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High-moisture corn grain silage inoculated with *Propionibacterium acidipropionici* and *Lactobacillus plantarum in different storage times*

Silagem de grão úmido de milho inoculada com *Propionibacterium acidipropionici* e *Lactobacillus plantarum* em diferentes tempos de armazenamento

Ensilaje de grano de maíz húmedo inoculado con Propionibacterium acidipropionici y Lactobacillus plantarum en diferentes tiempos de almacenamiento

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Abstract
The aim of this study was to evaluate the chemical composition, growth of microorganisms, and the aerobic stability of high-moisture corn grain silage inoculated with *Lactobacillus plantarum* + *Propionibacterium acidipropionici*. The experimental design was completely randomized in a 2 x 5 factorial arrangement with four replications (50 experimental units) and the treatments were: use or not of microbial inoculant (2 x 10^5 colony forming unit (CFU) g^{-1} *Lactobacillus plantarum* + *Propionibacterium acidipropionici*) and the storage length for 0, 1, 3, 7, and 14 d. Aerobic stability of silage was evaluated at 28 and 56 d of storage length. The lactic acid bacteria population was influenced by storage length and the greatest values were estimated at 8 d of storage length. Regardless of inoculant application, no enterobacteria were present from 3 d post-ensiling. With respect to mold growth, an interaction between inoculant and storage length was observed wherein molds were most abundant after 3 d of storage in silage that received inoculant. However, at 14 d of storage the use of bacterial inoculant reduced the occurrence of molds. pH values obtained after 3 d of ensiling were less than 4.0 for all the treatments. The high-moisture corn grain silage possessed good fermentative quality. Further, adequate pH values were achieved from the third day of ensilage and were not influenced by the presence of bacterial inoculant.
Keywords: Enterobacteria; Microbial additive; pH; Stability.

Resumo
O objetivo com este estudo foi avaliar a composição química, crescimento de microrganismos e estabilidade aeróbia de silagem de grão úmido de milho inoculada com *Lactobacillus plantarum* + *Propionibacterium acidipropionici*. O delineamento experimental foi inteiramente casualizado em um arranjo fatorial 2 x 5 com quatro repetições (50 unidades experimentais) e os tratamentos foram: uso ou não de inoculante microbiano (2 x 10^5 unidades formadoras de colônias (UFC) g^{-1} de *Lactobacillus plantarum* + *Propionibacterium acidipropionici*) e os tempos de armazenamento de 0, 1, 3, 7 e 14 dias. A estabilidade aeróbia da silagem foi avaliada aos 28 e 56 dias de armazenamento. A população de bactérias ácido láticas foi influenciada pelo tempo de armazenamento e os maiores valores foram estimados aos 8 dias após ensilagem. Independente do uso de inoculante, as enterobactérias não foram presentes a partir de três dias de ensilagem. Em relação ao desenvolvimento de fungos, ocorreu interação entre inoculante e tempo de armazenamento onde os fungos foram mais abundantes no tratamento com inoculante, no entanto, aos 14 dias de armazenamento o inoculante reduziu a ocorrência de fungos. Os valores de pH obtidos após três dias de ensilagem foram menores que 4.0 para todos os tratamentos. A silagem de grãos úmidos apresentou boa qualidade fermentativa, além de valores adequados de pH a partir do terceiro dia de ensilagem e não foi influenciado pela presença de inoculante bacteriano.
Palavras-chave: Aditivo microbiano; Enterobactéria; Estabilidade; pH.

Resumen
El objetivo de este estudio fue evaluar la composición química, crecimiento de microorganismos y estabilidad aeróbica de ensilaje de grano de maíz húmedo inoculado con *Lactobacillus plantarum* + *Propionibacterium acidipropionici*. El diseño experimental fue completamente aleatorio en un arreglo factorial 2 x 5 con cuatro repeticiones (50 unidades experimentales) y los tratamientos fueron: uso o no de inoculante microbiano (2 x 10^5 unidades formadoras de colonias (UFC) g-1 de *Lactobacillus plantarum* + *Propionibacterium acidipropionici*) y los tiempos de almacenamiento de 0, 1, 3, 7 y 14 días. Se evaluó la estabilidad aeróbica del ensilado a los 28 y 56 días de almacenamiento. La población de bacterias lácticas estuvo influenciada por el tiempo de almacenamiento y los valores más altos se estimaron a los 8 días después del ensilado. Independientemente del uso de inoculantes, las enterobacterias no estaban presentes después de tres días de ensilado. En cuanto al desarrollo de hongos, hubo interacción entre inoculante y tiempo de almacenamiento donde los hongos fueron más abundantes en el tratamiento con inoculante, sin embargo, a los 14 días de almacenamiento el inoculante redujo la ocurrencia de hongos. Los valores de pH obtenidos después de tres días de ensilado fueron inferiores a 4.0 para todos los tratamientos. El ensilado de grano húmedo mostró buena calidad fermentativa, además de valores adecuados de pH desde el tercer día de ensilado y no fue influenciado por la presencia de inoculante bacteriano.
Palabras clave: Aditivo microbiano; Enterobacterias; Estabilidad; pH.

1. Introduction

The use of high-moisture corn grain (HMC) silage has advantages compared to conventional grain which include harvest anticipation, the minimization of loss caused by transportation and reduced storage cost. In addition, its use improves grain quality maintenance and starch digestibility (Ferrareto et al., 2013; Kung et al., 2014).

Silage of high moisture corn grain is more susceptible to aerobic deterioration than grass and legume silages (Basso et al., 2012) and options are available for improving HMC fermentation as the use of microbial inoculants, which may facilitate silage conservation and therefore enhance its quality (Saylor et al., 2020).

Microbial additives with homofermentative or facultative heterofermentative bacteria as *Lactobacillus plantarum* have the potential to reduce silage pH (Restelatto et al., 2019) and inhibit the growth of undesirable microorganisms (Kung Jr. et al., 2003). However, the use of homolactic bacteria typically reduce the aerobic stability of silage (Silva et al., 2018). An alternative to improve the aerobic stability is the use of *Propionibacterium* sp., which can reduce silage deterioration, since this genus consume lactic acid and glucose, producing propionic and acetic acid as final fermentation products and both are compounds with antifungal action (Parizzi et al., 2012).

The combination of *P. acidipropionici* and *Lactobacillus plantarum* may improve the fermentative profile and quality of silages (Souza et al., 2020), however, few studies evaluated these additive in HMC silages, being important to evaluate the fermentation processes during ensiling and aerobic stability post-silo opening.

We hypothesized that the use of this bacterial inoculant can improve the aerobic stability and reduce pH of high moisture corn grain silage. Thus, the aim of this study was to evaluate the chemical composition, development of principal microorganisms of HMC silage, and assess the aerobic stability of silage inoculated with *L. plantarum* plus *P. acidipropionici*.

2. Methodology

The study was conducted in Marechal Cândido Rondon, PR, Brazil, (24°33'22"S and 54°03'24"W, 400 m altitude). The harvest was carried out when corn reached physiological maturity. Then, material was crushed and ensiled. A completely randomized design was employed for the experiment, which used a 2 x 5 factorial arrangement with four replications (50 experimental units). Treatments included silage: inoculated with commercial product containing *Lactobacillus plantarum* and *Propionibacterium acidipropionici* bacterial strains and a control that did not contain inoculant. Timepoints evaluated included 0 (before ensiling), 1, 3, 7, and 14 d of storage. The chemical composition and fermentative profile of samples were assessed, and the aerobic stability of silage was evaluated at 28 and 56 d of storage.

Inoculant was applied immediately before ensiling using a manual sprayer at a dose of two g of inoculant mixed within 1 L sterile water, and a total of 2 x 10^5 CFU per g mass was applied, in accordance with the manufacturer's recommendations (1 L for each 0.5 ton of mass. Control treatment received the same quantity of water (1 L for each 0.5 ton of mass). Silage was stored in experimental silos made of polyvinyl chloride (PVC), that were 50 cm length and 10 cm diameter, and silo caps were equipped with a Bunsen-type valve. In the lower part of each silo, 500 g of autoclaved and dried sand was placed, a cotton cloth functioned as a divider that facilitated liquid drainage. Approximately 3000 g of corn was ensiled in each silo and the material was manually compacted with a wooden stick and silo caps were sealed with adhesive tape, reaching a mean density of 764 kg wet grain/m^3.

When silos were opened, a 5-cm layer from the upper and lower portion of silage was discarded and the remaining material was homogenized and sampled for use in subsequent analyses. Samples used to evaluate the chemical composition were pre-dried in a forced-air oven at 55 °C for 72 h. Afterward, the samples were ground using a 30-mesh screen in a Willey type mill (Fortinox, Piracicaba, SP, Brazil). A subsample was dehydrated in an oven at 100 °C for 24 h, to determine dry matter

content (DM, method 934.01), crude protein (CP, method 981.10), ether extract EE, method 920.85) and ash (method 938.08) in accordance with protocols described by AOAC (1990). The determination of neutral detergent fiber (NDF) was performed according to Van Soest et al. (1991). Organic matter content was calculated as the difference between ash and total DM.

An aqueous extract of 25 g silage sample mixed with 450 mL deionized water was used to determine pH (Cherney & Cherney, 2003) using a pH meter (Tec 2- mp Tecnal, Piracicaba, SP, Brazil). The temperature of samples was measured using a digital skewer thermometer.

The microbial populations were determined in accordance with methods described into Silva et al. (1997) using selective culture technique. To perform the method, solutions containing 50 g silage samples and 450 mL sterilized distilled water were used. Bacterial, yeast, and mold populations were determined using serial dilutions of the original solution. For the evaluation of yeasts and molds, potato dextrose agar (PlantMedia) was used and the plates were incubated at 28 °C for 72 h, and molds were counted after 5 to 7 d. Lactic acid bacteria (LAB) were seeded on plates containing man, rogosa, and sharpe agar (MRS) (Himedia, M641) and incubated for 48 h at 37 °C. To assess the enterobacterial population, samples were seeded in depth on plates containing violet red bile agar (VRB) (Himedia, M049) and incubated at 35 °C for 24 hours, and to determine *Clostridium* spp., samples were surface seeded on plates with reinforced Clostridial agar (Himedia, M154) and incubated at 35 °C for 24 h under anaerobic conditions using jars and an incubator with a CO_2 gas system (TE 399 Tecnal; Tecnal Laboratory Equipment, Piracicaba, Brazil). For total bacterial count (TBC), plate count agar (PCA) (Himedia, M091) was used, incubated at 35 °C for 72 h. After incubation, colony counts were performed using a Quebec Counter, and plates containing between 30 and 300 CFU per plate were used. Microbial data were obtained by the average of plates.

To evaluate aerobic stability, samples were collected at 28 and 56 d of ensiling and 500 g samples were collected when silos were opened. Then, samples were packed in plastic trays and kept at room temperature (25.59 ± 6.0 °C). Temperature was measured as previously described every 12 h for 7 d and breaks in aerobic stability were identified when sample temperatures recorded were 2 °C above room temperature (O'Kiely et al., 2001). Microbial data were transformed into base 10 logarithm before statistical analyses. Data were analyzed in a 2 x 5 factorial arrangement using the procedure GLM of SAS (Statistical Analysis System, version 9.2; SAS Institute Inc., Cary, NC). The model used was:

$$Yijk = \mu + I + SLj + I \times SLij + eijk$$

Where: *Yijk* = dependent variable; μ = overall mean; *Ii* = fixed effect of inoculant; *Tij* = effect of storage length; I × SL*ij* = effect of interaction between inoculant and storage

length; *eijk* = random error.

Treatments were compared using the Tukey test. When significant for time, a regression analysis was performed with regression procedure of SAS program. Significance was declared at $P<0.05$.

3. Results

Dry matter and NDF content were greatest ($P=0.01$ and $P=0.04$) when the inoculant was added (Table 1), while OM, CP, and EE were not influenced by the addition of inoculant ($P>0.05$). The duration of storage did not affect the chemical composition of silage nor interaction inoculant x storage length for the silage chemical composition ($P>0.05$).

Table 1. Chemical composition (g kg^{-1} dry matter) of high moisture corn grain silage without (control) or with inoculant in different storage lengths.

Item		Time (days)					Mean	SEM[7]	Inoc[8]	Time	Inoc x SL[9]
		0	1	3	7	14					
DM[1]	Control	616	615	608	609	612	612[a]	1.268	>0.001	0.062	0.781
	Inoculant	607	605	597	602	608	604[b]				
OM[2]	Control	986	986	986	985	986	986	0.541	0.081	0.107	0.841
	Inoculant	985	985	986	985	986	985				
CP[3]	Control	113	111	112	111	112	112	0.132	0.807	0.529	0.389
	Inoculant	110	109	112	114	114	112				
NDF[4]	Control	127	106	99	92	108	106[b]	1.121	0.042	0.259	0.766
	Inoculant	126	135	126	110	122	124[a]				
EE[5]	Control	41.7	37.6	36.7	41.8	42.0	40.0	0.965	0.742	0.177	0.181
	Inoculant	40.0	45.1	36.7	45.7	35.7	40.6				

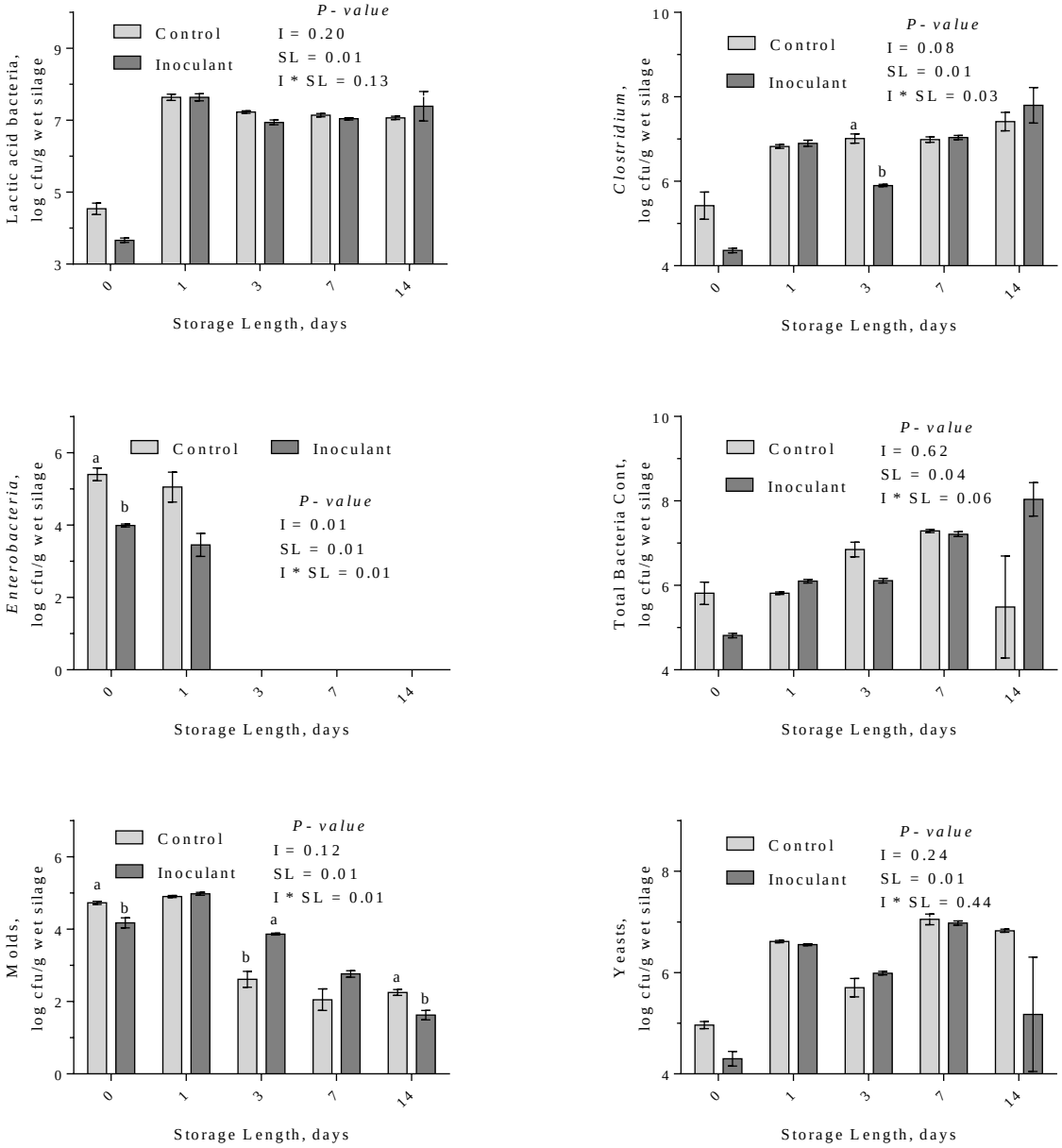
Different letters in columns differ by Tukey test at 5% probability. [1]DM: Dry Matter; [2]OM: Organic Matter; [3]CP: Crude Protein; [4]NDF: Neutral detergent fiber; [5]EE: Ether extract; [7]SEM: Standard error of mean; [8]Effect of inoculant. [9]Inoc x SL: Interaction between inoculant x storage length. Source: Authors.

Latic acid bacteria growth (Figure 1) was influenced only by storage length (P=0.01) and a quadratic effect was revealed via regression analysis, and the largest population was estimated to occur 9 d post-ensiling and consist of 7.81 log CFU g^{-1} silage. For bacteria of genus *Clostridium* sp., there was interaction between inoculant and storage length. After ensiling for 3 d, the lowest values were observed in treatment with inoculant. For other storage lengths, no effect of inoculant was observed.

When the population of enterobacteria was assessed, an interaction between inoculant and storage length was observed (P=0.01). Prior to ensiling, control treatment had the greatest degree of population growth, however, after 3 d, no enterobacteria was observed. Total bacteria count was influenced by storage length (P=0.04) and the population peaked 9 d post-ensiling at 7.36 log CFU g^{-1} silage.

With regard to molds, an interaction between inoculant and storage length was observed (P=0.01). After 3 d of storage lower mold levels were observed in samples of the control treatment relative to those that received inoculant. However, after 14 days, the use of bacterial inoculant was associated with a reduction in the mold population. The yeast population was influenced exclusively by storage length (P=0.01). Yeast peak 8 d after ensiling, and an estimated population of 7.0 log CFU g^{-1} was observed.

Figure 1. Microbiological profile of high moisture corn grain silage. Inoculant: *Lactobacillus plantarum* and *Propionibacterium acidipropionici* at 2 x 10^5 CFU per g of mass.

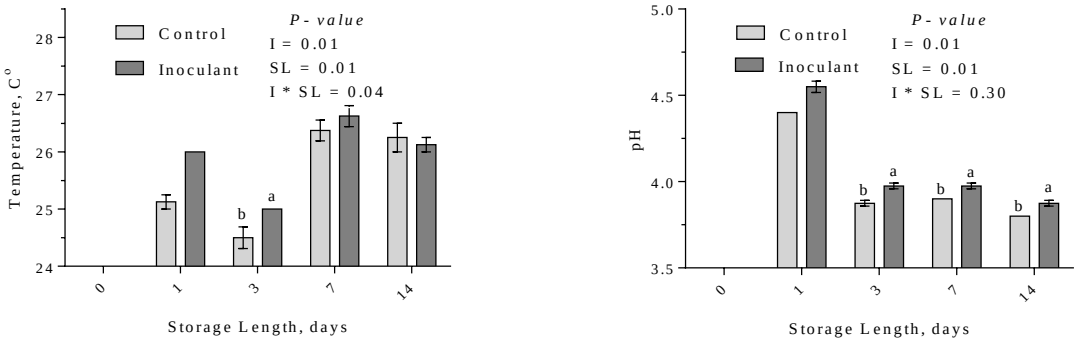


Control: without inoculation. I: inoculant effect; SL: storage length effect; I * SL: interaction between inoculant and storage length. Effect of inoculation in each day by Tukey test at 5% probability: different letters. Effect of storage length on: Lactic Acid Bacteria of inoculant and control = 5.525586 + 0.507835x - 0.028284x2 (R^2=0.36); *Clostridium* of inoculant treatment= 5.529615 + 0.176807x (R^2=0.49); *Clostridium* of control = 6.257212 + 0.094558x (R^2=0.34); *Enterobacteria* of inoculant = 2.785692 - 0.259538x (R^2=0.50); *Enterobacteria* of control = 3.900077 - 0.362654x (R^2=0.50); Total bacteria count of inoculant and control = 5.414282 + 0.433386x - 0.024141x2 (R^2=0.99); Molds of inoculant treatment = 4.575481 - 0.219096x (R^2=0.87); Molds of control = 4.949929 - 0.700879x + 0.036372x2 (R^2=0.83); Yeast of inoculant and control = 5.191025 + 0.448595x - 0.027907x2 (R^2=0.55). Source: Authors.

There was interaction between inoculant and storage length (P=0.04) in silage temperature at the moment of silos opening (Figure 2) and the greatest temperatures occurred in treatment that received inoculant when stored for 3 and 7 d. However, after 14 d of storage, no temperature differences were observed in treatment that received and did not receive inoculant (P>0.05). pH values were influenced by inoculant (P=0.01) and storage length (P=0.01). For the control treatment, the lowest

pH values occurred after 3, 7 and 14 d of storage. A quadratic effect was observed via regression analysis, and the lowest pH value (3.74) was estimated to occur in 10 d of storage.

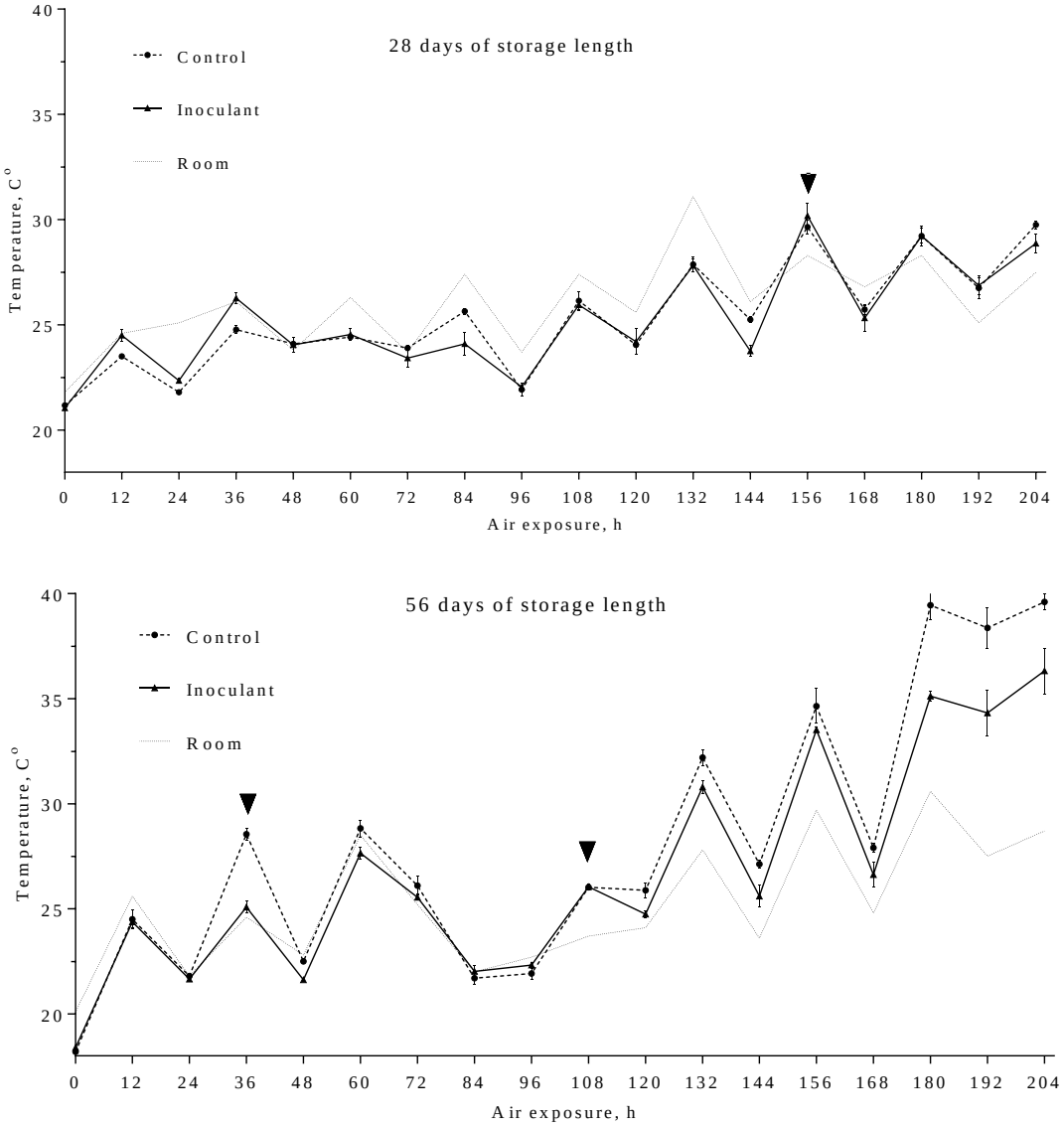
Figure 2. Temperature and pH of high moisture corn grain silage. Inoculant: *Lactobacillus plantarum* and *Propionibacterium acidipropionici* at 2 x 10^5 CFU per g of mass.



Control: without inoculation. I: inoculant effect; SL: storage length effect; I * SL: interaction between inoculant and storage length. Effect of inoculation in each day by Tukey test at 5% probability: different letters. Effect of storage length on: Temperature of inoculant = 24.668238 + 0.336986x - 0.016238x^2 (R^2=0.48); Temperature of control =25.014652 + 0.096434x (R^2=0.40); pH of inoculant and control =4.490972 - 0.147306x + 0.0072011x^2 (R^2=0.71). Source: Authors.

A break in aerobic stability occurred in the control treatment after 28 days of ensiling when exposed to air for 156 h (Figure 3). However, in the treatment with inoculant, no break in stability was observed until 204 h of exposure to air. After 56 days of ensiling, a break in aerobic stability occurred at 36 hours of exposure to air for control treatment and at 108 hours for inoculant treatment.

Figure 3. Temperature of high-moisture corn grain silage during air exposure after 28 or 56 days of storage length. Inoculant: *Lactobacillus plantarum* and *Propionibacterium acidipropionici* at 2 x 10^5 CFU per g of mass.



Control: without inoculation. ▼ indicate brake of aerobic stability. Source: Authors.

4. Discussion

The silage DM content determined in this study was close to the 622 g kg^{-1} value reported by Silva et al. (2019), additionally, a mean DM value for of 666 g kg^{-1} (n=62) was reported by Morais et al. (2017) for control treatments, similar to the value obtained in the present study. Factors that have the potential to affect the chemical composition of silage include harvest timing, moisture content at ensiling, particle size, silage density, proportion of cob, maize variety, fertilization level, soil, and climatic conditions (Carvalho et al., 2016; Krüger et al., 2020).

Lactic acid bacteria rapid accumulated starting at the first day at ensiling, consequently a rapid reduction in pH was observed, indicating that the stabilization of fermentation occurred until 14 days post-ensiling, however, the use of microbial inoculant was not associated with an increase in the LAB population. The occurrence of LAB in the present study were close to

those obtained by Silva et al. (2019) which reported a mean value of 7.74 log CFU g^{-1} of fresh forage for control treatment and Da Silva et al. (2015) who reported an average value of 7.93 CFU g^{-1} after 21 d of storage.

The occurrence of bacteria of the *Clostridium* genus was high throughout silage fermentation, with values that varied from 5.9 to 7.8 log CFU g^{-1} silage. The presence of this genus was likely a result of the previous contamination of the material in the field, since Clostridia are not normally part of the epiphytic forage population and contamination may be due the presence of soil or feces (Pahlow et al., 2003).

According to Muck (2010) clostridia can grow at lower pH values than enterobacteria and bacilli being more difficult to control. In addition, growth of clostridium is affected by the DM content of crops. To prevent clostridial growth in wet crops, a lower pH is required than that which is required in dry crops. This bacteria genus can cause losses in nitrogenous compounds in silage, in which is associated with reduced consumption by animals (Muck, 2010). In addition, may negatively affect animal health (Driehuis et al., 2018).

Ensiling HMC efficiently reduced enterobacteria population, in fact, the microorganisms were not observed in control and inoculated treatments from 3 d of ensiling. Enterobacteria are the principal competitors of lactic acid bacteria for the sugars in the crop, and pH values below five, are usually sufficient for reducing the enterobacterial population to undetectable levels within a few days (Muck, 2010). Total bacterial count tended to be similar to the other groups of bacteria evaluated, with exception of the enterobacteria. The use of the heterofermentative inoculant reduced the occurrence of molds at 14 days, which was a finding that was similar to that which was obtained by Filya et al. (2004) who obtained a reduction in the growth of molds and yeasts, in silages of wheat, sorghum, and corn plants. Propionic acid bacteria can ferment sugars and lactate to produce acetate and propionate, which inhibit the growth of yeasts and molds in silage (Rowghani et al., 2008).

According to Kung Junior et al. (2018) high mold population (>6 $\log^{10}$ CFU g^{-1} wet silage) are usually associated with aerobically spoiled silage. In this study, the mold population was lower than 6.0 log CFU g^{-1} and reduced linearly with storage duration to 1.6 log CFU g^{-1} at d 14. A quadratic effect was observed with regard to storage length in the control treatment, and the minimum value was estimated to occur after 12 d of storage. The presence of molds in silage is also associated with the production of mycotoxin, which damage animal health and can be transferred to milk and meat products (Avila & Carvalho, 2020; Vila Donat et al., 2018).

The enumeration of yeasts in silage is a useful tool because high population is usually associated with high concentrations of ethanol, and their presence may be associated with reduced aerobic stability (Muck, 2010). Kung et al. (1998) estimated that values lower than 6.0 log CFU g^{-1} for yeasts are necessary for enhancing the aerobic stability of silage. In this study, yeast population progressed significantly throughout fermentation. Yeast population between 5.2 and 7.0 log CFU g^{-1} after ensiling were observed, which may have influenced by the loss of the aerobic stability of silage.

The pH measured throughout the study (Table 3) indicate that the bacterial inoculant did not efficiently reduce pH values relative to the control treatment 3, 7, and 14 d post-ensiling. According to Woolford (1990) fermentation in wet grain silage may be more restricted than it is in most forage crops, since the material has relatively low moisture and soluble sugar content. However, in the present study, the pH values obtained from three d of storage length were equal to or below 4.0, which is a value recommended for both corn silage HMC (Kung et al., 2018). Da Silva et al. (2015) evaluated high-moisture corn silage (630 g kg^{-1} DM) and obtained pH values of 3.72 in the control treatment at 21 days of storage.

The addition of microbial inoculant increased the aerobic stability of silage relative to that of control treatment at both 28 and 56 days of storage. This effect may have been caused by the addition of *Propionibacteria* that can produce antifungal agents, acetate and propionate, which are capable of enhancing the aerobic stability of silage (Arriola et al., 2011). The results in the present study differ from those obtained by Filya et al. (2004) who evaluated whole crop silages of wheat, sorghum and maize adding an inoculant that contained *Propionibacteria acidipropionici* (1.0 x 10^6 CFU g^{-1} of fresh forage) and *Lactobacillus*

plantarum (1.0 x 10^6 CFU g^{-1} of fresh forage), either alone or in combination. The authors revealed that the addition of only *Propionibacteria* reduced yeast counts and promoted the maintenance of aerobic stability.

5. Conclusion

High-moisture corn grain silage has good fermentative quality and adequate pH values reach from the third d of storage independent of inoculant use, this additive also reduced molds population at 14 days of storage.

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