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Effects of xylanase on the fermentation profile and chemical composition of sugarcane silage

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Abstract

The current study aims to evaluate the effects of increasing levels of xylanase enzyme (XYL) on sugarcane silage fermentation, fermentative losses, chemical composition, dry matter (DM), neutral detergent fibre (NDF) degradation and aerobic stability. A completely randomized design trial was performed with five treatments and 50 experimental silos. Treatments were: 0, 100, 200, 300 and 400 mg of XYL per kg of DM. XYL contained 10 000 U/g. There was a quadratic effect of XYL on silage pH and acetic acid concentration: lower pH and higher acetic acid concentrations were found at intermediary levels of the enzyme. XYL decreased lactic acid concentration linearly. Furthermore, the enzyme had a quadratic effect on effluent and total losses, with higher losses at intermediary XYL levels. There was a quadratic effect of XYL on organic matter (OM), non-fibre carbohydrates (NFC) and crude protein (CP) content. In addition, a quadratic effect of XYL was observed on NDF content and degradation. Intermediary levels of XYL showed higher concentration of OM and NFC. The addition of XYL had no effect on silage temperature and pH after aerobic exposure. Thus, intermediate levels of XYL increased acetic acid and decreased silage pH. Besides positive effects on silage composition, intermediary XYL levels decreased NDF degradation.

Introduction

Sugarcane (*Saccharum* spp.) is one of the most agronomical and economically suitable forage sources for ruminants in tropical and sub-tropical regions. It shows high dry matter (DM) production (25–40 t/ha; Ávila *et al.*, 2009) and presents up to 571 g/kg total digestible nutrients after being ensiled. However, the fibre content of sugarcane exceeds 600 g/kg of DM (de Andrade *et al.*, 2016), which impairs digestibility (Aroeira *et al.*, 1993) and compromises animal feed intake and performance (Corrêa *et al.*, 2003).

Among enzymatic supplements, fibrolytic enzymes are an emerging additive used in ruminant diets to increase fibre digestion. This has been observed both *in vitro* (Bowman *et al.*, 2002; Kung *et al.*, 2002) and *in vivo* (Arriola *et al.*, 2017; Gandra *et al.*, 2017) trials. However, some studies (Sheperd and Kung, 1996; Daniel *et al.*, 2016) show no effects of exogenous enzymes on animal digestion and performance. Inconsistent results could be associated with enzyme dose, basal diet (Bowman *et al.*, 2002) and application method (total mixed ration, concentrate or silage) (Yang *et al.*, 1999).

Gandra *et al.* (2017) reported positive effects of xylanase (XYL) and cellulase addition on fibre digestibility when animals were fed with sugarcane silage, whereas no effect of these enzymes was observed in animals fed a maize silage diet. On the other hand, the addition of enzymes in grass (Mandebvu *et al.*, 1999; Dean *et al.*, 2005), sorghum (Xing *et al.*, 2009) and maize (Sheperd and Kung, 1996; Ying *et al.*, 2017) at ensiling increases silage digestibility. Degradation of xylan produces mainly acetic acid as an end-product during fermentation (Fred *et al.*, 1919; Dehghani *et al.*, 2012). Acetic acid reduces pH and inhibits the growth of spoilage organisms, being associated with increased aerobic stability (Danner *et al.*, 2003). To the authors' knowledge, there is no study in the literature evaluating the effect of XYL on ensiled sugarcane.

The aim of the current study was to determine the effects of increasing levels of XYL on sugarcane silage fermentation, gases and effluent losses, chemical composition, DM and neutral detergent fibre (NDF) degradation and aerobic stability. It was hypothesized that XYL improves DM and NDF degradation, alters parameters of fermentation and improves chemical composition and aerobic stability of sugarcane silage.

Materials and methods

The experiment was conducted between July and September of 2016, at the Group of Agriculture Study and Labor (GETAP) of Agricultural Sciences Center (CCA), Federal

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University of São Carlos (UFSCar), in Araras, Brazil: 22°18′ S latitude, 47°22′ W longitude and 665 m asl.

Treatments and experimental design

Sugarcane (Cultivar RB02-5799) was harvested and ensiled 11.5 months after planting (first cut), and solids concentration of sugarcane juice (BRIX) was 210 g/kg (Table 1). Evaluation of BRIX was performed using the refractometric method (990.35 of AOAC, 2000). Sugarcane was harvested manually from five batches and chopped in a forage harvester (90 z-10, JF, Itapira, Brazil). Particle size of processed sugarcane was analysed using the Penn State Particle Separator (Maulfair *et al.*, 2011). Immediately before ensiling, lyophilized enzyme (powder) was individually weighed, top-dressed to chopped sugarcane and hand mixed. The sugarcane was ensiled with specific density of 600 kg/m^3 in silos comprising of a plastic bucket, 28 cm in diameter, 25 cm high and equipped with a *Bunsen* valve to allow gas to escape. The trial was performed in a completely randomized design with five treatments and ten repetitions per treatment. Treatments were: 0, 100, 200, 300 and 400 mg of XYL/kg DM of sugarcane. Enzyme contained 10 000 U/g (Beijing Smile Feed Sci. & Tech. Co., Ltd.).

Fermentative profile and loss evaluation

Five kilograms of sand were placed at the bottom of a plastic bucket with a nylon screen separating the chopped sugarcane to be ensiled. The weight of empty and sealed silos at ensiling and after 60 days was recorded using a 5-g sensitivity scale (Mettler Toledo, Barueri, Brazil). Gas losses (GL) were obtained by difference between silo weight at ensiling (ESW) and opening (OSW), according to the following equation:

$$\text{GL (g/kg)} = \frac{\text{ESW (g)} - \text{OSW (g)}}{\text{EDM (kg)}}$$

where EDM was the ensiling DM. The difference between weight of empty silo before ensiling (EESW) and after opening (OESW) was considered effluent losses (EL), according to the following equation:

$$\text{EL (g/kg)} = \frac{\text{OESW (g)} - \text{EESW (g)}}{\text{EDM (kg)}}$$

Total DM losses were obtained by sum of gas and effluent production according to Jobim *et al.* (2007). Dry matter recovery (DMR) was calculated by the ratio between DM at silos after silos opening (ODM, kg) and ensiled DM:

$$\text{DMR} = \frac{\text{ODM (kg)}}{\text{EDM (kg)}}$$

After opening silos, a sample of 15 g was collected from each silo and diluted in 150 ml of distilled water (ratio 1 : 10). It was processed in a blender for 30 s (Cherney and Cherney, 2003) and then squeezed through four layers of cheesecloth to extract fluid for pH measurement using a digital potentiometer (LUCA-210, Lucadema, Sao José do Rio Preto, Brazil). Filtered samples were frozen for subsequent evaluation of ammonia-N (NH_3-N), acetate, propionate, ethanol and lactate.

Table 1. Chemical composition of sugarcane[a]

Item	Mean
Composition (g/kg)	
DM	277
OM	970
NDF	507
NFC[b]	426
ADF	289
EE	14.0
CP	23.6
$NEL_{3\times}$[c] (MJ/kg)	6.28
In situ degradation	
DM	0.602
NDF	0.376
Particle size distribution (g/kg as-fed)	
>19 mm	149
19–8 mm	290
8–4 mm	275
<4 mm	285

[a]First cut of RB025799 cultivar sugarcane, which showed 210 g/kg of soluble carbohydrates, at 11.5 months after plantation.
[b]NFC = 1000 – [(CP – neutral detergent insoluble protein) + EE + ash + NDF].
[c]Net energy of lactation, calculated according to NRC (2001).

Samples of silage extract were thawed to room temperature and centrifuged (500 ***g*** for 15 min). For NH_3-N content evaluation, extracts (2 ml) were diluted with sulphuric acid (1 N; 1 ml) and analysed by the colorimetric phenol-hypochlorite method (Broderick and Kang, 1980). Samples were analysed for lactic acid concentration using a spectrophotometric method (Pryce, 1969): samples were diluted in phosphoric acid solution and centrifuged (3000 ***g*** for 5 min); supernatant was homogenized with sulphuric acid and heated to 75 °C for 2.5 min; after cooling, colour reagent (4-phenylphenol, Sigma Aldrich, St. Louis, MO, USA) was added; the sample was heated to 90 °C for 1.5 min and, after cooling, read in the spectrophotometer at 560 nm. Other samples were acidified using formic acid in a 1 : 4 ratio for further ethanol, acetic, propionic and butyric acids analyses. Organic acids were determined by gas chromatography (GC-2010 Plus chromatograph, Shimadzu, Barueri, Brazil), equipped with an AOC-20i auto-sampler, Stabilwax-DA™ capillary column (30 m, 0.25 mm internal diameter, 0.25 µm df, Restek©) and a flame ionization detector. A 1 µl aliquot of each sample was injected with a split ratio of 40 : 1, using helium (He) as the carrier gas at linear velocity of 42 cm/s, in a chromatographic run of 11.5 min. The injector and detector temperatures were 250 and 300 °C, respectively, and the initial temperature of the column was 40 °C. The temperature ramp of the column started with a gradient from 40 to 120 °C at 40 °C/min rate, followed by a gradient from 120 to 180 °C at 10 °C/min rate and from 180 to 240 °C at 120 °C/min, keeping temperature at 240 °C for 3 min at the end. For quantification, a calibration of the method was made using diluted solutions of the WSFA-2 standard (Ref. 47056, Supelco©) and ethanol (Ref. 459828, Sigma-Aldrich©) analysed under the conditions described above. Peak detection and integration were performed using the GCsolution v. 2.42.00 software (Shimadzu©).

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Table 2. Effect of XYL level on sugarcane fermentation profile and losses

	XYL level						P^a	
Item	0	100	200	300	400	SEM	Lin	Qua
Fermentative profile								
pH	3.47	3.37	3.44	3.36	3.46	0.009	0.555	0.001
Ammonia N (g/kg CP)	11.1	11.6	11.4	11.8	12.2	0.33	0.304	0.829
Ethanol (g/kg DM)	6.46	5.83	6.28	6.57	6.33	0.094	0.471	0.445
Lactic acid (g/kg DM)	28.8	43.4	18.3	25.1	19.4	0.74	<0.001	0.161
Acetic acid (g/kg DM)	19.3	23.5	20.3	24.7	17.6	0.40	0.477	<0.001
Fermentative losses (g/kg DM)								
Gas	87	83	95	86	95	2.2	0.153	0.842
Effluent	170	241	246	173	170	9.7	0.262	0.003
Total	256	324	341	259	264	9.2	0.423	0.003
DM recovery	784	782	769	776	791	5.3	0.823	0.238

[a]Probabilities: Lin, linear; Qua, quadratic.

Chemical composition and in situ *degradation*

A sample (100 g) was collected from the centre of silos immediately after opening and frozen until analysis. Samples were pre-dried at 60 °C in a forced-air oven for 72 h and ground in a Wiley mill (SL-31, SolabCientífica, Piracicaba, Brazil) either through a 2-mm or a 1-mm sieve. Samples (1-mm processed) were analysed for DM (method 950.15, AOAC, 2000), ash (method 942.05, AOAC, 2000), crude protein (CP) ($N \times 6.25$; method 984.13, AOAC, 2000), ether extract (EE, method 920.39, AOAC, 2000), NDF using α-amylase without addition of sodium sulphite (Van Soest *et al.*, 1991) and acid detergent fibre (ADF) according to Van Soest *et al.* (1991). Net energy concentration of sugarcane was estimated according to NRC (2001) and non-fibre carbohydrate (NFC) according to Hall (2000). DM and NDF degradation were determined *in situ*. Samples (processed at 2-mm) were placed in bags of non-woven fabric tissue (5 × 5 cm and 100 g DM/m^2; Casali *et al.*, 2008) and incubated for 96 h in the rumen of two Holstein cows. After incubation, samples were washed in running tap water and analysed for NDF content as described previously.

Aerobic stability

For aerobic stability, samples of silage (3 kg) were placed in a plastic bucket and maintained in a controlled temperature room (22 ± 1.7; mean ± S.D.) for 5 days. Temperature of the silage centre was recorded every 8 h using an infrared digital thermometer (DT-8380, Tianjin Cheerman Technology Co. Ltd., Tianjin, China). Silage pH was evaluated every 24 h, using the method described previously. Aerobic stability was defined as the number of hours the silage remained <2 °C above the ambient temperature (Ranjit and Kung, 2000).

Statistical analysis

Data were analysed using PROC MIXED of SAS 9.3 (SAS Inst. Inc., Cary, NC, USA), according to the following model:

$$Y_{ij} = \mu + X_i + e_{ij}$$

with $e_{ij} \approx N(0, \sigma_e^2)$, where Y_{ij} is the value of dependent variable; μ is the overall mean; X_i is the fixed effect levels of XYL ($i = 1$ to 5); e_{ij} is the residual error and N stands for Gaussian distribution. Degrees of freedom were corrected by Kenward and Rogers (1997) method. XYL enzyme levels were tested for linear and quadratic effects using polynomial regression. Equations that describe the effect of XYL level were obtained for every variable.

Silage pH and temperature after aerobic exposition were analysed as repeated measures, according to the following model:

$$Y_{ijk} = \mu + X_i + \omega_{ij} + T_k + T \times X_{ki} + e_{ijk}$$

with $\omega_{ijk} N(0, \sigma_\omega^2)$ and $e_{ijk} MVN(0, R)$; where Y_{ijk} is the value of dependent variable; μ is the overall mean; X_i is the fixed effect levels of XYL enzyme ($i = 1$ to 5); T_k is time fixed effect ($k = 1$ to 5 for pH and $k = 1$ to 15 for temperature); ω_{ij} is the random effect of silo; $T \times X_{ki}$ is the interaction effect of time and XYL enzyme; e_{ijk} is the residual error; N stands for Gaussian distribution; σ_ω^2 is the variance associated with experimental silo; *MVN* stands for multivariate normal and R is the variance–covariance matrix of residuals due to the repeated measurements. It was evaluated the following variance–covariance matrix (CS, CSH, AR(1), ARH(1), TOEP, TOEPH, UN, FA(1) and ANTE(1)). The matrix was chosen using a Bayesian method. Statistical differences were defined as $P \leqslant 0.05$ and tendency towards significance was considered at $0.05 < P \leqslant 0.10$.

Results

The addition of XYL showed a quadratic effect ($P \leqslant 0.001$) on silage pH and acetic acid concentration (Table 2). According to regression, the lowest level of silage pH was found at an enzyme inclusion of 211 mg/kg DM (Table 3). Similarly, the highest acetic acid concentration was found at 185 mg/kg DM of XYL. Enzyme had no effect ($P \geqslant 0.445$) on ethanol concentration but linearly decreased lactic acid concentration ($P < 0.001$). Furthermore, there was a quadratic effect ($P = 0.003$) of XYL on effluent and total losses. The highest effluent and total losses were obtained at 178 and 184 mg/kg DM of XYL, respectively. However, there was no effect ($P \geqslant 0.153$) of enzyme on GL and DM recovery.

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Table 3. Regression coefficients and quadratic maxima for variables with linear and quadratic effects of XYL level

Item	Intercept	S.E.	Linear coefficient	SE	Quadratic coefficient	S.E.	Quadratic max/min[a]
Fermentative profile and losses							
pH	3.47	0.022	-8.3×10^{-4}	2.64×10^{-4}	1.97×10^{-6}	1.0×10^{-7}	211
Lactic acid (g/kg DM)	34	2.2	-3.71×10^{-2}	9.05×10^{-3}	–	–	–
Acetic acid (g/kg DM)	19	1.0	4.06×10^{-2}	1.21×10^{-2}	-1.1×10^{-4}	2.9×10^{-5}	185
Effluent (g/kg DM)	181	19.0	5.77×10^{-1}	2.25×10^{-1}	-1.62×10^{-3}	5.38×10^{-4}	178
Total losses (g/kg DM)	267	19.1	5.88×10^{-1}	2.26×10^{-1}	-1.59×10^{-3}	5.42×10^{-4}	185
Chemical composition (g/kg DM)							
OM	961	1.0	2.61×10^{-2}	1.16×10^{-2}	-7.0×10^{-5}	2.8×10^{-5}	186
NDF	742	10.2	-2.48×10^{-1}	1.20×10^{-1}	5.47×10^{-4}	2.88×10^{-4}	227
NFC	174	10.6	2.96×10^{-1}	1.26×10^{-1}	-6.8×10^{-4}	3.01×10^{-4}	218
CP	30	1.2	-3.38×10^{-2}	1.46×10^{-2}	9.2×10^{-5}	3.5×10^{-5}	184
DM degradation	0.287	0.0099	-1.99×10^{-4}	1.17×10^{-4}	5.16×10^{-7}	2.80×10^{-7}	193

[a]Level of XYL for maximal or minimal response = −linear coefficient/(2 × quadratic coefficient).

Table 4. Effect of XYL level on sugarcane silage chemical composition and ruminal *in situ* degradation

	XYL level						P^a	
Item	0	100	200	300	400	SEM	Lin	Qua
Chemical composition (g/kg DM)								
DM (g/kg as fed)	234	238	236	232	237	1.5	0.954	0.994
OM	961.0	964.3	962.2	963.9	960.4	0.45	0.603	0.013
NDF	745	713	726	711	731	4.8	0.386	0.063
ADF	460	440	440	445	447	3.9	0.472	0.141
NFC	171	208	192	211	182	5.0	0.461	0.027
CP	30.7	26.7	28.2	28.3	31.5	0.59	0.469	0.012
EE	14.1	17.1	16.5	13.4	15.4	0.69	0.831	0.437
In situ degradation								
DM	0.411	0.410	0.417	0.435	0.417	0.0421	0.216	0.494
NDF	0.297	0.251	0.273	0.290	0.282	0.0435	0.805	0.053

DM, dry matter; NDF, neutral detergent fibre; ADF, acid detergent fibre; NFC, non-fibre carbohydrates; CP, crude protein; EE, ether extract.
[a]Probabilities: Lin, linear; Qua, quadratic.

Although XYL had no effect ($P \geqslant 0.141$) on DM, ADF or EE content, it showed a quadratic affect ($P \leqslant 0.027$) on organic matter (OM), NFC and CP and tended to have a quadratic effect ($P = 0.063$) on NDF content (Table 4; Fig. 1). Intermediary levels of enzyme showed the highest concentration of OM (186 mg/kg DM) and NFC (218 mg/kg DM), with the lowest values of NDF (227 mg/kg) and CP (184 mg/kg DM; Table 3). Additionally, XYL had no effect ($P \geqslant 0.216$) on DM degradation and tended to quadratically affect ($P = 0.053$) NDF degradation (Fig. 1).

Besides the quadratic effect ($P = 0.001$) of XYL on silage pH at opening, which was still apparent ($P \leqslant 0.006$) 48 h after aerobic exposure (Fig. 2), in general, there was no XYL effect ($P \geqslant 0.543$) on silage pH after aerobic exposure (Table 5). Similarly, treatments showed no effect ($P = 0.791$) on silage temperature after aerobic exposure (Fig. 3) and, consequently, the time of stability was not affected ($P \geqslant 0.658$) by XYL (Table 5).

Discussion

Ensiled sugarcane had 426 g/kg of NFC, in the current study. According to McDonald *et al.* (1991), NFC is mainly sucrose, which can be fermented by lactic acid bacteria to produce organic acids and decrease pH. XYL had a quadratic effect on many variables related to silage fermentation, fermentative losses and chemical composition. He *et al.* (2015) previously reported a quadratic effect of XYL activity on degradation of some forages. These authors highlighted that the main factors associated with negative effects of elevated XYL levels are: blocking the enzyme binding sites (Nsereko *et al.*, 2000) and decreasing microbial attachment to substrate (Morgavi *et al.*, 2004). These mechanisms could have negated the enzyme effect at higher levels of application. Beauchemin *et al.* (1995) also observed improved fibre digestibility in cattle fed intermediate levels of XYL and cellulase but not at high levels of inclusion.

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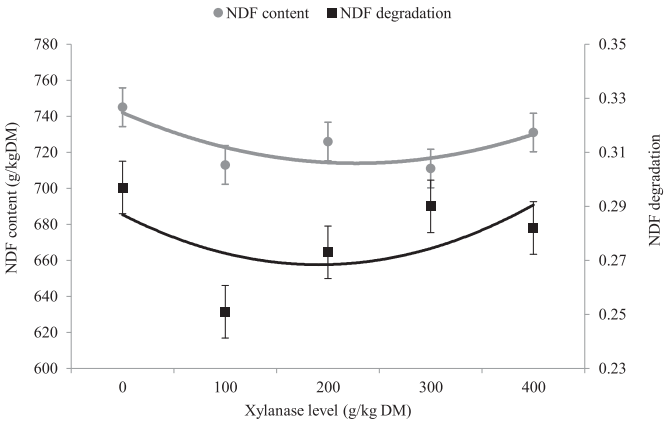

Fig. 1. Effect of XYL level on sugarcane silage NDF and degradation (mean ± SEM).

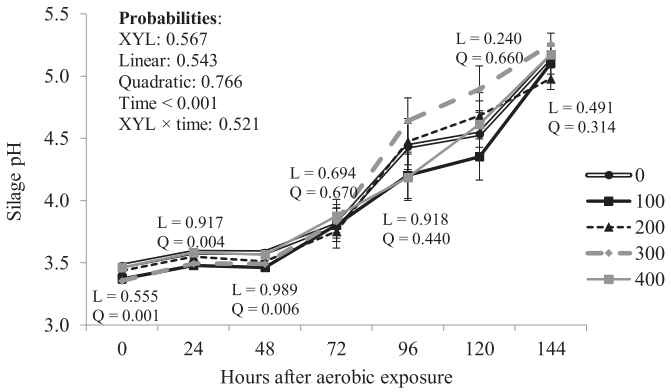

Fig. 2. Effect of XYL level on sugarcane silage pH after aerobic exposure (mean ± SEM).

Table 5. Effect of XYL level on sugarcane silage aerobic stability

	XYL level						P^a			
Item	0	100	200	300	400	SEM	Lin	Qua	Time	INT
Time of stability (h)	49	59	46	48	50	3.0	0.658	0.925		
pH	4.08	3.97	4.05	4.14	4.07	0.032	0.543	0.766	<0.001	0.521
Temperature (°C)	4.7	5.0	5.5	5.2	4.8	0.20	0.811	0.233	<0.001	0.752

[a]Probabilities: Lin, linear; Qua, quadratic; INT, treatment and time interaction effect.

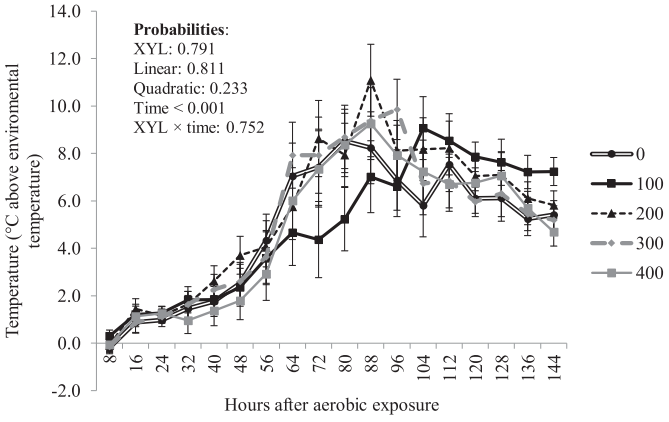

Fig. 3. Effect of XYL enzyme level on sugarcane silage temperature after aerobic exposure (mean ± SEM).

In the current study, intermediate levels of XYL increased acetic acid and decreased silage pH. Increased acetic acid could be associated with degradation of xylan (Fred *et al.*, 1919; Dehghani *et al.*, 2012), with a consequent decrease in NDF content. However, enzyme-induced release of polysaccharides available for microbial fermentation increased acid production (Eun and Beauchemin, 2008), resulting in decreased silage pH (Khota *et al.*, 2016). Similar reduction in pH was obtained when fibrolytic enzyme was applied to maize silage (Sheperd and Kung, 1996; Colombatto *et al.*, 2004), bermudagrass (Dean *et al.*, 2005) and alfalfa silages (Nadeau *et al.*, 2000).

Although it was hypothesized that increased acetic acid could prevent the growth of spoilage microorganisms (Daniel *et al.*, 2015), no effects were observed in ethanol concentration, and XYL decreased lactic acid concentration linearly in the silage. Other studies reported positive effects (Dehghani *et al.*, 2012; Ying *et al.*, 2017) or no effect (Sheperd and Kung, 1996; Mandebvu *et al.*, 1999) of fibrolytic enzymes in maize and grass silages. Differences between cited studies and the current work could be explained by the ensiled material. Higher levels of water-soluble carbohydrates, at intermediary enzyme levels could increase fermentative losses, following rapid fermentation (McDonald *et al.*, 1991).

According to Kung *et al.* (2007), rapid lactic acid production and silage pH decline results in a favourable environment for the proliferation of yeasts, increasing ethanol production and EL. In the current study, XYL had no effect on ethanol concentration; however, there was a quadratic effect of XYL on effluent and total losses, probably due to higher carbohydrate solubilization and fermentation by yeast. Dean *et al.* (2005) reported decreased DM losses on bermudagrass silage treated with a specific type of fibrolytic enzyme. However, it is important to highlight that increased acid production and decreased pH generally results in increased fermentative losses in sugarcane silage (Pedroso *et al.*, 2005).

When applied before feeding, XYL enzyme could alter the structure of the feed, making it more favourable to degradation (Beauchemin *et al.*, 1995). XYLs and cellulases have been found to be effective in degrading cell wall carbohydrates in forage and, therefore, reducing NDF content in silage (Sheperd and Kung, 1996; Colombatto *et al.*, 2004; Desta *et al.*, 2016; Khota *et al.*, 2016; Liu *et al.*, 2016). In the current study, intermediate levels of XYL decreased NDF content and degradation. Enzymes act on the most digestible content of the NDF (xylan), resulting in a silage with lower NDF, similar content of indigestible fraction, and proportionally higher indigestible fraction.

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Therefore, no improvement in nutritive value of silage was observed with XYL supply. Besides treatment effects on NDF silage concentration, contents of NDF and CP were higher in silage than in fresh sugarcane. This increased concentration is a consequence of NFC losses, increasing fibrous components of silage.

Aerobic deterioration of silage is a complex process that depends on many factors. During aerobic exposure, acids and other substrates are oxidized by aerobic bacteria, yeasts and moulds (McDonald *et al.*, 1991). Ying *et al.* (2017) reported increased aerobic stability in maize silage treated with fibrolytic enzyme and associated the increase in acetic acid concentration with improvements in aerobic stability. Danner *et al.* (2003) reported that the existence of acetic acid was able to inhibit the growth of yeasts and moulds to improve aerobic stability. Therefore, despite increased acetic acid concentration, XYL showed no effect on aerobic stability in the current study. Evaluating silage pH after aerobic exposure, it was observed that the quadratic effect found at silo opening was still apparent at 24 and 48 h after aerobic exposure, without major physiological and practical implications.

Conclusion

The use of fibrolytic enzymes in sugarcane silage alters fermentative profile and chemical composition, without affecting aerobic stability. Contrary to expectations, intermediate levels of XYL (around 200 mg/kg) decreases NDF degradation, despite reducing NDF content.

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Conflict of interest. The authors declare that there are no conflicts of interest.

Ethical standards. Not applicable.

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