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In vitro Effect of Increasing Levels of *Natuzyme*® on Fermentation Responses of Corn Silage Based Diet

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Authors' contributions

This work was carried out in collaboration between all authors and all authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: To evaluate the *in vitro* effect of an exogenous commercial enzyme blend (Natuzyme®) on fermentation responses and methane production of corn silage based diet.
Study Design: Completely randomized design.
Place and Duration of Study: Department of animal science, Faculty of agriculture, Ferdowsi University of Mashhad, between November 2012 and March 2013.
Methodology: Two hundred fifty mg of milled and dried corn silage based diet in 3 runs and four replicates was weighed into 125-ml serum bottles for an *in vitro* gas production trial. A solution of a commercial enzyme blend (Natuzyme®) was added 12 hour prior to commence of the incubation (96 h) to make treatments of 1.68 and 2.52 (g/kg). No added enzyme bottles were considered as control. Gas production parameters at 96 h incubation were estimated and half time of gas production ($t_{1/2}$) was calculated. Another gas test was run according to $t_{1/2}$. All the incubations for each treatment were terminated at $t_{1/2}$ and gas and methane volume recorded. Apparent dry matter degradability was assessed by centrifugation and ml methane per mg dry matter apparently degraded was calculated.
Results: Gas production parameters were not affected by addition of the enzyme blend. Supplementation of a corn silage based diet with the enzyme as 1.68 or 2.52 g/kg dry

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matter of the diet increased apparent dry matter degradability by 21% compared with control. Gas production, methane volume and volume of methane per mg of dry matter apparently degraded were not influenced by incrementing level of Natuzyme®.
Conclusion: Natuzyme® at the doses applied in the current experiment was capable of improving apparent dry matter degradability of corn silage based diet.

Keywords: Exogenous enzyme; gas production; degradability; methane.

1. INTRODUCTION

The use of exogenous fibrolytic enzyme products (EFE) to improve feed utilization by ruminants has attracted growing interest [1]. The primary objective of using feed enzymes is to enhance availability of nutrients that are locked within cell wall components. The use of fibrolytic enzymes as feed additives to improve degradation of fiber has been studied under *in vitro*, *in situ* and *in vivo* conditions, but the responses have been highly variable [2]. The effects of enzymes on digestion responses are influenced by several factors such as type and dose of enzyme, type of diet fed to animals, enzyme application method, and even the level of animal productivity [3]. Regarding the factors related to the diet, the effectiveness of fibrolytic enzymes has been shown to vary with forage [4,5], enzyme application method [6,7] and the component of the diet to which the enzyme is added [3]. To the best of our knowledge, there are few studies in which the attributable factors addressing the effect of exogenous fibrolytic enzymes on methane production are evaluated and discussed. This study was designed to evaluate the *in vitro* effect of an exogenous commercial enzyme blend (Natuzyme®) on fermentation responses and methane production of a corn silage based diet.

2. MATERIALS AND METHODS

2.1 Experimental Diet, Enzyme Mixture and Enzyme Administration

The composition of the tested diet is represented in Table 1. Samples of particle size of 1 mm were oven dried (Behdad Co., BC Oven 70, 3493, Iran) at 65ºC for 48 h [8] and 250 mg of each were weighed and placed in four replicates into 125-ml capacity serum bottles just before enzyme application. Enzyme was used at two concentrations (as recommended by the manufacturer) such as 1.68 and 2.52 g/kg DM (E1and E2, respectively). Enzyme 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 (E0). The suspension of DDW and enzyme was poured directly to feed samples in bottles 12 hours prior to the incubation. Bottles were closed with rubber caps and kept at room temperature (25℃). Natuzyme® was 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 (500units), protease (3000 units), and amylase (750 units) activities, as indicated by the manufacturer. Natuzyme® also contains hemicellulase, amyloglycosidase, pentosanase, pectinase and phytase activities. Natuzyme® is a micro-granulated enzyme product and possesses a wide pH range, stability and temperature tolerance.

2.2 *In vitro* Gas Production Technique

The gas production procedure was performed as described by Grings et al. [9]. Rumen inoculum was collected from three ruminally fistulated steers (580 ± 4.5 kg, body weight) prior to offering the morning feeding. Animals were fed 10.4 kg DM, a diet containing alfalfa hay (50%), wheat straw (20%), barley grain (15%), soybean meal (14%) and mineral-vitamin premix (1%). 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. A sample of 250 mg was weighed into a 125-ml serum bottles 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 in N-rich incubation medium and only sodium bicarbonate at 39.25 g/l in N-low medium), macro-mineral solution (5.7 g anhydrous Na2HPO4, 6.2 g anhydrous KH2PO4 and 0.6 g MgSO4·7H2O 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 CaCl2·2H2O, 10.0 g MnCl2·4H2O, 1 g CoCl2·6H2O and 8.0 g FeCl3·6H2O per 100 ml) was added.

Table 1. Ingredients and chemical composition of the corn silage based diet

Ingredients	Amount, DM (g/kg)
Alfalfa hay	109
Corn silage	383
Wheat straw	22
Corn grain	80
Barley grain	80
Wheat grain	22
Soybean meal, 44% CP	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	5
Chemical composition (g/kg)	
Ash	59
CP	166
NDF	340
Ether Extract	51

The medium was then reduced by addition of 41.7 ml reducing agent (40 ml deionized water, 1 ml 1N NaOH and 1 gNa2S·9H2O) 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 39º 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 [9]. 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 Ørskov and McDonald [10]:

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

where, y is the cumulative volume of produced gas at time t (h), A is the asymptotic gas volume(ml/250 mg DM), c is 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:

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

After the initial 96 h gas run, $t_{1/2}$ was calculated and a second incubation with the diet as substrate 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. Apparent substrate degradability was determined and calculated at $t_{1/2}$ by high speed centrifugation (13000 RPM, 20 min) of the incubation residue [11] followed by suspending into an iced water bath to stop fermentation. All blank samples were centrifuged (13000 RPM, 20 min) and supernatant collected, frozen and stored. Residue was weighed and used to correct apparent substrate degradability from the ruminal inoculum.

2.3 Calculations and Statistical Analyses

Data were statistically analyzed as a completely randomized design with three concentrations of enzymes as treatments on the following model:

$$y_i = \mu + E_i + e_i,$$

where, y = depended variable, μ = overall mean, E_i = effect of enzyme level and e_i = residual error. All the statistical analyses were performed using the general linear models procedures of SAS (1999, V. 8.2). Differences between means were assessed by Tukey test at $P = 0.05$.

3. RESULTS AND DISCUSSION

Results of asymptotic gas volume, halftime ($t_{1/2}$) of gas production and fractional constant rate are shown in Table 2. It has been indicated that the supplementation of the total mixed ration with Natuzyme® had no punctual effect on gas production parameters (A and c) at $t_{1/2}$. The use of fibrolytic enzymes as feed additives to improve degradation of fiber has been

studied under *in vitro*, *in situ* and *in vivo* conditions, but, results were inconsistant [2]. There are several factors such as enzyme doses [12] and type of diet [13] which may affect the fibrolytic activity of exogenous enzymes [3]. Cumulative gas production and the gas production rate were not significantly affected by enzyme supplements. Yang et al. [14] examined an enzyme product on a low forage diet at the amount of 50 mg/kg of total mixed ration (DM basis) using a gas test method. They did not find any significant response of cumulative gas production and gas production rate to enzyme supplements. They interpreted that according to the synergistic act of exogenous enzymes with inhabitant enzymes of the rumen, rumen inoculums must be obtained from animals fed with diets supplemented with fibrolytic enzymes. Giraldo et al. [15] stated that prolonged gas production trial would not make a marked effect of enzyme supplementation on fermentation parameters. Our results were confirming to previous results [5,16,17], which showed that the treatment with enzymes stimulated the initial phases of substrate degradation, but the effects were reduced as incubation time increased. Gonzalez-garcia et al. [18] conducted an experiment using a gas production technique to assess the effect of an enzyme preparation on fermentation parameters and found no trace of remarkable effect of enzyme supplementation on cumulative gas production which is in consistent with results of the present experiment. However, the constant rate of gas production was significantly reduced or the treated diet with enzyme preparation which was not observed in the present study. Results of apparent dry matter degradability, cumulative gas production, methane production and methane per mg of ADMD at $t_{1/2}$ are presented in Table 3. Dry matter degradability of the diet was increased significantly by 21 percent for E1 and E2 at $t_{1/2}$ compared to E0 (0.47 and 0.47 vs. 0.39) as the amount of dry matter degradability for E1 and E2 was exactly the same. Increase in diet degradability for E2 and E1 was same in level as compared to E0. Wallace et al. [19] showed that *in vitro* disappearance of dry matter and NDF was improved by 6.28% and 2.58%, respectively, after 36 h of incubation, when an exogenous fibrolytic enzyme blend was added to a diet having 0.57:0.43 forage to concentrate ratio. In our study, the enzyme mixture was applied 12 hours prior to the *in vitro* incubation as pre-treatment of the milled diet to allow for an enzyme-substrate interaction time as recommended by Beauchemin et al. [3]. Although it is hypothesized that a prolonged *in vitro* gas production trial would be a controversy in interpreting the overall results [5,16,17], it seems that under current conditions of the present study, $t_{1/2}$ could be a remarkable feature to be taken as the basis of the calculations [20,21].

Table 2. Asymptotic gas volume (A), halftime of gas production ($t_{1/2}$) and fractional constant rate (c, %/h) at $t_{1/2}$ for the test diet treated with different levels of powdered multi-enzyme (0.0, 1.68 and 2.52 g/kg substrate DM)

Parameters[1]	Enzyme Level			SEM[2]
	E0	E1	E2	
A (ml)	72.49	73.73	74.40	1.03
$t_{1/2}$ (h)	18.24	17.77	19.25	0.97
c (h^{-1})	0.038	0.039	0.036	0.003

[1]*Different superscript letters in each row indicate significant differences (P=.05).*
[2]*SEM = standard error of the means.*

Table 3. Apparent dry matter degradability at $t_{1/2}$ (*ADMD*), percentage of produced methane at $t_{1/2}$, gas production at $t_{1/2}$, volume of produced methane at $t_{1/2}$ and produced methane per mg of ADMD at $t_{1/2}$ (MD) with different levels of powdered multi-enzyme (0, 1.68 and 2.52 g/kg substrate DM).

Parameters[1]	Enzyme level			SEM[2]
	E0	E1	E2	
ADMD	0.39[b]	0.47[a]	0.47[a]	0.57
$GP_{t1/2}$ (ml)	43.69	43.68	43.10	0.37
Methane (ml)	5.10	4.69	5.17	0.24
MD (ml/mg ADMD)	0.022	0.020	0.022	0.001

[1]*Different superscript letters in each row indicate significant differences (P=.05).*
[2]*SEM = standard error of the means.*

Despite the amount of the other traits was numerically improved, enzyme supplements had no significant effect on ml of methane production, ml of gas production and amount of produced methane per mg dry matter apparently degraded at $t_{1/2}$. To date, few studies have investigated the effect of exogenous enzymes on methane production in the rumen, and their results are conflicting [14]. Giraldo et al. [15] reported that the treatment of a 0.7:0.3 grass hay:concentrate substrate with three fibrolytic enzymes increased NDFD by 15.9 to 26.4% and methane production by 14.3 to 24.6% in Rusitec fermenters. In contrast, Colombatto et al. [5] reported that adding a fibrolytic 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. [22] 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 [23].

4. CONCLUSION

Natuzyme® is an enzyme mixture generated to improve dry matter degradation in the gastrointestinal tract. *In vitro* findings of this experiment indicated that this product would have the ability to induce degradation of a corn silage based diet by 21 percent under current experimental conditions. Gas production parameters were not affected by enzyme supplement. Furthermore, volume of methane, gas production at $t_{1/2}$ and produced methane per mg of ADMD at $t_{1/2}$ were not a target of enzyme activity in this trial. These findings confirmed that the increase in dry matter degradability of the test diet used in the present study might not use for ATP production in bacteria as gas production, gas production rate and methane production are not influenced by the enzyme levels applied. However, there is still need to determine the incorporation of organic matter disappeared from the substrate into bacterial cell growth in rumen.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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