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1 **Effects of several commercial or pure lactic acid bacteria inoculants on fermentation and**
2 **mycotoxin levels in high-moisture corn silage**

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13

14 **ABSTRACT**

15 Effects of 3 commercial lactic acid bacteria (LAB) inoculants and 6 single strains of LAB on
16 fermentation and levels of aflatoxins and other mycotoxins in high-moisture corn (HMC) silage
17 were examined. Corn plants were inoculated with *Aspergillus flavus* at silk emergence and wet corn
18 kernels were harvested at black layer stage (dry matter [DM]: 704 ± 5.0 g/kg). Samples were
19 ground and ensiled directly (control group, CTR) or after treatment with one of the 9 LAB
20 inoculants. The commercial inoculants contained *Lentilactobacillus (Lent.) buchneri* LB1819 and
21 *Lactococcus lactis* O224 (SiloSolve FC; Chr. Hansen A/S, Hørsholm, Denmark), applied at a final
22 dose of 250,000 CFU/g or 1,250,000 CFU/g (SiloSolve FC_{1x} and SiloSolve FC_{5x}, respectively); and
23 *Lent. buchneri* and *Lactiplantibacillus (Lac.) plantarum* (Pioneer brand 11B91, Pioneer Hi-Bred
24 International, Des Moines, USA) applied at a final dose of 250,000 CFU/g. One pure strain of
25 *Levilactobacillus (Lev.) brevis* DSMZ 20054, two pure strains of *Lac. plantarum* (LP ATCC 8014
26 and LP PB) and three of *Lacticaseibacillus (La.) rhamnosus* (LR ATCC 7469, LR LR7 and LR RI)
27 were also applied at a final expected dose of 250,000 CFU/g. Wet corn kernels were ensiled in 20 L
28 mini-silos, compacted to a density of 389 kg DM/m³, and stored at room temperature. Conditions
29 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
30 molds and 6.81 ± 0.341 for yeasts. The mini-silos were opened after 30 or 120 days. From day 30 to
31 120 days, levels of acetic acid, propionic acid and 1,2 propanediol increased ($P < 0.05$) in the
32 SiloSolve FC_{1x}, SiloSolveFC_{5x} and Pioneer 11B91, and in LR LR7 groups. During this time, butyric
33 acid level increased 5-fold in CTR; by two to three-fold in pure strains groups; and only slightly in
34 the three commercial inoculants and the LR LR7 ($P < 0.05$). Aerobic stability increased ($P < 0.05$)
35 with storage time from 32 to 97 h, and the commercial inoculants and LR LR7 groups had longer
36 stability than CTR ($P < 0.05$). The storage time and the inoculants did not affect aflatoxin levels. No
37 zearalenone or tenuazonic acid were detected in the mini-silos, even if they contaminated the corn

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

40
41 **Keywords:** corn; volatile organic compounds; aflatoxin; aerobic stability.

42
43 **Abbreviations:** AF - aflatoxins; AFB1 - aflatoxin B1; AFB2 - aflatoxin B2; AFM1 - aflatoxin M1;
44 aNDFom - NDF assayed with sodium sulphite and a heat stable amylase and expressed exclusive of
45 residual ash; CFU - colony forming units; CP - crude protein; CTR - high moisture corn without
46 inoculant; DM - dry matter; DON - deoxynivalenol; GC - gas chromatography; GLM - general
47 linear model; HMC - high moisture corn; HPLC - high performance liquid chromatograph; LAB -
48 lactic acid bacteria; LB DSMZ 20054 - high moisture corn treated with *Levilactobacillus brevis*
49 DSMZ 20054; LP ATCC 8014 - high moisture corn treated with *Lactiplantibacillus plantarum*
50 ATCC 8014; LP PB - high moisture corn treated with *Lactiplantibacillus plantarum* PB; LR ATCC
51 7469 - high moisture corn treated with *Lacticaseibacillus rhamnosus* ATCC 7469; LR LR7 - high
52 moisture corn treated with *Lacticaseibacillus rhamnosus* LR7; LR RI - high moisture corn treated
53 with *Lacticaseibacillus rhamnosus* RI; VFA - volatile fatty acids; VOC - volatile organic
54 compounds; ZEA – zearalenone.

55
56 **1. Introduction**

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

62 ensiled forage (Gallo et al., 2021b). The AFB1 is the most toxic and carcinogenic of these
63 compounds (Placinta et al., 1999), and its ingestion by dairy cattle leads to its partial hydroxylation
64 in the liver to AFM1, a highly toxic and carcinogenic compound that can be secreted in milk
65 (Battacone et al., 2009; Masoero et al., 2007). Winn and Lane (1978) demonstrated that sufficient
66 production of AF in HMC at a relatively low temperature (25°C) following a short incubation time
67 (72 h) to be harmful to livestock. Thus, AF formation in silage at the farm level will often be a
68 problem in warm temperate regions (Ferrero et al., 2019).

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

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

89

90 **2. Materials and Methods**

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

95

96 *2.1 Corn harvesting, treatments and preparation of mini-silos*

97 A late-season corn hybrid (Pioneer Hi-Bred International, Inc., Des Moines, IA, USA) grown
98 during 2019 at the Centro Ricerche Zootecniche (CERZOO S.r.L., San Bonico, Italy) was
99 inoculated with a toxigenic strain of *A. flavus* (ITEM 8069, 1×10^5 spores/mL) at silk emergence
100 (Giorni et al., 2019). Approximately 8 tonnes of wet corn kernels were collected in each of four
101 plots from the same experimental field at the black layer stage (dry matter [DM]: 704 ± 5.0 g/kg)
102 and ground using a hammer mill (Hercules 130, Mifema, Spinadesco, Cremona, Italy) with a
103 working capacity of 15 tons/h and equipped with a 2-mm screen. To calculate the mean particle size
104 of each corn kernel batch, a representative amount (100 g) of the various grinds was run for 10 min
105 through a sieve shaker (Multidimensional Sieveshaker IG/1/S, Giuliani Tecnologie s.r.l., Torino,
106 Italy) that had 9 different screen sieves with nominal aperture sizes of 4.00, 3.50, 2.50, 1.50, 1.00,
107 0.75, 0.50, 0.25, and 0.125 mm, followed by a pan as reported by Gallo et al. (2016b). The mean
108 particle size was calculated using equation 1 of ASAE S319.3 method, as reported in ASABE
109 (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 (Pfoest 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 *Lactiplantibacillus plantarum*
119 (Pioneer® brand 11B91 HMC Inoculant, Pioneer Hi-Bred International, Des Moines, IA, USA;
120 Pioneer 11B91); (v) *Levilactobacillus brevis* DSMZ 20054 (Deutsche Sammlungen 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 preservation, 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/m³, and then sealed with plastic lids and
132 stored at $20.4^\circ\text{C} \pm 0.52$) 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.

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

136 *2.2 Chemical analysis and measurements*

137 After 30 or 120 days of storage times, fresh and fermented HMC samples were analyzed in
138 duplicate (except for DM) for chemicals. Samples were dried at 60°C in a forced air oven for 48 h,
139 milled and passed through a 1-mm screen using a laboratory mill (Thomas-Wiley, Arthur H.
140 Thomas Co., Philadelphia, PA, USA), and then stored for subsequent analysis. The DM was
141 determined by gravimetric loss of free water after heating at 105°C for 3 h (AOAC, 1995; method
142 945.15), with correction for volatile losses during oven drying using the equations of NorFor
143 (2011). Ash was determined as gravimetric residue after incineration at 550°C for 2 h (AOAC,
144 1995; method 942.05), and crude protein (CP; $N \times 6.25$) was determined using a Kjeldahl method
145 (AOAC, 1995; method 984.13). Neutral detergent (ND) fiber fraction (NDF) was determined using
146 the Ankom^{II} Fiber Analyzer (Ankom Technology Corporation, Fairport, NY, USA), as described by
147 Mertens (2002). The ND solution contained sodium sulfite and a heat stable amylase (activity:
148 17.400 Liquefon-U/mL, Ankom Technology) and NDF was corrected for residual ash (aNDFom).
149 Starch was determined by polarimetry (Polax 2L, Atago[®], Tokyo, Japan) and free total sugars (i.e.
150 sum of glucose, saccharose, lactose, fructose and maltose) were determined following phosphate
151 buffer and acetonitrile extraction, with quantification using a high-performance liquid
152 chromatography system (HPLC; Jasco, Tokyo, Japan) equipped with an LU-980 pump and an RI-
153 2031 refractive index detector (Gallo et al., 2016a).

154 Mycotoxin analysis was completed on fresh and fermented HMC samples to quantify AF (all
155 samples) and other mycotoxins (i.e., altenuene, alternol, alternol monomethyl ether, tentoxin,
156 tenuazonic acid, fusaric acid, zearalenone, deoxynivalenol [DON], PR toxin, mycophenolic acid,
157 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 N₂ 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

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

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

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

195 *2.3 Statistical analysis*

196 The LAB, yeast, and mold CFU were log₁₀-transformed prior to statistical analysis, and the
197 terms of the ANOVA (standard errors of the means and P values) refer to these log₁₀-transformed
198 values. Variables **quantified or calculated** at the two storage times were analyzed using a completely
199 randomized design, within a 2 × 10 factorial arrangement of treatments using the general linear
200 model (GLM) procedure of SAS (2003) as:

$$201 \quad Y_{ijkl} = \mu + B_i + T_j + D_k + (T \times D)_{jk} + e_{ijkl}$$

202 where: Y_{ijkl} is the dependent variable; μ is the overall mean; B_i is the fixed-effect of a plot ($i = 1-4$);
203 T_j is the fixed effect of an ensiling treatment ($j = 1-10$); D_k is the fixed effect of the storage time (k
204 = 30 or 120 days); $(j \times k)_{jk}$ is the first order interaction; and e_{ijkl} is residual error. When there was no
205 interaction, the means of the main factors are shown (e.g. storage time and treatments) and the

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

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

214

215 **3. Results**

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

219 Parameters **characterizing** high moisture corn silage were divided in those having the
220 interaction between the two tested effects (i.e., storage time and treatment) not significant (Table 2)
221 to those showing significant interaction (Table 3). Concerning chemical parameters, a DM
222 reduction from day 30 to day 120 was **quantified (storage time and treatment interaction, P =**
223 **0.0476)** in all treatments (Table 3), with a greater than 3.0% reduction in the CTR, SiloSolve FC_{1x}
224 or SiloSolve FC_{5x} groups and a lower than 2.0% reduction in LP ATCC 801 or LR LR7 groups. The
225 LB DSMZ 20054, LP ATCC 8014, LP PB, LR ATCC 7469, and LR LR7 treatments had a lower (**P**
226 **< 0.001**) CP content than CTR (Table 2).

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

230 happened only for some treatments after 120 days (i.e., LP ATCC 8014, LP PB, LR ATCC 7469,
231 LR LR7, LR RI, or Pioneer 11B91). As reported in Table 2, the ammonia level was higher ($P =$
232 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
233 LR7 and Pioneer 11B91 groups had less ($P < 0.001$) ethanol than CTR (7.0 and 8.1 vs. 11.0 g/kg
234 DM). Some other fermentation parameters had significant “storage time by treatment” effects
235 (Table 3). The 1,2 propanediol level remained low in both storage times in the CTR group (0.01-
236 0.02 g/kg DM), but increased ($P < 0.001$) 2-4 times from 30 to 120 days in the three commercial
237 LAB groups (SiloSolve FC_{1x}, SiloSolveFC_{5x}, Pioneer 11B91) and in the LR LR7 group. The 1,2
238 propanediol concentrations greater ($P < 0.001$) than CTR were **quantified** for SiloSolve FC_{1x},
239 SiloSolve FC_{5x} (only after 120 days), LR ATCC 7469 (only after 30 days), LR RI, or Pioneer
240 11B91. Acetic acid level increased ($P < 0.001$) 2-3 fold with storage time in CTR, SiloSolve FC_{1x},
241 SiloSolve FC_{5x}, LR LR7, or Pioneer 11B91. After 30 days, the acetic acid concentration **quantified**
242 in LR ATCC 7469 and LR LR7 was greater ($P < 0.001$) than CTR. After 120 days, SiloSolve FC_{1x},
243 SiloSolve FC_{5x}, LR LR7, or Pioneer 11B91 showed acetic acid value greater ($P < 0.001$) than CTR,
244 whereas values lower ($P < 0.001$) than CTR were **quantified** in both LP ATCC 8014 and LP PB. In
245 particular, and similarly to trends reported for acetic acid concentrations, the extent of propionic
246 acid increase between storage times ranged ($P < 0.001$) from 0.6 to 3.0 times in CTR or commercial
247 LAB (SiloSolve FC_{1x}, SiloSolveFC_{5x}, Pioneer 11B91) groups or approximately 0.4 in LR LR7
248 group. No increases from 30 to 120 days were **observed** for other treatments. Levels of lactic acid,
249 and total esters increased ($P = 0.008$ and $P = 0.003$, respectively) in all groups from 30 to 120 days
250 (except for total esters in LR ATCC 7469). The level of lactic acid increased ($P < 0.001$) with
251 storage time, and was below 20 g/kg DM at day 30 and **above** 20 g/kg DM at 120 days in all
252 groups. The highest lactic acid level ($P < 0.001$) at day 120 occurred in the LR LR7 group (32.1
253 g/kg DM). Analyses of changes in the lactic acid/acetic acid ratio indicated it remained constant in

254 most pure LAB groups over time, but decreased ($P < 0.001$) in the SiloSolve FC_{1x} group (4.18 to
255 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)
256 groups. Ratios above 10 were **calculated** in the LP ATCC 8014, LP PB, and LR RI groups at day
257 120.

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

268 Aerobic stability increased ($P = 0.001$) with storage time from 32 to 97 h. The three
269 commercial inoculant groups, and the LR LR7 group, had longer aerobic stability than CTR group
270 ($P = 0.029$). Except for LB DSMZ 20054 group, the LAB counts decreased (**storage time and**
271 **treatment interaction**, $P < 0.001$) from 30 to 120 days, and this decrease was more pronounced in
272 CTR, LR RI, LP ATCC 8014, and LP PB groups (Table 3); the exceptions were the LB DSMZ
273 20054 and the SiloSolveFC_{1x} groups. There was no evidence of molds on day 30 or day 120. Yeast
274 counts decreased ($P < 0.001$) with storage time (Table 2), and the LR LR7 group had fewer yeast
275 cells than the CTR group (2.26 vs. 4.73 log₁₀ CFU/g).

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

279

280 **4. Discussion**

281 The pre-ensiled wet corn meal had chemical traits typical of this type of forage and a sugar
282 content sufficient to support fermentation (Fijałkowska et al., 2020; Kung et al., 2018; Saylor et al.,
283 2021). Further, the mean particle size was in the normal range for this ensiled forage (Saylor et al.,
284 2020a).

285 In this study, different strains of the same species were tested for their ability to improve
286 fermentation profile of HMC silage, aerobic stability, and to reduce DM loss and mycotoxin
287 production after different storage times. In particular, an early (i.e., 30 days) and a long (i.e., 120
288 days) mini-silos opening time were tested to verify effects of LAB after these very different storage
289 times. Within the same LAB species, the strains can show different characteristics in terms of
290 fermentation efficiency, antimicrobial activity, resilience to different stresses and ability to resist in
291 harsh condition (e.g., low nutrients concentration) and to compete for the nutrients (Ávila and
292 Carvalho, 2020). The strain of *Lactiplantibacillus plantarum* ATCC 8014 was already tested to
293 determine its effect in improving corn silage quality (Abdul Rahman et al., 2017), whereas
294 *Lactiplantibacillus plantarum* PB has different characteristics with respect to the previous one. For
295 this reason, we planned to study the effect of the inoculation with *Lactiplantibacillus plantarum* on
296 fermentation characteristics of HMC silage. Furthermore, *La. rhamnosus* ATCC 7469 was
297 previously tested in corn silage by Guan et al. (2020), where it was reported that high temperature
298 affected the dynamic changes of these microorganisms in corn silage and resulted in a shift from
299 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

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

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

348 As stated by Oliveira et al. (2017), inoculation of silages with LAB usually reduces growth of
349 *Clostridium* spp. and consequent production of butyric acid, thereby improving milk yield probably
350 due to increase in DM intake (DMI) by cattle. In this work, one commercial LAB and one pure
351 strain (LR LR7) led only to slight increases in butyric acid (Saylor et al., 2020b) with storage time.
352 Further, the level of total aldehydes decreases during storage time, whereas total ketones increased
353 with HMC storage time in all groups, with higher concentrations in LB DSMZ 20054, LR ATCC
354 7469, and LP BP groups after 120 days. In particular, ketones derived from the metabolism of fatty
355 acid, substrates for LAB dehydrogenases, and from amino acid catabolism. According to Ganesan
356 and Weimer (2017), while mechanisms of microbial ketone production and diacetyl formation are
357 not yet elucidated, the low redox potential and microaerophilic conditions occurring into silage
358 might facilitate the biochemical reactions needed for ketone bacterial production. Intensive
359 proteolysis or lipolysis activity could release free amino acid or fatty acids, which represents the
360 substrates for production of ketone by bacterial community. The CTR and SiloSolve FC_{1x} groups
361 had the lowest levels of ketones after 120 days (<0.005 g/kg DM).

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

372 antifungal activity were phenyllactic acid, cyclic dipeptides, fatty acids, and organic acids (Behera
373 et al., 2018). In particular, *L. plantarum* is a facultative heterofermentative bacteria that in certain
374 conditions (i.e. depleted glucose concentration) can produce acetic acid, which is very efficient in
375 inhibiting molds in silage (Diepersloot et al., 2021; Saylor et al., 2021a). Recently, Møller et al.
376 (2021) showed the ability of two strains of *Lactiplantibacillus plantarum* to inhibit the production
377 of AFB1 when they were simultaneously inoculated with the mycotoxigenic fungus. Furthermore,
378 *Lactiplantibacillus plantarum* tested in this work showed probiotic characteristics *in vitro* and *in*
379 *vivo*, and antifungal activity on wheat artificially contaminated with *Fusarium* spp. (F. Fancello,
380 unpublished data).

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

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

397

398 **5. Conclusions**

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

408

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416

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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.**

¹AFB1, 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. Effects of storage time (ST) or addition of commercial or pure lactic acid bacteria (LAB) on parameters¹ 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
<i>Chemical parameters</i>																
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
<i>Mycotoxins</i>																
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.

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

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

³CTR, 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.

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

Table 3. Effects of storage time (ST) or addition of commercial or pure lactic acid bacteria (LAB) on parameters¹ 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	
<i>Chemical parameters</i>																
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.

¹The interaction ST*LAB was statistically significant (P<0.05) for all reported parameters.

²CTR, 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.