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Effects of several lactic acid bacteria inoculants on fermentation and mycotoxins in corn silage

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ABSTRACT

Effects of commercial and pure lactic acid bacteria (LAB) inoculants on fermentation and levels of aflatoxins and other mycotoxins in corn silage that was experimentally contaminated in the field with a toxigenic strain of *Aspergillus flavus* (ITEM 8069) were determined. Late-season corn hybrid plants were inoculated with *A. flavus* at silk emergence, and fresh-chopped corn was harvested at the ⅔ milk line stage (dry matter [DM]: 428±6.5 g/kg) and ensiled directly (control, CTR) or after treatment with 9 LAB inoculants. Commercial inoculants contained *Lactobacillus* (L.) *buchneri* LB1819 and *Lactococcus lactis* O224 or *Lactococcus lactis* SR3.54 (SiloSolve FC or SiloSolve MC, respectively in a proportion of 50:50 for two specific bacterial strains; Chr. Hansen A/S, Hørsholm, Denmark), *L. buchneri* and *L. plantarum* (bacterial strains proportion of 50:50, Pioneer brand 11C33, Pioneer Hi-Bred International, Des Moines, USA). One pure strain of *L. brevis* DSMZ 20054, two pure strains of *L. plantarum* (LP ATCC 8014 and LP PB) and three of *L. rhamnosus* (LR ATCC 7469, LR LR7 and LR RI) were also used. Each silage received an inoculant at final expected dose of approximately 250,000 CFU/g of freshly chopped forage, and was ensiled in 20 L mini-silos, compacted to density of 160 kg dry-mass/m^3, stored at room temperature, and opened after 30 or 120 days. The commercial and pure inoculants had very limited effects on fermentation relative to CTR. Ensiling time and LAB treatment had no effect on silage pH. However SiloSolve FC silage had lower ethanol levels than CTR group, while 1,2 propanediol level doubled as ensiling time increased and the high values were with SiloSolve FC and Pioneer CFT. Aerobic stability increased with ensiling time, with the longest stability in SiloSolve FC and *L. rhamnosus* ATCC 7469 silage. Lowest aflatoxin B1 (AFB1) concentrations were in the *L. rhamnosus* LR7 and *L. plantarum* ATCC 8014 silages (about 8 to 10 µg/kg DM) and the highest concentration was in *L. brevis* DSMZ 20054 silage (34.2 µg/kg DM). Results indicate that these silage inoculants had very limited effects on fermentation of corn silage.

Keywords: aerobic stability; volatile organic compounds; heterolactic fermentation; homolactic fermentation.

Abbreviations:

ADFom - ADF expressed exclusive of residual ash; ADICP – acid detergent insoluble CP; AF – aflatoxins; AFB1 - aflatoxin B1; AFB2 -aflatoxin B2; AFG1 - aflatoxin G1; AFG2 - aflatoxin G2; 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 - corn silage treated without inoculant; DM - dry matter; DON - deoxynivalenol; EE - ether extract; GC - gas chromatography; GLM - general linear model; HPLC - high performance liquid chromatograph; LAB - lactic acid bacteria; LB DSMZ 20054 - corn silage treated with *Lactobacillus brevis* DSMZ 20054; lignin(sa) - lignin determined by solubilization of cellulose with sulphuric acid; LP ATCC 8014 - corn silage treated with *Lactobacillus plantarum* ATCC 8014; LP PB - corn silage treated with *Lactobacillus plantarum* PB; LR ATCC 7469 - corn silage treated with *Lactobacillus rhamnosus* ATCC 7469; LR LR7 - corn silage treated with *Lactobacillus rhamnosus* LR7; LR RI - corn silage treated with *Lactobacillus rhamnosus* RI; $NDFD_{24}$ - NDF disappearance measured *in situ* after 24 hours of ruminal incubation; NDICP - Neutral detergent insoluble CP; TN - total N; VFA - volatile fatty acids; VOC - volatile organic compounds; ZEA – zearalenone.

1. Introduction

Utilization of high-quality silage that has no undesirable microorganisms or toxins is necessary to safeguard animal health and maximize their performance (Gallo et al., 2015a; Ogunade et al., 2018). Whole plant corn silage is widely used in dairy cow diets due to its high level of fermentable nutrients, such as fibers and starch, and the overall ease of ensiling (Gallo et al., 2016). However, growth of filamentous fungi and undesirable microorganisms, and their production of toxic metabolites such as mycotoxins, are difficult to control at field scale (Gallo et al., 2015a). Mycotoxins are secondary metabolites with low molecular weight that are mainly produced by fungi in the genera of *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria* spp., and several related fungi during plant growth and ensiling, particularly when oxygen is present in the ensiled mass (Keller et al., 2013; Gallo et al., 2021b).

Mycotoxins able to contaminate silage can be aflatoxins (AF), produced by two species in the *Aspergillus* section *Flavi* — *A. flavus* and *A. parasiticus* (Mauro et al., 2013). The naturally occurring AF are AFB1 and AFG1, and their di-hydro derivatives are AFB2 and AFG2. AFB1 is the most toxic and carcinogenic of these compounds (IARC, 2002), 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 into their milk (Fink-Gremmels and Diaz, 2005).

Corn silage can be a source of AF in warm regions, such as the southwestern USA (Ferrero et al., 2019), but in temperate regions corn silage is generally less contaminated than other feeds, such as corn grain, peanuts, cottonseed and high-moisture corn. Despite these issues, appropriate management of the agro-technical and conservation aspects related to production of corn silage is critical to prevent excessive AFB1 contamination. In particular, it is important to consider the risk of AF contamination from some field conditions, such proliferation of insects in stalk tunnels

(Masoero et al., 2010), stressful heat or drought events during ensiling and feed-out (Ferrero et al., 2019).

Inoculation of corn silage with lactic acid bacteria (LAB), such as *Lactobacillus buchneri* and *L. plantarum*, is a common practice used to improve the fermentation and increase bio-preservation and aerobic stability of silage because it delays development of spoilage yeasts and molds (Adesogan and Arriola, 2020; Muck et al., 2018). Antifungal effects of LAB are related to their production of a plethora of metabolites such as organic acids (e.g., propionic acid, acetic acid) and bacteriocins (e.g., peptides, proteinaceous) or phenolic compounds (phenyl lactic acid, n-decanoic and 3-hydroxydodecanoic acid) (Sadiq et al., 2019). Furthermore, LAB can cause yeast membrane depolarization and can co-aggregate with fungi, thus hampering their growth (Mieszkin et al., 2017). There is evidence that inhibition of fungal growth directly inhibits mycotoxin production due to reduction in the expression of genes needed for their synthesis (Gomaa et al., 2018; Sadhasivam et al., 2019).

The LAB can also adsorb mycotoxins onto the polysaccharides and peptidoglycans in their cell walls (Lahtinen et al., 2004; Gallo and Masoero, 2009). The ability of LAB to adsorb mycotoxins is affected by their molecular structure, concentration, number and the physiological state of the cells (i.e., viable or dead), and pH (Peltonen et al., 2000; Gratz et al., 2005; Wang et al., 2015; Ma et al., 2017; Sadiq et al., 2019). Further, the stability and fate of mycotoxins is determined by the nature of the bond between the mycotoxin and the LAB cell wall complex, which can be a covalent or ionic (Yiannikouris and Jouany, 2002; Moschini et al., 2008). Despite our general knowledge of the potential benefits of using LAB inoculants in silage, Ogunade et al. (2018) reported that few studies used mold-inhibiting chemical additives or microbial inoculants to reduce forage mycotoxin contamination. However, Ma et al. (2017) reported that, regardless of LAB inoculation, some silage bacteria reduced the concentration of corn silage spiked with AFB1 to a safe level within 3 days of

ensiling. In contrast, Ferrero et al. (2019) used mini-silos to determine whether *A. flavus* and AF production originated from the field or during the fermentation or aerobic deterioration of corn silage. They found that corn silage naturally contaminated with AF did not have different AF concentrations at the end of a long conservation period, even when fresh herbage was treated with *L. buchneri*, *L. hilgardii*, or their combination. They also found that *A. flavus* had the ability to survive in silage, and to revive when the pH, oxygen level and temperature became suitable for growth.

Thus, the scientific literature has contrasting results regarding the efficacy of different methods to control AF in silage. Our objective was to compare the fermentation and mycotoxin content of untreated corn silage harvested from corn plants infected with toxigenic fungi in the field, with the same silage treated with 3 commercial inoculants or 6 single LAB inoculants.

2. Materials and Methods

2.1. Silage preparation and treatments

A late-season corn hybrid (Pioneer Hi-Bred International, Inc., Des Moines, IA, USA) was grown during 2019 at the Centro Ricerche Zootecniche (CERZOO S.r.L., San Bonico, Italy) and inoculated with a toxigenic strain of *A. flavus* (ITEM 8069, 1×10^5 spores/mL) on 15 July at silk emergence (Giorni et al., 2019). The forage was harvested at the ⅔ milk line (dry matter [DM] content: 428 ± 6.5 g/kg) on 2 September, and then chopped to an 8 mm theoretical cut with a self-propelled forage harvester (John Deere 7780, John Deere, USA) that had a pick up mechanical kernel processor roll (roll gap: 2.3 mm, model 375plus).

Approximately 25 tonnes of freshly chopped corn forage was collected from each of four plots from the same experimental field (45°00'21.2"N 9°42'20.8"E). A tractor with a bucket loader and a weighbridge at the farm was used to weigh, mix, and manage each 2.5 tonnes mass of freshly

chopped forage. Each freshly chopped mass was divided into 10 approximately equal amounts, which were homogenized with a farm fork, and then individually sampled prior to application of the ensiling treatments (*i.e.*, 1 untreated control and 9 others treated with bacterial inoculants, described below). From each freshly chopped forage mass, two mini-silos (20 L plastic jars, in which about 7.5 kg of freshly chopped forage was weighed) were created and opened after ensiling times of 30 and 120 days. Thus there were 80 mini-silos, representing 4 field plots, 10 ensiling treatments (including no treatment), and 2 ensiling durations. Thus, the 10 ensiling treatments were:

1) untreated control (CTR, without inoculant);

2) commercial inoculant containing *Lactobacillus buchneri* LB1819 and *Lactococcus lactis* O224 (SiloSolve® FC, Chr. Hansen A/S, Hørsholm, Denmark);

3) commercial inoculant containing *Lactococcus lactis* SR3.54 (SiloSolve® MC, Chr. Hansen A/S, Hørsholm, Denmark);

4) commercial inoculant containing *Lactobacillus buchneri* and *Lactobacillus plantarum* (Pioneer® brand 11C33 Corn Silage Inoculant, Pioneer Hi-Bred International, Des Moines, IA, USA);

5) *Lactobacillus brevis* DSMZ 20054 (Deutsche SammLung von Mikroorganismen und Zellkulturen, German Collection of Microorganism of Cell Cultures) (LB DSMZ 20054);

6) *Lactobacillus plantarum* ATCC 8014 (American Type Culture Collection) (LP ATCC 8014);

7) *Lactobacillus rhamnosus* ATCC 7469 (LR ATCC 7469);

8) *Lactobacillus plantarum* PB (LP PB);

9) *Lactobacillus rhamnosus* LR7 (LR LR7);

10) *Lactobacillus rhamnosus* RI (LR RI).

To characterize the pre-ensiled freshly chopped whole plant corn, a sample from each of the 10 amounts of each four 2.5-tonne freshly chopped masses was collected before adding the treatments.

Samples from amounts assigned to the same treatment from different masses were pooled and 10 samples of freshly chopped whole plant were characterized for chemicals, pH, mycotoxin contamination and microbiologic counts (Table 1), as detailed below.

2.2 Application of bacterial inoculants

The 3 commercial inoculant products were used according to manufacturer recommendations, as freeze dried powders diluted in water 30°C at 2 mg/kg of fresh forage using a sprayer to a final expected dose of approximately 250,000 CFU/g freshly-chopped forage (Gallo et al., 2018). An equal amount of water without inoculant was sprayed in to CTR mass. The other 6 LAB strains were grown overnight at 37°C in 350 mL flasks containing 250 mL of MRS broth (Oxoid, Thermo Scientific, Basingstoke, England) until the end of the log-growth phase (~1×10^9 CFU/mL). Cultures were then centrifuged for 5 min at 4600 ×*g* at room temperature and the supernatant was discarded. The pellet was washed three times with a saline solution (0.89 g/100 mL NaCl), diluted in the same solution, and applied in the same manner and dose as the commercial products. Cell counts in MRS medium (Oxoid, Thermo Scientific, Basingstoke, UK) were used to confirm inoculum dose.

2.3 Preparation of samples and mini-silos

Each silage was thoroughly mixed with a wheel loader and shovels. Three representative samples (~1 kg each) were collected from different parts of each pile, stored in airtight bags, immediately frozen, and used for subsequent chemical, fermentative and mycotoxin analysis.

The freshly-chopped and mixed corn plants from each plot was weighed and transferred into 20 L plastic jars, compacted to a density of 160.3 ± 3.5 kg DM/m^3, and then carefully sealed with plastic lids and stored at 20.3°C ± 0.63. The mini-silos were opened and weighed after 30 and 120 days. After opening, the mass of each mini-silo was mixed thoroughly, and representative samples (~2 kg) were collected. Samples were then divided into 2 homogeneous subsamples (~1 kg each). One of these sub-samples was used for chemical and mycotoxin analysis as described by Gallo et al.

(2016), and the other subsample was stored at 4°C prior to measurements of lactic acid, ethanol, volatile fatty acids (VFA), volatile organic compounds (VOC, grouped as aldehydes, alcohols, ketones, esters), ammonia N, and aerobic stability (Gallo et al., 2018).

2.4 Chemical and biological analysis

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), ether extract (EE) was determined using AOAC method 920.29 (AOAC, 1995), and crude protein (CP; N × 6.25) was determined using a Kjeldahl method (AOAC, 1995; method 984.13). The soluble fraction of CP (expressed on a DM basis) was determined according to Licitra et al. (1996). Neutral detergent (ND), acid detergent (AD), and lignin (sulfuric acid [sa]) were sequentially determined using the Ankom^{II} Fiber Analyzer (Ankom Technology Corporation, Fairport, NY, USA), as described by Mertens (2002) for aNDFom, and as described by Van Soest et al. (1973) for ADFom and lignin(sa). The ND solution contained sodium sulfite and a heat stable amylase (activity: 17.400 Liquefon-U/mL, Ankom Technology; Mertens, 2020). All fiber fractions were corrected for residual ash. The proteins bound to the ND and AD residues (NDICP and ADICP) were quantified as described above using the Kjeldahl method. Starch was determined by polarimetry (Polax 2L, Atago®, Tokyo, Japan) and free sugars 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., 2016).

The $NDFD_{24}$ (expressed as the degradability coefficient on an aNDFom basis) was determined *in situ* using the rumens of 2 cannulated dry Holstein dairy cows (625 ± 10.2 kg body weight), fed at maintenance net energy level (NRC, 2001) with a total mixed ration (120 g/kg CP and 550 g/kg aNDFom on a DM basis) composed of alfalfa hay, grass hay, corn silage, beet pulp, and a protein–vitamin-mineral supplement (250, 450, 150, 50, and 100 g/kg DM, respectively). Animal care was in accordance with the European Commission Council Directive guidelines (EU, 2010) for animals used for experimental and other scientific purposes. Ground silage samples (about 300 mg) were inserted into acetone prewashed polyester/polyethylene filter bags (Ankom F57; ANKOM Technology Corporation) that were placed in string-net bags (10 by 15 cm and 15-mm pore size; 2 filter bags per sample for each sample) as described by Gallo et al. (2017). Ruminal incubation lasted 24 h, and was duplicated on a second occasion. At the end of each incubation, bags were rinsed in a household washing machine (3 washings of 2.5 min each, 2 with cold water and the last with distilled water, Smeg GW 3000; Smeg S.p.A – Instruments Division, Guastalla, Italy) and then dried at 55°C. The residual aNDFom was quantified on the dried bags as described by Gallo et al. (2017).

2.5 Measurement of mycotoxins

Measurements of AF were according to Gallo et al. (2010). Briefly, AF were extracted from 10 grams of dried samples with 100 mL of an acetone:water solution (70:30 vol/vol). The mixture was shaken at 150 r.p.m. for 45 min (Universal Table Shaker 709, Enrico Bruno srl, Torino, Italy), passed through Schleicher & Schuell 595 ½ filter paper (Dassel, Germany), and an aliquot of 5 mL was then purified on an immunoaffinity column (Aflatoxin Easi-extract, R-Biopharm Diagnostics Technologies, Glasgow, UK). The column was then washed with 5 mL of water and slowly eluted with 2.5 mL of methanol. The eluate was concentrated under a flow of N_2 and brought to 2 mL by

addition of an acetonitrile:water (41:59) solution, filtered into vials (Millipore Corporation, Bedford, MA, USA; HV 0.45 mm), and then analyzed by HPLC as described earlier.

Mycotoxins produced by *Alternaria* spp. (*i.e.*, altenuene, alternol, alternol monomethyl ether, tentoxin, tenuazoic acid), *Fusarium* spp. (*i.e.*, fusaric acid, zearalenone [ZEA], deoxynivalenol [DON]), *Penicillium roqueforti* (*i.e.*, PR toxin, mycophenolic acid, roquefortine C), and *Aspergillus fumigatus* (*i.e.*, gliotoxin) were extracted simultaneously from 3 g of a dried silage sample using a 30 mL extraction solution (Gallo et al.,2016). After shaking for 45 min, 4.0 g of anhydrous magnesium sulfate was added to obtain phase separation. After shaking for another 2 min and centrifugation at 4500 × *g* for 10 min at room temperature, the upper acetonitrile phase was recovered. This extract was diluted 5-fold (0.1 mL brought to 0.5 mL) by addition of acetonitrile:water 30:70 (v/v), and then passed through a filter (Millipore Corporation, Bedford, , USA; HV 0.45 µm). A 5 µL sample was injected into the HPLC-MS/MS system as described by Gallo et al. (2016). All analyses were in duplicate.

2.6 Measurement of volatile fatty acids, volatile organic compounds, and ammonia N

To quantify volatile organic acids, lactic acid, ethanol and other alcohols, aldehydes, esters, and ketones, a wet sample (about 50 g) was extracted using a Stomacher blender (Seward Ltd., West Sussex, Worthing, UK) for 3 min in distilled water (water:sample fresh weight ratio: 3:1). The mixture was filtered through gauze, and then two aliquots (2 mL each) of the resulting solution were added to 1 mL of an oxalic acid solution (15.2 g/L) and 1 mL of a mixture of pivalic acid (internal standard, 1 g/L) and formic acid (50 mL/L). To remove solid impurities, the mixtures were shaken for 10 min, centrifuged at 4500 × *g* for 10 min at room temperature, and the liquid phase was retained for determination of VFA and VOC using a gas chromatography (GC) system that was equipped with a flame ionization detector. Lactic acid was determined using HPLC (Carvalho et al., 2012). When a compound was undetectable, its detection limit was reported (*i.e.*, 0.1 g/kg DM).

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 (TN) basis, was determined after steam distillation using the Kjeldahl method described earlier. The pH of the previously filtered water mixture was also measured.

2.7 Determination of aerobic stability of silages

Aerobic stability of silages was determined using method of Ranjit and Kung (2000), and reported in days as in Gallo et al. (2018). About 3 kg of each sample was placed into a 20 L pail and allowed to undergo aerobic deterioration at 20.2 ± 0.82°C. During the test, environmental and silage mass temperatures were recorded every 2 h using a data logger (Microlite, Dostmann Electronic GmbH, Wertheim, Germany). A double layer of cheesecloth that allowed penetration of air was placed over the mini-silos to prevent drying and contamination. The aerobic stability test lasted 14 days, and the ensiled mass was considered aerobically unstable when the difference between the environmental and silage temperatures was 2°C or more.

2.8 Microbiological analysis

Microbial counts in the mini-silos were determined as reported by Gallo et al. (2018). A sample of about 30 g of silage was transferred into a sterile homogenization bag, suspended (1:10, w/v) in a peptone physiological salt solution (1 g/L of neutralized bacteriological peptone and 9 g/L of sodium chloride), and homogenized for 3 min in a Stomacher blender. Serial dilutions were prepared. The LAB, yeast, and mold numbers were then determined using a pour-plate technique with De Man, Rogosa and Sharpe (MRS) agar medium (LAB and yeasts; Oxoid, Basingstoke, UK) or 40 g/L of rose-bengal chloramphenicol agar (molds; Oxoid, Basingstoke, UK). Incubation was at 25°C for 3 days for LAB and yeasts, or 5 days for molds. The CFU's were enumerated separately,

according to their macro-morphological features. Mean CFU values of duplicate subsamples were recorded for total yeasts and molds on plates that yielded 10 to 100 CFU per Petri dish.

2.9 Statistical analysis

The LAB, yeast, and mold CFU's 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. Data measured at a single time point (i.e., mycotoxins with the exception of AF) were analyzed according to a completely randomized block design using the GLM procedure of SAS (2003), with the model:

$$Y_{ijk} = \mu + B_i + T_j + e_{ijk} \quad (1)$$

where: Y_{ijk} is the dependent variable; μ is the overall mean; B_i is the fixed-effect of plot (i = 4); T_j is the effect of an ensiling treatment (j = 10), and e_{ijk} is the residual error.

Data measured at different times were analyzed according to a completely randomized design, within a 2 × 10 factorial arrangement of treatments, also using the GLM procedure of SAS (2003), with the model:

$$Y_{ijkl} = \mu + B_i + T_j + D_k + (T \times D)_{jk} + e_{ijkl} \quad (2)$$

where: Y_{ijkl} is the dependent variable; μ is the overall mean; B_i is the fixed-effect of plot (i = 4); T_j is the fixed effect of an ensiling treatment (j = 10); D_k is the fixed effect of the time of ensiling (k = 30 or 120 days); $(_j \times _k)_{jk}$ is the first order interaction; and e_{ijkl} is the residual error. When there was no interaction and the treatment main effect was significant, which was overwhelming the case, the control mean was compared with other treatments using Dunnett's multiple comparison test, with a difference was considered significant if the P value was below 0.05. When the interaction was significant (*i.e.*, $P < 0.05$), biologically relevant, data were presented to show effects of ensiling time for each response.

3. Results

The freshly chopped uninoculated whole plants had pH, chemical traits, and $NDFD_{24}$ values typical of this forage type (Table 1). The microbial counts (log_{10} CFU/g) were 8.41 ± 0.143 for LAB, 6.05 ± 0.381 for molds, and 2.00 ± 0.001 for yeasts. There was evidence of contamination by AFB1 (20.2 ± 12.8 µg/kg DM), AFB2 (0.6 ± 0.3 µg/kg DM), DON (2706 ± 196.0 µg/kg DM), fusaric acid (184 ± 24.7 µg/kg DM), and tenuazoic acid (204 ± 11.2 µg/kg DM). However, there was no evidence of contamination by ZEA, fumonisins B1 and B2, mycophenolic acid, AFG1, AFG2, gliotoxin, alternol, alternol monomethyl ether, or tentoxin.

There were several significant effects of ensiling time and treatment type on the measured chemical parameters (Table 2), but most of these differences were judged to have little or no biological importance. However, there were relevant variations in the DM of the LR ATCC 7469 silage, which was higher than the CTR ($P < 0.05$). There was also a lower aNDFom content in the LB DSMZ 20054 and LR ATCC 7469 silages than in the CTR (both $P < 0.05$). The $NDFD_{24}$ varied little among combinations of experimental factors, and we considered the few statistically significant effects to have little or no biological relevance.

Ensiling time and treatment type had no effect on pH (Table 3) and levels of ammonia N and ethanol decreased with ensiling time by 0.40 to 0.60 ($P < 0.05$). However, there were no effects of treatment on ammonia N, and only the SiloSolve FC group had a lower ethanol level than the CTR ($P < 0.05$). The 1,2 propanediol level doubled as ensiling time increased ($P < 0.05$), with the highest values in the SiloSolve FC and Pioneer CFT groups (both $P < 0.05$ *versus* CTR).

Levels of VFA and lactic acid increased in all groups with ensiling time ($P < 0.05$), except for acetic acid. None of the 9 treatment groups had levels of VFA or lactic acid that differed from CTR.

Analysis of the sums of specific VOC groups indicated that the level of esters tended to increase with ensiling time ($P = 0.06$), and that aldehydes and ketones had a significant interaction

"ensiling day and treatment" ($P < 0.05$; Figure 1). Analysis of effects of ensiling time on levels of aldehydes indicated no effect in the CTR and SiloSolve FC groups, a decrease in the Pioneer CFT, and increases in the other 7 groups. Levels of ketones increased with ensiling time in 9 groups, but were constant in the SiloSolve FC group.

The level of alcohols decreased with ensiling time, and were 0.43 lower in SiloSolve MC than in CTR group ($P < 0.05$). The lactic/acetic and lactic/(acetic + ethanol) ratios increased with ensiling time, but there were no effects.

Aerobic stability increased with ensiling time from 73 to 197 h ($P < 0.05$). The SiloSolve FC and LR ATCC 7469 groups had longer aerobic stability than CTR (both $P < 0.05$). LAB counts tended to decrease with ensiling time ($P = 0.09$), and this decrease was more pronounced in CTR ($P < 0.05$) than in the SiloSolveFC and Pioneer CFT groups (both $P < 0.05$). Yeast counts increased with ensiling time ($P < 0.05$) but were unaffected by treatments ($P = 0.07$).

The AF levels in each mini-silo were quantified as well as those of other mycotoxins produced by *Aspergillus*, *Fusarium* and *Penicillium* spp. after 120 days of ensiling (Table 4). The AFB1 concentration showed a significant interaction "ensiling day and treatment" ($P < 0.05$; Figure 1): the AFB1 concentration of the LB DSMZ 20054 treatment was higher than control ($P<0.01$) after 120 days of ensiling, while the LR LR7 and LP ATCC 8014 treatments had lower concentrations than control ($P < 0.05$) at both ensiling times. The AFB2 level increased with ensiling time ($P < 0.05$), but the concentration did not vary among the different groups. The other detected mycotoxins (DON, tentoxin, tenuazoic acid, roquefortine C, mycophenolic acid, and fusaric acid) did not differ among the groups.

4. Discussion

Many studies have examined effects of bacterial inoculants, particularly homofermentative and heterofermentative LAB, on inhibition of growth of aerobic and undesirable anaerobic microorganisms in many silages. Indeed, a rapid pH decline during the first days of ensiling is crucial for inhibiting growth of undesirable microorganisms, reducing activity of proteases and deaminases from plant tissues and microorganisms, and prevention of DM loss (Ellis et al, 2016; Kung et al., 2018; Muck et al., 2018). A meta-analysis of 130 peer-reviewed studies by Oliveira et al. (2017) examined effects of LAB inoculation on silage quality and concluded that inoculation did not improve fermentation characteristics of corn, sorghum or sugarcane silages. These results are consistent with our findings in that no LAB inoculation substantively improved the quality of corn silage, even though low mass density (160 kg DM/m^3) was utilized. This is likely because whole plant corn with normal compositional traits and a sufficient amount of sugars to support silage fermentation was used (Kung et al., 2018; Fijałkowska et al., 2020). However, the different LAB did have few effects on the chemical composition, fermentation, and resultant levels of mycotoxin contamination. Our discussion below therefore focuses on the few isolated or specific effects of single LAB strains with respect to CTR.

4.1 *Effects of LAB on the chemical composition of corn silage*

The DSMZ 20054 (obligate heterofermentative *Lactobacillus brevis*) treated forage was the only LAB that relevantly modified silage composition. In particular, this silage had a low level of aNDFom, a high starch content and high DM losses relative to CTR group. This is consistent with Michlmayr et al. (2013) with showed that this strain hydrolyzed depolymerization products of arabinoxylans and arabinans, the main components of hemicellulose, which is a main component of aNDFom. In general, this enzymatic activity is rare among LAB, mainly occurring in obligate heterofermentative *Lactobacillus* spp.

4.2 *Effects of LAB on fermentation compounds in corn silage*

As stated by Oliveira et al. (2017), inoculation of silages with LAB usually reduces growth of *Clostridia* and molds, and production of butyric acid, biogenic amines and ammonia N, thereby improving milk yield probably due to increase in DM intake (DMI) by cattle. The pH values after 30 and 120 days of ensiling were low in all silages, and so the higher concentration of NH_3-N at 30 than 120 days was more likely due to LAB proteolytic activity than that of *Clostridia*, although future research should investigate this possibility.

Two of the commercial inoculants (i.e., SiloSolve FC, Pioneer CFT) resulted in high silage levels of 1,2-propanediol. This is consistent with both inoculants containing *L. buchneri*, which is well known to degrade lactic acid into acetate and 1,2-propanediol causing increased silage pH after long ensiling times (Muck et al., 2018). Indeed, previous research showed no production of this alcohol when ensiling for 5 (Schmidt et al., 2009) or 42 days (Kleinschmit and Kung, 2006). In contrast to the latter, our mini-silos produced detectable 1,2-propanediol after 30 days of ensiling. This topic requires future investigation.

The ethanol content was lower in CTR than in SiloSolve FC, although these groups had no differences in yeast count. The content of butyric acid increased with time of ensiling, with no differences among silages. It is likely that the low pH, low energy loss, and low DM content of the silages were related to the low level of *Clostridia* contamination because the metabolic activity of this bacterium is strongly related to the butyrate content of corn silage (Kung et al., 2018). However, the possibility of other butyrate-producing microorganisms in the silages cannot be excluded. The silages had significant differences in the levels of ketones (Figure 1), possibly due the different lipolytic activity of the inoculants. Indeed, ketone production occurs when lipolysis releases free fatty acids that are then oxidized to β-ketoacids and decarboxylated to ketones (McSweeney and Sousa, 2000). The high ketone concentrations were in the SiloSolve MC silage and most of the silages with single LAB strains, except SiloSolve FC and LB DSMZ 20054.

Similarly, the higher content of total aldehydes in silage inoculated with SiloSolve MC and *L. plantarum* may be due to greater aminotransferase and decarboxylase activity of these strains, because this is the main pathway of aldehyde synthesis in LAB. There is also a possible role of proteases in *L. plantarum* because these can degrade the protein matrix surrounding starch granules. Furthermore, aldehydes levels were higher than CTR for SiloSolve MC and the majority of the single LAB inoculants. In contrast, aldehydes were lower than CTR for SiloSolve FC, Pioneer CFT and LB DSMZ 20054.

As expected, the aerobic stability of silage increased about 2.5-fold from 30 to 120 days of ensiling. The highest aerobic stability relative to CTR was in the SiloSolve FC and LR ATCC 7469 silages, with values > 100 h. The lowest aerobic stability was in the CTR and LB DSMZ 20054 groups, with values < 65 h. The ability of *L. buchneri* to produce acetic acid and 1,2-propandiol is a likely reason for the higher aerobic stability of silage treated with this microorganism (Oude Elferink et al., 2001; Kleinschmit and Kung, 2006). The higher aerobic stability provided by *L. rhamnosus* could be due to its production of antimicrobial compounds that inhibit yeast and mold proliferation, as well as other microorganisms that contribute to silage deterioration. *L. plantarum* is the most commonly used silage inoculant because it produces abundant lactic acid, inhibits deleterious epiphytic microbes, and preserves the nutritional quality of ensiled forages (Muck, 2013; Saylor et al., 2020). However, differences in the fermentation patterns of the two *L. plantarum* inoculants examined: the LP PB silage had a lower content of starch and a higher content of fiber than the LP ATCC 8014 group. The higher amylolytic activity of the LP PB silage could be related to higher activity of genes that code for α-amylase in this bacterium (Fancello Francesco, University of Sassari, unpublished data). Other studies also reported higher amylolytic activity of these bacterial species (Plaza-Vinuesa et al., 2019).

L. rhamnosus and *L. brevis* are only infrequently used as individual silage inoculants. Li and Nishino (2011) examined effects of *L. rhamnosus* as a silage additive and reported that they depended on the LAB strain and type of forage. In general, they found that inoculation of commercial *L. rhamnosus* had marginal effects on the fermentation products and the microbial community of whole-crop corn silage. Similarly, there were no changes in the fermentation profile of silages inoculated with *L. rhamnosus* with respect to CTR.

Previous studies examining effects of an *L. brevis* silage inoculant (Duniere et al., 2017; McAllister et al., 2018; Fijałkowska et al., 2020) reported it to be the main LAB species during the early stage of ensiling (Danner et al., 2003; Zheng et al., 2017), although it was associated with silage deterioration (Li and Nishino, 2011). Two studies have shown *L. brevis* to inhibit pathogenic bacteria and reduce concentrations of toxin-producing fungi, thereby helping to preserve the nutritive value of silage (Xu et al., 2017; Fijałkowska et al., 2020).

4.3 *Effects of LAB additions on mycotoxin contamination of corn silage*

The content of AFB1 was unaffected by ensiling time and this suggests no bio-conversion of this "parent molecule" due to epiphytic microbes. This does not apply to other complex microbial environments, such as the rumen-reticulum where bacteria can rapidly convert AFB1 into AFM1 or aflatoxicol (Moschini et al., 2008; Masoero et al., 2009; Gallo et al., 2010). On the contrary, we found that the concentration of AFB2 tended to increase with ensiling time, possibly because activity by mycotoxigenic *Aspergillus* section *Flavi* strains continued during ensiling, probably because oxygen persisted in some porous silage niches. To the best of our knowledge, this is the first report that an ensiling treatment did not increase the level of AFB1, but did increase the level of AFB2. This finding requires confirmation and further investigation, for instance by use of molecular techniques to quantify the specific fungi responsible (McAllister et al., 2018). In contrast,

Ferrero et al. (2019) observed an increase in AFB1 during silage conservation and during exposure of the ensiled corn mass to air, and that AFB2 did not increase during storage as silage.

Under the conditions of our experiments, the commercial inoculants and *L. plantarum* only slightly reduced levels of AFB1 and AFB2, indicating they are not suitable for this purpose. The lowest values of AFB1 occurred at 120 days of ensiling in the LR LR7 silage and the highest was in the LB DSMZ 20054 silage (Figure 1). These results are consistent with those of other authors, who found the lowest AFB1 level was in silage treated with *L. rhamnosus* XH753 while demonstrating the capacity of this bacterium to inhibit mycotoxin accumulation, especially under aerobic conditions (Guan et al., 2020). Furthermore, Dogi et al. (2013) found that silage inoculation with *L. rhamnosus* RC007 provided strong inhibition of fungal growth and mycotoxin production, especially *Fusarium graminearum* and *Aspergillus parasiticus*. Oluwafemi et al. (2010) found that some strains of LAB were able to partially degrade AFB1 in corn grain (31% to 46% reduction) and Ma et al. (2017) reported that some strains of LAB in *L. plantarum*, *L. buchneri*, and *P. acidilactici* species can bind to AFB1 *in vitro*.

In addition to examining AF, production of other important and often legally regulated mycotoxins in silage, mainly those produced by different strains of *Alternaria*, *Fusarium*, and *Penicillium* were studied. *Alternaria* and *Fusarium* are often categorized as field fungi, and they may appear on whole corn plants before harvest due to agricultural practices, climatic conditions and other factors (Medina et al., 2014; Battilani et al., 2013). However, most mycotoxigenic fungi can be eliminated during ensiling, even though species such as *A. fumigatus*, *Penicillium Roqueforti*, *P. paneum*, *F. oxysporum*, and *Monascus ruber* can tolerate high levels of organic acids and carbon dioxide, and low levels of oxygen, or find microenvironments within silage that supports their growth (Gallo et al., 2015b; Gallo et al., 2021b).

The freshly chopped whole-plant corn that we used was contaminated by mycotoxins in addition to AF, such as DON, fusaric acid, and tenuazoic acid After 120 days of ensiling, the DON level exceeded the 3000 µg/kg DM in the LP ATCC 8014, LP PB LR ATCC 7469 and LR LR7 silages, and the level in the LR LR7 group was 4373 µg/kg DM. These levels of DON are common in silage. Recent studies reported that DON dietary intake estimable from these contamination levels of corn silage reduced milk yield of lactating dairy cows by 1.34 kg/cow *per* day (Gallo et al., 2020) as well as rumen microbiota activity (Gallo et al., 2021a). Further, ingestion of DON at this level drastically interfered with milk quality, diet digestibility, metabolic variables and the immunity of lactating dairy cows. Even though the level of tenuazoic acid (from *Alternaria spp.*) was higher in CTR silage than in most other silage, the high concentration of this mycotoxin was in silage treated with LP ATCC 8014. Analysis of mycotoxins produced by *Penicillium* indicated that the level of roquefortine C was very low (<10 µg/kg DM) in CTR silage, and in all other silages except LP PB. Levels of mycophenolic and fusaric acids had some relevant biological differences among the silages, but high intragroup variability prevented statistical support.

Some previous studies reported that LAB increased mycotoxin production, possibly because fungi reacted to LAB as a stress trigger (Dalié et al., 2012). In particular, Gallo et al. (2018) and Saylor et al. (2020) recently reported that the LAB inoculated during silage fermentation interacted with the fungal population and changed the mycotoxin profile with respect to untreated silages, and that LAB increased some mycotoxins produced by *Fusarium* spp. and *Alternaria* spp. and decreased those produced by *Penicillium* spp.

5. Conclusions

The examined silage inoculants, both commercial products and pure LAB inoculants, had limited effects in modifying the chemical and fermentative traits of corn silage. Exceptions were SiloSolve

FC resulted in a lower ethanol level that CTR and that 1,2 propanediol level doubled as ensiling time increased, with the highest values in the SiloSolve FC and Pioneer CFT. In addition, aerobic stability increased with the ensiling time, and the longest stability was in the SiloSolve FC and *L. rhamnosus* ATCC 7469 silages. The pure LAB inoculants *L. rhamnosus* LR7 and *L. plantarum* PB reduced the AFB1 concentration of corn silage by about 25% compared to pre-ensiled forage. Analysis of levels of other mycotoxins indicated that LAB inoculation interacted with the fungal population to change the mycotoxin profile relative to untreated silage, thereby increasing levels of some mycotoxins and decreasing levels of others.

Future investigations should further examine the relationship between LAB and mycotoxinegic fungi during ensiling in an effort to develop methods to produce safer silage for diets of cattle.

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Figure

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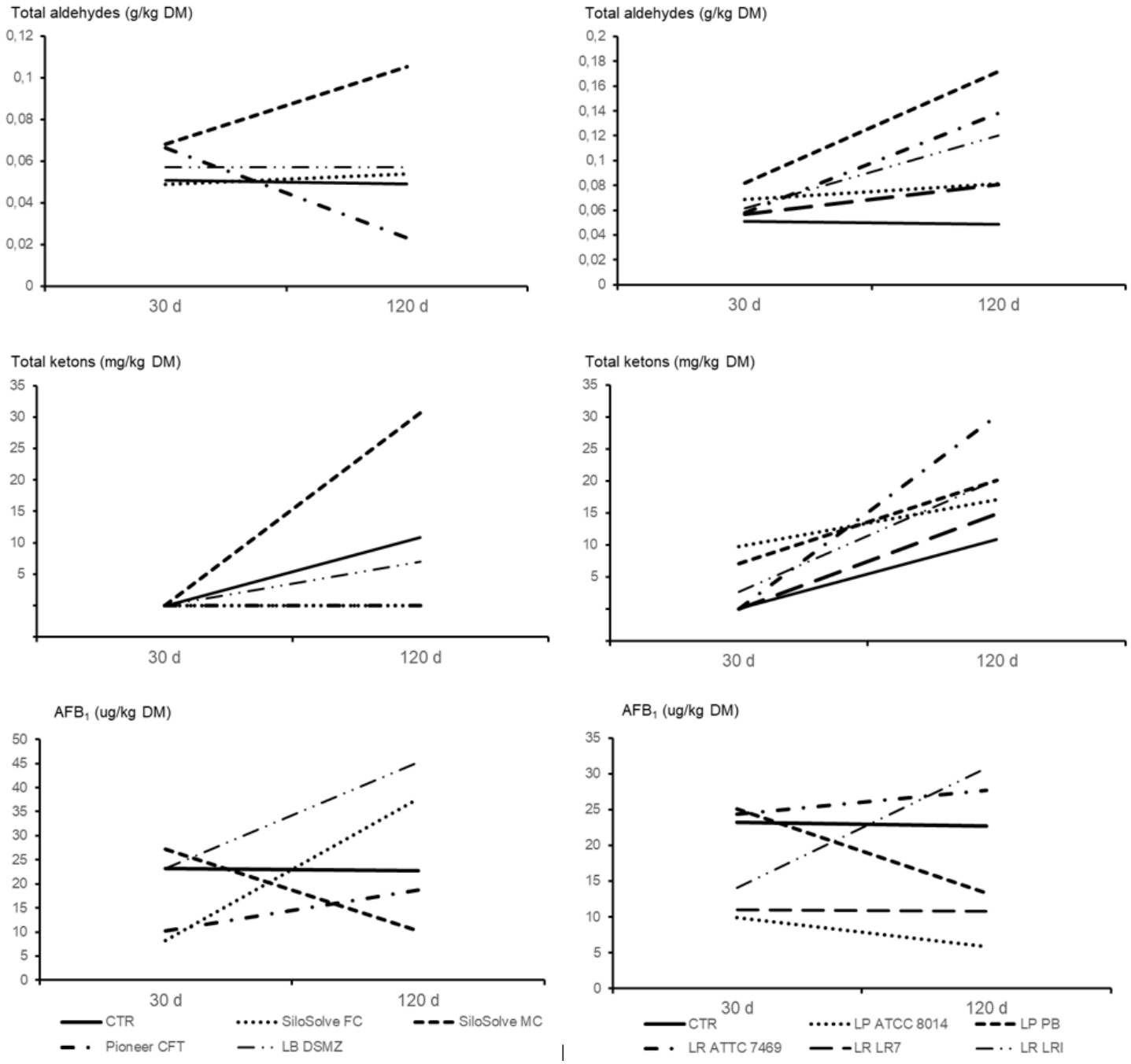


Figure 1.

Effect of ensiling length and addition of commercial mixtures of bacteria, or only LAB, on total aldehydes, ketones and aflatoxin B1 (AFB1) concentrations of corn silages (ensiling day × treatment interaction significant at $P < 0.05$; CTR, SiloSolve FC, SiloSolve MC, Pioneer CFT and LB DSMZ 50054 treatments in graphs on the left side of the figure; CTR, LP ATCC 8014, LP PB, LR ATTC 7469, LR LR7 and LR RI treatments in graphs on the right side of the figure).

Table
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Table 1

Characteristics (mean ± SD) of freshly chopped whole corn plants (*n*=10 samples[1]).

		Mean	SD
Dry matter	g/kg as fed	428	6.5
Ash	g/kg DM	48	1.7
CP	g/kg DM	79	2.2
NDICP	g/kg DM	14	0.4
ADICP	g/kg DM	9	0.4
Soluble CP	g/kg DM	32	1.4
aNDFom	g/kg DM	371	16.6
ADFom	g/kg DM	250	9.6
Lignin(sa)	g/kg DM	29	0.8
$NDFD_{24}$	g/g aNDFom	0.565	0.0097
Ether extract	g/kg DM	24	0.2
Starch	g/kg DM	281	14.4
Sugars	g/kg DM	71	9.0
pH		5.47	0.101

NDICP, Neutral detergent insoluble CP; ACIDP, Acid detergent insoluble CP; $NDFD_{24}$, NDF disappearance measured *in situ* after 24 hours of ruminal incubation.

[1] A sample from each of the 10 amounts of each four 2.5-tonne freshly chopped masses was collected before adding the treatments. Samples from amounts assigned to the same treatment from different masses were pooled and 10 samples of freshly chopped whole plant were analyzed.

Table 2

Effect of ensiling length and addition of commercial mixtures of bacteria, or only LAB, on chemical parameters of the corn silage.

		Ensiling length (days)		Treatments[1]										Effects[2] of model (P < 0.05)			SEM
		30	120	CTR	SiloSolve FC	SiloSolve MC	LB DSMZ 20054	LP ATCC 8014	LP PB	LR ATCC 7469	LR LR7	LR RI	Pioneer CFT	Days	Treatment	Days × Treatment	
Dry matter	g/kg as fed	434	427	425	450	429	428	412	416	457[b]	431	435	425	0.109	<0.05	0.445	10.3
Ash	g/kg DM	54	55	55	54	55	54	55	56	53[a]	55	55	55	<0.05	<0.05	0.094	0.7
CP	g/kg DM	81	80	81	82	81	81	81	79	79	81	80	80	<0.05	0.076	0.093	0.9
NDFIP	g/kg DM	13	13	13	14	13	13	13	14	13	13	13	14	0.583	0.381	0.334	0.4
ADFIP	g/kg DM	8	9	9	8	8	9	9	9	8	8	8	9	<0.05	0.157	0.124	0.2
Soluble CP	g/kg DM	37	38	38	39	37	37	38	37	38	38	37	38	0.091	0.145	0.135	0.7
aNDFom	g/kg DM	363	372	375	369	364	358[a]	371	383	356[a]	358	367	374	<0.05	<0.05	0.352	6.4
ADFom	g/kg DM	249	242	251	254	244	238	247	251	244	237	242	248	<0.05	0.060	0.660	5.8
Lignin(sa)	g/kg DM	29	28	29	29	28	28	29	29	28	28	28	28	<0.05	0.280	0.244	0.5
$NDFD_{24}$	g/g aNDFom	0.540	0.545	0.538	0.531	0.542	0.544	0.543	0.552[b]	0.540	0.542	0.551[b]	0.543	<0.05	<0.05	<0.05	0.0043
Ether extract	g/kg DM	29	28	28	28	29	29	29	29	29	29	29	28	<0.05	0.642	0.120	0.5
Starch	g/kg DM	286	307	284	291	304	309[b]	290	282	305	304	303	294	<0.05	<0.05	0.640	8.7
Sugars	g/kg DM	12	13	13	12[a]	12	12	13	14	12[a]	13	13	12	<0.05	<0.05	0.072	0.5

CP, crude protein; NDICP, neutral detergent insoluble CP; ADICP, acid detergent insoluble CP; $NDFD_{24}$, NDF disappearance measured *in situ* after 24 hours of ruminal incubation.

[1] CTR, control; LB DSMZ 20054, *Lactobacillus brevis* DSMZ 20054; LP ATCC 8014, *Lactobacillus plantarum* ATCC 8014; LP PB, *Lactobacillus plantarum* PB; LR ATCC L, *Lactobacillus rhamnosus* ATCC L; LR LR7, *Lactobacillus rhamnosus* LR7; LR RI, *Lactobacillus rhamnosus* RI. Silosolve FC, Silosolve MC and Pioneer CFT were commercial mixtures of bacteria.

[2] Block (plot) effect resulted significant at a P < 0.05 for ash.

Superscripts indicated that the specific mean was lower (i.e., a) or higher (i.e., b) than CTR at a P < 0.05, as evaluated with Dunnett's multiple comparison test.

Table 3

Effect of ensiling length and addition of commercial mixtures of bacteria, or only LAB, on fermentative parameters and microbiological counts of the corn silage.

		Ensiling length (days)		Treatments[1]										Effects of model[2] (P < 0.05)			SEM
		30	120	CTR	SiloSolve FC	SiloSolve MC	LB DSMZ 20054	LP ATCC 8014	LP PB	LR ATCC 7469	LR LR7	LR RI	Pioneer CFT	Days	Treatment	Days × Treatment	
Fermentative parameters																	
pH		3.96	3.93	3.89	4.07	4.00	3.93	3.95	3.92	3.89	3.90	3.94	3.99	0.246	0.159	0.164	0.065
Ammonia N	g/kg TN	64.9	26.8	54.2	52.3	56.5	46.7	38.9	38.1	35.4	38.1	40.5	59.0	<0.05	0.131	0.262	9.93
Ethanol	g/kg DM	5.3	3.4	5.7	2.8[a]	4.5	4.6	5.5	5.6	3.7	3.9	3.6	3.9	<0.05	<0.05	0.206	0.93
1,2 propanediol	g/kg DM	0.2	0.4	0.1	0.6[b]	0.1	0.2	0.2	0.1	0.2	0.1	0.1	1.2[b]	<0.05	<0.05	0.265	0.13
Acetic acid	g/kg DM	8.0	8.5	7.7	6.6	5.9	7.9	7.8	6.9	7.8	6.9	12.1	12.5	0.701	0.387	0.816	3.05
Propionic acid	mg/kg DM	17.3	73.5	28.8	100.8	48.0	28.9	43.8	34.7	35.7	31.8	44.0	57.6	<0.05	0.253	0.218	26.65
Iso-butyric acid	mg/kg DM	0.1	21.9	2.7	29.6	18.4	5.1	14.8	8.8	3.5	2.1	8.4	16.2	<0.05	0.237	0.237	10.72
Butyric acid	mg/kg DM	20.9	39.2	31.4	32.0	31.3	25.2	26.1	29.6	35.9	25.2	30.3	33.5	<0.05	0.922	0.601	7.91
Iso-valeric acid	mg/kg DM	0.1	31.6	0.3	4.7	2.7	0.5	2.5	1.0	0.5	0.5	1.2	2.0	<0.05	0.218	0.218	16.87
Valeric acid	mg/kg DM	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	-	-	-	-
Lactic acid	g/kg DM	25.4	35.7	30.8	24.1	27.2	33.0	34.4	33.4	31.4	31.0	30.5	29.9	<0.05	0.150	0.088	2.69
Total aldehydes	g/kg DM	0.06	0.09	0.05	0.05	0.09	0.06	0.08	0.13	0.10	0.07	0.09	0.04	<0.05	<0.05	<0.05	0.018
Total alcohols[3]	g/kg DM	5.4	3.5	5.8	3.1[a]	4.6	4.6	5.6	5.6	3.8	3.9	3.7	3.9	<0.05	<0.05	0.163	0.92
Total ketones	mg/kg DM	1.9	15.1	5.4	0.1	15.3	3.5	13.4	13.6	15.1	7.4	11.4	0.1	<0.05	<0.05	<0.05	3.62
Total esters	mg/kg DM	66.8	98.5	126.3	48.6	83.0	67.3	89.6	79.6	64.5	122.0	67.2	78.1	0.062	0.545	0.488	37.17
Lactic/acetic		3.29	5.86	4.01	4.17	5.95	4.36	6.08	5.08	4.03	4.53	4.90	2.70	<0.05	0.196	0.478	1.172
Lactic/acetic+ethanol		1.96	4.17	2.37	3.20	3.97	2.98	3.98	2.93	2.72	2.97	3.39	2.15	<0.05	0.701	0.736	1.011
DM loss	%	0.9	3.3	0.8	1.8	1.8	1.4	0.8	0.8	1.2	0.3	1.1	4.7	0.540	0.276	0.137	0.43
Aerobic stability	hours	73	197	64	105[b]	87	64	89	67	123[b]	97	101	98	<0.05	<0.05	0.278	43.6
Microbiological counts																	
LAB	log_{10} cfu/g	7.67	7.34	7.29	8.25[b]	8.16	7.15	7.05	6.86	7.01	7.39	7.57	8.35[b]	0.09	<0.05	0.198	0.427
Molds	log_{10} cfu/g	3.27	2.86	4.19	3.03	3.71	3.69	2.73	2.36	2.38	2.75	3.38	2.42	0.166	0.066	0.483	0.640
Yeasts	log_{10} cfu/g	5.38	6.41	6.24	5.53	6.20	5.81	5.86	6.13	5.96	5.32	6.09	5.80	<0.05	0.877	0.466	0.604

nd, not detected.

[1] CTR, control; LB DSMZ 20054, *Lactobacillus brevis* DSMZ 20054; LP ATCC 8014, *Lactobacillus plantarum* ATCC 8014; LP PB, *Lactobacillus plantarum* PB; LR ATCC L, *Lactobacillus rhamnosus* ATCC L; LR LR7, *Lactobacillus rhamnosus* LR7; LR RI, *Lactobacillus rhamnosus* RI. SiloSolve FC, SiloSolve MC and Pioneer CFT were commercial mixtures of bacteria.

[2] Block (plot) effect resulted no significant at a P < 0.05 for any parameters.

[3] Total alcohols less methanol, total ketones less 2-butanone.

Superscripts indicated that the specific mean was lower (i.e., a) or higher (i.e., b) than CTR at a P < 0.05, as evaluated with Dunnett's multiple comparison test.

Table 4

Effect of ensiling length and addition of commercial mixtures of bacteria, or only LAB, on mycotoxin contamination during fermentation of the corn silage

		Ensiling length (days)		Treatments[1]									Effects[2] of model (P < 0.05)			SEM
		30	120	CTR	SiloSolve FC	SiloSolve MC	LB DSMZ 20054	LP ATCC 8014	LP PB	LR ATCC 7469	LR LR7	LR RI	Pioneer CFT	Days	Treatment	Days × Treatment	
AFB1	µg/kg DM	17.64	22.31	22.96	22.97	18.78	34.19	7.85	19.25	26.04	10.89	22.43	14.44	0.140	<0.05	<0.05	6.970
AFB2	µg/kg DM	0.56	1.04	0.82	0.79	0.71	0.63	0.23	0.94	1.23	1.10	0.94	0.59	<0.05	0.568	0.284	0.432
DON	µg/kg DM	-	-	2215	2629	2802	2988	3155	3488	3721	4373	2563	2655	-	0.181	-	408.8
Tentoxin	µg/kg DM	-	-	<0.5	<0.5	<0.5	15.25	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	-	0.203	-	2.787
Tenauzoic acid	µg/kg DM	-	-	250.5	5.0	89.9	88.3	331.4	87.9	98.9	238.9	113.4	55.9	-	0.210	-	39.31
Roquefortine C	µg/kg DM	-	-	1.78	<0.05	<0.05	0.71	0.81	20.31	<0.05	<0.05	7.86	1.50	-	0.545	-	2.313
Mycophenolic acid	µg/kg DM	-	-	<5	1885	86	<5	<5	<5	<5	<5	<5	<5	-	0.825	-	768.3
Fusaric acid	µg/kg DM	-	-	328.4	266.8	402.2	210.9	225.0	289.0	254.7	267.8	274.4	2100.0	-	0.185	-	337.13

[1] CTR, control; LB DSMZ 20054, *Lactobacillus brevis* DSMZ 20054; LP ATCC 8014, *Lactobacillus plantarum* ATCC 8014; LP PB, *Lactobacillus plantarum* PB; LR ATCC L, *Lactobacillus rhamnosus* ATCC L; LR LR7, *Lactobacillus rhamnosus* LR7; LR RI, *Lactobacillus rhamnosus* RI. SiloSolve FC, SiloSolve MC and Pioneer CFT were commercial mixtures of bacteria.

[2] Block (plot) effect resulted significant for a P < 0.05 for AFB1 and AFB2.

Conflict of interest

All authors declare no potential conflicts of interest.

Author Statement

Antonio Gallo: Conceptualization, Investigation, Data curation, Methodology, Project administration, Supervision, Software, Visualization, Writing - review & editing original draft preparation.

Francesco Fancello: Data curation, Formal analysis, Methodology, Validation, Visualization, Writing - review & editing.

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