentation in the rumen (Hoover and Stokes, 1991). Ammonia is the principal Nitrogenous (N) source in rumen, the concentration of ammonia-N was affected not only by feed protein but also by energy level and microbial assimilation. In the present study, the amount of ammion-N is sufficient in *in vitro* batch culture experiment, the fibrolytic enzyme decreased the concentration of ammonia-N in the fermentation fluid. Appropriate supplementation of fibrolytic enzyme promoted cellulose degradation and increased fermentable carbohydrates which provided enough energy to microorganism for their protein synthesis.

Effects of fibrolytic enzyme on rumen microbes: Nsereko *et al*. (2002) reported that fibrolytic enzyme addition increased the number of total bacteria in dairy cow fed with F:C 52:48 diet. In the present study, fibrolytic enzyme increased total bacteria in F:C 80:20 substrate. *Fibrobacter succinogenes* is one of the most important cellulolytic bacteria. Giraldo *et al*. (2007, 2008b) observed that fibrolytic enzyme increased the number of cellulolytic bacteria in a high forage substrate (F:C 70:30). Chung *et al*. (2012) found that fibrolytic enzyme addition in lactating Holstein cow tended to increase ($p = 0.06$) the number of *Fibrobacter succinogenes* in F:C 48:52 diet. In the present study, the exogenous fibrolytic enzyme increased the number of *Fibrobacter succinogenes* in the high forage F:C 80:20 substrate. Suggested that fibrolytic enzyme hydrolyzed the structural carbohydrate to small particle and increased the availability of feed nutrients to ruminal microbes (Wang *et al*., 2001).

In addition to the importance of propionate formation as a fermentation end-product increased, *Fibrobacter succinogenes* is a representative rumen bacteria of succinogenes and propionate producer (Kobayashi, 2010). In the present trial, the number of *Fibrobacter succinogenes* was increased by the supplementation of fibrolytic enzyme, explaining for the molar proportion of propionate was increased by fibrolytic enzyme.

Dong *et al*. (1999) reported that fibrolytic enzyme increased the number of methanogens which was similar to the result that the number of methanogens was elevated by the addition of fibrolytic enzyme. Although, there was an ecosystem relationship between the methanogens and protozoa (Tokura *et al*., 1999), Wang *et al*. (2001) observed that fibrolytic enzyme had no effect on protozoa of diet with chopped alfalfa hay: steam-rolled barley grain 50:50. Chung *et al*. (2012) testified that fibrolytic enzyme addition did not affect

protozoa of diet with F:C 48:52 for lactating Holstein cow. Dehghani *et al.* (2011) reported that fibrolytic enzyme had no effect on the number of protozoa when the cow fed with F:C 40:60 diet. In the present study, fibrolytic enzyme did not affect the number of protozoa in the F:C 80:20 substrate but had a tendency to increase the number of protozoa (p = 0.066). No significant difference was detected for microbial populations in the F:C 50:50 and 20:80 substrates indicating that the effects of fibrolytic enzyme on microbial populations depended on the F:C ratio substrates.

CONCLUSION

Exogenous fibrolytic enzyme addition increased IVDMD, total VFA concentration and modulated the microbial populations indicating that fibrolytic enzyme supplementation could improve the *in vitro* ruminal fermentation. The greater response found for the high forage (F:C 80:20) and medium forage (F:C 50:50) substrates compared with the low forage (F:C 20:80) substrate would indicated that effects of fibrolytic enzyme on ruminal fermentation could depend on the nature of the incubated substrate.

ACKNOWLEDGEMENT

This research was supported by the National Key Technologies R&D Program of China (2012BAD12B02, 2012BAD39B05), Program of International S&T Cooperation of China (2010DFB34230).

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