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Exogenous enzyme on *in vitro* gas production and ruminal fermentation of diet containing high level of concentrate[1]

Enzimas exógenas sobre produção de gás e fermentação ruminal in vitro de dieta contendo alta nível de concentrado

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SUMMARY

Exogenous enzyme preparations (fibrolytic activity (FIB), 0, 0.6, 1.2, 1.8, and 2.4 mg/ml liquid volume incubated; amylolytic activity (AMZ), 0, 0.05, 0.10, 0.15, and 0.20 mg/ml liquid volume incubated; proteolytic activity (PRO), 0, 0.05, 0.10, 0.15, and 0.20 mg/ml liquid volume incubated) were incubated *in vitro*. Their fermentation effects were assessed based on accumulated gas production, kinetic parameters, and fermentation profile using the technique of gas fermentation. Ruminal liquid was obtained from two rumen cannulated Santa Inês sheep, fed a diet with roughage-to-concentrate ratio of 20:80. Accumulated gas production was during 96 h of incubation, measured at 18 different times. After incubation, pH, dry matter degradability (DMD), organic matter *in vitro* digestibility (OMD), metabolisable energy (ME), partitioning factor (PF_{96}), gas yield (GY_{24}), short chain fatty acids (SCFA), and microbial protein production (MCP) were evaluated. Increasing FIB dose linearly decreased ($P<0.05$) *lag time* without affecting others kinetic parameters. However, FIB increased the accumulated gas production, resulting in improved DMD, OMD, ME, GY_{24} and SCFA. The addition of AMZ decreased linearly ($P<0.05$) *lag time* and increased ($P<0.05$) gas production on initial times of incubation without altering the fermentation profile. The inclusion of PRO did not affect ($P>0.05$) the evaluated parameters. The addition of these exogenous enzyme preparations with fibrolytic activity altered ruminal fermentation *in vitro* of diets containing high levels of concentrates.

Keywords: amylolytic, degradability, fibrolytic, proteolytic

RESUMO

Preparações de enzimas exógenas (atividade fibrolítica (FIB); 0,0; 0,6; 1,2; 1,8 e 2,4 mg/ml do volume de líquido incubado; atividade amilolítica (AMZ); 0,0; 0,05; 0,10; 0,15 e 0,20 mg/ml do volume de líquido incubado; atividade proteolítica (PRO); 0,0; 0,05; 0,10; 0,15 e 0,20 mg/ml do volume de líquido incubado) incubado *in vitro*. Os efeitos de fermentação foram avaliados com base na produção de gás acumulado, parâmetros cinéticos e, perfil de fermentação usando a técnica de fermentação *in vitro*. O líquido ruminal foi obtido de dois ovinos Santa Inês canulados no rúmen, alimentados com dieta relação volumoso:concentrado de 20:80. A produção de gás acumulada foi durante 96 h de incubação, mensurados em 18 tempos diferentes. Após a incubação foi avaliado pH,

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digestibilidade da material seca (DMS), digestibilidade da material orgânica (DMO), energia metabolizável (EM), fator de partição (FP_{96}), rendimento de gás (RG_{24}), ácidos graxos de cadeia curta (AGCC), e produção de proteína microbiana (PPM). O incremento de doses linear de FIB diminuiu (P<0,05) o *lag time* sem afetar outros parâmetros cinéticos. No entanto, adição de FIB aumentou a produção de gás acumulado, resultando em incremento na DMS, DMO, EM, RG_{24} e AGCC. A adição de AMZ diminuiu linearmente (P<0,05) *lag time* e incrementou (P<0,05) produção de gás nos tempos iniciais de incubação sem alterar o perfil de fermentação. A inclusão de PRO não afetou (P>0,05) os parâmetros avaliados. As adições de preparações enzimáticas exógenas com atividades fibrolíticas alteram fermentação ruminal *in vitro* de dieta de alta níveis de concentrado.

Palavras-chaves: amilolítica, degradabilidade, fibrolítica, proteolítica

INTRODUCTION

Modern feedlot diets are balanced by taking into account many different nutrients (COLE et al., 2012). Studies have aimed to maximize the use of feed ingredients at the ruminal level. There are still big unknowns to be explored regarding the inclusion of exogenous enzymes in feedlot diets.

Exogenous enzymes can promote improvements on ruminal kinetics parameters (ELGHANDOUR et al., 2013), and nutrient digestibility (YANG et al., 2011; TANG et al., 2008), and can allow the manipulation of final products from fermentation (TRICARICO et al., 2005; 2008) without changing the composition of the diet being fed. This occurs due to mechanisms that still have to be better understood (YANG et al., 2011; RANILLA et al., 2008), how differences in enzyme activity, application rate and composition, mode and time of enzymatic contact, ruminal activity *in vitro* and enzyme stability in this environment, and enzyme-food specificity (BEAUCHEMIN et al., 2004; ADESOGAN, 2014).

The initial evaluation of these mechanisms can be established through the *in vitro* gas production technique, using semiautomatic methods (MAURÍCIO et al., 1999; THEODOROU et al., 1994) or computerized methods (TEDESCHI et al., 2009). This technique evaluates the ruminal kinetics parameters such as fermentation rate, extension and profile (ELGHANDOUR et al., 2013; VÁRADYOVÁ et al., 2005), in a short period of time (MAURÍCIO et al., 2003), which allows to understand the effects of including exogenous enzyme on ruminant diets, helping nutritionists to identify ideal enzyme preparations and effective dosages before creating profitable commercial products for the ruminant production industry (EUN & BEAUCHEMIN, 2007).

The objectives of this study were to evaluate dosages of exogenous enzyme preparations with fibrolytic, amylolytic, and proteolytic activity on diets containing high levels of concentrates through gas production, kinetic parameters, and fermentation profile using the technique of *in vitro* fermentation.

MATERIAL AND METHODS

The diet used on *in vitro* incubations was a typical diet containing high levels of concentrates, for our conditions in which roughage-to-concentrate ratio is about 20:80, and the diet is composed of corn silage, ground corn, soybean meal, soybean hulls, and mineral mixture (Table 1).

Table 1. Formulation and chemical composition of the feedlot diet

Ingredients, g/kg DM	diet
Corn silage	200.0
Ground corn	540.0
Soybean meal	120.0
Soybean hulls	105.0
Mineral mix[1]	35.0
Chemical composition	
DM, g/kg	685.1
OM, g/kg da MS	941.4
CP, g/kg da MS	127.4
NDF, g/kg da MS	265.0

[1]Mineral mix = security levels: calcium 198g; phosphoro 60g; sodium 117g; magnesium 5.1g; sulfur 12.6g; iodine 17.7mg; iron 425mg; selenium 10.4mg; cobalt 80mg; manganese 527mg; fluorine 600mg; copper 1000mg and zinc 3000mg.

Roughage and concentrate samples were oven dried at 55°C for 72 h, grounded in a Wiley mill using 1 mm screen and stored for further determination of chemical components and *in vitro* gas production.

Five dosages of each enzyme preparation were evaluated according to its activity. For the fibrolytic preparation (FIB; Fibrozyme, Alltech Inc., Nicholasville, KY), the dosages were 0, 0.6, 1.2, 1.8, and 2.4 mg/ml liquid volume incubated. For the amylolytic preparation (AMZ; Amaize, Alltech Inc., Nicholasville, KY), the dosages were 0, 0.05, 0.10, 0.15, and 0.20 mg/ml liquid volume incubated. For the proteolytic preparation (PRO; VEG PRO, Alltech Inc., Nicholasville, KY), the dosages were 0, 0.05, 0.10, 0.15, and 0.20 mg/ml liquid volume incubated. These levels were set according to manufacturer's daily intake recommendations for beef cattle, and extrapolated for *in vitro* incubation according to Tricarico et al. (2005), calculating the enzyme amount in relation to liquid medium (concentration mg/ml liquid volume incubated), not necessarily relating to the enzyme-to-substrate ratio in a practical diet.

Ruminal fluid was collected from two Santa Inês sheep (60 a 70 kg body weight) fitted with permanent rumen cannula and fed a TMR diet with 20:80 roughage:concentrate ratio (same diet incubate *in vitro*), previously adapted for 15 d. The animals were kept in individual sheltered pens, equipped with feeding trough and drinker.

Rumen fluid was collected from each animal before the morning feeding, filtered through four cheese cloth layers and stored in an insulated bottle without leaving empty spaces, and immediately send to Animal Nutrition Laboratory/ Federal University of Mato Grosso. For each 125 ml amber glass flask, we weighted 0.5 g of diet, and enzyme preparations added according previously cited dosages (mg enzyme/ ml liquid). All methodological procedures used should be right supported by Mauricio et al. (1999). Sequentially, 40 ml of buffer solution were added for each flask according to Goering & Van Soest (1970), followed by 10 ml of particle-free rumen fluid, resulting in a proportion inoculum:buffer of 1:4 (v/v). Flasks were immediately closed with rubber caps and

aluminium ring and maintained at 39 ºC in constant agitation.

We used a total of 126 flasks in triplicates for each enzymatic dosage and for blanks (only ruminal fluid and buffer solution) in three runs in different weeks, being incubated for 96 h. Produced gas volume was registered on times 1, 2, 3, 4, 5 6, 8, 10, 12, 18, 24, 30, 36, 42, 48, 60, 72, and 96 h of incubation utilizing the semiautomatic reading technique described by Theodorou et al. (1994) and Mauricio et al. (1999). At the end of each incubation, flasks were opened to measure pH (pH meter, pH METER TC-2, Tecnal), and were filtered to obtain the non-fermentable residue, for the determination of disappeared substrate.

At the end of incubation, the contents of each bottle were filtered through quantitative paper filter (Whatman no. 54, 11 cm, particle retention from 20-25 µm). Fermentation residues were dried at 105ºC overnight to estimate DM disappearance with loss in weight after drying being the measure of undegradable DM.

The feed samples were submitted to chemical analysis in accordance with the procedures of AOAC (1990) for dry (DM, method # 930.15), ash (method # 924.05), and crude protein (CP, method # 984.13). The aNDFom were determined by methods of Van Soest et al. (1991). In NDF analysis, samples were treated with a heat stable alpha amylase, without addition of sodium sulphite and exclusive of residual ash.

The fibrolytic enzyme preparation containing xylanase and cellulase activities (Fibrozyme TM, Alltech Inc., Nicholasville, KY, USA) were previously checked for enzymatic activities suppliers guarantee levels, in accordance with the methodologies proposed by Colombatto & Beauchemin (2003). Enzymatic activity of the amylolytic preparation is 600 FAU/g, and was previously checked for guarantee levels according to methodologies proposed by Biely et al. (1985) and Tricarico et al. (2008). Enzymatic activity of the proteolytic preparation is a minimum of protease 7.500 u HUT/g and a minimum of cellulase of 45 u CMCU/g, and was previously checked according to methodologies proposed by Food Chemicals Codex (2010).

Gas production kinetic parameters (ml/g DM) were estimated using NLIN option of SAS (version 9.3), according to the model proposed by Schofield et al. (1994):

$$V_t = V_1/(1 + \exp(2 - 4^{**}_1(t - L))) + V_2/(1 + \exp(2 - 4^{**}_2(t - L)))$$

where V_t is the total gas volume at time t; V_1 is asymptotic cumulative gas volume (ml/g DM), k_1 is the rate (/h) parameters for the first pool (rapid), and V_2 and k_2 is corresponding parameters for the second pool (slow); L is the latency, and t is incubation time (h). It was used the interactive process of Marquardt algorithm for adjustments.

The metabolisable energy (ME, MJ/kg DM) and *in vitro* organic matter digestibility (OMD, g/kg OM) were estimated according to Menke et al. (1979) as:

$$EM = 2.20 + 0.136\,GP(ml/0.2\ g\ MS) + 0.057\,CP(mg/0.2\ g\ MS)$$

$$OMD = 148.8 + 8.89\,GP + 4.5\,CP(mg/0.2\ g\ MS) + 0.651\,ash(mg/0.2\ g\ MS)$$

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where GP is the gas production in ml from 200 mg of dry sample, incubated for 24 h.

The partitioning factor at 96 h of incubation (PF_{96}; a measure of fermentation efficiency) was calculated as the ratio of DM degradability *in vitro* (DMD, mg) to the volume (ml) of GP at 96 h (i.e., DMD/total gas production (GP_{96})) according to Blümmel et al. (1997).

Gas yield (GY_{24}) was calculated as the volume of gas (ml gas/g DM) produced after 24 h of incubation divided by the amount of DMD (g) according to Salem et al. (2014) as:

$$Gas\ Yield\ (GY_{24}) = GP(ml/g\ DM)/DMD(g)$$

A short chain fatty acid concentration (SCFA) was calculated according to Getachew et al. (2002) as:

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

where GP is the 24 h net gas production (ml/g DM). Microbial CP biomass production was calculated according to Blümmel et al. (1997) as:

$$MCP\ (mg/g\ DM) = DMD\ (mg) - (GP \times 2.2\ mg/ml)$$

where GP is the 24 h net gas production (ml/g DM), and 2.2 mg/ml is a stoichiometric factor which expresses mg of C, H and O required for the production of SCFA gas associated with production of 1 ml of gas.

Data from *in vitro* gas production and fermentation parameters were analysed in a completed randomized design using the MIXED procedure of SAS (version 9.3). The statistical model was:

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

where Y_{ij} is the dependent variable, μ the overall mean, ENZ_i is the effect of enzyme level, and E_{ij} is the residual error term.

The levels of enzymatic preparations were considered fixed effects. Before the statistical analyses, it was obtained the means from data of each week, which was used as experimental unit (UDÉN et al., 2012). The LSMEANS option was used to generate individual means for each treatment. Orthogonal contrasts were used to partition specifically the effects of enzyme levels on linear, quadratic, cubic and quartic. Cubic and quartic effects were not significant. In all analysis, significances were declared at $P<0.05$.

RESULTS AND DISCUSSION

Addition of FIB linearly decreased ($P<0.05$) latency (*L*), but there were no differences ($P>0.05$) in the degradation rates k_1 and k_2, and the volume of gas production from rapid and slow degrading pools, (V_1 and V_2), respectively (Table 2). Increasing the dose of FIB linearly increased ($P<0.05$) the accumulated gas production (GP, ml/g DM) for evaluated times (Table 2).

Table 2. *In vitro* rumen gas kinetics and cumulative gas production in response to fibrolytic enzyme dose (mg/ml liquid volume incubated)

Enzyme	Gas production parameters [a]					*In vitro* gas production (ml/g DM)					
Dosage	V_1	k_1	L	V_2	k_2	GP_6	GP_{12}	GP_{24}	GP_{48}	GP_{72}	GP_{96}
0.0	136.8	0.093	2.36	154.4	0.023	76.5	149.2	215.7	267.6	287.3	291.2
0.6	140.7	0.093	2.15	167.5	0.025	83.3	159.1	230.8	285.0	305.1	308.2
1.2	148.0	0.095	1.82	177.5	0.024	93.4	173.0	246.8	304.2	323.1	325.4
1.8	148.9	0.096	1.50	183.5	0.024	100.9	180.8	255.1	311.0	328.8	332.3
2.4	158.4	0.096	1.34	189.1	0.025	110.7	195.3	271.5	330.0	347.5	347.5
SEM	9.282	0.005	0.273	18.158	0.004	2.925	2.609	15.579	19.259	15.708	13.244
P-value											
Linear	NS	NS	**	NS	NS	**	**	*	*	*	**
Quadratic	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

V_1 is the volume for the rapid pool (first pool); k_1 is rate for the rapid pool (first pool); L is the latency; V_2 is the volume for the slow pool (second pool); k_2 is the rate for the slow pool (second pool).
NS: Not significant, *P<0.05 and **P<0.01.

The comportment of the gas production curve with include FIB is presented in Figure 1a. The DMD, OMD, ME, GY_{24} and SCFA (P<0.05, linear effect) increased in response to FIB dose, and PF_{96} decreased (P<0.05, linear effect), but not effect (P>0.05) pH and MCP (Table 3).

The rumen fluid used in the present work was from animals fed with high concentrate diets (20:80) it may be inferred that there was reduced proportion of fibrolytic microorganisms (and their enzymes) according observed by Tajima et al. (2001), caused by reduced amount of substrate (fibrous carbohydrates) as well low rumen pH (in this study pH of the rumen fluid was 5.53 ± 0,081).

Thus, the addition of fibrolytic enzymes increased the digestion of the fibre, as evidenced by increase on total gas production. This is enzymes promote greater hydrolytic capacity of ruminal liquid (BEAUCHEMIN et al., 1999; 2004), with a possible better synergistic relationship between exogenous enzymes and ruminal microbiota (NSEREKO et al., 2002), due to the interaction occurs via cross-feeding, for release of polysaccharides readily utilized by microorganisms (TRICARICO et al., 2008).

The animals kept in intensive production systems where diets have high concentrate proportions, adding fibrolytic enzymes can increase the fibre digestion in the rumen, with possibility increase microbial protein synthesis and VFA production, favourable increasing in animal production. Russell et al. (1992) proposed that the yield of fibrolytic microbial population of the rumen is reduced by 2.5% for each reduction unit forage fibre, when its percentage is lower than 20%, as a result of reduction in population growth on pH lower than 6.0. Although the percentage of roughage and the energy contribution from fibre carbohydrates in diets containing high levels of concentrates is small, several authors (RUSSELL, 2002; KRAUSE et al., 2003) highlight the need to use strategies that could improve fibre digestion in these conditions, in order to increase the energy supply and microbial protein synthesis.

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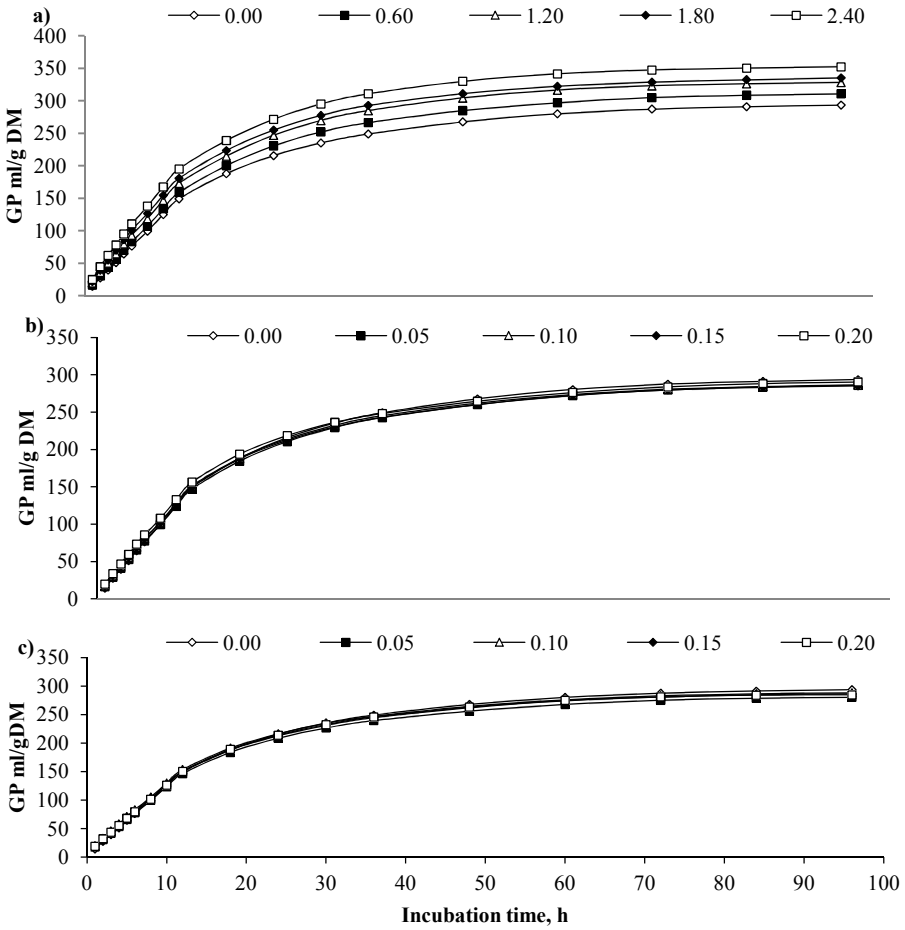

Figure 1. Cumulative gas production profiles (ml gas/g DM) from *in vitro* fermentation of tree exogenous enzyme at five doses (mg/ml liquid volume incubated): a Fibrolytic enzyme, b Amylolytic enzyme and c Proteolytic enzyme

Table 3. *In vitro* rumen fermentation profile in response to exogenous enzyme dose (mg/ml liquid volume incubated)[1]

Enzyme dosage	pH	DMD	OMD	ME	PF96	GY24	SCFA	MCP
				Fibrolytic Enzyme				
0.0	6.60	817	0.616	9.5	2.8	263	4.78	342
0.6	6.60	852	0.642	9.9	2.8	270	5.12	344
1.2	6.57	857	0.668	10.4	2.6	287	5.47	314
1.8	6.57	873	0.682	10.6	2.6	292	5.66	311
2.4	6.57	887	0.709	11.0	2.5	306	6.02	289
SEM	0.026	13.948	0.027	0.423	0.080	14.415	0.344	22.443
P-value								
Linear	NS	**	*	*	*	*	*	NS
Quadratic	NS	NS	NS	NS	NS	NS	NS	NS

[1] DMD is dry matter degradability (mg/g DM); OMD is *in vitro* organic matter digestibility (g/g DM incubated at 24h); ME is metabolisable energy (MJ/kg DM); PF96 is the partitioning factor at 96h of incubation (mg DM:ml gas); GY24 is gas yield at 24h (ml gas/g DMD); SCFA is short chain fatty acids (mmol/g DM); MCP is microbial CP production (mg/g DM)
NS: Not significant, *P<0.05 and **P<0.01.

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Thus, the use of fibrolytic enzymes presents the potential of promoting improvement in fiber digestion in animals fed high concentrates diets. The importance improvement fiber digestion fed high concentrates diets are represented for positive impact OMD, Hales et al. (2017) observed that reduce of 10% in fiber digestion, could decrease 2% in OMD, condition of use corn oil in high concentrates diets. Montgomery et al. (2008), affirm that reduce OMD infer microbial efficiency and minor flow N microbial for intestinal.

In high concentrates diets even there is participate of fiber have aim ruminal health, for rumination stimulation , equilibrium of pH, keep ruminal movements, and reduces acidosis risks (NAGARAJA & TITGEMEYER, 2007), an increase fiber digestion, that not reduce your paper in provide ruminal health, could provide condition acceptable for major performance animal.

However, it is fundamental to evaluate the economic impact of the use of fibrolitic enzymes in diets containing high levels of concentrates.

Addition of AMZ declined L linearly (P<0.05) without modifying (P>0.05) others gas production parameters (k_1, k_2, V_1, and V_2, Table 4). However, increasing AMZ, linearly increased (P<0.05) initial GP, GP_6 and GP_{12}, without altering others times (Table 4).

Table 4. *In vitro* rumen gas kinetics and cumulative gas production in response to amylolytic and proteolytic enzyme dose (mg/ml liquid volume incubated)

Enzyme	Gas production parameters [a]					*In vitro* gas production (ml/g DM)					
Dosage	V_1	k_1	L	V_2	k_2	GP_6	GP_{12}	GP_{24}	GP_{48}	GP_{72}	GP_{96}
Amylolytic Enzyme											
0.00	136.8	0.093	2.36	154.3	0.023	76.5	149.2	215.7	267.6	287.3	291.2
0.05	137.4	0.090	2.06	146.6	0.022	77.5	147.0	210.6	259.9	279.7	284.0
0.10	140.6	0.089	1.84	143.4	0.022	80.9	151.2	213.9	261.9	280.5	284.0
0.15	140.8	0.088	1.77	143.2	0.022	80.5	149.8	212.3	260.4	279.9	283.9
0.20	148.7	0.086	1.51	140.1	0.022	85.5	156.4	218.6	264.6	283.7	288.8
SEM	6.118	0.006	0.200	5.655	0.003	2.446	2.117	12.475	14.506	10.846	7.768
P-value											
Linear	NS	NS	**	NS	NS	*	*	NS	NS	NS	NS
Quadratic	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Proteolytic Enzyme											
0.00	136.8	0.093	2.36	154.4	0.023	76.5	149.2	215.8	267.6	287.3	291.1
0.05	139.1	0.086	1.79	139.6	0.022	78.2	146.2	208.5	256.0	275.2	278.8
0.10	146.1	0.086	1.58	140.5	0.022	83.1	153.5	216.3	264.6	283.1	286.6
0.15	141.7	0.085	1.62	143.0	0.021	81.3	150.4	212.5	261.8	280.6	284.7
0.20	141.4	0.086	1.75	142.6	0.022	79.7	150.3	213.8	263.0	280.9	284.0
SEM	5.805	0.005	0.233	6.134	0.003	2.152	1.376	12.662	13.812	10.931	8.287
P-value											
Linear	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Quadratic	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

V_1 is the volume for the rapid pool (first pool); k_1 is rate for the rapid pool (first pool); L is the latency; V_2 is the volume for the slow pool (second pool); k_2 is the rate for the slow pool (second pool).
NS= not significant, *P<0.05 and **P<0.01.

The comportment of the gas production curve with include AMZ is presented in Figure 1b.

The PRO enzymatic preparation not affected (P>0.05) the gas production parameters and accumulated gas production in of diet containing high level of concentrate (Table 4). The comportment of the gas production curve with include PRO is presented in Figure 1c. Increasing of the dose of AMZ and PRO, resulted in no effect (P>0.05) on fermentation profile (Table 5).

It may be inferred that the absence of response to amylolytic enzymes adding is due to fact that probably there is no limitation of amylolytic microorganisms and their enzymes on rumen inoculum used in this study. Klingerman et al. (2009), observed in diets dairy cows, apparent digestibility of DM, OM, CP, ADF, NDF and starch not influence with additional inclusion of amylase enzymes (Amaize, Alltech Inc., Nicholasville, KY), and those enzymes did not affect milk production. In the studies of Tricarico et al. (2005) and Hristov et al. (2008), amylase enzymes did not affect the digestibility of nutrients in the total tract.

Table 5. *In vitro* rumen fermentation profile in response to exogenous enzyme dose (mg/ml liquid volume incubated) [1]

Enzyme dosage	pH	DMD	OMD	ME	PF96	GY24	SCFA	MCP
				Amylolytic Enzyme				
0.00	6.60	821	0.616	9.5	2.8	263	4.78	347
0.05	6.67	847	0.607	9.4	3.0	248	4.67	384
0.10	6.67	833	0.613	9.5	2.9	256	4.74	362
0.15	6.60	816	0.610	9.4	2.8	260	4.70	350
0.20	6.63	843	0.621	9.6	2.9	259	4.84	362
SEM	0.026	19.367	0.024	0.365	0.045	11.362	0.298	15.263
P-value								
Linear	NS	NS	NS	NS	NS	NS	NS	NS
Quadratic	NS	NS	NS	NS	NS	NS	NS	NS
				Proteolytic Enzyme				
0.00	6.60	821	0.616	9.5	2.8	263	4.78	347
0.05	6.67	826	0.604	9.3	2.9	253	4.62	367
0.10	6.67	845	0.617	9.5	2.9	256	4.79	370
0.15	6.63	831	0.611	9.4	2.9	256	4.71	364
0.20	6.63	841	0.613	9.5	2.9	254	4.74	371
SEM	0.029	18.120	0.022	0.344	0.051	10.961	0.281	15.587
P-value								
Linear	NS	NS	NS	NS	NS	NS	NS	NS
Quadratic	NS	NS	NS	NS	NS	NS	NS	NS

[1] DMD is dry matter degradability (mg/g DM); OMD is *in vitro* organic matter digestibility (g/g DM incubated at 24h); ME is metabolisable energy (MJ/kg DM); PF96 is the partitioning factor at 96h of incubation (mg DM:ml gas); GY24 is gas yield at 24h (ml gas/g DMD); SCFA is short chain fatty acids (mmol/g DM); MCP is microbial CP production (mg/g DM).
NS = Not significant, *P<0.05 and **P<0.01.

Thus, it might can be stated that the digestion of starch already happens near to maximum, even when amylolytic enzymes are not supplemented, or occurs a competition for substrate, in which the enzyme may compete with the microbial population for binding sites of the target nutrient, not promoting increase dry matter digestibility (MORGAVI et al., 2000).

It must be emphasized that the main species of rumen amylolytic bacteria also present high proteolytic capacity (RUSSELL, 2002), in order to digest the protein matrix which surrounds the starch granules, especially in the maize and sorghum. In this way it may be infers that the concentration of proteolytic enzymes in the inoculum used in this study was not limiting for digestion of dietary protein, thus helping to explain the lack of response with respect to addition of proteolytic enzymes.

In according with Chen et al. (1995), added a fungal-derived enzyme complex with amylolytic and proteolytic activities, improvement DM, OM, CP, and NDF digestibility, for lactating cows, but did not affect milk production or DMI. Vera et al. (2012) and, Eun & Beauchemin (2005a) in diet low forage, added protease increase a digestibility of nutrients, but in diet with barley silage or barley dry rolled. However, in studies has different responses, as Colombatto et al. (2003) and, Eun & Beauchemin (2005b), did not affect with added protease in diets with corn silage, due some fiber components such as lignin can also inhibit specific proteolytic enzymes.

The understanding of the lack of effect for proteolytic enzymes, is not well defined. Several factors may interfere with the effectiveness of the action of exogenous enzymes, being them, differences in enzyme activity, application rate and composition, mode and time of enzymatic contact, ruminal activity in vitro and enzyme stability in this environment, and enzyme-food specificity (BEAUCHEMIN et al., 2004; ADESOGAN, 2014).

The addition of exogenous FIB enzymes improve *in vitro* gas production and fermentation profile of a mixture of feeds, representing diets containing high levels of concentrates. Increasing the enzyme dose of AMZ increase the accumulated gas production only during the initial times. The PRO enzymes not promote effects on ruminal fermentation *in vitro*.

The authors declare that they have no conflict of interest.

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