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Mycological diversity in whole-plant corn silages inoculated with *Lentilactobacillus buchneri* or *Lentilactobacillus farraginis*

(*Diversidade micológica em silagens de milho planta inteira inoculadas com* Lentilactobacillus buchneri *ou* Lentilactobacillus farraginis)

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ABSTRACT

The objective was to investigate the effect of wild lactic acid bacteria (LAB) strains on the fungal and chemical composition of whole-plant corn silage. Three treatments were evaluated: CON—without inoculation, LB—inoculated with *Lentilactobacillus buchneri* (CCMA 1366), and LF—inoculated with *L. farraginis* (CCMA 1362). The silages were stored for 29, 103, and 193 d. The highest acetic acid (19.7 g/kg DM) and 1,2-propanediol (6.3 g/kg DM) concentrations were found in the LF, the highest aerobic stability was observed in this silage after 29 d (158.3 h). The yeast population was below the detection level at 29 d in the LF and 103 and 193 d in all silages. Seven genera of filamentous fungi (FF) were identified before ensiling; none of these were found after ensiling. At 29 d, *Monascus pilosus* was identified in the LB after aerobic exposure but did not produce citrinin. At 103 and 193 d, there was *Aspergillus fumigatus* predominance. *A. flavus* was identified at 103 d in the CON and LB; none produced B_1, B_2, G_1, or G_2 toxins. LAB strains modified the silages' chemical and FF composition, and LF increased aerobic stability and reduced FF's yeast population and diversity.

Keywords: *Aspergillus*, β-Tubulin, filamentous fungi molecular identification, MALDI-TOF-MS, silage quality

RESUMO

O objetivo foi investigar o efeito de cepas selvagens de bactérias do ácido lático (BAL) sobre a composição fúngica e química da silagem de planta inteira de milho. Três tratamentos foram avaliados: CON - sem inoculação, LB - inoculado com Lentilactobacillus buchneri *(CCMA 1366) e LF - inoculado com* L. farraginis *(CCMA 1362). As silagens foram armazenadas por 29, 103 e 193 dias. As maiores concentrações de ácido acético (19,7g/kg MS) e 1,2-propanodiol (6,3g/kg MS) foram encontradas na silagem LF, e a maior estabilidade aeróbia foi observada nessa silagem após 29 dias (158,3h). A população de leveduras ficou abaixo do nível de detecção aos 29 dias na silagem LF e aos 103 e 193 dias em todas as silagens. Sete gêneros de fungos filamentosos (FF) foram identificados antes da ensilagem; nenhum deles foi encontrado após a ensilagem. Aos 29 dias,* Monascus pilosus *foi identificado na silagem LB após exposição aeróbia, mas não produziu citrinina. Aos 103 e 193 dias, houve predominância de* Aspergillus fumigatus. A. flavus *foi identificado aos 103 dias nas silagens CON e LB; nenhum produziu toxinas B1, B2, G1 ou G2. As cepas de BAL modificaram a composição química, e de FF das silagens; LF aumentou a estabilidade aeróbia e reduziu a população e a diversidade de leveduras FF.*

Palavras-chave: Aspergillus, *β-tubulina, identificação molecular de fungos filamentosos, MALDI-TOF-MS, qualidade de silagem*

INTRODUCTION

One of the biggest problems related to whole-plant corn silage (WPCS) fermentation is the growth of yeasts and filamentous fungi (FF) (Ferrero *et al*., 2019; Ogunade *et al*., 2018). These microorganisms' growth is more intense in tropical regions (Driehuis *et al*., 2018) and is directly related to dry matter (DM) loss, reduced nutritional quality and possible contamination with mycotoxins. Contamination with mycotoxins can cause damage to animal health, hence reducing the animal performance

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(Ferrero *et al*., 2019). The best way to avoid mycotoxins' production is to inhibit the growth that produces them.

Bacterial inoculants have been studied to minimize fermentative losses, reduce the presence of toxic metabolites, aerobic deterioration and the presence of undesirable microorganisms, thus improving the overall quality of the silage (Amaral *et al*., 2020; Costa *et al*., 2021). The species *Lentilactobacillus buchneri* has been the most used and studied species to reducing the growth of fungi and increasing the aerobic stability of silages. This ability is strain-specific; therefore, different strains may perform differently (Amaral *et al*., 2020; Costa *et al*., 2021). Other heterofermentative LAB species have shown promising results in inhibiting fungi (Amaral *et al*., 2020; Costa *et al*., 2021). Recently, a wild strain of *L. farraginis* (CCMA 1362) was evaluated in elephant grass silage and WPCS harvested with high DM (454.0 g/kg) (Amaral *et al*., 2020; Costa *et al*., 2021). In both studies, inoculation of this strain reduced the DM losses and yeast population while increasing the aerobic stability of the silage. Therefore, we aimed to investigate the effect of wild strains of *L. buchneri* (CCMA 1366) and *L. farraginis* (CCMA 1362) on the fungal and chemical composition of WPCS.

MATERIALS AND METHODS

The strains *L. buchneri* (CCMA1366) and *L. farraginis* (CCMA 1362) were selected from 88 strains isolated from WPCS (Costa *et al*., 2021). The inoculants (20mL of each culture) were mixed with pure distilled water (380mL) and sprayed in the forage (20kg). The same volume of pure distilled water (400mL) was added to the control treatment. Samples of each culture were collected to check the population of viable cells (plating in MRS ágar; M641I, HiMedia®). The population was 8 and 11 log colony-forming units (CFU)/mL for the *L. buchneri* and *L. farraginis*, respectively. The inoculated population was 5 and 8 log CFU/g of forage for the *L. buchneri* and *L. farraginis* strains, respectively.

Corn (hybrid RB9077 VT PRO Riber – KWS Sementes S.A, Brazil) were harvested and chopped, resulting in medium-sized particles 10.65±3.6mm. Plastic containers (30 L) were used as experimental silos, three replicates were prepared totaling 27 experimental units . The forage was compacted to a density of 701±21kg/m^3 and stored for 29, 103, and 193 d at ambient temperature (18°C - 28°C).

After 29 and 193 d of storage, the silos were opened, and 15 kg of silage were exposed to the air at ambient temperature. Data loggers (Impac, model MI-IN-D-2-L; São Paulo, Brazil) were inserted into the silage mass. The aerobic stability was defined as the number of hours the silage remained stable before rising more than 2ºC above the ambient temperature. The ambient temperature averages in 29, and 193d were 28.1ºC and 28.5ºC, respectively. After 24h of aerobic exposure, samples were collected to assess pH, a_w, yeast, FF count, isolation, and identification of FF. An aqueous extract was prepared (25g of fresh plant or silagein 225mL of distilled water) to measure the pH (Digimed®DM 20 Potentiometer; Digicrom Instrumentos, SP, Brazil). 2 mL aliquots were acidified from this aqueous extract with 10 µL of H_2SO_4 50% (v/v) and frozen to analyze the concentration of ethanol, 1,2-propanediol, and lactic, acetic, propionic, and butyric acids by high-performance liquid chromatography (HPLC). The aw of the fresh plant and silages were verified using Aqua-Lab equipment (Decagon Devices, Inc., USA). To determine the DM contents, the samples were dried at 105°C for 24 h.

Serial dilutions were prepared using 25g of the sample and 225mL of sterile 0.1% peptone water. Aliquots of 0.1 mL of the dilutions were plated in the Dichloran Rose Bengal chloramphenicol medium (DRBC; Himedia®), Dichloran glycerol 18% medium (DG18; Himedia®) and Potato Dextrose agar medium (PDA; Himedia®). The plates were incubated at 25ºC. The counting was performed after 2 and 5 d of incubation for FF (counting and isolating) and yeasts (counting), respectively.

Each FF colony type was macroscopically characterized (mycelium structure, size, conidia, color of reverse, and mycelium). The square root of the number of colonies counted for each type was restreaked and purified on a malt agar medium (MA; Acumedia, Lansing, MI, USA). For morphological identification, all isolates were transferred to specific culture media

according to Klich (2002), and Pitt and Hocking (1999). Finally, macroscopic characters were studied under different media and growth conditions (Habibi and Afzali 2021).

The FF isolates were identified and statistical clustering according to their protein profile. All isolated were grown for 72h on plates using MEA. With the help of a pipette tip, part of the fungal growth was placed directly onto the MALDI-TOF stainless steel plate. One µL of the matrix solution a-cyano-4-hydroxycinnamic acid (CHCA) previously prepared into a final concentration of 10mg CHCA/mL of organic solution [ethanol/acetonitrile/trifluoroacetic acid (10%), 1:1:1] was added and gently mixed. The samples were dried at room temperature and analyzed by MALDI-TOF MS (UltrafleXtreme MALDI-TOF MS—Bruker Daltonics; Bremen, Germany). The clustering (Biotyper 3.0 software, Bruker Daltonics) was performed separately for the fungi isolated before ensiling (Supplementary Fig. 1), at the silos opening (Supplementary Fig. 2), and isolated after 24 h of aerobic exposure (Supplementary Fig. 3).

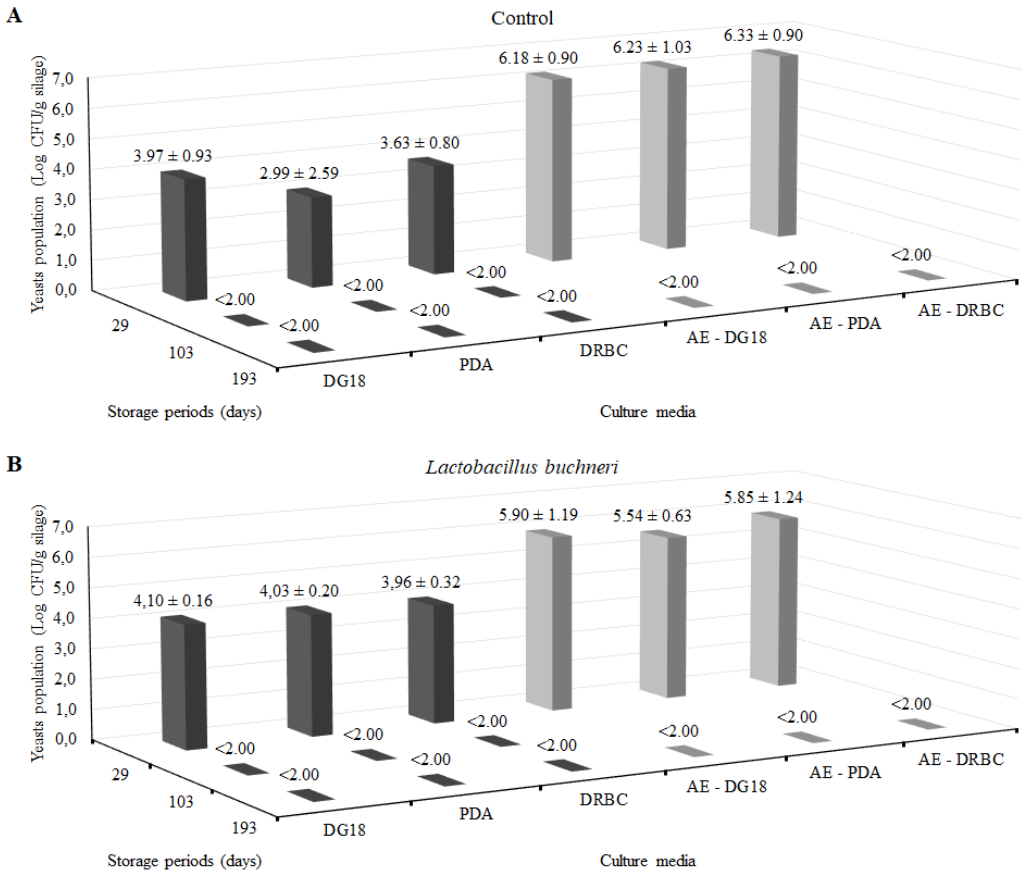


Figure 1. Yeast population (mean of three replicates ± standard deviation) in whole-plant corn silage without inoculant (control) (A) or inoculated with *Lentilactobacillus buchneri* (CCMA1366) (B) at different storage periods and after 24 h of aerobic exposure in each storage period.
*Culture media: DG18 - Dichloran glycerol 18%; PDA - Potato dextrose ágar; DRBC - Dichloran rose bengal chloramphenicol.
** There was no growth of yeast in the silages inoculated with *Lentilactobacillus farraginis* (CCMA1362).

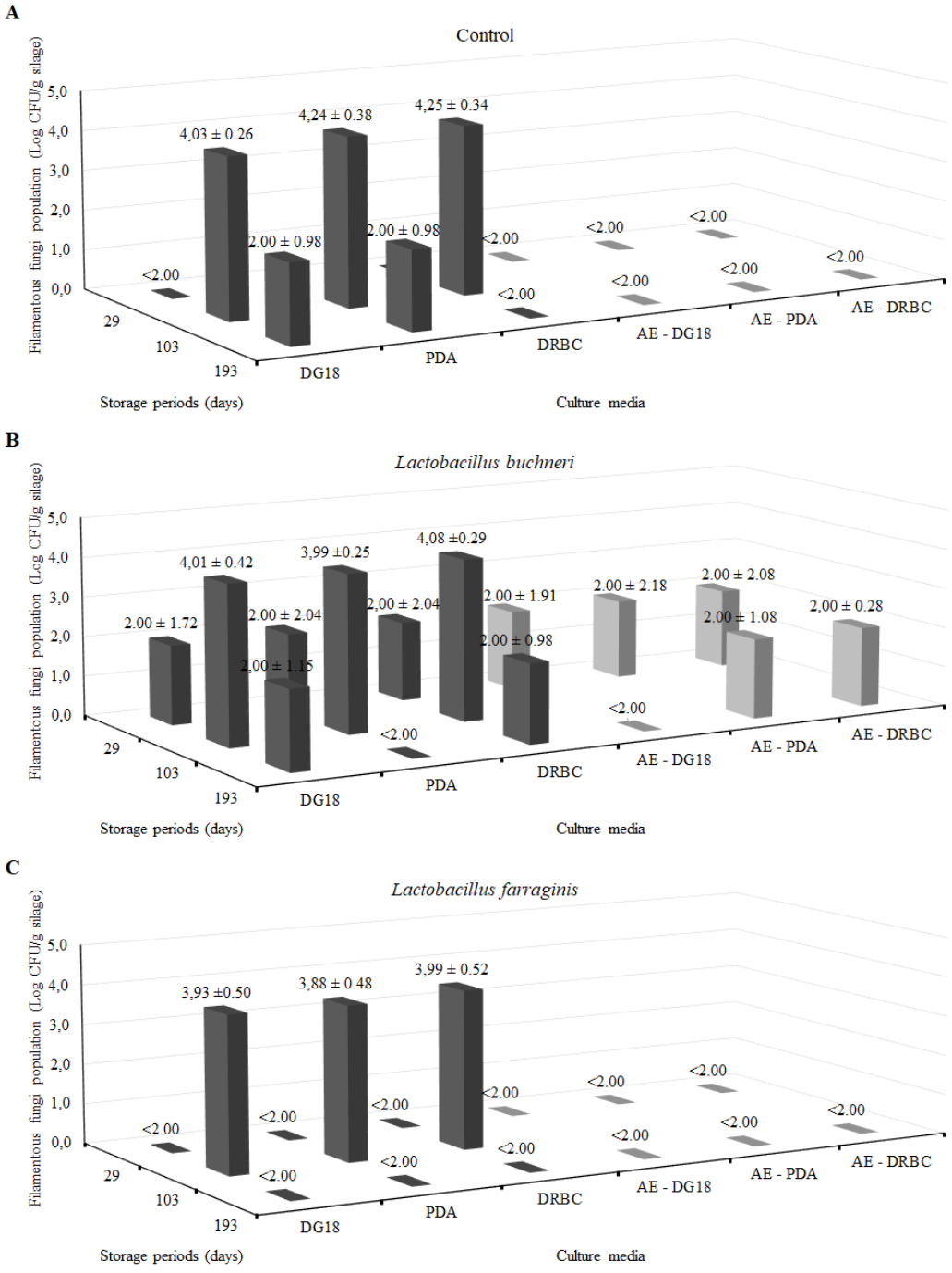
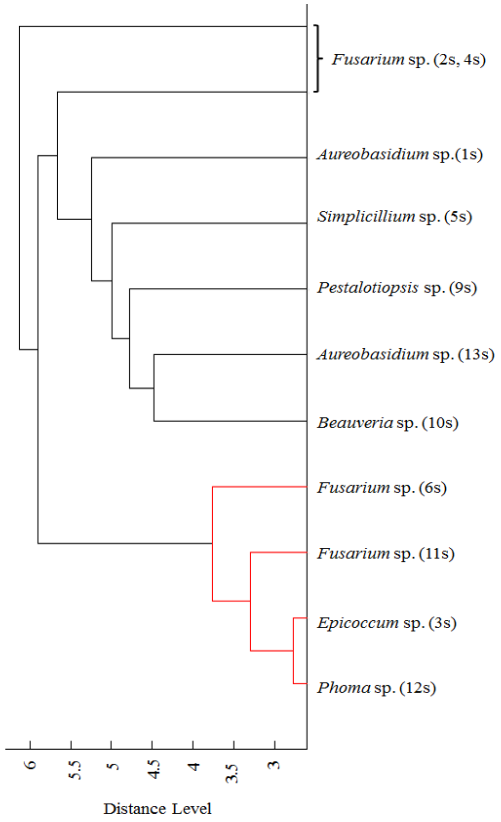


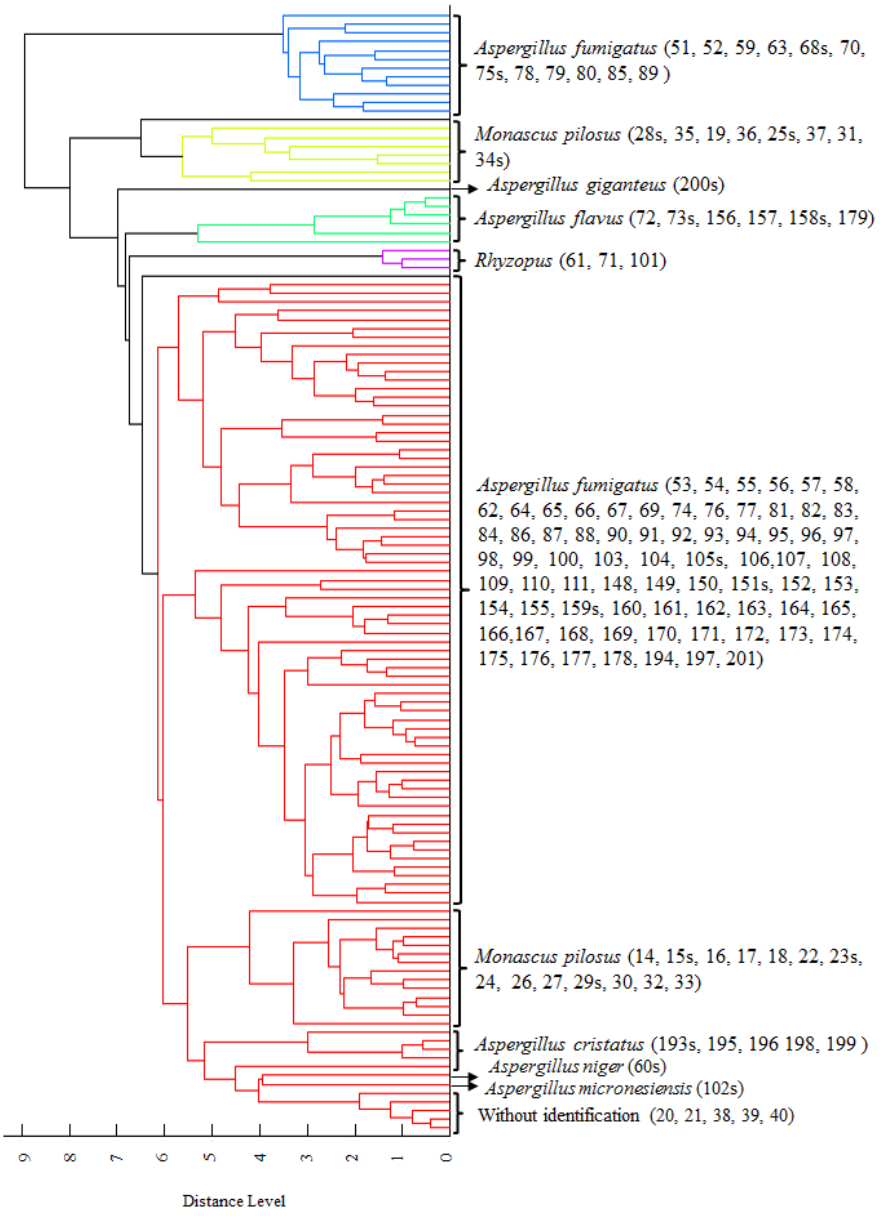
Figure 2. Filamentous fungi population (mean of three replicates ± standard deviation) in whole-plant corn silage without inoculant (control) (A) or inoculated with *Lentilactobacillus buchneri* (CCMA1366) (B) or *Lentilactobacillus farraginis* (CCMA1362) (C) at different storage periods and after 24 h of aerobic exposure in each storage period.
*Culture media: DG18 - Dichloran glycerol 18%; PDA - Potato dextrose ágar; DRBC - Dichloran rose bengal chloramphenicol.

According to FF grouping results by the MALDI-TOF technique, some isolates were selected from the different groups formed (Supplementary Figs. 1, 2, and 3); these were identified from the sequencing of the ITS and β-Tubulin regions. The fungi were grown in malt extract broth (MEB; Merck, Darmstadt, Germany) for 72 h, and the mycelium was recovered by filtration. A portion of the fungal growth (approximately 0.1mg) was added to a centrifuge tube containing glass beads (0.5mm). Three hundred µL of breaking buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris pH 8, 1 mM EDTA pH 8) was added to the mycelium and then was homogenized in a vortex (1 min). Then, it was added 300 µL phenol/chloroform/isoamyl alcohol (25:24:1) and again vortexed for 15 min. The tubes were sonicated for 5 min. Three hundred µL of TE (10mM Tris, 1 mM EDTA pH 7.6) was added, and the bead/mycelium mixture was centrifuged for 10 min at 16,000 × *g* at 4°C after which the aqueous phase was collected. The DNA was precipitated with two volumes of 100% iced ethanol and centrifuged at 16,000 × *g* at 4°C for 10 min; then, the supernatant was discarded. Five hundred µL of 70% iced ethanol was added to wash the pellet and centrifuged at 16,000 × *g* at 4°C for 2 min. The supernatant was discarded, and the pellet was dried and resuspended in 50µL of sterile ultra-pure water. For the ITS region, the primers used for DNA amplification were: ITS1 Forward (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 Reverse (5'-TCCTCCGCTTATTGATATGC-3'). For β-Tubulin region, they were: Bt2a Forward (5'–GGTAACCAAATCGGTGCTGCTTTC – 3') and Bt2b Reverse (5' – ACCCTCAAGTTGTAGTGACCCTTGGC - 3'). The PCR products were sent for sequencing to the Myleus Facility (MG, BRA). The sequences were compared with the GenBank database using the Blast tool. The sequenced FF were conserved and deposited in the Culture Collection of Microorganisms of the Department of Food Science (CCDCA)- WDCM 1081 of the Federal University of Lavras, Brazil.

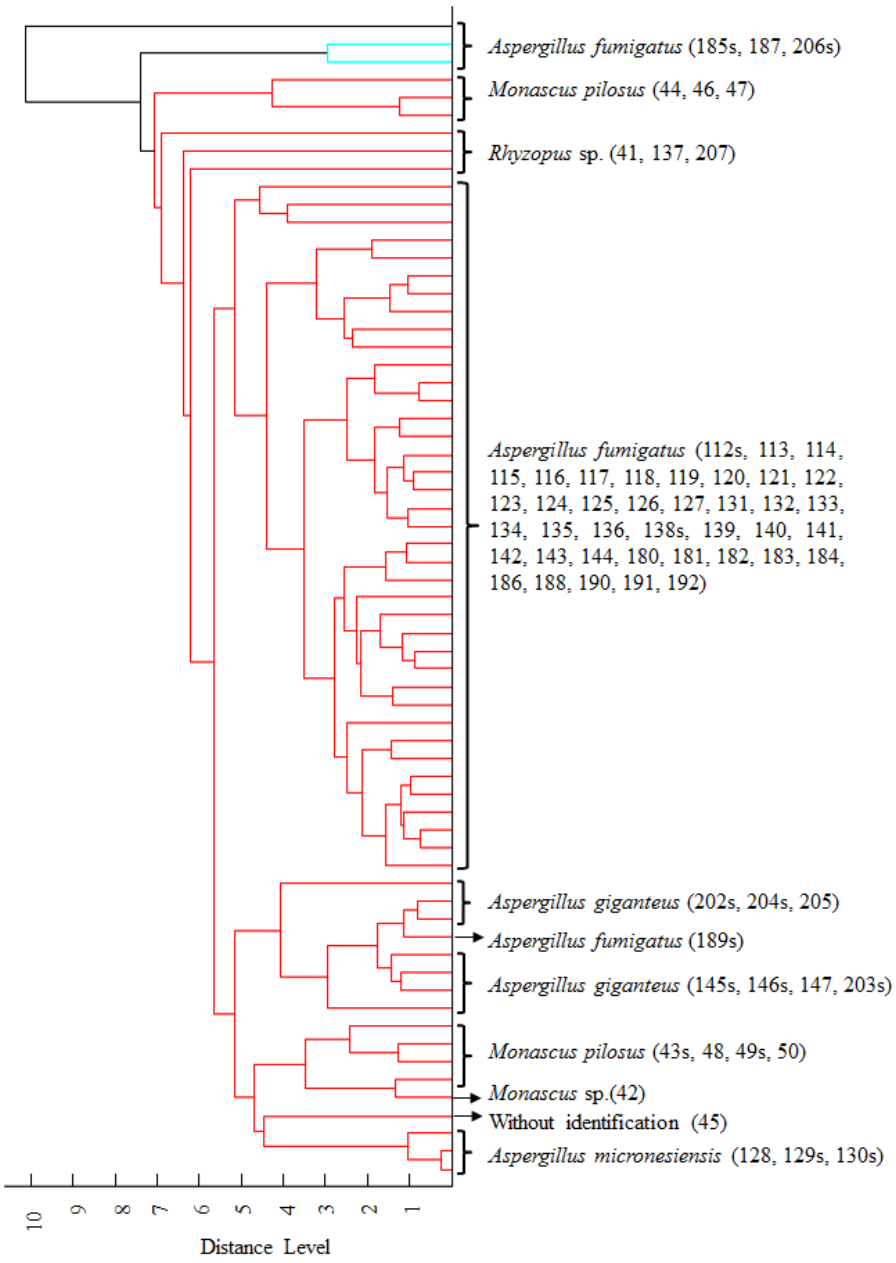
The toxigenic potential of isolates belonging to the genus *Aspergillus* section Flavi was evaluated using a thin layer chromatography. The isolates were inoculated in yeast extract sucrose medium (YES; containing the following components: yeast extract 20 g; saccharose 150 g; $ZnSO_4 \cdot 7H_2O$ 0.1 g; $CuSO_4 \cdot 5H_2O$ 0.05 g; agar 20 g; distilled water 1000 mL) and incubated for 10 d at 25ºC . Standard aflatoxin B1, B2, G1, and G2 solutions were used (Sigma-Aldrich®) on thin chromatography plates (Merck-Silica gel 60, 20 × 20) and as a mobile phase TEF - Toluene; ethyl acetate; formic acid 90% (60:30:10). To confirm toxin production, the plate was exposed to UV light with λ 366 nm in a Chromatovisor CAMAG (Uf-Betrachter). The isolates were considered producers if they presented a retention factor (RF) and fluorescence in the same color on the plate concerning the standards.



Supplementary Figure 1. Dendrogram derived from a protein cluster analysis of filamentous fungi isolated from whole-plant corn before ensiling using MALDI-TOF MS. The dendogram was created using BioTyper MSP Dendogram Creation (Bruker Daltonics). Numbers in brackets are the strain identification. Strains number followed by the letter 's' indicate that the strain was sequenced.



Supplementary Figure 2. Dendrogram derived from a protein cluster analysis of filamentous fungi isolated from whole-plant corn silage after 29, 103 and 193 d of ensiling using MALDI-TOF MS. The dendogram was created using BioTyper MSP Dendogram Creation (Bruker Daltonics). Numbers in brackets are the strain identification. Strains number followed by the letter 's' indicate that the strain was sequenced.



Supplementary Figure 3. Dendrogram derived from a protein cluster analysis of filamentous fungi isolated from whole-plant corn silage after 29, 103 and 193 d of ensiling and 24 h of aerobic exposure using MALDI-TOF MS. The dendogram was created using BioTyper MSP Dendogram Creation (Bruker Daltonics). Numbers in brackets are the strain identification. Strains number followed by the letter 's' indicate that the strain was sequenced.

The isolates of *Monascus* genus were grown on coconut agar (containing the following components: coconut milk 400g; agar 12g; distilled water 400mL). The plates were incubated at 25ºC for 10 d to verify citrinin production by the isolate. Citrinin production was confirmed in ultraviolet light at λ 366nm in a Chromatovisor CAMAG (Uf-Betrachter). The indicator of citrinin production was observed by the yellow-green fluorescence around the fungal colonies.

The experiment was conducted in a completely randomized design in a factorial arrangement (3 × 3) with three silages (control, without inoculant—CON; *L. buchneri*—LB; and *L. farraginis*—LF) for three storage periods (29, 103 and 193 d) with three replicates. The data obtained after the aerobic exposition (pH, w_a, hours of aerobic stability, maximum temperature, and time to reach maximum temperature) were analyzed in a factorial arrangement (3 × 2) with three silages (CON, LB, and LF) in two storage periods (short 29 d and long 193 d) with three replicates. The residuals of the variance analysis for each variable were tested for normality, independence, and homogeneity (Shapiro-Wilk test $P > 0.05$ and inspection of residual plots). Once the normality, independence, and homogeneity assumptions were attended to, variance analysis was carried out on the data. The ANOVA was performed using the *fat2.dic* function of the *ExpDes.pt* package of the statistical program R (R Development Core Team, 2021). The data of MS, pH, a_w and fermentation products were analysed using the following model: $Y_{ijk} = \mu + S_i + T_j + (S \times T)_{ij} + \varepsilon_{ijk}$, where: μ = overall mean; S_i = silage effect (i = without inoculant, with *L. buchneri* or with *L. farraginis*); T_j = storage period effect (j = 29, 103 or 193 d) (j = 29 or 193 d, for aerobic exposure data); $(S \times T)_{ij}$ = effect of the interaction between silage and storage period; and ε_{ijk} = error term. The means of each inoculant were compared only with the control to evaluate the effects of inoculants. The Dunnett test made a comparison between the means with the *pairw.anova* function of the *asbio* package function in the statistical program R, the contrasts (with $P < 0.05$) were performed for comparisons between the silages and control. The effects of adding strains were tested using the following contrasts: CON *vs.* LB and CON *vs.* LF. Tukey's test at 5% probability was used to compare the means throughout the storage period.

The data on yeasts and FF count did not present a normal distribution for the residues in the ANOVA; therefore, descriptive analysis graphics were made with the means of the silages throughout the storage period. The microorganisms' populations were transformed into log before statistical analysis.

RESULTS

The corn plant showed 314g/k of dry matter (as feed). pH and w_a were 5.58 and 0.9914, respectively. The population of yeasts and FF in the DG18, PDA, and DRBC culture media were 6.15, 6.04, 5.95, and 4.78, 4.60, and 4.60 log CFU/g, respectively.

There was no effect of inoculation and storage period on the silages' DM concentrations (Table 1). There was significant interaction ($P<0.01$) between silages and storage period on pH. At 29 d of storage, LB silage showed a higher ($P<0.01$) pH than CON silage, but at 103 and 193 d of storage, the pH of LF silages were higher ($P<0.01$) than CON. The CON and LB silages' pH decreased with the storage period and were the lowest at 193 d. In the LF silage, the pH was higher at 103 d and a further reduction at 193 d of storage was observed (Table 1). The a_w varied over the storage period ($P = 0.04$), with a higher value at 103 d than 29 d (Table 1).

The LF silage showed a lower ($P = 0.01$) mean lactic acid concentration than the CON silage, while the *L. buchneri* addition did not alter ($P = 0.67$) this variable (Table 2). The average lactic acid concentration increased from 29 d (46.3g/kg DM) to 103 d (53.2g/kg DM) of storage, remaining stable until the evaluation at 193 d. The LB (11.4 g/kg DM) and LF (19.7g/kg DM) silages had a higher mean concentration of acetic acid compared with the CON (7.9g/kg DM). Acetic acid concentrations increased after 193 d, regardless of the silage treatment. The ratio of lactic acid to acetic acid was lower ($P < 0.01$) in LF silages than in the CON silage at all storage periods, while in the LB silage, this ratio was lower ($P < 0.01$) than in the CON silage at 29 and 193 d. The ratio of lactic acid to acetic acid decreased with the increase in the storage period only in the CON treatment. This ratio remained stable in LB and LF silages throughout the evaluation period (Table 1). The butyric acid concentration increased ($P < 0.01$) during the storage period, and the addition of inoculants did not change this variable about the control. The 1,2-propanediol concentration was 9 and 21 times higher in the silages inoculated with LB and LF, respectively, compared with the CON ($P < 0.01$) (Table 1). There was no treatment effect on ethanol concentration ($P = 0.26$).

In the silages exposed to air for 24 h, the pH were higher (3.69) at 29 d than at 193 d (3.54) (Table 2). The water activity reduced (P = 0.02) in this period and was higher in the LF than in uninoculated silage (P = 0.02) (Table 2).

Table 1. Chemical composition, pH values, water activity, and final fermentation products of whole-plant corn silages inoculated with *Lentilactobacillus buchneri* (LB) or *Lentilactobacillus farraginis* (LF) at different storage periods

Variable	Silage (S)	Storage periods (SP) (days)			Mean	SEM	*P*-value		
		29	103	193			S	SP	S × SP
Dry matter (g/kg as fed)	CON	276.1	291.4	264.5	277.3	3.37	0.66	0.13	0.30
	LB	269.8	286.6	286.5	281.0				
	LF	282.7	281.5	256.2	273.5				
	Mean	276.2	286.5	269.1					
pH	CON	3.54a*	3.50a	3.43b	3.49	0.004	<0.01	<0.01	<0.01
	LB	3.60a	3.52b	3.46c	3.53				
	LF	3.53b	3.63a	3.53b	3.56				
	Mean	3.56	3.55	3.47					
	P-value for contrasts								
	CON vs. LB	0.01	0.60	0.17					
	CON vs. LF	0.88	<0.01	<0.01					
Water activity	CON	0.9853	0.9893	0.9857	0.9868	0.0004	0.19	0.04	0.77
	LB	0.9843	0.9873	0.9860	0.9859				
	LF	0.9873	0.9887	0.9877	0.9879				
	Mean	0.9857b	0.9884a	0.9864ab					
Lactic acid (g/kg DM)	CON	41.1	59.7	66.3	52.4	1.17	0.05	<0.01	0.31
	LB	48.2	52.5	56.0	52.3				
	LF	41.5	47.3	48.2	45.7				
	Mean	43.6b	53.2a	53.5a					
	P-value for contrasts								
	CON vs. LB				0.67				
	CON vs. LF				0.01				
Acetic acid (g/kg DM)	CON	5.1	9.1	9.7	7.9	0.37	<0.01	0.01	0.22
	LB	11.2	9.7	13.2	11.4				
	LF	18.1	20.2	20.8	19.7				
	Mean	11.4b	13.0ab	14.6a					
	P-value for contrasts								
	CON vs. LB				0.01				
	CON vs. LF				<0.01				
Lactic/ Acetic ratio	CON	8.15a	6.57ab	5.82b	6.84	0.157	<0.01	0.14	0.05
	LB	4.34a	5.45a	4.36a	4.72				
	LF	2.29a	2.34a	2.33a	2.32				
	Mean	4.92	4.79	4.17					
	P-value for contrasts								
	CON vs. LB	0.01	0.11	0.02					
	CON vs. LF	<0.01	<0.01	<0.01					
Butiric acid (g/kg DM)	CON	0.0	0.4	2.9	1.0	0.10	0.87	<0.01	0.99
	LB	0.0	0.5	3.1	1.2				
	LF	0.1	0.5	2.9	1.1				
	Mean	0.0b	0.5b	2.9a					
1,2-propanediol (g/kg DM)	CON	0.0	0.5	0.1	0.3	0.24	<0.01	0.54	0.25
	LB	1.7	2.4	4.2	2.7				
	LF	6.6	6.2	6.0	6.3				
	Mean	2.8	3.0	3.4					
	P-value for contrasts								
	CON vs. LB				<0.01				
	CON vs. LF				<0.01				
Ethanol (g/kg DM)	CON	15.3	15.4	15.4	15.4	0.33	0.26	0.23	0.17
	LB	19.2	16.0	14.9	16.7				
	LF	15.9	15.5	16.2	15.8				
	Mean	16.8	15.6	15.5					

* For each row, mean values with different lowercase letters are significant at $P < 0.05$ by Tukey test

Table 2. Characteristics of whole-plant corn silages inoculated with *Lentilactobacillus buchneri* (LB) or *Lentilactobacillus farraginis* (LF) stored for 29 and 193 d after aerobic exposition

Variable	Silage (S)	Storage periods (SP) (days)		Mean	SEM	*P*-value		
		29	193			S	SP	S × SP
pH (after 24h*)	CON	3.66	3.50	3.57	0.001	0.08	<0.01	0.06
	LB	3.75	3.52	3.64				
	LF	3.68	3.59	3.64				
	Mean	3.69a**	3.54b					
Water activity (after 24h*)	CON	0.9867	0.9800	0.9833	0.0002	0.02	0.02	0.18
	LB	0.9900	0.9833	0.9867[1]				
	LF	0.9900	0.9900	0.9900[2]				
	Mean	0.9889a	0.9844b					
Aerobic stability (h)	CON	37.7b	191.0a	114.3	36.27	<0.01	<0.01	<0.01
	LB	35.3b	194.0a	114.7				
	LF	158.3b	>216a	187.2				
	Mean	77.1	200.3					
	P-value for contrasts							
	CON vs. LB	0.96	0.95					
	CON vs. LF	<0.01	0.10					
Maximum temperature (MT) (°C)	CON	39.1	40.9	40.0	2.19	0.01	0.59	0.35
	LB	40.8	40.1	40.4[3]				
	LF	34.2	30.7	32.5[4]				
	Mean	38.0	37.3					
Time to reach MT (h)	CON	214.7a	>216.0a	215.3	0.54	<0.01	<0.01	<0.01
	LB	215.3a	214.7a	215.0				
	LF	215.3a	207.3b	211.3				
	Mean	215.1	212.7					
	P-value for contrasts							
	CON vs. LB	0.82	0.48					
	CON vs. LF	0.82	<0.01					

* Storage period plus 24h of aerobic exposure

** For each row, mean values with different lowercase letters are significant at $P < 0.05$ by Tukey test

[1] Comparison of control silage with silage inoculated with *L. buchneri* ($P = 0,31$); [2] Comparison of control silage with silage inoculated with *L. farraginis* ($P = 0,03$); [3] Comparison of control silage with silage inoculated with *L. buchneri* ($P = 0,95$); [4] Comparison of control silage with silage inoculated with *L. farraginis* ($P < 0,01$).

There was an interaction between the type of inoculant and the storage period on the aerobic stability ($P < 0.01$). Aerobic stability increased with increasing storage period in both silages. After 29 d of storage, aerobic stability was higher in the silage inoculated with *L. farraginis* when compared to the control silage. However, at 193 d, this difference was not observed, even though the LF silage did not lose aerobic stability in this period (Table 2). LB silage was not more stable than control silage when exposed to air. The maximum temperature reached by the LF silage (32.5° C) was lower than the control (40.0° C). The time to reach the maximum temperature was not different between the silages after 29 days of storage; however, after 193 d, the LF silage reached the maximum temperature with less time of exposure to air than the control silage (Table 2).

The yeasts and FF populations in corn before ensiling were 6.00 and 4.60 log CFU/g, respectively. Figs. 1 and 2 show the populations (average of replicates plus standard deviation) of yeasts and FF in each culture medium. At 29 d of storage, the CON silage showed a yeast mean population of 3.53 log CFU/g, ranging from 2.99 (PDA) to 3.97 log CFU/g (DG18) (Fig. 1A). After 24 h of aerobic exposure, the yeast population in the CON increased to 6.25 log CFU/g, with the population in each medium being numerically similar (Fig. 1A). In the LB silage, the yeast population was 4.03 log CFU/g at 29 d of storage, increasing to 5.76 log CFU/g after 24 h of aerobic exposure (Fig. 1 B). At 103 and 193 d, the yeast population in all silages was below the detection limit (<2.0 log CFU/g), and even after aerobic exposure (193 d), there was no yeast growth. The yeast population in the LF

silage was below the detection limit for all storage periods and after aerobic exposure.

After 29 d of storage, the FF population (average of replicates) was below the detection limit (<2.0 log CFU/g) in the CON and LF silages (Fig. 2A and 2C). In the LB silage, the FF population was 2 log CFU/g, remaining constant after 24 hours of aerobic exposure (Fig. 2B). At 103 d of storage, the FF population in the CON silage was 4.17 log CFU/g, with a variation of 0.22 log CFU/g between the culture media used. In the LB and LF silages, the FF population was 4.03 and 3.93 log CFU/g respectively and the growth in the different culture media was numerically similar.

At 193 d of storage, the FF population in the CON and LB silages was 2 log CFU/g (Fig. 2A and 2B). In the LB silage, there was no change in this population after 24 h of aerobic exposure (Fig. 2B), while in the CON silage, it was below the detection limit (< 2.0 log CFU/g). In the LF silage, the FF population was below the detection limit at the silos opening and after aerobic exposure (Fig. 2C).

Two hundred and two isolates of FF (isolated from DG18, PDA, and DRBC culture media) belonging to 10 different genera were obtained (Tables 3 and 4). Seven genera were identified in the whole-plant corn before ensiling. After 29 d of storage, two species were isolated only in the LB silage: the *Monascus pilosus* species with a population (average of the culture media used) of 3.50 log CFU/g and an unidentified morphotype with an average population of 2.69 log CFU/g (Table 4). The fungi diversity observed in these silages was the same after 24 h of aerobic exposure (Table 3). After 29 d of storage and 24 h of aerobic exposure, no isolates were observed in the CON and LF silages.

Table 3. Diversity of filamentous fungi in whole-plant corn silages inoculated with *Lentilactobacillus buchneri* (LB) or *Lentilactobacillus farraginis* (LF) at different storage periods and after 24 h of aerobic exposure at 29 and 193 d of storage periods

	Silages	Fungi diversity	Population in different media‡ (Log CFU/g)		
			DG18	PDA	DRBC
0	Fresh corn	*Aureobasidium sp.*	4.00	-	4.30
		Beauveria sp.	-	-	4.30
		Epicoccum sp.	4.00	-	-
		Fusarium sp.	4.85	-	4.30
		Pestalotiopsis sp.	-	6.30	-
		Phoma sp.	-	-	4.30
		Simplicillium sp.	4.00	-	-
29	LB	*M.* pilosus*	3.51	3.51	3.50
		Not identified	-	2.78	2.60
29 AE†	LB	*M. pilosus*	3.60	3.78	3.90
		Not identified	-	3.30	-
103	CON	*A.** fumigatus*	4.08	4.19	4.21
		A. flavus	2.78	2.60	-
		Rhizopus oryzae	-	2.30	-
	LB	*A. fumigatus*	4.45	4.29	4.27
		A. flavus	2.30	-	-
		A. niger	-	2.30	-
		A. micronesiensis	-	-	2.30
		R. oryzae	-	2.30	2.30
	LF	*A. fumigatus*	4.13	4.21	4.25
193	CON	*A. cristatus*	2.00	-	-
		A. fumigatus	-	2.00	-
	LB	*A. fumigatus*	2.00	-	-
		A. giganteus	2.47	-	2.00
193 AE	LB	*A. giganteus*	-	2.30	2.30

* *Monascus*; ** *Aspergillus*; *** *Rhizopus*

‡ The plate with the largest population and visual diversity of colonies was used for isolation and identification of filamentous fungi.

† Storage period plus 24h of aerobic exposure (AE)

Table 4. Number of each filamentous fungi species isolated from whole-plant corn and silages in the different media used

Fungi diversity	Access code*	Collection code**	Culture media		
			DG18	PDA	DRBC
Before ensiling					
Aureobasidium sp.	KX023301.1	CCDCA 11554, CCDCA 11559	1	0	1
Beauveria sp.	MH231203.1	CCDCA 11558	0	0	1
Epicoccum sp.	MK809036.1	CCDCA 11556	1	0	0
Fusarium sp.	MK841428.1	CCDCA 11555, CCDCA 11557	3	0	1
Pestalotiopsis sp.	MK862236.1		0	1	0
Phoma sp.	KR261531.1		0	0	1
Simplicillium sp.	MH990628.1		1	0	0
After ensiling					
Aspergillus cristatus	MK696342.1		1	0	0
Aspergillus flavus	MH781287.1	CCDCA 11571, CCDCA 11582, CCDCA 11583	4	2	0
Aspergillus fumigatus	MH536091.1	CCDCA 11566, CCDCA 11567, CCDCA 11570, CCDCA 11572, CCDCA 11574, CCDCA 11578, CCDCA 11584 a 11586	42	42	43
Aspergillus giganteus	FR775339.1	CCDCA 11579, CCDCA 11580, CCDCA 11587 a 11590	4	5	3
Aspergillus micronesiensis	KP987047.1	CCDCA 11573, CCDCA 11575, CCDCA 11576	0	0	4
Aspergillus niger	MH208814.1	CCDCA 11568	0	1	0
Monascus pilosus	AB607170.1	CCDCA 11560 a 11565	8	12	10
Rhizopus oryzae	KX035094.1	CCDCA 11569	0	2	1
Not identified			0	4	2
Total isolates			65	69	67
Number of different species			9	8	10

*Database accession number of Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi)
** Deposit code in the Culture Collection of Microorganisms of the Department of Food Science (CCDCA) of the Federal University of Lavras, Brazil.

At 103 d of storage, four *Aspergillus* genus species were identified when opening the silos, *Aspergillus fumigatus*, *A. flavus*, *A. niger*, and *A. micronesiensis. A. fumigatus* was isolated in all silages with populations between 4.08 and 4.45 log CFU/g of silage. The species *A. flavus* was isolated in the CON and LB silages at silo open, with average populations of 2.69 and 2.30 log CFU/g, respectively. *A. niger* was isolated only in LB silage, while *Aspergillus micronesiensis* was isolated from the LB silage at silo opening (2.3 log CFU/g). *Rhizopus oryzae* was isolated in the CON and LB silages at the silo opening.

After 193 d of storage, *A. fumigatus* was isolated from the CON and LB silages (Table 3). The population of this species was close to the detection limit (2.0 log CFU/g). In the CON silage, *A. cristatus* was isolated from DG18 medium with a population of 2 log CFU/g. In the LB silage, *A. giganteus* was isolated at silo opening and after aerobic exposure.

The FF diversity before ensiling was different in the media used, four genera from the DG18 medium (*Aureobasidium* sp., *Epicoccum* sp., *Fusarium* sp., and *Simplicillium* sp.), and four genera from the DRBC medium (*Aureobasidium* sp., *Beauveria* sp., *Fusarium* sp. and *Phoma* sp.). Only one was isolated from the PDA medium (*Pestalotiopsis* sp.) (Table 4). After ensiling, 83.7% of the isolates grew in the three culture media used, and these isolates belong to the three predominant species in the silage (*A. fumigatus*, *A. giganteus* and *M. pilosus*). Except for silages after 193 d of storage, *A. fumigatus* was isolated from the three culture media used, with similar populations. The *Rhizopus* and *A. niger*'s growth occurred only in the PDA medium, whereas the growth of *A. flavus* occurred in the DG18 and PDA media. *Aspergillus micronesiensis* was isolated only in DRBC media. The total number of isolates and species found within each culture media used was numerically similar (Table 4).

The six isolates identified as *A. flavus* did not produce aflatoxins B1, B2, G1, and G2 because they did not show fluorescence spots of the tested mycotoxin patterns. Instead, the isolates belonging to the genus *Monascus* were tested on coconut agar to produce citrinin, and none of them showed toxigenic potential for the tested toxin.

DISCUSSION

The observed pH values indicated adequate fermentation with the capacity to inhibit most undesirable microorganisms. In the silages inoculated with obligate heterofermentative strains, the pH values were low concerning the commonly observed. However, low pH values were also observed in the corn silages inoculated with *L. buchneri* (NCIMB 40788) (3.78) and *L. hilgardii* (CNCM I-4785) (3.69) (Ferrero *et al*., 2018). With 29 d, only the LB silage showed a higher pH than the CON silage, while at 103 and 193 d, this occurred only in the LF silage. Costa *et al*. (2021) observed that at 32 d of storage, inoculation with *L. buchneri* (CCMA 1366) resulted in silages with a higher pH value than the control silage, while silage inoculated with *L. farraginis* (CCMA 1362) resulted in a similar pH to control throughout the study period (100 d). In elephant grass silage, inoculation with other strains of the *L. farraginis* species resulted in silages with pH values similar to the control silage during 60 d of storage (Amaral *et al*., 2020). After 193 d of storage, the pH remained low and decreased from 103 d for all silages, showing that conditions to inhibit undesirable microorganisms were maintained even after a prolonged storage period. The pH observed after aerobic silage exposure, can still be considered appropriate pH for WPCS (3.7–4.0).

A_w increased with the storage period, this increase might be due to fungi growth, which, when oxidizing the sugars, produces CO_2 and water or by condensation of water vapor inside the silo. This factor was not limited to the growth of inoculated bacteria and FF and yeasts because most microorganisms require a minimum water activity of 0.9 to grow.

The mean concentration of lactic acid in the LF silage was lower than the CON silage, but in LB silage, this did not occur (52.3g/kg DM), even with the smallest inoculated *L. buchneri* population. Costa *et al*. (2021) observed that, the inoculation of these strains in corn silages with high DM (454g/kg DM) resulted in the opposite behaviour, the silages inoculated with *L. buchneri* showed a lower concentration of lactic acid than the control at 10, 32, and 100 d of storage, while the silages inoculated with *L. farraginis* (CCMA 1362), showed a lower concentration of lactic acid than the control only after 100 d. The inoculation of the *L. buchneri* strain (CCMA 1366) reduced the lactic acid concentration in elephant grass silages by 20.3%, while the inoculation of *L. farragines* did not affect the concentration of this acid (Amaral *et al*., 2020). Despite the lower concentration of this acid in the LF silage, the values are within the values found in WPCS (Ferrero *et al*., 2018). These results confirmed that the metabolism of the strains is different in each forage and silage situation (DM concentration or cutting season).

The increase in acetic acid concentration concerning the CON was expected because of the heterofermentative metabolism observed in whole-plant corn and elephant grass silages inoculated with these strains (Amaral *et al*., 2020; Costa *et al*., 2021). Both lactic and acetic acid have important functions in forage conservation, the first one being lowering the pH value and the second inhibiting the fungi growth. Thus, the lactic acid/acetic acid ratio is an interesting criterion to verify the efficiency of an inoculant and silages with a high lactic acid/acetic acid ratio may sometimes be more aerobically unstable. Inoculated silages had a lower lactic acid/acetic acid ratio than the CON silages at all ensiling periods except in the LB silage at 103 d of storage.

The butyric acid concentration increased over the storage period in all silages, with an average of 2.9 g/kg DM after 193 d, a relatively high value, considering that most studies with corn silage did not detect this acid (Ferrero *et al*., 2018, 2019; Wang *et al*., 2018). This concentration may be related to water activity at 193 d (above 0.985) that favors the growth of butyric acid-producing bacteria, especially genus *Clostridium* (a_w 0.952 to 0.971). The high concentration of butyric acid observed in this period is indicative of *Clostridium* growth, however, the pH values were within the limits considered adequate for

inhibition of most species of this genus (Driehuis *et al.*, 2018). Thus, one possibility is that other butyric acid-producing bacteria may have contributed to these results. Özcelik *et al*. (2016) confirmed butyric acid production in MRS broth by species such as *Lactiplantibacillus plantarum* and *Lactobacillus acidophilus*. LAB's ability to produce butyric acid is strain-specific and dependent on the substrate type. The LF and LB silages presented an average of 21 and 9 times more 1,2-propanediol than the CON silage (0.3 g/kg of DM), respectively. The inoculation of this same strain of *L. buchneri* (CCMA 1366) resulted in elephant grass and corn silages with concentrations below the detection level and with 0.17 g/kg DM of 1,2-propanediol, respectively, however, the concentration was below the level of detection when *L. farraginis* was inoculated (Amaral *et al*., 2020; Costa *et al*., 2021). Some heterofermentative LAB of the *L. buchneri* group can convert lactic acid to acetic acid and 1,2-propanediol, and this conversion occurs after a storage period (about 30–60 d). In the current study, there is an indication that this conversion occurred after 29 d.

At 29 d of storage, inoculation with *L. farraginis* increased silage stability by 120.6 h. This effect was also observed in corn (Costa *et al*., 2021) and elephant grass (Amaral *et al*., 2020) silages inoculated with this species. Heterofermentative LAB increases aerobic stability mainly due to the production of antifungal compounds.

Yeasts and FF's epiphytic populations were within the usually observed range (Ferrero *et al*., 2018; Wang *et al*., 2018). The inoculants modified the silage fermentation differently compared with the control. Yeast growth was inhibited at all storage periods, and after aerobic exposure in LF silage, this result may be associated with the higher concentration of acetic acid in these silages than CON silage. In the LB silage, a higher concentration of acetic acid than CON was also observed; however, yeast growth inhibition was observed only after 103 d of storage. These data suggest that other modes of action, together with the acetic acid, can contribute to the inhibition of undesirable microorganisms in silages. For example, after aerobic exposure for 24 h, the CON and LB silages with 29 d of storage showed an increase of 43.4% and 30% in the yeast population, respectively.

After 103 d of storage, the yeast population was below the detection limit in all silages. As the storage period increases, a reduction in readily fermentable substrates and an increase in the concentration of antifungal compounds can occur, creating unfavorable conditions for yeast growth. At 193 d of storage, no increase in the yeast population was observed after aerobic, probably because of the low yeast population in the silages. Ferrero *et al*. (2018) observed a reduction in the yeast population and an increase in aerobic stability with an increase in the storage period (15, 30, 100, and 250 d), showing that controlling the yeast population during the anaerobic phase is an efficient way to increase the aerobic stability of silages.

The FF population was close to or below the detection limit in all silages after 29 d of storage. At 103 d, the FF developed, but at 193 d of storage, the population decreased again, indicating that the conditions in this period did not favor their growth. It is known that water activity, pH, substrate availability, fermentation products, prolonged exposure to anaerobic conditions and low pH can interfere with FF growth. In addition, there is the possibility of antifungal metabolites production by LAB species.

Three different culture media were used to study the FF diversity in silages. The use of culture media with different water activity values (DRBC and PDA with a_w: 0.99 and DG18 a_w: 0.95) favors the isolation of the greatest diversity of biological species. Likewise, the use of complementary identification methods makes the result more accurate. The identification methodology in this study was based on macro and micromorphological characteristics, protein profile (MALDI-TOF MS) and ITS and β-Tubulin gene sequencing.

The genus *Aureobasidium* is a yeast-like fungus identified in barley before ensilage and in corn silage, however, the occurrence of this genus in silages is not common. FF of the genus *Fusarium* were isolated from the fresh plant; these fungi are commonly associated with crop infestations still in the field (Driehuis *et al*., 2018). Fungi of

the genus *Phoma* are phytopathogens, while those of the genus *Beauveria* are pathogens of insects; both are geographically disseminated. The *Epicoccum* genus is composed of endophytic fungi this genus also was identified in the fresh corn plant, and as in the current work, it was not persistent after ensiling (Drouin *et al*., 2019). The *Simplicillium* and *Pestalotiopsis* genera are rarely reported in WPCS and are generally characterized as phytopathogenic fungi. None of these genera persisted after ensiling, probably because they do not tolerate anaerobic conditions and the acidic environment.

Fungi of the genus *Monascus* were identified on corn silages this genus can resist silage conditions (Wambacq *et al*., 2016), and in the present study, the species *M. pilosus* was identified by culture-dependent technique only in LB silages with 29 d of storage and persisted in these silages after 24 h of aerobic exposure. Some species of the genus *Monascus* can coproduce the citrinin mycotoxin, which has nephrotoxic effects, thus representing a risk to animal and human health; the isolates belonging to this genus did not produce citrinin.

The *Aspergillus* genus was dominant after 103 d of storage, with *A. fumigatus* being found in the largest population in all silages. The strains of *L. buchneri* and *L. farraginis* that were evaluated could not inhibit the growth of this fungus in the silage because the population of this species was similar in the silages treated with the different inoculants. *Aspergillus fumigatus* was also identified in all silage samples obtained from six different regions in the south of Minas Gerais, Brazil (Carvalho *et al*., 2016). This species is common in silages, especially those produced in warm climates; it has high resistance to silage conditions such as low pH and low oxygen concentration, which allows it to survive in silage for longer than other FF (Carvalho *et al*., 2016; Keller *et al*., 2013; Wambacq *et al*., 2016). The presence of *A. fumigatus* in silages represents a risk to animal and human health because it has the potential for the mycotoxins production such as gliotoxins and fumagillin (Guruceaga *et al*., 2020). In addition, *A. fumigatus* has a remarkable ability to invade the host immune system, making it an effective opportunistic pathogen responsible for causing severe aspergillosis cases. This fungal species has also been associated with hemorrhagic bowel syndrome (HBS). The only fungus isolated from silages inoculated with *L. farraginis* (CCMA1362) was *A. fumigatus*, indicating that inoculation with this LAB could inhibit the growth of other fungi species. The inoculation of this strain (CCMA1362) inhibited the FF growth in the WPCS with high DM (454.0g/kg DM) (Costa *et al*., 2021). However, other strains of the same species could not inhibit fungi growth in elephant grass silage (Amaral *et al*., 2020). The antifungal capacity of LAB is known; however, learning more about the inhibition mechanism is crucial and needed.

The *A. flavus* species was isolated from the CON and LB silages at 103 d of storage and was previously isolated from corn silages produced in a warm climate (Ferrero *et al*., 2019; Keller *et al*., 2013). Some strains of this species can produce aflatoxins, which, besides being toxic to animals, can compromise the safety of food products of animal origin (Driehuis *et al*., 2018). The strains of the *A. flavus* species isolated in the current study could not produce aflatoxins B1, B2, G1, and G2 *in vitro*. The activation of the genes involved in mycotoxin biosynthesis responds to different environmental stimuli and/or stress conditions (Wambacq *et al*., 2016). Thus, the presence of the genes involved in the synthesis of mycotoxins is not an obligate indicative of the toxin production by a fungus. Ferrero *et al*. (2019) analyzed the presence of four genes involved in the aflatoxin biosynthesis in FF isolated from corn silage, finding that although 64% of the isolates have four genes, only 43% of them produced aflatoxins *in vitro*.

The species *A. niger* was isolated from LB silage in a low population. Ochratoxigenic strains of this specie were isolated from corn silage produced in Brazil (Keller *et al*., 2013). This mycotoxin (ochratoxin) is highly relevant because of its known nephrotoxic, hepatoxic, and neurotoxic effects. However, *A. niger* is rarely a producer of ochratoxin A, and when this occurs, it usually does so in low concentrations, meaning they are not an important source of ochratoxin A (Cabañes and Bragulat, 2018). *Rhizopus oryzae* were isolated in low populations in the CON and LB silages; there are reports of this genus in silages, but little is known about the effects of their growth in this feed (Alonso *et al*., 2013). In addition, some species of the *Rhizopus* genus are

associated with mycotoxin degradation (Wambacq *et al*., 2016).

Penicillium and some *Aspergillus* species are considered 'moderate xerophiles'. Therefore, it was expected that these genera would grow more in the DG18 medium; however, this was not observed. There was no difference between the species number and the number of fungi isolates between the three media used. However, Schenck *et al*. (2019) reported a more significant number of genera when using the medium MEA than DG18. In whole-plant corn samples before ensiling, the genus *Fusarium* was only isolated in DG18 medium; the presence of this genus is more common before silage, its growth is favored in conditions of high humidity, and the ensiling conditions are not favorable for its growth (Ogunade *et al*., 2018). Although the DG18 medium favors the growth of fungi that grow at low a_w, in the current study, the genus *Fusarium* could be isolated from this medium; the same was observed in wrapped forage bales with high DM (Schenck *et al*., 2019).

CONCLUSION

Inoculating *L. buchneri* (CCMA 1366) and *L. farraginis* (CCMA 1632) strains to silages resulted in sufficient acids to lower the pH value to inhibit yeast growth. The addition of wild LAB strains significantly affected the fungal composition during the ensiling and aerobic exposure. *Aspergillus fumigatus* and other *Aspergillus* species are the most important FF associated with WPCS in warm climates. Inoculation with the *L. farraginis* (CCMA 1632) strain resulted in the rapid inhibition of yeast growth, highest aerobic stability and less FF diversity in WPCS. Further studies are needed to evaluate the inhibition mechanisms of FF and the yeasts promoted by this strain, and its results in larger scale silos.

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