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Influence of Exogenous Enzymes on *In Vitro* Gas Production Kinetics and Dry Matter Degradability of a High Concentrate Diet[#]

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ABSTRACT

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This study was conducted to evaluate the influence of an exogenous enzyme mixture on *in vitro* gas production (GP), *in vitro* dry matter degradability (DMD), metabolizable energy (ME) and short chain fatty acid (SCFA) production in growing lambs fed a high concentrate diet. ZADO® (ENZ) is a powdered, commercially available multi-enzyme feed additive produced from *Ruminococcus flavefaciens*. Four levels of ENZ (*i.e.*, 0, 5, 10 and 20 mg/g DM; or EO, E5, E10 and E20, respectively) were applied directly to the substrate inside the incubation bottles before addition of buffer medium and rumen fluid, and the treatments were assayed in triplicate runs. Addition of ENZ linearly increased ($P<0.05$) GP at 6 and 96 h of incubation and tended ($P=0.08$) to linearly increase GP at 12, 48 and 72 h of incubation. Asymptotic GP was increased linearly ($P=0.05$) as the level of ENZ increased and the lag time decreased linearly ($P=0.003$). Concurrently, DMD increased linearly ($P<0.001$) as the level of ENZ increased, but level of ENZ had no effect on SCFA and ME. Finally, level of ENZ had no influence on rate of gas production. Results suggest that this enzyme preparation has potential to improve efficiency of utilization of high concentrate diets fed to growing lambs.

Key words: Exogenous enzymes, *In vitro* gas production, Degradability, Lambs.

INTRODUCTION

Use of enzymes as feed additives in ruminant diets has attracted considerable interest in recent years. However, there is increasing evidence indicating that the mode

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of action of exogenous enzymes in ruminants combines pre- and post-feeding effects (Colombatto *et al.*, 2007). Of these pre-feeding effects, enzyme application rate and available enzyme/feed interaction time appear as important (Yang *et al.*, 2000). Responses to enzyme application level have been somewhat variable, with quadratic responses most commonly observed (Beauchemin *et al.*, 2003) in which maximum benefits occur at intermediate enzyme addition levels. Other reports have emphasized the importance of the enzyme/feed interaction time, since addition of enzyme in animal feeds may create a stable enzyme/feed complex which protects free enzymes from proteolysis in the rumen (Kung *et al.*, 2000).

Improvements in ruminant production with supplemental fibrolytic enzymes are generally attributed to increased ruminal fiber digestion, but the mechanism by which this increase occurs are not completely understood. Numerous potential mechanisms have been proposed (Beauchemin *et al.*, 2003), including pre-ingestive and ruminal effects such as direct hydrolysis (Moharrery *et al.*, 2009), structural changes in the fiber, increased ruminal microbial attachment (Colombatto *et al.*, 2003), stimulation of ruminal microbial populations and synergism with ruminal microbial enzymes.

As published opinions of scientists vary, no firm conclusions can be drawn about the effects of exogenous enzymes in animal nutrition. However, effects of exogenous enzymes on *in vitro* fermentation kinetics depend on the source of enzyme (Salem *et al.*, 2012), level and activity of the enzyme (Jalilvand *et al.*, 2008) and the physical chemistry properties of substrate (Kung *et al.*, 2000; Moharrery *et al.*, 2009). Synergistic effects between exogenous enzymes and ruminal micro-organisms and enzymes (Morgavi *et al.*, 2000), and/or the ruminal environment (Colombatto *et al.*, 2003) have also been reported.

Recent research has shown that xylanase-based products used as an additive to non-lactating dairy cow diets had no effects on potential dry matter (DM) and acid detergent fiber (ADF) disappearance (Phakachoed *et al.*, 2012). Pre-treatment of forage with fibrolytic enzymes can solubilize some fiber and improve digestibility at short incubation times (Moharrery *et al.*, 2009). It appears that effective enzymes work best by removing structural barriers which retard microbial colonization of digestible fractions (Colombatto *et al.*, 2003) to increase rate of degradation. Exogenous fibrolyitc enzymes also seem to work better at close to neutral ruminal pH (Colombatto *et al.*, 2007).

However, there are some inconsistencies on effects of exogenous enzyme levels on ruminal fermentation kinetics. Some research has shown that efficiencies of forage utilization were increased at increasing levels of exogenous enzymes (Miller *et al.*, 2008) whereas others suggest that exogenous enzymes produced better results at a particular level, rather than showing a dose response (Jalilvand *et al.*, 2008). The objective of the present research was to evaluate the dose response of exogenous enzyme addition on *in vitro* gas production kinetics and energy utilization of growing lambs fed a high concentrate diet.

MATERIALS AND METHODS

Substrates and enzyme product

Three individual samples of a high concentrate lamb diet (HCD, Table 1) were randomly collected in triplicate from different fields. Samples were dried at 70°C for 48 h in a forced air oven to constant weight, ground in a hammer mill to pass a 1 mm sieve and stored in plastic bags for subsequent determination of chemical components and *in vitro* GP mixed with four doses of ZADO® enzyme preparation mixture (ENZ), a patented (Patent No.: 22155, Cairo, Egypt) commercially available multi-enzyme feed additive in a powder form produced from *Ruminococcus flavefaciens* and manufactured by the Academy of Scientific Research and Technology in Cairo (Egypt). Prior to our research, the enzyme mixture was assayed for several enzymatic activities and found to contain (per g ENZ) 7.1 units of endoglucanase, 2.3 units of xylanase, 61.5 units of α-amylase and 29.2 units of protease activity. Doses of ENZ inclusion were (per g DM of HCD): control (0 mg, no enzyme - E0), low (5 mg - E5), medium (10 mg - E10) and high (20 mg - E20).

In vitro incubation

Rumen fluid was collected by stomach tube from 4 growing lambs (Katahdin×Pelibuey, live weight 24±0.3 kg) fed HCD *ad libitum* (Table 1). Samples (1 g) of substrate (*i.e.,* HCD) were weighed into 120 ml serum bottles. Enzyme (ENZ) doses (*i.e.*, 0, 5, 10, 20 mg/g DM) were added directly on to the substrate immediately before addition of buffer medium and rumen fluid.

Table 1. Ingredient and chemical composition of the growing lamb's diet in g/kg DM (adapted from Salem *et al.*, 2012)

Ingredient composition	
Soybean meal	220
Alfalfa hay	150
Sorghum grain	550
Fishmeal	35
Mineral/vitamin premix[1]	25
Salt	20
Chemical composition	
Organic matter	911
Crude protein	219
Ether extract	119
Neutral detergent fiber	141
Acid detergent fiber	59
Lignin	21

[1]Mineral/vitamin premix (25) (Vitamin A (12 000 000 IU), Vitamin D3 (2 500 000 IU), Vitamin E (15 000 IU), Vitamin K (2.0 g), Vitamin B1 (2.25 g), Vitamin B2 (7.5 g), Vitamin B6 (3.5 g), Vitamin B12 (20 mg), Pantotenic acid (12.5 g), Folic acid (1.5 g), Biotin (125 mg), Niacin (45 g), Fe (50 g), Zn (50 g), Mn (110 g), Cu (12 g), I (0.30 g), Se (200 mg), Co (0.20 g).

Ruminal contents were obtained from each sheep immediately before morning feeding, mixed and strained through 4 layers of cheesecloth into a flask with an O_2-free headspace. Ten ml of particle-free ruminal fluid was then added to each serum bottle and 40 ml of buffer solution (Goering and Van Soest, 1970), with no trypticase immediately added in a proportion 1: 4 (v/v).

A total of 108 bottles, 3 bottles for each ENZ dose (*i.e.*, 0, 5, 10, 20 ml/g DM) in triplicate samples of HCD in 3 runs for different weeks plus 3 bottles as blanks (*i.e.,* rumen fluid only), were incubated for 96 h. Once all bottles were filled, they were immediately closed with rubber stoppers, shaken and placed in the incubator at 39°C. The volume of gas produced was recorded at incubation times of 2, 4, 6, 8, 10, 12, 24,48, 72 and 96 h after inoculation using the Reading Pressure Technique (RPT; DELTA OHM, Italy) of Mauricio *et al*. (1999). At the end of the incubation at 96 h, bottles were uncapped and the pH was measured immediately using a pH meter (GLP 22, Crison Instruments, Barcelona, Spain). The fermentation was then stopped by swirling the bottles in ice.

Dry matter degradability

At the end of incubation (*i.e.* 96 h), contents of each serum bottle were filtered under vacuum through glass crucibles with a sintered filter (coarse porosity no. 1, pore size 100 to 160 μm, Pyrex, Stone, UK). Fermentation residues were dried at 105°C overnight to estimate potential DM disappearance. Loss in weight after drying was used as the measure of undegradable DM. The DM degradability at 96 h of incubation (*i.e.,* dry matter degradability; mg/g DM) was calculated as the difference between DM content of substrate and its undegradable DM.

Chemical analyses and assay of enzymatic activity

Samples of HCD were analysed for DM (934.01), ash (942.05), N (954.01) and EE (920.39) according to AOAC (1997). Neutral detergent fiber (NDF; Van Soest and Mason, 1991), acid detergent fiber (ADF) and lignin (AOAC, 1997; 973.18) analyses were carried out using an ANKOM 200 Fiber Analyzer Unit (ANKOM Corporation, Macedon, NY, USA). The NDF was assayed without use of an alpha amylase but with sodium sulfite in the NDF. Both NDF and ADF are expressed without residual ash.

Endoglucanase activity of ZADO® was assayed by liberating glucose from carboxymethyl cellulose, which was determined calorimetrically using alkaline copper reagent as described by Robyt and Whelan (1972). One unit of endoglucanase catalyzes liberation of one mmol of glucose/min from sodium carboxymethyl cellulose at 40°C and pH 4.5. The α-amylase was assayed by its ability to produce reducing groups from starch, which were measured by reduction of 3,5-dinitrosalicylic acid (Bernfeld, 1955). One unit of α-amylase catalyzes liberation of one mmol of reducing groups/min from soluble starch at 25°C and pH 6.0, calculated as maltose equivalents. Protease activity was determined by hydrolysis of dimethyl casein (DMC) and the liberated amino acids

were determined using 2,4,6-trinitrobenzene sulfonic acid (Lin *et al.*, 1969). One DMC unit catalyzes cleavage of one mmol of peptide bond/min from DMC at 25°C and pH 7.0 expressed in terms of newly formed terminal amino groups. Xylanase catalyzes hydrolysis of xylan from oat spelt, and the reducing groups liberated were determined using alkaline copper reagent (Robyt and Whelan, 1972). One unit catalyzes the liberation of one mmol reducing groups/h from xylan at 37°C and pH 5.5, expressed as xylose equivalents.

Calculations

To estimate kinetic parameters of GP results (ml/g DM) were fitted using the NLIN option of SAS (2002) according to the France *et al.* (2000) using the model:

$$GP = b \times (1 - e^{-c^{(t-L)}})$$

Where, GP is the volume of GP at time t; b is the asymptotic GP (ml/g DM); c is the rate of GP (/h), and L (h) is the discrete lag time prior to GP.

Metabolizable energy (ME, MJ/kg DM) was estimated according to Menke *et al.* (1979) as:

$$ME = 2.20 + 0.136\ GP\ (ml/0.5g\ DM) + 0.057\ CP\ (g/kg\ DM)$$

Where, GP is net GP in ml from 200 mg of dry sample after 24 h of incubation.

Short chain fatty acids concentration (SCFA) was calculated according to Getachew *et al.* (2002) as:

$$SCFA\ (mmol/200\ mg\ DM) = 0.0222\ GP - 0.00425$$

Where, GP is the 24 h net gas production (ml/200 mg DM).

Statistical analyses

Data of *in vitro* ruminal GP and fermentation parameters were analysed as a randomized design using the PROC MIXED procedure of SAS (2002). Data of each of the 3 runs within the same sample of HCD were averaged prior to statistical analysis. Mean values of each individual sample within each HCD sample (3 samples of each) were used as the experimental unit. The statistical model was:

$$Y_{ij} = \mu + Z_j + \varepsilon_{ij}$$

Where, Y_{ijk} represents every observation of the i^{th} lamb diet when incubated in the j^{th} ENZ doses, Z_j is the ENZ doses and ε_{ijk} is the experimental error. Tukey's test was used for the multiple comparisons among mean values for each HCD sample and linear and quadratic effects were calculated at $P < 0.05$.

RESULTS

Cumulative *in vitro* GP (ml/g DM) from the HCD treated with different levels of ENZ and incubated in rumen liquor of growing lambs is shown in Table 2. In general,

lower levels of ENZ application tended to be more effective in increasing fermentation kinetics of HCD of growing lambs in the early stages of incubation, whereas higher levels of ENZ were more effective at the mid- and late stages of incubation. Specifically, ENZ linearly increased ($P<0.05$) GP at 6 and 96 h of incubation and tended ($P=0.08$) to linearly increase GP at 12, 48 and 72 h of incubation without quadratic effects.

Table 2. Cumulative *in vitro* gas production (ml/g DM) from a high concentrate diet treated with different levels of enzyme (ENZ) and incubated in rumen liquor from growing lambs

Time (h)	ENZ level†				SEM	P Linear
	E0	E5	E10	E20		
6	74.5	81.1	83.7	87.5	2.22	0.006
12	162.4	196.8	168.5	174.7	3.34	0.048
24	259.1	265.3	263.0	271.1	4.45	0.122
48	320.1	323.5	323.9	332.4	4.73	0.076
72	331.9	334.1	336.0	344.4	5.82	0.057
96	334.1	336.1	338.5	346.8	6.98	0.052

†ENZ: ZADO® enzyme added at 0 (E0), 5 (E5), 10 (E10) and 20 (E20) mg/g DM, respectively.

The GP parameters (b, c and L phase), *in vitro* DM degradability (DMD), metabolizable energy (ME) and short chain fatty acids (SCFA) of the HCD fed to growing lambs with different levels of ENZ is in Table 3. The asymptotic GP increased linearly ($P<0.05$) as the level of ENZ increased and the lag time decreased linearly ($P=0.003$) as the level of ENZ increased. Concurrently, DMD increased linearly ($P<0.001$) as level of ENZ increased, but level of ENZ had no effect on SCFA and ME. Additionally, level of ENZ had no influence on the rate of GP. Generally, there were not any quadratic effects on the fermentation parameters.

Table 3. Gas production parameters†, *in vitro* dry matter degradability (DMD, %), metabolizable energy (ME, MJ/kg DM) and short chain fatty acids (SCFA, mmol) of the high concentrate diet fed to growing lambs with different levels enzyme (ENZ)

	ENZ levels‡				SEM	P Linear
	E0	E5	E10	E20		
Asymptotic, ml/g DM	334.7	336.5	339.1	347.3	5.83	0.050
Rate of gas,/h	0.069	0.071	0.067	0.068	0.0051	0.406
Lag time, h	2.34	2.12	1.78	1.73	0.114	0.003
DMD	70.9	80.9	82.0	84.3	0.96	<.001
ME	10.2	10.4	10.3	10.7	1.25	0.123
SCFA	1.15	1.17	1.16	1.20	0.135	0.122

†Parameters of gas productions were estimated according to the model of France *et al.* (2000).

‡ENZ, ZADO® enzyme added at 0 (E0), 5 (E5), 10 (E10) and 20 (E20) mg/g DM, respectively.

DISCUSSION

The substrate incubated with ENZ was similar to the diet fed to growing lambs to evaluate effects of ENZ on GP kinetics of similar diets to reflect the properties of the reaction of ENZ to those feeds. The GP and DMD appeared to be related to the level of ENZ applied (Tables 2 and 3), which is consistent with numerous recent studies which have shown that adding exogenous fibrolytic enzymes to ruminant diets improved digestion of DM and fiber *in vitro* (Moharrery *et al.*, 2009) and *in vivo* (Salem *et al.*, 2012). Eun *et al.* (2007) reported that *in vitro* GP and the DM degradation rate were useful in identifying changes in substrate availability due to enzyme addition.

Effects of ENZ levels on GP

Gas production *in vitro* appears related to the chemical composition of the feed, in particular to the fiber content and its structural polysaccharides (Jalilvand,*et al.*, 2008). As the results of our study suggest, the particular ENZ used here has potential as an influence to GP kinetics. Cumulative GP after 6 h of incubation increased ($P<0.01$) with ENZ addition compared to E0 but, as level of ENZ had no effect on GP at other incubation times, this suggests that the *Ruminococcus flavefaciens* in the ENZ might have caused stimulation of *in vitro* ruminal fermentation at early incubation times, but not at mid times. The initial fermentation of high concentrate diets, in ruminal fluid is generally enzyme-limited. However, addition of ENZ at 72 h ($P=0.057$) and 96 h ($P=0.052$) increased GP during the later period of fermentation, which may be a reflection of an increase in bacterial numbers, and hence, hydrolytic capacity of the ruminal fluid. This view is similar to previous hypotheses that exogenous ENZ increased fibrolytic activity due to increased numbers of ruminal microbes (Colombatto *et al.*, 2003), and increased bacterial attachment and synergistic effects with hydrolysis of ruminal micro-organisms. Another report showed that a fibrolytic enzyme preparation increased numbers of cellobiose-utilizing, xylanolytic and amylolytic bacteria, but had no effect on numbers of cellulolytic bacteria (Nsereko *et al.*, 2002) and that the population density of *Ruminobacter amylophilus* was increased by the high enzyme treatment, while *Selenomonas ruminantium* tended to increase linearly with increasing levels of enzyme (Chung *et al.*, 2012).

That the asymptotic GP was increased linearly as the level of ENZ increased indicates that the higher level of ENZ improved rumen fermentation kinetics. However, these results contrast with Jalilvand *et al.* (2008), who found that effects of addition of ENZ to different forages on GP kinetics were negligible, which suggests that quality of the diet is important, and that effects of ENZ depend on the fiber content, structural polysaccharide composition of the substrate and the differences in enzyme composition.

Enzymes produced by a variety of microbes are capable of degrading lignocellulosic materials to SCFA, but require substantive rumen retention time (Kumar *et al.*, 2009). In our study, the lag time decreased linearly as the level of ENZ increased, indicating that ENZ could degrade complex substrates to simpler ones at early stages of fermentation to allow faster ruminal microbial colonization and

fermentation (Colombatto *et al.*, 2003). Adding the ENZ at more than 2.55 l/g of DM did not increase rate of GP further, suggesting that the maximal level of stimulation was already achieved at lower ENZ concentrations. Enzyme addition tended to affect asymptotic GP, but had no effect on rate of GP, which is consistent with previous studies using other types of enzymes (Jalilvand *et al.*, 2008).

Effect of ENZ levels on DMD, ME and SCFA

The ENZ linearly increased ($P<0.001$) DMD) of this HCD, which is consistent with Miller *et al.* (2008) but contrasts with Jalilvand *et al.* (2008) who reported a quadratic effect with increasing level of ENZ. This ENZ effect may be due to the efficiency of ENZ addition which could degrade the complex lignocelluloses substrate of HCD to simpler compounds which might change the surface structure of substrates or destroy the chemical bond of lignocelluloses, making them more accessible to ruminal microbial degradation and/or promote colonization of ruminal microbes and efficiency of fermentation. This linear increase of DMD with increasing levels of ENZ is consistent with previous studies using the same enzyme mixture (Gado *et al.*, 2011; Gado *et al.*, 2009; Salem *et al.*, 2013) and other exogenous enzymes (Mattéotti *et al.*, 2012).Recent work has demonstrated that supplementing diets of ruminants with exogenous enzymes can improve DM intake, feed utilization and animal performance by enhancing DM degradation *in situ* (Krueger and Adesogan, 2008), *in vitro* (Colombatto *et al.*, 2007; Moharrery *et al.*, 2009) and *in vivo* (Salem *et al.*, 2012, 2013).

While the mechanism by which our ENZ improved the digestion of forage is still poorly understood, several modes of action are suggested. These include that ENZ increased ruminal microbial colonization on the surface of feed particles (Yang *et al.*, 2000) to enhance attachment and improve access to the matrix surface by ruminal microbes to accelerate rate of digestion (Jalilvand *et al.*, 2008), or that ENZ enhanced the hydrolytic ability of ruminal microbes due to enzyme activities or increased synergism with rumen microbial enzymes (Morgavi *et al.*, 2000). Unfortunately, we did not measure enzyme activities in this particular study, which does not allow to draw a final conclusion.

That the ENZ had no impact on ME and SCFA suggests that energy was not the limiting nutrient in our HCD. Beauchemin *et al.* (2003) showed that ENZ increased digestible energy intake of animal when the substrate was fiber-rich material and energy was the limiting nutrient in the diet. The lack of ENZ effect on SCFA suggests that exogenous enzymes did not change the diversity of the ruminal microbial communities enough to affect SCFA, which contrasts with Gado *et al.* (2009) and Salem *et al.* (2013) who reported that supplementation of enzymes increased total SCFA concentrations.

Results suggest that our exogenous enzyme mixture produced from *Ruminococcus flavefaciens* holds potential to improve efficiency of utilization of high concentrate diets fed to growing lambs, as evidenced by increased gas production, *in vitro* dry matter degradability and reduced lag time. *In vivo* studies are required to confirm these findings.

Addition of exogenous enzymes to ruminant diets is one of the effective additives in animal nutrition. Enzymes have a potential impacts on improving the nutritive utilization of high concentrate diet fed to growing animals due improving the ruminal activities. Research is needed to understand the mode of action of this enzyme product which could play an important role in future ruminant production system.

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