Effects of Exogenous Fibrolytic Enzyme on *in vitro* Ruminal Fiber Digestion and Methane Production of Corn Stover and Corn stover Based Mixed Diets

Liyuan Zhao[1], Yongjia Peng[1], Jiakun Wang[1], Jianxin Liu[1 *]

MoE Key Laboratory of Molecular Animal Nutrition, College of Animal Sciences, Zhejiang University, Hangzhou 310058, China
E-mail: liujx@zju.edu.cn; Tel: +86 571 88982097; Fax: +86 571 88982930

Abstract: Introduction: Many *in vitro* studies have showed that fibrolytic enzyme can improve fiber degradation of roughage, but the effect on methane mitigation is likely to be inconsistent. **Objective:** The aim of this study was to investigate the effect of commercial exogenous fibrolytic enzymes on *in vitro* rumen fermentation and methane production with corn stover as substrate. **Design and Methods:** Two *in vitro* experiments were conducted using reading pressure technique system. In Exp.1, synergetic effect of cellulase (CEL) (0, 10, 20 and 30 U/g DM) and xylanase (XYL) (0, 20, 40 and 60 U/g DM) were evaluated on corn stover by a 4 × 4 factorial design. In Exp.2, the effect of two chosen enzymes were evaluated on 3 corn stover based mixed diets by a 3 × 3 factorial design. **Results:** In Exp.1, a single 30U/g DM of CEL and combination of 10 U/g DM of CEL with 60 U/g DM of XYL were screened out based on higher digestibility of dry matter and neutral detergent fiber. In Exp.2, enzyme supplement did not affect fiber digestion, but decreased methane production (P<0.01). **Conclusion:** Fibrolytic enzyme supplement potentially improved fiber digestion of corn stover in this *in vitro* experiment, and had positive effect on methane mitigation.
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1. Introduction

For keeping the rumen health, forage is an essential component in ruminant diet. But the high quantities of cellulose and hemicellulose in the cell wall of forage usually limit the efficient utilization of nutrition by ruminants. Exogenous fibrolytic enzyme has been used as diet supplement, which can overcome this limitation by improving plant cell wall digestibility and the efficiency of feed utilization (Gado *et al*., 2009; Khattab *et al*., 2011; Salem *et al*., 2013, 2015a; Abdel-Aziz *et al*., 2015; Valdes *et al*., 2015) .

Many *in vitro* studies have been performed to evaluate the effect of fibrolytic enzyme on the digestion of low (Díaz *et al*., 2014) and high quality forages (Eun *et al*., 2007). Positive correlation was found between activities of endoglucanase and exoglucanase and improvement in neutral detergent fiber degradability for alfalfa hay and corn silage in Eun *et al*. (2007) study. Similarly, enzyme additives increased neutral detergent fiber digestibility (NDFD) and acid detergent fiber digestibility (ADFD) of corn silage (Phakachoed *et al*., 2013). As for the low quality forage, Krueger and Adesogan (2008) reported multi-enzyme cocktails improved efficiency of ruminal fermentation of mature bermudagrass but did not increase dry matter digestibility (DMD) or NDFD. Moreover, it was reported that the effectiveness of enzymes varied with the incubated forage (Díaz *et al*., 2013), indicating it is necessary to evaluate the effect of enzyme on fiber digestion when substrate varied. Corn stover is a normal forage, and its production can reach to more than 2.65 billion tons each year in China. The effect of fibrolytic enzyme on corn stover has not been well determined.

Methane emitted from ruminant contributes to greenhouse gases. Some fibrolytic enzyme could decrease the ratio of acetate: propionate (Eun *et al*., 2007). Since hydrogen is consumed through propionate production, and methane production might decrease concurrently. Beauchemin, *et al*. suggested that dietary supplementation with fibrolytic enzyme might be a new strategy to reduce methane production (Beauchemin *et al*., 2008). However, very few studies have been reported regarding the effect of enzyme on methane production. Giraldo, *et al*. attempted to treat the substrate with the enzyme, and found enhanced methane production simultaneous with increased fiber degradation and volatile fatty acids (VFA) (Giraldo *et al*., 2007). Above all, it still not very confirmed that whether fibrolytic enzyme could decrease methane production at the same time of increasing fiber digestion. Therefore more research needs to be conducted to evaluate the effect of fibrolytic enzyme on methane production.

The objective of this study was to analyze the effect of exogenous fibrolytic enzyme on *in vitro* ruminal fermentation and methane production of corn

stover. Our hypothesis were: Exp.1 the cellulase (CEL), xylanase (XYL) and their combination might positively affect the fiber digestion of corn stover, and decreased methane production per fiber degradation. Exp. 2 the effects of chosen enzyme combination from Exp.1 were also effective on the corn stover based mixed diets.

2. Material and Methods

2.1 Enzyme and substrates

In Exp1, CEL was purchased from Wuhan Sunny Animal Pharmacy Co., Ltd, and xylanase was supplied by Dyadic International, INC. (Jupiter, Florida 33477). All activities were measured at pH 6.6 and 39ºC in order to resemble optimal ruminal conditions. The xylanase activity was assayed using 1% (w/v) oat spelt xylan as a substrate following the procedure described by Bailey *et al*. (1992), while endoglucanase and exoglucanase activities were assayed using 1% (w/v) carboxymethylcellulose (CMCase) sodium and 1% (w/v) cellulose following the method described by Wood and Bhat (1988), the 0.1 mL of diluted enzyme solutions were incubated at 39ºC together with 0.9 mL of 0.1 M sodium citrate phosphate buffer (PH=6.6) and 1 mL of the substrate solutions. The enzyme activities were expressed as μmol of sugar released min^{-1} mL^{-1} of the enzyme products at 39ºC, pH 6.6. and shown in Table 1.

Table 1. Protein concentration and enzymatic activities of the enzymes products

Enzyme†	Protein concentration‡ mg/mL	Enzyme activity§ U/mL		
		Xylanase	Endoglucanase	Exoglucanase
CEL	17.7	777	365	56
XYL	110.5	35499	885	122

† CEL = Cellulase; XYL = Xylanase.
‡ Protein content was expressed as mg of protein mL^{-1} the enzyme products.
§ Enzyme activities were expressed as μmol of sugar released min^{-1} mL^{-1} of the enzyme products at 39ºC, pH 6.6.

The corn stover was used as the substrate, and was grounded by a miller (DFT-50, Lin-tai Machinery Co., Ltd) to pass a 2 mm screen. Chemical composition of corn stover was (DM %): 5.79 crude proteins, 70.82 NDF, and 39.58 ADF.

In Exp2, two enzymes treatment (EC: CEL of 30 endoglucanase units/g of substrate DM and ECX: CEL of 10 endoglucanase units/g of substrate DM + XYL of 60 xylanase units/g of substrate DM) were screened from Exp1. Three corn stover based mixed diets were used as substrates: 1) CSC (corn: soybean meal: corn stover = 25:15:60), 2) CSCC (corn: soybean meal: corn stover: corn silage =25:15:30:30) and 3) CSCCA (corn: soybean meal: corn stover: corn silage: alfalfa = 25:15:30:24:6). The chemical composition of the mixed diet is shown in Table 2.

Table 2. Chemical composition of mixed diets

Diets†	Composition‡, %			
	DM	CP	NDF	ADF
CSC	91.3	13.0	50.1	28.0
CSCC	92.7	13.8	44.2	24.6
CSCCA	92.5	14.2	43.7	25.0

† Diets: CSC = mixture of corn, soybean meal and corn stover at ratios of 25%, 15% and 60%; CSCC = mixture of corn, soybean meal, corn stover and corn silage at ratios of 25%, 15%, 30% and 30%; CSCCA = mixture of corn, soybean meal, corn stover, corn silage and alfalfa at ratios of 25%,15%, 30%, 24% and 6%.
‡ DM, Dry matter. CP, Crude protein. NDF, neutral detergent fiber. ADF, acid detergent fiber.

2.2 Experiment design

In Exp1, the experiment was carried out according to a 4 × 4 factorial design with CEL and XYL as main effects. The addition levels of CEL were 0, 10, 20 and 30 units/g of substrate DM, and the XYL addition levels were 0, 20, 40 and 60 units/g of substrate DM according to previous results and economic effect (Mao *et al*., 2013).

In Exp2, the experiment was carried out according to a 3 × 3 factorial design with enzyme and substrate as main effects. The enzymes included E0 (no enzyme), EC and ECX. The substrates included CSC, CSCC and CSCCA.

2.3 In vitro fermentation

Approximately 1 L of rumen content was collected from 3 donor sheep before feeding in the morning, the donor sheep were fed with a mixed diet of Chinese wildrye and concentrate mixture (70:30, w/w) fed twice daily. Ingredients (% DM) of concentrate mixture included corn (50), wheat bran (15), soybean meal (15), rapeseed meal (13), dicalcium phosphate (2.0), salt (1.5), calcium carbonate (1.5) and vitamin-trace mineral premix (2), and were formulated to provide (per kg of DM) 1,200,000 IU of vitamin A, 280,000 IU of vitamin D, 5000 mg of vitamin E, 14,000 mg of Zn, 100 mg of Se, 200 mg of I, 3,000 mg of Fe, 60 mg of Co, 3,500 mg of Mn, and 3,000 mg of Cu. The rumen fluid was then transported to the laboratory within 1 h under anaerobic condition, and strained through four layers of cheese cloth into pre-warmed insulated flask.

Ten ml of rumen fluid was added into a 120 mL-bottle containing 40 mL of buffered medium and 1 g of substrate at 39°C. *In vitro* incubations were conducted in two consecutive runs, each involving triplicates of samples. In each round of incubation, three blanks were included simultaneously to correct the gas values for gas released form endogenous substrates. Triplicate of a hay as reference was also included. Incubation was repeated when the difference between two rounds in gas volumes from the reference hay was large than 10%. *In vitro* gas

test was performed with the semi-automated Reading Pressure Technique (RPT) system (Mauricio *et al*., 1999). The total incubation time for Exp.1 was 48 h, and for Exp.2 was 24 h.

Table 3. Effect of cellulase (CEL) and xylanase (XYL) addition on *in vitro* dry matter (DM), neutral detergent fiber (NDF) degradation and methane production of corn stover.

CEL (U/g of DM)	XYL (U/g of DM)	Degradability (%)		Methane (mL/g)	
		DM	NDF	per DMD	per NDFD
0	0	46.7	34.6	22.3	41.5
0	20	47.3	35.3	21.2	40.3
0	40	46.4	34.2	21.7	40.5
0	60	45.9	33.1	18.8	35.2
10	0	46.6	34.8	21.3	39.3
10	20	47.4	35.3	20.8	38.1
10	40	44.9	31.5	20.3	39.0
10	60	49.3	37.7	21.2	39.1
20	0	45.4	32.8	19.3	36.6
20	20	47.6	35.6	20.9	38.6
20	40	47.9	36.1	20.0	37.3
20	60	47.9	36.2	20.0	37.2
30	0	48.5	36.9	21.8	40.4
30	20	46.3	34.5	20.7	37.3
30	40	47.1	35.0	20.6	38.3
30	60	48.5	36.6	20.2	37.6
Pooled SEM		0.75	1.10	0.09	0.77
Effect					
CEL		Ns	ns	**	**
Linear		Ns	ns	*	*
Quadratic		ns	ns	**	**
XYL		ns	ns	**	**
Linear		ns	ns	**	**
Quadratic		ns	ns	**	*
CEL*XYL		**	**	**	**

** $P<0.01$; * $P<0.05$; ns = not significant.

2.4 Chemical analysis

During the gas test, head space gas pressure and methane concentration were measured at 6, 12, 24, and 48 h (only in Exp.1). Five mL gas was sampled for methane measurement, and analyzed by a gas chromatography (GC-2010, Shimadzu Corporation, Kyoto, Japan) equipped with a Flame Ionization Detector and a capillary column (HP-INNOWAX, 19091N-133, Agilent Technologies, Santa Clara, CA, USA), and the temperature of the injector/detector and the column was 100ºC and 38ºC, respectively, as described by Hu *et al*. (Hu *et al*., 2005) with minor modification.

After incubation, culture fluid was sampled to determine pH, ammonia nitrogen (NH_3-N) and VFA. NH_3-N concentration was determined by the spectrometer (SpectraMax M5, Molecular Devices) using the colorimetry with the NH_4Cl solution as a standard (Feng and Gao, 1993). The VFA were determined using the gas chromatography with the same column, 2 uL of fluid sample was injected with a syringe, and the temperature of the injector/detector and the column was 220ºC and 80ºC, respectively. Nitrogen was used as a carrier (Hu *et al*., 2005).

The contents in the bottle were completely rinsed out and filtered through filter bags. The bags were put into an oven at 65°C for 48 h to determine DMD. The sample of residues was then taken from filter bags for analysis of NDFD.

2.5 Statistical analyses

All the statistical analyses were conducted using the GLM procedures (SAS, 1999). Data were analyzed as two-way analysis of variance. Multiple comparisons of means among treatments were carried out by the Duncan's multiple range tests. For Exp.1, the responses (linear or quadratic) to increasing concentrations of CEL and XYL were examined by orthogonal polynomial contrast.

3. Results

3.1 Synergistic effects of cellulose and xylanase on in vitro fermentation of corn stover (Exp.1).

3.1.1 Fiber degradation and methane production

As showed in Table 3, *in vitro* degradability of DM and NDF were not affected by addition of CEL or XYL. While both degradations of DM and NDF were potentially linear increased in response to CEL addition (linear P=0.07 and P=0.07 respectively for DMD and NDFD). The interaction effects were observed on both DMD and NDFD (P<0.01).

Methane per DMD or per NDFD were decreased in response to either XYL addition (linear, P<0.05, quadratic, P<0.01) or CEL addition (linear, P<0.01, quadratic, P<0.05) (Table 3).

Table 4. Effects of cellulase (CEL) and xylanase (XYL) addition on *in vitro* fermentation parameters of corn stover.

CEL U/g of DM	XYL U/g of DM	pH	Total VFA mmol/L	Molar proportion mol/100mol			A:P	NH_3-N mg/dL
				Acetate	Propinate	Butyrate		
0	0	6.35	78.5	69.5	23.1	7.4	3.0	14.4
0	20	6.41	79.8	69.0	25.1	6.0	2.8	14.1
0	40	6.40	78.8	70.1	23.8	6.2	3.0	14.8
0	60	6.44	74.6	70.0	22.9	7.1	3.1	14.5
10	0	6.35	79.8	69.0	24.1	6.9	2.9	14.2
10	20	6.41	79.3	69.3	23.8	6.9	2.9	14.3
10	40	6.48	75.0	69.1	21.6	9.3	3.2	15.1
10	60	6.35	83.7	69.0	23.8	7.2	2.9	14.5
20	0	6.39	77.5	68.4	24.2	7.4	2.9	15.1
20	20	6.39	81.1	69.5	23.4	7.0	3.0	15.4
20	40	6.40	79.7	69.0	23.7	7.3	2.9	14.9
20	60	6.39	79.6	68.8	23.5	7.7	2.9	14.8
30	0	6.45	78.8	65.9	24.3	9.8	2.7	16.0
30	20	6.43	74.6	67.8	23.1	9.1	2.9	15.2
30	40	6.41	80.3	68.7	23.2	8.1	3.0	14.6
30	60	6.37	78.4	68.3	23.3	8.4	2.9	15.7
Pooled SEM		0.02	2.18	0.87	0.64	0.78	0.10	1.01
Effect								
CEL		ns	ns	*	ns	**	ns	ns
Linear		ns	ns	**	ns	**	ns	ns
Quadratic		ns	ns	*	ns	*	ns	ns
XYL		ns	ns	ns	ns	ns	ns	ns
Linear		ns	ns	ns	ns	ns	ns	ns
Quadratic		ns	ns	ns	*	ns	ns	ns
CEL*XYL		**	ns	ns	ns	ns	ns	ns

** P<0.01; * P<0.05; ns = not significant.

3.1.2 In vitro fermentation parameters

As shown in Table 4, most fermentation parameters (final pH, total VFA, acetate:propionate ratio (A: P) and ammonia nitrogen) of corn stover were not affected by CEL or XYL addition. Supplementation of CEL decreased acetate portion (P<0.05) but increased butyrate proportion portion (linear, P<0.05; quadratic, P<0.01). Addition of XYL only affected the proportion of propionate in a quadratic way (P<0.05).

3.2 Evaluation of enzyme combination on in vitro fermentation of corn stover based mixed diet (Exp.2)

3.2.1 Fiber degradation and methane production

As shown in table 5, enzyme treatment did not affect the DMD and NDFD of three mixed diets (P>0.05). Interaction effects between substrate and enzyme were detected in both DMD and NDFD (P<0.01). DMD and NDFD varied between three diets. For DMD, CSCC > CSCCA > CSC; for NDFD, CSCC > CSC > CSCCA.

In regard to methane production, enzyme supplementation significantly decreased the methane production (both expressed as ml per DM and per NDF) (P<0.01). Methane production was also affected by diets (P<0.01), with the lowest production in CSCCA. Interaction effects between substrate and enzyme were found in methane per DMD (P<0.01), but not in methane per NDFD (P>0.05).

Table 5. Effects of CEL and XYL addition on *in vitro* dry matter (DM), neutral detergent fiber (NDF) degradation and methane production of mixed substrates

Substrate	Enzyme †	Degradability (%)		Methane (mL/g)	
		DM	NDF	per DMD	per NDFD
CSC	E0	74.4^{c}	46.8^{b}	12.0^{a}	42.3^{abc}
	ECX	73.0^{d}	45.6^{c}	10.0^{bc}	35.4^{de}
	EC	73.5^{cd}	46.0^{bc}	9.9^{bc}	35.1^{de}
CSCC	E0	76.1^{b}	46.7^{b}	12.2^{a}	47.8^{a}
	ECX	77.5^{a}	48.9^{a}	10.4^{b}	37.2^{cde}
	EC	76.3^{b}	46.2^{bc}	8.7^{d}	32.3^{e}
CSCCA	E0	75.8^{b}	42.5^{e}	9.9^{bc}	46.5^{a}
	ECX	76.0^{b}	42.0^{e}	9.6^{c}	45.8^{ab}
	EC	76.4^{b}	43.7^{d}	8.7^{d}	39.8^{bcd}
Pooled SEM		0.26	0.37	0.19	2.06
Effect					
Substrate		**	**	**	**
Enzyme		ns	Ns	**	**
Substrate*Enzyme		**	**	**	ns

** P<0.01; * P<0.05; ns = not significant.

† Enzyme: E0 = no enzyme addition; ECX = CEL of 10 units/g DM + XYL of 60 units/g DM; EC = CEL of 30 units/g DM.

3.2.2 In vitro fermentation parameters

As shown in table 6, majority of fermentation parameters were affected by enzyme supplementation. The terminal pH value, total VFA, the molar proportion of acetate, propionate and butyrate were increased by enzyme addition (P<0.01). The ratio of acetate vs. propionate was increased in response to enzyme treatment (P<0.05). Significant effects of substrate were also detected on pH, total VFA, the proportion of acetate, propionate and butyrate (P<0.01), but no difference was found among three substrate on A: P and NH_3-N (P>0.05).Except for NH_3-N, other fermentation parameters were affected by the interaction of substrate and enzyme (P<0.01).

4. Discussions

4.1 Effect of exogenous fibrolytic enzyme on fiber degradation of corn stover

The application of fibrolytic enzyme is one of most useful treatment to improve forage digestibility and ruminant performance (Gado *et al*., 2009; Kholif and Aziz, 2014; Alsersy *et al*., 2015; Elghandour *et al*., 2015). As Jung, *et al*. reported that a one-unit increase in NDF digestibility *in vitro* was associated with a 0.14 kg increase in 3.5% fat-corrected milk and a 0.12 kg increase in DMI (Jung *et al*., 2004), suggested that NDFD was an important parameter of evaluating the digestion of forage. Newly study also indicated the degradation of NDF and ADF were the more useful criteria of differentiating the enzymes compared with DM and total GP (Phakachoed *et al*.,

2015). Therefore, the degradation of DM, NDF and ADF were used to be the first parameter in this study, and also used to adjust the methane production value.

Previous study had shown that the in situ degradation of NDF and ADF of corn stover were increased by adding enzyme extract from C. *flavigena* (Hernández-Cruz *et al*., 2009). Meanwhile, Eun, *et al*. reported the *in vitro* degradation of NDF and ADF from alfalfa hay and corn silage was increased by exogenous fibrolytic enzymes in a dose-dependent manner (Eun *et al*., 2007). Krueger and Adesogan (2008) also showed that application of enzyme to forage increased fiber degradation. In this study (Exp.1), in consistent results were found, no significant effect of CEL or XYL was obtained on *in vitro* DMD and NDFD of corn stover. However, the DMD and NDFD seemed to be potentially higher at high dose of CEL treatment than that at low dose of CEL. Thus, taking CEL of 30 units of per DM as the candidate enzyme for Exp. 2 might obtain better effect. The interaction of cellulase and xylanase affected the DMD and NDFD of corn stover (Exp.1), suggesting that the combination of CEL and XYL had better effect on substrate digestion than single enzyme. The synergistic effect of CEL and XYL was also reported in the study of Eun, *et al*. (2006), the authors found that both endoglucanase and xylanase could cause more apparent effect on *in vitro* ruminal degradation of ammoniated rice straw when compared with only endoglucanase was supplied. In this study, when XYL was added in maximum dose, three doses of CEL combined with XYL showed almost same effect on core stover digestion, from the point of economic benefit view, CEL of 10 units/g plus XYL of 60 units/g substrate was used for Exp.2 as the factor ECX.

Table 6 Effects of CEL and XYL addition on *in vitro* fermentation parameters of mixed substrate

Substrate	Enzyme	pH	Total VFA mmol/L	Molar proportion mol/100mol			A:P	NH_3-N mg/dL
				Acetate	Propionate	Butyrate		
CSC	E0	6.19^{de}	157.2^{g}	60.7^{a}	17.7^{a}	21.6^{f}	3.4^{cd}	29.3^{de}
	ECX	6.32^{a}	171.4^{f}	57.8^{b}	15.8^{bc}	26.4^{de}	3.7^{ab}	31.8^{a}
	EC	6.25^{bc}	190.4^{e}	57.6^{b}	16.2^{b}	26.2^{e}	3.6^{c}	29.9^{bc}
CSCC	E0	6.15^{e}	203.1^{d}	56.1^{c}	15.9^{bc}	27.9^{cd}	3.5^{c}	27.5^{e}
	ECX	6.21^{cd}	203.1^{d}	54.0^{d}	15.7^{bc}	30.3^{b}	3.4^{c}	29.6^{cd}
	EC	6.24^{bcd}	231.3^{b}	52.8^{d}	15.3^{c}	32.0^{a}	3.5^{bc}	32.0^{bcd}
CSCCA	E0	6.21^{cd}	218.9^{c}	55.5^{c}	16.2^{b}	28.3^{b}	3.4^{cd}	31.5^{cd}
	ECX	6.28^{ab}	222.5^{bc}	56.2^{c}	17.2^{a}	26.6^{de}	3.3^{d}	32.9^{ab}
	EC	6.24^{bcd}	321.2^{a}	55.9^{c}	15.2^{c}	28.9^{bc}	3.7^{a}	30.9^{bcd}
Pooled SEM		0.008	5.36	0.29	0.12	0.37	0.02	0.38
Effect								
Substrate		**	**	**	**	**	ns	ns
Enzyme		**	**	**	**	**	*	ns
Substrate*Enzyme		**	**	**	**	**	**	ns

** $P<0.01$; * $P<0.05$; ns = not significant.

Enzyme-feed specificity is often considered as an important determinant of enzyme action in feed nutrition (Beauchemin *et al*., 2004; Gado *et al*., 2009; Salem *et al*., 2015b). In the study of Giraldo, *et al*., the effects of exogenous fibrolytic enzymes on *in vitro* ruminal fermentation of three substrates (high, middle and low percent of forage ratios) were evaluated, the authors found both aNDF and hemicellulose content for high forage substrate were decreased by all enzymes, but only one and two enzyme affected middle and low forage substrate respectively, which indicated the nature of substrate influenced the effect of enzyme on fiber content (Giraldo *et al*., 2004). In this study, three types of diet were tested, effect of diet as well as interaction between diet and enzyme were found in DMD and NDFD, suggesting the efficient effect of enzyme in response to forage type of substrate with different NDF content. Similarly, Soltan, *et al*. also pointed out that enzyme should match the substrate to make sure the enzyme's maximum effects (Soltan *et al*., 2013).

4.2 Effect of exogenous fibrolytic enzyme on methane production of corn stover

By now, the effects of exogenous enzymes on ruminal methane production were not investigated well, and the results were inconsistent. Arriola, *et al*.

reported that dietary fibrolytic enzyme supplementation decreased methane production which was calculated according to the fermentation balance calculations (Arriola *et al*., 2011). On the contrary, Chung, *et al*. found an increase in enteric methane production for exogenous enzyme addition (Chung *et al*., 2012). Giraldo, *et al*. (2007) reported that exogenous pure cellulases augmented methane production. And our previous study with rice straw also detected increased methane production in response to CEL treatment (Mao *et al*., 2013). In this study, inconsistent results were detected, addition of CEL and XYL decreased the production of methane per DMD and NDFD. Compared with previous studies, one thing should be noted, that is the way of expressing methane production is different. Here, decreased methane production was found when expressed as production of per DMD and per NDFD, while the total absolute methane production was not changed. This is consistent with the results of Beauchemin, *et al*., which demonstrated increased absolute methane production and decreased methane per kg of milk (Beauchemin *et al*., 2008). Therefore, even though enzyme treatments increase the methane production, the relative amount is more make sense when the forage digestibility was considered.

4.3 Effect of exogenous fibrolytic enzyme on in vitro fermentation of corn stover

Since total VFA contribute around 70% to the caloric requirements of ruminants (Bergman, 1990), VFA is an important parameter for *in vitro* fermentation, which can be used to estimate the supplied energy from diet (Togtokhbayar *et al*., 2015). In this study, enzyme treatment did not affect the VFA concentration of corn stover, but when corn stover was mixed with concentrate, positive effect of fibrolytic enzyme on improving the energy utilization of diets was suggested from the increased VFA found in Exp.2. Moreover, as implied from the work of Eun, *et al*., enzyme addition could affect the population and metabolic pathways of specific microbes which utilize corresponding substrates (Eun *et al*., 2007). It can be also inferred that the effect of enzyme might more efficient to stimulate the microbial fermentation of soluble carbohydrates when substrate was changed from single to complicate, more relative microbes might be promoted mutually. Here, the significant interaction effect between substrate and enzyme were also detected with VFA (Exp.2), which also suggested the complication of enzyme effect on rumen fermentation.

As for the individual VFA, the molar proportion of acetate was decreased in response to CEL (Exp.1) and enzymes (Exp.2), and the butyrate proportion were concurrently increased. This was similar with Krueger and Adesogan (2008) and Díaz, *et al*. (2013), who indicated the fermentation became more gluconeogenic and improved energetic efficiency of substrates. Meanwhile, butyrate is one the main VFA generated from fiber fermentation, increased molar proportion of butyrate was found in CSC and CSCC substrates, these were inconsistent with higher ADF content in these two substrates. The ratio of acetate and propionate reflected the fermentation pattern, in the current study, enzyme increased the A:P of CSC and CSCCA substrates, suggested the fermentation pattern of these two substrates were changed. Looking into the individual portion of acetate and propionate, different reasons were found under this increased A:P between CSC and CSCCA, for CSC, both acetate and propionate were decreased, and propionate decreased more; for CSCCA, only propionate was decreased, and acetate did not change.

The concentration of NH_3-N was not affected by enzyme supplement, which was similar with one recently *in vitro* study (Romero *et al*., 2015), the authors reported that no influence of enzyme treatment on NH_3-N content of Bermuda grass haylage. As they suggested it was difficult to induce significant change in such high level of NH_3-N (above 40 mg/dL) with enzyme addition.

5. Conclusion

Enzyme supplementation failed to improve fiber digestion of corn stover in this in vitro experiment, but had positive effect on methane mitigation. Meanwhile, the efficiency of enzyme varied among substrates, suggesting the match issue between enzyme and substrate should be noticed in future study.

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References

1. Abdel-Aziz NA, Salem AZM, El-Adawy MM, Camacho LM, Kholif AE, Elghandour MMY, Borhami BE. Biological treatments as a mean to improve feed utilization in agriculture animals-An overview. Journal of Integrative Agriculture 2015; 14(3): 534–543
2. Alsersy H, Salem AZM, Borhami BE, Olivares J, Gado HM, Mariezcurrena MD, Yacuot MH, Kholif AE, El-Adawy M and Hernandez SR. Effect of Mediterranean saltbush (*Atriplex*

halimus) ensilaging with two developed enzyme cocktails on feed intake, nutrient digestibility and ruminal fermentation in sheep. Animal Science Journal, 2015, 86: 51–58.
3. Arriola K G, Kim S C, Staples C R and Adesogan A T. Effect of fibrolytic enzyme application to low- and high-concentrate diets on the performance of lactating dairy cattle. Journal of Dairy Science, 2011, 94(2):832-841.
4. Bailey M J, Biely P, Poutanen K. Interlaboratory testing of methods for assay of xylanase activity. J Biotechnol 1992, 23(3): 257-270.
5. Beauchemin K A., Colombatto D and Morgavi D P. A rationale for the development of feed enzyme products for ruminants. Canadian Journal of Animal Science, 2004, 84(1):23-36.
6. Beauchemin K A., Kreuzer M, O'Mara F and McAllister T A. Nutritional management for enteric methane abatement: A review. Animal Production Science, 2008, 48(2):21-27.
7. Bergman E N. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiological reviews, 1990, 70(2): 567-590.
8. Chung, Y. H., Zhou, M., Holtshausen, L., Alexander, T. W., McAllister, T. A., Guan, L. L.,Oba M and Beauchemin, K. A. A fibrolytic enzyme additive for lactating Holstein cow diets: ruminal fermentation, rumen microbial populations, and enteric methane emissions. Journal of Dairy Science, 2012, 95(3): 1419-1427.
9. Díaz A, Ranilla M J , Giraldo L A , Tejido M L and Carro M D. Treatment of tropical forages with exogenous fibrolytic enzymes: Effects on chemical composition and *in vitro* rumen fermentation. Journal of Animal Physiology and Animal Nutrition, 2014, doi: 10.1111/jpn.12175
10. Elghandour MMY, Salem AZM, Martínez Castañeda JS, Camacho LM, Kholif AE, Vázquez Chagoyán JC. Direct fed microbes: A tool for improving the utilization of low quality roughages in ruminants. Journal of Integrative Agriculture 2015; 14(3): 526–533
11. Eun J S, Beauchemin K A and Schulze H. Use of exogenous fibrolytic enzymes to enhance *in vitro* fermentation of alfalfa hay and corn silage. Journal of Dairy Science, 2007, 90(3): 1440-1451.
12. Eun J S, Beauchemin K A, Hong S H and Bauer M W. Exogenous enzymes added to untreated or ammoniated rice straw: Effects on *in vitro* fermentation characteristics and degradability. Animal Feed science and Technology , 2006, 131(1):87-102.
13. Eun J S, Beauchemin K A. Assessment of the efficacy of varying experimental exogenous fibrolytic enzymes using *in vitro* fermentation characteristics. Animal Feed science and Technology , 2007, 132(3): 298-315.
14. Feng Z C, Gao M. The improved method in determining the ammonia-N in rumen fluid by colorimetry. Inner Mongolia. Anim. Sci, 1993, 14: 40-41.
15. Gado, H.M., Salem, A.Z.M., Robinson, P.H., Hassan, M. 2009. Influence of exogenous enzymes on nutrient digestibility, extent of ruminal fermentation as well as milk production and composition in dairy cows. Animal Feed Science and Technology, 2009, 154(1-2),36-46.
16. Giraldo L A, Ranilla M J, Tejido M L and Carro M D. Influence of exogenous fibrolytic enzymes and fumarate on methane production, microbial growth and fermentation in rusitec fermenters. British Journal of Nutrition, 2007, 98(4):753-761.
17. Giraldo L A, Tejido M L, Ranilla M J and Carro M D. Effects of exogenous fibrolytic enzymes on *in vitro* ruminal fermentation of substrates with different forage:concentrate ratios. Animal Feed science and Technology, 2008, 141(3):306-325.
18. Hernández-Cruz L, Ramírez-Bribiesca J E, Guerrero-Legarreta M I, Hernández-Mendo O, Crosby-Galvan M M and Hernández-Calva L M. Effects of crossbreeding on carcass and meat quality of mexican lambs. Arquivo Brasileiro de Medicina Veterinária e Zootecnia, 2009, 61(2):475-483.
19. Hu W L, Liu J X, Ye J A, Wu Y M and Guo Y Q. Effect of tea saponin on rumen fermentation in vitro. Animal Feed science and Technology, 2005, 120(3):333-339.
20. Jung H G, Raeth-Knight M, Linn J G. Forage fiber digestibility: Measurement, variability, and impact.Proc. 65th Minnesota Nutr. Conf., St. Paul, MN. Univ. Minnesota, Minneapolis. 2004: 105-125.
21. Khattab HM, Gado HM, Kholif AE, Mansour AM, Kholif AM. The potential of feeding goats sun dried rumen contents with or without bacterial inoculums as replacement for berseem clover and the effects on milk production and animal health. International Journal of Dairy Science 2011; 6: 267-277.
22. Kholif AM, Aziz HA. Influence of feeding cellulytic enzymes on performance, digestibility and ruminal fermentation in goats. Journal of Animal Nutrition and Feed Technology 2014; 14: 121-136.

23. Krueger N A. and Adesogan A T. Effects of different mixtures of fibrolytic enzymes on digestion and fermentation of bahiagrass hay. Animal Feed science and Technology, 2008, 145(1): 84-94.
24. Mao H L, Wu C H, Wang J K and Liu J X. Synergistic effect of cellulase and xylanase on in vitro rumen fermentation and microbial population with rice straw as substrate. Anim Nutr Feed Technol, 2013, 13:477-487.
25. Mauricio R M,Mould F L,Dhanoa M S, Owen E, Channa K S and Theodorou M K. A semi-automated *in vitro* gas production technique for ruminant feedstuff evaluation. Animal Feed Science and Technology,1999,79(4): 321-330.
26. Phakachoed N, Suksombat W, Colombatto D and Beauchemin K A. Use of fibrolytic enzymes additives to enhance *in vitro* ruminal fermentation of corn silage. Livestock Science, 2013, 157: 100-112.
27. Romero J J, Zarate M A, Adesogan A T. Effect of the dose of exogenous fibrolytic enzyme preparations on preingestive fiber hydrolysis, ruminal fermentation, and in vitro digestibility of bermudagrass haylage. Journal of Dairy Science, 2015, 98(1): 406-417.
28. Salem AZM, Buendía-Rodríguez G, Elghandour MMM, Mariezcurrena Berasain MA, Peña Jiménez FJ, Pliego AB, Chagoyán JCV, Cerrillo MA, Rodríguez MA. Effects of cellulase and xylanase enzymes mixed with increasing doses of Salix babylonica extract on in vitro rumen gas production kinetics of a mixture of corn silage with concentrate. Journal of Integrative Agriculture 2015a; 14(1): 131-139.
29. Salem AZM, Alsersy H, Camacho LM, El-Adawy MM, Elghandour MMY, Kholif AE, Rivero N, Alonso MU, Zaragoza A. Feed intake, nutrient digestibility, nitrogen utilization and ruminal fermentation activities of sheep fed Atriplex halimus treated with three developed enzyme cocktails. Czech Journal of Animal Science Czech Journal of Animal Science 2015b; 60, (2): 80–88.
30. Salem AZM, Gado HM, Colombatto D, Eghandour MMY. Effect of exogenous enzymes on nutrient digestibility, ruminal fermentation and growth performance in beef steers. Livest Sci 2013; 154: 69–73.
31. Soltan Y, Abdalla A, Silva L, Natel A, Morsy A and Louvandini H. Response of different tropical pasture grass species to treatments with fibrolytic enzymes in terms of *in vitro* ruminal nutrient degradation and methanogenesis. Animal Nutrition and Feed Technology, 2013, 13(3):551-568.
32. Togtokhbayar N, Cerrillo S M A, Jigjidpurev S, Shinekhuu J, Urantulkhuur D, Nergui D, Elghandour M M Y, Odongo N E and Kholif A E. Effect of exogenous xylanase on rumen in vitro gas production and degradability of wheat straw. Animal Science Journal 2015, doi: 10.1111/asj.12364.
33. Valdes K I, Salem A Z M., Lopez S., Alonso M U, Rivero N, Elghandour M M Y, Domínguez I A, Ronquillo M G and Kholif A E. Influence of exogenous enzymes in presence of Salix babylonica extract on digestibility, microbial protein synthesis and performance of lambs fed maize silage. Journal of Agricultural Science, 2015, doi:10.1017/S0021859614000975.
34. Wood T M, Bhat K M. Methods for measuring cellulase activities. Methods in enzymology, 1988, 160: 87-112.

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