FULL ARTICLE

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Chitosan level effects on fermentation profile and chemical composition of sugarcane silage

Efeitos de níveis de quitosana sobre o perfil fermentativo e a composição química da silagem de cana-de-açúcar

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ABSTRACT

This study aimed to evaluate the effects of increasing levels of chitosan (CHI) on sugarcane fermentation profile and losses, chemical composition, and *in situ* degradation. Treatments were: 0, 1, 2, 4, and 8 g of CHI/kg of dry matter (DM). Twenty experimental silos (PVC tubing with diameter 28 cm and height 25 cm) were used. Sand (2 kg) was placed at the bottom of each silo to evaluate effluent losses, and silos were weighed 60 d after ensiling to calculate gas losses. Samples were collected from the center of the silo mass to evaluate silage chemical composition, *in situ* degradation, fermentation profile, and mold and yeast count. Data were analyzed as a completely randomized design, and the treatment effect was decomposed using polynomial regression. Chitosan linearly increased acetic acid and NH_3-N concentration, while yeast and mold count, and ethanol concentration decreased. Intermediary levels of CHI (from 4.47 to 6.34 g/kg DM) showed the lower values of effluent, gas, and total losses. There was a quadratic effect of CHI on the content of non-fiber carbohydrates, neutral and acid detergent, and *in situ* DM degradation. The lowest fiber content was observed with levels between 7.01 and 7.47 g/kg DM, whereas the highest non-fiber carbohydrate content and *in situ* DM degradation were found with 6.30 and 7.17 g/kg DM of CHI, respectively. Chitosan linearly increased acetic acid and NH_3-N concentration, whereas it linearly reduced ethanol concentration and count of yeast and mold. Thus, intermediary levels of CHI, between 4.47 and 7.47 g/kg of DM, decrease fermentation losses and improve the nutritional value of sugarcane silage.

Keywords: Acetic acid. Chitin. Degradation. Ethanol. Neutral detergent fiber.

RESUMO

Foram avaliados os efeitos do aumento dos níveis de quitosana (CHI) sobre o perfil e as perdas fermentativas, a composição química e degradação *in situ* da silagem de cana-de-açúcar. Os tratamentos foram: 0, 1, 2, 4 e 8 g de CHI / kg de matéria seca (MS). Foram utilizados vinte silos experimentais (tubos de PVC com 28 cm de diâmetro e 25 cm de altura). Areia (2 kg) foi adicionada na porção inferior de cada silo para avaliar as perdas por efluentes e os silos foram pesados 60 dias após a ensilagem para calcular as perdas por gases. Amostras foram coletadas do centro da massa do silo para avaliar a composição química, degradação *in situ*, perfil fermentativo e a contagem de fungos e leveduras da silagem. Os dados foram analisados como um delineamento inteiramente casualizado e o efeito do tratamento foi decomposto usando regressão polinomial. A CHI aumentou linearmente a concentração de ácido acético e N-NH_3, enquanto diminuiu a contagem de leveduras e bolores e a concentração de etanol. Os níveis intermediários de CHI (de 4,47 a 6,34 g/kg MS) mostraram os menores valores de perdas por efluentes, gases e totais. Houve efeito quadrático da CHI sobre o teor de carboidratos não fibrosos, fibra em detergente neutro e ácido e sobre a degradação *in situ* da MS. Os menores teores de fibras foram observados com níveis de CHI entre 7,01 e 7,47 g/kg MS, enquanto que os maiores teores de carboidratos não fibrosos e degradação *in situ* da MS foram encontrados com 6,30 e 7,17 g/kg MS de CHI, repectivamente. A CHI aumentou linearmente as concentrações de ácido acético e

$N\text{-}NH_3$, enquanto reduziu linearmente a concentração de etanol e a contagem de fungos e leveduras. Desta forma, níveis intermediários de CHI, entre 4,47 e 7,47 g / kg de MS, diminuem as perdas fermentativas e melhoram o valor nutricional da silagem de cana-de-açúcar.

Palavras-chave: Ácido acético. Quitina. Degradação. Etanol. Fibra em detergente neutro.

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Introduction

Sugarcane (*Saccharum officinarum L.*) ensiling results in high dry matter (DM) losses due to fermentation of sucrose by yeasts (Daniel et al., 2015). Yeast population converts the water-soluble carbohydrates in fermentable end-products, which are characterized by volatile organic compounds, mainly ethanol (Ávila et al., 2010). Furthermore, the high ethanol production and DM losses enhance fibrous component content and compromise the nutritional value of silage (Muck et al., 2018). Studies evaluating the fermentation process and use of microbial inoculants (Santos et al., 2015), calcium oxide (Jacovaci et al., 2017) and chitosan (CHI) (Gandra et al., 2016; Del Valle et al., 2018) reported improved fermentation pattern and nutritional value of sugarcane silage.

Among the strategies used to manipulate the sugarcane silage fermentation process, the addition of CHI could inhibit undesirable fermentation (Del Valle et al., 2018). Chitosan is a polymer obtained from chitin, which composes the exoskeleton of crustaceans and insects, and has antimicrobial activity against fungi and bacteria (Tachaboonyakiat, 2017). Del Valle et al. (2018) reported increased DM recovery, lower ethanol production, and total losses, and improved *in situ* DM degradation of sugarcane silage treated with CHI. Furthermore, CHI improved *in vitro* neutral detergent fiber (NDF) degradation and chemical composition (Gandra et al., 2016) of the sugarcane silage.

Although there is a positive effect of CHI on sugarcane conservation, to the best of our knowledge, there is no study evaluating the effects of different levels of CHI. Paiva et al. (2017) studied increasing levels of CHI in the diet of lactating dairy cows (up to almost 7.3 g/kg DM) and reported a linear increase in milk yield and crude protein (CP) digestibility. In other recent studies, Del Valle et al. (2018) evaluated 6 g of CHI/kg of DM, whereas Gandra et al. (2016) used 10 g/kg as fed (36 g/kg DM) as an additive in sugarcane silage. In this context, we hypothesized that increasing levels of CHI linearly increase DM recovery, non-fiber carbohydrates and DM degradation of sugarcane silage. The aim of this study was to evaluate the effects of CHI levels on sugarcane silage parameters such as fermentation profile and losses, microbiology analyses, nutritional composition, and *in situ* DM and NDF degradation.

Material and Methods

The experiment was conducted at the Agrarian Sciences Center (CCA) of the Federal University of São Carlos (UFSCar) in Araras, Brazil. The area is located at 22°18' 32" S latitude, 47° 22' 52" W longitude and 665 m altitude. The local climate is classified as subtropical humid.

Sugarcane crops (variety RB83-5054) with approximately eight months of growth (first cut) from four different fields were manually harvested and chopped in a forage harvester (90 z-10, JF, Itapira, Brazil). Average silage composition was: 231 g/kg DM; 961 g/kg organic matter, 561 g/kg NDF, 359 g/kg NFC, 336 g/kg ADF, 30.7 g/kg CP, 11.4 g/kg EE, and 175 g/kg Brix. The trial was performed in a completely randomized design with six treatments replicated four times. Treatments were: 0, 1, 2, 4, and 8 g of CHI/kg of DM. Chitosan was obtained from Fragon (São Paulo, Brazil) and contained 0.697 of density, pH 10.1, and concentration of heavy metals lower than 1 mg/kg, besides the absence of countable fungi, yeasts, and bacteria.

Silos were made of PVC tubing (diameter 28 cm and height 25 cm) and equipped with *Bunsen* valves to avoid gas penetration and allow gas to escape (Del Valle et al., 2018). Sand (2 kg) was placed at the bottom of each silo, separated from forage by a nylon screen to determine effluent losses. Sugarcane was compacted (around 600 kg/m^3), sealed, weighed, and stored at room temperature for 60 d.

Silos were weighed before opening to calculate gas loss. After opening, the top layer of silage (5-cm) was

discarded. Silage was removed from the silos and one sample (300 g) was collected after homogenization. One subsample (100 g) was frozen for chemical composition and *in situ* degradation analysis. Another subsample (15 g) was diluted with 150 mL of distilled water and processed in a blender for 30 sec (Yan et al., 2019). These samples were then filtered through four layers of cheesecloth, and pH was immediately measured (LUCA-210, Lucadema, São José do Rio Preto, Brazil). Filtered samples were frozen for further evaluation of NH_3-N, organic acids, and ethanol.

Silage extracts were thawed at room temperature and centrifuged (500 × g for 15 min). For NH_3-N, one subsample (2 mL) was mixed with sulfuric acid (1 mL 1 N) and analyzed by colorimetric phenol-hypochlorite method. One fluid sample (100 µL) was used to determine lactic acid concentration using spectrophotometric method (Pryce, 1969). The analyses of organic acids and ethanol concentration were performed as described by Del Valle et al. (2018). The samples were acidified using formic acid at a 1:4 ratio and the concentrations of ethanol, acetic, propionic and butyric acids were determined using a gas chromatograph (GC-2010 Plus Chromatograph, Shimadzu, Barueri, Brazil) equipped with AOC-20i auto-sampler, Stabilwax-DA™ capillary column (30 m, 0.25 mm ID, 0.25 µm *df*; Restek©), and a flame ionization. Temperatures of injector and detector were 250 and 300^0 C, respectively. Helium was used as a carrier gas with a linear velocity of 42 cm/s, in a chromatographic run of 11.5 min. Peak detection and integration were made using the GC solution v. 2.42.00 software (Shimadzu©).

Mold and yeast count was performed according to American Health Association (2001). Each sample (10 g, as fed) was mixed with 90 mL of sterilized peptone water (1%, w/v) and different dilution ratios were plated on dichloran rose bengal chloramphenicol agar, and plates were incubated at 28 °C for 6 d. Water activity (WA) was evaluated using a benchtop water activity meter (Aqualab 4T, Decagon Devices Inc., Pullman, WA, USA), at 25 °C.

Samples were dried at 60^0 C in a forced air oven for 72 h and ground in a knife mill (SL-31, Solab Científica, Piracicaba, Brazil) with a 1-mm screen. Dry matter (method 950.15), ash (method 942.5), crude protein (CP, N × 6.25; method 984.13), ether extract (EE; method 920.39), and acid detergent fiber (ADF; method 973.18) analyses were performed according to Association of Official Analytical Chemists (2000). The NDF content was analyzed using α-amylase without the addition of sodium sulfite (Van Soest et al., 1991). Non-fiber carbohydrates were calculated as 1000 - (NDF + ash + CP). Two cannulated dairy cows, previously adapted to a diet with forage-to-concentrate ratio of 60:40 (DM basis), were used for *in situ* degradation assay. Samples (about 500 mg of the sample, processed in a 2-mm screen knife mill) were placed in non-woven fabric bags (TNT; 5 × 5 cm and 100 g/m^2; Casali et al., 2008) and incubated for 96 h (Del Valle et al., 2018). After removal, bags were washed in running water and were analyzed for DM and NDF content as previously described.

Gas losses (GL) were calculated by the difference between silo weight at ensiling (ESW) and at the opening (OSW), according to the following equation:

$$GL\left(\frac{g}{kg}\right)=\frac{ESW\,(g)-OSW\,(g)}{EDM\,(kg)}$$

Where EDM is the ensiled dry matter. The difference between empty silo weight before ensiling (EESW) and after opening (OESW) was considered effluent losses:

$$EL\left(\frac{g}{kg}\right)=\frac{OESW\,(g)-EESW\,(g)}{EDM\,(kg)}$$

Total DM losses were obtained by the sum of gas and effluent production as performed by Del Valle et al. (2019). Dry matter recovery (DMR) was calculated according to the equation:

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

Where, the ratio between DM at silos after opening (ODM, kg) and ensiled dry matter.

Data were analyzed by SAS 9.3 (SAS Inst. Inc., Cary, NC), according to the following model:

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

With $e_{ij}\approx N\left(0,\sigma_i^2\right)$, where Y_{ij} is the value of the dependent variable; µ is the overall mean; C_i is the fixed effect of chitosan level (i = 1 to 5); e_{ij} is the residual error; N stands Gaussian distribution. Chitosan level effects were studied using polynomial regression to evaluate the following effects: 1) linear, 2) quadratic, and 3) cubic effect of CHI level. Equations that describe the effect of CHI level were obtained using the solution function of PROC MIXED. As CHI levels were non-equidistant, orthogonal contrasts were obtained using PROC IML of SAS.

Results and Discussion

Increasing doses of CHI linearly increased (P = 0.01; Table 1) acetic acid concentration and did not affect (P = 0.33) silage pH. Del Valle et al. (2018) reported a positive effect of chitosan on sugarcane silage pH and related this effect with decreased fermentation extension. Controversially, in the present study, there was no CHI effect (P = 0.13) on lactic acid concentration. As lactic acid is a stronger acid than acetic (pKa 3.86 *vs.* 4.76; Muck, 2010), besides increased

Table 1. Fermentation profile of sugarcane silage treated with increasing levels of chitosan

Item	Treatments[a]					SEM	Probabilities[b]			
	CON	C1	C2	C4	C8		Treat.	Linear	Quad.	Cub.
Fermentative profile										
pH	3.75	3.72	3.69	3.69	3.74	0.010	0.33	0.94	0.14	0.72
NH_3-N, mg/dL	1.29	1.65	1.94	3.74	4.17	0.077	<0.01	<0.01	0.14	0.12
Ethanol, g/kg DM	23.3	28.8	14.8	16.7	14.8	0.94	0.02	<0.01	0.11	0.76
Acetic, g/kg DM	21.6	27.8	24.2	26.9	26.6	0.45	0.04	0.01	0.12	0.36
Lactic, g/kg DM	16.8	14.7	18.5	17.3	15.9	0.33	0.13	0.85	0.13	0.54
Propionic, mg/kg DM	393	385	381	361	320	36.8	0.99	0.56	0.95	0.99
Butyrate, mg/kg DM	146	143	139	134	148	2.4	0.41	0.89	0.19	0.53
Water activity	0.958	0.965	0.974	0.968	0.961	0.0035	0.60	0.94	0.21	0.51
Yeast and mold[c]	3.88	3.07	3.96	2.15	2.56	0.231	0.08	0.02	0.27	0.36

[a]CON: control, without additives; C1, C2, C4, and C8: sugarcane silage with 1, 2, 4 and 8 g of chitosan per kg of silage DM; [b]Probabilities: linear, quadratic and cubic effect of chitosan level; [c]log CFU/g as fed.

acetic acid concentration, absence of CHI effect on lactic acid resulted in no impact on silage pH. Moreover, increased level of acetic acid was also reported by Gandra et al. (2016) and Del Valle et al. (2018). It was previously suggested that metal linked CHI could act as an electron acceptor (Goy et al., 2009) and improves lactic acid conversion to acetic acid, by a similar mechanism found in heterolactic bacteria (Rabelo et al., 2019).

Acetic acid is the major organic acid associated with growth inhibition of spoilage microorganisms in silage (Danner et al., 2003). In the present study, the increasing levels of CHI linearly decreased (P = 0.02) yeast and mold count. The antifungal effect of CHI is related to the capacity of suppressing sporulation and spore germination (Hernandez-Lauzardo et al., 2008), and perhaps even higher in sugarcane silages compared to other crops because CHI antifungal activity is increased at lower pH values (Kong et al., 2010). Gandra et al. (2016) also reported decreased aerobic bacteria and fungi on sugarcane silage treated with CHI. As yeasts are essentially ethanol producers microorganisms (Abrão et al., 2017), increasing levels of CHI linearly reduced (P <0.01) ethanol concentration in the silage. Also, CHI linearly increased (P < 0.01) NH_3-N silage concentration. Increased NH_3-N level could be a result of N present in chitosan, which is mainly converted to soluble protonated form when environmental pH is below that of chitosan pKa (6.3) (Goy et al., 2009).

The addition of CHI showed a quadratic decrease ($P \leq 0.02$) in fermentation losses (Table 2). Decreased fermentation losses observed with intermediary levels of CHI seems associated with reduced ethanol production. However, a higher level of CHI results in a low improvement in fermentation losses, with losses in C8-treated silages higher than that observed in C4-treated ones. Therefore, regressions allowed us to estimate the lowest fermentative losses with CHI level between 4.47 and 6.34 g/kg DM (Table 3). In a previous study from our research group, Del Valle et al. (2018) used 6 g/kg DM based on a pilot study. We agree that levels higher than those evaluated in the present study have a minimal additional effect on fermentation losses and could reduce the technical and financial feasibility of this additive. Besides the quadratic effect of CHI on fermentation losses, DM recovery linearly increased with increasing levels of CHI (P = 0.01). This effect is associated with a linear increase (P = 0.02) in DM content of silage, which changes the inflection point of the curve out of the rated range. Increased DM content could be a consequence of lower silage ethanol concentration. According to McDonald et al. (1991), yeast largely ferments sugar causing 49% loss of substrate as CO_2 and water. As silo drainage is not always perfect, silage with higher ethanol production generally shows decreased DM content.

The NDF and ADF contents were lower, whereas DM degradation was higher with intermediary levels of CHI, resulting in a quadratic effect ($P \leq 0.04$) on these variables (Table 4). The lowest NDF and ADF contents were found using 7.01 and 7.47 g of CHI/kg DM, respectively (Table 3). The highest level of NFC and DM degradation were found with 6.30 and 7.17 g of CHI/kg DM, respectively. The lower fermentation losses had improved NDF and ADF content on silage treated with CHI. According to Del Valle et al. (2018), CHI decreased fiber concentration of silage, with a positive effect on NFC content and DM degradation. Moreover, lower fiber content resulted from an inhibition of yeasts by CHI, which decreased effluent losses (Lopes & Evangelista, 2010), increased DM recovery, and improved DM degradation. Higher DM recovery and increases in NDF degradation on sugarcane treated for CHI were reported by Gandra et al. (2016).

Table 2. Fermentation losses of sugarcane silage treated with increasing levels of chitosan

Item	Treatments[a]					SEM	Probabilities[b]			
	CON	C1	C2	C4	C8		Treat.	Linear	Quad.	Cub.
Fermentative losses, g/kg DM										
Effluent	163	169	160	130	153	5.0	<0.01	0.01	<0.01	0.07
Gas	138	144	132	107	105	1.6	<0.01	<0.01	0.02	0.06
Total	308	310	292	238	258	5.5	<0.01	0.01	<0.01	0.06
DM recovery	760	723	768	797	803	5.6	0.09	0.01	0.41	0.09

[a]CON: control, without additives; C1, C2, C4, and C8: sugarcane silage with 1, 2, 4 and 8 g of chitosan per kg of silage DM; [b]Probabilities: linear, quadratic and cubic effect of chitosan level.

Table 3. Regression coefficients and quadratic maximum or minimum for variables with linear and quadratic effects of chitosan

Item	Intercept	SE	Linear coefficient	SE	Quadratic coeficient	SE	Quadratic Max./min[a]
NH_3-N, mg/dL	1.27	0.065	0.362	0.0187			
Ethanol, g/kg DM	22.4	2.36	- 0.963	0.3065			
Acetic acid, g/kg DM	24.4	0.957	0.362	0.232			
Yeast and mold, CFU/g	3.66	0.460	- 0.146	0.0673			
Effluent losses, g/kg DM	165	4.62	- 15.0	2.41	1.68	0.281	4.47
Gas losses, g/kg DM	161	8.43	-18.8	3.96	1.49	0.375	6.34
Total losses, g/kg DM	305	7.49	-27.6	2.98	2.71	0.304	5.10
Dry matter recovery, g/kg	756	7.15	6.21	2.227			
Dry matter (DM), g/kg as-fed	188	1.66	1.23	0.528			
Neutral detergent fiber, g/kg DM	766	14.2	-51.9	8.19	3.70	1.079	7.01
Acid detergent fiber, g/kg DM	444	8.83	-27.2	4.93	1.82	0.709	7.47
Non-fiber carbohydrate, g/kg DM	161	13.4	56.4	7.66	-4.48	0.879	6.30
DM degradation, g/kg	494	10.7	30.8	4.54	-2.15	0.494	7.17

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

Table 4. Chemical composition and *in situ* degradation of sugarcane silage treated with increasing levels of chitosan

Item	Treatments[a]					SEM	Probabilities[b]			
	CON	C1	C2	C4	C8		Treat.	Linear	Quad.	Cub.
Chemical composition, g/kg DM										
Dry matter, g/kg as fed	189	183	190	195	197	1.3	0.09	0.02	0.68	0.21
Neutral detergent fiber	769	720	673	620	585	6.5	<0.01	<0.01	0.01	0.75
Acid detergent fiber	444	418	397	364	343	4.0	0.01	0.01	0.04	0.99
Non-fiber carbohydrate	155	211	264	310	328	5.7	<0.01	<0.01	<0.01	0.47
Crude protein	36.0	28.2	25.0	31.9	28.4	0.92	0.09	0.25	0.18	0.03
Ether extract	9.98	11.0	7.77	8.60	8.90	0.764	0.77	0.62	0.47	0.99
In situ degradation, g/kg										
Dry matter	498	515	535	583	602	5.0	<0.01	<0.01	0.02	0.35
Neutral detergent fiber	347	326	309	327	318	5.5	0.45	0.37	0.30	0.12

[a]CON: control, without additives; C1, C2, C4, and C8: sugarcane silage with 1, 2, 4 and 8 g of chitosan per kg of silage DMlant; [b]Probabilities: linear, quadratic and cubic effect of chitosan level.

Conclusions

Chitosan linearly increased acetic acid concentration, reduced ethanol production, and improved silage DM recovery. Furthermore, lower fermentation losses were observed using 4.47 to 6.34 g of CHI/kg DM. Lastly, intermediate levels of CHI reduced fiber content (7.01 g/kg DM) and increased DM degradation (7.17 g/kg DM). Therefore, CHI levels between 4.47 e 7.47 g/kg DM are recommended for sugarcane ensiling.

Conflict of Interest Statement

The authors declare no conflict of interests in the current manuscript.

Ethics Statement

The procedures were approved by the Animals Ethics committee of UFSCar (protocol number: 5665301117).

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