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Antonio Gallo[*1], Francesco Fancello[2], Francesca Ghilardelli[1], Severino Zara[2], and Mauro Spanghero[3]

[1] *Department of Animal Science, Food and Nutrition (DIANA), Faculty of Agricultural, Food and Environmental Sciences, Università Cattolica del Sacro Cuore, 29122, Piacenza, Italy.*

[2] *Department of Agricultural Sciences, University of Sassari, 07100 Sassari, Italy.*

[3] *Department of Agriculture, Food, Environment and Animal Science, University of Udine, 33100 Udine, Italy.*

[*]Corresponding author: antonio.gallo@unicatt.it

ABSTRACT

Effects of 3 commercial lactic acid bacteria (LAB) inoculants and 6 single strains of LAB on fermentation and levels of aflatoxins and other mycotoxins in high-moisture corn (HMC) silage were examined. Corn plants were inoculated with *Aspergillus flavus* at silk emergence and wet corn kernels were harvested at black layer stage (dry matter [DM]: 704 ± 5.0 g/kg). Samples were ground and ensiled directly (control group, CTR) or after treatment with one of the 9 LAB inoculants. The commercial inoculants contained *Lentilactobacillus* (*Lent.) buchneri* LB1819 and *Lactococcus lactis* O224 (SiloSolve FC; Chr. Hansen A/S, Hørsholm, Denmark), applied at a final dose of 250,000 CFU/g or 1,250,000 CFU/g (SiloSolve FC_{1x} and SiloSolve FC_{5x}, respectively); and *Lent. buchneri* and *Lactiplantibacillus* (*Lac.*) *plantarum* (Pioneer brand 11B91, Pioneer Hi-Bred International, Des Moines, USA) applied at a final dose of 250,000 CFU/g. One pure strain of *Levilactobacillus* (*Lev.*) *brevis* DSMZ 20054, two pure strains of *Lac. plantarum* (LP ATCC 8014 and LP PB) and three of *Lacticaseibacillus* (*La.*) *rhamnosus* (LR ATCC 7469, LR LR7 and LR RI) were also applied at a final expected dose of 250,000 CFU/g. Wet corn kernels were ensiled in 20 L mini-silos, compacted to a density of 389 kg DM/m^3, and stored at room temperature. Conditions before ensiling were pH 5.87±0.169, and (log_{10} CFU/g) of 7.68±0.473 for LAB, 5.71±0.121 for molds and 6.81±0.341 for yeasts. The mini-silos were opened after 30 or 120 days. From day 30 to 120 days, levels of acetic acid, propionic acid and 1,2 propanediol increased ($P < 0.05$) in the SiloSolve FC_{1x}, SiloSolveFC_{5x} and Pioneer 11B91, and in LR LR7 groups. During this time, butyric acid level increased 5-fold in CTR; by two to three-fold in pure strains groups; and only slightly in the three commercial inoculants and the LR LR7 ($P < 0.05$). Aerobic stability increased ($P < 0.05$) with storage time from 32 to 97 h, and the commercial inoculants and LR LR7 groups had longer stability than CTR ($P < 0.05$). The storage time and the inoculants did not affect aflatoxin levels. No zearalenone or tenuazonic acid were detected in the mini-silos, even if they contaminated the corn

plants. Results indicate that different silage inoculants affected fermentation of HMC and its contamination by mycotoxins, although no inoculant had an effect on levels of aflatoxins.

Keywords: corn; volatile organic compounds; aflatoxin; aerobic stability.

Abbreviations: AF - aflatoxins; AFB1 - aflatoxin B1; AFB2 -aflatoxin B2; AFM1 - aflatoxin M1; aNDFom - NDF assayed with sodium sulphite and a heat stable amylase and expressed exclusive of residual ash; CFU - colony forming units; CP - crude protein; CTR - high moisture corn without inoculant; DM - dry matter; DON - deoxynivalenol; GC - gas chromatography; GLM - general linear model; HMC - high moisture corn; HPLC - high performance liquid chromatograph; LAB - lactic acid bacteria; LB DSMZ 20054 - high moisture corn treated with *Levilactobacillus brevis* DSMZ 20054; LP ATCC 8014 - high moisture corn treated with *Lactiplantibacillus plantarum* ATCC 8014; LP PB - high moisture corn treated with *Lactiplantibacillus plantarum* PB; LR ATCC 7469 - high moisture corn treated with *Lacticaseibacillus rhamnosus* ATCC 7469; LR LR7 - high moisture corn treated with *Lacticaseibacillus rhamnosus* LR7; LR RI - high moisture corn treated with *Lacticaseibacillus rhamnosus* RI; VFA - volatile fatty acids; VOC - volatile organic compounds; ZEA – zearalenone.

1. Introduction

A common problem on dairy farms is the presence of aerobically unstable high-moisture corn (HMC) silage during the feed-out phase of fermentation. During this phase the HMC has warm areas that allow the proliferation of mold, increasing the risk of mycotoxin contamination (Gallo et al., 2016a). Among the several groups of mycotoxins, four aflatoxins (AF) produced by two species in the *Aspergillus* section *Flavi*, *A. flavus* and *A. parasiticus* (Mauro et al., 2013), can occur in

ensiled forage (Gallo et al., 2021b). The AFB1 is the most toxic and carcinogenic of these compounds (Placinta et al., 1999), and its ingestion by dairy cattle leads to its partial hydroxylation in the liver to AFM1, a highly toxic and carcinogenic compound that can be secreted in milk (Battacone et al., 2009; Masoero et al., 2007). Winn and Lane (1978) demonstrated that sufficient production of AF in HMC at a relatively low temperature (25°C) following a short incubation time (72 h) to be harmful to livestock. Thus, AF formation in silage at the farm level will often be a problem in warm temperate regions (Ferrero et al., 2019).

Inoculation of forage with lactic acid bacteria (LAB; *Lentilactobacillus buchneri*, *Levilactobacillus brevis*, or *Lactiplantibacillus plantarum*) before ensiling, is a common practice to improve the fermentation quality and increase the bio-preservation and aerobic stability (Adesogan and Arriola, 2020; Muck et al., 2018). However, the antifungal effects of this practice are uncertain, particularly, in silage from HMC as it is an oxygen-rich microenvironment in which caked and clumpy areas develop visible green-grey mold indicative of mycotoxin production, including AF and several *Fusarium*-produced mycotoxins (Cheli et al., 2013; Gallo et al., 2015; 2021b). Use of inoculants containing obligate heterofermentative bacteria, such as *Lentilactobacillus buchneri*, can increase the aerobic stability of HMC silage (Kristensen et al., 2010; Muck et al., 2018) and rehydrated corn grain silage (da Silva et al., 2019). Despite the potential benefits of using LAB inoculants in silage, Ogunade et al. (2018) reported that very few studies have examined the use of mold-inhibiting chemical additives or microbial inoculants to reduce mycotoxin contamination. Some researchers have investigated effects of storage time on mycotoxin detoxification, as reviewed by Cheli et al. (2013). For instance, Martinez Tuppia et al. (2017) demonstrated a significant decrease in fumonisin B_1 (a mycotoxin produced by *Fusarium* spp.) during ensiling, and that isolated *Lactobacillus* strains from HMC could metabolize fumonisin B1 to a less toxic form: hydrolyzed fumonisin B1.

Our objective was to compare fermentation parameters and mycotoxin content of HMC silage samples from plants that were infected in the field with a toxigenic strain of *A. flavus* and received either no treatment or treatment with LAB inoculants from commercial mixtures or single strains.

2. Materials and Methods

The experiment was conducted at the same time and location as the trial with whole corn plants described in our previous publication (Gallo et al., 2021a). Experimental design, treatments and analyses were identical for most aspects between the two experiments and therefore only basic descriptions of methods are reported here.

2.1 Corn harvesting, treatments and preparation of mini-silos

A late-season corn hybrid (Pioneer Hi-Bred International, Inc., Des Moines, IA, USA) grown during 2019 at the Centro Ricerche Zootecniche (CERZOO S.r.L., San Bonico, Italy) was inoculated with a toxigenic strain of *A. flavus* (ITEM 8069, 1×10^5 spores/mL) at silk emergence (Giorni et al., 2019). Approximately 8 tonnes of wet corn kernels were collected in each of four plots from the same experimental field at the black layer stage (dry matter [DM]: 704 ± 5.0 g/kg) and ground using a hammer mill (Hercules 130, Mifema, Spinadesco, Cremona, Italy) with a working capacity of 15 tons/h and equipped with a 2-mm screen. To calculate the mean particle size of each corn kernel batch, a representative amount (100 g) of the various grinds was run for 10 min through a sieve shaker (Multidimensional Sieveshaker IG/1/S, Giuliani Tecnologie s.r.l., Torino, Italy) that had 9 different screen sieves with nominal aperture sizes of 4.00, 3.50, 2.50, 1.50, 1.00, 0.75, 0.50, 0.25, and 0.125 mm, followed by a pan as reported by Gallo et al. (2016b). The mean particle size was calculated using equation 1 of ASAE S319.3 method, as reported in ASABE (2006). In particular, the mean particle size of each material retained on a sieve was calculated on a

110 weight basis as the geometric mean of the diameter of the openings in the two adjacent sieves in the
111 stack (Pfost and Headley, 1976). The harvested crop from each plot were divided into 10
112 approximately equal sub-fractions, individually sampled before ensiling, and then subjected to one
113 of the following 10 ensiling treatments: (*i*) untreated control (CTR, no inoculant); (*ii*) label dose of
114 commercial inoculant containing *Lentilactobacillus buchneri* LB1819 and *Lactococcus lactis* O224
115 (SiloSolve® FC, Chr. Hansen A/S, Hørsholm, Denmark; SiloSolve FC_{1x}); (*iii*) five-times label dose
116 of commercial inoculant containing *Lentilactobacillus buchneri* LB1819 and *Lactococcus lactis*
117 O224 (SiloSolve® FC, Chr. Hansen A/S, Hørsholm, Denmark; SiloSolve FC_{5x}); (*iv*) label dose of
118 commercial inoculant containing *Lentilactobacillus buchneri* and *Lactiplantibacilluss plantarum*
119 (Pioneer® brand 11B91 HMC Inoculant, Pioneer Hi-Bred International, Des Moines, IA, USA;
120 Pioneer 11B91); (*v*) *Levilactobacillus brevis* DSMZ 20054 (Deutsche SammLung von
121 Mikroorganismen und Zellkulturen, LB DSMZ 20054); (*vi*) *Lactiplantibacillus plantarum* ATCC
122 8014 (American Type Culture Collection) (LP ATCC 8014); (*vii*) *Lactiplantibacillus plantarum* PB
123 (LP PB); (*viii*) *Lacticaseibacillus rhamnosus* ATCC 7469 (LR ATCC 7469); (*ix*) *Lacticaseibacillus*
124 *rhamnosus* LR7 (LR LR7); or (*x*) *Lacticaseibacillus rhamnosus* RI (LR RI). The final dose applied
125 was 250,000 CFU/g for each treatment, with the exception of the third group (SiloSolve® FC_{5x}),
126 which had a dose of 1,250,000 CFU/g. In particular, the concentration of microbial cells of
127 commercial inoculants were calculated based on manufacturing instruction, whereas for single
128 inoculants the concentrations were quantified in the lab by plate count in MRS agar (Gallo et al.,
129 2021a).

130 For each corn kernel preservations, two mini-silos (20 L plastic jars, filled with about 11.1 ± 0.31
131 kg of wet corn, compacted to a density of 389 ± 4.6 kg DM/m^3, and then sealed with plastic lids and
132 stored at 20.4°C ± 0.52) were created, and opened after 30 or 120 days of storage times. Thus, there
133 were 4 field plots, 10 experimental treatments (including CTR), 2 storage times, and 80 mini-silos.

After opening on day 30 or 120, the mass of each mini-silo was mixed thoroughly and representative samples (~2 kg) were collected for analysis.

2.2 Chemical analysis and measurements

After 30 or 120 days of storage times, fresh and fermented HMC samples were analyzed in duplicate (except for DM) for chemicals. Samples were dried at 60°C in a forced air oven for 48 h, milled and passed through a 1-mm screen using a laboratory mill (Thomas-Wiley, Arthur H. Thomas Co., Philadelphia, PA, USA), and then stored for subsequent analysis. The DM was determined by gravimetric loss of free water after heating at 105°C for 3 h (AOAC, 1995; method 945.15), with correction for volatile losses during oven drying using the equations of NorFor (2011). Ash was determined as gravimetric residue after incineration at 550°C for 2 h (AOAC, 1995; method 942.05), and crude protein (CP; N × 6.25) was determined using a Kjeldahl method (AOAC, 1995; method 984.13). Neutral detergent (ND) fiber fraction (NDF) was determined using the Ankom^{II} Fiber Analyzer (Ankom Technology Corporation, Fairport, NY, USA), as described by Mertens (2002). The ND solution contained sodium sulfite and a heat stable amylase (activity: 17.400 Liquefon-U/mL, Ankom Technology) and NDF was corrected for residual ash (aNDFom). Starch was determined by polarimetry (Polax 2L, Atago®, Tokyo, Japan) and free total sugars (i.e. sum of glucose, saccharose, lactose, fructose and maltose) were determined following phosphate buffer and acetonitrile extraction, with quantification using a high-performance liquid chromatography system (HPLC; Jasco, Tokyo, Japan) equipped with an LU-980 pump and an RI-2031 refractive index detector (Gallo et al., 2016a).

Mycotoxin analysis was completed on fresh and fermented HMC samples to quantify AF (all samples) and other mycotoxins (i.e., altenuene, alternol, alternol monomethyl ether, tentoxin, tenuazonic acid, fusaric acid, zearalenone, deoxynivalenol [DON], PR toxin, mycophenolic acid, roquefortine C, gliotoxin) using fresh samples and samples collected after 120 days. AF were

158 extracted from samples that were dried at 65 °C in a forced air oven for 48 h, milled and passed
159 through a 1-mm screen using a laboratory mill (Thomas-Wiley, Arthur H. Thomas Co.,
160 Philadelphia, PA, USA). The AF were extract from each dried samples (i.e.10 g) with 100 mL of an
161 acetone:water solution (70:30 vol/vol). The solution was shaken at 150 r.p.m. for 45 min (Universal
162 Table Shaker 709, Enrico Bruno srl, Torino, Italy), then filtered using Schleicher & Schuell 595 ½
163 filter paper (Dassel, Germany) and 5 mL of this was purified on an immunoaffinity column
164 (Aflatoxin Easi-extract, R-Biopharm Diagnostics Technologies, Glasgow, UK) (Gallo et al., 2010).
165 The column was washed using 5 mL of water and slowly eluted with 2.5 mL of methanol. The
166 eluate was concentrated under a flow of N_2 and brought to 2 mL by addition of an acetonitrile:water
167 (41:59) solution, filtered into vials (Millipore Corporation, Bedford, MA, USA; HV 0.45 mm), and
168 then analyzed by HPLC as described earlier.
169 The other mycotoxins were extracted from 3 g of dried samples using 30 mL extraction solution
170 (Gallo et al., 2016a). Then, 4.0 g of anhydrous magnesium sulfate was added after shaking for 45
171 min the solution previously prepared, to obtain phase separation. After shaking for another 2 min
172 and centrifugation at 4500 × g for 10 min at room temperature, the upper acetonitrile phase was
173 recovered. This extract was diluted 5-fold (0.1 mL brought to 0.5 mL) by addition of
174 acetonitrile:water 30:70 (v/v), and then passed through a filter (Millipore Corporation, Bedford
175 USA; HV 0.45 µm). A 5 µL sample was injected into the HPLC-MS/MS system as described by
176 Gallo et al. (2016a).
177 The fermentation parameters were volatile fatty acids (VFA), lactic acid, volatile organic
178 compounds (VOC; grouped as aldehydes, alcohols, ketones, or esters), ammonia, and pH (Gallo et
179 al., 2021a). To quantify VFA, or VOC, 50 g of wet samples were extracted with Stomacher blender
180 (Seward Ltd., West Sussex, Worthing, UK) for 3 min in distilled water (water:sample fresh weight
181 ratio: 3:1). After filtering using gauze, an aliquot was centrifuged at 3000 r.p.m. for 15 min at room

temperature. Liquid phase added with internal standard (i.e. pivalic acid) was injected in a gas chromatographic-flame ionization detector (GC/FID) system that was equipped with a capillary column DB-WAX UI (60m × 0.250 mm; 0.25 µm; Agilent Technologies S.p.A., Milano, Italy). Lactic acid instead was determined using HPLC (Carvalho et al., 2012).

The ammonia N concentration was determined in about 20 g of fresh sample in a slurry mix composed of 150 mL of distilled water and magnesium oxide (10 g/sample; NorFor, 2011). The ammonia N concentration, expressed on a total N basis, was determined after steam distillation using Kjeldahl method (AOAC, 1995) as previously described.

The aerobic stability was determined using the method of Ranjit and Kung (2000) with slight modification (Gallo et al., 2021a). Counts of microbes (LAB, yeasts, molds) in the fresh and fermented samples were done as described by Gallo et al. (2018). The DM loss was calculated at mini-silos opening as the ratio between the remaining DM (weight) after ensiling relative to original DM ensiled (Queiroz et al., 2013).

2.3 Statistical analysis

The LAB, yeast, and mold CFU were $\log_{10}$-transformed prior to statistical analysis, and the terms of the ANOVA (standard errors of the means and P values) refer to these $\log_{10}$-transformed values. Variables quantified or calculated at the two storage times were analyzed using a completely randomized design, within a 2 × 10 factorial arrangement of treatments using the general linear model (GLM) procedure of SAS (2003) as:

$$Y_{ijkl} = \mu + B_i + T_j + D_k + (T \times D)_{jk} + e_{ijkl}$$

where: Y_{ijkl} is the dependent variable; µ is the overall mean; B_i is the fixed-effect of a plot (i = 1–4); T_j is the fixed effect of an ensiling treatment (j = 1–10); D_k is the fixed effect of the storage time (k = 30 or 120 days); $(_j \times _k)_{jk}$ is the first order interaction; and e_{ijkl} is residual error. When there was no interaction, the means of the main factors are shown (e.g. storage time and treatments) and the

control was compared with other treatments using Dunnett's multiple comparison test. When an interaction was detected, the means of the combination of the main factors were shown and within each storage time, the control was then compared to other treatments using Dunnett's multiple comparison test. Within each treatment, the mean value obtained at 30 days was compared with mean value obtained at 120 days with t student test.

Data quantified at a single storage time (i.e., mycotoxins other than AF) were analyzed as in the above model, but without a fixed effect of storage time (D_k) and the first order interaction. For all the above statistical comparisons the significance was declared if the P value was < 0.05.

3. Results

Chemical composition, pH, particle size and microbial counts of the pre-ensiled wet corn meal (Table 1) were within normal ranges for this forage, while there was evidence of contamination by several mycotoxins (AFB1, AFB2 , DON , ZEA , fusaric acid and tenuazonic acid).

Parameters characterizing high moisture corn silage were divided in those having the interaction between the two tested effects (i.e., storage time and treatment) not significant (Table 2) to those showing significant interaction (Table 3). Concerning chemical parameters, a DM reduction from day 30 to day 120 was quantified (storage time and treatment interaction, P = 0.0476) in all treatments (Table 3), with a greater than 3.0% reduction in the CTR, SiloSolve FC_{1x} or SiloSolve FC_{5x} groups and a lower than 2.0% reduction in LP ATCC 801 or LR LR7 groups. The LB DSMZ 20054, LP ATCC 8014, LP PB, LR ATCC 7469, and LR LR7 treatments had a lower (P < 0.001) CP content than CTR (Table 2).

The pH decreased (storage time and treatment interaction, P = 0.002) in the CTR and LR ATTC 7469 groups from day 30 to day 120, but slightly increased (P < 0.001) in LP PB (Table 3). After 30 days, a pH lower (P < 0.001) than CTR was observed for all treatments, while this

happened only for some treatments after 120 days (i.e., LP ATCC 8014, LP PB, LR ATCC 7469, LR LR7, LR RI, or Pioneer 11B91). As reported in Table 2, the ammonia level was higher ($P = 0.014$) in the SiloSolve FC_{5x} group than in the CTR group (62.9 *vs.* 46.0 g/kg total N) and the LR LR7 and Pioneer 11B91 groups had less ($P < 0.001$) ethanol than CTR (7.0 and 8.1 *vs.* 11.0 g/kg DM). Some other fermentation parameters had significant "storage time by treatment" effects (Table 3). The 1,2 propanediol level remained low in both storage times in the CTR group (0.01-0.02 g/kg DM), but increased ($P < 0.001$) 2-4 times from 30 to 120 days in the three commercial LAB groups (SiloSolve FC_{1x}, SiloSolveFC_{5x}, Pioneer 11B91) and in the LR LR7 group. The 1,2 propanediol concentrations greater ($P < 0.001$) than CTR were quantified for SiloSolve FC_{1x}, SiloSolve FC_{5x} (only after 120 days), LR ATCC 7469 (only after 30 days), LR RI, or Pioneer 11B91. Acetic acid level increased ($P < 0.001$) 2-3 fold with storage time in CTR, SiloSolve FC_{1x}, SiloSolve FC_{5x}, LR LR7, or Pioneer 11B91. After 30 days, the acetic acid concentration quantified in LR ATCC 7469 and LR LR7 was greater ($P < 0.001$) than CTR. After 120 days, SiloSolve FC_{1x}, SiloSolve FC_{5x}, LR LR7, or Pioneer 11B91 showed acetic acid value greater ($P < 0.001$) than CTR, whereas values lower ($P < 0.001$) than CTR were quantified in both LP ATCC 8014 and LP PB. In particular, and similarly to trends reported for acetic acid concentrations, the extent of propionic acid increase between storage times ranged ($P < 0.001$) from 0.6 to 3.0 times in CTR or commercial LAB (SiloSolve FC_{1x}, SiloSolveFC_{5x}, Pioneer 11B91) groups or approximately 0.4 in LR LR7 group. No increases from 30 to 120 days were observed for other treatments. Levels of lactic acid, and total esters increased ($P = 0.008$ and $P = 0.003$, respectively) in all groups from 30 to 120 days (except for total esters in LR ATCC 7469). The level of lactic acid increased ($P < 0.001$) with storage time, and was below 20 g/kg DM at day 30 and above 20 g/kg DM at 120 days in all groups. The highest lactic acid level ($P < 0.001$) at day 120 occurred in the LR LR7 group (32.1 g/kg DM). Analyses of changes in the lactic acid/acetic acid ratio indicated it remained constant in

most pure LAB groups over time, but decreased ($P < 0.001$) in the SiloSolve FC_{1x} group (4.18 to 2.23) and increased ($P < 0.001$) in the LR ATCC 7469 (5.26 to 7.37) or LR RI (9.56 to 11.40) groups. Ratios above 10 were calculated in the LP ATCC 8014, LP PB, and LR RI groups at day 120.

The level of butyric acid increased 5-fold in CTR, but by only two to three-fold in the groups with pure strains. The level of total aldehydes decreased ($P = 0.008$) by approximately 20% from day 30 to day 120 (Table 2), but there were no differences among groups. The level of total ketones increased (storage time and treatment interaction, $P < 0.001$) with HMC storage time in all groups, except CTR, SiloSolve FC_{1x} and Pioneer 11B91 (Table 3). In particular, higher ($P < 0.001$) concentrations of ketones than CTR were in the LB DSMZ 2005 at both storage times or in LR ATCC 7469, and LP BP groups after 120 days. The total esters increased ($P = 0.003$) by two- to three-folds in all treatments, with the exception of LR ATCC 7469 group, for which ester concentrations remained almost constant from 30 to 120 days. The DM loss was unaffected by storage time or treatment, and was less than 20 g/kg in all groups (Table 2).

Aerobic stability increased ($P = 0.001$) with storage time from 32 to 97 h. The three commercial inoculant groups, and the LR LR7 group, had longer aerobic stability than CTR group ($P = 0.029$). Except for LB DSMZ 20054 group, the LAB counts decreased (storage time and treatment interaction, $P < 0.001$) from 30 to 120 days, and this decrease was more pronounced in CTR, LR RI, LP ATCC 8014, and LP PB groups (Table 3); the exceptions were the LB DSMZ 20054 and the SiloSolveFC_{1x} groups. There was no evidence of molds on day 30 or day 120. Yeast counts decreased ($P < 0.001$) with storage time (Table 2), and the LR LR7 group had fewer yeast cells than the CTR group (2.26 *vs.* 4.73 $\log_{10}$ CFU/g).

Storage time, and LAB treatments, had no effect on the AFB1 and AFB2 concentrations (Table 2). Similarly, DON did not differ among treatments. In contrast, the fusaric acid level increased (P = 0.014) in the Silosolve FC_{1x} and Silosolve FC_{5x} groups.

4. Discussion

The pre-ensiled wet corn meal had chemical traits typical of this type of forage and a sugar content sufficient to support fermentation (Fijałkowska et al., 2020; Kung et al., 2018; Saylor et al., 2021). Further, the mean particle size was in the normal range for this ensiled forage (Saylor et al., 2020a).

In this study, different strains of the same species were tested for their ability to improve fermentation profile of HMC silage, aerobic stability, and to reduce DM loss and mycotoxin production after different storage times. In particular, an early (i.e., 30 days) and a long (i.e., 120 days) mini-silos opening time were tested to verify effects of LAB after these very different storage times. Within the same LAB species, the strains can show different characteristics in terms of fermentation efficiency, antimicrobial activity, resilience to different stresses and ability to resist in harsh condition (e.g., low nutrients concentration) and to compete for the nutrients (Ávila and Carvalho, 2020). The strain of *Lactiplantibacillus plantarum* ATCC 8014 was already tested to determine its effect in improving corn silage quality (Abdul Rahman et al., 2017), whereas *Lactiplantibacillus plantarum* PB has different characteristics with respect to the previous one. For this reason, we planned to study the effect of the inoculation with *Lactiplantibacillus plantarum* on fermentation characteristics of HMC silage. Furthermore, *La. rhamnosus* ATCC 7469 was previously tested in corn silage by Guan et al. (2020), where it was reported that high temperature affected the dynamic changes of these microorganisms in corn silage and resulted in a shift from homo- to hetero-fermentative LAB community. Further, the *La. rhamnosus* RI strain was isolated

300 from olive oil and it showed interesting characteristics related to a potential probiotic activity
301 (Fancello et al., 2020). Furthermore, *La. rhamnosus* LR7 also showed the ability to inhibit fungal
302 growth (Fancello, unpublished data).

303 All inoculants tested reduced the pH with respect to the CTR group, even if the dynamics
304 differed between treatments. In the commercial inoculants, which included *Len. buchneri*, the
305 increase in pH from 30 to 120 days could be due to the ability of this microorganism to convert
306 lactic acid to acetic acid, 1,2-propanediol, and ethanol (Driehuis et al., 1999). The lactate could also
307 be metabolized by yeast or certain LAB that oxidize lactate to pyruvate by lactate oxidase or NAD^+-
308 independent lactate dehydrogenase in aerobic conditions, leading in both cases to an increase in pH.
309 Regarding the CP reduction that occurred with ensiling, it is possible that some strains of
310 commercial products antagonized growth of proteolytic bacteria, such as enterobacteria and
311 clostridia, thus reducing degradation of CP (Ávila and Carvalho, 2020). As for the ammonia, the
312 decrease obtained with storage time appeared consistent with our previous results on corn silage
313 (Gallo et al., 2021a). In particular, the SiloSolve, at the two tested doses (i.e., FC_{1x} and FC_{5x}),
314 showed a higher proteolytic activity than CTR, probably due to the presence of *Lactococcus lactis*
315 (Ardö et al., 2017). In particular, *Lactococcus lactis* is known to have an efficient proteolytic
316 system compared to other LAB, as recently reported by Kieliszek et al. (2021) and Canon et al.
317 (2021). Further, Saylor et al. (2020a) studied the effects of microbial inoculation and particle size
318 on fermentation profile, aerobic stability, and ruminal *in situ* starch degradation of HMC ensiled for
319 a short period. The HMC inoculated with SiloSolve FC had the greatest ammonia-N concentration
320 with respect to other treatments (i.e., control or a commercial microbial inoculant containing
321 *Lactobacillus plantarum* CH6072 and *Enterococcus faecium* CH212), according to the results
322 obtained in this work. However, further trials should be carried out to confirm that the great
323 proteolysis is due to inoculation of *Lactococcus lactis* strains. Indeed, different LAB strains showed

proteolytic activity during silage fermentation process, and we are aware that further studies are needed to better understand entity of protein degradation activity by LAB growing in specific conditions. In addition, a protein matrix in the endosperm of corn grains surrounds the starch granules (Kotarski et al., 1992), and this could inhibit digestion by microorganisms (McAllister et al., 1993). For HMC, in which LAB populations dominate, this phenomenon is likely more pronounced owing to the low ability of LAB to degrade this protein matrix. However, there is evidence that ensiling of HMC (Hoffman et al., 2012) leads to degradation of the hydrophobic starch-protein matrix due to proteolytic activity of microorganisms and plant enzymes, leading to increased starch digestibility by animals.

The high content of 1,2 propanediol in commercial LAB groups (i.e. SiloSolve FC_{1x}, SiloSolveFC_{5x}, Pioneer 11B91) was likely due to *Len. buchneri* (Muck et al., 2018), independently by mean particle size of HMC (Saylor et al., 2020a). The high level of this compound in the LR LR7 group might be due to its ability to produce (S)-lactaldehyde, one of two products resulting from degradation of the methylpentoses, L-fucose and L-rhamnose (normally present in plants), that are metabolized by an analogous series of reactions. Anaerobically, (S)-lactaldehyde is converted to (S)-propane-1,2-diol by a reversible reaction catalyzed by EC 1.1.1.77 (lactaldehyde reductase; Rodionova et al., 2013). Notably, there were higher levels of 1,2 propanediol, acetic acid and propionic acid in the LR7 group relative to the *La. rhamnosus* LR ATCC 7469 and LR RI groups. Recently, LeBlanc et al. (2017) showed that *La. rhamnosus* GG led to significant production of propionate (89 µM) when grown in MRS (i.e., Man, Rogosa and Sharpe) medium. These results, and our data, show that the ability to produce propionate in *La. rhamnosus* is strain–specific. The higher production of acetic acid by this strain should be due to the differentially expression during storage period of phosphotransacetylase and acetate kinase enzymes that are involved in its synthesis (Hatti-Kaul et al., 2018).

As stated by Oliveira et al. (2017), inoculation of silages with LAB usually reduces growth of *Clostridium* spp. and consequent production of butyric acid, thereby improving milk yield probably due to increase in DM intake (DMI) by cattle. In this work, one commercial LAB and one pure strain (LR LR7) led only to slight increases in butyric acid (Saylor et al., 2020b) with storage time. Further, the level of total aldehydes decreases during storage time, whereas total ketones increased with HMC storage time in all groups, with higher concentrations in LB DSMZ 20054, LR ATCC 7469, and LP BP groups after 120 days. In particular, ketones derived from the metabolism of fatty acid, substrates for LAB dehydrogenases, and from amino acid catabolism. According to Ganesan and Weimer (2017), while mechanisms of microbial ketone production and diacetyl formation are not yet elucidated, the low redox potential and microaerophilic conditions occurring into silage might facilitate the biochemical reactions needed for ketone bacterial production. Intensive proteolysis or lipolysis activity could release free amino acid or fatty acids, which represents the substrates for production of ketone by bacterial community. The CTR and SiloSolve FC_{1x} groups had the lowest levels of ketones after 120 days (<0.005 g/kg DM).

The aerobic stability increased in all commercial products and LR LR7 compared to CTR (Gallo et al., 2018, 2021a). We also tested some pure strains of *Lactiplantibacillus plantarum* to verify their ability to improve fermentative characteristics and aerobic stability of high-moisture corn silage, but these effects were not obtained either here or in a previous trial on corn silage (Gallo et al., 2021a). Despite these results, *Lactiplantibacillus plantarum* should be retained as highly versatile bacteria, due to its nomadic lifestyle, meaning its ability to survive and adapt to different environmental conditions (Martino et al., 2016; Guan et al., 2020). For this reason, several authors studied the antifungal activity of *Lactiplantibacillus plantarum* strains isolated from different grass (Ström et al., 2002; Prema et al., 2010; Valan Arasu et al., 2013; Arasu, et al., 2014; Vijayakumar, et al., 2015) or legume (Muthusamy, et al., 2020) silages. The main metabolites implicated in their

antifungal activity were phenyllactic acid, cyclic dipeptides, fatty acids, and organic acids (Behera et al., 2018). In particular, *L. plantarum* is a facultative heterofermentative bacteria that in certain conditions (i.e. depleted glucose concentration) can produce acetic acid, which is very efficient in inhibiting molds in silage (Diepersloot et al., 2021; Saylor et al., 2021a). Recently, Møller et al. (2021) showed the ability of two strains of *Lactiplantibacillus plantarum* to inhibit the production of AFB1 when they were simultaneously inoculated with the mycotoxigenic fungus. Furthermore, *Lactiplantibacillus plantarum* tested in this work showed probiotic characteristics *in vitro* and *in vivo*, and antifungal activity on wheat artificially contaminated with *Fusarium* spp. (F. Fancello, unpublished data).

In this study, both storage time and LAB treatments had no effect on AFB1 and AFB2 concentrations as previously showed (Ferrero et al. ,2019), and DON concentrations did not change among treatments. Indeed, the higher level of fusaric acid in Silosolve FC_{1x} and Silosolve FC_{5x} groups were in line with previous results (Gallo et al., 2018; Saylor et al., 2020b). Corn silage inoculated with the same commercial product containing *Lent. buchneri* and *Lactococcus lactis* (i.e., Silo Solve FC) reduced contamination of *Penicillium*-related mycotoxins and increased fusaric acid contamination after 32 d of ensiling with respect to untreated control (Gallo et al., 2018). Further, ZEA and tenuazonic acid were in fresh corn but were undetectable after ensiling. Recent studies also demonstrated reductions of toxins during ensiling. For example, Møller et al. (2021) showed reductions of at least 50% in levels of AFB1, ochratoxin A and zearalenone following inoculation with *L. brevis* 2QB422 and other six lab strains *in vitro*. Furthermore, Martinez Tuppia et al. (2017) isolated LAB strains from HMC silages that metabolized fumonisin B1 to the less toxic form, hydrolyzed fumonisin B1. Such LAB strains may also be effective in degrading fumonisin B1 in silages from grasses, legumes, or whole crop-cereals. The mechanism of mycotoxin reductions

remain to be elucidated, and more researches are needed to identify other microbes that can degrade or detoxify these toxins during silage.

5. Conclusions

Examination of effects of nine LAB inoculants, three commercial products and six pure LAB strains, indicated that none of them lowered AF levels in HMC silage. Our measurements of other mycotoxins indicated that zearalenone and tenuazonic acid were not detected at day 30 or day 120, even when they were present in freshly harvested kernels. The characteristics of fermentation of HMC silage differed among LAB groups. In particular the commercial inoculants and LR LR7 increased levels of acetic acid and 1,2 propanediol and improved aerobic stability of HMC silage. Thus, our study indicates that the three commercial LAB inoculants, and the LR LR7 inoculant, improved the fermentation profile and reduced mycotoxin contamination, but did not affect levels of AF in HMC silage.

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Authors Contribution - Sample CRediT author statement

Antonio Gallo: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Roles/Writing - original draft, Writing - review & editing.

Francesco Fancello: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Roles/Writing - original draft.

Francesca Ghilardelli: Data curation, Investigation, Methodology, Visualization, Writing - review & editing.

Severino Zara: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing - review & editing.

Mauro Spanghero: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Supervision, Visualization, Roles/Writing - original draft.

Conflict of interest

All authors declare no potential conflicts of interest.

Table 1. Chemical composition, microbial counts and mycotoxin contaminations of pre-ensiling high moisture corn (HMC) silage (n = 4 samples, one for each plot of experimental field).

Item[1]	Mean	SD
Dry matter, g/kg as fed	704	4.5
Ash, g/kg DM	12.4	0.40
CP, g/kg DM	81.7	2.70
aNDFom, g/kg DM	135	5.0
Starch, g/kg DM	610	11.5
Sugar, g/kg DM	31	0.9
pH, dmnl	5.87	0.171
LAB, $\log_{10}$ cfu/g	7.68	0.467
Yeasts, $\log_{10}$ cfu/g	6.81	0.339
Molds, $\log_{10}$ cfu/g	5.71	0.115
AFB1, µg/kg DM	19.4	5.00
AFB2, µg/kg DM	0.77	0.196
DON, µg/kg DM	493	71.6
ZEA, µg/kg DM	20	16.5
Fusaric acid, µg/kg DM	72	10.8
Tenuazonic acid, µg/kg DM	315	10.8
Mean particle size, mm	1013	129.0

cfu, colony forming units; dmnl, dimensionless.

[1]AFB1, aflatoxin B1; AFB2, aflatoxin B2; CP, crude protein; DM, dry matter; DON, deoxynivalenol; aNDFom, NDF assayed with sodium sulphite and a heat stable amylase and expressed exclusive of residual ash; ZEA, zearalenone.

Table 2

Table 2. Effects of storage time (ST) or addition of commercial or pure lactic acid bacteria (LAB) on parameters[1] measured in high moisture corn silage.

Item[2]	Storage time (days)		Treatments[3]										SEM	Effects of model (P < 0.05)		
	30	120	CTR	SiloSolve FC_{1x}	SiloSolve FC_{5x}	LB DSMZ 20054	LP ATCC 8014	LP PB	LR ATCC 7469	LR LR7	LR RI	Pioneer 11B91		Days	Treatment	Days × Treatment
Chemical parameters																
Ash, g/kg DM	14.9	14.9	14.6	14.8	14.9	14.8	15.0	15.0	14.8	15.0	15.0	15.0	0.22	0.589	0.571	0.488
CP, g/kg DM	88.4	86.5	89.8	88.0	88.9	86.4[a]	85.7[a]	87.0[a]	86.8[a]	86.9[a]	87.3	87.7	1.00	0.004	<0.001	0.840
aNDFom, g/kg DM	127	130	132	128	127	131	127	128	127	127	128	133	3.3	0.052	0.454	0.483
Starch, g/kg DM	606	611	606	610	610	605	614	609	612	611	610	601	4.7	0.017	0.293	0.602
DM loss, g/kg DM	1.1	1.2	1.3	1.1	1.1	1.2	0.9	0.9	2.0	0.9	1.0	1.2	0.34	0.072	0.482	0.364
Ammonia N, g/kg total N	57.6	37.3	46.0	53.0	62.9[b]	47.5	38.5	40.1	48.2	45.0	45.5	47.7	5.96	<0.001	0.014	0.098
Ethanol, g/kg DM	9.4	10.2	11.0	9.1	9.5	13.0	10.4	10.5	8.8	7.0[a]	10.7	8.1[a]	0.91	0.050	<0.001	0.072
Total aldehydes, g/kg DM	0.053	0.041	0.045	0.033	0.031	0.056	0.057	0.052	0.048	0.043	0.057	0.048	0.0010	0.008	0.100	0.610
Total alcohols, g/kg DM	9.5	10.3	11.1	9.3	9.6	13.0	10.4	10.5	8.9	7.3[a]	10.8	8.2[a]	0.91	0.041	<0.001	0.067
Aerobic stability, hours	32	97	42	92[b]	89[b]	49	57	43	42	92[b]	62	78[b]	4.2	0.001	0.029	0.289
Yeasts, log_{10} cfu/g	5.23	3.02	4.73	3.56	3.82	4.24	4.31	4.24	4.98	2.26[a]	4.42	4.69	0.452	<0.001	<0.001	0.093
Mycotoxins																
AFB1, µg/kg DM	22.0	24.2	15.8	22.4	19.3	16.9	17.4	17.2	17.9	17.1	17.6	19.4	1.83	0.348	0.631	0.361
AFB2, µg/kg DM	1.44	1.42	0.77	1.034	0.92	0.85	1.02	0.97	0.93	0.97	0.94	0.89	0.092	0.456	0.617	0.351
DON[4], µg/kg DM	-	-	715	687	719	784	795	637	704	681	717	728	57.2	-	0.864	-
Fusaric acid[4], µg/kg DM	-	-	50	117[b]	112[b]	76	65	61	52	67	90	75	16.2	-	0.014	-

Superscripts indicate that the specific mean is lower (i.e., a) or higher (i.e., b) than CTR at a P < 0.05, as evaluated with Dunnett's multiple comparison test.
cfu, colony forming units; Molds were not detected in any samples.

[1]The interaction ST*LAB was not statistically significant (P>0.05) for all reported parameters.

[2]CP, crude protein; DM, dry matter; NDFom, NDF assayed with sodium sulphite and a heat stable amylase and expressed exclusive of residual ash..

[3]CTR, control; LB DSMZ 20054, *Levilactobacillus brevis* DSMZ 20054; LP ATCC 8014, *Lactiplantibacillus plantarum* ATCC 8014; LP PB, *Lactiplantibacillus plantarum* PB; LR ATCC 7469, *Lacticaseibacillus rhamnosus* ATCC 7469; LR LR7, *Lacticaseibacillus rhamnosus* LR7; LR RI, *Lacticaseibacillus rhamnosus* RI.

[4]DON and fusaric acid were measured only in mini-silos opened after 120 days of ensiling.

Table 3

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Table 3. Effects of storage time (ST) or addition of commercial or pure lactic acid bacteria (LAB) on parameters[1] measured in high moisture corn silage.

Item		Storage time (days)	Treatments[2]									SEM	Effects of model (P < 0.05)		
			CTR	SiloSolve FC$_{1x}$	SiloSolve FC$_{5x}$	LB DSMZ 20054	LP ATCC 8014	LP PB	LR ATCC 7469	LR LR7	LR RI	Pioneer 11B91		Days	Treatment	Days × Treatment
Chemical parameters																
Dry matter	g/kg as fed	30	714*	709*	711*	713*	711*	711*	718*	714*	710*	716*	2.1	<0.001	<0.001	0.0476
		120	691	686	690	698	700^{b}	693	697	701^{b}	692	700^{b}				
pH	dmnl	30	4.25*	4.06^{a}	4.08^{a}	4.10^{a}	3.92^{a}	3.96*^{a}	4.01*^{a}	3.97^{a}	3.95^{a}	4.09^{a}	0.022	0.685	<0.001	0.002
		120	4.14	4.10	4.11	4.15	3.97^{a}	4.04^{a}	3.94^{a}	3.97^{a}	3.92^{a}	4.07^{a}				
1,2 propanediol	g/kg DM	30	0.01	0.74*^{b}	0.64*	0.06	0.02	0.02	0.67^{b}	1.10^{b}	0.02	0.90^{b}	0.209	<0.001	<0.001	<0.001
		120	0.02	3.37^{b}	1.83^{b}	0.17	0.04	0.05	0.52	1.88^{b}	0.02	2.46^{b}				
Acetic acid	g/kg DM	30	2.00*	3.20*	3.44*	2.31	1.33	1.35	3.68^{b}	4.53*^{b}	1.99	3.40*	0.401	<0.001	<0.001	<0.001
		120	3.89	10.55^{b}	7.48^{b}	3.28	2.02^{a}	2.04^{a}	3.95	7.67^{b}	2.38	8.85^{b}				
Propionic acid	g/kg DM	30	0.019*	0.028*	0.025*	0.021	0.019	0.021	0.035	0.083*^{b}	0.024	0.028*	0.0048	<0.001	<0.001	<0.001
		120	0.047	0.083^{b}	0.043	0.029	0.022^{a}	0.026^{a}	0.045	0.115^{b}	0.030	0.069^{b}				
Lactic acid	g/kg DM	30	9.7*	12.7*	14.3*^{b}	15.1*^{b}	17.3*^{b}	16.0*^{b}	17.7*^{b}	15.5*^{b}	19.0*^{b}	12.9*	1.18	<0.001	<0.001	0.008
		120	20.5	23.3	23.5	22.8	27.5^{b}	25.6^{b}	27.7^{b}	32.1^{b}	26.8^{b}	20.9				
Lactic/acetic		30	4.92	4.18*	4.31	6.62	13.0^{b}	11.9^{b}	5.26*	3.51	9.56*^{b}	3.91	0.581	0.333	<0.001	0.010
		120	5.40	2.23^{a}	3.17	7.05	13.7^{b}	12.6^{b}	7.37	4.35	11.4^{b}	2.36^{a}				
Butyric acid	g/kg DM	30	0.134*	0.129	0.153	0.193*	0.168*	0.228*	0.216*	0.134	0.211*	0.145	0.0763	<0.001	<0.001	<0.001
		120	0.770	0.198^{a}	0.206^{a}	0.487	0.461	0.690	0.679	0.209^{a}	0.420	0.206^{a}				
Total ketones	g/kg DM	30	0.001	0.002	0.002*	0.009*^{b}	0.005*	0.002*	0.004*	0.001*	0.011*^{b}	0.004	0.0023	<0.001	<0.001	0.001
		120	0.003	0.004	0.011	0.023^{b}	0.014^{b}	0.020^{b}	0.023^{b}	0.011	0.019^{b}	0.010				
Total esters	g/kg DM	30	0.204*	0.155*	0.194*	0.206*	0.178*	0.136*	0.274	0.180*	0.166*	0.210*	0.0310	<0.001	0.014	0.003
		120	0.353	0.493	0.424	0.369	0.312	0.295	0.303	0.302	0.297	0.352				
LAB	log$_{10}$ cfu/g	30	8.05*	8.10*	8.66*^{b}	8.14	8.18*	8.07*	8.56*^{b}	8.89*^{b}	8.55*^{b}	8.57*^{b}	0.141	<0.001	<0.001	<0.001
		120	6.97	8.53^{b}	8.17^{b}	7.75^{b}	7.05	7.03	7.83^{b}	8.46^{b}	6.52	8.09^{b}				

Within each treatment, the symbol "*" means the average value at 30 days significantly differed at a P < 0.05 by average value at 120 days, as evaluated by *t* student test.
Superscripts indicate that the specific mean is lower (i.e., a) or higher (i.e., b) than CTR at a P < 0.05 within the same storage time (i.e., 30 or 120 days), as evaluated with Dunnett's multiple comparison test.
cfu, colony forming units; Molds were not detected in any samples.
[1]The interaction ST*LAB was statistically significant (P<0.05) for all reported parameters.
[2]CTR, control; LB DSMZ 20054, *Levilactobacillus brevis* DSMZ 20054; LP ATCC 8014, *Lactiplantibacillus plantarum* ATCC 8014; LP PB, *Lactiplantibacillus plantarum* PB; LR ATCC 7469, *Lacticaseibacillus rhamnosus* ATCC 7469; LR LR7, *Lacticaseibacillus rhamnosus* LR7; LR RI, *Lacticaseibacillus rhamnosus* RI.