# *In vitro* Estimation of Microbial Nitrogen Production and Ruminal Fermentation Responses to Levels and Applying Duration of a Commercial Enzyme Mixture

**Research Article**

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## ABSTRACT

The objective of the present experiment was to quantify the gas production parameters, methane emission and microbial nitrogen production of a conventional total mixed ration including corn silage, alfalfa hay, wheat straw and concentrate (390, 110, 22 and 478 g/kg of diet DM, respectively) treated with different levels of Natuzyme® and pre-incubation time after enzyme administration. Two hundred fifty mg of milled and dried basal diet (n=4) was weighed into a 125 mL serum bottles for an *in vitro* gas production trial. A solution of a commercial enzyme blend (Natuzyme®) was added at 0, 12 and 24 h prior to starting the incubation (96 h) to make treatments of 0.84, 1.68 and 2.52 g/kg, respectively. No added enzyme bottles were considered as control. Half time of gas production ($t_{1/2}$) was calculated. Then, another gas test was run for each treatment and terminated at $t_{1/2}$ to measure gas volume, methane emission and solid residues. Dose rate of Natuzyme® did not make any significant differences in gas production parameters and fermentation responses except for microbial nitrogen production. Pre-treatment of the basal diet at 24 h incubation significantly ($P<0.05$) lowered dry matter degradability and accumulated gas, but percentage and amount of produced methane, produced methane by mg degraded dry matter and microbial nitrogen production was significantly increased. Twelve and twenty four h pre-treatment of the basal diet significantly ($P<0.05$) increased accumulated gas by 16% and 8%, respectively. However, fractional constant rate was significantly ($P<0.05$) decreased by 31% and 13%, respectively, using twelve and twenty four h pre-treatment of the basal diet. However, a specific pre-treatment time must be taken into consideration to allow the enzyme mixture to bind to the feed particles.

**KEY WORDS** degradability, enzyme, methane, microbial nitrogen.

## INTRODUCTION

Exogenous enzymes are commonly used to improve the nutrient value of feeds for non-ruminants (Kung *et al.* 2000). The use of fibrolytic enzymes in ruminant diets has received considerable research interest in dairy cattle, although performance responses were variable (Beauchemin *et al.* 1999; Beauchemin *et al.* 2000; Yang *et al.* 1999). The nutritional benefit from the supplementation appears to depend on many factors, most of which have yet to be evaluated (Almaraz *et al.* 2010). The effectiveness of enzymes varies with diet (Beauchemin *et al.* 1995) and with the component of the diet to which the enzyme is added (Hristov *et al.* 1998; Krause *et al.* 1998). Beauchemin *et al.* (2003) declares that the effect of exogenous enzymes are influenced by various factors such as type and dose of enzyme, type of diet fed to animals, enzyme application method and even the level of animal productivity. The use

of exogenous fibrolytic enzymes holds promise as a means of increasing forage utilization and improving the productive efficiency of ruminants (Beauchemin *et al.* 2003) and moreover, exogenous fibrolytic enzymes are thought to improve fiber degradation in the rumen by acting synergistically with the rumen microflora (Morgavi *et al.* 2000), thereby increasing the hydrolytic capacity within the rumen environment (Beauchemin *et al.* 2004; Faramarzi-Garmroodi *et al.* 2013; Parand *et al.* 2014). It has been of great interest to recognize the impact of fibrolytic enzymes on chemical reactions occurring *in vitro*, specially on dry matter disappearance, neutral detergent fiber degradability and volatile fatty acids production. Microbial biomass is the major source of protein for the ruminant host animal and prediction of microbial production can be crucial in ruminant nutrition (Beever, 1993). Also, methane is a by-product of digestion in the rumen which is emitted in different amounts in various feeding systems. However, *in vitro* studies focusing on the measurement of methane and microbial nitrogen production are few and the need for discussing on the topics is inevitable. The objective of the present study was to determine the *in vitro* microbial nitrogen production, methane emission, gas production parameters as influenced by different doses of a commercial enzyme mixture (Natuzyme®) and pre-incubation time after enzyme administration.

## MATERIALS AND METHODS

### Experimental diet, enzyme mixture and enzyme administration

The composition of the tested diet is presented in Table 1. Samples of particle size of 1 mm (Foss, Cyclotec[TM] 1093) were oven dried (Behdad Co., BC Oven 70, 3493, Iran) at 65 ˚C for 48 h (AOAC, 1990) and 250 mg of each were weighed and placed into a 125 mL capacity serum bottles just before Natuzyme® application (n=4). Natuzyme® is a powdered multi-enzyme commercially available feed additive (M/s Bioproton, 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, pectinase and phytase activities. It is a micro-granulated enzyme product and possesses a wide pH range, stability and temperature tolerance. The enzyme was used at four concentrations (as recommended by the manufacturer) including 0.0 (as control), 0.84, 1.68 and 2.52 g/kg DM. It was mixed with double distilled water (DDW) to maintain the moisture content of the test feed in the serum bottles equal to 45% on weight basis. The control bottles were added DDW with no enzyme.

The suspension of DDW and enzyme was poured directly to feed samples in bottles atzero, 12 and 24 hours prior to the incubation. Bottles were closed with rubber caps and kept at room temperature (25 ˚C).

**Table 1** Ingredients (DM) and the chemical composition of the test feed

| Ingredients | Amount (g/kg DM) |
|---|---|
| Alfalfa hay | 109 |
| Corn silage | 383 |
| Wheat straw | 22 |
| Corn grain | 80 |
| Barley grain | 80 |
| Wheat grain | 22 |
| Soybean meal, 44% crude protein | 70 |
| Wheat bran | 60 |
| Cottonseed meal | 60 |
| Wheat residue | 24 |
| Sugar beet pulp | 44 |
| Fish meal | 16 |
| Fat powder | 5 |
| Calcium carbonate | 4 |
| Magnesium oxide | 1 |
| Mineral supplement[1] | 5 |
| **Chemical composition (g/kg)** | |
| Crude protein | 166 |
| Neutral detergent fiber | 340 |
| Ether extract | 51 |
| Ash | 59 |

[1] The mineral supplement contained: Ca: 196 g kg^{-1}; P: 90 g kg^{-1}; Mg: 20 g kg^{-1}; Na: 40 g kg^{-1} and Se: g kg^{-1}.

### *In vitro* gas production technique

The gas production procedure was performed as described by Grings *et al.* (2005). Rumen inoculum was collected from three ruminally fistulated steers (580±4.5 kg, body weight) prior to the morning feeding. Animals were fed 10.4 kg DM, a diet containing alfalfa hay, wheat straw, barley grain, soybean meal and mineral-vitamin premix (50, 20, 15, 14 and 1 percent, respectively). Ruminal content was immediately blended and strained through four layers of cheesecloth to eliminate large feed particles and transferred to the laboratory in a pre-warmed thermos. Bottles were incubated in 3 runs and 4 replicates. The filtrate was then mixed with carbonate buffer (containing ammonium bicarbonate at 4 g/L) and sodium bicarbonate (35 g/L), macro-mineral solution (5.7 g anhydrous $Na_2HPO_4$, 6.2 g anhydrous $KH_2PO_4$ and 0.6 g $MgSO_4 \cdot 7H_2O$ per liter) and deionized water in a ratio of 1:1:0.5:1.5 and 0.1 mL micro-mineral solution (13.2 g $CaCl_2 \cdot 2H_2O$, 10.0 g $MnCl_2 \cdot 4H_2O$, 1 g $CoCl_2 \cdot 6H_2O$ and 8.0 g $FeCl_3 \cdot 6H_2O$ per 100 mL) was added. The medium was then reduced by addition of 41.7 ml reducing agent (40 mL deionized water, 1 mL 1 N NaOH and 1 g $Na_2S \cdot 9H_2O$) per liter. Twenty milliliters of medium were dispensed into a 125 mL glass serum bottle whose top were stopped with rubber and aluminum cabs and placed in a 39 ˚C water bath for 96 h. Blank samples

were also incubated simultaneously to make correction in gas production, if any, from the medium. Rumen liquor was handled under a constant stream of $CO_2$ and all containers used were pre-warmed at 39 °C and filled with $CO_2$. Gas production was measured at 2, 4, 6, 8, 10, 12, 24, 48, 72 and 96 h 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 serum bottles (Grings *et al.* 2005). The transducer was then removed leaving the needle in place to permit venting. Pressure values were corrected for the amount of substrate organic matter (OM) incubated and the gas released from negative controls. In order to prevent accumulation of produced gases, the gas in the head space of each bottle was released. After subtraction of gas production from blank bottles, data were fitted to the exponential model of Orskov and McDonald (1979):

$$y = A \times (1 - e^{-ct})$$

Where:
y: cumulative volume of produced gas at time t (h).
A: asymptotic gas volume (mL/250 mg DM).
c: fractional constant rate (mL/h).

Halftime of gas production ($t_{1/2}$) [i.e., the time (h) when half of the asymptotic gas volume (A; mL) was produced] was calculated as:

$$t_{1/2} = \ln 2/c$$

### *In vitro* apparent and true degradability measurements
After the initial 96 h gas run, $t_{1/2}$ was calculated and a second incubation using the same samples already mentioned was conducted to obtain degradability measures at substrate-specific times (i.e., $t_{1/2}$ for each substrate). Collection and handling of ruminal fluid was the same as that described for the 96 h incubations. The only difference with the previous section was that 500 mg of DM of the experimental diet was placed in 3 runs and 3 replicates and 40 mL of the medium was dispensed in serum bottles.
The incubations were terminated at $t_{1/2}$ and the volume of gas was recorded. Methane volume in each bottle at $t_{1/2}$ was recorded using Biogas Detector Device (SR2-Bio, SEWERIN, UK). True substrate degradability of the diet (Goering and Van Soest, 1970) at $t_{1/2}$ was measured by refluxing the incubation residue with neutral detergent (ND) solution (prepared without sodium sulfite) for 1 h with subsequent recovery of the truly undegraded substrate in sintered glass crucibles of porosity '*c*' (i.e., 40-60 m). Apparent substrate degradability was determined and calcu-

lated at $t_{1/2}$ by high speed centrifugation (13000 RPM, 20 min) of the incubation residue (Blummel and Lebzien, 2001) followed by suspending into an iced water bath to stop fermentation. All blank samples were centrifuged (13000 RPM, 20 min) and supernatant collected, freezed and stored. Residue was weighed and used to correct apparent substrate degradability from the ruminal inoculum.

### Microbial nitrogen determinations and chemical analysis
The nitrogen determination of all materials was carried out by the apparatus behr (distillation unit S5) and samples were titrated by TitroLine (easy). Microbial nitrogen production of diets at $t_{1/2}$ was estimated directly by using the nitrogen content of the apparently degraded residue remaining after centrifugation (pellet N) and neutral detergent fiber nitrogen (NDFN) in diets at $t_{1/2}$, using the following equation (Grings *et al.* 2005):

Microbial nitrogen production at $t_{1/2}$= pellet Nat $t_{1/2}$ - blank pellet Nat 0 h incubation - NDFN at $t_{1/2}$

The dry matter (DM) of the samples was determined by oven drying at 65 °C to a constant weight. Neutral detergent fiber was determined as described by Van Soest *et al.* (1991). Ash content of the feed was determined by burning the dried samples in the furnace at 370 °C for 3 hours. Oil content determination of the test feed was done using automatic Soxhlet extraction (Soxtec system 1043, Foss Tecator, Seweden) (AOAC, 1990).

### Calculations and statistical analysis
Differences of *in vitro* measurements were examined by analysis of variance using the SAS (2002) general linear model procedure considering dose rate and time of administration as fixed effects in a completely randomized design. Data were analyzed as a factorial design with four concentrations of enzyme (0.0, 0.84, 1.68 and 2.52 mg/g DM) and three pre-incubation times after enzyme administration (0.0, 12 and 24 h) and the interaction enzyme × time included in the model. When a significant effect of treatment (P<0.05) was detected, differences between means were assessed by Tukey test P < 0.05.

$$Y_{ijk} = \mu + A_i + B_j + (AB)_{ij} + e_{ijk}$$

Where:
Y: represents the dependent variable.
$\mu$: overall mean.
$A_i$: main effect of the enzyme dose rate.
$B_j$: main effect of pre-incubation time.
$(AB)_{ij}$: interaction between enzyme dose rate and preinc-

ubation time.

$e_{ijk}$: residual error.

## RESULTS AND DISCUSSION

Estimated gas production parameters are indicated in Table 2. Pre-treatment of the basal diet with enzyme 12 and 24 h prior to incubation significantly increased the potential gas production by 16% and 8%, respectively. Simultaneously, dose rate of the enzyme mixture did not show any significant effect on the potential and rate of gas production (Table 2). The rate of gas production was drastically lowered by 12 h pre-treatment in comparison to 0.0 h. Likely, this pattern was seen for 24 h pre-treatment which was decreased by 9.5%.

Data in Table 3 demonstrates the effect of pre-treatment and Natuzyme® on the basal diet dry matter degradability (DMD), accumulated gas (GP), percentage and amount of produced methane, produced methane by mg degraded dry matter (MD) and microbial nitrogen production (MNP) at $t_{1/2}$. Pre-treatment of the basal diet at 12 and 24 h before incubation significantly ($P<0.05$) affected all the above parameters. None of the above parameters was affected by the dose rate of Natuzyme® except for MNP which was decreased in each time preparation. Twenty four hours pre-treatment of the basal diet significantly ($P<0.05$) decreased the DMD and GP. However, an opposite impact was seen for percentage of methane, amount of methane, MD and MNP which were raised by approximately 72%, 5%, 90% and 22%, respectively. Data of the present study indicated that potential of the gas production was affected significantly ($P<0.05$) by pre-treatment of the basal diet (Table 2). Dose rate of Natuzyme® did not alter the cumulative gas production and the gas production rate. There has been indicated that the application of the enzyme mixture in advance to the incubation by rumen fluid is logical due to the creation of a stable enzyme-feed complex (Kung *et al.* 2000), but others have demonstrated an alteration in the fiber structure which is a possibility for microbial colonization and synthesis (Newbold, 1997; Nsereko *et al.* 2000; Giraldo *et al.* 2007). Yang *et al.* (2000) added an enzyme mixture to a mid-forage total mixed rations (TMR) and found no effect on cumulative gas production and the gas production rate. Present findings confirmed the results of Almaraz *et al.* (2010) who examined two levels of a fibrolytic enzyme on TMR and found no effect on total gas production. In addition, our results are in contrast with Jalilvand *et al.* (2008) who reported that the fermentation rate was adversely affected by enzyme addition and the level of enzyme addition showed higher fractional gas production rates and half times as enzyme level increased.

Lack of response to different doses of Natuzyme® in this study may be due to several reasons. Firstly, it is proven that the capability of an enzyme mixture at improving the dry matter degradability, gas production parameters or other *in vitro* data depend on enzyme components and optimum ratio of cellulase and xylanase activity as the main substances involved in degrading cell wall components. Secondly, high levels of enzyme addition can be less effective than low levels, and the optimal level of enzyme supplementation may depend on the diet. Lack of response to low levels of enzyme addition may indicate an insufficient supply of enzyme activity (Beauchemin *et al.* 2003). However, the rationale for reduced efficiency of added enzymes at high levels of incorporation is not clear (Beauchemin *et al.* 2003). Last but not least, enzyme-feed specificity is a factor worthy of considering. An object meaning that different feeds such as alfalfa hay, corn silage or barley grain, may be more degraded by specific individual enzymes such as cellulases, xylanases or endoglucanases.

Results of the present study are in consistent with Jalilvand *et al.* (2008), who treated three substrates (alfalfa hay, corn silage and wheat straw) with Natuzyme® at three dose rates (3, 6 or 9 g enzyme/kg DM substrate). The results on gas production measured over a 96h period clearly showed that only the 3 g/kg DM inclusion rate increased volume of gas produced for the wheat straw, measured in calibrated syringes.

No effects of enzymes were reported on the alfalfa hay or corn silage and authors concluded that adding enzyme at higher dose rates may not be beneficial.

None of the measured or estimated parameters were affected by dose rate of Natuzyme® except for MNP and MD which were significantly ($P<0.05$) raised by any of the administration rates. Most studies have not found exogenous enzymes to improve the extent of in situ or *in vitro* dry matter digestion (Feng *et al.* 1996; Hristov *et al.* 1996). Baloyi (2008) found no effects on gas production and *in vitro* dry matter and neutral detergent fiber degradability when an exogenous fibrolytic enzyme was added to forage hays and mixed feed substrates. Dhiman *et al.* (2002) tested a trial in which fifty dairy cows in early lactation were offered a diet with forage portion treated with cellulase, xylanase and ferulic acid esterase prior to feeding. They found no effect on improvement of feed intake, milk yield or milk energy output. However, authors have revealed results contrasting with the findings of researchers discussed above. Eun and-Beauchemin (2007) assessed the potential of different endoglucanases and xylanases exhibiting different biochemical properties using the cumulative GP at 18 hours. Their results revealed that EFE on alfalfa hay might improve the gas production.

**Table 2** Gas production parameters of un / pre-treated total mixed rations (TMR) (0, 12 and 24 h prior to incubation) with ascending doses of Natuzyme® (DR)

| Time | | | | | | | | | | | | | SEM | P-value | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.0 | | | | 12 | | | | 24 | | | | | | | |
| DR | 0 | 0.84 | 1.68 | 2.52 | 0 | 0.84 | 1.68 | 2.52 | 0 | 0.84 | 1.68 | 2.52 | | Time | DR | Time × DR |
| Parameters | | | | | | | | | | | | | | | | |
| A (mL) | 61.61[a] | 63.69[bcd] | 65.01[b] | 62.85[d] | 72.49[e] | 73.64[e] | 73.73[e] | 74.40[e] | 68.85[fg] | 70.06[f] | 68.80[fh] | 67.36[gh] | 1.01 | < 0.001 | 0.21 | 0.40 |
| c (mL h⁻¹) | 0.053[a] | 0.052[a] | 0.054[a] | 0.054[a] | 0.038[bc] | 0.036[b] | 0.039[c] | 0.036[b] | 0.049[d] | 0.047[de] | 0.045[e] | 0.049[d] | 0.001 | < 0.001 | 0.25 | 0.10 |
| $t_{1/2}$ (h) | 13.07 | 13.32 | 12.83 | 12.83 | 18.24 | 19.25 | 17.77 | 19.25 | 13.86 | 14.74 | 15.40 | 13.86 | - | - | - | - |

SEM: standard error of the means.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).

**Table 3** Dry matter degradability (DMD), cumulative gas production (GP), percentage and amount of methane, mL methane per mg DMD (MD) and microbial nitrogen production (MNP) of pre-treated TMR (0.0, 12 and 24 h prior to incubation) with ascending doses of Natuzyme® (DR) at $t_{1/2}$

| Time | | | | | | | | | | | | | SEM | P-value | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.0 | | | | 12 | | | | 24 | | | | | | | |
| DR | 0 | 0.84 | 1.68 | 2.52 | 0 | 0.84 | 1.68 | 2.52 | 0 | 0.84 | 1.68 | 2.52 | | Time | DR | Time × DR |
| Item | | | | | | | | | | | | | | | | |
| DMD (%) | 42.73[a] | 40.73[a] | 45.40[a] | 34.06[b] | 45.40[a] | 42.06[a] | 45.40[a] | 40.06[ab] | 25.40[c] | 17.40[d] | 17.40[d] | 25.40[c] | 3.41 | < 0.001 | 0.29 | 0.19 |
| GP (mL) | 48.37[a] | 43.54[bc] | 43.64[bc] | 46.27[ab] | 41.63[c] | 42.25[c] | 42.68[c] | 43.49[cb] | 31.12[de] | 33.09[fd] | 28.89[e] | 31.68[fde] | 1.42 | < 0.001 | 0.28 | 0.20 |
| Methane (%) | 0.136[a] | 0.133[ab] | 0.123[abc] | 0.136[a] | 0.116[bc] | 0.110[c] | 0.110[c] | 0.120[abc] | 0.193[d] | 0.210[d] | 0.200[d] | 0.193[d] | 0.009 | < 0.001 | 0.82 | 0.74 |
| Methane (mL) | 6.60[a] | 5.86[abc] | 5.39[cbe] | 6.33[ae] | 4.90[db] | 4.63[e] | 4.69[e] | 5.22[fb] | 6.01[gacf] | 7.02[a] | 5.78[abc] | 6.12[gacf] | 0.52 | 0.002 | 0.46 | 0.61 |
| MD (mL/mg DMD) | 0.031[ae] | 0.028[a] | 0.023[a] | 0.043[bc] | 0.021[a] | 0.022[a] | 0.020[a] | 0.027[a] | 0.047[b] | 0.080[d] | 0.066[e] | 0.050[b] | 0.006 | < 0.001 | 0.30 | 0.003 |
| MNP (mg/250 mg DM) | 0.53[a] | 1.19[bec] | 1.09[b] | 1.23[c] | 1.38[d] | 1.45[d] | 1.22[ec] | 1.24[ec] | 1.80[f] | 1.57[g] | 1.58[g] | 1.43[d] | 0.05 | < 0.001 | 0.01 | < 0.001 |

SEM: standard error of the means.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).

Eun *et al.* (2007) also found that EFE substantially improved the cumulative gas production and fiber degradation of alfalfa hay and corn silage at 24 hours. In another study, Kung *et al.* (2002) found that the *in vitro* gas production from forages treated with EFE was significantly higher than untreated forage.

Evidence exist that the mode of action of exogenous enzymes in ruminants is a combination of pre and post-feeding effects (McAllister *et al.* 2001; Colombatto *et al.* 2003). The pre-feeding effects include an enzyme-substrate pre-incubation interaction period. Almaraz *et al.* (2010) reported that several researchers had previously suggested that pre-incubation of the diet with the enzyme is of importance (Krueger *et al.* 2008).

The enzyme requires an adsorption and binding time to the substrate to allow for protection against proteolytic breakdown in the rumen (Beauchemin *et al.* 2003). The resultant stable enzyme-feed complex can then potentially degrade the relevant plant tissue in the rumen (Kung *et al.* 2000).

When enzymes are directly infused into the rumen instead of inclusion via the feed, no improvements indegradation were observed (Lewis *et al.* 1996) which serves as further justification for allowing a pre-incubation interaction period. Indeed, Moharrery *et al.* (2009) reported improved *in vitro* DMD and NDF om digestibility after 8 h incubation in rumen fluid where forages were pre-treated (24 h prior to incubation) with EFE. Feng *et al.* (1992) reported that pre-treatment of dry grass with exogenous enzymes improved *in vitro* fiber digestion.

Improvements in volatile fatty acids (VFA) production and NDF digestion were reported by Lewis *et al*. (1996) when exogenous enzymes were sprayed onto a grass hay-barley diet prior to feeding. Cruywagen and Goosen (2004) reported improved weight gain (6.75 and 7.13 kg) and feed conversion ratios (0.15 and 0.16 kg gain/kg DM intake) when wheat straw was pre-treated with exogenous enzyme ABO374 for 18 h before feeding to growing lambs at high and medium levels of enzyme application, respectively.

To date, few studies have investigated the effects of exogenous enzymes on methane production in the rumen and their results are conflicting. Dong *et al.* (1999) found that the treatment of grass hay with a preparation including cellulase and xylanase activities increased cellulose digestibility and methane production in Rusitec fermenters by 15 and 43%, respectively. Giraldo *et al.* (2007) reported that the treatment of a 0.7:0.3 grass hay:concentrate substrate with three fibrolytic enzymes increased neutral detergent fiber degradability by 15.9-26.4% and methane production by 14.3-24.6% in Rusitec fermenters. In contrast, Colombatto *et al.* (2003) reported that adding a proteolytic enzyme to a 0.6:0.4 forage:concentrate substrate increased NDF degradability by 43% without affecting methane production in dual-flow continuous fermenters and McGinn *et al.* (2004) found no effect of the same enzyme on fiber digestibility and methane production in steers fed a barley silage based diet. Differences in the response observed in different studies are probably related to the type of enzyme used, but also to the different experimental conditions, since methane production is affected by many factors, such as the type of

diet, microbial populations and ruminal pH (Eun *et al.* 2004).

Grings *et al.* (2005) compared microbial biomass production (MBP) and efficiency of microbial production (EMP) by *in vitro* and *in vivo* methods. They acclaimed that MBP and EMP estimated at substrate specific $t_{1/2}$ was higher than when estimated at 24 h. In addition, estimates of MBP obtained at $t_{1/2}$ were well related to microbial crude protein production per MJ of metabolizable energy intake *in vivo* when metabolizable energy was calculated using gas production values.

Giraldo *et al.* (2008) found that treating high forage diet with exogenous fibrolytic enzyme stimulated the *in vitro* numbers of microbes and enhanced the fibrolytic activity. Nsereko *et al.* (2002) revealed results declaring the improvements in fiber digestion by fibrolytic enzyme addition to the feed and concomitant increase in the quantity of microbial crude protein.

## CONCLUSION

Results of the present study revealed that Natuzyme® did not improve DMD or methane production. On the other hand, time of administration took the role of the main variate in raising MNP. It is hypothesized that the enzyme mixture needs some time to make the enzyme-substrate complex, if not, once the feed and enzyme enter the rumen fluid, not any opportunity could be considered for the enzyme to bind and hydrolyze the feed particles. Nevertheless, literature has mentioned that to which part of the feed, enzyme is added is of great importance.

## ACKNOWLEDGEMENT

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