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Additive containing homo and heterolactic bacteria on the fermentation quality of maize silage

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ABSTRACT. This trial evaluated the addition of *Lactobacillus plantarum*, *L. brevis* and *Enterococcus faecium* combo additive against a control treatment. The silages were made in laboratory silos that were stored for 30, 60, 90 or 120 days before opening. We evaluated the chemical composition of the forage before and after ensiling and the fermentative losses of silages. The additive decreased ($p < 0.01$) effluent production (11.4 kg ton^{-1}) compared to control silage (14.0 kg ton^{-1}), but it increased ($p < 0.01$) the Neutral Detergent Fiber (NDF) and Acid Detergent Fiber (ADF) from 45.6% and 24.5% to 47.0 and 25.1% for control and additive silages, respectively. The storage periods affected ($p < 0.01$) effluent production, Dry Matter Losses (DML), NDF, ADF and pH variables. Fermentative losses were very low because of the adequate characteristics of maize for ensilage.

Keywords: aerobic stability, effluent, gases, *Lactobacillus brevis, Lactobacillus plantarum*, losses.

Aditivo contendo bactérias homo e heteroláticas sobre a qualidade fermentativa da silagem de milho

RESUMO. O presente ensaio experimental avaliou inoculação de aditivo composto por *Lactobacillus plantarum*, *L. brevis* e *Enterococcus faecium* em relação à silagem sem aditivo (Controle). As silagens foram confeccionadas em silos experimentais e armazenadas por 30, 60, 90 ou 120 dias antes da abertura dos silos. Foi avaliada a composição bromatológica da forragem antes e após a ensilagem, e as perdas fermentativas durante o processo. O aditivo avaliado reduziu ($p < 0,01$) a produção de efluente (11,4 kg t^{-1}) em relação à silagem Controle (14,0 kg t^{-1}). A inoculação com aditivo elevou os teores de Fibra em Detergente Neutro (FDN) e Fibra em Detergente Ácido (FDA), de 45,6 e 24,5% para 47,0 e 25,1% para as silagens controle e com aditivo, respectivamente. Os tempos de armazenamento influenciaram ($p < 0,01$) a produção de efluente, a perda de matéria seca (PMS), os teores de FDN e FDA, e o pH das silagens. As silagens apresentaram bons parâmetros de qualidade e as perdas fermentativas observadas foram bastante reduzidas, dadas as adequadas características da cultura do milho para a ensilagem.

Palavras-chave: estabilidade aeróbia, efluente, gases, *Lactobacillus brevis, Lactobacillus plantarum*, perdas.

Introduction

Although the silage pH quickly reduce and stabilize for about three to seven days after harvest (BOLSEN et al. 1992; STOKES; CHEN, 1994), a period of 21 to 30 days has been widely reported as adequate time for fermentation. But the fermentation proceeds beyond seven days, with significant increases of lactic acid, ethanol and ammonia nitrogen (NH_3-N) between seven and 120 days after harvest (BOLSEN et al., 1992). Ward and Ondarza (2008) verified the need for at least 120 days for the complete fermentation. Thus, some microbial processes have been shown to occur during prolonged storage.

During fermentation, the forage undergoes a series of processes that may cause nutrient losses through effluent and gases, which are undesirable and should be avoided in order to prevent feed quality loss. Losses caused by inadequate conservation may amount 40% (McDONALD et al., 1991). Therefore, studies using microbial additives are carried on aiming to minimize these losses. Inoculation with homolactic microorganisms (*Streptococcus* sp., *Lactobacillus* sp., *Leuconostoc* sp., *Pediococcus* sp., and *L. plantarum*) lead to lactic acid production during fermentation, reducing pH and controlling the proliferation of undesirable bacteria (YANG et al., 2010). Heterolactic microorganisms (*L. buchneri, L. brevis, Propionibacterium acidipropionici*)

produce other organic acids, besides the lactic acid, and are used to inhibit yeast growth and ensure greater aerobic silage stability after the silos opening (PAHLOW et al., 2003). The combination of homo and heterolactic bacterial strains aims to obtain a synergetic effect and avoid fermentative losses during the entire ensilage process.

The objective of this study was to investigate the fermentative losses and chemical composition of maize silage inoculated with or without an additive containing homo and heterolactic bacteria at different storage periods.

Material and methods

The trial was conducted in Castro, Paraná State, Brazil situated at 24º47'S, 050º00W and 1008 m. The climate is classified as 'Cfb' (Köppen classification). The data were collected at Centro de Pesquisas em Forragicultura (CPFOR) of the Federal University of Paraná, Curitiba, Paraná, Brazil. The maize crop (DKB 330) was planted on January 16, 2009, under no-till system with 45 cm spacing between rows and 3.1 seed per linear meter. We applied 240 kg ha^{-1} of the fertilizer 12-27-06 (N-P-K) to the crop, in addition to 246 kg ha^{-1} cover fertilization of 22-00-21. The crop was harvested at 111 days after planting.

The forage was harvested with a self propelled harvester regulated for 15 mm particle size. We established two treatments: Control (no additives) and Additive (commercial inoculant comprised of *Lactobacillus plantarum*, *L. brevis* and *Enterococcus faecium* – 1 x 10^5 colony-forming units (CFU) per fresh matter gram). The inoculant (Biostabil - Biomin®) was diluted in distilled water (one liter ton^{-1}) and uniformly applied onto the fresh forage prior to silos filling. We applied the same quantity of distilled water to the control treatment. Experimental silo compaction was carried out immediately after inoculant application in order to reach 600 kg per cubic meter.

Maize plants used in this test were taken from the same crop to compose both treatments. Five replicates per treatment were used, and each silo was considered an experimental unit. The silos were 20 L plastic buckets of 360 mm high and 290 mm wide with approximately 15 kg of silage capacity, equipped with an apparatus to determine gravimetric losses as described by Jobim et al. (2007). The silos were sealed with adhesive tape and stored at room temperature for four distinct periods: 30, 60, 90 or 120 days.

At each opening the silos were weighed again to determine gravimetric loss. Gas losses was calculated by the difference between initial and final DM weight in the experimental silos. The effluent amount was calculated by the initial and final silo weight containing sand in the bottom, according to Jobim et al. (2007). The DML was calculated by DM weight at ensiling and at opening, subtracting the produced effluent.

The silage was removed and homogenized in plastic bags for sampling and chemical and pH assessments (three aliquots per experimental unit). One aliquot was dried in a forced-ventilation oven at 55°C for 72 hours and processed in a Wiley mill using a 1-mm sieve. Another aliquot was used for pH determination after silo opening, in aqueous extract, where 25 g of silage was added to 0.225 L of deionized water and the pH was determined in a potentiometer model WTW 330i. Pressed juice was extracted from a third aliquot using a hydraulic press, acidified and frozen for analysis of fermentation end products.

The chemical analyses were carried out at the Laboratório de Nutrição Animal of the Federal University of Paraná. The DM, crude protein (CP) and ether extract (EE) contents were determined according to AOAC (1980) and NDF and ADF according to Van Soest et al. (1991), using thermostable α-amylase without sulfite, in a sequential method using ANKOM Fiber Analyser (ANKOM® Technology Corp.) as described by Holden (1999). Determinations of volatile fatty acids (acetic, propionic and butyric acids), lactic acid and ethanol were performed in the Laboratório de Análise de Alimentos of the São Paulo University (FMZ-USP) in Pirassununga, São Paulo, Brazil, according to ERWIN et al. (1961), using gas chromatography column containing silica glass 30 mx 0.53 min. The readings were performed by injecting 1.0 microliter sample into the chromatograph using standard solution as the basis for calculating the concentration of the end products of the fermentation of silage. Calculations of concentrations of fermentation products were performed on computer comparing the silage samples with the standard solution.

Aerobic stability was evaluated by controlling the temperature of silages exposed to air, according to Kung Jr. et al. (2000). Silage samples were kept in open buckets in the laboratory at 25 ± 1ºC for 5 days. The temperature was measured every 15 min. by a data logger. The aerobic stability (AS) was defined as the number of hours to increase 2°C the

temperature of silage. The thermal accumulation (AC5d) was the sum of the difference between silage and room temperature during 5 days.

Data were analyzed as repeated measures using the MIXED procedure of SAS (2001). The covariance structures tested were: components of variance (CV), compound symmetry (CS), first order autoregressive (AR1) and unstructured (UN). The structure chosen was the one with the lowest value for the Akaike Information Criterion (AIC). The effect of treatment within bucket was used as the error term to test the effect of treatment. The effect of storage period was compared by contrasts (linear and quadratic).

Results and discussion

The average composition of maize at ensiling was: 30.7% DM; 7.7% CP; 49.0% NDF; 24.6% ADF; 2.1% EE and 3.2% ash. This composition was in line with values described in the literature for high quality maize crops. Borreani et al. (2007) observed values of 34% DM; 6.3% CP; 41.2% NDF; 22.0% ADF; 2.0% EE and 4.0% ash, in maize plants used for silage production. The chemical composition of silages, fermentative losses and aerobic stability are shown in Table 1.

The effluent production, from overflow of cytoplasmatic content, showed quite low values, with an average of 12.7 kg ton^{-1} of fresh matter (FM). Possibly the lower effluent production in the Additive treatment can be attributed to the rapid pH decrease with the reduction of cell rupture by plant enzymes (McDONALD et al., 1991). The gas production did not differ ($p = 0.88$) between the treatments, with similar values for the Control (4.5% of DM) and Additive (4.4% of DM) treatments. Assessing scientific studies published between 1990 and 1995, Kung Jr. et al. (2003) observed DML reduction due to additives in only 35% of the trials. Thus, the results of our study are in line with those presented in most of the studies reported in literature.

The average DM content for both treatments was reduced from 30.7% at ensiling to 29.7% after 120 storage days. The DM decreased due to the fermentative process, plant cells respiration and anaerobic metabolism of microorganism during ensiling, where CO_2 and water are produced. Likewise, forage NDF contents showed average reduction from 49.0% in the fresh forage to 48.2% after 120 storage days. The NDF reduction of 0.8 percent unit indicates that part of the fiber was solubilized, probably the hemicellulose fraction. Herrmann et al. (2011) reported NDF reduction of 5.8 percent units in silages stored for 365 days. This effect may be considered positive to the process for providing soluble carbohydrate to fermentative microorganisms and raising silage intake by animals.

Silage inoculation altered ($p < 0.01$) the contents of fiber components (NDF and ADF) and ash, and did not affect the other chemical variables analyzed. The higher contents of these components found in the additive silage are possibly related to the consumption of part of soluble components during the metabolism of inoculated bacteria, leading to the increase of insoluble components. However, no inoculation effect was observed ($p = 0.06$) on silage pH. Thus, the soluble components loss in the effluent may explain these differences.

The authors suggest that in combined additives, the competition between microorganisms may reduce acid production resulting in higher pH.

This effect was not observed in this current study, where both silages showed pH 3.7. Although the inoculation of homolactic bacteria generally results in low pH in the silage due to lactic acid production (KUNG JR. et al., 2003), the epiphytic bacteria population can be large enough to perform the fermentative process.

In assessing 221 scientific papers on microbial additives in maize silage, Kung Jr. et al. (2003) observed that additive inoculation provided positive effects on pH of 60% of the studies. The authors verified that in less than 35% of the studies the inoculation reduce DML. These data are in line with the outputs found in the current study.

Table 1. Fermentative losses, chemical composition and aerobic stability of maize silages without additives (Control) or inoculated with *Lactobacillus plantarum*, *L. brevis* and *Enterococcus faecium* (Additive)

	Storage period (d)											
	30		60		90		120			p[3]		
Variable[1]	Control	Additive	Control	Additive	Control	Additive	Control	Additive	SEM[2]	A	S	A×S
Effluent[4] kg t^{-1}	6.04	4.22	11.21	6.38	18.75	14.57	19.95	20.45	1.08	0.01	< 0.01	0.09
Gases[6] % DM	1.87	4.26	4.13	3.94	5.52	4.47	6.53	4.86	1.17	0.88	0.16	0.35
DM losses[4] %	2.45	4.67	5.21	4.56	7.31	5.88	8.47	6.83	1.15	0.65	< 0.01	0.33
Dry matter[6] %	30.34	29.59	29.71	29.74	29.28	29.61	29.73	29.52	0,17	0.25	0.04	0.03
CP % DM	7.18	7.63	7.49	7.47	7.55	7.25	7.31	7.15	0.28	0.98	0.85	0.55
NDF[4;5] % DM	45,83	45.96	43.97	46.86	45.38	45.75	46.74	49.64	0.38	< 0.01	< 0.01	< 0.01
ADF[4;5] % DM	24.33	24.68	23.96	24.93	24.57	24.39	24.97	26.44	0.26	< 0.01	< 0.01	0.02

EE % DM	2.47	2.42	3.02	2.55	2.61	2.38	2.47	2.66	0.15	0.22	0.14	0.21
Ash % DM	2.92	3.47	3.13	3.35	3.15	3.40	3.07	3.52	0.09	< 0.01	0.68	0.20
Ethanol[4] % DM	1.48	1.54	1.85	1.72	2.30	2.11	2,31	2.38	0.05	0.18	< 0.01	0.02
Acetic acid[4] % DM	1.66	1.79	1.87	1.84	1.92	1.90	1.90	1.90	0.06	0.70	0.02	0.53
Propionic acid[4,5] % DM	0.04	0.04	0.05	0.05	0.06	0.06	0.06	0.05	0.00	0.22	< 0.01	< 0.01
Butiric acid[4] % DM	0.00	0.00	0.00	0.00	0.01	001	0.00	0.00	0.00	0.47	< 0.01	0.87
Lactic acid % DM	6,87	8,14	6,93	7,87	8,45	8,05	8,04	6,76	0.35	0.53	0.12	< 0.01
pH[4,5]	3.71	3.71	3.70	3.66	3.72	3.67	3.75	3.79	0.00	0.06	< 0.01	< 0.01
AS[4,5] h	46.40	45.37	30.40	30.40	41.40	40.30	35.10	32.12	1.40	0.23	< 0.01	0.76
AC5d[4] ºC	8.12	7.98	15.51	13.86	16.33	17.08	24.05	25.28	1.43	0.96	< 0.01	0.75

[1]DM: dry matter; CP: crude protein; NDF: neutral detergent fiber; ADF: acid detergent fiber; EE: ether extract; AS: aerobic stability; AC5d: thermal accumulation during 5 days; [2]SEM: standard error of the mean; [3]A: additive effect; S: storage period effect; A×S: interaction between additive application and storage period; [4]Linear effect ($p < 0.01$); [5]Quadratic effect ($p < 0.01$); 6Linear effect ($p < 0.05$).

The different storage periods affected the fermentative losses, chemical composition and aerobic stability of the silages (Table 1). The increase of storage period for all times (30, 60, 90 and 120 days) caused an increase of effluent production. This effect results from silage secondary fermentations that increase overflow of cell contents, and from fermentative DML, which leads to moisture increasing of silages. Even though the values observed may be considered low. Oliveira et al. (2010) reported effluent losses above 20 kg ton^{-1} of FM during 60-day fermentation of maize silages.

No effect of storage period was observed on gas production, although there was a trend to increase it ($p = 0.16$) due to storage. This effect was significant for DML, which showed higher values at 90 and 120 days after ensiling. The higher losses in longer storage periods may be related to secondary fermentations, normally caused by heterolactic bacteria (McDONALD et al., 1991). These microorganisms are characterized by CO_2 production in the conversion of lactic acid and carbohydrates to acetic and propionic acids, resulting in DML (FILYA, 2003).

The NDF and ADF variables showed higher values at 120 storage days (48.2% and 25.7% of DM) compared to the other periods. This is explained because NDF and ADF become proportionally more concentrated due to DML through gases and effluents in the fermentation process. However, although there was a significant interaction between additive and storage period (Table 1), with linear and quadratic effect for NDF and ADF, the biological explanation can not be correlated with the variables assessed in the current study. The pH remained below 3.8 in all storage periods, which is considered ideal for maize silages (KLEINSCHMIT et al., 2005). Silages stored for 120 days showed pH slightly higher (Figure 1) which may be related to lactic acid degradation in secondary fermentations (McDONALD et al., 1991; FILYA, 2003).

The contents of organic acids and ethanol in maize silages are shown in Table 1. No treatment effects were detected for volatile fatty acids, ethanol or lactic acid. Huisden et al. (2009) observed ethanol contents of 0.94 and 0.59% of DM for control and inoculated maize silages. MARI et al. (2009) reported 0.7% of DM of ethanol for control or heterolactic bacteria inoculated silages. In our study, the average the values were considered high for both treatments (1.99 and 1.94% DM).

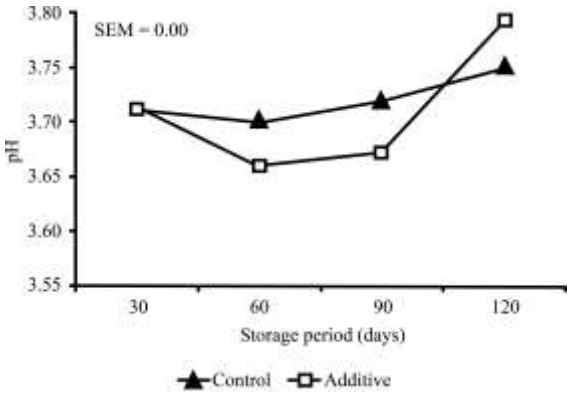

Figure 1. pH value of maize silages on different storage period. Linear and quadratic effect ($p < 0.01$) are significant for interaction between treatment and storage period.

The additive did not affect acid content in the silages. This effect was also reported by Kleinschmit
et al. (2005) assessing bacterial inoculants. MARI et al. (2009) evaluated heterolatic bacteria and did not observe difference in acetic acid contents (2.24 and 2.41% DM for control and inoculated silages, respectively). Kung Jr. et al. (1993) observed increase of acetic acid production (2.36 and 1.82% DM) for maize silages with *L. brevis* and without additives. In the current trial, *L. brevis* was inefficient for improving acetic acid content, and the strong homolactic fermentation

prevailed for both treatments.

The high values of lactic acid were also observed by Luther et al. (1986) and Meeske and Basson (1998). However, the lactic acid content in silages does not show the quantity of acid produced during the fermentative process, given that part of this acid may have been metabolized in secondary fermentations (MOON, 1983).

The increase of storage period led to high ethanol (Figure 2) and propionic acid content. Der Bedrosian et al. (2012) reported that the ethanol content generally increases until 45-90 days of storage for silages and remained constant thereafter. A similar effect occurred in this trial, where there seems to be a constant in ethanol content starting 90-120 days of storage. This effect may be attributed to secondary fermentation by heterolactic microorganisms and yeasts, which may have been allowed by the low acetic acid content.

Aragón et al. (2012) compared the effect of additives on maize silages, using the same dosages and additives from the current trial, and found average levels of 1.12% ethanol, whereas in our study was verified an average higher level (1.95% of DM).

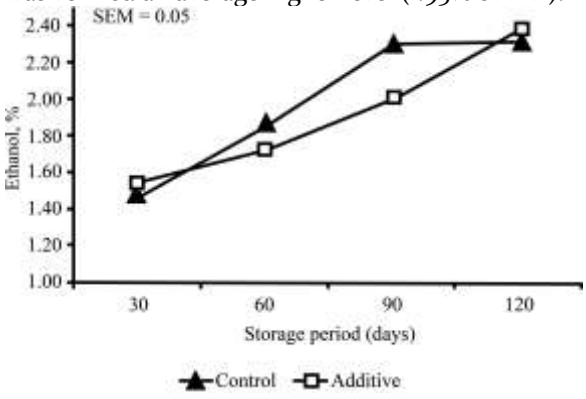

Figure 2. Ethanol content of maize silages on different storage period. Significant effect ($p < 0.01$) of storage period and significant linear effect ($p = 0.02$) of interaction between treatment and storage period.

The acetic acid content was influenced by storage period in this trial (Figure 3). Der Bedrosian et al. (2012) observed that acetic acid increased from 0.98% to 1.71%, for silages stored by 45 and 365 days. Aragón et al. (2012) observed 3.15% of acetic acid in maize silages inoculated with a *Lactobacillus plantarum, L. brevis* and *Enterococcus faecium* combo inoculant. The authors pointed out this increase as desirable since acetic acid is a good antifungal agent. Maize silages do not usually show butyric acid in their composition due to the low pH that inhibits clostridia growth (HU et al., 2009). Only traces of butyric and propionic acids were detected (Table 1), showing the absence of clostridia and silages with high fermentative quality.

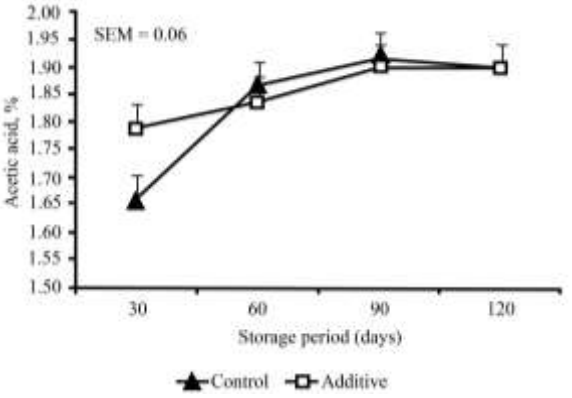

Figure 3. Acetic acid content of maize silages on different storage period. Significant effect ($p = 0.02$) of storage period.

The lactic acid content was not different among periods. Herrmann et al. (2011) found that long storage period decreased lactic acid and increased acetic acid concentrations compared to silages stored for a shorter period. Some strains of lacticacid bacteria are capable of using lactic acid in anaerobic conditions when glucose becomes a limiting substrate for their metabolism (LINDGREN et al., 1990). However, this effect was not observed in this trial.

The aerobic stability (AS) was similar for both treatments where the inoculation with heterolactic microorganism was not effective in inhibiting the growth of spoilage microorganisms. Danner et al. (2003) evaluated maize silages inoculated with *L. buchneri*, *L. brevis* and *L. plantarum* and found values of 274, 72 and 26 hours of AS, respectively. Aragón et al. (2012) found in maize silage inoculated with *Lactobacillus plantarum, L. brevis* and *Enterococcus faecium* values of 72 hours of AS. In this trial, the positive effect of the heterolactic microorganisms (*L. brevis*) on AS was not verified (Figure 4).

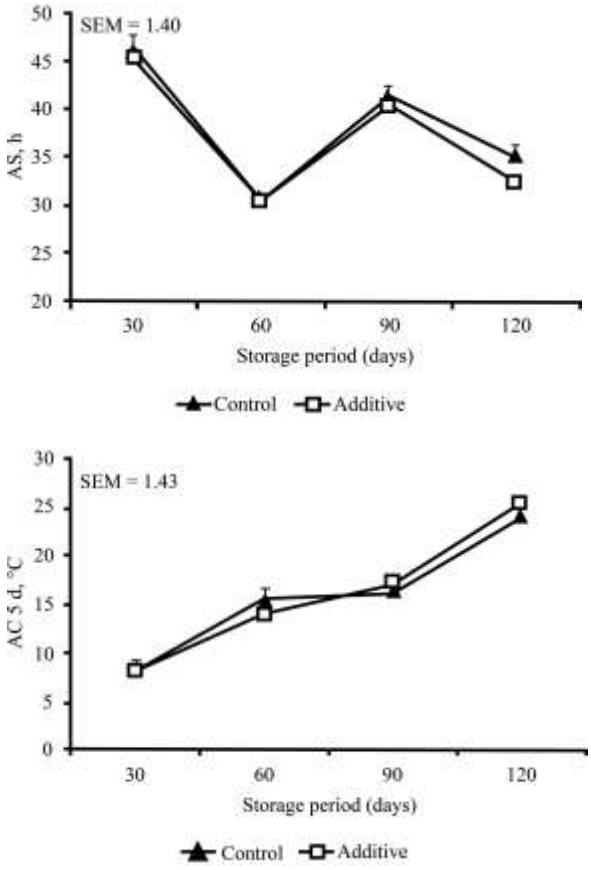

Figure 4. Aerobic stability (AS) and thermal accumulation during 5 days (AC5d) of maize silages on different storage period. Linear and Quadratic effect ($p < 0.01$) for AS and linear effect ($p < 0.01$) for AC5d.

The storage period influenced ($p < 0.01$) the AS and thermal accumulation during 5 days (AC5d). The AC5d was consistent with the lower aerobic stability. The silages showed low aerobic stability, probably due the high concentration of lactic acid and sugars which are preferentially utilized by spoilage microorganisms (KUNG Jr.; RANJIT, 2001; MUCK, 2004). The pH of silages (Figure 1) increased from 90-120 days of storage indicating that these silages were less stable aerobically (Figure 4).

The results found in the current study allow to state that maize silages produced from high quality forage at correct DM content and well stored have excellent quality and adequate fermentative characteristic, regardless of the microbial inoculation of additives.

Conclusion

The microbial additive was inefficient to reduce fermentative losses and to improve chemical composition or aerobic stability of maize silage. The silages showed a good fermentation pattern and reduced losses in all storage periods, and can be used 30 days after ensiling.

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