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Fermentation characteristics in hay from *Cynodon* and crop stubble treated with exogenous enzymes[1]

Características fermentativas dos fenos de *Cynodon* e restolhos de culturas tratados com enzimas exógenas

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ABSTRACT - The effect of treatment with xylanase and β-glucanase was evaluated for gas production and the ruminal degradation of nutrients from the hay of Tifton 85 grass and the stubble of maize, sorghum, peanut, sunflower and sesame crops. Two commercial fibrolytic enzymes were used (*Dyadic xylanase* PLUS - Xylanase; *BrewZyme* LP-β-glucanase), added to the hay at doses of 7.5 units of endoglucanase and 0.46 units of xylanase per 500 mg/gDM, for the cellulase and xylanase products respectively. The chemical composition of the hay was determined for no enzyme application and 24 hours after enzyme treatment, and the *in vitro* gas production and *in situ* microbial degradation was estimated for dry matter, organic matter, neutral detergent fibre and truly-degradable organic matter after 24 hours of incubation in the rumen. Enzyme treatment of the hay from Tifton 85 grass and the stubble of maize, sorghum, sunflower, peanut and sesame crops with the exogenous fibrolytic enzymes β-glucanase and xylanase influences *in vitro* gas production, and the *in situ* degradation of dry matter, organic matter, neutral detergent fibre and truly-degradable organic matter in the rumen. This variation can be attributed to differences in the chemical composition of the hay from the grass and the crop stubble, and to the different ways the enzymes act upon the cell wall.

Key words: *Arachis hypogaea*. β-glucanase. *Helianthus annuus*. *Sesamum indicum*. *Sorghum* bicolor. Xylanase.

RESUMO - Avaliou-se o efeito do tratamento com xilanase e β-glucanase sobre a produção de gases e degradação ruminal dos nutrientes dos fenos de capim-Tifton 85 e restolhos das culturas do milho, sorgo, amendoim, girassol e gergelim. Foram usadas duas enzimas fibrolíticas comerciais (*Dyadic xylanase* PLUS – Xilanase; *BrewZyme* LP - β-glucanase), adicionadas aos fenos nas doses de 7,5 unidades de endoglucanase e de 0,46 unidades de xilanase por 500 mg/gMS para os produtos celulase e xilanase, respectivamente. Determinou-se a composição química dos fenos sem aplicação de enzimas e 24 horas após o tratamento enzimático e estimou-se a produção de gases *in vitro* e a degradação microbiana *in situ* da matéria seca, matéria orgânica e fibra em detergente neutro e a matéria orgânica verdadeiramente degradável após 24 horas de incubação no rúmen. O tratamento enzimático dos fenos de capim-Tifton 85 e dos restolhos das culturas do milho, sorgo, girassol, amendoim e gergelim com as enzimas fibrolíticas exógenas β-glucanase e xilanase influencia de forma variável a produção de gases *in vitro*, a degradação *in situ* da matéria seca, matéria orgânica, fibra em detergente neutro e a matéria orgânica verdadeiramente degradável no rúmen. Essa variação pode ser atribuída às diferenças na composição químicas dos fenos da gramínea e dos restolhos de culturas e à diferente forma de ação das enzimas sobre a parede celular.

Palavras-chave: *Arachis hypogaea*. β-glucanase. *Helianthus annuus*. *Sesamum indicum*. *Sorghum bicolor*. Xilanase.

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INTRODUCTION

Forage plants are the main source of energy for ruminants due to the action of enzymes produced by microorganisms present in the rumen, which act on the carbohydrates of the fibrous fraction and degrade them. However, when enzyme activity on the substrates is low, the process of degradation of the cellulose and hemicellulose may be slow and incomplete, limiting the energy availability for the animals (SÁNCHEZ, 2009).

Interest in evaluating the addition of exogenous fibrolytic enzymes as an additive to ruminant forages has been increasing in recent years, with the aim of improving the benefits of the diet. These enzymes can be produced by bacteria or fungi, and when in contact with the substrate can potentiate the degradation of structural polysaccharides and increase the rate of fibre degradation.

It can be seen in the literature that the results obtained by the use of these enzymes are influenced among other factors, by the way the enzymes are supplied, by the levels of application, by the stability and activity of the enzymes in the rumen environment, and by the type of substrate. Forages with a higher fibre content may provide a greater number of degradation sites for enzyme action (CYSNEIROS *et al*., 2013). According to Martins *et al*. (2007), the chemical and morphological composition of forage, and the requirement of specific enzymes for the degradation of fibre, may compromise the performance of certain enzymes on degradation of the cell-wall components. Such aspects make it difficult to identify the mode of action of these enzymes in ruminants, and in part justify the variation seen in the responses.

The aim of this study was to evaluate the effect of treating hay from Tifton 85 grass and the stubble of maize, sorghum, sunflower, peanut and sesame crops with the exogenous fibrolytic enzymes xylanase and β-glucanase on *in vitro* gas decay and *in situ* degradation.

MATERIAL AND METHODS

This research was developed at the Animal Science Department of the Centre for Agricultural Sciences (CCA) at the Federal University of Piauí (UFPI), in Teresina, in the State of Piauí. Hay from Tifton 85 grass and from the stubble of maize, sorghum, sunflower, peanut and sesame crops were produced at the Centre for Agricultural Sciences, UFPI. The commercial fibrolytic enzymes (*Dyadic xylanase* PLUS - Xylanase; *BrewZyme* LP-β-glucanase) were mixed with the hay at doses of 7.5 units of endoglucanase and 0.46 units xylanase per 500 mg of hay DM for the cellulase and xylanase products respectively (SOLTAN *et al*., 2013b).

The levels of DM (method 934.01), OM (method 942.05), CP (method 954.01) and EE (method 920.39) were analysed as per the AOAC (2012). The total carbohydrates (TCH) were obtained with the equation 100 - (CP% + EE% + MM%), and the non-fibre carbohydrates (NFC) from the difference between TCH and NDF (SNIFFEN *et al*., 1992). NDF, ADF and lignin (Lig) were determined as per Van Soest, described and simplified by Souza *et al*. (1999). Hemicellulose and cellulose were calculated from the difference between NDF and ADF, and between ADF and lignin respectively.

The kinetics of rumen fermentation, and the ruminal microbial degradation of OM and NDF from the hays, were determined by the *in vitro* technique of gas production, as per Bueno *et al*. (2005). Two mestizo bovines fitted with rumen cannulae, and with a live weight of around 450 kg, were used as donors of ruminal inoculum, and submitted to a diet composed of Tifton 85 hay and a concentrate containing maize and soybean bran, in a bulk to concentrate ratio of 60:40, with free access to water and mineral salt in order to meet the requirements for maintenance (NRC, 2001). The animals were treated following the guidelines of the Ethical Committee on Animal Experimentation of UFPI (Process No 23111.019808/2014-12).

In order to describe the kinetics of ruminal fermentation, gas pressure readings (psi) were taken at 4, 8, 12, 18 and 24 hours after the start of incubation, and the gas volume estimated with the equation proposed by Azevêdo *et al*. (2008): $V = 0.11171p^2 + 4.7659p$, where: V = volume of gas (mL/gMS) and p=pressure (psi). After 24 hours incubation, fermentation was stopped, and the vials placed in a tray with ice water. The non-degraded residue was then recovered and an aliquot of the liquid contained in the vials was used to determine the pH, with the aid of a digital potentiometer, and the concentration of ammoniacal nitrogen ($N\text{-}NH_3$), as per Nogueira and Souza (2005).

Degradation of the organic matter (DOM) was calculated by the ratio between the truly-degraded organic matter in the rumen (TDOM) and the incubated OM. To estimate the TDOM, the residue was treated with a neutral detergent solution for 24 h, as per Blümmel *et al*. (1997). At the end of this period, the bags were washed with warm distilled water and acetone, and placed in a forced air circulation oven at 105 °C for 24 hours. They were then weighed, and incinerated in a muffle oven at 550 °C for 4 hours.

Degradation of the neutral detergent fibre (DNDF) was calculated by the difference between the NDF content of the initial sample and that recovered after incubation. The partitioning factor (PF) was calculated from the ratio between the TDOM and the volume of gas produced after 24 h incubation (BLÜMMEL *et al*., 1997).

Table 1 - Chemical composition of hay from Tifton 85 grass (TGH) and from the stubble of maize (MAS), sorghum (SOS), peanut (PES), sunflower (SUS) and sesame (SES) treated with exogenous enzymes[1]

Forage	Enzyme	DM	OM	CP	EE	MM	NDF	ADF	Hem	Cel	Lig	TCH	NFC
TGH	No enzyme	88.2	92.2	11.1	1.2	7.8	69.5	37.4	32.1	30.0	7.4	79.9	10.4
	Xylanase	89.5	91.2	11.9	1.1	8.3	69.9	37.2	32.7	29.9	7.3	78.6	8.7
	β-glucanase	89.5	91.6	13.3	1.1	8.4	69.0	38.5	30.5	31.0	7.5	77.1	8.1
MAS	No enzyme	87.9	89.3	10.5	0.9	10.7	68.1	39.9	28.2	33.6	6.3	78.3	10.2
	Xylanase	89.4	86.6	11.1	0.8	13.4	65.1	40.5	24.6	34.0	6.5	75.0	9.9
	β-glucanase	88.2	87.6	12.5	0.8	12.4	66.5	39.6	26.9	33.7	5.8	74.4	7.8
SOS	No enzyme	88.3	92.6	8.5	1.5	7.4	63.1	40.7	22.4	28.2	12.6	82.7	19.6
	Xylanase	89.0	91.5	8.5	1.5	8.5	59.3	36.3	23.0	24.3	12.0	81.7	22.3
	β-glucanase	87.1	89.8	9.3	1.5	8.1	62.8	35.1	27.7	22.7	12.4	81.2	18.5
PES	No enzyme	87.2	88.3	13.6	1.5	11.7	53.8	38.8	15.0	23.9	14.9	73.0	19.2
	Xylanase	88.2	84.1	11.9	1.3	15.9	58.0	46.1	11.8	28.3	17.9	70.3	12.3
	β-glucanas	87.9	91.9	14.2	1.5	10.2	53.0	42.1	10.9	26.2	15.9	73.9	20.9
SUS	No enzymes	86.6	85.0	10.0	1.5	15.0	49.4	45.4	3.9	28.4	17.0	73.5	24.2
	Xylanase	87.9	90.2	10.0	1.4	9.8	51.9	47.5	4.4	26.9	20.6	78.7	26.8
	β-glucanas	87.2	84.2	10.2	1.4	15.8	50.9	45.4	5.6	29.1	16.2	72.2	21.3
SES	No enzyme	88.3	90.0	9.5	0.8	10.0	62.9	47.3	15.6	37.5	9.7	79.8	16.9
	Xylanase	88.9	91.6	9.7	0.8	10.7	60.8	50.5	10.3	40.0	10.5	78.6	17.8
	β-glucanase	88.4	90.0	11.2	0.8	10.0	60.5	49.7	10.8	37.8	11.9	78.3	17.8

[1]DM = dry matter; OM = organic matter; CP = crude protein; EE = ether extract; MM = mineral matter; NDF = neutral detergent fibre; ADF = acid detergent fibre; Hem = hemicellulose; Cel = cellulose; Lig = lignin TCH = total carbohydrates; NFC = non-fibre carbohydrates

The data were analysed according to a randomised block design, using mixed models, these being the treatment considered as a fixed-effects model (5 degrees of freedom - DF), the control, and the random enzyme effect (3 DF), and also the random block, using the MIXED procedure of the SAS (2000) software.

RESULTS AND DISCUSSION

Treatment of the hay from Tifton 85 grass (TGH) and peanut stubble (PES) with the enzyme β-glucanase resulted in higher (P<0.05) *in vitro* gas production (ml/gOM and mL/gNDF) (Table 2), indicating greater efficiency of the microbial fermentation; gas production however was lower than that obtained for TGH by Soltan *et al*. (2013a), of between 126 and 144 mL/gDM.

The high proportion of ADF (cellulose and lignin) in the PES may have contributed to the better response to the action of β-glucanase (MARTINS *et al.*, 2008). An increase of 24.1% was seen in gas production (mL/gDM) in the treatment with β-glucanase, higher than that obtained by Soltan *et al*. (2013a) for leucaena, of 13.3%, and Gemeda and Hassen (2015) for maize stubble, of 7.1%.

The enzyme treatments with β-glucanase and xylanase resulted in greater (P<0.05) gas production (ml/gOM and mL/gNDF) from the *in vitro* fermentation of the corn stubble. In this case, the β-glucanase and xylanase may have contributed to solubilisation of the lignin of the fibrous fraction, and to a decrease in hemicellulose and cellulose crystallinity, facilitating the subsequent microbial enzyme attack (CASTRO, 2010).

Enzyme treatment of the hay from sorghum stubble (SOS) with β-glucanase resulted in greater (P<0.05) gas production (mL/gOM), followed by the treatment with xylanase, compared to untreated SOS. The increase in gas production as a function of the earlier action of β-glucanase and xylanase, reached 28.4% and 14.1% respectively in relation to the gas production resulting from the *in vitro* fermentation of SOS with no previous enzyme treatment.

The effect of these enzymes at equivalent doses in the treatment of hay from Tifton 85 grass and from sugarcane bagasse, was evaluated by Soltan *et al*. (2013ab), who found an increase in gas production

Table 2 - *In vitro* fermentation parameters of hay from Tifton 85 grass (TGH) and from the stubble of maize (MAS), sorghum (SOS), peanut (PES), sunflower (SUS) and sesame (SES) treated with exogenous enzymes[1]

Forage	Enzyme	Parameter				
		GP (mL/gMS)	GP (mL/gMO)	GP (mL/gFDN)	pH	N-NH_3 (mg/100mL)
TGH	No enzyme	77.87 b	22.36 b	7.88 b	7.0 a	121.64 a
	Xylanase	74.82 b	25.18 b	9.95 b	7.1 a	151.98 a
	β-glucanase	83.66 a	38.41 a	27.00 a	7.0 a	142.33 a
MAS	No enzyme	87.13 b	29.92 b	16.69 b	7.0 a	145.44 a
	Xylanase	94.68 a	37.44 a	20.75 a	7.0 a	140.31 a
	β-glucanase	87.46 b	38.17 a	23.09 a	7.0 a	143.73 a
SOS	No enzyme	79.95 c	31.85 c	10.30 a	7.0 a	139.07 a
	Xylanase	90.57 b	36.35 b	12.20 a	7.0 a	140.00 a
	β-glucanase	99.89 a	40.90 a	13.88 a	7.0 a	134.66 a
PES	No enzyme	89.02 b	43.40 b	14.80 b	7.1 a	140.47 a
	Xylanase	83.58 b	40.62 b	12.12 b	7.1 a	137.98 a
	β-glucanase	110.45 a	57.97 a	23.66 a	7.0 a	144.04 a
SUS	No enzyme	63.43 c	23.74 b	2.38 b	7.1 a	142.64 a
	Xylanase	89.15 a	34.51 a	11.13 a	7.0 a	140.62 a
	β-glucanase	72.13 b	27.37 b	7.42 a	7.1 a	144.82 a
SES	No enzyme	54.21 b	16.64 a	5.19 b	7.1 a	146.22 a
	Xylanase	59.35 b	19.51 a	9.97 a	7.1 a	148.87 a
	β-glucanase	66.21 a	20.50 a	2.85 b	7.1 a	147.93 a
P		<0.0001	<0.0001	<0.0001	0.0871	0.0977
SEM		3.6210	2.9748	1.9780	0.0727	9.2468

[1]GP (gas production); P = statistical probability; SEM = standard error of the mean. Mean values followed by different letters in a column for each type of hay differ (P<0.05) by Tukey test

(mL/gDM), albeit less expressive. This variation may result from the action of β-glucanase on surface cellulose chains, providing numerous additional sites for attack by the cellobiohydrolases, with the understanding that each hydrolytic event catalysed by one β-glucanase results in new sites for the cellobiohydrolases (OGEDA; PETRI, 2010).

The enzyme treatment of the hay from sunflower stubble (SUS) with xylanase increased (P<0.05) gas production (ml/gOM and mL/gNDF) by 45.4% and 367.6% respectively, which can be attributed to the removal of the xylan branches and the depolymerisation of the remaining fibre, resulting in an increase in the degradation of the fibrous carbohydrates (OGEDA; PETRI, 2010). The increase in gas production was greater than that obtained by Díaz *et al*. (2015) for *Pennisetum clandestinum*, of 17.4%. In addition, the fibre (NDF) from the SUS also showed susceptibility to the enzyme action of β-glucanase, with an increase (P<0.05) of 211.8% in gas volume.

Enzyme treatment of the stubble from the sesame crop (SES) with β-glucanase resulted in greater (P<0.05) gas production (ml/gDM) from the *in vitro* fermentation of the SES, which indicates the greater efficiency of this enzyme in forage with fibre of a higher cellulose content. The fibre (NDF) from the SES showed susceptibility to the enzyme action of xylanase, with an increase (P<0.05) of 91.1% in gas volume. Similarly, Dineshkumar *et al*. (2014) obtained an increase in gas production when treating brachiaria grass with cellulase.

The earlier enzyme treatment of the hay from Tifton 85 grass and from the stubble of maize, sorghum, peanut, sunflower and sesame crops had no influence (P >0.05) on the pH or the concentration of ammoniacal nitrogen (N-NH_3) of the incubation medium, as also seen by Arriola *et al*. (2011), Díaz *et al*. (2015) and Loures *et al*. (2005).

Enzyme treatment of hay from the Tifton 85 grass with β-glucanase increased (P<0.05) the DDM, DOM,

DNDF and TDOM *in situ* by 43.2%, 58.6%, 158.5% and 55.2% respectively, followed by the treatment with xylanase (Table 3). The cellulose content of the Tifton 85 hay may have contributed to the action of β-glucanase, due to the increases in degradation being related to the greater enzyme activity and crystallisation of the cell wall (BEAUCHEMIN *et al.*, 2003). When treating Tifton 85 hay with cellulase, Soltan *et al*. (2013a) obtained a lower value for DOM (39.8%), which indicates greater efficiency for the β-glucanase treatment adopted in this study. Díaz *et al*. found an increase in DDM and DNDF of only 8.8% and 8.0% respectively, for Marvel grass (*Dichanthium aristatum*) treated with the enzyme cellulase.

The enzyme treatment of hay from the maize stubble with β-glucanase increased (P<0.05) the DDM, DOM, DNDF by 21.5%, 28.0% and 37.2% respectively, followed by the treatment with xylanase (Table 3), indicating that these enzymes acted on the cell wall (NDF) and the cellulose and hemicellulose fractions, favouring the *in situ* degradation of the NDF by the microorganisms of the rumen environment. There was no difference (P>0.05) in the DDM or TDOM of the MAS treated with β-glucanase or xylanase. Despite the similarity between the stubble from the maize and sorghum, the earlier enzyme treatment of the stubble from the sorghum crop (SOS) had no influence on the DDM, DOM, DNDF or TDOM *in situ* (P>0.05) (Table 3).

The enzyme treatment of hay from the peanut stubble (PES) with β-glucanase increased (P<0.05) the DOM, DNDF and TDOM *in situ* by 7.5%, 28.2% and 12.0% respectively (Table 3). The higher cellulose than hemicellulose content of the PES, favoured the action of β-glucanase on the fibrous carbohydrates, with breakage of glycosidic bonds in the cellulose chains, promoting the degradation of NDF (OGEDA; PETRI, 2010). This increase in DNDF corresponds to twice that obtained by Soltan *et al*. (2013a) for DNDF of leucaena treated with the enzyme cellulase.

Table 3 - *In situ* ruminal degradation of dry matter (DDM), organic matter (DOM) and neutral detergent fibre (DNDF), truly-degraded organic matter (TDOM) and partitioning factor (PF) in hay from Tifton grass 85 and from the stubble of maize (MAS), sorghum (SOS), peanut (PES), sunflower (SUS) and sesame (SES) treated with exogenous enzymes[1]

Forage	Enzyme	Parameter				
		DDM (g/kg)	DOM (g/kg)	DNDF (g/kg)	TDOM (g)	PF (g/mL)
TGH	No enzyme	328.01 c*	285.05 c	101.33 c	122.2 c	1.56 b
	Xylanase	395.29 b	363.09 b	168.27 b	143.8 b	2.09 a
	β-glucanase	469.87 a	451.95 a	261.91 a	189.7 a	2.28 a
MAS	No enzyme	371.90 b	340.72 c	192.27 b	136.2 b	1.57 b
	Xylanase	441.59 a	392.37 b	216.20 b	160.5 a	1.69 ab
	β-glucanase	451.68 a	436.28 a	263.86 a	168.9 a	1.93 a
SOS	No enzyme	396.10 a	397.17 a	127.52 a	156.5 a	1.95 a
	Xylanase	428.38 a	400.51 a	131.07 a	169.6 a	1.87 ab
	β-glucanase	422.54 a	410.00 a	139.59 a	164.2 a	1.65 b
PES	No enzyme	526.50 a	487.44 b	166.55 b	181.0 b	2.04 b
	Xylanase	552.70 a	470.75 b	150.48 b	160.3 b	2.35 a
	β-glucanase	546.96 a	523.90 a	213.48 a	202.7 a	1.83 b
SUS	No enzyme	434.83 b	372.83 b	37.65 b	134.2 b	2.13 a
	Xylanase	494.31 a	456.29 a	114.55 a	176.7 a	1.95 a
	β-glucanase	441.40 b	374.62 b	72.94 ab	143.4 b	2.00 a
SES	No enzyme	361.02 a	302.70 a	78.59 b	122.3 a	2.25 a
	Xylanase	353.63 a	311.46 a	171.60 a	133.7 a	2.20 a
	β-glucanase	350.49 a	309.60 a	44.47 b	123.5 a	2.24 a
P		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
SEM		18.4633	22.9715	23.3154	10.2	0.1076

[1]P = statistical probability; SEM = standard error of the mean. Mean values followed by different letters in a column for each type of forage differ (P<0.05) by Tukey test

The enzyme treatment of the hay from the sunflower stubble (SUS) with xylanase increased (P <0.05) the DDM, DOM, DNDF and TDOM *in situ* by 13.7%, 22.4%, 204.2% and 31.7% respectively (Table 3). The cell wall presents regions of high crystallinity, increasing the resistance of the cellulose to enzymatic attack by the cellulase (OGEDA; PETRI, 2010). The increase in DDM and DNDF of the SUS with xylanase was higher than that found by Díaz *et al*. (2015) for kikuyu grass (*Pennisetum clandestinum*) treated with xylanase, of 5.7% for DDM and 11.6% for DNDF.

The PF found in this study ranged from 1.56 to 2.35 gTDOM/mL gases, within the range recommended by Makkar (2004), of up to 4.4 g TDOM/mL gases.

Enzyme treatment of the forages had a varying influence on the partitioning factor (PF). It is important to consider the interpretation of the PF in studies evaluating the nutritional value of food, considering that the higher the PF, the greater the need for TDOM to produce an equivalent volume of gas. The PF for TGH was higher (P<0.05) during enzyme treatment.

The treatment with xylanase increased (P<0.05) the FP of the PES; treatment with β-glucanase increased (P<0.05) the FP of the MAS, showing no difference (P>0.05) from the treatment with xylanase, although the FP of the MAS treated with xylanase showed no difference from the untreated MAS, while the enzyme treatment reduced the PF of the SOS (P<0.05) (Table 3). Soltan *et al*. (2013a) also obtained an increase in PF for hay from Tifton 85 grass treated with cellulose; this was lower than that seen in this study when the Tifton 85 hay was treated with xylanase, 34.0%, and with β-glucanase, 46.0%.

CONCLUSIONS

The enzyme treatment of the hay from the Tifton 85 grass and the stubble of the maize, sorghum, sunflower, peanut and sesame crops with the exogenous fibrolytic enzymes β-glucanase and xylanase, has a variable effect on *in vitro* gas production, and *in situ* degradation of DM, OM, NDF and TDOM. This variation can be attributed to differences in the chemical composition of the hay from the grass and crop stubble, and to the different ways the enzymes act on the cell wall.

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