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EFFECT OF FIBROLYTIC ENZYME PRODUCTS AT DIFFERENT LEVELS ON *IN VITRO* RUMINAL FERMENTATION OF LOW QUALITY FEEDS AND TOTAL MIXED RATION

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ABSTRACT

This study aimed to evaluate the effects of cellulase and xylanase plus enzymes on *in vitro* ruminal fermentation of *Eragrostis curvura* hay, maize stover and a total mixed ration (TMR) at six levels of application. The feed samples were incubated for 2, 12, 24, 32, 48, 72, h in an *in vitro* batch culture with buffer and ruminal fluid, and the fibrolytic enzymes associated with the six levels of application. Gas production was measured using a pressure transducer connected to a data tracker. Degradability of fiber was measured after 48 hrs of incubation. Increased level of enzyme inclusion increased the volume of gas, the total volatile fatty acid (VFA) production and fibre disappearance, but the rate of increment associated with each mg of additional enzyme application was reduced beyond 1-2mg/g DM. Considering the enzymes cost and their efficiency at different rates on gas production and NDF degradability the medium levels of applications (1-2mg/g DM) were found to be the most efficient.

Key words: *Eragrostis curvula, fibrolytic enzymes, gas production, in vitro, maize stover, TMR.*

INTRODUCTION

In tropical and subtropical farming systems, forage plants are the major sources of energy for ruminants. The fibre is a major component of the forage dry matter. The fibre has low energy and low digestibility co-efficiency (Hatfiled *et al.*, 1999; Azzaz *et al.*, 2012) since the plant cell wall is an interwoven matrix of polymers that form complex and dynamic structures. These dynamic structures are barriers against microbial invasion and limit their access to the digestible cell wall networks of plants (Krueger *et al.*, 2008; McDonald *et al*., 2011). Moreover, the rumen environment affects fibre digestion (McDonald *et al*., 2011).

Plant cell walls typically consist of about 35-50% cellulose, 20-35% hemicelluloses, and 10-25% lignin in the dry mass (Sticklen, 2008). Over past decades, various chemical treatment options significantly improved the cell wall digestibility (McDonald *et al*., 2011). But, despite all these efforts more than 50% of fibre fraction is still not readily digested. Therefore, the efficient use of fibrous feeds in ruminant production systems is still limited (Hatfield *et al*., 1999).

The inclusion of exogenous fibrolytic enzymes (EFE) as an alternative way of improving fibre digestibility in fibrous feeds have resulted in positive outcomes (Adesogan *et al*., 2007; Krueger *et al*., 2008; Azzazet *et al*., 2012). Cellulase and xylanase are two major ruminant diet enzyme groups that break down the cellulose and xylans found in the plant cell wall components, respectively (Beauchemin *et al*., 2003, Lynd *et al*., 2005). Many authors reported an increase in the digestion of fibre and improved animal performances (Adesogan *et al*., 2007; Bala *et al*., 2009). However, others reported either a negative or no effect at all (Bowman *et al*., 2003; Vicini *et al*., 2003; Baloyi, 2008).

The activities of enzymes vary with the proportion of concentrate in the diet (Giraldo *et al.*, 2008), enzyme doses (Jalilvand *et al*., 2008), rumen pH (Yang *et al*., 2002), moisture content of the feed (Wang *et al.*, 2002), and methods of supplementation (Krueger *et al*., 2008). Moreover, the optimal level of inclusion is dependent on the diet under consideration. Thus the optimum rate of inclusion of a given enzyme preparation for the various feeds needs to be determined. The relationship between enzyme activity and forage utilization could in turn help to explain and determine the conditions most likely to result in positive responses of animals. The objective of this study was to evaluate the effects of fibrolytic cellulase and xylanase enzymes on *in vitro* digestibility and rumen fermentation characteristic of *Eragrostsis curvula*, maize stover and a total mixed ration (TMR).

MATERIALS AND METHODS

The cellulase and xylanase plus enzymes used in the study (Dyadic International Inc., Florida, USA) were concentrated liquids of acid cellulase (E.C. 3.2.1.4) and acid-neutral endo-1, 4-β-D-xylanase (E.C. 3.2.1.8), respectively. They were produced by the fermentation of non-GMO *Trichoderma longibrachiatum* (formerly *Trichoderma reesei*). *Eragrostis curvula*, maize stover and formulated total mixed ration (TMR) were used as

test feeds. The TMR contains hominy chop (26.8%), wheat bran (7.9%), *Eragrostis* hay (14.85%), alfalfa hay (14.85%), sun flower oil meal (21.8%), soya meal (3.96%), molasses (6.94%), lime stock (1%), dicalcium phosphate (0.5%), salt (0.5%), sodium bicarbonate (0.5%) and a remix (0.4%). They were treated at 0, 0.5, 1, 2, 3, 4 and 5 mg/g DM for cellulose and 0, 0.25, 0.5, 1, 2, 3, 4 mg/g DM for xylanase.

Feed sample collection, preparation and chemical analysis: Samples of test feeds were dried at 55°C for 48 h in the forced draft oven and ground with a Wiley Mill fitted with a 1 mm screen to be used for in this study. Feed samples were analyzed for DM and total ash using the method of AOAC (2002). Fat content was determined using ether extraction in the Tecator Soxtec (HT6) system (AOAC, 2002). The neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) contents were determined using an ANKOM200/220 Fiber Analyzer (ANKOM Technology, Fairport, NY) based on the method describe by Van Soest *et al*., (1991). Sodium sulphite and heat-stable amylase were used in the analysis of NDF. Crude protein was measured according to the combustion method (AOAC, 2002) for nitrogen on a Leco FP-428 Nitrogen and Protein analyzer (Leco Corporation, St. Joseph, MI, USA).

Enzyme assay (Enzyme activity determination): Enzyme activities were determined using single polysaccharides as a substrate in triplicate with the inclusion of control. Xylanase activity was assayed using 1% (w/v) Birchwood xylan as a substrate following the procedure described by Bailey *et al.* (1992). Endo-glucanase and Exo-glucanase enzyme activities were assayed following the method described by Wood and Bhat (1988). The enzymes were studies at 4 different pH (4, 4.8, 5.8, and 6.0). One unit of activity was defined as the amount of enzyme required for releasing 1µmol equivalent of glucose or xylose per minute per gram of enzyme under the condition of assay.

***In vitro* gas production measurement**

Collection of rumen fluid from donor sheep: The rumen fluid was collected from three ruminally cannulated Merino wethers fed on *ad libtum* amount of alfalfa hay before the morning feeding. Approximately 500 ml of the ruminal fluid were collected from each donor animal, mixed, strained through four layers of cheesecloth and then transferred to pre-heated thermos flasks. In the laboratory, the flasks were emptied into an industrial blender while being purged with CO_2 to maintain anaerobic conditions (Grant and Mertens, 1992). After blending, the rumen fluid was transferred to a large glass beaker that was kept inside a 39°C water bath being continuously purged with CO_2 and continuously stirred as recommended by Goering and Van Soest (1970). Thereafter, the required amount of rumen fluid was added to the buffer solution in the respective incubation vessels at a ratio of one part rumen fluid to four parts of buffer solution. This rumen buffer solution was used for the *in vitro* gas production and *in vitro* NDF digestibility studies.

Reducing buffer solution: The rumen buffer solution, macro mineral solution and micro mineral solution were prepared in large quantities and utilized as needed. The micro mineral solution was stored in a dark glass bottle in order to maintain the quality of the solution. In the morning, before the commencement of the experiment, the appropriate amounts of distilled water, rumen buffer solution, macro and micro mineral solutions were mixed with the tryptose and prepared 0.1% (wt/vol) resazurin. Enzyme solution was prepared based on required dose rate(s) for specific experimental treatments in order to deliver the desired amount of enzyme in a 1ml aliquot. Appropriate amounts of L-cysteine hydrochloride and sodium sulphide were weighted and directly added to the rest of the solution once all chemicals were dissolved. As soon as the reducing agent was added, the buffer solution was placed in a 39°C water bath and bubbled with CO_2. The serum bottles were then sealed with a rubber stopper and left at 39°C until the buffer solution was clear, indicating that the solution was sufficiently reduced.

Measurement of gas production: A semi- automated system was used to measure gas production through *in vitro* incubation at 39°C, according to Theodorou *et al.* (1994). The system consists of digital data tracker (tracker 220 series indicators, Omega Engineering, Inc., Laval, QC, Canada) connected to pressure transducer (PX4200-015GI from Omega Engineering, Inc., Laval, QC, Canada) with needle at the tip. About 500 mg of respective feed sample was weighed into 120ml serum bottle, and 1ml of the appropriate enzyme treatment was directly pipetted onto the substrate and incubated for 24 hours. Then 42 mL of rumen fluid + medium was added under a stream of CO_2 to each of the serum bottles and closed with rubber stoppers and crimp seal caps. Needle was inserted through rubber stopper of each serum bottles for about 5 seconds to release small amount of gas that might have build up and create starting point for incubation. All serum bottles were returned to the incubator, and the rotary shaker was turned on at 120 rpm. Gas pressure was taken at 2, 4, 8, 12, 16, 24, 32, 48, 54, 72 h of incubation. To quantify the gas production derived from the culture medium and the ruminal inoculums, two blank were included in every analysis. Two replicates and four different runs were executed for every treatment. The pressure and volume values of each reading time were registered, and added to the values of the previous readings. Thus, the cumulative pressure and volume of the fermentation gases were obtained. Fermentation was terminated after 72 h by removing serum bottles from incubator and placing them on ice.

Supernatants were taken immediately, pipetted and stored at -20°C until analysed for ammonia N (McDonald *et al*., 1960) and volatile fatty acid (Ottenstein and Bartley, 1971)

***In vitro* degradability:** To evaluate *in vitro* NDF degradability at 48 h of fermentation, ruminal fluid samples and DM residuals were collected from two bottles per treatment. All the serum bottle contents were transferred into gush crucibles and using vacuum filter system the fluid was filtered and dried in an oven at 55°C for 48 h after which DM disappearance was determined. The blank corrected sample weight was referred as apparent undegradable DM, and the degradability was calculated as the ratio of degradable DM to that of substrate DM incubated. About 0.25 mg of dried sample was transferred into ANKOM filter bags for NDF determination and later estimation of the NDF disappearance. Total degradable DM and NDF were derived from the difference between the weight of DM incubated and NDF residues as indicated by formulas described in manufactures manual.

Calculations and statistical analysis: Glucose and xylose equivalents (mg) = a + bx
Where x is the absorbance obtained after correction for the enzyme and the substrate blanks.
Metabolizable energy (ME, MJ/kg DM) were estimated according to Menke and Steingass (1988) as: ME (MJ/kg DM) = 2.20 + 0.136 IVGP24 (ml/0.5 g DM) + 0.057 CP (% DM)
Rate and extent of gas production was determined for each feed by fitting gas production data to the non-linear equation: $y = b\ (1 - e^{-ct})$ (Ørskov and Mcdonald, 1979), where y = the gas production at time t; b = the slowly fermentable fraction (g kg DM^{-1}), and c = the rate (% h^{-1}) of fermentation of fraction b.

The experimental design used in this study was a completely randomized design. The data were statistically analyzed using the 'GLM' option of SAS (2004), and differences among means were determined using Tukey test. *In vitro* incubation times were used to fit non-linear regression models using the 'NLIN' procedure (SAS, 2004).

RESULTS

Chemical composition: The nutrient compositions of test feeds are shown in table 1. TMR contains high crude protein (CP) and lower cell wall contents (ADF, NDF, ADL and cellulose) among the three test feeds. High proportions of ADF and ADL were recorded for Maize stove while *Eragrostis curvula* contains high values of NDF and cellulose. Maize stove contains low amounts of acid detergent insoluble nitrogen (ADIN), and potentially available nitrogen (NDIN) while TMR contains high proportion of ADIN and NDIN.

Enzyme activity: The enzymes activity profile determined at different pH and a temperature of 39°C is presented in table 2. High enzyme activity was observed at pH of 4.8 while enzyme activity declines as pH increases for both enzymes.

***In vitro* Gas Production:** The effects of application of different levels of cellulase and xylanase enzymes on cumulative gas production pattern of the three test feeds are shown on table 3. The addition of cellulase and xylanase enzymes increased the cumulative gas production recorded at various time intervals. The cumulative gas production increased with increasing levels of enzymes application at quadratic rate for all test feeds, except at the lowest rate of application.

Table 1. Chemical composition of test feeds (Mean±SE) treated with two commercial enzyme products (cellulase and xylanase PLUS) at different level of enzyme treatments.

Chemical components	Test feeds composition g/kg DM		
	Eragrotis curvula	**Maize stover**	**TMR**
DM	945.1±8.4^{a}	922.5±0.5^{b}	938.6±0.05^{a}
Ash	37.5±0.4^{b}	22.8±0.1^{c}	74.8±0.26^{a}
OM	907.6±0.4^{a}	901.4±8.2^{a}	863.8±0.24^{b}
EE	10.8±0.2^{b}	8.71±0.18^{c}	59.9±1.4^{a}
CP	31.1±0.03^{b}	20.5±0.18^{c}	196.9±0.47^{a}
ADF	502.7±2.3^{b}	521.9±3.9^{a}	202.9±4.04^{c}
NDF	844.5±3.2^{a}	811.5±17.7^{b}	296.0±6.06^{c}
ADL	76.3±1.32^{b}	108.7±1.5^{a}	34.9±0.40^{c}
ADIN(% CP to NDF)	9.78±0.3^{b}	2.70±0.1^{c}	116.4±0.4^{a}
NDIN(% CP to NDF)	14.2±0.3^{b}	10.4±0.1^{c}	148.4±0.04^{a}
Cellulose	426.4±1.32^{a}	413.24±2^{b}	168.0±4.4^{c}
ME(MJ/kg calculated)	25.7±0.03^{b}	25.9±0.01^{b}	45.6±0.03^{a}

Means with different letters (superscripts) within a row differ significantly at indicated P value, P<0.05

Table 2. The activities of two experimental enzyme used in the study and the amounts of released sugar (U/ml) at different pH.

pH	Cellulase activity		Hemi-cellulase activity (Xylanase assay)
	Endo-glucanase assay	*Exo-glucanase assay*	
4	4232.2±24.4^{b}	3±0.24^{b}	1677.2±11.0^{d}
4.8	4484.3±32.8^{a}	4.3±0.12^{a}	2497.6±9.2^{a}
5.8	3373.6±29.2^{c}	1.5±0.5^{c}	1831.5±10.2^{b}
6	2141.9±28.2^{d}	1.0±0.2^{d}	1737.2±11.8^{c}

Means with different letters (superscripts) across a column differ significantly at indicated P value, P<0.05

Table 3. Gas production (ml/500mg DM) of feeds treated with two commercial cellulase and xylanase (PLUS) enzyme products at five levels.

Enzymes and levels	*Gas production at different time intervals*														
	Eragrostis curvula					*Maize stover*					*Total mixed ration (TMR)*				
	2	**12**	**24**	**48**	**72**	**2**	**12**	**24**	**48**	**72**	**2**	**12**	**24**	**48**	**72**
Cellulase															
0	1.16^{b}	2.65^{f}	6.5^{g}	21.9^{g}	33.8^{g}	1.16^{c}	4.59^{c}	8.53e	24.7^{f}	37.4^{f}	2.15^{e}	26.1^{f}	44.9^{g}	56.2^{g}	67.0^{g}
0.5	1.21^{b}	3.54^{e}	7.4^{f}	22.8^{f}	34.5^{f}	1.76^{ab}	5.17^{c}	9.07^{ed}	25.3^{f}	37.8^{f}	2.81^{d}	26.9^{e}	45.7^{f}	58.7^{f}	70.3^{f}
1	1.05^{b}	4.62^{d}	9.32^{e}	27.3^{e}	39.7^{e}	1.59^{b}	5.60^{c}	9.93^{d}	26.9^{e}	40.2^{e}	3.65^{b}	28.6^{d}	48.2^{e}	62.8^{e}	75.3^{e}
2	1.83^{a}	5.99^{c}	12.4^{d}	31.9^{d}	46.1^{d}	1.82^{ab}	7.20^{b}	13.6^{c}	33.2^{d}	48.2^{d}	3.27^{c}	29.0^{c}	49.5^{d}	65.8^{d}	79.1^{d}
3	1.8^{a}	5.95^{c}	9.82^{c}	32.7^{c}	47.7^{c}	1.87^{ab}	8.90^{a}	12.8^{bc}	38.2^{c}	53.2^{c}	2.83^{d}	29.4^{c}	50.8^{c}	68.3^{c}	82.4^{c}
4	1.76^{b}	6.78^{b}	11.5^{b}	36.1^{b}	51.0^{b}	1.85^{ab}	9.93^{a}	14.6^{a}	40.8^{b}	56.6^{b}	3.66^{b}	31.1^{b}	53.3^{b}	73.7^{b}	87.8^{b}
5	*1.79^{b}*	8.4^{a}	14.9^{a}	41.1^{a}	60.2^{a}	2.14^{a}	9.93^{a}	15.9^{a}	42.2^{a}	61.3^{a}	4.99^{a}	33.2^{a}	56.2^{a}	78.7^{a}	94.1^{a}
SEM	*0.53*	*1.32*	*1.92*	*4.61*	*6.27*	*0.65*	*1.63*	*2.01*	*4.94*	*6.25*	*0.93*	*1.61*	*2.66*	*5.31*	*6.34*
Linear(P)	*<0.04*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.05*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.03*	*<0.001*	*<0.001*	*<0.001*	*<0.001*
Quadratic(P)	*<0.02*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.02*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.02*	*<0.001*	*<0.001*	*<0.001*	*<0.001*
Xylanase															
0	1.16^{d}	2.65^{f}	6.50^{e}	21.9^{f}	33.8^{f}	1.16^{d}	4.59^{e}	8.51^{de}	24.7^{f}	37.4^{e}	2.15^{c}	26.1^{e}	44.9^{f}	56.2^{f}	67.0^{f}
0.25	1.21^{d}	3.54^{e}	6.57^{e}	22.0^{f}	33.9^{f}	1.79^{c}	5.47^{d}	8.51^{de}	23.9^{f}	37.6^{e}	2.83^{b}	26.1^{e}	44.9^{f}	56.3^{f}	67.1^{f}
0.5	1.28^{d}	3.62^{e}	6.66^{e}	22.9^{e}	34.6^{e}	1.57^{c}	5.26^{de}	8.30^{e}	24.6^{f}	38.4^{e}	2.83^{b}	26.9^{d}	45.7^{e}	58.7^{e}	70.4^{e}
1	1.83^{c}	4.33^{d}	8.20^{d}	26.1^{d}	39.4^{d}	2.68^{b}	5.26^{ed}	9.13^{d}	27.1^{e}	43.7^{d}	3.66^{b}	28.6^{c}	48.3^{d}	62.9^{d}	75.3^{d}
2	1.83^{c}	5.57^{c}	10.3^{c}	29.8^{c}	43.2^{c}	2.18^{bc}	6.61^{c}	11.3^{c}	30.9^{d}	49.2^{c}	2.83^{b}	28.6^{c}	49.1^{c}	65.4^{c}	78.7^{c}
3	2.48^{b}	6.82^{b}	12.4^{b}	33.6^{b}	46.9^{b}	2.6^{b}	7.76^{b}	13.3^{b}	34.6^{b}	53.7^{b}	3.66^{b}	30.2^{b}	51.6^{b}	69.1^{b}	83.2^{b}
4	*3.12^{a}*	10.2^{a}	19.01^{a}	46.9^{a}	62.7^{a}	3.43^{a}	10.8^{a}	19.6^{a}	47.6^{a}	60.4^{a}	3.66^{b}	31.1^{a}	53.3^{a}	73.7^{a}	88.6^{a}
SEM	*0.57*	*1.65*	*3.08*	*6.04*	*6.99*	1.25	*1.45*	*2.8*	*7.26*	*7.23*	*0.95*	*1.31*	*2.37*	*5.03*	*6.3*
Linear	*<0.04*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.05*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.03*	*<0.001*	*<0.001*	*<0.001*	*<0.001*
Quadratic	*<0.02*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.02*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.02*	*<0.001*	*<0.001*	*<0.001*	*<0.001*

Means with different letters (superscripts) within a column differ significantly at indicated P value, P<0.05 for each enzymes.

The potential extent of gas production (b), the gas production rate (c) and the effective gas production (EGP) estimated or calculated based on gas production data for the three test feeds were different at the different application levels of cellulase and xylanase enzymes (Table 4). For all test feeds, potential extent of gas production (b) increased with increasing levels of enzyme application, and the highest b values were obtained at application level of 5mg/g DM cellulase and 4mg/g DM xylanase enzymes. For the three test feeds, application of cellulase and xylanase improved the gas production rate (c) when compared to the control. However, there was no clear pattern of improvement observed for increased levels of applications.

Volatile Fatty Acids (VFA) and ammonia-N profiles: The ammonia- N and VFA profiles recorded for cellulase and xylanse treatments are shown in table 5 and 6. Generally, for all test feeds, acetate and total VFA concentration seems to be higher for the enzyme treated samples compared to the control samples. Among the enzyme treated feeds, the production of acetate and total VFA increased with increasing application level of enzymes. Propionate production increased with increasing levels of cellulase enzyme application for *E. curvula* and maize stover. While, in case of TMR, propionate production seems to decrease with increasing levels of application of the two enzymes. There seems to be an increase of iso-butryic and butyric acids production while no clear pattern was observed for valeric acid production in all the three test feeds. The acetate to propionate ratio (A:P) increased with increasing application level of both enzyme for *E. curvula* and TMR test feeds. The ammonia-N production from the roughage test feeds seems to be unaffected by application of cellulase and xylanase enzymes, while higher concentration of ammonia was recorded for the TMR test feed due to cellulase and xylanase application.

***In vitro* NDF degradability:** The NDF degradability of the three test feeds treated with different levels of cellulase and xylanase enzymes are shown in table 7. The addition of cellulase and xylanase enzymes at more than 0.5 mg/g DM increased NDF degradability of the *E. curvula* and maize stover. In case of TMR, cellulase increased NDF degradation at an application rate higher than 0.5 mg/g DM while similar increase in NDF degradability was recorded for xylanase at application rate greater than 0.25 mg/g DM. The NDF degradability has increased at quadratic rate with increasing application levels of cellulase and xylanase enzymes.

Table 4. The kinetics of gas production (ml/g DM) of feeds treated with two commercial cellulase and xylanase (PLUS) enzyme products at six levels.

Enzyme and levels of application		*Eragrostis curvula*			Maize stover			Total mixed ration(TMR)		
		b	C	ED	b	c	ED	b	c	ED
Cellulase	0	13.2^{g}	0.010^{f}	2.19^{g}	15.7^{f}	0.012^{f}	3.10^{f}	41.4^{g}	0.037^{b}	17.5^{g}
	0.5	14.4^{f}	0.010^{f}	2.4^{f}	18.9^{d}	0.029^{a}	6.90^{b}	40.9^{f}	0.039^{a}	18.3^{f}
	1	16.4^{e}	0.010^{e}	2.75^{e}	18.1^{e}	0.028^{b}	6.57^{e}	43.7^{e}	0.036^{c}	18.4^{e}
	2	20.1^{d}	0.016^{b}	4.89^{c}	20.5^{c}	0.013^{f}	4.29^{f}	46.9^{d}	0.033^{d}	18.7^{d}
	3	19.7^{c}	0.0199^{a}	5.59^{a}	25.2^{b}	0.027^{c}	8.84^{a}	51.9^{c}	0.032^{e}	20.2^{c}
	4	21.1^{b}	0.0126^{c}	4.24^{d}	30.2^{a}	0.015^{d}	6.86^{c}	52^{b}	0.032^{f}	20.4^{b}
	5	28.4^{a}	0.0109^{d}	5.09^{b}	30.2^{a}	0.014^{e}	6.60^{d}	60.6^{a}	0.031^{g}	23.3^{a}
	Linear	*<0.001*	*0.16*	*<0.001*	*<0.001*	*0.16*	*<0.001*	*<0.001*	*<0.01*	*<0.001*
	Quadratic	*<0.001*	*0.23*	*<0.001*	*<0.001*	*0.08*	*<0.001*	*<0.001*	*<0.01*	*<0.001*
Xylanase	0	13.2^{g}	0.010^{f}	2.10^{g}	15.1^{f}	0.010^{b}	3.096^{e}	41.1^{d}	0.037^{b}	18.3^{d}
	0.25	13.5^{f}	0.011^{e}	2.37^{f}	15.1^{f}	0.010^{b}	2.52^{g}	41.2^{d}	0.037^{b}	18.3^{d}
	0.5	13.9^{e}	0.011^{e}	2.52^{e}	17.1^{e}	0.010^{b}	2.94^{f}	41.1^{d}	0.039^{a}	17.5^{f}
	1	16.6^{d}	0.020^{d}	4.75^{d}	17.8^{d}	0.011^{a}	3.14^{d}	43.1^{c}	0.036^{c}	18.0^{e}
	2	17.8^{c}	0.026^{b}	6.09^{b}	21.4^{c}	0.010^{b}	3.56^{c}	49.9^{b}	0.03^{d}	18.7^{c}
	3	20.6^{b}	0.021^{c}	6.08^{c}	22.9^{b}	0.010^{b}	3.83^{b}	49.9^{b}	0.03^{d}	19.2^{a}
	4	28.8^{a}	0.029^{a}	10.6^{a}	33.6^{a}	0.011^{a}	5.96^{a}	52.3^{a}	0.03^{d}	18.9^{b}
	Linear	*<0.001*	*0.1*	*<0.001*	*<0.001*	*0.19*	*<0.001*	*<0.001*	*<0.02*	*<0.001*
	Quadratic	*<0.001*	*0.12*	*<0.001*	*<0.001*	*0.13*	*<0.001*	*<0.001*	*<0.02*	*<0.001*

Means with different letters (superscripts) within a column differ significantly at indicated P value, P<0.05 for each enzymes

Table 5. Total and individual volatile fatty acid (mM/L) production, acetate to propionate ratio (A:P), and ammonia–N (mg/L), in supernatant after 72 h incubation of 500 mg DM of feed with cellulase enzyme

Feed and levels of enzymes use	Ammonia nitrogen and volatile fatty acids							
	NH_3-N	***Acetic***	***Propionic***	***Iso butyric***	***Butyric***	***Valeric***	***Total VFA***	***A:P ratio***
Eragrostis Curvula 0	117.1	23.0	7.29	0.73	2.63	1.08	34.74	3.155
0.5	115.9	23.05	7.33	0.74	2.68	1.08	34.87	3.147
1	110.4	33.42	10.13	1.03	3.80	1.36	49.74	3.299
2	108.8	34.09	10.30	1.01	3.71	1.44	50.56	3.311
3	108.2	37.20	10.40	1.33	5.16	1.16	55.25	3.576
4	107.4	38.49	10.55	1.43	5.25	1.47	57.20	3.647
Maize stove 0	107.6	36.8	13.86	1.00	4.59	1.45	57.7	2.654
0.5	102.0	38.64	13.98	1.08	5.00	1.61	60.31	2.764
1	95.2	47.25	18.68	1.51	6.84	1.98	76.26	2.529
2	91.9	52.76	15.09	1.60	7.19	1.60	78.24	3.497
3	90.2	55.63	14.18	1.52	6.16	1.62	79.10	3.923
4	90.1	50.01	19.38	1.62	6.91	2.02	79.95	2.580
TMR 0	194.1	42.08	26.50	1.47	4.28	2.81	77.14	1.588
0.5	191.6	54.53	19.38	2.19	9.68	3.12	88.90	2.814
1	232.7	54.67	19.91	2.20	9.21	3.05	89.04	2.746
2	211.6	57.64	23.98	1.72	7.58	2.03	92.96	2.404
3	205.8	58.98	25.19	1.83	8.08	1.20	95.28	2.342
4	202.7	58.63	21.10	2.37	10.44	3.35	95.89	2.779

Table 6. Total and individual volatile fatty acid (mM/L) production, acetate to propionate ratio (A:P), and ammoniaN (mg/L), in supernatant after 72 h incubation of 500 mg DM of feed with xylanase enzyme.

Feed and levels of enzymes use	Ammonia nitrogen and volatile fatty acids							
	NH_3-N	*Acetic*	*Propionic*	*Iso butyric*	*Butyric*	*Valeric*	*Total VFA*	*A:P ratio*
Eragrostis curvula 0	117.1	23.0	7.29	0.73	2.63	1.08	34.74	3.155
0.5	116.6	29.36	9.16	0.93	3.37	1.33	44.15	3.21
1	112.7	34.87	10.59	1.11	3.92	1.64	52.13	3.29
2	109.7	35.53	9.99	1.32	5.16	0.90	52.90	3.56
3	108.8	37.71	11.15	1.48	5.49	1.48	57.31	3.38
4	108.8	39.20	10.80	1.48	5.76	1.30	58.54	3.63
Maize stover 0	107.6	36.8	13.86	1.00	4.59	1.45	57.7	2.654
0.5	89.7	50.26	12.01	1.58	6.01	0.72	70.57	4.19
1	90.2	43.12	17.85	1.44	6.69	1.96	71.06	2.42
2	88.5	44.10	17.48	1.34	6.57	1.93	71.42	2.52
3	85.4	51.46	11.84	1.47	5.90	1.21	71.87	4.35
4	83.8	52.83	11.55	1.62	6.07	0.90	72.97	4.57
TMR 0	194.1	42.08	26.50	1.47	4.28	2.81	77.14	1.59
0.5	192.0	49.26	17.55	2.07	9.30	2.99	81.17	2.81
1	208.0	50.57	18.18	2.10	9.32	2.92	83.08	2.78
2	203.2	51.14	18.84	2.09	9.02	3.00	84.09	2.71
3	205.3	55.95	23.59	1.57	7.79	1.94	90.84	2.37
4	200.3	61.21	26.29	1.93	7.82	1.55	98.81	2.33

Table 7. Effects of exogenous fibrolytic enzymes on the *in vitro* 48-h NDF degradability (%) of feeds at different levels.

Enzyme levels	Eragrostis curvula	Maize stover	TMR
Cellulase 0	33.7±0.88^{f}	39.4±0.27^{f}	59.5±0.27^{e}
0.5	35.2±0.35^{f}	40.0±0.91^{f}	60.1±0.46^{e}
1	39.7±1.32^{e}	40.9±0.20^{e}	62.7±0.86^{d}
2	44.6±0.91^{d}	44.6±0.79^{d}	65.1±0.44^{c}
3	47.2±0.45^{c}	49.0±0.09^{c}	68.1±0.03^{b}
4	48.9±0.91^{b}	53.8±0.44^{b}	75.4±0.06^{a}
5	51.0±0.29^{a}	56.1±1.06^{a}	80.2±1.02^{a}
Linear (P)	*<0.001*	*<0.001*	*<0.001*
Quadratic (P)	*<0.001*	*<0.001*	*<0.001*
Xylanase 0	33.7±0.9^{d}	39.4±0.27^{d}	59.5±0.3^{f}
0.25	33.9±0.6^{e}	40.4±0.33^{d}	60.0±0.36^{f}
0.5	34.8±0.2^{e}	41.4±0.44^{d}	62.9±0.51^{e}
1	37.5±0.4^{d}	45.7±1.85^{c}	66.0±1.00^{d}
2	41.2±0.8^{c}	49.1±0.57^{b}	68.8±0.24^{c}
3	45.7±1.8^{b}	53.9±0.79^{a}	74.2±0.62^{b}
4	48.9±0.9^{a}	55.9±1.25^{a}	77.2±0.53^{a}
Linear (P)	*<0.001*	*<0.001*	*<0.001*
Quadratic (P)	*<0.001*	*<0.001*	*<0.001*

DISCUSSION

Enzyme activity: In this study, the xylanase and endo-glucanase activities for the studied enzymes are relatively high when compared to other values reported elsewhere (Colombatto *et al*., 2003, Enu and Beauchemin, 2007). However, it is difficult to extrapolate these results due to differences in sources of enzymes and differences in analytical procedures. These enzymes showed optimum performance at a pH of 4.8 as recommended by the manufacturer. It appears that ruminants may not benefit optimally at a higher pH of the rumen unless applied at a higher level to compensate for the loss of efficacy at a higher pH. It is important to note that most of the enzymes were not produced with the intention of feeding ruminants.

Gas production, degradability and VFA production: In this study the enzyme treatment at different levels of application significantly (P<0.05) increased gas production for all the test feeds. Gas production is an indirect measure of feed degradation, particularly the carbohydrate component (Menke *et al*., 1979). The increment in gas production at quadratic rate throughout the fermentation shows continuous degradation of the feeds over time. This indicates a continuous effect of the studied enzymes on the degradation of incubated feeds. In contrast to our finding, Tricario (2001) and Colombatto *et al*. (2003), reported short term effect of enzymes on degradation of feeds, with limited effects during fermentation. This continuous effect might be partly due to the pre-incubation effect that may form stable enzyme-feed complex. The stable enzyme-feed complex increases the resistance of the enzymes to proteolysis and lengthens their residence during latter fermentation periods (Kung *et al*., 2000; Yang *et al*., 2000).

The gas production increases at quadratic rate with increasing levels of enzyme application. Higher gas production indicates higher feed degradability. There is a significant and positive correlation between gas production data and the *in vitro* degradation data. Our finding with regard to *in vitro* degradability supports earlier finding (Menke *et al*., 1979), on gas production. Moreover, the results obtained for *in vitro* gas production and degradability might be attributed to high activities of xylanase and endo-glucanases enzymes activities. In agreement with our result, many authors noticed an increase in fibre degradability of diets or feedstuffs with enzyme supplementation (Eun and Beauchemin, 2007; Giraldo *et al*., 2008; Pinos- Rodríguez *et al*., 2008).

The observed increases in the production of total VFA, acetate, butyric, iso butyric as well as substrate DM and fibre disappearance increases the flow of microbial-N and microbial colonization of the substrate, resulting in enhanced fibre degradation. Similar to our finding, increased total VFA and acetate concentration was reported by Ranilla *et al*. (2008) from lucerne hay, barley straw, and isolated NDF cell walls with Fibrozyme. Pinos- Rodríguez *et al*. (2002), also reported increased total VFA from lucerne and rye grass based diet with Fibrozyme. Increased total VFA, acetate and propionate were reported by Giraldo *et al*. (2008) from different proportions of forage in the diet and grass hay with addition of fibrolyitc enzyme from *Trichoderma viride*, *Aspergillus niger* and *Trichoderma longibrachiatum.* Increased acetate, butyrate and methane production was reported by Giraldo *et al.* (2007) from grass hay and diet

with two different proportion of concentrate treated with mixed fibrolytic enzymes from *Trichoderma longibrachiatum* and Fumarate. Decreased acetate and increased proportion of propionate was reported by Krueger and Adesogan (2008) from Bahiagrass hay with the addition of cellulase and xylanase when combined with ferulic acid esterase.

The decrease in ammonia-N for *Eragrostis curvula* and maize stover might be due to insufficient N to support the increase in microbial growth while the higher N in TMR has sufficiently supported the microbial growth that has substantially improved fermentation of fiber. Enzymes results in subtle changes to the cell wall structure and facilitates microbial access to the cell contents, as a result the N located in these structures might be exposed and digested with better extent (Colombatto *et al*., 2003). The improvement of protein digestion by enzymes has also been reported by various authors (Colombatto *et al*., 2003, Pinos-Rodriguez *et al*., 2002, Pinos-Rodriguez *et al*., 2008).

This study showed that cellulase and xylanase enzymes could improve fibre degradation and the rate of degradation of these feeds. But, the mechanism of this improvement is not clearly known. The improvement in the attachment of microorganisms to the plant cell (Nsereko *et al*., 2000, Wang *et al*., 2001), an alteration in the fibre structure due to the enzyme effects (Giraldo *et al*., 2008), coupled with the increased colonization, that would have shorten the lag time could be possible reasons for the observed improvement. When enzymes act on the structures of plant cell walls, the access of the microbes to the potentially fermentable fibre is enhanced (Sutton *et al*., 2003; Elwakeel *et al*., 2007). In addition, the 24hr pre -incubation of feed sample with enzymes in our study might have enhanced the attachment of enzymes to the cell wall component and improved fermentation of the feeds. The positive effect of pre-feeding treatment was elaborated by many researchers due to the enzyme-substrate pre-incubation interaction period (Elwakeel *et al*., 2007; Krueger and Adesogan, 2008; Alvarez *et al*., 2009). Considering the enzymes cost and their efficiency at different rates on gas production and NDF degradability the medium levels of applications (1-2mg/g DM) were found to be the most efficient.

Conclusion: This study showed that the inclusion of cellulase and xylanase enzymes improved the fermentation and degradability of *Eragrostis curvala*, maize stover and total mixed ration. Pre-treatment of these low quality forages with cellulase at 5mg/g DM and xylanase at 4mg/g DM improved i*n vitro* ruminal fermentation and degradability of NDF. However, with application of dose rates the response level per unit of enzyme deceased beyond 1-2mg/g DM application. Thus, these levels needs to be further evaluated under *in vivo* condition to measure their effect in terms of feed intake, digestibility, rumen fermentation parameter, microbial protein synthesis and performance of target animals.

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