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Lactobacillus plantarum LPBR01 as inoculant in sugarcane silage

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ABSTRACT. Sugar cane is highly productive (dry matter.hectare^{-1}), but after ensiling process nutritional quality is affected, thus additives are needed to control or minimize losses. This study aimed to evaluate if *Lactobacillus plantarum* LPBR01 strain used as silage inoculant for sugar cane can control fermentation losses. Sugar cane samples (72) were divided in two treatments with three replicates, control (no *Lactobacillus*) and treatment silage with *Lactobacillus* (10^6 CFU g^{-1} of silage). Nutritional composition of samples in different periods of fermentation (0, 7, 15, 30 and 45 days) was estimated by determining levels of dry matter (DM), crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), hemicellulose (HEM), mineral matter (MM) and acid detergent lignin (ADL). Fermentative profile of the silage was characterized by determining sugars, ammoniacal nitrogen, acidity and pH at 0, 12, 24, 36, 48, 60 and 72 hours. Inoculation of sugar cane silage with *Lactobacillus plantarum* LPBR01 strain presented no significant results ($p \leqslant 0, 5$) however, interaction between treatment and day ($p \leqslant 0, 5$) could be observed for the levels of ADF. The *Lactobacillus plantarum* LPBR01 strain was not efficient to control the fermentation losses that occur in the silages of sugar cane at the concentration used in this study.

Keyword: Fermentation; microbiological inoculant; bromatology.

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Introduction

Sugar cane (*Saccharum officinarum*) cultivation in Brazil has been recently expanded mainly due to high yield per area. Production varies according to fertilization, but can reach over 140 tons of green matter per hectare (Ambrosano et al., 2011). Thus, it has become an alternative for animal nutrition and when processed as silage, it can provide a more convenient source.

Silage production is an alternative to improve daily work, but sugar cane is harvested and preserved when it reaches its physiological maturity, which is the stage when the content of saccharose is higher (Carvalho et al., 2015). The main limitation for sugar cane silage production is the production of ethanol (Santos et al., 2008), which occurs due to the high content of soluble carbohydrates, resulting in a fast proliferation of yeasts, because these microorganisms metabolize carbohydrates by fermentation producing ethanol when in anaerobiosis (Mendes et al., 2008). The production of ethanol causes loss of dry matter and energy, thus the use of inoculants to minimize these losses can be an alternative (Schmidt et al., 2011).

Lactobacillus plantarum are microorganisms compatible with several substrates, as well as sugar cane. These microorganisms are ideal to be used as inoculants because of their vigorous growth, the ability to compete and dominate other microorganisms, homofermentative, acid tolerant and continuous growth throughout a fermentation process (Valeriano et al., 2009).

The strain of *Lactobacillus plantarum* LBPR01, chosen for this study, was isolated from a coffee husk silage and showed great potential as a starter for fermentation and acidification, reducing the initial time of the process from 24 hours to 12 hours with a high production of lactic acid and low production of butyric, acetic and propionic acids (Pereira et al., 2016). This property can influence the development of yeast, decreasing the production of ethanol, resulting in minor losses of dry matter and energy.

Therefore, this work evaluates the *Lactobacillus plantarum* LPBR01 strain as inoculant for silage of sugar cane.

Material and methods

The present study was carried out between May and September 2017, at Universidade Tecnológica Federal do Paraná – Campus Dois Vizinhos (UTFPR-DV), silages of sugar cane supplied by a farmer of the town of Boa Esperança do Iguaçu-PR, located in a region of 521 meters of altitude, classified as humid subtropical climate, mesothermal with no defined dry season, according to the Köppen classification, with average temperatures of 22°C (Alvares et al., 2013).

The strain of *Lactobacillus plantarum* LPBR01, was supplied lyophilized by the Biological Process Studies Group of UFPR, Curitiba, isolated from coffee husks silage. The microorganisms were activated in culture medium based on molasses and yeast extract.

Harvest and grinding of sugar cane were fully mechanized, with a particle size of approximately 3 cm^{-1}. Immediately after the cut, material was stored in plastic bags and was inoculated with *Lactobacillus plantarum* (10^6 CFU g^{-1}) through sprinkled individually in each microsilo.

Plastic bags of 12 microns were used to assemble the 72 (12 times of fermentation * triplicate * two treatments) micro silos. Bags were inserted into 4 L^{-1} beckers, with the edges of the bag out and a flat base wood that would compress 1.4 kg of the sample resulting in 550 kg m^{-3}. Later, microsilos were placed in dark plastic bags, and as fermentation time or day was reached (0, 12, 24, 36, 48, 60 and 72 hours or 0, 7, 15, 30 and 45 days) material was frozen at -20°C until analysis.

Nutritional composition analyzes were performed in samples of 0, 7, 15, 30 and 45 days. Samples were thawed at room temperature, 5 cm from the edge was discarded, and the remaining, homogenized and a sampled (sub-sample) for analysis. The sub-samples were dried in a forced ventilation oven (55ºC) for 72 hours, milled in Willey mills with a 1 mm sieve.

Dry matter (DM) was determined by drying in a Pasteur oven at 105ºC for 16 hours, ashes by burning at 600ºC for 4 hours (Method 942.05, Association Official Analytical Chemist [AOAC], 2005) and the crude protein (CP) was estimated indirectly from total nitrogen (N) determined by Kjeldahl method (Method 984.13, AOAC (2005).

Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined according Van Soest (1965), adapted by Senger et al. (2008), using 16 microns polyester bags and 40 minutes of autoclave at 110°C. Hemicellulose was calculated from the difference between NDF and ADF content. Concentration of acid detergent lignin (ADL) was determined with ADF residue treated with 72% sulfuric acid (Method 973.18; AOAC (2005).

Samples with fermentation time of 0, 12, 24, 36, 48, 60 and 72 hours were analysed for sugars, acidity and pH. Titratable acidity and pH were determined with 25 g^{-1} samples processed with approximately 150 mL^{-1} of distilled water in an industrial blender for one minute, then distilled water was added until 250 mL^{-1} was reached. These samples were filtered, and the pH of the aqueous extract was analyzed with the use of a pH meter (Van Soest, Robertson, & Lewis, 1991).

Acidity, expressed in percentage of lactic acid in DM was performed according to the method described by AOAC (2005). Sugars were estimated by DNS (3.5 dinitro-salicylic acid) method (Miller, 1959). Ammoniacal nitrogen was determined adding 200 mL^{-1} of H_2SO^4 in 25 g^{-1} of silage, stored 48 hours under refrigeration and then filtered and frozen (Zanine, Santos, Ferreira, Pinto, & Pereira, 2007) according to the methodology by (Weatherburn, 1967).

Data obtained was submitted to analysis of variance and when significant ($p \leqslant 0.05$) averages were compared by the Tukey test ($p \leqslant 0.05$), and when relevant, regression analysis was performed using SAS software, version 9.4 (SAS Institute, Cary, NC).

Results and discussion

Treatment didn't result in differences at the pH ($p > 0.05$), as well as there was no interaction between fermentation time and treatment (Figure 1).

According Muck (2010) in order to lower the metabolism of yeasts, *Enterobacter* and *Clostridium*, pH must decrease as fast as possible. After 72 hours of fermentation, both treatments showed a similar pH. However, according to França et al. (2011), both would be a suitable pH for forage quality preservation, and values above five are indicative of a low quality.

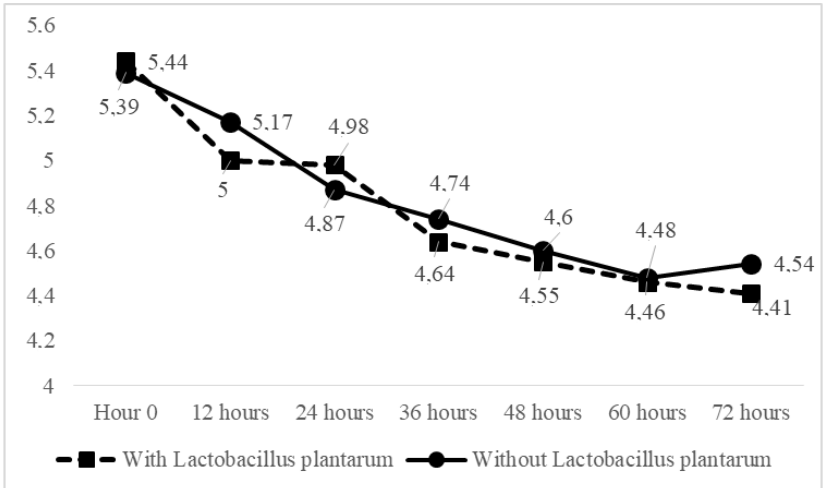

Figure 1. pH values found in sugarcane silage inoculated with *Lactobacillus plantarum* LPBR01 and the control.

When a silage presents pH values above five, it is an indicative that bacteria of the genus *Clostridium* has been developed and conservation is impaired. However, the production of lactic acid and the increase of pH, reduces the nutritional value as a result of the catabolism of amino acids and production of undesirable substances from secondary fermentation, such as the butyric acid (Castro et al., 2006).

Sugar cane has high sugar content and low buffering power, resulting in ideal conditions for a fast development of lactic acid bacterium and low pH. Van Soest (1994) mentions that pH stabilization depends on the production of lactic acid bacterium, as well as the amount of organic acids. The decrease of pH happens according to the increase in acidity that occurred as a function of fermentation time (Figure 2). The variable lactic acid was submitted to regression analysis, which can be described by the equation y= 0.0025+0.001751x (R^2 0.92; $p > 0.05$), showing an increase of acidity as a function of time, as was already expected (Figure 2).

Lactobacillus plantarum is an optional heterofermentative and the analyzes of acidity, was not enough to define if its metabolism is heterofermentative or homofermentative when inoculated in sugar cane. However, the control treatment also presented a small variation in the levels of lactic acid, possibly because silage samples were not sterile containing a natural bacterium load, with several genres and metabolisms.

The values found may not correspond to the total production of lactic acid during the fermentation time because some of it, although more frequent in aerobic conditions, can be used by bacterium and yeasts producing ethanol and organic acids (Schmidt et al., 2011).

Sugars can be used for fermentation by both bacterium and yeast producing ethanol, which reduces the availability of sugars as a function of time (Figure 3). The values found showed no statistically significant difference ($p > 0.05$).

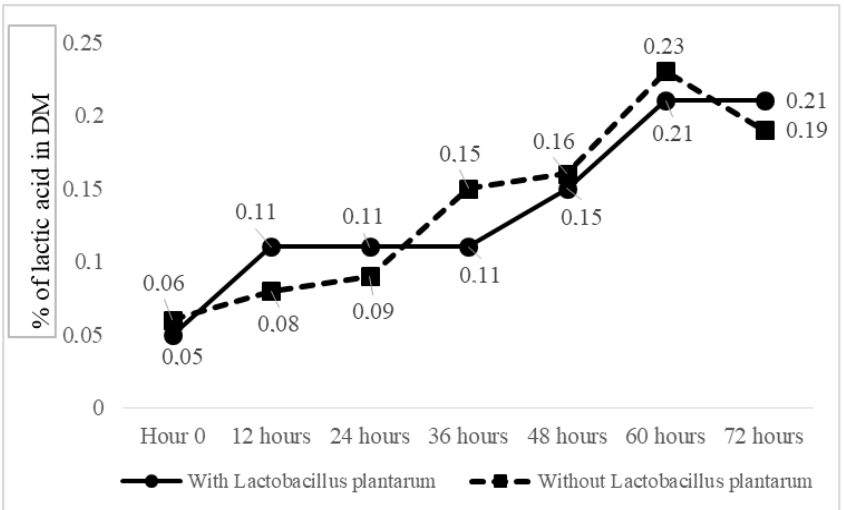

Figure 2. Lactic acid found in DM of sugar cane silage inoculated with *Lactobacillus plantarum* LPBR01 and the control treatment.

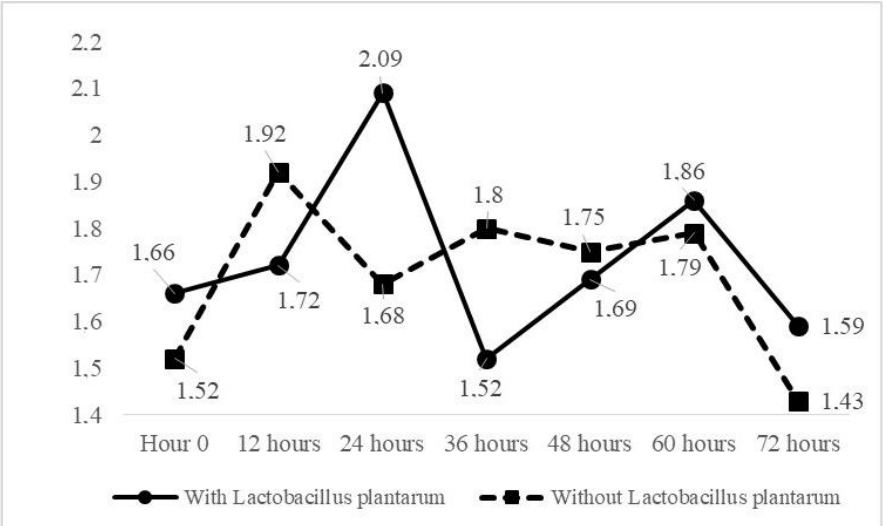

Figure 3. Sugars found in DM of sugarcane silage inoculated with *Lactobacillus plantarum* LPBR01 and the control treatment.

The rapid decrease of pH and the gradual increase of lactic acid indicate an increase of lactic acid bacterium and, thus these sugars (glucose and fructose) were available, then microorganisms metabolized them (Santos, Prado, Pertierra, & Palacios, 2018).

The variation in the sugar levels can be justified by the high activity of the bacterium, which use sugars, but also release molecules of carbohydrates. The inoculants are used primarily by interfering in factors such as pH and acidity to minimize losses of the nutritional compounds, which are important for animal feed.

In this study the nutritional composition showed no significant statistical difference ($p \leqslant 0.05$) when comparing the treatment with the inoculation *Lactobacillus plantarum* LPBR01 and the control treatment, but there was a difference according to the days of fermentation, as shown in Table 1.

Levels of dry matter presented in Table 1 demonstrate incremental losses, mainly because of gases and effluents release. In both treatments there was loss of dry matter, which allows inferring that microorganisms were not efficient.

The levels of CP showed gradual increases according to the days of fermentation. Valeriano et al. (2009) attributed these increases, which were also found in his study with the use of *Lactobacillus plantarum* on silage of sugar cane, to the use of soluble carbohydrates by microorganisms in both treatments. According to Valeriano et al. (2009), the levels of ash are usually very low in sugar cane and these values vary greatly according to the variety of sugar cane used, fertilization and the maturity point at the time of harvest. Whereas Pedroso et al. (2007) observed that the levels of ash in the sugar cane silage increase with fermentation, which also occurred in this study, this same author implies this result to the loss of nutrients as gases and effluent during silage processing.

Hemicellulose is an important digestible fraction in ruminant feed and can be calculated by the difference between the levels of NDF and ADF. It ranged from 11.0% in DM at time 0, and 29.5% on the 7th day, and then there were decreases of its content. This may occur due to degradation by enzymes and acid hydrolysis from the plant.

Table 1. Average of the bromatological components of sugarcane silage inoculated with *Lactobacillus plantarum*.

Day	DM	CP	MM	NDF	HEM	LDA	N-NH_3
0	27.26^{a}	3.11^{a}	3.91^{b}	54.89^{b}	10.97^{b}	7.06^{b}	1.82^{a}
7	23.97^{b}	3.88^{a}	4.92^{ab}	73.91^{a}	29.47^{a}	9.43^{a}	1.65^{ab}
15	23.46^{b}	3.98^{a}	5.29^{a}	76.03^{a}	20.34^{ab}	9.32^{a}	1.44^{bc}
30	21.05^{ab}	3.82^{a}	5.54^{a}	72.47^{a}	21.05^{ab}	9.22^{ab}	1.43^{bc}
45	22.64^{b}	4.20^{a}	4.45^{ab}	74.67^{a}	18.44^{ab}	9.63^{a}	1.32^{c}
R^2	0.654	0.335	0.627	0.681	0.480	0.532	0.708
CV	6.713	18.537	11.519	6.560	35.344	11.68	58.578

DM (%): dry matter; CP (%): Crude protein; MM (%); mineral matter; NDF (%): neutral detergent fiber; HEM (%): Hemicellulose; ADL(%): Lignin and NH_3-N (%): ammoniacal nitrogen. All expressed on the basis of dry matter. Values followed by the same letter in column, did not differ among themselves, by the Tukey test at 5% probability.

The levels of lignin increased according to the days of fermentation. Lignin can form complexes with the cellulose and hemicellulose, impairing digestion and utilization of nutrients by the animal (Harbers, Brazle, Raiten, & Owensby, 1980).

The values found for loss of ammoniacal nitrogen, ranged between 1.8 and 1.3% in DM, ammoniacal nitrogen is a product of the fermentation by *Clostridium* bacteria, thus these levels indicate that the activities of these bacteria were inhibited.

Ohshima and McDonald (1978) reported that levels of ammoniacal nitrogen must be below 8% to 11%, which would indicate a good quality silage that is not in intense proteolysis. The percentages found are an indicative that an intense rate of proteolysis of the material did not take place, as well as observed in the analysis of pH, according to the method used, the values fell fast due to the low protein content of sugar cane.

The ADF analysis showed interaction between treatment and day of fermentation ($p > 0.05$), as well as a significant difference among the days of fermentation.

The ADF was lower at the end of 45 days when it was inoculated with *Lactobacillus plantarum* LPBR01 but it was not statistically different from the initial value. The values of ADF involve cellulose and lignin, which in the case of treatment with the inoculation of strain of *Lactobacillus plantarum* suggests that the bacteria maintained these levels in DM whereas the control treatment showed an alteration of this percentage in relation to the first day of fermentation until the 45th day.

The levels of NDF and ADF found were high; however, Schmidt et al. (2011), that inoculated *Lactobacillus buchneri* on sugar cane silage, obtained similar results to those found in the present study for the levels of NDF (Table 1), and ADF (Table 2), however in his work the results were not influenced by the addition of the inoculant. In addition, Pedroso et al. (2007) observed that the levels of NDF and ADF increased in dry matter, as a result of soluble carbohydrates loss as gases and effluents during silage processing.

The increases of NDF and lignin show that inoculation of *Lactobacillus plantarum* on sugar cane silage was not enough to control these increases, as well as the loss of DM. NDF is composed by cellulose, hemicellulose and lignin, and is a limiting factor of food intake by the animal.

Table 2. ADF present in DM of sugarcane silage inoculated or not with *Lactobacillus plantarum*.

Days	Treated with *L. plantarum*	Treated without *L. plantarum*
0	49.60 ^{bA}	38.24 ^{cB}
7	54.44 ^{aA}	52.17 ^{bA}
15	57.48 ^{aA}	54.67 ^{aA}
30	55.41 ^{aA}	53.17 ^{bA}
45	50.06 ^{bB}	62.86 ^{Aa}
R^2	0.679	
CV	7.801	

Values followed by the same letter letters do not differ in columns. Values followed by the same letter did not differ in the lines, by Tukey test at 5% probability.

Conclusion

The use of LPB-R01 strain of *Lactobacillus plantarum* could not control fermentation losses that occur during fermentation process of sugarcane silage, therefore it is not recommended as inoculant for this material.

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