Increase in aflatoxins due to *Aspergillus* section *Flavi* multiplication during the aerobic deterioration of corn silage treated with different bacteria inocula

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ABSTRACT

The growth of *Aspergillus flavus* and the production of aflatoxins (AF) during the aerobic deterioration of corn silage represent a problem for animal and human health. This experiment was conducted to evaluate whether the presence of *A. flavus* and AF production originate from the field or additional AF are produced during the fermentation phase or during aerobic deterioration of corn silage. The trial was carried out in northern Italy on corn at a dry matter (DM) level of 34%. The fresh herbage was either not treated (C) or treated with a *Lactobacillus buchneri* (LB) NCIMB 40788 [(at 3×10^5 cfu/g of fresh matter (FM))], *Lactobacillus hilgardii* (LH) CNCM I-4785 (at 3×10^5 cfu/g of FM), or their combination (LB+LH; at 1.5×10^5 cfu/g of FM of each strain) ensiled in 20-L silos and opened after 250 d of ensiling. After silo opening, the aerobic stability was evaluated and samples were taken after 7 and 14 d of air exposure. The pre-ensiled material, the silages at silo opening, and the aerobically exposed silages were analyzed for DM content, fermentative profiles, microbial count, nutritive characteristics, DM losses, and AFB₁, AFB₂, AFG₁, and AFG₂ contents. Furthermore, a subsample of colonies with macromorphological features of *Aspergillus* section *Flavi* was selected for AF gene pattern characterization and in vitro AF production. The presence of *A. flavus* was below the detection limit ($<1.00 \log_{10}$ cfu/g) in the fresh forage before ensiling, whereas it was found in 1 out of 16 silage samples at silo opening at a level of $1.24 \log_{10}$ cfu/g. The AF were found in both the fresh forage and at opening in all the samples, with a predominance of AFB₂ (mean value of $1.71 \mu\text{g/kg}$ of DM). The inocu-

lation of lactic acid bacteria determined a reduction in the lactic-to-acetic ratio compared with the control. A larger amount of acetic acid resulted in a lower yeast count and higher aerobic stability in the treated silages than in the control ones. At the beginning of aerobic deterioration, the yeasts increased to over $5 \log_{10}$ cfu/g, whereas the molds were close to the value observed at silo opening. When the inhibiting conditions were depleted (pH and temperature higher than 5 and 35°C , respectively), both the total molds and *A. flavus* reached higher values than 8.00 and $4.00 \log_{10}$ cfu/g, respectively, thus determining the ex novo production of AFB₁ during aerobic deterioration, regardless of treatments. The analysis of gene pattern showed that 64% of the selected colonies of *A. flavus* showed the presence of all 4 AF gene patterns, and 43% of the selected colonies were able to produce AF in vitro. During air exposure, after $1,000^\circ\text{C}\cdot\text{h}$ have been cumulated, starch content decreased (below 10% DM) and concentration of neutral detergent fiber, acid detergent fiber, hemicelluloses, crude protein, and ash increased. The inoculation with LB and LB+LH increased the aerobic stability of the silages and delayed the onset of aerobic microbial degradation, which in turn indirectly reduced the risk of *A. flavus* outgrowth and AFB₁ production after silage opening.

Key words: aerobic deterioration, aflatoxin, mycotoxins, silage, fermentation quality

INTRODUCTION

Producing high-quality and microbiologically safe silage while avoiding DM losses as much as possible is a challenge for any livestock producer (Borreani et al., 2018). Aerobic deterioration increases DM losses, reduces the nutritive value of silage, and increases risks to animal and human health due to the growth of pathogenic microorganisms and production of endotoxins and mycotoxins (Pahlow et al., 2003). Mycotoxins are secondary metabolites with low molecular weight

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that are mainly produced by fungi belonging to the *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria* genera (Keller et al., 2013). Mold and mycotoxin contamination of several feed and forages represent an important problem for human and animal health (Driehuis, 2013; Spadaro et al., 2015). Furthermore, this contamination causes increases in food production costs as a result of the increased need for testing, lower prices received for contaminated loads, potential lawsuits from consumers, and decreased livestock performance (Mitchell et al., 2016). Only a few of the detected mycotoxins in the milk supply chain are responsible for significant changes in food safety, and among these the most harmful are aflatoxins (AF; Murphy et al., 2006). Aflatoxins can be produced by such species of *Aspergillus* section *Flavi* as *A. flavus* and *A. parasiticus* (Varga et al., 2011). The potential ability of *A. flavus* strains to produce AF has been analyzed in several studies; for example, through the analysis of the production of sclerotia and the presence of aflatoxin biosynthesis gene pathways and the in vitro production of AF (Cotty, 1989; Abbas et al., 2005; Criseo et al., 2008). The incidence of toxigenic *A. flavus* from different matrices has been found to range from 50 to 70% (Nesci and Etcheverry, 2002; Mauro et al., 2013; Prencipe et al., 2018).

The AF that occur naturally are aflatoxin B₁ (AFB₁), G₁ (AFG₁), and their dihydro derivatives B₂ (AFB₂) and G₂ (AFG₂). Aflatoxin B₁ is the most toxic and carcinogenic (IRAC, 2012), and when it is ingested through contaminated rations to lactating animals it is in part hydroxylated in the liver to aflatoxin M₁ (AFM₁). Aflatoxin M₁ appears in milk within 12 h from ingestion, with a mean carryover of around 3.5% (Veldman et al., 1992). The World Health Organization (WHO, 2002) evaluated the 2 maximum concentrations of aflatoxin M₁ that had been proposed by the Codex Committee on Food Additives and Contaminants, 50 and 500 ng/kg (for the European Union and United States, respectively), and concluded that, based on worst-case assumptions, the projected risk of liver cancer attributable to AFM₁ would be very small if either of these maximum levels were implemented. To reduce the risk of AFM₁ contamination, the daily ingestion of AFB₁ should be limited to 40 and 400 µg/cow per day for the European Union and the United States, respectively (Veldman et al., 1992). Corn silage is the main source of AF in warm regions (i.e., southwestern United States), whereas in temperate region corn is generally less contaminated than other feeds (e.g., corn grain, peanuts, and cottonseed). However, the large use of corn silage on dairy farms throughout the world imposes the need for a careful management of the factors that could increase AFB₁ contamination in the field or during silage conservation.

Aflatoxins in corn silage can be derived from both the field and produced ex novo during aerobic deterioration (Garon et al., 2006; González-Pereyra et al., 2008; Cavallarin et al., 2011). The preharvest contamination of corn crops is well known and has been studied and reviewed by Guo et al. (2008), whereas very few studies have analyzed the postharvest contamination of corn silage for several reasons; for example, the difficulties of recovering AF in the silage matrix (Garon et al., 2006; Cavallarin et al., 2011), the possible increase in concentration of AF due to DM losses during conservation or the feed-out phase, the possible ex novo production by *Aspergillus* during ensiling or the feed-out phase, and the possible degradation or detoxification by lactic acid bacteria (Ahlberg et al., 2015). Furthermore, it is not easy to separate the pre- and postharvest fungal contamination of whole corn silage, and only a few unclear indications have been reported in literature (Lacey, 1989).

Inoculation with different lactic acid bacteria (LAB) has been used over the years to improve silage fermentation or aerobic stability by delaying the development of yeasts and spoilage molds (Muck et al., 2018). Furthermore, it has been documented that LAB can degraded or immobilize aflatoxins during ensiling by binding to their surface (el-Nezami et al., 1998; Oluwafemi et al., 2010; Ma et al., 2017), thereby contributing to improved safety of feed and food. Ogunade et al. (2018) reported that only a few studies have used additives to reduce forage mycotoxin contamination. In one of these studies (Queiroz et al., 2012), the inoculation of corn plants infested with southern rust with a mixture of *Pediococcus pentosaceus* and *Lactobacillus buchneri* at ensiling increased aerobic stability and prevented production of aflatoxins. Some other studies reported that limiting aerobic spoilage by inoculating corn silage with *L. buchneri* can reduce aflatoxin production (Iglesias et al., 2005; Cavallarin et al., 2011). More recently, Ma et al. (2017) reported that, regardless of LAB inoculation, certain silage bacteria can reduce the concentration of AFB₁ that was spiked in corn silage to a safe level within 3 d of ensiling.

Thus, owing to the lack of clear information in literature, the aims of our study were (1) to evaluate whether the presence of *A. flavus* and aflatoxin production in corn silage originates from the field environment or growth of *A. flavus* takes place and additional aflatoxins are produced during storage or air exposure after silo opening; (2) to evaluate the effect of different LAB inocula used to improve the aerobic stability of corn silage on reducing *A. flavus* growth and aflatoxin production during fermentation and air exposure; and (3) to characterize the toxigenic potential of *A. flavus* strains isolated from corn silages.

MATERIALS AND METHODS

Crop and Ensiling

The trial was carried out on a commercial farm located in Rocca de' Baldi in the western Po plain of northwest Italy (44°27'18"N, 7°43'19"E, 408 m above sea level). Corn hybrid (P1517W, Pioneer Hi-Bred Italia Srl, Gadesco Pieve Delmona, Cremona, Italy) was sown in April 2015 at an intended planting density of 75,000 seeds/ha. The whole corn crop was harvested at around the 50% milk line stage and with a DM content of around 34%. Fresh forage was chopped using a precision forage harvester (Claas Jaguar 950, equipped with an 8-row Orbis head, Claas, Harsewinkel, Germany) to a theoretical cutting length of 12 mm. The field was divided in 4 plots, which were subsequently harvested separately, and the crop was chopped to obtain 4 replicates. The fresh herbage of each plot was divided into four 70-kg piles. The piles were either not treated (C) or treated with different LAB strains and their combinations. The LAB strains were *L. buchneri* (LB) NCIMB 40788 [Lallemand Animal Nutrition, BP 59, Cedex, France; theoretical rate of 300,000 cfu/g of fresh matter (FM)], *Lactobacillus hilgardii* (LH) CNCM I-4785 (Lallemand Animal Nutrition; theoretical rate of 300,000 cfu/g of FM), and their combination (LB+LH; theoretical rate of 150,000 cfu/g of FM of each strain). The microbial inoculants were diluted in sterilized water and applied using a hand sprayer at a rate of 4 mL/kg of forage by spraying uniformly onto the forage, which was constantly hand mixed. The same amount of water was added to the C treatment. To add the targeted amount of LAB, the inocula were plated on de Man, Rogosa, Sharpe agar (Merck, Whitehouse Station, NY), with the addition of natamycin (0.25 g/L) and, on the basis of the measured concentration of LAB, an appropriate amount was used to achieve the desired application rate.

The fresh forage was sampled before ensiling after the inoculum had been applied. The untreated and treated forage was then ensiled (11 to 13 kg of wet forage) in 20-L plastic silos equipped with a lid that only enabled the release of gas. The forage was packed by hand, and the final packing densities, on a wet basis, were 627 ± 26 kg of FM/m³. All the laboratory silos were filled within 3 h. The silos were weighed, conserved at ambient temperature ($20 \pm 1^\circ\text{C}$), and opened after 250 d. At opening, each silo was weighed and the content was mixed thoroughly and subsampled to determine the DM content, the chemical composition, the fermentation profile, and the microbial counts. After sampling, the silages were subjected to an aerobic stability test, which involved monitoring the temperature increases

due to the microbial activity in the samples exposed to air. About 3 kg from each silo were allowed to aerobically deteriorate at room temperature ($20 \pm 1^\circ\text{C}$) in 17-L polystyrene boxes (290 mm diameter and 260 mm height). A single layer of aluminum foil was placed over each box to prevent drying and dust contamination, but also to allow the air to penetrate. The room and silage temperatures were measured hourly by means of a data logger. Aerobic stability was defined as the number of hours the silage remained stable before its temperature increased by 2°C above room temperature. From silo opening to 14 d of air exposure of silages, peak temperature, hours to reach peak temperature, interval to reach 35°C , and time with temperature greater the 35°C were also calculated to better describe the optimum temperature for growth of *A. flavus* in absence of inhibitory conditions. The silage was sampled after 7 and 14 d of aerobic exposure to quantify the chemical, fermentative, and microbial changes in the silage during exposure to air, as reported by Tabacco et al. (2011). Other samplings were conducted on d 21 and 28 on the silages that did not show any increase in temperature at 14 d of air exposure. The DM losses due to fermentation were calculated as the difference between the weight of the forage placed in each plastic silo at ensiling and the weight of the silage at the end of conservation, corrected for the DM content of the forage and its respective silage. The DM losses were calculated after 7 and 14 d of exposure to air using the ash content, as reported by Borreani et al. (2018). Small increases in the ash content of deteriorated silage represented large percentage unit increases in DM loss, as can be seen when the equation for calculating DM losses according to the ash content is used: $\text{DM loss (\%)} = [1 - (\text{ash silage at opening} / \text{ash silage after 7 or 14 d of air exposure})] \times 100$.

Sample Preparation and Analyses

The pre-ensiled material and the silage were split into 5 subsamples at both the opening of the silos and before the exposure to air periods (at 7 and 14 d for all silages and at 21 and 28 d, when needed). One subsample was analyzed immediately for DM content by oven drying at 80°C for 24 h. Dry matter was corrected according to Porter and Murray (2001) to consider the volatile compound losses that can take place at 80°C . The second subsample was oven-dried at 65°C to a constant weight and air equilibrated, weighed, and ground in a Cyclotec mill (Tecator, Herndon, VA) to pass a 1-mm screen. The dried samples were analyzed for the total nitrogen according to the Dumas method (method number 992.23; AOAC International, 2005) using a Nitrogen analyzer Primacs SN (Skalar, Breda, the Neth-

erlands), for CP (total N $\times$ 6.25), for ash by ignition (method number 942.05; AOAC International, 2005), and for water-soluble carbohydrates by the phenol sulphuric acid method according to DuBois et al. (1956). Neutral detergent fiber was analyzed using a Raw Fiber Extractor (FIWE, VELP Scientifica, Usmate Velate, Italy) with the addition of heat-stable amylase (A3306, Sigma Chemical Co., St. Louis, MO) and expressed on a DM basis, including residual ash, as described by Van Soest et al. (1991). Acid detergent fiber was analyzed and expressed on a DM basis, including residual ash (Robertson and Van Soest, 1981).

A third fresh subsample was used to determinate the water activity (a_w), pH, NO_3 , NH_3 -N contents, and the buffering capacity. The a_w was measured at 25°C on a fresh sample using an AquaLab Series 3TE (Decagon Devices Inc., Pullman, WA), which adopts the chilled-mirror dew point technique. The fresh forage was extracted for pH, NO_3 , and NH_3 -N determination using a Stomacher blender (Seward Ltd., Worthing, UK) for 4 min in distilled water at a 9:1 water-to-sample material (fresh weight) ratio. The total nitrate concentration was determined in the water extract through semiquantitative analysis, using Merckoquant test strips (Merck, Darmstadt, Germany; detection limit 100 mg of NO_3 /kg of DM). The ammonia nitrogen content and pH were determined using specific electrodes. The buffering capacity was determined in the water extract, as described by Playne and McDonald (1966).

A fourth subsample was extracted, using a Stomacher blender, for 4 min in 0.05 mol/L of H_2SO_4 at a 5:1 acid-to-sample material (fresh weight) ratio. An aliquot of 40 mL of silage acid extract was filtered with a 0.20- μm syringe filter and used for quantification of the fermentation products. The lactic and monocarboxylic acids (acetic, propionic and butyric acids) were determined, by means of HPLC in the acid extract (Canale et al., 1984). Ethanol and 1,2-propanediol were determined by means of HPLC, coupled to a refractive index detector, on a Aminex HPX-87H column (Bio-Rad Laboratories, Richmond, CA). The fifth subsample was used for the microbial analyses.

Microbial Analysis, Fungal Isolation, Macromorphology, and Sclerotia Production

To conduct the microbial counts, 30 g of sample was transferred into a sterile homogenization bag, suspended 1:10 wt/vol in a peptone salt solution (1 g of bacteriological peptone and 9 g of sodium chloride per liter), and homogenized for 4 min in a laboratory Stomacher blender (Seward Ltd.). Serial dilutions were prepared and the mold and yeast numbers were determined using the pour plate technique with 40.0 g/L of

yeast extract glucose chloramphenicol agar (YGC agar, Difco, West Molesey, Surrey, UK) after incubation at 25°C for 3 and 5 d for yeast and mold, respectively. The yeast and mold colony-forming units were enumerated separately, according to their macromorphological features, on plates that yielded 1 to 100 cfu. The LAB were determined on de Man, Rogosa, Sharpe agar with added natamycin (0.25 g/L) by incubating petri plates at 30°C for 3 d under anaerobic conditions, according to Spoelstra et al. (1988). As LAB are facultative anaerobe bacteria, anaerobic incubation was chosen to improve the selectivity of the medium against *Bacillus* spp.

A subsample of 5 to 10 colonies per sample, with macromorphological features of *A. section Flavi*, was selected for characterization (Table 1). The strains were maintained as a monoconidial culture on 30% glycerol and stored at -80°C . A macro-morphological analysis of each isolate was performed on yeast extract sucrose agar (YES), malt extract autolysate agar (MEA), and czapek yeast autolysate agar (CYA) and observed after 7 d of incubation at 25°C in the dark, according to Samson et al. (2004).

The strains were inoculated on CYA agar and incubated for 14 d at 30°C in the dark to evaluate the sclerotia production (Horn et al., 1996; Horn and Dörner, 1998). The sclerotial size was evaluated by measuring a reticule with a Nikon Eclipse 55i Microscope (Nikon Inc., Garden City, NY) connected to a Samsung color monitor (Samsung Corporation, Seoul, Korea) using 10 $\times$ magnification. The strains were identified as small strains (S), with a sclerotial size <400 μm in diameter, or large strains (L), with sclerotia >400 μm in diameter (Horn, 2003).

Molecular Identification

The isolates were grown on potato dextrose broth (27 g, Merck, Germany; in 1 L of H_2O) and incubated at 28°C in the dark. We extracted DNA from the mycelium using an Omega E.Z.N.A. Fungal DNA Mini Kit (Omega Bio-tek Inc., Norcross, GA) according to the manufacturer's instructions (<http://omegabiotek.com/store/wp-content/uploads/2013/09/D3390-Fungal-DNA-Mini-Kit-Combo-May-2013-Online-1.pdf>). Partial amplification of the calmodulin gene was obtained using the *cmd5* and *cmd6* primer pair (Hong et al., 2006). We carried PCR out in a total volume of 25 μL containing 2.5 μL of buffer 10 $\times$, 0.5 μL of MgCl_2 , 0.75 μL of deoxynucleotide triphosphates (10 mM), 1 μL of each primer (10 mM), 0.2 μL of Taq DNA polymerase (Qiagen, Hilden, Germany), and 20 ng of template DNA. A thermal cycling program was performed according to Samson et al. (2014). Amplification was

Table 1. Sampling time, accession numbers of the calmodulin sequences, growth on yeast extract sucrose agar (YES), malt extract autolysate agar (MEA) and Czapek yeast autolysate agar (CYA) media, sclerotia production, and aflatoxin (AF) gene patterns of the strains of *Aspergillus* section *Flavi* used in this study

Strain name	Accession number	Species	Sampling time	YES ¹	MEA ¹	CYA ¹	Sclerotia type ²	AF genes pattern ³				AFB ₁ production in vitro ⁴
								<i>aflR</i>	<i>omtA</i>	<i>ver-1</i>	<i>nor-1</i>	
8931/2	KY886401	<i>Aspergillus flavus</i>	Silo opening	4.17	3.57	3.27	S and L	+	+	+	+	++
8931/3	KY886402	<i>A. flavus</i>	Silo opening	3.90	3.80	3.38	—	+	+	+	+	—
8931/7	KY886381	<i>A. flavus</i>	Silo opening	4.13	3.97	3.53	L	+	+	+	+	—
8959	KY886377	<i>A. flavus</i>	7-d air exposure	4.20	3.77	3.60	—	—	—	—	—	—
9002	KY886382	<i>A. flavus</i>	14-d air exposure	4.13	3.53	3.63	S	+	+	+	+	+
9005	KY886383	<i>A. flavus</i>	14-d air exposure	4.03	3.77	3.33	S	+	+	+	+	++
9006	KY886384	<i>A. flavus</i>	14-d air exposure	4.10	3.60	3.44	S	+	+	+	+	++
9010	KY886385	<i>A. flavus</i>	14-d air exposure	4.33	3.70	3.61	S and L	+	+	+	+	—
9011	KY886386	<i>A. flavus</i>	14-d air exposure	4.17	3.87	3.52	—	+	+	+	+	Weak
9012	KY886387	<i>A. flavus</i>	14-d air exposure	4.03	3.83	3.56	—	+	+	+	+	—
9015/1	KY886378	<i>A. flavus</i>	14-d air exposure	4.00	3.93	3.54	L	—	—	—	—	—
9015/2	KY886379	<i>A. flavus</i>	14-d air exposure	3.67	3.87	3.56	L	—	—	—	—	—
9015/3	KY886380	<i>A. flavus</i>	14-d air exposure	3.93	3.73	3.60	—	—	—	—	—	—
9016	KY886388	<i>A. flavus</i>	14-d air exposure	4.17	3.70	3.41	S and L	+	+	+	+	+
8931/1	KY886389	<i>Aspergillus oryzae</i> var. <i>effusus</i>	Silo opening	4.20	3.77	3.30	—	—	—	+	+	—
8931/4	KY886390	<i>A. oryzae</i> var. <i>effusus</i>	Silo opening	3.93	3.83	3.60	—	—	—	+	+	—
8931/5	KY886391	<i>A. oryzae</i> var. <i>effusus</i>	Silo opening	4.13	3.83	3.67	—	—	—	+	+	—
8931/6	KY886392	<i>A. oryzae</i> var. <i>effusus</i>	Silo opening	4.13	3.67	3.47	—	+	+	+	+	—
8976	KY886393	<i>A. oryzae</i> var. <i>effusus</i>	7-d air exposure	4.13	3.63	3.62	—	—	—	+	+	—
9004	KY886394	<i>A. oryzae</i> var. <i>effusus</i>	14-d air exposure	4.00	3.77	3.47	—	+	+	+	+	—

¹Colony diameters (mean cm) of strains grown on YES, CYA, and MEA at 25°C for 7 d in the dark.

²Sclerotia production on CYA after 14 d at 30°C in the dark. S = small sclerotia (<400 µm diameter); L = large sclerotia (>400 µm diameter); — = no sclerotia production.

³+ = amplification in quadruplex PCR; — = no amplification in quadruplex PCR.

⁴Weak <100 µg/kg of DM; + = 101–1,000 µg/kg of DM; ++ >1,001 µg/kg of DM.

verified by means of electrophoresis on 1% agarose Tris-acetate EDTA gel, and gel images were acquired using a Gel Doc 1000 System (Bio-Rad Laboratories, Hercules, CA). The PCR products were purified using a QIAquick PCR purification Kit (Qiagen) and sent to Macrogen Inc. (Amsterdam, the Netherlands) for sequencing in both directions. The consensus sequences were obtained using the DNA Baser program (Heracle Biosoft S.R.L., Arges, Romania) and compared using the BLAST program, with the sequences deposited in the RefSeq database of the National Centre for Biotechnology Information (NCBI; <https://www.ncbi.nlm.nih.gov/>) for species identification. All the sequences are deposited in the GenBank database (<https://www.ncbi.nlm.nih.gov/genbank/>) with the accession numbers listed in Table 1.

Aflatoxin Gene Detection and Aflatoxin Production In Vitro

The presence of 4 genes, 3 structural genes (*nor1*, *ver1*, and *omtA*) and 1 regulatory gene (*aflR*), involved in the aflatoxin biosynthesis pathway, which have been studied extensively and used as a diagnostic tool for the differentiation of aflatoxin producing and nonproducing fungi, was verified through a quadruplex PCR assay. Quadruplex PCR was performed using the primers listed in Supplemental Table S1 (<https://doi.org/10.3168/jds.2018-15468>). A PCR reaction was carried out in a total volume of 50 μ L containing 5 μ L of buffer 10 $\times$, 1 μ L of $MgCl_2$, 2 μ L of dNTP (10 mM), 1 μ L of each primer (10 mM), 0.4 μ L of Taq DNA polymerase (Qiagen), and 100 ng of template DNA. A thermal cycling program was performed according to Criseo et al. (2008), with some minor modifications. The PCR products were separated by gel electrophoresis using a Tris-borate EDTA buffer with 2% agarose and 5 μ L of SYBRSafe (Invitrogen, Waltham, MA) at 80 V/cm for 4 h. A Get Pilot 1 kb Plus Ladder (100–10,000 bp, Qiagen) was used as a molecular marker and an aflatoxigenic *A. parasiticus* strain AFCAL11 (from the collection of Agroinnova, University of Turin, Italy) was used as positive control. The gel profiles were visualized under UV transilluminator using the Quantity One program (BioRad Labs, Segrate, Italy). The experiment was repeated in triplicate.

The production of aflatoxin was tested for each isolate using a YES medium according to Visagie et al. (2014), with 20 g/L of yeast extract, 150 g/L of sucrose, 0.5 g/L of $MgSO_4$ (Merck, Germany), and 1 mL of trace elements (1 g of $ZnSO_4 \cdot 7H_2O$ and 0.5 g of $CuSO_4 \cdot 5H_2O$ in 100 mL of distilled water). Strains were inoculated with 3 mycelia plugs from a 7-d-old culture (4 mm diameter) on 50 mL of medium and incubated

in the dark at 35°C. After 7 d, the cultures were filtered and extracted to establish the aflatoxin production. The samples were extracted and analyzed according to Prencipe et al. (2018).

Aflatoxin Analysis

The aflatoxins were extracted according to the method reported by Cavallarin et al. (2011). Sample extracts were stored at $-20^\circ C$ until HPLC analysis. The HPLC apparatus consisted of a Dionex P680 pump (Dionex, Sunnyvale, CA) equipped with a Rheodyne Model 7725i injection valve (Rheodyne, Rohnert Park, CA), a Dionex RF-2000 fluorimetric detector (excitation wavelength = 365 nm, emission wavelength = 435 nm for AFB_1 , AFB_2 , AFG_1 , and AFG_2), a Dionex TCC-100 thermostatted column compartment, and a Chromeleon6 data handling system (Dionex). The analytical column was a ProdigyODS 2 (150 $\times$ 4.6 mm, 5- μ m particles; Phenomenex, Torrance, CA), which was preceded by a SecurityGuard (Phenomenex) guard column.

Statistical Analysis

The microbial counts were $\log_{10}$ transformed and presented on a wet weight basis. The values below the detection limit for yeasts and molds (detection levels = 10 cfu/g of silage) were assigned a value corresponding to half of the detection limit to calculate the average value.

The data were analyzed for their statistical significance, via ANOVA, with their significance reported at a 0.05 probability level using the General Linear Model of SPSS (v 24.0, IBM Corp., Armonk, NY). Data were analyzed using the presence or absence of *L. buchneri* (LB+ or LB-) and *L. hilgardii* (LH+ or LH-) as fixed factors with 4 replications. The used statistical model was $Y_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \varepsilon_{ijk}$, where Y_{ijk} = observation, μ = overall mean, α_i = LB effect (i = presence or absence of LB), β_j = LH effect (j = presence or absence of LH), $\alpha\beta_{ij}$ = LB $\times$ LH effect, and ε_{ijk} = error. The measured AF were pooled together for silo opening, 7 d of air exposure, and 14 d of air exposure, and were corrected for the DM losses. An unpaired t -test was used to compare the mean values of the measured AF and DM loss-corrected values.

RESULTS

Fermentative, Chemical, and Microbial Parameters

The chemical and microbial parameters and the AF contamination of the corn forage before ensiling

are summarized in Table 2. The chemical values were typical of corn harvested at the 50% milk line stage. The DM content was 34% and the NDF, starch, and CP were 42.5, 32.3, and 7.8% of DM, respectively. The yeast and mold counts were 6.46 and 5.26 log₁₀ cfu/g, respectively. At ensiling, the *A. flavus* count was below the detection limit and the AF were present with mean values of 0.055, 1.050, 0.095, and 0.044 µg/kg of DM for AFB₁, AFB₂, AFG₁, and AFG₂, respectively. Table 3 shows the fermentative parameters after 250 d of conservation at silo opening and after 7 and 14 d of air exposure. All the silages were well fermented, with the main acids found at silo opening being lactic and acetic acid; butyric acid was under the detection limit (0.01 g/kg of DM). A slight amount of propionic acid was found in the LB+LH silages. The presence of LB and LH affected the pH, with higher values in the inoculated silages than in the control ones. The LB and LH inocula affected the lactic-to-acetic ratio greatly, and this resulted in values higher than 4 in the C silages and values that ranged from 1 and 2 in the treated silages. The LB+LH silages presented about the half of lactic acid and 2.5 fold the acetic acid compared to C silage. The 1,2-propanediol was present in all the treated silages and ranged from 7.1 to 12.9 g/kg of DM, whereas it was not detected in the C silages. After 7 d of air exposure, the lactic acid content decreased and pH increased in all the silages, except for LB+LH treatment. The acetic acid content decreased in C and LH silages, whereas it remained stable in the presence of LB. Ethanol only remained in LB+LH treatment. After 14 d of air exposure, no fermentative products were found in C, LB, or LH silages, whereas 23.4 g/kg of DM of lactic acid and 17.9 g/kg of DM of acetic acid were found in the LB+LH silages.

The aerobic stability, the temperature indices (as observed from silo opening to 14 d of air exposure), DM losses, microbial count, and AF concentrations after 250 d of conservation at silo opening and after 7 and 14 d of air exposure are reported in Tables 4 and 5. At opening, C silages showed lower DM losses and LAB count than treated silages. The yeast count was under the detection limit in the treatments containing the LB strain. The aerobic stability was affected by the treatments, with the highest value (365 h) in LB+LH silages. At opening, the mold count was below the detection limit or around 1.00 log₁₀ cfu/g in all the silages, and the average *A. flavus* count was under the detection limit in 15 out of 16 silage samples. Only 1 LH sample showed the presence of *A. flavus*, at a level of 1.24 log₁₀ cfu/g. The addition of LB and LH did not affect the AFB₁, AFB₂, or AFG₂ contents, whereas AFG₁ was lower in the treatments containing

the LH strains. After 7 d of air exposure, the yeast count increased in all the silages, with the highest value observed in C silage. The addition of LB and LH affected the mold count, with higher values in C than in the treated silages. Aflatoxin was not influenced by the treatments during air exposure, except for AFG₁ after 7 d in C silage. In air-exposed silages after 14 d, the *A. flavus* count increased in all the treatments and reached 3.32 log₁₀ cfu/g in LB silages. Aflatoxin G₁ was not detected after 14 d of air exposure in any treatment, and the other AF did not show any significant difference between treatments. After 14 d of air exposure, 2 out of 4 LB+LH samples were still aerobically stable, with the *A. flavus* count below the detection limit; these samples were left to deteriorate and were sampled after 21 and 28 d. The average *A. flavus* count in these samples was 1.33 log₁₀ cfu/g and 2.74 log₁₀ cfu/g after 21 and 28 d, respectively.

During air exposure, when the cumulated hourly difference between silage and ambient temperatures reached 1,000°C·h, the yeast had completely depleted the fermentative products, regardless of the treatment and LAB inoculum, causing a rise in silage pH greater than 4 (with most of the values ranging from 5.5 to 7) and in the silage temperatures around 30 to 40°C (Figure 1). A cumulated 1,000°C·h temperature difference was reached at different times during aerobic exposure, the lowest being for C silages after 176 h and the highest for LB+HB silages after 484 h. When all the inhibiting conditions (pH and the presence of undissociated organic acids) were depleted, the total molds and *A.*

Table 2. Chemical and microbiological characteristics of the herbage before ensiling

Parameter ¹	Value	SD
DM (%)	34.0	1.94
pH	5.80	0.32
Buffering capacity (mEq/kg of DM)	56	9.31
Water activity	0.996	0.00
Nitrate (mg/kg)	<100	—
NDF (% of DM)	42.5	1.94
ADF (% of DM)	22.1	1.43
CP (% of DM)	7.84	0.20
Ash (% of DM)	5.54	0.18
Soluble carbohydrates (% of DM)	7.37	0.89
Starch (% of DM)	32.3	1.95
LAB (log ₁₀ cfu/g)	7.71	0.56
Yeasts (log ₁₀ cfu/g)	6.46	0.29
Molds (log ₁₀ cfu/g)	5.26	0.20
<i>Aspergillus flavus</i> (log ₁₀ cfu/g)	<1.00	—
Aflatoxin B ₁ (µg/kg of DM)	0.055	0.077
Aflatoxin B ₂ (µg/kg of DM)	1.050	1.498
Aflatoxin G ₁ (µg/kg of DM)	0.095	0.147
Aflatoxin G ₂ (µg/kg of DM)	0.044	0.059

¹Values represent the average of 16 replications and the standard deviation. LAB = lactic acid bacteria.

Table 3. Fermentative parameters of corn silage at opening (after 250 d) and after 7 and 14 d of air exposure

Item ¹	Treatment	DM (%)	DM corrected (%)	pH	NH ₃ -N (g/kg of DM)	Lactic acid (g/kg of DM)	Acetic acid (g/kg of DM)	Lactic-to-acetic ratio	Propionic acid (g/kg of DM)	1,2-Propanediol (g/kg of DM)	Ethanol (g/kg of DM)
Opening											
C	(LB- LH-)	36.4	37.3	3.57	1.05	60.7	13.4	4.5	<0.01	<0.01	8.9
LB	(LB+ LH-)	32.8	34.2	3.78	0.94	47.9	29.5	1.6	<0.01	9.9	13.5
LH	(LB- LH+)	34.1	35.4	3.69	1.84	52.5	26.0	2.0	<0.01	7.1	11.3
LB+LH	(LB+ LH+)	34.4	36.0	3.88	2.01	35.4	33.4	1.1	1.3	12.9	14.1
SEM		1.094	1.088	0.018	0.082	NS	1.208	0.128	—	0.600	0.989
Significance ²											
LB		NS	NS	***	NS	***	***	***	—	***	***
LH		NS	NS	***	***	**	***	***	—	***	NS
LB × LH		NS	NS	NS	NS	NS	**	***	—	***	NS
7 d											
C	(LB- LH-)	36.1	36.2	5.84	—	10.7	<0.01	—	<0.01	<0.01	<0.01
LB	(LB+ LH-)	32.1	32.9	4.21	—	34.9	29.5	—	<0.01	7.7	<0.01
LH	(LB- LH+)	34.2	34.6	4.25	—	29.0	10.3	—	<0.01	5.6	<0.01
LB+LH	(LB+ LH+)	34.1	35.5	3.91	—	35.0	32.4	—	1.2	12.6	11.3
SEM		1.145	1.158	0.276	—	3.720	2.499	—	—	0.351	—
Significance ²											
LB		NS	NS	***	—	***	***	—	—	***	—
LH		NS	NS	**	—	*	*	—	—	***	—
LB × LH		NS	NS	*	—	*	NS	—	—	NS	—
14 d											
C	(LB- LH-)	29.8	29.8	5.93	—	<0.01	0.2	—	<0.01	<0.01	<0.01
LB	(LB+ LH-)	28.0	28.1	6.39	—	<0.01	0.8	—	<0.01	<0.01	<0.01
LH	(LB- LH+)	29.9	29.9	6.53	—	<0.01	0.4	—	<0.01	<0.01	<0.01
LB+LH	(LB+ LH+)	34.9	35.7	4.81	—	23.4	17.9	—	1.1	5.6	4.9
SEM		1.136	1.369	0.373	—	—	4.209	—	—	—	—
Significance ²											
LB		NS	0.17	NS	—	—	*	—	—	—	—
LH		*	0.02	NS	—	—	NS	—	—	—	—
LB × LH		*	0.02	**	—	—	NS	—	—	—	—

¹C = control; LB = *Lactobacillus buchneri*; LH = *Lactobacillus hilgardii*.

²LB = effect of LB; LH = effect of LH; LB × LH = interaction of LB and LH.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 4. Hours of aerobic stability and temperature indices of corn silage during air exposure

Opening ¹	Treatment	Aerobic stability 2°C (h)	Peak temperature ³ (°C)	Interval to peak temperature ³ (h)	Interval to 35°C ³ (h)	Interval with temperature >35°C ³ (h)
C	(LB- LH-)	102	51	213	117	180
LB	(LB+ LH-)	138	46	260	163	143
LH	(LB- LH+)	124	48	251	139	182
LB+LH	(LB+ LH+)	365	35	332	294	37
SEM		50.1	3.33	7.80	12.8	18.2
Significance ²						
LB		*	*	***	***	***
LH		*	*	***	***	*
LB × LH		NS	NS	*	**	*

¹C = control; LB = *Lactobacillus buchneri*; LH = *Lactobacillus hilgardii*.

²LB = effect of LB; LH = effect of LH; LB × LH = interaction of LB and LH.

³As observed from silo opening to 14 d of air exposure.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

flavus quickly started to increase (Figure 2). The DM losses in all the silages increased during exposure to air and reached the highest value in C silage and the lowest in LB+LH silage after 14 d. As the actual concentration of AF in the silage samples should take into account in the amount of DM losses, Figure 3 reports the AFB₁, AFB₂, AFG₁, and AFG₂ concentrations from ensiling to the end of conservation as well as after 7 and 14 d of air exposure for both the potential amount of AF derived from the field (corrected for DM losses during conservation and air exposure), hypothesizing no new biosynthesis or degradation, and their actual measured contents. The AFB₁ increased during silage conservation and during air exposure, with higher values observed after 14 d of conservation, whereas AFB₂ did not increase significantly during conservation compared with its increase due to DM losses. On the other hand, the AFG₁ content decreased during conservation and subsequent exposure to air. Aflatoxin G₂ was not affected by the ensiling process or aerobic deterioration, even though it apparently increased when the DM losses were not taken into account.

The nutritional analyses after 250 d of conservation at silo opening and after 7 and 14 d of air exposure are reported in Table 6. At opening, no differences between treatments were found among the studied nutritional parameters. After 7 d of air exposure, a slight increase of starch was observed in C, LB, and LH silages, as well as a reduction in NDF. The CP increased after 14 d of exposure to air. After 1,000°C·h, when mold activity was evident, the starch content corrected for DM losses decreased greatly, reaching values below 10% of DM (Figure 1). After 14 d of exposure to air, an increase of NDF, ADF, hemicellulose, CP, and ash was observed, and this was more evident in C, LB, and LH silages than in LB+LH silages.

Strain Identification, Macro-Morphology, and Sclerotia Production

Representative subsamples of the isolated strains of *Aspergillus* section *Flavi* obtained from silage at opening, after 7 d of air exposure, and after 14 d of air exposure were used for the analysis. A data set of 532 bp for the partial sequence of the calmodulin gene was obtained for each strain. The majority of isolates (14/20) resulted to belong to the *A. flavus* species, whereas other isolates (6/20) were identified as *A. oryzae* var. *effusus* and showed 100% identity with the RefSeq deposit in GenBank (Table 1). A second subsample of *Aspergillus* section *Flavi*, obtained from silage after 21 d of exposure to air (4 isolates) and after 28 d of exposure to air (4 isolates) was used for the analysis (data not shown). All 4 isolates from the LB+LH samples at 21 d of exposure to air belonged to the *A. oryzae* var. *effusus* species (9026/1, 9026/2, 9026/3, and 9026/4). After 28 d of exposure to air, 2 out of 4 isolates were identified as *A. flavus* (9031/1 and 9031/2) and 2 as *A. oryzae* var. *effusus* (9031/3 and 9031/4).

The typical morphology of *Aspergillus* section *Flavi* was observed with yellow to green conidia, as well as a mean diameter of 40.7, 37.6, and 35.0 mm for YES, MEA, and CYA, respectively. The *A. oryzae* var. *effusus* strains showed poor sporulation, with a white surface and irregular margins, as well as a mean diameter of 40.9, 37.5, and 35.2 mm for YES, MEA, and CYA, respectively (Table 1).

Nine out of 14 *A. flavus* produced sclerotia, whereas none of the strains of *A. oryzae* var. *effusus* were able to produce them (Table 1). The 8931/2, 9010, and 9016 strains produced both S and L sclerotia, the 8931/7, 9015/1, and 9015/2 strains produced L sclerotia, whereas the 9002, 9005, and 9006 strains produced S

Table 5. Microbial count, DM losses, *Aspergillus flavus* count, and aflatoxin B₁, B₂, G₁, and G₂ of corn silage at opening (after 250 d) and after 7 and 14 d of air exposure

Item ¹	Treatment	Lactic acid bacteria (log ₁₀ cfu/g)	Yeast (log ₁₀ cfu/g)	Mold (log ₁₀ cfu/g)	DM losses (% of DM)	<i>Aspergillus flavus</i> (log ₁₀ cfu/g)	Samples with <i>A. flavus</i>	Aflatoxin B ₁ (µg/kg of DM)	Aflatoxin B ₂ (µg/kg of DM)	Aflatoxin G ₁ (µg/kg of DM)	Aflatoxin G ₂ (µg/kg of DM)
Opening											
C	(LB– LH–)	6.18	2.17	<1.00	2.3	<1.00	0/4	0.190	0.492	0.215	<0.01
LB	(LB+ LH–)	8.43	<1.00	<1.00	3.0	<1.00	0/4	0.106	2.038	0.057	0.037
LH	(LB– LH+)	7.44	1.41	0.90	3.0	0.69	1/4	0.314	2.436	0.013	0.039
LB+LH	(LB+ LH+)	8.58	<1.00	1.05	3.6	<1.00	0/4	0.137	1.878	<0.01	0.039
SEM		0.098	0.289	0.251	0.058	—	—	0.086	1.152	0.049	0.020
Significance ²											
LB		***	***	NS	***	—	—	NS	NS	NS	NS
LH		***	NS	NS	***	—	—	NS	NS	*	NS
LB × LH		***	NS	NS	NS	—	—	NS	NS	NS	NS
7 d											
C	(LB– LH–)	—	8.03	4.98	7.0	1.36	1/4	0.148	0.871	0.094	<0.01
LB	(LB+ LH–)	—	3.81	1.34	5.8	<1.00	0/4	0.705	0.551	<0.01	0.053
LH	(LB– LH+)	—	6.56	1.14	6.0	<1.00	0/4	0.348	1.512	<0.01	0.132
LB+LH	(LB+ LH+)	—	3.24	1.46	6.0	<1.00	0/4	0.292	1.639	<0.01	0.129
SEM		—	0.777	0.658	2.164	—	—	0.192	1.248	—	0.068
Significance ²											
LB		—	***	*	NS	—	—	NS	NS	—	NS
LH		—	NS	*	NS	—	—	NS	NS	—	NS
LB × LH		—	NS	**	NS	—	—	NS	NS	—	NS
14 d											
C	(LB– LH–)	—	6.14	8.49	39.6	2.23	2/4	0.972	1.656	<0.01	<0.01
LB	(LB+ LH–)	—	3.74	8.10	28.6	3.32	3/4	3.588	1.415	<0.01	0.203
LH	(LB– LH+)	—	5.24	8.04	30.2	2.61	2/4	0.860	1.851	<0.01	0.092
LB+LH	(LB+ LH+)	—	2.38	4.56	18.9	1.25	1/4	0.586	2.273	<0.01	0.071
SEM		—	0.860	0.792	3.633	0.999	—	1.242	1.417	—	0.094
Significance ²											
LB		—	**	*	**	NS	—	NS	NS	—	NS
LH		—	NS	*	*	NS	—	NS	NS	—	NS
LB × LH		—	NS	NS	NS	NS	—	NS	NS	—	NS

¹C = control; LB = *Lactobacillus buchneri*; LH = *Lactobacillus hilgardii*.

²LB = effect of LB; LH = effect of LH; LB × LH = interaction of LB and LH.

P* < 0.05; *P* < 0.01; ****P* < 0.001.

Figure 1. Scatter plot of fermentative products in relation to hourly accumulated temperature (T) rise (°C·h) above the ambient temperature over air exposure (A); of pH in relation to the hourly accumulated temperature rise (°C·h) above the ambient temperature over air exposure (B); starch content corrected for DM losses during air exposure in relation to the hourly accumulated temperature rise (°C·h) above the ambient temperature over air exposure (C); and silage temperature in relation to the hourly accumulated temperature rise (°C·h) above the ambient temperature over air exposure (D).

sclerotia. The 8959, 8931/3, 9012, and 9015/3 strains did not produce any sclerotia.

Aflatoxin Production In Vitro and Aflatoxin Gene Detection

After 14 d of incubation at 35°C in the dark on YES, 6 out of 14 *A. flavus* were able to produce AFB₁, whereas none of the *A. oryzae* var. *effusus* strains seemed to be able to produce them (Table 1). Quadruplex PCR for gene detection produced the expected size amplicons in the positive control, with around 1,000, 800, 500, and 400 bp for the *aflR*, *omtA*, *ver1*, and *nor1* genes, respectively (Figure 4). All the aflatoxigenic *A. flavus* strains showed a complete molecular pattern with the 4 analyzed genes. The nonaflatoxigenic *A. flavus* strains showed different banding pattern results. No DNA amplification was found for the *A. flavus* 8959, 9015/1, 9015/2, and 9015/3 strains for any of the genes. As far as the *A. oryzae* var. *effusus* strains are concerned,

quadruplex PCR showed 3 banding patterns: strains 8976, 8931/1 and 8931/4 with 2 bands, corresponding to the *ver1* and *nor1* genes, a second group with 3 bands corresponding to *aflR*, *ver1*, and *nor1* amplification (8931/5 and 8931/6 strains), and 1 strain (9004) with the complete pattern.

DISCUSSION

In light of the potential risk of mycotoxin contamination, we considered it important to obtain information about the type and distribution of AF in corn silage, which is the main source of forage for lactating dairy cows in Europe and North America. Furthermore, a possible effect of different LAB inocula on the growth of spoilage molds has also been evaluated. The longer the anaerobic phase of silage is, the greater the reduction in yeast and mold counts (Borreani et al., 2014); this implicates that silage is not a favorable environment for mold development during conservation if anaerobiosis

Figure 2. Scatter plot between the total mold and *Aspergillus flavus* counts and the hourly accumulated temperature rise ($^{\circ}\text{C}\cdot\text{h}$) above the ambient temperature over air exposure.

is maintained (Borreani et al., 2018). In the present experiment, the presence of *A. flavus* was found to be below the detection limit ($<1.00 \log_{10} \text{cfu/g}$) in the fresh forage before ensiling, in which the mold count was observed to be higher than $5 \log_{10} \text{cfu/g}$. At silo opening, after a long ensiling period (250 d), the mold count was around or below $1.00 \log_{10} \text{cfu/g}$ of silage, whereas *A. flavus* was found in 1 out of 16 silage samples ($1.24 \log_{10} \text{cfu/g}$, which represents around 45% of the total molds of that sample). This could suggest that *A. flavus* was already present in the field and that it survived after the anaerobic conservation period, whereas many other mold species did not. This hypothesis is also supported by the presence of AF in the herbage before ensiling, which means that *A. flavus* must have developed on the crop during the growing cycle, albeit at a low level, and synthesized AF.

Over the last decade, the Po plain environment has been characterized by warmer and drier summers than in previous decades, and these conditions could have favored the development of *A. flavus* on the corn crops and AF synthesis. This could explain the increased frequency of AFB₁ contamination observed on corn grain and silages in the last few years (Decastelli et al., 2007). The presence of *A. flavus* on corn crops at a low level at our latitudes could be explained by the occurrence of some periods with higher ambient temperatures than 35°C in summer, as this is the optimal growth temperature for the fungus and these increased temperatures generally cause it to be the predominant species in

tropical and subtropical climates (Cheli et al., 2013). In this regard, González-Pereyra et al. (2008) found *A. flavus* as the predominant species, followed by *Aspergillus fumigatus* and *Aspergillus niger*, in different farm corn silages in the tropical environment of central Argentina. Keller et al. (2013) found *A. flavus* as the predominant *Aspergillus* species at both ensiling and at silo opening in Brazil, and El-Shanawany et al. (2005), in a farm survey, found *A. flavus* as the dominant species in corn silage in Egypt. On the other hand, Garon et al. (2006) and Spadaro et al. (2015) did not find *A. flavus* in corn silage in the temperate climates of France or northern Italy, but instead reported the presence of *A. parasiticus* and *A. fumigatus*. However, Richard et al. (2007) detected *A. flavus* in farm corn silage in France after 11 mo of conservation. This indicates that the fungus is able to survive during the anaerobic conservation phase of corn silage, albeit at a low level, and could grow when the environmental conditions become more suitable for its growth, such as during the feed-out phase or in peripheral areas of the silage, where temperatures increase as a consequence of a deteriorating microflora activity, which depletes the acidic conditions.

In the present experiment, even though *A. flavus* was below the detection limit in the fresh forage before ensiling and it was present at a low level at silage opening, AF were found in both the fresh forage and at opening in all the samples, with a predominance of AFB₂. The presence of AF has been found at silo opening in different countries of the world, as reviewed by Alonso

et al. (2013), with higher concentrations in warmer climates (Carvalho et al., 2016; Ogunade et al., 2018) than in colder ones (Driehuis et al., 2008; Cavallarin et al., 2011).

In our experiment, the inoculation with heterolactic bacteria, which was aimed at improving the aerobic stability of silage during the feed-out phase, and the long ensiling duration (250 d) influenced the fermentative profile of the silages, with a dominant homolactic fermentation (lactic-to-acetic ratio >4) in the control silages and a heterolactic fermentation (lactic-to-acetic ratio <2) in LB- and LH-treated silages, as previously reported by Kleinschmit and Kung (2006). The 1,2-propanediol found in LB silages, as previously reported by Oude Elferink et al. (2001), and in LH silages was in agreement with the results of Assis et al. (2014). The fermentation process, combined with the longer ensiling duration, greatly reduced the yeast and mold count compared with those observed at harvesting. Furthermore, the inocula containing LB showed a reduction in yeast to below the detection limit, as reported by Kleinschmit and Kung (2006). The use of heterolactic inocula causes greater DM losses during fermentation,

and greater DM losses could cause a higher concentration of nondegraded components (e.g., ashes). Therefore, to obtain a better understanding of the fate of different AF in silage, due to the fact that they could be produced both in the field and during ensiling and at the same time could be degraded or bound by LAB microbial activity (Oluwafemi et al., 2010) or bound by other silage bacteria (Ma et al., 2017), it is important to keep in mind that they could concentrate in silage as a consequence of DM losses. Such a scenario is evident in Figure 3, where it appears that the AFB₁ increased slightly during the fermentation process and increased significantly after 7 and 14 d of exposure to air. Aflatoxin B₂ and AFG₂ were not affected by the anaerobic fermentation phase or by the subsequent exposure to air, and their higher concentration in deteriorated silages could mainly be attributed to the DM losses. On the other hand, AFG₁ was partially degraded during the ensiling process and almost completely disappeared after 14 d of air exposure. Cavallarin et al. (2011) analyzed the presence of AF in both fresh forage and after ensiling in silage stored under different plastic films and, during feed out, they found that AF were absent

Figure 3. Concentration of aflatoxin (AF) B₁, AFB₂, AFG₁, and AFG₂ from ensiling to end of conservation and after 7 and 14 d of air exposure (exp); data from different treatments are pooled together. In gray is the potential amount of aflatoxins derived from the field corrected for DM losses hypothesizing no new biosynthesis or degradation; in black is the actual measured content. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 6. Nutritional analyses of corn silage at opening (after 250 d) and after 7 and 14 d of air exposure

Item ¹	Treatment	Starch (% of DM)	NDF (% of DM)	ADF (% of DM)	Hemicellulose (% of DM)	CP (% of DM)	Ash (% of DM)
Opening							
C	(LB– LH–)	34.6	37.0	19.4	17.6	8.5	4.66
LB	(LB+ LH–)	33.3	39.4	21.1	18.3	8.5	5.16
LH	(LB– LH+)	33.1	39.2	20.9	18.3	8.8	5.14
LB+LH	(LB+ LH+)	35.0	38.7	20.5	18.2	8.5	5.02
SEM		0.94	1.275	0.81	0.19	0.495	0.158
Significance ²							
LB		NS	NS	NS	*	NS	NS
LH		NS	NS	NS	NS	NS	NS
LB × LH		NS	NS	NS	NS	NS	NS
7 d							
C	(LB– LH–)	39.3	35.0	18.8	16.3	7.6	4.88
LB	(LB+ LH–)	36.5	37.3	20.0	17.3	8.1	5.31
LH	(LB– LH+)	36.4	38.1	20.0	18.1	8.4	5.29
LB+LH	(LB+ LH+)	35.7	38.0	20.1	17.9	8.6	5.14
SEM		0.628	0.799	0.525	0.146	0.314	0.169
Significance ²							
LB		*	NS	NS	***	NS	NS
LH		*	*	NS	***	***	NS
LB × LH		NS	NS	NS	**	NS	NS
14 d							
C	(LB– LH–)	18.1	56.4	35.3	21.1	17.7	7.27
LB	(LB+ LH–)	26.8	51.5	31.0	20.5	12.8	6.91
LH	(LB– LH+)	26.0	50.1	30.5	19.7	13.4	6.96
LB+LH	(LB+ LH+)	33.5	42.1	23.4	18.7	9.6	5.90
SEM		2.859	2.734	1.937	0.523	0.869	0.424
Significance ²							
LB		*	*	**	***	NS	NS
LH		*	**	**	*	NS	NS
LB × LH		NS	NS	NS	NS	NS	NS

¹C = control; LB = *Lactobacillus buchneri*; LH = *Lactobacillus hilgardii*

²LB = effect of LB; LH = effect of LH; LB × LH = interaction of LB and LH.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

in the center of the bunker silos, whereas they were present with values of up to 6 µg/kg of DM in the top layer of the bunker. In contrast to the results of the present study and those of Cavallarín et al. (2011), Garon et al. (2006) observed, in farm-scale silos, a decrease in the AFB₁ content as the ensiling duration increased. This could be attributable to a detoxification effect during fermentation, as reported by Oluwafemi et al. (2010), who observed that some strains of LAB are able to partially degrade AFB₁ in corn grain via a biological pathway, with a reduction range from 31 to 46%. Ahlberg et al. (2015), reviewing the ability of different LAB species and strains to bind AF in different food matrices, reported binding effects ranging from 0 to 90%, but their review did not report any research results concerning inoculation trials with *L. buchneri* or *L. hilgardii*. Ma et al. (2017) reported the capacity of binding AFB₁ in vitro by some strains of LAB when applied at 10⁹ cfu/mL (*Lactobacillus plantarum*, *L. buchneri*, and *Pediococcus acidilactici*); however, those authors failed to find the same effect on corn silage artificially contaminated with AFB₁, although they

concluded that some silage bacteria could have reduced the AFB₁ to a safe content within 3 d of ensiling regardless of LAB inoculation. In the present experiment, even though the conservation period was 250 d long, AF concentrations did not decrease compared with AF present in the fresh forage.

One of the most relevant issues of corn silage around the world is its susceptibility to aerobic deterioration, which negatively influences the silage nutritive value, animal performance, and farm profitability (Borreani et al., 2018). To better understand the role of air on the reduction of the nutritional value and on the increase in DM losses during aerobic deterioration, silages were left to deteriorate in laboratory trials for up to 14 d. This time period was chosen to represent the average age of silages in the peripheral areas of farm bunker silos at risk to exposure to air when a feed-out rate of 0.7 to 1.4 m/wk is adopted (Borreani and Tabacco, 2008; Weinberg et al., 2009), which results in a potential aerobic exposure of the silage of 10 to 20 d. In our experiment, the inocula improved aerobic stability compared with the control, and this is in agreement with previous

studies about the efficiency of *L. buchneri* (Arriola et al., 2011; Comino et al., 2014) and *L. hilgardii* (Assis et al., 2014). In agreement with the findings reported by Tabacco et al. (2011), the deteriorated silages of the present study showed an increase in the yeast and mold counts and a reduction of their nutritive value, with a substantial decrease in starch and an increase in the fiber and ash concentrations.

At the beginning of aerobic deterioration, the mold count values were close to those observed at silo opening, whereas the yeasts increased to values higher than $5 \log_{10}$ cfu/g; the developing yeast depleted the fermentative products (first the ethanol and then the acetic and lactic acid) and caused an increase in the pH and silage temperature (Figure 1). Irrespective of the treatment, when the hourly cumulated temperature reached $1,000^{\circ}\text{C}$, all the inhibiting conditions for mold growth (undissociated organic acids) were depleted and the mean pH was 6.19 ± 0.62 . At the same time, the silage temperature increased to over 35°C (with an average value of $38.7 \pm 6.3^{\circ}\text{C}$). These lowered inhibiting conditions allowed the growth of both the total molds and *A. flavus*, which reached values higher than 8.00 and 4.00 $\log_{10}$ cfu/g, respectively. Those conditions were observed in both the control and LAB-treated silages after about 70 h from the time at which the temperature started to rise above the ambient temperature (data not shown), with LB- and LH-treated silage showing a longer period of aerobic stability. This means that all the silages (both the control and LAB-inoculated ones) are prone to the development of *A. flavus* during exposure to air, and this development takes place when silage stability has already been compromised (pH and temperature higher than 5 and 35°C , respectively). Cavallarin et

al. (2011) provided evidence of AF accumulation in whole-crop corn silage as a result of aerobic deterioration, which had previously been hypothesized by other authors that had found higher contaminations of AF in peripheral areas of commercial silos, which are known to be the most prone to aerobic deterioration (Rosiles, 1978; Richard et al., 2009). The results of this experiment have also shown that the use of LAB inocula, which are able to shift silage fermentation toward a more heterolactic pathway, could delay the onset of aerobic deterioration after exposure to air of the silage, and, as a consequence, could stave off *A. flavus* development and AF synthesis. Results are in agreement with data of Queiroz et al. (2012), who found that when corn infested with southern rust was inoculated with a mixture of *P. pentosaceus* and *L. buchneri* at ensiling, its aerobic stability increased and the production of AF was prevented compared with an untreated control. Our results are also in agreement with Cavallarin et al. (2011), who reported that inoculation with *L. buchneri* delayed the onset of aerobic deterioration and the synthesis of AF. In the present experiment we also observed, as previously reported by Cavallarin et al. (2011), that, when deterioration have took place, the total AF concentration in *L. buchneri*-treated silages increased more than in the control or in *L. plantarum*-inoculated silage.

The environment resulting from aerobic deterioration allowed *A. flavus* to grow and AFB₁, which was produced ex novo during ensiling and air exposure, to increase. To verify the aflatoxigenic potential of the selected colonies of *A. flavus*, 4 genes involved in the aflatoxin biosynthesis pathway were analyzed through a quadruplex PCR and an in vitro assay was performed. Nine

Figure 4. Agarose gel electrophoresis of quadruplex PCR products for strains used in this study. Lane M = molecular marker 1 kb Plus Ladder (Qiagen, Hilden, Germany); 1 = *Aspergillus flavus* 8959; 2 = *Aspergillus oryzae* var. *effusus* 8976; 3 = *A. oryzae* var. *effusus* 8931/1; 4 = *A. flavus* 8931/2; 5 = *A. flavus* 8931/3; 6 = *A. oryzae* var. *effusus* 8931/4; 7 = *A. oryzae* var. *effusus* 8931/5; 8 = *A. oryzae* var. *effusus* 8931/6; 9 = *A. flavus* 8931/7; 10 = *A. flavus* 9002; 11 = *A. oryzae* var. *effusus* 9004; 12 = *A. flavus* 9005; 13 = *A. flavus* 9006; 14 = *A. flavus* 9010; 15 = *A. flavus* 9011; 16 = *A. flavus* 9012; 17 = *A. flavus* 9015/1; 18 = *A. flavus* 9015/2; 19 = *A. flavus* 9015/3; 20 = *A. flavus* 9016; 21 = negative control; 22 = positive control.

out of 14 strains of *A. flavus* showed the presence of the complete gene pattern and, of these strains, 6 were able to produce AF. According to Criseo et al. (2001), some atoxigenic strains could have a quadruplet pattern and other markers are necessary to discriminate aflatoxigenic strains from atoxigenic ones. Furthermore, the occurrence of S sclerotia was positively related to the production of AF in vitro, as previously reported by Cotty (1989). On the other hand, some *A. flavus* strains isolated from the corn silage in our study did not reveal the presence of the complete AF gene pattern and were unable to produce AF in vitro. This observation could be a starting point for future investigations on the selection of atoxigenic *A. flavus* to occupy the same niche as the naturally occurring epiphytic toxigenic *A. flavus*, as recently proposed as a strategy to reduce AF contamination in the field (Mauro et al., 2018).

During aerobic deterioration, the molds also degraded starch and part of the hemicelluloses, thus contributing to an increase of the less degradable part of NDF. This was previously described by Tabacco et al. (2011), who reported that when molds started to grow, the nutritional value of the silages decreased, with a strong reduction in the starch content, which represents the main energy component of corn silage, an increase in NDF, and a reduction in its degradability.

CONCLUSIONS

Ensiling corn that was naturally contaminated by aflatoxin did not change its aflatoxin concentration at the end of a long conservation period. *Aspergillus flavus* showed ability to survive in the anaerobic silage and revive when the inhibiting conditions in terms of low pH and temperature were lost. Both the control and inoculated silages deteriorated during aerobic exposure and showed an increase in the *A. flavus* count and AFB₁ concentration. Inoculation with LB and LB+LH increased the aerobic stability of the silages and delayed the onset of aerobic microbial degradation, this, in turn, could indirectly reduce the risk of *A. flavus* outgrowth and AFB₁ production after silage opening.

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