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Effect of propionic acid and *Lactobacillus plantarum* UFLA SIL 1 on the sugarcane silage with and without calcium oxide

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Alkaline agents reduce the fiber content, but results in a buffering of the silage that can benefit the growth of undesirable micro-organisms. The association of alkaline agents and additives capable of inducing a decrease in the initial pH may be interesting. Our aim was to evaluate the effects of a wild strain of *Lactobacillus plantarum* (UFLA SIL 1) and the addition of propionic acid on the fermentation and aerobic stability of sugarcane silages treated with and without calcium oxide. Propionic acid reduced the pH value, the number of yeasts, filamentous fungi and clostridia. The *L. plantarum* reduced DM losses in silages without calcium oxide, without changing other microbiological and fermentative parameters. The addition of calcium oxide reduced the neutral detergent fiber (NDF), the concentration of ethanol and acetic acid and increased the populations of clostridia, filamentous fungi and yeasts. Calcium oxide lengthened aerobic stability and reduced the temperature during aerobic exposition. Addition of propionic acid or the strain of *L. plantarum* was not able to improve the microbiological quality of the silage treated with calcium oxide. The addition of calcium oxide improved aerobic stability; however, this addition resulted in lower silage quality observed by higher counts of undesirable micro-organisms.

Key words: Aerobic stability, anaerobic fermentation, silage additives, lactic acid bacteria, yeasts.

INTRODUCTION

Sugarcane (*Saccharum* spp.) has a high productivity per hectare [Brazilian average of 76.4 ton of fresh matter (FM) ha^{-1}] (FAOSTAT, 2011); thus, yields a large energy production per planted area. During the fermentation of sugarcane silage, the yeasts population is high, which results in a high DM loss (Kung and Stanley, 1982), lower aerobic stability (Ávila et al., 2009) and a large decrease in the energy content of the food. Sugarcane has fiber content around 450 g kg^{-1} and, this fiber is poorly digestible (Correa et al., 2003). Alkaline agents such as calcium oxide have been added to reduce the fiber content of sugarcane silage (Balieiro Neto et al., 2007). The alkali addition caused hydrolysis of the bonds between lignin and plant cell wall components (Van Soest, 1994). However, this addition results in a buffering of the silage and a high final pH that can benefit the growth of undesirable micro-organisms during ensiling. Thus, the association between calcium oxide and other additives capable of inducing a pronounced decrease in the initial pH of silage may be of interest in reducing the fiber content in the feed, thereby improving its nutritional quality without the risk of undesirable fermentation.

The use of weak organic acids is an option to aid in controlling the growth of yeasts and filamentous fungi

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(Britt et al., 1975; Moon, 1983; Woolford, 1975) and decreasing the pH of silage more rapidly. Among these acids, propionic acid has been tested in silage and promotes reduction in the population of yeast and losses during fermentation (Carvalho et al., 2012; Selwet, 2008). The use of specific strains for each forage species ensures the best inoculation performance (Ávila et al., 2010). Specific strains of lactic acid bacteria (LAB) isolated from sugarcane silage can improve silage treated with calcium oxide (Carvalho et al., 2012). *Lactobacillus plantarum* is facultative heterofermentative LAB and is effective in rapidly reducing the pH. It may be used as an inoculant in sugarcane silage with added calcium oxide.

The objective of this study was to evaluate the effects of applying a wild isolate of *L. plantarum* (UFLA SIL 1) (Ávila et al., 2009) and of adding propionic acid on the effect of sugarcane silage treated with and without calcium oxide.

MATERIALS AND METHODS

Forage and application of treatments

Silages were prepared with freshly cut, 12-month-old sugarcane [206 g DM Kg^{-1} (±0.44), 510 g neutral detergent fiber (NDF) Kg^{-1} DM (±1.79), pH = 5.75 (±0.01)]. The sugarcane was manually harvested and chopped using a laboratory-type chopper (PP-47, Pinheiro, Itapira, SP, Brazil) into 30-mm lengths. Three mini-silos (plastic buckets, 15 L capacity) were individually prepared for each of the following treatments: untreated silage, inoculated with *L. plantarum* (UFLA SIL1), propionic acid addition, calcium oxide addition, inoculated with *L. plantarum* (UFLA SIL1) combined with propionic acid addition, inoculated with *L. plantarum* (UFLA SIL1) combined with calcium oxide addition, propionic acid addition combined with calcium oxide addition, and inoculated with *L. plantarum* (UFLA SIL1) combined with calcium oxide addition and propionic acid addition. The *Lactobacillus* strain (UFLA SIL1) used was previously isolated from sugarcane silage (Ávila et al., 2009) and grown on De Man Rogosa Sharpe broth (Oxoid CM361, Basingstoke, Hampshire, England). The inoculants were enumerated on De Man Rogosa Sharpe agar (Oxoid CM361, Basingstoke, Hampshire, England) to determine the amount of inoculant required to meet the targeted inoculation rate (5.0×10^5 cfu g^{-1} of FM). The De Man Rogosa Sharpe broth with the strain were mixed with deionized water and sprayed onto the forage.

The additives calcium oxide (quicklime micronized) (Qualical, Carbotex Química LTDA, Araçariguama, SP, Brazil) and propionic acid (Sigma, St. Louis, MO, USA) were applied at 10 g kg^{-1} or 10 ml kg^{-1} based on FM. For better homogenization, all treatments were diluted in distilled water to a final application volume of 200 ml for each 7 kg of forage. Untreated forage was sprayed with deionized water such that equal amounts of water were added to all treatments.

To prevent cross contamination between treatments, first deionized water was applied, then calcium oxide, then propionic acid and finally the microbial inoculants, using one sprayer for each treatment. Each silo was packed with approximately 7 kg of wet forage to achieve a packing density of 600 kg of FM per m^3. The weights of the empty and full silos were recorded. The silos were covered with a black plastic film (0.15 mm), which was then covered with a layer of sand, and the silos were sealed with adhesive tape.

After sealing, the silos were maintained at room temperature (on average, 25°C) and protected from sunlight and rain. After 180 days of ensiling and before opening, the full silos were weighed. DM loss was calculated using the weights and DM contents of the fresh matter and silage.

Analytical procedures

To obtain the aqueous extract, 40 g sample of fresh forage or sugarcane silage was blended in 360 ml of 0.1% sterile peptone water and homogenized using an orbital mixer for 20 min. The pH of each sample was then determined. Aliquots of the aqueous extracts (2 ml) were acidified with 10 µL of 50% (vol/vol) H_2SO_4 and frozen before analysis of the fermentation end products. Acetic, propionic, butyric and lactic acids, glycerol and ethanol present in the aqueous extracts were detected using high-performance liquid chromatography. The apparatus (Shimadzu Corp., Tokyo, Japan) was equipped with a dual detection system consisting of an ultraviolet detector (UV-Vis SPD-10Ai) and a refractive index detector (RID 10A). An ion exclusion column from Shimadzu (Shim-pack SCR-101H; 7.9 mm × 30 cm) operating at 50°C was used for the chromatographic separation. The mobile phase consisted of a 100 mM perchloric acid solution pumped at a flow rate of 0.6 ml min^{-1}. The acids were detected based on their UV absorbance (210 nm), and ethanol and glycerol were identified using the refractive index detector.

The DM contents of each sample were determined using a forced-draft oven at 60°C for 72 h. The dried samples were ground in a Wiley-type grinder using a sieve mesh of 1.0 mm and stored in labeled plastic pots. Neutral detergent fiber was analyzed using the sulfite method described by Van Soest et al. (1991) using an Ankom (ANKON® Technology Corp.) fiber analyzer.

Microbiological analyses

The other portion of aqueous extracts was used for enumeration of micro-organisms. Serial ten-fold dilutions were prepared to quantify the microbial groups present in the silages. Yeasts and filamentous fungi were enumerated on Dichloran Rose Bengal Chloramphenicol Medium (DRBC, Difco; Becton Dickinson, Sparks, MD, USA), and the plates were incubated at 28°C for 72 h. Clostridia spores were enumerated on DRCB (Differential Reinforced Clostridial Broth, Himedia) agar plus (16 g L^{-1}) and, prior to plating, the samples were maintained at 80°C for 10 min. The plates were incubated for 7 days at 30°C in an anaerobic chamber. The inoculant strain was biochemically characterized using API 50 CHL (BioMerieux SA) for lactobacilli.

Assessment of the aerobic stability of silages

Three kilograms of well-mixed silage from each silo were placed into clean containers, and thermometers were inserted into the center of each silage mass. The containers were stored in a controlled temperature room at 25°C (±1.5°C). The silage temperature was recorded every 8 h. During the evaluation of aerobic stability, silage samples were collected after 48, 96 and 144 h of exposure to air for enumeration of yeasts and filamentous fungi and to measure the pH and DM content, as described earlier. It was considered aerobically unstable the silage whose temperature exceeded 2°C in room temperature.

Statistical analysis

Anaerobic fermentation was evaluated using a completely randomized

Table 1. Chemical composition of sugarcane silage treated with calcium oxide, microbial inoculant or propionic acid after 180 days of storage.

Variables (g Kg^{-1} dry matter - DM)	WC[1]	NC[2]	*P*	WA[3]	NA[4]	*P*	WI[5]	NI[6]	P	SEM[7]
DM	175	146	<0.01	161	160	0.87	164	157	0.09	0.46
NDF[8]	616	725	<0.01	686	655	<0.01	669	671	0.79	0.60
DM loss	410	378	0.23	389	400	0.67	368	421	0.05	1.78
Lactic acid	66.2	36.0	<0.01	54.8	47.5	0.37	45.0	57.2	0.14	0.553
Acetic acid	28.5	51.4	0.03	54.3	25.5	0.01	35.8	44.0	0.39	0.663
Propionic acid	3.2	4.2	0.18	7.3	0.1	<0.01	4.1	3.3	0.25	0.049
Glycerol	74.7	42.6	0.04	65.8	51.5	0.33	65.5	51.7	0.35	1.001
Ethanol	2.8	21.5	<0.01	11.8	12.4	0.86	13.8	10.4	0.37	0.254

[1]With calcium oxide; [2]without calcium oxide; [3]with propionic acid; [4]without propionic acid; [5]with microbial inoculant; [6]without microbial inoculant; [7]Standard error of the means for the main effect; [8]Neutral detergent fiber.

design with three replications. The treatments were arranged in the following factorial arrangement: 2 (with or without the addition of calcium oxide) × 2 (with or without the addition of propionic acid) × 2 (with or without the addition of *L. plantarum* UFLA SIL 1). In total it was eight combinations and 24 experimental units. All microbial data were transformed to log10 and presented on a FM basis, whereas, chemical data were presented on a DM basis. Data regarding the anaerobic phase of fermentation were analyzed using the GLM procedure of SAS (SAS Institute, 2001) according to the model for a 2 × 2 × 2 factorial treatment design considering the following interactions:

$$Yjkl = \mu + Ij + Ck + Al + (I \times C)jk + (C \times A)kl + ejkl,$$

Where, μ = the overall mean; Ij = the effect of inoculation with *L. plantarum* (SIL UFLA 1) (j = with inoculant, without inoculant); Ck = the effect of calcium oxide (k = with calcium oxide, without calcium oxide); Al = the effect of propionic acid (l = with propionic acid, without propionic acid); (I × C)jk = the effect of interaction between the inoculant and calcium oxide; (C × A)kl = the effect of interaction between calcium oxide and propionic acid and ejkl = an error term.

To evaluate the aerobic stability, the same model was utilized. The data on yeast and filamentous fungi populations and pH value were analyzed as repeated measures using the MIXED procedure of SAS (SAS Institute, 2001) according to the same model, considering the effect of sampling time. The covariance structure used was that with the highest value for the Akaike information criterion.

RESULTS

Chemical composition of the silages and DM loss

When compared with other treatments, the addition of calcium oxide increased the DM content and reduced the NDF content in the silages after 180 days of fermentation (Table 1). The addition of propionic acid resulted in silages with a higher content of NDF 686 (g kg DM). The treatment with *L. plantarum* (UFLA SIL1) reduced the DM loss from 42.1 to 36.8% of the initial quantity of ensiled DM ($p = 0.05$) and tended to increase the contents of DM ($p = 0.09$). The contents of the final products of fermentation were affected by calcium oxide and propionic acid. The treatment with calcium oxide increased the concentration of lactic acid and glycerol and reduced the concentration of ethanol and acetic acid in the silage. The addition of propionic acid increased the acetic and propionic acid contents in the silage (Table 1).

Microbiological composition and pH at silos opening

An interaction ($p = 0.01$) was found between the treatments with calcium oxide and propionic acid in relation to the pH values and yeast, filamentous fungi, and clostridial populations after 180 days of storage (Table 2). Treatment with propionic acid was able to reduce the pH of the silages with or without calcium oxide; however, in the silages with calcium oxide, the decrease was more pronounced (from 4.87 to 4.18). The populations of yeasts, filamentous fungi and clostridia were higher in the silages with calcium oxide (Table 2). Propionic acid reduced the yeast population when applied with calcium oxide (Table 2). Propionic acid was able to reduce the population of clostridia in the absence of calcium oxide to 3.06 (log cfu g^{-1} of silage); however, propionic acid plus calcium oxide did not show this effect (Table 2).

Characteristics of sugarcane silage during aerobic exposure

There was interaction between the treatment with calcium oxide and time of aerobic exposure with respect to DM content ($p = 0.06$) (Table 3). The silages treated with calcium oxide exhibited higher DM content, which increased with the period of air exposure (Table 4). The addition of calcium oxide or propionic acid also affected the pH of the silages after opening, demonstrating an interaction between these factors ($p = 0.01$) (Table 3). The addition of propionic acid was able to maintain low pH values during the entire evaluation period ($p < 0.01$ for

Table 2. pH and microbial composition of sugarcane silage treated with calcium oxide, microbial inoculant or propionic acid after 180 days of storage.

Variable	WC[1]	NC[2]	WA[3]	NA[4]	WI[5]	NI[6]	WC		NC		WC		NC		SEM[7]
							WA	NA	WA	NA	WI	NI	WI	NI	
pH	4.52	3.64	3.86	4.30	4.04	4.11	4.18	4.87	3.54	3.74	4.52	4.53	3.57	3.70	0.104
P	<0.01		<0.01		0.42		0.01				0.50				
Yeasts (log cfu[8] g^{-1} silage)	5.65	3.34	3.14	5.85	4.66	4.33	5.34	5.97	1.00	5.73	5.71	5.59	3.60	3.07	0.637
P	<0.01		<0.01		0.57		<0.01				0.72				
Filamentous fungi (log cfu g^{-1} silage)	5.84	2.96	3.33	5.48	4.44	4.36	5.94	5.75	1.00	5.20	6.13	5.55	2.74	3.18	0.445
P	<0.01		<0.01		0.87		<0.01				0.21				
Clostridia (log cfu g^{-1} silage)	6.94	4.34	4.99	6.29	5.74	5.54	6.93	6.96	3.06	5.61	7.23	6.67	4.25	4.42	0.389
P	<0.01		<0.01		0.62		<0.01				0.36				

[1]With calcium oxide; [2]without calcium oxide; [3]with propionic acid; [4]without propionic acid; [5]with microbial inoculant; [6]without microbial inoculant; [7]standard error of the means of two-way interactions (calcium oxide and propionic acid or calcium oxide and microbial inoculant); [8]Colony forming units.

Table 3. Probability (*P*) for the purposes contained in the model during the evaluation of aerobic stability.

Variable	C[1]	A[2]	I[3]	H[4]	*P* value for interaction				
					C × A	C × I	C × H	A × H	I × H
DM	<0.01	0.78	0.49	<0.01	0.70	0.73	0.06	0.89	0.76
pH	<0.01	<0.01	0.58	<0.01	0.01	0.95	0.16	<0.01	0.47
Yeast	<0.01	<0.01	0.52	<0.01	<0.01	0.42	0.03	0.22	0.65
Fungi[5]	<0.01	<0.01	0.84	0.02	<0.01	0.04	<0.01	0.33	0.93

[1]Calcium oxide; [2]propionic acid; [3]microbial inoculant; [4]time (h) of evaluation of aerobic stability; [5]filamentous fungi.

Table 4. Characteristics of sugarcane silage treated with or without calcium oxide, propionic acid and microbial inoculant during assessment of its aerobic stability.

Variable	Hours after silo opening	WC[1]	NC[2]	With calcium oxide		Without calcium oxide		With calcium oxide		Without calcium oxide		SEM[7]
				WA[3]	NA[4]	WA	NA	WI[5]	NI[6]	WI	NI	
	0	175	146	178	172	144	148	179	171	149	143	
	48	179	164	177	180	166	161	181	177	168	159	
DM (g Kg^{-1})	96	184	166	188	179	166	166	183	184	167	164	0.704
	144	195	177	196	193	176	179	193	196	179	175	
	Mean			184.8	181.0	163.0	163.5	184.0	182.0	165.8	160.3	
pH	0	4.53	3.61	4.18	4.87	3.54	3.74	4.52	4.53	3.57	3.71	0.340

Table 4. Contd

	48	5.12	4.69	4.58	5.67	3.65	5.73	5.10	5.14	4.67	4.71	
	96	6.71	5.54	6.41	7.02	3.71	7.38	6.59	6.83	5.49	5.59	
	144	7.18	5.99	6.94	7.42	3.90	8.07	6.96	7.41	5.81	6.16	
	Mean			5.53	6.25	3.70	7.06	5.79	5.98	4.89	5.04	
	0	5.65	3.34	5.34	5.97	0.94	5.73	5.71	5.59	3.60	3.07	
	48	5.40	5.11	4.54	6.27	2.71	7.51	5.10	5.70	4.99	5.22	
Yeasts (log[8] cfu g^{-1} silage)	96	6.16	5.63	5.72	6.59	3.48	7.77	5.80	6.51	5.51	5.74	0.419
	144	6.40	6.03	6.01	6.79	4.32	7.74	6.13	6.68	6.09	5.97	
	Mean			5.40	6.41	2.86	7.19	5.69	6.12	5.05	5.00	
	0	5.84	2.96	5.94	5.75	0.72	5.20	6.13	5.55	2.74	3.18	
	48	5.54	3.74	5.24	5.84	1.59	5.89	5.58	5.49	3.61	3.88	
Fungi[9] (log cfu g^{-1} silage)	96	6.18	3.80	6.60	5.77	2.57	5.03	6.72	5.64	3.01	4.59	0.419
	144	5.49	4.70	5.20	5.77	3.01	6.39	5.71	5.28	4.54	4.85	
	Mean			5.75	5.78	1.97	5.63	6.04	5.49	3.48	4.13	

[1]With calcium oxide; [2]without calcium oxide; [3]with propionic acid; [4]without propionic acid; [5]with microbial inoculant; [6]without microbial inoculant; [7]standard error of the means of two-way interactions (calcium oxide and propionic acid or calcium oxide and microbial inoculant); [8]colony forming units; [9]filamentous fungi.

Table 5. Time required to lose stability, maximum temperature and time required to reach maximum temperature in sugarcane silage during aerobic exposure.

Variable	WC[1]	NC[2]	WA[3]	NA[4]	WI[5]	NI[6]	SEM[7]	*P* value				
								C	A	I	C × A	C × I
Time required to lose aerobic stability (h)	30.7	14.7	25.3	20.0	24.0	21.3	5.72	0.07	0.69	0.68	0.76	0.61
Maximum temperature (ºC)	38.7	40.4	39.7	39.4	39.0	40.0	0.49	0.03	0.65	0.18	0.10	0.22
Time required to reach the maximum temperature (h)	86.0	47.3	71.3	62.0	66.7	66.7	8.23	0.01	0.45	0.35	0.67	0.45

[1]With calcium oxide. [2]without calcium oxide. [3]with propionic acid. [4]without propionic acid. [5]with microbial inoculant. [6]without microbial inoculant. [7]standard error of the means of two-way interactions (calcium oxide and propionic acid or calcium oxide and microbial inoculant).

the interaction between propionic acid and evaluation time), even in the silages treated with calcium oxide (p = 0.01 for the relationship between calcium oxide and propionic acid) (Table 4). The pH was always higher in the silages treated with calcium oxide, and this increased with the evaluation period (Table 4). The treatment with calcium oxide, propionic acid and evaluation time affected the yeast and filamentous fungi content of the silages. Treatment with calcium oxide increased the population of yeasts and filamentous fungi in the silage as the aerobic stability evaluation period increased (Table 4). The addition of propionic acid reduced the population of these micro-organisms in the silage

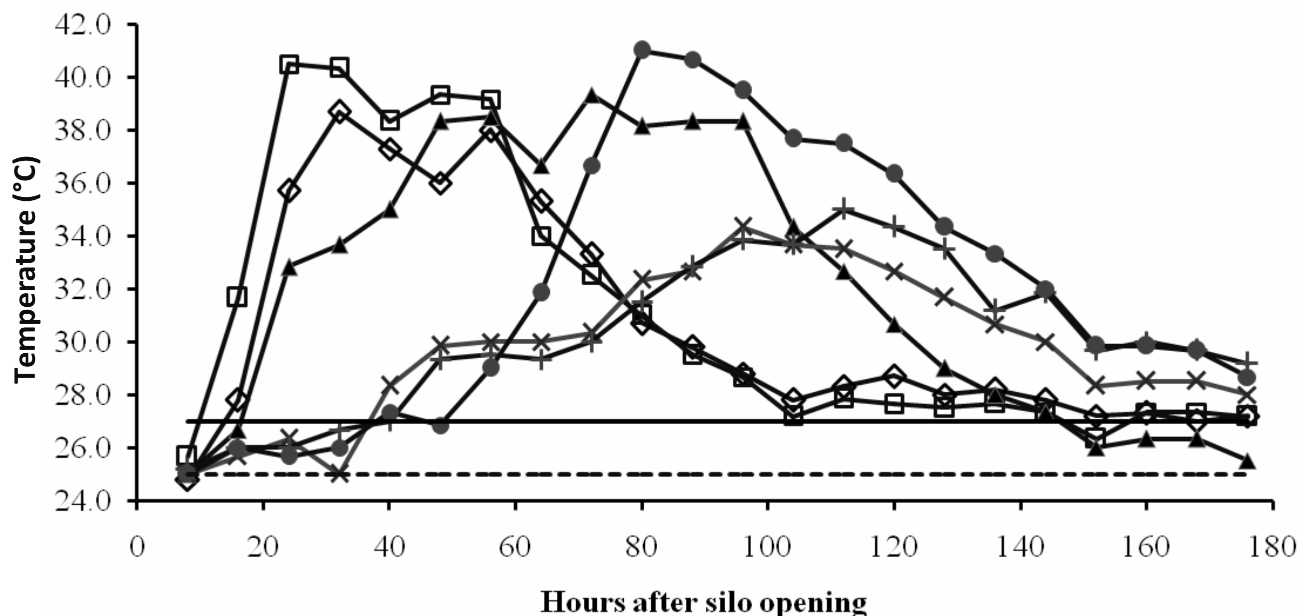

Figure 1. Sugarcane silages after 180 days of ensilage: temperatures during the assessment of aerobic stability. Treatments: control (◊), microbial inoculant (▯), propionic acid (▲), calcium oxide (×), calcium oxide combined with microbial inoculant (+), calcium oxide combined with propionic acid (●), environmental temperature (---), and aerobic stability (:).

in the presence and in the absence of calcium oxide (Tables 3 and 4). The addition of the microbial inoculant in the silage treated with calcium oxide caused an increment in the population of filamentous fungi from 5.49 to 6.04 log cfu g^{-1}; whereas, in the silage without calcium oxide, the addition of this inoculant caused a reduction in the population of filamentous fungi from 4.13 to 3.48 log cfu g^{-1}.

Calcium oxide was the only additive that affected the silage temperature during the aerobic stability evaluation. The time required for stability loss ranged from 14.7 h (silages without calcium oxide) to 30.7 h (silages with calcium oxide). The silages without calcium oxide exhibited a maximum temperature of 40.4°C after 47.7 h of aerobic exposure. The silages with calcium oxide exhibited a slightly lower maximum temperature (38.7°C), but the time necessary to achieve this temperature was much greater (86 h).

Evaluation of the aerobic stability of the silages based on temperature data obtained during aerobic exposure showed that the silage with the microbial inoculant had shorter aerobic stability. In this treatment, the temperature of the silage surpassed 40°C during the first 24 h of aerobic exposure (Figure 1).

The treatment with propionic acid did not increase the stability of the silage, although, it did reduce the yeast and filamentous fungi population (Tables 4 and 5). Treatment with calcium oxide or calcium oxide combined with microbial inoculant promoted higher reductions in temperature and higher aerobic stability in the sugarcane silage (Figure 1).

DISCUSSION

The DM content of silages ranged on average from 146 (silages without calcium oxide) to 175 g kg^{-1} of DM (silages with calcium oxide). These values are below the minimum (193 g kg^{-1}) found in a review of 26 trials of sugar cane silage (Zopollatto et al., 2009). This result may be due to the low initial forage DM content. The NDF values were lower in silages with calcium oxide (616 g kg^{-1}) compared with others treatments.

These values were within the usual range for sugarcane silage (Zopollatto et al., 2009). The increase in the DM content occurred in the treatment with calcium oxide resulted from an increase of non-fermentable DM in these silages because, of every 1000 g of ensiled sugarcane, 10 g of calcium oxide were applied, which represents the mineral matter. The silage with this additive exhibited a reduction in NDF content of 725 to 616 g kg DM after 180 days of ensiling.

The reduction in the fiber content in these silages is attributable to alkaline hydrolysis of the plant cell wall components caused by the addition of alkali (Van Soest, 1994). The capacity of the calcium oxide to promote a decrease in the poorly digestible sugarcane fiber component of the forage (Correa et al., 2003) has been established in other studies, where a better nutritional food quality was found (Balieiro Neto et al., 2007; Carvalho et al., 2012). This reduction in fibrous components is the main purpose behind the addition of calcium oxide to sugarcane silage.

According to the carbohydrate fermentation profile, the

wild inoculant strain was identified as *L. plantarum* (99% identification based on API software). Classified as facultative heterofermentative facultative bacteria, this group of micro-organisms use the glycolytic pathway to produce mainly lactic acid from the fermentation of hexose sugars (Axelsson, 1998). The wild strain was able to use the main carbohydrates present in sugarcane (sucrose, glucose and fructose) to produce acid. Despite this, treatment with *L. plantarum* (UFLA SIL 1) did not affect the production of metabolites in the silages (Table 1). Inoculation with *L. plantarum* (UFLA SIL 1) reduced the losses of DM during anaerobic fermentation of the silage, although, this additive did not have any effect on the population of filamentous fungi, yeasts and clostridia or on the acidic profile after 180 days of fermentation (Tables 1 and 2). The reduction in the loss of DM found with this wild strain is important for the nutritional and economical status of the sugarcane, where the loss of DM during the ensiling is represented mainly by a loss of sucrose and a consequent reduction in the nutritional quality of silage. Other authors found different results when this species of LAB was inoculated in various forage materials. Ávila et al. (2010) observed a greater population of yeasts in sugarcane silage inoculated with this *L. plantarum* strain (UFLA SIL 1) than control silage. These authors found higher concentrations of lactic acid and lower concentrations of propionic acid and ethanol in the treated silages than in control silages. Thus, it can be inferred that the ensiling conditions also affected the performance of the inoculated strain.

In relation to the fermentative parameters, the calcium oxide buffering capacity during the ensiling may have stimulated a high production of lactic acid (Table 1). According to Klosterman et al. (1960), the concentrations of organic acids in silage are related to the moisture content of the forage and with the buffering during the fermentation. High concentrations of lactic acid in sugarcane silage treated with calcium oxide were also found by Carvalho et al. (2012). The increase in the concentration of glycerol occurred in the silages treated with calcium oxide may be associated with microbial metabolism, in particular, to the anaerobic fermentation of yeasts (Table 1). Glycerol is intermediate to various metabolic processes in several organisms (Smidt et al., 2012), and this compound is an important source of carbohydrates and energy. Although, calcium oxide caused an increase in the population of clostridia, the presence of butyric acid (the main metabolite associated with this microorganism) was not detected in any silage. The addition of calcium oxide reduced the concentration of ethanol and increased the concentration of lactic acid in the silage; however, this treatment did not reduce the DM losses during the fermentation studied here (Table 1). It is possible that the extension of the fermentation represented by a high microbial population is the main cause of the losses in these silages. The losses of DM found in this study (37.8 and 42%) were greater than the maximum quoted by Zopollatto et al. (2009), which reported a maximum DM loss of 35.3% in sugarcane silage. The low concentration of DM initially occurred in the forage may also have been responsible for the high loss of DM.

In this work, it was verified that the addition of calcium oxide favor the increase of the populations of yeasts, filamentous fungi and clostridia in the silage (Tables 2 and 4). The buffering capacity caused by this additive may have stimulated the increase in the population of these micro-organisms; in addition, the possibility of lignin hydrolysis, which is also caused by the addition of calcium oxide, may have promoted increased nutrient availability for microbial growth. Organic acid additives, such as propionic acid, are considered as fermentation inhibitors; used as antimicrobials and/or acidifiers, therefore, they may modify the microbiota and, consequently, the fermentation of the silage (Kung Junior et al., 2003). Selwet (2008) also found a decrease in yeasts and filamentous fungi when propionic acid (0.4% FM) was added to corn silage. A decrease in the population of LAB, yeasts and clostridia was found here and by Carvalho et al. (2012) when propionic acid (1% FM) was added to sugarcane silage. An improvement in silage quality occurred when propionic acid was used together with calcium oxide, resulting in reductions in pH and the populations of clostridia, yeasts and filamentous fungi (Table 2). Although, this effect was reduced, the application of propionic acid maintains its antimicrobial effect when applied together with calcium oxide. The addition of the *L. plantarum* strain (UFLA SIL 1) did not affect the temperature, pH, DM content or yeast population of the silage. When the microbial inoculant was used with calcium oxide, the population of filamentous fungi increased, and when the microbial inoculant was added without calcium oxide, the population of filamentous fungi decreased (Table 4) (an interaction occurred between the treatments with calcium oxide and microbial inoculant on the population of filamentous fungi) (Table 3).

Due to the metabolic characteristics of the inoculated strain, a favorable response of its inoculation on the aerobic stability of the silage was not expected.

The lactic acid produced by the *L. plantarum* can be used as substrate for the growth of some yeast when the silage is exposed to air (Danner et al., 2003). Filya et al. (2006) did not observe an improvement in the aerobic stability or the quality of corn or sorghum silage inoculated with *L. plantarum*. Silages treated with calcium oxide exhibited higher values of pH, and silages treated with propionic acid exhibited lower values of pH, even when these silages had received calcium oxide (Tables 3 and 4), and these results prove the acidifying effect of propionic acid (Kung Junior et al., 2003). Silages treated with calcium oxide also exhibited increased DM content, which increased with the aerobic stability evaluation time. The initial increase in the DM content observed when

using this treatment was due to an increase in non-fermentable DM (calcium oxide) because the increase in the DM content during the aerobic stability evaluation period is due to dehydration of the silage resulting from the evaporation of water. Treatment with calcium oxide increased the pH values and favored an increase of filamentous fungi and yeasts during the aerobic stability evaluation. This result is due to the greater increase of these micro-organisms under aerobic conditions and higher pH (5.0 to 6.0) than under anaerobic conditions and low pH (McDonald et al., 1991). However, treatment with propionic acid maintained low pH values and reduced the presence of filamentous fungi and yeasts, which demonstrates the antimicrobial effect of propionic acid as previously found by Carvalho et al. (2012), Moon (1983), Sebastian et al. (1996) and Selwet (2008). The action of the propionic acid in improving the microbiological quality of sugarcane silage treated with calcium oxide also became evident during evaluation of the aerobic stability. Silages treated with calcium oxide exhibited greater microbial populations, although, this treatment reduced the temperature of the silage, thereby increasing its aerobic stability (Table 5 and Figure 1). Other groups of micro-organisms, such as bacteria of the *Bacillus* genus (Li and Nishino, 2011; Woolford, 1990), *Enterobacter* (Li and Nishino, 2011) or *Clostridium* (Borreani and Tabaco, 2010), which were not quantified in this work, were most likely associated with the aerobic deterioration found in the silages that were not treated with calcium oxide. Another factor that is possibly responsible for the increased aerobic stability length of silages treated with calcium oxide is the high population of micro-organisms present at the opening of the silos. As their population was already high, there were no large increases in the number of filamentous fungi and yeasts.

As the exothermic reactions occurring during microbial growth are mainly responsible for increases in the temperature of silage (Hunter, 1917), the temperature was lower in silage where there was less of an increase in the number of micro-organisms. Further studies aimed at identifying and elucidating the metabolism of the species present in these silages is necessary for further understanding. Borreani and Tabaco (2010) evaluated corn silages stored in farm silos and found greater populations of yeasts, filamentous fungi and clostridial spores in the peripheral areas of these silos than in the central area. Samples from the peripheral areas exhibited higher temperatures, and there was a positive correlation between the presence of these micro-organisms and the temperature of the silage. No findings were found for the other treatments on the time required for stability loss, the maximum temperature reached or the time to reach this temperature (Table 5).

Conclusion

The addition of calcium oxide improved aerobic stability, however, resulted in lower silage quality observed by higher counts of undesirable microorganisms. The application of propionic acid or wild strain of *L. plantarum* was not able to improve the microbiological quality of the sugarcane silage treated with calcium oxide. Accordingly, the use of calcium oxide as sugarcane silage additive is not recommended. More detailed studies related to the identification of the total microbiota and its metabolic products must be conducted in sugarcane silage treated with various additives.

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