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The Effects of Short-Time Delayed Sealing on Fermentation, Aerobic Stability and Chemical Composition on Maize Silages

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Abstract: Despite the efforts to improve the methodological standards of silage trials, many factors that can influence the results of lab-scale studies need to be better understood. This study aimed to determine the effects of short-time delayed sealing and inoculation with a blend of *Lentilactobacillus buchneri* and *Lactiplantibacillus plantarum* on fermentation, aerobic stability, and chemical composition of silages. Whole-crop maize was treated with or without a commercial inoculant and ensiled (29.3% dry matter) for 55 days in 8.8 L PVC silos that were sealed immediately (up to 30 min delay) or after a delay (90, 150, or 210 min between chopping and sealing) with five replicates each. The increasing air exposure before sealing increased fermentation losses and reduced silage nutritional value. Crude protein and ash were significantly affected by inoculation, with control treatments showing higher ash and lower protein values. Lignin, neutral detergent fiber, and acid detergent fiber were only affected by the delay period. The longer the sealing delay, the higher the gas production, and the lower the starch values and lactic acid content observed in samples. Inoculation was inefficient in reducing total dry matter losses, but it increased aerobic stability, acetic acid, and ethanol contents of silages and reduced effluent loss. Control silages had higher total dry matter loss during the aerobic exposure than inoculated silages. The results confirmed that the delay periods tested were long enough to negatively interfere with the chemical composition of silages, especially the fibrous fraction content.

Keywords: aerobic exposure; corn silage; delayed sealing; *Lactiplantibacillus plantarum*; *Lentilactobacillus buchneri*

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1. Introduction

Whole-plant maize silage (WPMS) has become the predominant forage used in both dairy and beef cattle diets in Brazil [1,2], and numerous studies have shed light on strategies to improve the nutritional value of WPMS to produce high-quality feed [3].

In the last 30 years, relevant literature has been published focusing on developments on management practices of silage production from the field (pre-ensiling conditions) to the post-opening phase [3–5]. One of the most frequent subjects is the role of additives on the fermentative pattern and their effect on quality and aerobic stability of silages [6].

Inoculation of crops with strains of lactic acid bacteria (LAB) at the time of harvest has been the predominant type of additive for ensiling [7]. In Brazil, approximately one-fourth of the farmers use inoculants [1,2]. Combination inoculants of obligate and facultative heterofermentative lactic acid bacteria (LAB; e.g., *Lentilactobacillus buchneri* and *Lactiplantibacillus plantarum*), such as the commercial inoculant Pioneer 11C33 (11C33), aim

to provide the aerobic stability benefits of *L. buchneri* with the silage fermentation efficiency of homofermentative LAB [4,6].

Despite the efforts to improve the methodological standards for silage research, many factors that can influence the results of lab-scale trials need to be better understood, bringing into question the need for standardization of analytical procedures carried out experimentally and that enable the suitable comparison of results obtained from different research groups.

Among the knowledge obtained on silage making is the necessity of shortening the initial aerobic phase in the silo immediately after harvest [8]. After chopping, the presence of air in the forage mass delays the onset of fermentation, encouraging growth of undesirable microbes and leading to secondary fermentations [9]. The slow silo filling and delayed sealing negatively affect the silage quality once dry matter (DM) losses and quality changes occur during these phases [10–13], and although some losses are unavoidable, good management practices can reduce them [14].

Much research effort has been placed on understanding the changes that occur during the initial aerobic phase focusing on farm-scale practices [7,8]. However, as far as we know, no efforts to understand the impact of pre-ensiling conditions over the results of experimental trials in lab-scale silos have been described in the scientific literature. Recent meta-analyses compared research data on the chemical composition of WPMS produced for different experimental purposes (i.e., research groups that studied maize silage or that only used maize silage as a form of roughage) and reported differences among studies [15].

The assembly of experiments with silages is complex and laborious. Preparing the packaging for filling and sealing the experimental silos takes time, which can be a cause of variation among treatments. This delay is among the main limitations in tests evaluating additives in silages, and there is no methodology or way to cancel this effect, other than the standardization of the silage process. Thus, the comparison of experimental results that disregard the time of exposure to air before ensiling is questionable and, therefore, care must be taken when comparing studies that may have been exposed to very different experimental conditions.

We hypothesized that different delay periods may interfere with the evaluation of laboratory trials, leading to unreliable results. Therefore, the objective of this study was to compare the effects of short-time delayed ensiling on silage fermentation, aerobic stability, and chemical composition, as well as their effects on the results of inoculation with a blend of *Lentilactobacillus buchneri* and *Lactiplantibacillus plantarum* [16].

2. Materials and Methods

2.1. Study Site and Experiment Set-Up

The study was conducted in Pinhais, located in the State of Paraná, southern Brazil, in the Forage Research Center—CPFOR (25°32′05″ S, 49°12′23″ W, 906 m altitude, Köpen-Geiger climate type Cfb) at the Federal University of Paraná. The soil in the area is a Dystrophic Red Latosol (Rhodic Hapludox) [17]. Soil fertility was improved by adding 500 kg ha^{-1} of 10-20-20 NPK fertilizer in the whole experimental area. The used area consisted of 120 m^2 with ten 12 m rows spaced at 50 cm and 80,000 plants per hectare. Urea (80 kg N ha^{-1}) was added as a top dressing at 28 days after seeding.

The experiment was performed as a completely randomized design following a 4 × 2 factorial scheme: four delay periods (the time-lapse between chopping forage and silo sealing), with and without the use of a commercial inoculant (inoculated and non-inoculated control), with five replicates, totalizing 40 experimental units (silos). Maize (*Zea mays* L.) seeds, hybrid P4285VYHR (Pioneer, Corteva Agriscience™, Wilmington, DE, USA), and maize silage inoculant 11C33 (Pioneer, Corteva Agriscience™, Wilmington, DE, USA) were used in the field trial.

The eight treatments were (i) non-inoculated with 30 min delay (*T1C* hereafter); (ii) non-inoculated with 90 min delay (*T2C*); (iii) non-inoculated with 150 min delay (*T3C*); (iv) non-inoculated with 210 min delay (*T4C*); (v) inoculated with 11C33 and with 30 min delay

(*T1A*); (vi) inoculated with 11C33 and with 90 min delay (*T2A*); (vii) inoculated with 11C33 and with 150 min delay (*T3A*); and (viii) inoculated with 11C33 and with 210 min delay (*T4A*).

2.2. Ensiling

Whole-plant maize was manually harvested at 29.3% of DM and chopped in a stationary chopper (Super 15 T, Menta Ltd., Cajuru, SP, Brazil) with a 10 mm screen. Then, the processed forage was split in two small piles for each time-lapse tested. From the two piles, one received the inoculant, and the other (control pile) received no additive, only water (2 L t^{-1}). These piles were 4 m apart to avoid inoculant cross-contamination by runoff.

The inoculant was diluted in deionized water and manually sprayed and mixed into the forage at the rate of 2 L t^{-1}, following the manufacturer's instructions, reaching a final cell density of 1×10^5 colony-forming units g^{-1} of forage. To guarantee the effectiveness of the pre-programmed control (30 min) and delay times (90, 150, and 210 min), a trained staff of ten people worked simultaneously during the field trial assembly.

The chopped forage was packed into forty PVC silos (8.8 L). After packing to a density of approximately 550 kg DM m^{-3}, the silos were closed with a proper cap and completely sealed with the liquid plastic glue Selabond® (Carvalho and Ferreira Inc., Curitiba, PR, Brazil). The cap of each silo was equipped with a mobile apparatus to measure the volume of gas produced during fermentation (GProd), according to the work of Bueno et al. [18]. Additionally, the total gravimetric DM losses (TDML), gas losses (Gloss), and effluent losses (Eloss) were determined [19,20]. Silos were stored indoors at ambient temperature (average 22.7 $\pm$ 1 °C) for 55 days.

2.3. Chemical Composition, Gas Production, and Fermentative Profile of Silages

The PVC silos were weighed at the time of ensiling and before opening in order to estimate gravimetric DM losses according to the work of Jobim et al. [19]. Subsamples of 300 g were collected just before ensiling (fresh chopped forage), and after 55 days of storage for the analysis of pH, as described by Kung et al. [21], DM content (method number 934.01) [22], and chemical composition. Samples were oven dried at 60 °C for 48 h and ground in a Wiley forage mill (Arthur H. Thomas, Philadelphia, PA, USA) to pass a 1 mm screen.

Ground samples were sent to a specialized laboratory (3RLAB, Chapecó, SC, Brazil) for determination of DM, crude protein (CP), acid and neutral detergent fiber (ADF, NDF), lignin, starch, ether extract (EE), and ash by near-infrared reflectance spectroscopy (NIRS) using a scanning monochromator NIRSystems 6500 (NIRSystems, Silver Spring, MD, USA) according to the calibrations set by Rock River Laboratory (Rock River Laboratory Inc., Watertown, WI, USA). The content of ethanol-soluble carbohydrate (ESC) of the fresh forage was determined by the phenol-sulfuric method of Hall [23]. All chemical analysis was expressed on a dry weight basis and analyzed in duplicate.

Concentrations of volatile fatty acids (VFA) were obtained using high-performance liquid chromatography (HPLC, model LC-20A, Shimadzu, Kyoto, Kansai, Japan). HPLC columns used in the analysis included Rezex RHM 300 $\times$ 7.8 (Phenomenex, Torrance, CA, USA) and the analytical parameters recommended by the manufacturer (Mobile Phase: H_2SO_4 5.0 mmol L^{-1}; flow rate: 0.6 mL^{-1} min; column temperature: 65 °C).

2.4. Microbiological Analyses

For microbial counts, 25 g of fresh forage (pre-ensiled material) or silage was placed in sterile plastic bags containing 225 mL of Ringer solution (25%), as described by Restelatto et al. [20], with modifications. Briefly, the material was homogenized at 150 rpm for 4 min in a Stomacher shaker (Marconi-MA 440/CF, Sao Paulo, SP, Brazil) and then filtered with 3 layers of cheesecloth. Then, the extract was used for serial decimal dilution preparation (10^{-2} to 10^{-6}) in sterile tubes containing 9 mL of Man Rogosa Sharpe (MRS—Merck, Darmstadt, Germany) medium for lactic acid bacteria (LAB) count and 9 mL of Ringer solution (25%)

for yeast and mold count. From each dilution phase, 1 mL of the dilutions was transferred to 3M Petrifilm plates (3M®, Saint Paul, MN, USA). LAB count was performed in 3M Petrifilm AC plates, after incubation at 30 °C for 48 ± 4 h in anaerobic jars. Yeast and mold counts were performed in 3M Petrifilm YM plates, after incubation at 23.5 °C for 72 and 120 h, respectively.

2.5. Aerobic Stability Test

One sample of each silo was used to evaluate aerobic stability (AS) according to the work of Kung and Ranjit [24]. The sample (3 kg) was kept in a 20 L bucket, and its temperature was measured every 5 min during nine days via dataloggers (EL-USB-1, Lascar Electronics Inc., Erie, PA, USA) inserted into the silage mass center. The pH was measured at 0, 24, 96, 144, and 192 h after silo opening with a digital pH meter (Gehaka PG1800, Kaufmann group, Sao Paulo, SP, Brazil) according to the work of Kung et al. [25]. We sampled and weighed silage at the beginning and at the end of the trial to estimate the DM and total DM loss during aerobic exposure (TDMLAS). Aerobic stability was defined as the number of hours that the silage remained stable before reaching 2°C above ambient temperature (23 ± 1 °C) after silo opening [26,27].

2.6. Statistical Analyses

Data were subjected to preliminary exploratory analyses to check for normality and homocedasticity. Then, after meeting the ANOVA assumptions, we performed a two-way ANOVA using the MIXED procedure from SAS (SAS Institute, Inc., Cary, NC, USA), using the following model: $\hat{Y}ijk = \mu + Ai + Tj + (AT)ij + \varepsilon ijk$, where

$\hat{Y}ijk$ = observation "k" at level "i" of the additive and level "j" at delay period;

μ = general mean associated with all observations;

Ai = effect of additive "i", with i = 1 and 2;

Tj = effect of delay period "j", with j = 1, 2, 3, and 4;

$(AT)ij$ = effect of the interaction of level "i" of the additive with level "j" of the delay period;

εijk = random error with mean 0 and variance δ 2.

Tukey's test was applied considering the level of 5% ($p < 0.05$) of significance. When the interaction was verified, post-analysis was performed to investigate whether the effect occurred due to the delay period, to the additive, or both. To establish the correlation between the chemical and fermentative variables, a principal component analysis (PCA) with a biplot was adopted and generated using the software PAST 3.03 [28].

3. Results

3.1. Changes in pH and Chemical Composition of Silages

The effects of treatments on the pH and chemical composition of silages are presented in Table 1. The pH and DM values were affected by the interaction between the delay times and inoculation ($p < 0.05$). The DM content of silages decreased with the increasing delay period, and this decrease was significantly lower in the inoculated treatments ($p < 0.05$). For the control silages, *T2C* presented the highest DM content when compared to the other treatments ($p < 0.05$).

There was no interaction among delay times and inoculation for CP, ash, and lignin ($p > 0.05$). CP and ash variables were significantly affected by inoculation ($p < 0.05$), with control treatments showing higher ash and lower CP values. On the other hand, the ADF, NDF, and lignin contents increased along with increasing delay times ($p < 0.05$). Starch content was influenced by the interaction between time and inoculation ($p < 0.05$). Indeed, the longer the sealing delay, the lower the starch values observed in samples ($p < 0.05$). Moreover, starch content was also influenced by inoculation, and *T1A* had the highest value for this variable ($p < 0.05$).

Table 1. Effects of delay period and inoculation on pH and chemical composition of silages.

Item [2]	Additive (A)	Delay Period (T) [1]				Mean	SEM	*p*-Value		
		30	90	150	210			T	A	T × A
DM	Control	28.61 a	29.16 bA	28.51 b	28.51 bA	28.70 A	0.0370	0.0016	<0.001	<0.001
	Inoculated	28.78 a	28.25 bB	28.32 b	28.16 bB	28.38 B				
	Mean	28.70 a	28.71 a	28.41 b	28.33 b					
pH	Control	3.77 bc	3.88 aA	3.85 ab	3.68 cB	3.79	0.0085	0.0019	0.3756	0.001
	Inoculated	3.83	3.81 B	3.79	3.80 A	3.81				
	Mean	3.80 ab	3.85 a	3.82 a	3.74 b					
CP	Control	8.08 a	8.11 a	8.13 a	7.66 bB	8.21 A	0.0267	0.0120	<0.001	0.0371
	Inoculated	8.17	8.21	8.26	8.19 A	7.99 B				
	Mean	8.12 ab	8.16 a	8.20 a	7.93 b					
NDF	Control	47.94 bc	46.64 cB	49.27 b	52.64 aA	49.12	0.2307	<0.001	0.7963	0.0047
	Inoculated	46.99 b	49.54 aA	48.97 ab	50.48 aB	49.00				
	Mean	47.47 b	48.09 b	49.13 b	51.56 a					
ADF	Control	27.78 b	27.67 bB	28.88 b	30.97 a	28.83	0.1518	<0.001	0.5470	0.0138
	Inoculated	27.20 b	29.25 aA	28.51 ab	29.59 a	28.64				
	Mean	27.49 c	28.46 bc	28.70 b	30.28 a					
Ash	Control	3.07	3.36	3.28	3.12	3.21 A	0.0441	0.6546	0.0336	0.4386
	Inoculated	2.97	2.97	2.98	3.10	3.01 B				
	Mean	3.02	3.17	3.13	3.11					
Lignin	Control	3.90	3.92	4.05	4.32	4.05	0.0244	<0.001	0.4540	0.1248
	Inoculated	3.86	4.15	4.12	4.22	4.09				
	Mean	3.88 c	4.04 bc	4.09 ab	4.27 a					
Starch	Control	20.83 abB	22.53 aA	19.00 bB	15.85 cB	19.55 B	0.2089	<0.001	0.0320	<0.001
	Inoculated	22.68 aA	20.08 bB	20.67 abA	18.59 bA	20.51 A				
	Mean	21.76 a	21.30 ab	19.83 b	17.22 c					

[1] Delay period: the time-lapse between chopping forage and silo sealing in min. [2] Chemical composition is presented as percentage (%). A: additive; T: time; T × A: interaction additive × time; SEM: standard error of mean; DM: dry matter; CP: crude protein; NDF: neutral detergent fiber; ADF: acid detergent fiber. Lowercase letters in the same row and capital letters in the same column indicate significant differences by Tukey's test ($p < 0.05$).

3.2. Fermentation Profile and Influence of Silage on Gas Dynamics and Effluent Losses

The lactic acid, acetic acid, and ethanol contents of the silages were not affected by the interaction between the delay times and inoculation with the Pioneer® 11C33 brand (Table 2). The lactic acid content decreased with increasing delay sealing ($p < 0.05$), and the inoculated silages presented average values of 0.9% less of this acid when compared to the control silages, although no statistical difference was found ($p = 0.0613$). Acetic acid and ethanol contents were higher in the inoculated treatments ($p < 0.05$), and butyric acid was not detected in the tested silages (Table 2).

For the TDML variable, an interaction was observed between the delay sealing and inoculation ($p < 0.05$). For the inoculated silages, higher values of losses were obtained in the longest delay times ($p < 0.05$). The inoculation affected TDML, especially in the *T4A* treatment. For the control silages, *T2C* showed the lowest losses, being significantly different from the other treatments ($p < 0.05$).

The silages produced with the longest delay time for sealing presented higher GProd (Table 2). In general, inoculated silages showed higher GProd than control silages ($p < 0.05$). The silages exhibited GProd for eight days after sealing and, although the volume of gas produced during fermentation was small, it had a significant treatment effect (Table 2). The highest GProd was recorded in the first 24 h, and this rate decreased until the eighth day. Control silages presented accumulated GProd of 5.70 L^{-1} kg^{-1} DM, and 6.02 L^{-1} kg^{-1} DM was observed for the inoculated silages. The inoculated silages showed lower ELoss values compared to the control silages ($p < 0.05$). Gravimetric estimates of GLoss were small but showed a significant effect for the interaction between the factors tested, delay period, and inoculation ($p < 0.05$). Inoculation and increasing delay sealing resulted in silages with increased gloss ($p < 0.05$).

Table 2. Effect of ensiling time and inoculant on fermentation products, DM loss, gas production, and effluents.

Item [2]	Additive (A)	Delay Period (T) [1]				Mean	SEM	*p*-Value		
		30	90	150	210			T	A	T × A
Lactic acid	Control	9.29	9.29	8.51	7.48	8.65	0.1316	0.0046	0.0613	0.5445
	Inoculated	8.66	8.22	8.06	7.55	8.13				
	Mean	8.98 a	8.76 a	8.29 ab	7.52 b					
Acetic acid	Control	1.68	1.64	1.58	1.47	1.59 B	0.0360	0.1389	<0.001	0.9815
	Inoculated	2.62	2.54	2.47	2.32	2.49 A				
	Mean	2.15	2.09	2.03	1.90					
Ethanol	Control	0.26	0.28	0.29	0.29	0.28 B	0.0244	0.6339	<0.001	0.3461
	Inoculated	0.65	0.59	0.47	0.47	0.55 A				
	Mean	0.45	0.44	0.38	0.38					
TDML	Control	3.05 a	1.18 bB	3.28 a	3.36 aB	2.71 B	0.1343	0.0123	<0.001	0.0014
	Inoculated	3.08 b	4.41 abA	4.15 ab	4.73 aA	4.09 A				
	Mean	3.06 ab	2.79 b	3.71 ab	4.04 a					
GProd (L^{-1} kg^{-1} DM)	Control	5.45	5.70	5.68	6.17	5.75 B	0.0565	0.0174	0.0241	0.6664
	Inoculated	5.97	5.91	5.89	6.32	6.02 A				
	Mean	5.71 b	5.81 ab	5.78 b	6.25 a					
Eloss	Control	2.49	2.46	1.71	1.91	2.14 A	0.1271	0.1862	0.0029	0.2638
	Inoculated	0.76	1.91	1.14	1.40	1.30 B				
	Mean	1.63	2.19	1.43	1.65					
Gloss	Control	2.81 a	0.98 bB	3.13 a	3.17 aB	2.52 B	0.1320	0.0073	<0.001	0.0018
	Inoculated	3.00 b	4.22 abA	4.03 ab	4.63 aA	3.97 A				
	Mean	2.90 ab	2.60 b	3.58 ab	3.90 a					

[1] Delay period: the time-lapse between chopping forage and silo sealing in min. [2] Items are presented as percentage of dry matter (% DM) unless otherwise noted. A: additive; T ×A: interaction additive × time; T: time; SEM: standard error of mean; TDML: total dry matter losses; Gprod: gas production; Eloss: effluent losses; Gloss: gas losses. Lowercase letters in the same row and capital letters in the same column indicate significant differences by Tukey's test ($p < 0.05$).

3.3. Microbial Counts and Aerobic Stability

The treatments did not influence the microbial counts of silages (Table 3). Although there was a decrease in the yeast population with inoculation, no statistical difference was found between treatments ($p = 0.1049$).

Table 3. Microbial counts of silages.

Item [2]	Additive (A)	Delay Period (T) [1]				Mean	SEM	*p*-Value		
		30	90	150	210			T	A	T × A
LAB	Control	7.68	7.59	>8.0	>8.0	>8.0	0.57	0.2722	0.0614	-
	Inoculated	>8.0	>8.0	>8.0	-	>8.0				
	Mean	>8.0	>8.0	>8.0	>8.0					
Yeasts	Control	4.86	5.15	5.38	4.52	4.98	0.09	0.5950	0.1049	0.7352
	Inoculated	3.30	3.78	3.90	3.78	3.69				
	Mean	4.08	4.47	4.64	4.15					
Molds	Control	3.78	3.90	3.30	3.48	3.62	0.03	0.8547	0.6211	0.6211
	Inoculated	3.78	3.30	3.30	3.90	3.57				
	Mean	3.78	3.60	3.30	3.69					

[1] Delay period: The time-lapse between chopping forage and silo sealing in min. [2] Items are presented as log colony-forming units (log CFU g^{-1}). A: additive; T × A: interaction additive × time; T: time; SEM: standard error of mean; LAB: lactic acid bacteria.

For the AS and $TDML_{AS}$ variables, the interaction between the delay time and inoculation was significant ($p < 0.05$). In addition, the inoculated silages did not show a break in AS during the evaluation period of 213 h (Table 4). Moreover, AS was influenced by the delay times; however, only for control silages ($p < 0.05$). Control silages had higher $TDML_{AS}$ than inoculated silages ($p < 0.05$).

Table 4. Effects of delay period and inoculation on aerobic stability and on total dry matter loss during aerobic exposure.

Item [2]	Additive (A)	Delay Period (T) [1]				Mean	SEM	*p*-Value		
		30	90	150	210			T	A	T × A
AS	Control	62.03 cB	80.35 bB	90.20 aB	89.13 aB	80.45 B	0.1662	<0.001	<0.001	<0.001
	Inoculated	213.00 A	213.00 A	213.00 A	213.00 A	213.00 A				
	Mean	137.51 c	146.68 b	151.65 a	151.07 a					
$TDML_{AS}$	Control	7.84 abA	8.71 aA	6.19 b	6.56 ab	7.33 A	0.2363	0.2530	0.0011	0.0433
	Inoculated	5.47 B	5.46 B	6.71	4.75	5.60 B				
	Mean	6.66	7.09	6.45	5.65					

[1] Delay period: the time-lapse between chopping forage and silo sealing in min. [2] AS: aerobic stability (hours for silage to increase temperature in 2 °C above room temperature); TDMLAS: total dry matter loss during the aerobic exposure (%). A: additive; T × A: interaction additive × time; T: time; SEM: standard error of mean. Lowercase letters in the same row and capital letters in the same column indicate significant differences by Tukey's test ($p < 0.05$).

3.4. Association between Chemical and Fermentative Parameters of Silages

The PCA graph is presented in Figure 1. The inoculated samples were spread on the upper half of the graph, while control samples were grouped together on the bottom half. It was evident that there was the formation of exclusive clusters as a function of the delay period, especially in the non-inoculated (control) samples. Moreover, the inoculation seemed to have diminished the delay period effect, especially in the longer periods (*T2A*, *T3A*, and *T4A*), since these samples are grouped together on the left-upper-hand side of the graph; meanwhile, their control counterparts are spread out around the bottom half.

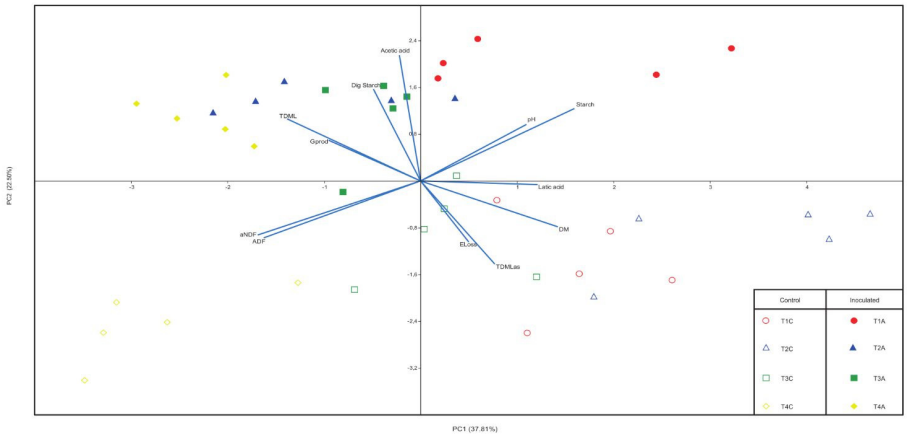

Figure 1. Biplot of principal component analysis (PCA) with respect to the association between chemical and fermentative parameters of silages. *T1C*: non-inoculated with 30 min delay; *T2C*: non-inoculated with 90 min delay; *T3C*: non-inoculated with 150 min delay; *T4C*: non-inoculated with 210 min delay; *T1A*: inoculated with 11C33 and with 30 min delay; *T2A*: inoculated with 11C33 and with 90 min delay; *T3A*: inoculated with 11C33 and with 150 min delay; *T4A*: inoculated with 11C33 and with 210 min delay.

The angle in PCA included between the arrows pointing at two variables determined the correlation between the parameters: sharp angles defined positive correlations, orthogonal angles defined no correlations, and obtuse angles defined negative correlations [29]. pH and starch were positively correlated and delimited the *T1A* group samples. Those parameters were negatively correlated with NDF and ADF located at the opposite direction, and the latter ones defined the *T4C* group. Acetic acid and dig-starch were positively correlated and defined the *T2A* and *T3A* sample groups, while TDML and GProd defined the *T4A* group. It is estimated that acetic acid, dig-starch, TDML, and GProd were more affected by inoculation than by the delay periods, as they were mostly influenced by PC2

(Figure 1). A direct and positive correlation was observed between the lactic acid and DM parameters, which along with ELoss and $TDML_{AS}$ clustered together close to PC1 and defined *T1C*, *T2C*, and *T3C* groups, corresponding to 37.81% of the sample's variability. Finally, there was a negative correlation between acetic acid production and $TDML_{AS}$ and ELoss.

4. Discussion

Farm-scale silos are often exposed to air for a few days until sealing, and this exposure is frequently associated with changes in the ensiled material [10,14]. In laboratory trials, after forage chopping, different delayed sealing times are commonly adopted by research groups, without further questioning about their harmful influence on the obtained data. The present study measured the influence of air exposure on silage quality, on a laboratory scale experimental routine, finding that the period of 210 min of air exposure was enough to promote qualitative and fermentative changes in silages. However, time intervals even greater than 210 min between chopping and sealing are common when setting up a silage trial, depending on the teamwork, machinery, and field distances.

4.1. Changes in pH and Chemical Composition of Silage

Analyzing the effects of air exposure on the treatments tested, we observed that the pH values of the evaluated silages were within the range commonly reported in the literature for well-preserved silages [15]. However, we found increased lignin contents with delayed sealing. It is important to consider that during the exposure of fresh forage to air, lignin concentrations were not affected by respiration, but there was a decrease in the soluble carbohydrate content that could proportionally increase the lignin values, since the results are expressed as percentages. Although lignin is not a carbohydrate, it is closely related to cell wall polysaccharides [30], and because its matrix presents polymer distribution around the cellulose microfibrils, fiber digestibility is worsened [16,31].

In the present study, inoculation decreased the ash content of silages. This probably indicates a better fermentation pattern when compared to control silages. In agreement with our findings, Coelho et al. [32] also found an effect of co-inoculation with *Lactiplantibacillus plantarum* and *Propionibacterium acidipropionici* on the reduction of ash content in re-ensiled maize silages. Although soluble carbohydrates from the cell content are the main substrate for microbes during ensiling, in a minor proportion, the constituents of the cell wall may also be cleaved and used for silage fermentation [33]. Likewise, the lower ash levels in treated silages are possibly related to the consumption of plant cell wall constituents by the stimulated metabolism of inoculated bacteria [18,30,32], and the extent of this breakage is likely favored in silages with higher moisture content [34].

Increased delayed sealing caused a reduction in silage CP content, indicating increased proteolysis since protein degradation remains as long as the oxygen in the silo is not consumed and the pH is not lowered [9,35,36]. Proteolysis refers to the enzymatic breakdown of proteins to soluble nonprotein nitrogen (NPN) forms such as peptides, free amino acids, and ammonia [37]. This loss of true protein may significantly reduce the efficiency of nitrogen utilization by ruminants [38,39]. Here, we did not measure soluble-NPN in silages tested, but this parameter is frequently correlated with CP reduction in silages as a result of proteolysis during ensiling [35]. However, in maize silages (both whole-plant maize and grain silages), proteolysis has been positively associated with starch digestibility due to the degradation of prolamins that surround the starch granules [40]. Such an increase in starch digestibility often results in greater synthesis of ruminal microbial protein and, in turn, higher N use efficiency [38,39]. Moreover, inoculated silages had higher CP content than the control treatments; as the use of additives is intended to preserve the nutrients in silage, its effect was considered positive.

Delayed sealing increased the NDF and ADF concentrations and reduced starch contents at silo opening. Similar findings were previously described [10,12,41,42]. The NDF and ADF increases may be a result of the respiration converting sugars into CO_2 [32,41].

For instance, Coelho et al. [32] found increased percentages of NDF in re-ensiled silages, and Yang [43] affirms that the exhaustion of WSC (85% reduction) after ensiling resulted in increases on structural carbohydrate concentrations represented by NDF and ADF contents on silages tested. It stands out that the increase in ADF contents indicates a worse nutritional value of silages, since there is a negative correlation between ADF contents and feed degradability. Furthermore, there are data showing that delaying sealing by 4 days led to a decline of up to 65% in WSC in silages [10].

Most of the bacteria responsible for silage fermentation are not associated with amylolytic activity, and a decrease in starch content is generally not expected due to the ensiling process. However, starch loss during fermentation has been reported in total mixed ration (TMR) silages and ranged between 17% and 23% [34,44]. In this study, starch hydrolysis may have been caused by the continued hydrolytic activity of the plant enzyme before sealing, yielding additional carbohydrate substrates needed for lactic acid fermentation [43]. The reduction in starch content may have also been caused by the metabolism of aerobic microorganisms, specifically fungi [12]. In rehydrated corn grain silages, Junges et al. [45] reported that bacterial enzymes were primarily responsible for proteolysis (60%), followed by plant proteases (30%). The quality of maize silages is evaluated according to their energy contents, as they are characterized by high quantities of starch and low contents of CP. From this perspective, the decrease in the amount of starch with increasing delay sealing in the evaluated silages characterizes a sharp drop in the nutritional value of the feed. However, this drop was mitigated by the inoculant that prevented starch decrease in the treated silages.

4.2. Fermentation Profile and Influence of Silage on Gas Dynamics and Effluent Losses

The fermentation products (lactate, acetate, and ethanol) of the experimental silages were within the typical range previously reported in the literature for well-preserved silages [13,15]. This fact is supported by the lack of detection of butyric acid, a final product of saccharolytic clostridial fermentation [11]. Indeed, previous studies show that maize silages usually have very low contents of butyric acid in their composition, due to the low pH that this material can quickly reach during fermentation [26].

LAB are generally considered oxygen-tolerant anaerobes with a fermentative metabolism, meaning that most of them can grow under aerobic conditions [46]. However, oxygen influences LAB metabolism [46–48], and similar to our findings, other studies have reported that air infiltration results in the decline of lactic acid concentration in maize silage [10,11,13] and also in barley silage [12]. The shift from fermentative to respiratory metabolism triggers in LAB an early entrance into the stationary phase along with marked changes in final products profiles, rerouting pyruvate away from lactate or from lactate and ethanol, respectively, in homofermentative and heterofermentative lactobacilli, resulting in acetate accumulation [46–48].

Decreased contents of lactic acid in silages is often associated with both prevented anaerobiosis and lactic acid fermentation caused by a delay in sealing [10]. Additionally, prolonged respiration processes by plant enzymes and aerobic microorganisms competing with LAB for WSC during the pre-sealing phase causes a loss of substrates for LAB that ended up contributing to reduce lactic acid concentrations even more [13]. Studying the effects of high ensiling temperatures, surface moisture at harvest, and delayed sealing for 3 h, Kim and Adesogan [11] reported that hot-wet-delay silages had the lowest proportion of lactic acid to acetic acid ratio and total concentrations of VFA.

Exposure to air before ensiling for 210 min resulted in silages with lower lactic acid concentrations than the immediately ensiled silage (30 min delay). The total absence of air is an essential factor because the extended respiration of plants exposed to air, due to delay sealing, causes the consumption of available carbohydrates for the natural fermentation of lactic acid, and consequently, the content of soluble carbohydrates is reduced with the increasing exposure time, increasing nutrient losses and decreasing the amount of lactic acid as a fermentation product.

In the present study, the concentrations of acetic acid and ethanol remained unchanged among the different periods of delayed sealing and were affected only by the additive. Indeed, the use of heterofermentative inoculant in maize silage under our experimental conditions increased the production of acetic acid and ethanol in treated silages, as previously reported [6,49].

The silages underwent great changes with increasing delay sealing; among them, we observed DM reduction as a result of greater losses represented by the parameters TDML, Eloss, and GProd. Bruning et al. [10] reported DM losses of up to 11% with increasing delay sealing. Likewise, Nutcher et al. [42] observed that a 1-day delay in sealing increased OM losses by 27.2% in the top 45 cm of maize silage under farm-scale conditions compared to immediate sealing (156 vs. 123 g kg^{-1} loss of OM). TDML and GProd were higher in inoculated silages *T2A*, *T3A*, and *T4A*; however, the use of the inoculant was effective in reducing ELoss in treated silages. The higher TDML and GProd in the inoculated silages corroborates with the higher levels of ethanol and acetic acid found in this material, resulting in greater losses of CO_2 to the environment through the LAB heterolactic fermentation pathway [50].

Gprod continued for eight days after sealing and had a significant treatment effect, with increased delayed sealing raising GProd values. Gas is produced from residual oxygen respiration, as well as when forage carbohydrates are fermented to acetate and butyrate, with fermentation to propionate occurring only from buffering of VFA [51]. In the present study, GProd increase with delay sealing can be associated with increased aerobic microbial population due to the extended air exposure [10,12]. In general, gas dynamics were similar to previous reports in maize and sugarcane silages in which gas production achieved its peak in the first few days of fermentation [18,52,53]. The first 24 h after sealing was defined by the highest GProd value, and this rate decreased until the eighth day. This scenario is typical of a high initial respiratory activity by plant cells and aerobic microorganisms, which is followed by a stabilization of the microbial activity resulting in GProd rate decrease [18–20].

4.3. Microbial Counts and Aerobic Stability of Silage

Microbial counts were not influenced by treatments under our experimental conditions. The absence of statistical significance in LAB numbers among treatments tested was probably due to the typically high numbers of epiphytic LAB (<10^5 colony-forming units g^{-1}) in the fresh crop [13,54]. However, it is important to emphasize that the counting plate technique does not represent the microbial dynamics throughout the assay but the number of viable colony-forming units at silo opening. In fact, we observed that inoculated silages produced more ethanol and acetic acid than control silages, which means that changes in LAB composition may still have occurred during the ensiling process. The bacterial succession in WPMS has been previously studied and is often dominated by *Leuconostoc* and *Weissella* before ensiling, and when favorable conditions, occur they are replaced by *Lactobacillus* during ensiling [55]. Regarding AS, we observed that delayed sealed silages (150 and 210 min of delay) were more stable upon air exposure. Conflicting results have been described on the effects of delayed sealing on AS [10–13]. Here, an increased aerobic stability was observed in inoculated silages. This fact can be correlated with the higher concentration of acetic acid in this material, which might lead to the inhibition of yeast and mold growth [5]. Likewise, for the variable $TDML_{AS}$, we observed a significant effect of the additive. Higher $TDML_{AS}$ on control silages is consistent with the higher lactic acid content found in this material, since yeasts involved in silage deterioration mainly consume lactic acid to obtain energy [56]. Reductions on lactic acid contents causes an increase in pH, triggering the degradation of the ensiled material [3,6].

Ensiling is a process mainly driven by the succession of bacterial groups regarding their divergence in diversity and dominance. In general, LAB competes with undesirable aerobic microbes for the water-soluble carbohydrates, and when their development prevails, it results in a drop in pH, limiting the occurrence of aerobic and basophilic microbes [6]. It

has been demonstrated that inoculated silages commonly exhibit a lower bacterial diversity when compared to natural silages [57], meaning that inoculation with LAB could enhance or at least accelerate the natural dominance of LAB over other bacteria. On the other hand, an unsuccessful fermentation process is characterized by an increasingly complex microbial community after fermentation [58]. Therefore, inoculation with commercial products such as inoculant 11C33 has both an immediate impact (with the production of lactic acid by *L. plantarum*) as well as a long-term impact (with the production of acetic acid by *L. buchneri*), with the objective to promote an effect on the post-opening phase by enhancing aerobic stability and reducing silage total dry matter loss upon aerobic exposure. Here, the inoculant successfully tested preserved CP and starch contents in treated silages when compared with control silages. Moreover, inoculated silages had higher contents of acetic acid that finally resulted in enhanced AS and reduced silage $TDML_{AS}$.

4.4. Association between Chemical and Fermentative Parameters of Silage

In analyzing the PCA, the correlations found between the plotted parameters in our study corroborated with the above-mentioned results regarding the chemical and fermentative profile of silages evaluated. In short, starch defined *T1A* group samples, which corroborates with starch reduction with increasing delay periods [10,32]. The negative correlation with NDF and ADF parameters are in accordance with WSC exhaustion as a result of the extended air exposure (210 min delay samples) [18,43]. The correlation of acetic acid with inoculated samples addresses its higher content in this material, as a result of the heterolatic inoculation [5]. Likewise, the enhanced aerobic stability found in inoculated silages is visually clear by the negative correlation established among acetic acid, $TDML_{AS}$, and ELoss variables. In fact, ELoss and $TDML_{AS}$ parameters were more predominant in control silages and therefore defined this sample position over the PCA graph.

In general, the chemical composition of evaluated silages allows us to understand that the delay periods tested in the present study were long enough to negatively interfere in some of the results. For example, in assuming a trial where the control treatment was carried out immediately after harvesting and processing (0–90 min), and when the inoculated treatment was carried out from 150 to 210 min later. If we focus only on the variables where there was no additive effect (e.g., NDF, ADF, and lignin), it would be concluded that the additive would have increased the fibrous fraction content, which is not true. It is important to emphasize that the delay periods tested here, and even those much longer than these, are commonly adopted by research groups, and without further questioning their influence on the data obtained, the conclusions made consequently compromise their recommendations. In order to ensure the most accurate conclusions on silage studies, it is recommended that all treatments are carried out concomitantly, and in the shortest time possible, according to the objectives of the trial in question. More importantly, the reader needs to be warned about the time spent in the process of making both treated and control silages in order to be sure of its conclusions. Further investigations are encouraged to broaden the database also by using data from other silage types (e.g., sugarcane and wet-grain silages). Moreover, the study of the microbial community might provide more concrete insights over the effect of the inoculant tested on the chemical composition and fermentative profile of silages subjected to increasing sealing delay periods.

5. Conclusions

Increasing the time of air exposure before sealing increases fermentation losses and reduces the quality of silages. The different times tested affected the results of experimental trials that assess the effects of silage inoculants, leading to wrong conclusions. The inoculant tested was inefficient in reducing total dry matter losses, but it markedly increased the aerobic stability, reduced effluent loss, and improved the nutritional value of the treated silages.

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