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Effect of Level and Duration of Application of a Commercial Enzyme Mixture on *In Vitro* Ruminal Fermentation Responses of a Mid-Forage Total Mixed Ration

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ABSTRACT

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In vitro gas production technique was used to evaluate ruminal fermentation characteristics of a mid-forage total mixed ration (TMR; containing 39.4% corn silage, 6.2% wheat straw and 54.4% concentrate) which was pre-treated with a commercial enzyme mixture (Natuzyme®) applied at the rate of 0.0, 0.84, 1.68 and 2.52 g/kg DM (E0.0, E0.84, E1.68 and E2.52, respectively) and at different times (0, 12 and 24 hr before to the start of the *in vitro* incubation, namely hr0, hr12 and hr24, respectively). Increasing levels of enzyme from 0.84 to 2.52 g/kg substrate DM linearly ($P<0.001$) increased gas production volume (GP_{96}). *In vitro* dry matter disappearance (IVDMD) increased quadratically ($P<0.05$) with increasing level of enzyme and was highest at E1.68. Fermentation efficiency (FE) increased quadratically ($P<0.001$) with increasing level of enzyme and the highest ($P<0.05$) FE was at E0.84. Methane production at ($t_{1/2}$) increased ($P<0.05$) linearly with increasing level of enzyme. Gas production volume (GP_{24}) responded quadratically ($P<0.001$) as the time of enzyme administration increased from hr 0 to hr12 and hr24 and was highest at hr12. IVDMD and FE decreased linearly ($P<0.001$) as time of pre-incubation increased from 0h to 24 h. Results suggest that the enzyme used and the times of pre-treatment are advantageous to improve *in vitro* fermentation of a mid-forage ration.

Key words: Enzyme, Gas production, *In vitro* fermentation.

INTRODUCTION

Forages are low cost and important energy sources for ruminants but their quality and availability is not always constant throughout the year. One of the most

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common methods for preserving forages for whole-year feeding is ensiling. Furthermore, the digestion of forages in the rumen is relatively slow and incomplete, limiting animal performance and increasing feed cost of livestock production (Bassiouni *et al.*, 2010). The availability of new methods of enzyme production such as biotechnological approaches has led to the hypothesis that enzymes (e.g. cellulases and xylanases) which degrade plant cell walls can be used to hydrolyse forage fibre and increase their digestibility. Beauchemin *et al. (*2003) reported that application of fibrolytic enzymes improved forage utilization and productive efficiency of ruminants. They also found that the improvement in animal performance due to addition of enzyme additives could be attributed mainly to improvements in ruminal fibre digestion resulting in an increase of digestible energy intake.

However, responses to enzyme supplementation differ for dry forage, fresh forage, and silage (Beauchemin *et al.*, 1995; Feng *et al.*, 1996). A number of studies have shown that the addition of fibrolytic enzymes to grass or alfalfa hay before ensiling reduced the concentration of plant structural carbohydrates compared with untreated silages (Henderson and McDonald, 1977; van Vuuren *et al.*, 1989; Jacobs and McAllan, 1991; Kung *et al.*, 1992; Stokes, 1992). Such enzymatic action on silage may enhance *in vivo* digestion. However, the animal responses to some commercial enzyme products have been variable as reviewed by Beauchemin *et al.* (2001). This inconsistency of responses to enzyme supplementation may be due to a number of factors, including diet composition, type of enzyme used, level of enzyme provided, enzyme stability in the digestive tract, and method of application (Yang *et al.*, 2000).

Furthermore, results from various studies indicated that responses to the level of enzyme addition are non-linear both *in vitro* (Colombatto *et al.*, 2003a,b) and *in vivo* (Lewis *et al.*, 1999; Kung *et al.*, 2000). The objective of the present study was to determine the effect of enzyme dose and pre-treatment duration of a commercial enzyme mixture on *in vitro* ruminal fermentation responses of a mid-forage total mixed ration.

MATERIALS AND METHODS

Experimental diet, enzymes mixture and enzymes administration

The experimental TMR contained (g/kg DM) corn silage, 394; wheat straw, 62; corn grain, 96; barley grain, 84; wheat grain, 22; soybean meal, 79; wheat barn, 62; cotton seed meal, 59; wheat residue, 50; sugar beet pulp, 56; fish meal, 17; fat powder, 5; calcium bicarbonate, 4; magnesium oxide, 4 and mineral additive, 6. Triplicated dried and ground (1 mm) samples of the test feed (300 mg) was weighed and placed in 125 ml serum bottles. To avoid undesirable variances, the needed amounts of enzyme equivalent 0, 0.84, 1.68 and 2.52 g/kg (referred as E0.0, E0.84, E1.68 and E2.52) was mixed with double distilled water in such a manner to maintain the moisture content of test feed in the serum bottles equal to approximately 40% on weight basis and pipetted directly on to feed samples in the serum bottles at 0,

12 and 24h before incubation (referred as hr0, hr12 and hr24). The bottles were closed with rubber stoppers and aluminium caps and kept at room temperature until half an hour before incubation time when the bottles were placed in a water bath at 39°C until incubation to avoid heat shock to micro-organisms. The enzyme was a powdered multi-enzyme commercially available feed additive product named Natuzyme® (Bioproton, Queensland, Australia) containing (per gram of enzyme preparation) cellulase (4200 units), xylanase (2500 units), ß-glucanase (500 units), protease (3000 units) and amylase (750 units) activities, as indicated by the manufacturer. Natuzyme® also contains hemicellulase, amyloglycosidase, pentosanase, pectinaseandphytase activities.

Determination of in vitro gas production parameters

Rumen fluid was obtained from three ruminally fistulated steers (580±4.5 kg, BW) before the morning feeding and immediately strained through four layers of cheesecloth to eliminate large feed particles and transferred to the laboratory in a pre-warmed thermos. The fluid were diluted (1:2 v/v) with a culture medium containing macro and micro mineral solutions, resazurin and a bicarbonate buffer solution prepared as described by Menke and Steingass (1988). The medium was kept at 39°C in a water bath and saturated with CO_2. Under an anaerobic condition, 30 ml of buffered rumen fluid was dispensed with a pipette pump into each of the pre-warmed 125 ml serum bottle containing the test feed and enzyme mixture. Each bottle was then sealed with rubber stopper and aluminium cap as above and placed in a shaking water bath set at 39°C for 96 h. The experiment was repeated in three runs, and in each run bottles containing only buffered rumen fluid were included as blank.

Gas production was measured at 2, 4, 6, 8, 10, 12, 24, 48, 72 and 96h of the incubation by inserting a 23 gauge (0.6 mm) needle attached to a pressure transducer (model PX4200-015GI, Omega Engineering Inc., Laval, Que., Canada) connected to a visual display (Data Track, Christchurch, UK) into the head space of the serum bottles. The transducer was then removed leaving the needle in place to permit venting. Pressure values and gas released from negative controls were used to generate volume estimates as reported by Mauricio *et al*. (1999) and to prevent accumulation of gas produced; head space gas of each bottle was released. Gas production data were modelled as described by Ørskov and McDonald (1979) using non-linear regression (NLIN procedure of SAS (1999, V. 8.2)) to obtain estimates for potential gas production (A, ml/g DM), time to reach half the asymptote ($t_{1/2}$, h) and fractional rate of gas production (μ, %/h) at specific times.

Determination of in vitro dry mater disappearance, fermentation efficiency and methane production

Experimental procedures were similar to those described above with the following exceptions. At $t_{1/2}$, total gas production (as described earlier) and percent of methane was measured in each bottle using Biogas Detector Device (SR2-BIO Sewerin, UK)

and bottles were immediately transferred on to an ice bath to stop fermentation. The contents of each bottle was filtered through 46 μm pore size filters and residuals were oven dried at 60°C for 72h and used to calculate IVDMD and FE as DM disappeared in terms of miligrams per milliliter methane produced at $t_{1/2}$.

Calculations and statistical analyses

Data were statistically analysed separately as a 4×3 factorial arrangement of completely randomized design with four concentrations of enzyme (0.0, 0.84, 1.68 and 2.52 g/kg substrate DM) and three times of administration (0, 12 and 24h prior to incubation) using the following statistical model:

$$y_{ijkl} = \mu + E_i + A_j + (EA)_{ij} + e_{ijk},$$

where y=depended variable, μ=overall mean, E_i=effect of enzyme level, A_j=effect of administration time, $(EA)_{ij}$=effects of the interaction between enzyme level and administration time and e_{ijk}=residual error. All the statistical analyses were performed using the general linear models procedures of SAS (1999, Version 8.2). Differences between means were assessed by Tukey test and treatment effects were declared significant if $P<0.05$. Additionally, orthogonal polynomial contrast was used to examine responses to increasing level of enzyme (linear, quadratic, and cubic) or administration time (linear and quadratic).

RESULTS

Parameters describing *in vitro* gas production of test diet treated with different levels of the enzyme and used at different administration times are shown in Tables 1 and 2. Time to reach half the asymptote ($t_{1/2}$, h) and fractional rate of gas production (μ, %/h) were not affected ($P>0.05$) by enzyme levels (Table 1). Potential gas production (A, ml/g DM) and gas production volume (GP_{96}) was higher ($P<0.05$) for E0.0 compared to E0.84, but potential gas production for other levels of enzyme did not differ. Increasing levels of enzyme from 0.84 to 2.52 g/kg substrate DM linearly ($P<0.001$) increased gas production volume (GP_{96}). Responses to increasing time of administration was both linear ($P<0.001$) and quadratic ($P<0.001$) for *in vitro* gas production volume (GP_{96}), time to reach half the asymptote ($t_{1/2}$) ($P<0.05$ and $P<0.01$) and fractional rate of gas production (μ, %/h) after 96h incubation ($P<0.05$ and $P<0.001$), but potential gas production (A), decreased linearly ($P<0.001$) as the time of enzyme administration increased from hr 0 to hr12 and hr 24, respectively (Table 2). Time to reach half the asymptote gas production increased ($P<0.01$) quadratically and was highest ($P<0.05$) at hr12 prior administration time compared to hr 0 and hr 24. Time hr 0 was not different from other times of administration. Also, hr 24 had higher ($P<0.05$). Fractional rate of gas production decreased ($P<0.001$) quadratically and was lowest ($P<0.05$) at hr12 administration time.

Table 1. *In vitro* gas production volume (GP_{96}), potential gas production (A), time to reach half the asymptote ($t_{1/2}$) and fractional rate of gas production (μ, %/h) after 96h incubation for test diet treated with different levels of enzyme

Parameters	Enzyme level (g/kg substrate DM)					Contrast[1]		
	0.0	0.84	1.68	2.52	SEM[2]	L	Q	C
GP_{96} (mL)	82.2[a]	75.2[b]	79.8[ab]	80.3[ab]	1.49	***	NS	*
A (mL)	84.2[a]	76.7[b]	82.1[ab]	78.6[ab]	1.71	NS	NS	*
$t_{1/2}$ (h)	15.0	14.9	14.8	15.4	0.42	NS	NS	NS
μ (h^{-1})	0.047	0.047	0.047	0.046	0.001	NS	NS	NS

[a,b,c]different superscripts following means within the same row indicate differences at $P<0.05$;

[1]Contrasts: L, Q and C=linear, quadratic and cubic effects of enzyme level;

[2]SEM - standard error of the mean; [2]FE=mg DM disappeared/ml methane produced at $t_{1/2}$;

*, ** and ***=$P<0.05$, 0.01 and 0.001, respectively. NS=not significant ($P>0.05$).

Table 2. *In vitro* gas production volume (GP_{96}), potential gas production (A), time to reach half the asymptote ($t_{1/2}$) and fractional rate of gas production (μ, %/h) after 96h incubation for test diet treated with powdered multi-enzyme at different times of administration

Parameters	Administration time (hr)				Contrasts[1]	
	0	12	24	SEM[2]	L	Q
GP_{96} (mL)	88.3[a]	80.0[b]	69.8[c]	1.29	***	***
A (mL)	89.8[a]	80.9[b]	70.5[c]	1.48	***	NS
$t_{1/2}$ (h)	15.2[ab]	16.0[a]	13.9[b]	0.37	*	**
μ (h^{-1})	0.046[b]	0.044[b]	0.050[a]	0.001	*	***

[a,b,c]different superscripts following means within the same row indicate differences at $P<0.05$;

[1]Contrasts: L and Q=linear and quadratic effects of administration time;

[2]SEM - standard error of the mean;

*, ** and ***=$P<0.05$, 0.01 and 0.001, respectively. NS=not significant ($P>0.05$).

Fermentation characteristics of test diet treated with different levels of enzyme at different administration times are shown in Tables 3 and 4. *In vitro gas* production volume responded linearly ($P<0.001$) and cubic ($P<0.05$) to increasing levels of enzyme. Enzyme level E1.68 had the highest *in vitro* gas production volume compared to others. IVDMD increased quadratically ($P<0.05$) with increasing level of enzyme and was highest at E1.68. Fermentation efficiency increased quadratically ($P<0.001$) with increasing level of enzyme and the highest fermentation efficiency was at E0.84 compared to other enzyme levels ($P<0.05$). Methane production at ($t_{1/2}$) increased ($P<0.05$) linearly with increasing level of enzyme.

Gas production volume (GP_{24}) responded quadratically ($P<0.001$) as the time of enzyme administration increased from hr 0 to hr 12 and hr 24 and was highest at hr 12 (Table 4). IVDMD and FE decreased linearly ($P<0.001$) as time of pre-incubation increased from hr 0 to hr 12 and hr 24 and percent of methane production increased linearly ($P<0.001$) and quadratic ($P<0.001$).

Table 3. *In vitro* gas production volume (GP), IVDMD, fermentation efficiency (FE) and percent of methane at ($t_{1/2}$) for test diet treated with different levels of powdered multi-enzyme

Parameters	Enzyme level (g/kg substrate DM)					Contrasts[1]		
	0	0.84	1.68	2.52	SEM[2]	L	Q	C
GP (mL)	36.3[c]	36.2[c]	48.3[a]	44.6[b]	0.23	***	NS	*
IVDMD (%)	31.9[c]	35.1[ab]	35.4[a]	34.1[ab]	0.26	NS	*	NS
FE[3] (mg/mL)	13.5[b]	15.3[a]	9.6[c]	8.8[c]	0.40	*	***	NS
Methane (%)	12.3[c]	15.7[b]	17.6[a]	18.9[a]	0.36	*	NS	NS

[a,b,c]different superscripts following means within the same row indicate differences at $P<0.05$;

[1]Contrasts: L, Q and C=linear, quadratic and cubic effects of enzyme level;

[2]SEM - standard error of the mean; [3]FE=mg DM disappeared/ml methane produced at $t_{1/2}$;

*, ** and ***=$P<0.05$, 0.01 and 0.001, respectively. NS=not significant ($P>0.05$).

Table 4. *In vitro* gas production volume (GP_{24}), IVDMD and fermentation efficiency (FE) after 24h incubation for test diet treated with powdered multi-enzyme at different times of administration

Parameters	Administration time (hr)				Contrast[1]	
	0	12	24	SEM[2]	L	Q
GP (mL)	45.5[b]	47.5[a]	31.6[c]	0.20	***	***
IVDMD (%)	38.4 [a]	34.3 [b]	29.7 [c]	0.22	***	NS
FE[3] (mg/mL)	15.3[a]	12.9[b]	7.2[c]	0.35	***	NS
Methane (%)	11.6[b]	12.2[b]	27.1[a]	0.31	***	***

[a,b,c]different superscripts following means within the same row indicate differences at $P<0.05$;

[1]Contrasts: L and Q=linear and quadratic effects of administration time;

[2]SEM - standard error of the mean; [3]FE=mg DM disappeared/ml methane produced at $t_{1/2}$;

*, ** and ***=$P<0.05$, 0.01 and 0.001, respectively. NS=not significant ($P>0.05$).

DISCUSSION

In the present experiment, *in vitro* gas production volume at $t_{1/2}$ and after 96h of incubation responded to increasing levels of enzyme in the same manner. Dawson and Tricario (1999) and Giraldo *et al*. (2007b) also reported that effects of fibrolytic enzyme *in vitro* were generally larger during the initial stages of degradation. Excluding E0.84, incremental levels of enzyme did not affect potential gas production or *in vitro* gas production volume at 96 hr of incubation. This is in agreement with the results reported by Jalilvand *et al*. (2008) who using several straws treated with three levels (3, 6, 9 g per kg DM) of the same enzyme as in the current experiment also reported that asymptotic gas production was not affected by enzyme level. The lack of effect on final gas production using exogenous fibrolytic enzyme was also reported by others (Mould *et al.*, 1999; Colombatto *et al.*, 2003a, b; Jalilvand *et al.*, 2008).

Since the rate at which enzymes show optimal effects depends on forage type (Beauchemin *et al.*, 1995), and that responses to incremental levels of enzymes are typically non-linear (Beauchemin *et al.*, 1995; Kung *et al.*, 2000), the decrease in gas production volume (GP_{96}) and potential gas production (A) after 96 hr incubation using E0.84 and increase in *in vitro* gas production volume (GP) and fermentation efficiency (FE) at $t_{1/2}$ using E1.68 in this experiment may be related to the nature of test diet and the observed non-linear effects.

In the present experiment, fractional rate of gas production and time at which half of the gas production was achieved was not affected by enzyme levels in agreement with the findings of Yang *et al.* (2000) and Kung *et al.* (2002) who reported that the gas production rate was not affected by fibrolytic enzyme supplementation. In contrast, other studies (Wallace *et al.*, 2000) have shown that cellulase and other commercial fibrolytic enzymes can increase the cumulative gas production and rates of *in vitro* fermentation of grass and corn silage. Results from other Studies have also shown that the effects of using fibrolytic enzyme supplements in ruminant diets are not always consistent (Rode *et al.*, 1999; Yang *et al.*, 1999), presumably due to diet composition, type of enzyme used, level of enzyme provided, enzyme stability and method of application (Yang *et al.*, 2000).

In the present study, effect of enzyme level on IVDMD was non-linear and E0.84 showed lower IVDMD compared to E0.0 and E2.25. Similarly, Almaraz *et al.* (2010) reported a quadratic response of *in vitro* dry matter disappearance as enzyme level in the diet was increased. Previous studies (Lewis *et al.*, 1999; Wang *et al.*, 2001; Giraldo *et al.*, 2004) have shown that a pre-treatment of feed with enzymes before incubation with ruminal fluid enhanced the beneficial effects of enzymes on ruminal fermentation. As pointed out by Colombatto *et al.* (2003a), the mode of action is not clear. Some authors have suggested that this could be due to the creation of a stable enzyme-feed complex (Kung *et al.*, 2000), but others have indicated the possibility of alteration in the fibre structure, which would stimulate microbial colonization (Newbold, 1997; Nsereko *et al.*, 2000; Giraldo *et al.*, 2007b). Forwood *et al.* (1990) reported that IVDMD was increased when forage were pre-incubated with enzymes at room temperature for various periods of time. These findings highlighted the importance of adsorption and binding of the enzyme to substrate before exposing feed-enzyme complex to rumen microbial community to allow proper attachment and protection against degradation by rumen proteases (Forwood *et al.*, 1990; Beauchemin *et al.*, 2003). However, the effects of duration of pre-treatment with exogenous enzymes on fermentation characteristics of feeds have not been investigated in depth, and in the current study, increasing the time of administration generally had negative effect on the potential gas production, *in vitro* gas production volume at 96 hr of incubation and IVDMD both at 96 hr and $t_{1/2}$.

Only a few studies have investigated the effects of exogenous enzymes on methane production, and the results are conflicting. Treating forage-based substrates

with fibrolytic enzymes has been reported to increase methane production (Giraldo *et al.,* 2007a). Dong *et al*. (1999) found that the treatment of grass hay with cellulase and xylanase enzymes in a Rusitec system increased cellulose digestibility and methane production by 15 and 43%, respectively. In present study, methane production was increased by 27.1, 42.4 and 53.2% for E0.84, E1.68 and E2.52, respectively compared with E0.0. Increasing the pre-treatment duration also increased percent of methane production but the negative effect of incremental levels of enzyme and effects of increasing time of enzyme administration on percent of methane production may be misleading without considering the basics of *in vitro* gas production technique. The *in vitro* gas production technique intends to measure the potential conversion of different nutrient fractions (monosaccharaides, polysaccharides, pectin, starch, cellulose and hemicellulose) to CO_2, VFA and CH_4 (Getachew *et al.*, 2004). In fact, when a feedstuff is incubated with buffered rumen fluid, it is first degraded and the degraded fraction may either be fermented to produce gas and fermentation acids, or incorporated into microbial biomass (Rymer *et al.*, 2005). Consequently, mg DM disappeared/ml methane produced (advocated as FE) can be a better index for investigating the effect of treatments on methane production. In the present experiment, although E0.84 had higher percentage of methane compared to E0.0 and lower *in vitro* gas production and IVDMD than E1.68, it had the highest FE. In other word, using E0.84, less portion of the DM that disappeared was converted to methane and overall fermentation was improved slightly. Additionally, hr12 had less FE than hr0 although percent of methane production did not show significant difference.

CONCLUSION

Application of Natuzyme® at 0.84 g/kg DM increased the fermentation efficiency via reduced methane production per unit of disappeared DM. Additionally, gas production volume (GP_{24}) increased quadratically as the time of enzyme administration increased from hr 0 to hr 24 and was highest at hr 12 and *in vitro* dry matter disappearance and fermentation efficiency at $t_{1/2}$ decreased linearly as time of pre-incubation increased from hr 0 to hr 24. The present results suggest that Natuzyme® applied at the rate of 0.0, 0.84, 1.68 or 2.52 g/kg DM, has the potential to modify *in vitro* gas production parameters and dry matter disappearance, fermentation efficiency and percentage of methane production at $t_{1/2}$.

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