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

This is the author's manuscript

Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/1687728 since 2022-12-06T10:59:33Z

Published version:

DOI:10.3168/jds.2018-15468

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03 September 2024

1 **Interpretative summary**

2 **Increase in aflatoxins due to *Aspergillus* section *Flavi* multiplication during the aerobic**
3 **deterioration of corn silage treated with different bacteria inocula.** *By Ferrero et al.*

4 Aflatoxins produced by *Aspergillus flavus* are toxic molecules which, when ingested by animals
5 through contaminated feed, are partially transferred to the milk. This experiment has evaluated
6 whether *A. flavus* and aflatoxins originated from the field or whether growth of *A. flavus* took place
7 and additional aflatoxins were produced during the ensiling of corn. Aflatoxins were found both at
8 harvest and after ensiling. A multiplication of *A. flavus* and an increase in aflatoxin B_1 was observed
9 during exposure of the corn to air. The aerobic deterioration decreased the nutritional and hygienic
10 value of the silages. Inoculation with *Lactobacillus buchneri* alone or in combination with *L.*
11 *hilgardii* delayed the onset of aerobic microbial degradation, and the risk of *A. flavus* outgrowth and
12 AFB_1 production after silage opening.

13

14 **RUNNING HEAD: *ASPERGILLUS* SECTION *FLAVI* IN SILAGE**

15

16 **Increase in aflatoxins due to *Aspergillus* section *Flavi* multiplication during the aerobic**

17 **deterioration of corn silage treated with different bacteria inocula**

18

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36

37 **ABSTRACT**

38

39 The growth of *Aspergillus flavus* and the production of aflatoxins (AF) during the aerobic
40 deterioration of corn silage represent a problem for animal and human health. This experiment was
41 conducted to evaluate whether the presence of *A. flavus* and AF production originate from the field
42 or additional AF are produced during the fermentation phase or during aerobic deterioration of corn
43 silage. The trial was carried out in Northern Italy on corn at a dry matter (DM) level of 34%. The
44 fresh herbage was either not treated (C) or treated with a *L. buchneri* (LB) NCIMB 40788 [(at $3*10^5$
45 cfu/g fresh matter (FM)], *L. hilgardii* (LH) CNCM I-4785 (at $3*10^5$ cfu/g FM) or their combination
46 (LB+LH) (at $1.5*10^5$ cfu/g FM of each one), ensiled in 20 L silos and opened after 250 d of
47 ensiling. After silo opening, the aerobic stability was evaluated and samples were taken after 7 and
48 14 d of air exposure. The pre-ensiled material, the silages at silo opening and the aerobically
49 exposed silages were analyzed for DM content, fermentative profiles, microbial count, nutritive
50 characteristics, DM losses and aflatoxin B_1, B_2, G_1 and G_2 contents. Furthermore, a subsample of
51 colonies with macromorphological features of *A.* section *Flavi* was selected for AF gene pattern
52 characterization and in vitro AF production. The presence of *A. flavus* was below the detection limit
53 (< 1.00 $\log_{10}$ cfu/g) in the fresh forage prior to ensiling, whereas it was found in 1 out of 16 silage
54 samples at silo opening at a level of 1.24 $\log_{10}$ cfu/g. The AF were found in both the fresh forage
55 and at opening in all the samples, with a predominance of AFB_2 (mean value of 1.71 μg/kg DM).
56 The inoculation of lactic acid bacteria (LAB) determined a reduction in the lactic-to-acetic ratio
57 compared to the control. A larger amount of acetic acid resulted in a lower yeast count in the LB
58 containing silages and higher aerobic stability in the treated silages than in the control ones. At the
59 beginning of aerobic deterioration, the yeasts increased to over 5 $\log_{10}$ cfu/g, whereas the molds
60 were close to the value observed at silo opening. When the inhibiting conditions were depleted (pH
61 and temperature higher than 5 and 35°C, respectively), both the total molds and *A. flavus* reached
62 higher values than 8.00 and 4.00 $\log_{10}$ cfu/g, respectively, thus determining the *ex-novo* production

of AFB_1 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 four AF gene patterns, and 43% of the selected colonies were able to produce aflatoxins in vitro. During air exposure, after 1000°C h have been cumulated, starch content decreased (below 10% DM) and concentration of NDF, ADF, hemicelluloses, CP 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 AFB_1 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 dry matter (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 which 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 represents an important problem for human and animal health (Driehuis, 2013; Spadaro et al., 2015). Furthermore, this contamination causes the rise of food production costs as the result of the increased need for testing, the lower prices received for contaminated loads, the potential lawsuits from consumers, and the 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 are carcinogenic, mutagenic, teratogenic and immunosuppressive secondary metabolites of *Aspergillus* fungi (Eaton and Gallagher, 1994).~~ 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 B_1 (**AFB_1**), G_1 (**AFG_1**) and their dihydro derivatives B_2 (**AFB_2**) and G_2 (**AFG_2**). AFB_1 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 M_1 (**AFM_1**). Aflatoxin M_1 appears in milk within 12 h from ingestion, with a mean carry-over of around 3.5% (Veldman et al., 1992). ~~The European Union has fixed the limit for AFM1 in milk as 50 ng/kg (ppt) (Commission Regulation (EC) N. 466/2001), whereas the USA has fixed it at tenfold higher level than the EU (500 ng/kg).~~ The World Health Organization (WHO, 2002) evaluated the two maximum concentrations of aflatoxin M_1 that had been proposed by the Codex Committee on Food Additives and Contaminants, 50 and 500 ppt (for EU and USA, respectively), concluding that based on worst-case assumptions, the projected risk of liver cancer attributable to aflatoxin M_1 would be very small if either of these maximum levels were implemented. In order to reduce the risk of AFM_1 contamination, the daily ingestion of AFB_1 should be limited to 40 µg and 400 µg per cow per day for the EU and the USA, respectively (Veldman et al., 1992). Corn silage is the main source of AF in warm regions (i.e. Southwestern US) whereas in temperate ones it is generally less contaminated than other feeds (e.g. corn grain, peanuts and cottonseed). However, the large use of corn silage on dairy farms through the world imposes the need for a careful management of the factors that could increase AFB_1 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 pre-harvest 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 post-harvest contamination of corn silage for several reasons, such as 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 post-harvest 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) ~~inocula~~ has been used over the years to improve silage fermentation and/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 contribute 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 lactic acid bacterial inoculation, certain silage bacteria can reduce the concentration of AFB_1 that was spiked in corn silage to a safe level within 3 d of ensiling.

140 Thus, owing to the lack of clear information in literature, the aims of this study were: i) to
141 evaluate whether the presence of *A. flavus* and aflatoxin production in corn silage originates from
142 the field environment or growth of *A. flavus* take place and additional aflatoxins are produced
143 during storage or air exposure after silo opening; ii) to evaluate the effect of different LAB inocula
144 used to improve the aerobic stability of corn silage on reducing *A. flavus* growth and aflatoxin
145 production during fermentation and air exposure; and iii) to characterize the toxigenic potential of
146 *A. flavus* strains isolated from corn silages.
147
148 **MATERIALS AND METHODS**
149
150 ***Crop and Ensiling***
151 The trial was carried out on a commercial farm located in Rocca de' Baldi (CN) in the
152 western Po plain, North West Italy (44°27’18"N, 7°43’19"E, 408 m above sea level). Corn hybrid
153 (P1517W, Pioneer Hi-Bred Italia Srl, Gadesco Pieve Delmona, Cremona, Italy) was sown in April
154 2015, at an intended planting density of 75,000 seeds/ha. The whole corn crop was harvested at
155 around the 50% milk-line stage and with a DM content of around 34%. Fresh forage was chopped
156 using a precision forage harvester (Claas Jaguar 950, equipped with an 8-row Orbis head, Claas,
157 Harsewinkel, Germany) to a theoretical cutting length of 12 mm. The field was divided in four
158 plots, which were subsequently harvested separately and the crop was chopped in order to obtain
159 four replicates. The fresh herbage of each plot was divided into four 70-kg piles. The piles were
160 either not treated (**C**) or treated with different LAB strains and their combinations. The LAB strains
161 were *L. buchneri* (**LB**) NCIMB 40788 (Lallemand Animal Nutrition, BP 59, Cedex, France)
162 [(theoretical rate of 300,000 cfu/g fresh matter (FM)], *L. hilgardii* (**LH**) CNCM I-4785 (Lallemand
163 Animal Nutrition) (theoretical rate of 300,000 cfu/g FM) and their combination (**LB**+**LH**)
164 (theoretical rate of 150,000 cfu/g FM of each one). The microbial inoculants were diluted in
165 sterilized water and applied using a hand sprayer, at a rate of 4 ml/kg of forage, by spraying

166 uniformly onto the forage, which was constantly hand mixed. The same amount of water was added
167 to the C treatment. In order to add the targeted amount of LAB, the inocula were plated on MRS
168 agar (Merck, Whitehouse Station, NY), with the addition of natamycin (0.25 g/L) and, on the basis
169 of the measured concentration of LAB, an appropriate amount was used to achieve the desired
170 application rate.
171 The fresh forage was sampled prior to ensiling after the inoculum had been applied. The untreated
172 and treated forage was then ensiled (11 to 13 kg of wet forage) in 20 L plastic silos equipped with a
173 lid that only enabled the release of gas. The forage was packed by hand, and the final packing
174 densities, on a wet basis, were 627±26 kg FM/m^3. All the laboratory silos were filled within three
175 hours. The silos were weighed, conserved at ambient temperature (20 ± 1°C) and opened after 250
176 d. At opening, each silo was weighed, and the content was mixed thoroughly and sub-sampled to
177 determine the DM content, the chemical composition, the fermentation profile and the microbial
178 counts. After sampling, the silages were subjected to an aerobic stability test, which involved
179 monitoring the temperature increases due to the microbial activity in the samples exposed to air.
180 About three kilograms from each silo were allowed to aerobically deteriorate at room temperature
181 (20 ± 1°C) in 17 L polystyrene boxes (290 mm diameter and 260 mm height). A single layer of
182 aluminum foil was placed over each box to prevent drying and dust contamination, but also to allow
183 the air to penetrate. The room and silage temperatures were measured hourly by means of a data
184 logger. Aerobic stability was defined as the number of hours the silage remained stable before its
185 temperature increased by 2°C above room temperature. From silo opening to 14 d of air exposure of
186 silages, peak temperature, hours to reach peak temperature, interval to reach 35°C and time with
187 temperature greater the 35°C were also calculated to better describe the optimum temperature for
188 growth of *A. flavus* in absence of inhibitory conditions. The silage was sampled after 7 d and 14 d
189 of aerobic exposure in order to quantify the chemical, fermentative and microbial changes in the
190 silage during exposure to air, as reported by Tabacco et al. (2011). Other samplings were
191 conducted, on d 21 and d 28, on the silages that did not show any increase in temperature at 14 d of

192 air exposure. The DM losses due to fermentation were calculated as the difference between the
193 weight of the forage placed in each plastic silo at ensiling and the weight of the silage at the end of
194 conservation, corrected for the DM content of the forage and its respective silage. The DM losses
195 were calculated after 7 and 14 d of exposure to air using the ash content, as reported by Borreani et
196 al. (2018). Small increases in the ash content of deteriorated silage represent large percentage unit
197 increases in DM loss, as can be seen when the equation for calculating DM losses according to the
198 ash content is used: DM loss (%) = [1 − (ash silage at opening/ash silage after 7 or 14 d of air
199 exposure)] × 100.
200
201 ***Sample Preparation and Analyses***
202 The pre-ensiled material and the silage were split into five subsamples, at both the opening
203 of the silos and before the exposure to air periods (at 7 and 14 d for all silages, and at 21 and 28 d
204 when needed). One sub-sample was analyzed immediately, for the DM content, by oven drying at
205 80°C for 24 h. Dry matter was corrected according to Porter and Murray (2001), in order to
206 consider the volatile compound losses that can take place at 80°C. The second subsample was oven-
207 dried at 65°C to a constant weight and was air equilibrated, weighed and ground in a Cyclotec mill
208 (Tecator, Herndon, VA, USA) to pass a 1 mm screen. The dried samples were analyzed for the total
209 nitrogen (TN), according to the Dumas method (method number 992.23, AOAC, 2005), using a
210 Nitrogen analyzer Primacs SN (Skalar, Breda, The Netherlands), for crude protein (CP) (total N x
211 6.25), for ash by ignition (method number 942.05, AOAC, 2005), and for water soluble
212 carbohydrates (WSC), by the phenol sulphuric acid method, according to DuBois et al. (1956).
213 Neutral detergent fiber (NDF) was analyzed, using a Raw Fiber Extractor (FIWE, VELP
214 Scientifica, Usmate Velate, Italy), with the addition of heat-stable amylase (A3306, Sigma
215 Chemical Co., St. Louis, MO) and expressed on a DM basis, including residual ash, as described by
216 Van Soest et al. (1991). Acid detergent fiber (ADF) was analyzed and expressed on a DM basis,
217 including residual ash (Robertson and Van Soest, 1981).

218 A third fresh sub-sample was used to determinate the water activity (a_w), pH, nitrate (NO_3),
219 the ammonia nitrogen (NH_3-N) contents and the buffering capacity. The water activity was
220 measured at 25°C on a fresh sample using an AquaLab Series 3TE (Decagon Devices Inc., Pullman,
221 WA), which adopts the chilled-mirror dew point technique. The fresh forage was extracted for pH,
222 NO_3 and NH_3-N determination, using a Stomacher blender (Seward Ltd, Worthing, UK), for 4 min
223 in distilled water at a 9:1 water-to-sample material (fresh weight) ratio. The total nitrate
224 concentration was determined in the water extract, through semi-quantitative analysis, using
225 Merckoquant test strips (Merck, Darmstadt, Germany; detection limit 100 mg NO_3/kg DM). The
226 ammonia nitrogen content and pH were determined using specific electrodes. The buffering
227 capacity was determined in the water extract, as described by Plaine and McDonald (1966).
228 A fourth sub-sample was extracted, using a Stomacher blender, for 4 min in H_2SO_4 0.05
229 mol/L at a 5:1 acid-to-sample material (fresh weight) ratio. An aliquot of 40 ml of silage acid
230 extract was filtered with a 0.20-μm syringe filter and used for quantification of the fermentation
231 products. The lactic and monocarboxylic acids (acetic, propionic and butyric acids) were
232 determined, by means of high performance liquid chromatography (HPLC), in the acid extract
233 (Canale et al., 1984). Ethanol and 1,2-propanediol were determined by means of HPLC, coupled to
234 a refractive index detector, on a Aminex HPX-87H column (Bio-Rad Laboratories, Richmond, CA).
235 The fifth subsample was used for the microbial analyses.
236
237 ***Microbial Analysis, Fungal Isolation, Macro-Morphology and Sclerotia Production***
238 In order to conduct the microbial counts, 30 g of sample was transferred into a sterile
239 homogenization bag, suspended 1:10 w/v in a peptone salt solution (1 g of bacteriological peptone
240 and 9 g of sodium chloride per liter) and homogenized for 4 min in a laboratory Stomacher blender
241 (Seward Ltd, London, UK). Serial dilutions were prepared, and the mold and yeast numbers were
242 determined using the pour plate technique with 40.0 g/L of Yeast Extract Glucose Chloramphenicol
243 Agar (YGC agar, DIFCO, West Molesey, Surrey, UK) after incubation at 25°C for 3 and 5 d for

244 yeast and mold, respectively. The yeast and mold colony forming units (cfu) were enumerated
245 separately, according to their macromorphological features, on plates that yielded 1 to 100 cfu. The
246 LAB were determined on MRS agar with added natamycin (0.25 g/L), by incubating Petri plates at
247 30°C for 3 d under anaerobic conditions, according to Spoelstra et al. (1988). Since LAB are
248 facultative anaerobe bacteria, anaerobic incubation was chosen to improve the selectivity of the
249 media against *Bacillus* spp.

250 A subsample of 5 to 10 colonies per sample, with macromorphological features of *A. section*
251 *Flavi,* was selected for characterization (Table 1). The strains were maintained as a monoconidial
252 culture on 30% glycerol and stored at -80°C. A macro-morphological analysis of each isolate was
253 performed on Yeast Extract Sucrose agar (YES), Malt Extract Autolysate agar (MEA) and Czapek
254 Yeast Autolysate agar (CYA), and observed after 7 days of incubation at 25°C in the dark,
255 according to Samson et al. (2004).

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

262

263 ***Molecular Identification***

264 The isolates were grown on Potato Dextrose Broth (27 g PDB, Merck, Germany; 1 L H_2O)
265 and incubated at 28°C in the dark. DNA was extracted from the mycelium using an Omega
266 E.Z.N.A. Fungal DNA Mini Kit (Omega Bio-tek Inc., Norcross, GA, USA), according to the
267 manufacturer's instructions. Partial amplification of the calmodulin gene was obtained using the
268 cmd5 and cmd6 primer pair (Hong et al., 2006). PCR was carried out in a total volume of 25 µl
269 which contained: 2.5 µl of Buffer 10 X, 0.5 µl of $MgCl_2$, 0.75 µl of dNTPs (10 mM), 1 µl of each

270 primer (10 mM), 0.2 µl of Taq DNA polymerase (Qiagen, Hilden, Germany) and 20 ng of template
271 DNA. A thermal cycling program was performed according to Samson et al. (2014). Amplification
272 was verified by means of electrophoresis on 1% agarose TAE gel, and gel images were acquired
273 using a Gel Doc 1000 System (Bio-Rad Laboratories, Hercules, CA, USA). The PCR products were
274 purified using a QIAquick© PCR purification Kit (Qiagen) and sent to Macrogen, Inc. (Amsterdam,
275 The Netherlands) for sequencing in both directions. The consensus sequences were obtained using
276 the DNA Baser program (Heracle Biosoft S.R.L., Arges, Romania) and compared, using the
277 BLAST program, with those deposited in the RefSeq database of the National Centre for
278 Biotechnology Information (NCBI) for species identification. All the sequences are deposited in the
279 GenBank database with the accession numbers listed in Table 1.
280
281 ***Aflatoxin Gene Detection and Aflatoxin Production* in Vitro**
282 The presence of four genes, three structural genes (*nor-1*, *ver-1* and *omtA*) and one regulatory
283 gene (*aflR*), which were involved in the aflatoxin biosynthesis pathway, and which have been
284 studied extensively and used as a diagnostic tool for the differentiation of aflatoxin producing and
285 non-producing fungi, was verified through a quadruplex PCR assay. Quadruplex PCR was
286 performed using the primers listed in supplementary Table S1. A PCR reaction was carried out in a
287 total volume of 50 µl which contained: 5 µl of Buffer 10 X, 1 µl of $MgCl_2$, 2 µl of dNTPs (10 mM),
288 1 µl of each primer (10 mM), 0.4 µl of Taq DNA polymerase (Qiagen) and 100 ng of template
289 DNA. A thermal cycling program was performed according to Criseo et al. (2008), with some
290 minor modifications. The PCR products were separated by gel electrophoresis using a TBE buffer
291 with 2% agarose and 5 µl of SYBRSafe® (Invitrogen, Waltham, MA, USA) at 80 V/cm for 4 hours.
292 A Get Pilot 1 kb Plus Ladder (100-10000 bp, Qiagen) was used as a molecular marker and an
293 aflatoxigenic *A. parasiticus* strain AFCAL11 (from the collection of Agroinnova, University of
294 Turin, Italy) was used as positive control. The gel profiles were visualized under UV

transilluminator using the Quantity One program (BioRad Labs, Segrate (MI), Italy). The experiment was repeated in triplicate.

The production of aflatoxin was tested for each isolate using a YES Broth medium, according to Visagie et al. (2014): 20 g/L Yeast extract, 150 g/L Sucrose, 0.5 g/L $MgSO_4$ (Merck, Germany) and 1 mL trace elements (1 g $ZnSO_4.7H_2O$ and 0.5 g $CuSO_4.5H_2O$ in 100 mL distilled water). Strains were inoculated with three mycelia plugs from a seven-day-old culture (4 mm diameter) on 50 mL of medium and incubated in the dark at 35°C. After 7 days, the cultures were filtered and extracted to establish the aflatoxin production. The samples were extracted and analyzed according to Prencipe et al. (2018). ~~Ten mL of ethyl acetate (Merck) was added twice, the mixture was shaken for 1 min, and then the ethyl acetate extracts were collected in a flask. The final extract was evaporated to dryness in a rotary evaporator. The residue was dissolved in 0.5 mL methanol-water (50:50 v/v) for the HPLC-MS/MS analysis.~~

~~Analyses were performed using a Varian Model 212-LC micro pump (Palo Alto, CA, USA), equipped with a Varian autosampler Model 410 Prostar, coupled with a Varian 310-MS triple quadrupole mass spectrometer, with an electrospray ion source operating in positive ionization mode. Chromatographic separation was performed in isocratic mode on a Pursuit XRs Ultra C18 (100 mm x 2.0 mm, 2.8 µm, Varian) column using water acidified with 0.05% formic acid (Sigma Aldrich) and methanol (Merck) 40:60 *v/v* as eluents, and the flow rate was set at 0.2 mL min^{-1} for 15 min. The monitoring reaction mode (MRM) transitions used for the analyses were: 313>285 (CE 14 eV) and 313>241 (CE 34 eV) for AFB_1, 315>287 (CE 18 V) and 315>243 (CE 38 eV) for AFB_2, 329>243 (CE 18 V) and 329>311 (CE 18 eV) for AFG_1, and 331>245 (CE 24 V) and 331>313 (CE 23 eV) for AFG_2, 404>239 (CE 26 eV) and 404>221 (CE 36 eV) for OTA, 327>245 (CE 16 eV) and 327>263 (CE 12 eV) for GT.~~

319 ***Aflatoxin Analysis***

320 The aflatoxins were extracted according to the method reported by Cavallarin et al. (2011).
321 Sample extracts were stored at −20°C until HPLC analysis. ~~A 10 g portion of silage (fresh weight)
322 was weighed in an Erlenmeyer flask. Mycotoxins were extracted with 80 mL of a acetone/water
323 mixture (85:15). The mixture was shaken in a horizontal shaker for 30 min. The obtained extract
324 was then filtered through Whatman No. 1 folded filter paper (Whatman, Maidstone, UK). A volume
325 of 5 mL of the filtered extract was diluted in 100 mL of phosphate buffer saline (PBS) and then
326 filtered through a glass microfiber filter (Whatman). The pH of the filtered extract was measured
327 and adjusted to 7.0 with 0.1 mol/L NaOH. A volume of 20 mL of diluted extract was placed in an
328 AflaTest TM immunoaffinity column (IAC) by means of gravity. Elution of the mycotoxins was
329 performed by adding a 0.5 mL portion of methanol, which was followed by the addition of a second
330 1 mL portion of methanol after 1 min. The eluted extract was evaporated in a Speed Vac
331 Concentrator (Savant Technologies, Rockville, MD, USA) at 45°C. The aflatoxins were derivatised
332 by adding 500 µL of trifluoroacetic acid (TFA) at 70°C for 1 h. The TFA was evaporated in a Speed
333 Vac Concentrator, and the pellet was reconstituted with 500 µL of the HPLC mobile phase. Sample
334 extracts were stored at −20°C until HPLC analysis.~~ The HPLC apparatus consisted of a Dionex
335 P680 pump (Dionex, Sunnyvale, CA, USA) equipped with a Rheodyne Model 7725i injection valve
336 (Rheodyne, Rohnert Park, CA, USA), a Dionex RF-2000 fluorimetric detector (λex = 365 nm, λem
337 = 435 nm for AFB_1, AFB_2, AFG_1, AFG_2), a Dionex TCC-100 thermostatted column compartment
338 and a Chromeleon®6 data handling system (Dionex). The analytical column was a ProdigyODS 2
339 (150×4.6 mm, 5 µm particles) (Phenomenex, Torrance, CA, USA), which was preceded by a
340 SecurityGuard (Phenomenex) guard column. ~~Twenty microliters of reconstituted extract was
341 injected into the chromatographic system through a full loop injection system. The system was run
342 isocratically with a mobile phase containing water acetonitrile methanol (60:20:30, v/v/v), at a
343 flow rate of 1 mL/min. The linearity of the working standard solutions was determined by
344 conducting two analyses of five concentration levels between 0.1 and 0.5 ng/mL for AFB_1 and~~

345 ~~AFG_1, and between 0.025 and 0.125 ng/mL for AFB_2 and AFG_2. The detection limits (LODs) were~~

346 ~~determined from the spiked samples, on the basis of signal-to-noise ratios of 3:1.~~

347

348 ***Statistical Analysis***

349 The microbial counts were log_{10} transformed and were presented on a wet weight basis. The

350 values below the detection limit for yeasts and molds (detection levels: 10 cfu/g of silage) were

351 assigned a value, corresponding to half of the detection limit, in order to calculate the average

352 value.

353 The data were analyzed for their statistical significance, via analysis of variance, with their

354 significance reported at a 0.05 probability level, using the General Linear Model of the Statistical

355 Package for Social Science (v 24.0, SPSS Inc., Chicago, Illinois, USA). Data were analyzed using

356 the presence or absence of *L. buchneri* (LB+ or LB-) and *L. hilgardii* (LH+ or LH-) as fixed factors,

357 with 4 replications. The used statistical model was as follows: $Y_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \varepsilon_{ijk}$, where

358 Y_{ijk} = observation, μ = overall mean, α_i = LB effect (i = presence or absence of LB), β_j = LH effect

359 (j = presence or absence of LH), $\alpha\beta_{ij}$ = LB × LH effect, and ε_{ijk} = error. The measured aflatoxins

360 were pooled together for silo opening, 7 d of air exposure and 14 d of air exposure, and were

361 corrected for the DM losses. An unpaired t-test was used to compare the mean values of the

362 measured aflatoxins and DM loss corrected ones.

363

364 **RESULTS**

365

366 ***Fermentative, Chemical and Microbial Parameters***

367 The chemical and microbial parameters and the AF contamination of the corn forage, prior to

368 ensiling, are summarized in Table 2. The chemical values were typical of corn harvested at the 50%

369 milk-line stage. The DM content was 34% and the NDF, starch and CP were 42.5, 32.3 and 7.8% of

370 DM, respectively. The yeast and mold counts were 6.46 and 5.26 log_{10} cfu/g, respectively. At

371 ensiling, the *A. flavus* count was below the detection limit and the aflatoxins were present with
372 mean values of 0.055, 1.050, 0.095 and 0.044 μg/kg DM for AFB_1, AFB_2, AFG_1 and AFG_2,
373 respectively. Table 3 shows the fermentative parameters after 250 d of conservation at silo opening
374 and after 7 and 14 d of air exposure. All the silages were well fermented with the main acids found
375 at silo opening were lactic and acetic acid, and butyric acid was under the detection limit (0.01 g/kg
376 DM). A slight amount of propionic acid was found in the LB+LH silages. The presence of LB and
377 LH affected the pH, with higher values in the inoculated silages than in the control ones. The LB
378 and LH inocula affected the lactic-to-acetic ratio to a great extent, and this resulted in higher values
379 than 4 in the C silages and values that ranged from 1 and 2 in the treated silages. The LB+LH
380 silages presented about the half of lactic acid and 2.5-fold the acetic acid than the C silage. The 1,2-
381 propanediol was present in all the treated silages and ranged from 7.1 to 12.9 g/kg DM, whereas
382 was not detected in the C silages. After 7 d of air exposure, the lactic acid content decreased and pH
383 increased in all the silages, except for LB+LH treatment. The acetic acid content decreased in C and
384 LH silages, whereas it remained stable in the presence of LB. Ethanol only remained in LB+LH
385 treatment. After 14 days of air exposure, no fermentative products were found in C, LB or LH
386 silages, while 23.4 g/kg DM of lactic acid and 17.9 g/kg DM of acetic acid were found in the
387 LB+LH silages. The aerobic stability, the temperature indices, as observed from silo opening to 14
388 d of air exposure, DM losses, microbial count and aflatoxin concentrations, after 250 d of
389 conservation at silo opening and after 7 and 14 d of air exposure, are reported in Tables 4 and 5. At
390 opening, C silages showed lower DM losses and LAB count than treated silages. The yeast count
391 was under the detection limit in the treatments containing the LB strain. The aerobic stability was
392 affected by the treatments, with the highest value (365 h) in LB+LH silages. At opening, the mold
393 count was below the detection limit or around 1.00 $\log_{10}$ cfu/g in all the silages, and the average *A.*
394 *flavus* count was under the detection limit in 15 out of 16 silage samples. Only one LH sample
395 showed the presence of *A. flavus*, at a level of 1.24 $\log_{10}$ cfu/g. The addition of LB and LH did not
396 affect the AFB_1, AFB_2 or AFG_2 contents, whereas AFG_1 was lower in the treatments containing the

397 LH strains. After 7 d of air exposure, the yeast count increased in all the silages, with the highest
398 value observed in C silage. The addition of LB and LH affected the mold count, with higher value
399 in C than in the treated silages. Aflatoxin was not influenced by the treatments during air exposure,
400 except for AFG_1 after 7 d in C silage. In 14 d air exposed silages, the *A. flavus* count increased in all
401 the treatments and reached 3.32 $\log_{10}$ cfu/g in LB silages. Aflatoxin G_1 was not detected after 14 d
402 of air exposure in any treatment, and the other aflatoxins did not show any significant difference
403 between treatments. After 14 d of air exposure, 2 out of 4 LB+LH samples were still aerobically
404 stable, with the *A. flavus* count below the detection limit. These samples were left to deteriorate and
405 were sampled after 21 and 28 d. The average *A. flavus* count in these samples was 1.33 $\log_{10}$ cfu/g
406 and 2.74 $\log_{10}$ cfu/g after 21 and 28 d, respectively. During air exposure, when the cumulated
407 hourly difference between silage and ambient temperatures (dT) reached 1000°C h, the yeast had
408 completely depleted the fermentative products, regardless of the treatment and LAB inoculum,
409 causing a rise in silage pH, which was greater than 4 (with most of the values ranging from 5.5 to
410 7), and in the silage temperatures, which were around 30 to 40°C (Figure 1). A cumulated 1000°C h
411 dT was reached at different times during aerobic exposure, the lowest being for C silages after 176
412 h and the highest for LB+HB silages after 484 h. When all the inhibiting conditions (pH and the
413 presence of undissociated organic acids) were depleted, the total molds and *A. flavus* quickly started
414 to increase (Figure 2). The DM losses in all the silages increased during exposure to air and reached
415 the highest value in C silage and the lowest in LB+LH silage after 14 d. Since the actual
416 concentration of AF in the silage samples should take into account in the amount of DM losses,
417 Figure 3 reports the AFB_1, AFB_2, AFG_1, and AFG_2 concentrations from ensiling to the end of
418 conservation and after 7 and 14 d of air exposure, for both the potential amount of aflatoxins
419 derived from the field, corrected for DM losses during conservation and air exposure, hypothesizing
420 no new biosynthesis or degradation, and their actual measured contents. The AFB_1 increased during
421 silage conservation and during air exposure, with higher values observed after 14 d of conservation,
422 whereas AFB_2 did not increase significantly during conservation, compared to its increase due to

423 DM losses. On the other hand, the AFG_1 content decreased during conservation and the subsequent
424 exposure to air. Aflatoxin G_2 was not affected by the ensiling process or aerobic deterioration, even
425 though it apparently increased when the DM losses were not taken into account.

426 The nutritional analyses after 250 d of conservation at silo opening and after 7 and 14 d of air
427 exposure are reported in Table 6. At opening, no differences between treatments were found among
428 the studied nutritional parameters. After 7 day of air exposure, a slight concentration of the starch
429 was observed in C, LB, and LH silages, as well as a reduction in NDF. The crude protein increased
430 after 14 d of exposure to air. After 1000°C h, when mold activity was evident, the starch content,
431 corrected for DM losses, decreased greatly, until reaching values below 10% on DM (Figure 1).
432 After 14 d of exposure to air, a concentration of NDF, ADF, hemicelluloses, CP and ash was
433 observed, and this was more evident in C, LB and LH silages than in LB+LH silages.

434

435 ***Strain Identification, Macro-Morphology and Sclerotia Production***

436 A representative subsample of the isolated strains of *Aspergillus* section *Flavi* obtained from
437 silage at opening, after 7 d of air exposure and after 14 d of air exposure, was used for the analysis.
438 A dataset of 532 bp for the partial sequence of the calmodulin gene was obtained for each strain.
439 The majority of isolates (14/20) resulted to belong to the *A. flavus* species, while the other isolates
440 (6/20) were identified as *A. oryzae* var. *effusus,* and they showed 100% identity with the RefSeq
441 deposit in GenBank (Table 1). A second subsample of *Aspergillus* section *Flavi,* obtained from
442 silage after 21 d of exposure to air (4 isolates) and after 28 d of exposure to air (4 isolates), was
443 used for the analysis (data not shown). All 4 isolates from the LB+LH samples at 21 d of exposure
444 to air belonged to the *A. oryzae* var. *effusus* species (9026/1, 9026/2, 9026/3 and 9026/4). After 28 d
445 of exposure to air, 2 out of 4 isolates were identified as *A. flavus* (9031/1 and 9031/2) and 2 as *A.*
446 *oryzae* var. *effusus* (9031/3 and 9031/4).

447 The typical morphology of *A. section Flavi* was observed, with yellow to green conidia, as
448 well as a mean diameter of 40.7, 37.6 and 35.0 mm for YES, MEA and CYA, respectively. The *A.*

449 *oryzae* var. *effusus* strains showed poor sporulation, with a white surface and irregular margins, as
450 well as a mean diameter of 40.9, 37.5 and 35.2 mm for YES, MEA and CYA, respectively (Table
451 1).

452 Nine out of fourteen *A. flavus* produced sclerotia, while none of the strains of *A. oryzae* var.
453 *effusus* were able to produce them (Table 1). The 8931/2, 9010 and 9016 strains produced both S
454 and L sclerotia, the 8931/7, 9015/1 and 9015/2 strains produced L sclerotia, while the 9002, 9005
455 and 9006 strains produced S sclerotia. The 8959, 8931/3, 9012 and 9015/3 strains did not produce
456 any sclerotia.

457

458 ***Aflatoxin Production in vitro and Aflatoxin Gene Detection***

459 After 14 d of incubation at 35°C in the dark on YES, 6 out 14 *A. flavus* were able to produce
460 AFB_1, whereas not one of the *A. oryzae* var. *effusus* strains seemed to be able to produce them
461 (Table 1). Quadruplex PCR for gene detection produced the expected size amplicons in the positive
462 control, with around 1000 bp, 800 bp, 500 bp and 400 bp for the *aflR*, *omt-A*, *ver-1* and *nor-1* genes,
463 respectively (Figure 4). All the aflatoxigenic *A. flavus* strains showed a complete molecular pattern
464 with the four analyzed genes. The non-aflatoxigenic *A. flavus* strains showed different banding
465 pattern results. No DNA amplification was found for the *A. flavus* 8959, 9015/1, 9015/2 and 9015/3
466 strains for any of the genes. As far as the *A. oryzae* var. *effusus* strains are concerned, quadruplex
467 PCR showed three banding patterns: strains 8976, 8931/1 and 8931/4 with 2 bands, corresponding
468 to the *ver-1* and *nor-1* genes, a second group with three bands corresponding to *aflR*, *ver-1* and *nor-*
469 *1* amplification (8931/5 and 8931/6 strains) and one strain (9004) with the complete pattern.

470

471 **DISCUSSION**

472

473 In light of the potential risk of mycotoxin contamination, it has been considered important to
474 obtain information about the type and distribution of AF in corn silage, which is the main source of

475 forage for lactating dairy cows in Europe and North America. Furthermore, a possible effect of
476 different LAB inocula on the growth of spoilage molds has also been evaluated. The longer the
477 anaerobic phase of silage is, the greater the reduction in yeast and mold counts (Borreani et al.,
478 2014~~; Ferrero et al., 2018~~). This implicates that silage is not a favorable environment for mold
479 development during conservation, if anaerobiosis is maintained (Borreani et al., 2018). In the
480 present experiment, the presence of *A. flavus* has been found to be below the detection limit (< 1.00
481 $\log_{10}$ cfu/g) in the fresh forage prior to ensiling, in which the mold count was observed to be higher
482 than 5 $\log_{10}$ cfu/g. At silo opening, after a long ensiling period (250 d), the mold count was around
483 or below 1.00 $\log_{10}$ cfu/g of silage, whereas *A. flavus* was found in 1 out of 16 silage samples (1.24
484 $\log_{10}$ cfu/g, which represents around 45% of the total molds of that sample). This could suggest that
485 *A. flavus* was already present in the field and that it survived after the anaerobic conservation
486 period, whereas many other mold species did not. This hypothesis is also supported by the presence
487 of AF in the herbage prior to ensiling, which means that *A. flavus* must have developed on the crop
488 during the growing cycle, albeit at a low level, and synthesized AF. Over the last decade, the Po
489 plain environment has been characterized by warmer and drier summers that in the previous
490 decades, and these conditions could have favored the development of *A. flavus* on the corn crops
491 and AF synthesis. This could explain the increased frequency of AFB_1 contamination observed on
492 corn grain and silages in the last few years (Decastelli et al., 2007; Anfossi et al., 2009). The
493 presence of *A. flavus* on corn crops at a low level, at our latitudes, could be explained by the
494 occurrence, of some periods with higher ambient temperatures than 35°C in summer, as this is the
495 optimal growth temperature for the fungus, and these increased temperatures generally cause it to
496 be the predominant species in tropical and subtropical climates (Cheli et al., 2013). In this regard,
497 Gonzales and Pereira (2008) found *A. flavus* as the predominant species, followed by *A. fumigatus*
498 and *A. niger*, in different farm corn silages in the tropical environment of Central Argentina. Keller
499 et al. (2013) found *A. flavus* as the predominant *Aspergillus* species, at both ensiling and at silo
500 opening, in Brazil and El-Shanawany et al. (2005), in a farm survey, found *A. flavus* as the

501 dominant species in corn silage in Egypt. On the other hand, Garon et al. (2006) and Spadaro et al.
502 (2015) did not find *A. flavus* in corn silage in the temperate climates of France or northern Italy, but
503 instead reported the presence of *A. parasiticus* and *A. fumigatus*. However, Richard et al. (2007)
504 detected *A. flavus* in farm corn silage in France after eleven months of conservation. This indicates
505 that the fungus is able to survive during the anaerobic conservation phase of corn silage, albeit at a
506 low level, and could grow when the environmental conditions become more suitable for its growth,
507 such as during the feed-out phase, or in peripheral areas of the silage, where temperatures increase
508 as a consequence of a deteriorating microflora activity, which depletes the acidic conditions.

509 In the present experiment, even though *A. flavus* was below the detection limit in the fresh
510 forage prior to ensiling and it was present at a low level at silage opening, aflatoxins were found in
511 both the fresh forage and at opening in all the samples, with a predominance of AFB_2. The presence
512 of AF has been found, at silo opening, in different countries of the world, as reviewed by Alonso et
513 al. (2013), with higher concentrations in warmer climates (Carvalho et al., 2016; Ogunade et al.,
514 2018) than in colder ones (Driehuis et al., 2008; Cavallarin et al., 2011).

515 In our experiment, the inoculation with heterolactic bacteria, which was aimed at improving
516 the aerobic stability of silage during the feed-out phase, and the long ensiling duration (250 d)
517 influenced the fermentative profile of the silages, with a dominant homolactic fermentation (higher
518 lactic-to-acetic ratio than 4) in the control silages and a heterolactic fermentation (lower lactic-to-
519 acetic ratio than 2) in LB and LH treated silages, as previously reported by Kleinschmit and Kung
520 (2006)~~ and Ferrero et al. (2018)~~. The 1,2-propanediol was found in LB silages, as previously
521 reported by Oude Elferink et al. (2001), and in LH silages, in agreement with the results of Assis et
522 al. (2014). The fermentation process, combined with the longer ensiling duration, greatly reduced
523 the yeast and mold count compared to those observed at harvesting. Furthermore, the inocula
524 containing LB determined a reduction in yeast to below the detection limit, as reported by
525 Kleinschmit and Kung (2006). The use of heterolactic inocula determines greater DM losses during
526 fermentation, and greater DM losses could determine a higher concentration of non-degraded

527 components (e.g. ashes). Therefore, in order to obtain a better understanding of the fate of the
528 different aflatoxins in silage, due to the fact that they could be produced both in the field and during
529 ensiling, and at the same time could be degraded or bound by LAB microbial activity (Oluwafemi
530 et al., 2010) or bound by other silage bacteria (Ma et al., 2017), it is important to keep in mind that
531 they could concentrate in silage as a consequence of occurring DM losses. This is evident in Figure
532 3, where it appears that the AFB_1 increased slightly during the fermentation process and increased
533 significantly at 7 and 14 d of exposure to air. Aflatoxin B_2 and AFG_2 were not affected by the
534 anaerobic fermentation phase or by the subsequent exposure to air, and their higher concentration in
535 deteriorated silages could mainly be attributed to the DM losses. On the other hand, AFG_1 was
536 partially degraded during the ensiling process and almost completely disappeared after 14 d of air
537 exposure. Cavallarin et al. (2011) analyzed the presence of AF in both fresh forage and after
538 ensiling in silage stored under different plastic films and, during feed out, they found that AF were
539 absent in the center of the bunker silos, while they were present with values of up to 6 μg/kg DM in
540 the top layer of the bunker. In contrast to the results of the present study and those of Cavallarin et
541 al. (2011), Garon et al. (2006) observed, in farm-scale silos, a decrease in the AFB_1 content as the
542 ensiling duration increased. This could be attributable to a detoxification effect during fermentation,
543 as reported by Oluwafemi et al. (2010), who observed that some strains of LAB are able to partially
544 degrade AFB_1 in corn grain via a biological pathway, with a reduction range from 31 to 46%.
545 Ahlberg et al. (2015), reviewing the ability of different LAB species and strains to bind aflatoxins in
546 different food matrices, reported binding effects ranging from 0 to 90%, but this review did not
547 report any research results concerning inoculation trials with *L. buchneri* or *L. hilgardii*. Ma et al.
548 (2017) reported the capacity of binding AFB1 in vitro by some strains of lactic acid bacteria when
549 applied at 10^9 cfu/mL (*L. plantarum*, *L. buchneri*, and *Pediococcus acidilactici*), but they failed to
550 find the same effect on corn silage artificially contaminated with AFB_1, even if they concluded that
551 some silage bacteria could have reduced the AFB_1 to a safe content within 3 d of ensiling,
552 regardless of LAB inoculation. In the present experiment, even if the conservation period was 250 d

553 long, aflatoxin concentrations did not decrease compared to aflatoxin present in the fresh forage.
554 ~~Cavallarin et al. (2011) analyzed the presence of AF in both fresh forage and after ensiling in silage~~
555 ~~stored under different plastic films and treated with different LAB inocula. The authors found that,~~
556 ~~at silo opening, AF were absent in the treated silages and in the center of the bunker silos, while~~
557 ~~they were present with values of up to 6 ppb in the top layer of the bunker. In the same experiment,~~
558 ~~Cavallarin et al. (2011) also provided evidence of aflatoxin accumulation in whole crop corn silage~~
559 ~~as a result of aerobic deterioration, which had previously been hypothesized by other authors, who~~
560 ~~had found higher contaminations of AF in peripheral areas of commercial silos throughout the~~
561 ~~world (Rosiles, 1978; Richard et al., 2009).~~
562 One of the most relevant issues of corn silage at a world level is its proneness to aerobic
563 deterioration, which negatively influences the silage nutritive value, animal performance, and farm
564 profitability (Borreani et al., 2018). In order to better understand the role of air on the reduction of
565 the silage nutritional value and on the increase in DM losses during aerobic deterioration, silages
566 were left to deteriorate in laboratory trials for up to 14 d. This time period was chosen to represent
567 the average age of silages in the peripheral areas of farm bunker silos at risk to exposure to air when
568 a feed-out rate of 0.7 to 1.4 m/wk is adopted (Borreani and Tabacco, 2008; Weinberg et al., 2009),
569 which results in a potential aerobic exposure of the silage of 20 to 10 d. In our experiment, the
570 inocula improved aerobic stability compared to the control, and this is in agreement with previous
571 studies about the efficiency of *L. buchneri* (Arriola et al., 2011; Comino et al., 2014) and *L.*
572 *hilgardii* (Assis et al., 2014). In agreement with the findings reported by Tabacco et al. (2011), the
573 deteriorated silages of the present study showed an increase in the yeast and mold counts and a
574 reduction of their nutritive value, with a substantial decrease in starch and an increase in the fiber
575 and ash concentrations.
576 At the beginning of aerobic deterioration, the mold count values were close to those observed
577 at silo opening, whereas the yeasts increased to higher values than 5 $\log_{10}$ cfu/g; the developing
578 yeast depleted the fermentative products (first the ethanol and then the acetic and lactic acid) and

579 determined an increase in the pH and silage temperature (Figure 1). Irrespective of the treatment,
580 when the hourly cumulated temperature reached 1000°C, all the inhibiting conditions for mold
581 growth (undissociated organic acids) were depleted, and the mean pH was 6.19 ± 0.62. At the same
582 time, the silage temperature increased to over 35°C (with an average value of 38.7 ± 6.3°C). These
583 lowered inhibiting conditions allowed the growth of both the total molds and *A. flavus*, which
584 reached higher values than 8.00 and 4.00 $\log_{10}$ cfu/g, respectively. Those conditions were observed
585 in both the control and LAB treated silages after about 70 hours from the time at which the
586 temperature started to rise above the ambient temperature (data not shown), with LB and LH treated
587 silage showing a longer period of aerobic stability. This means that all the silages (both the control
588 and LAB inoculated ones) are prone to the development of *A. flavus* during their exposure to air,
589 and this development takes place when silage stability has already been compromised (pH and
590 temperature higher than 5 and 35°C, respectively). Cavallarin et al. (2011) provided evidence of
591 aflatoxin accumulation in whole crop corn silage as a result of aerobic deterioration, which had
592 previously been hypothesized by other authors throughout the world, who had found higher
593 contaminations of AF in peripheral areas of commercial silos, which are known to be the most
594 prone to aerobic deterioration (Rosiles, 1978; Richard et al., 2009). The results of this experiment
595 have also shown that the use of LAB inocula, which are able to shift silage fermentation toward a
596 more heterolactic pathway, could delay the onset of aerobic deterioration after exposure to air of the
597 silage, and, as a consequence, could stave off *A. flavus* development and aflatoxin synthesis. Results
598 are in agreement with data of Queiroz et al. (2012) who found that when corn infested with southern
599 rust was inoculated with a mixture of *Pediococcus pentosaceus* and *L. buchneri* at ensiling, its
600 aerobic stability increased and the production of aflatoxins was prevented compared to an untreated
601 control. Results are also in agreement with Cavallarin et al. (2011) who reported that inoculation
602 with *L. buchneri* delayed the onset of aerobic deterioration and the synthesis of aflatoxins. In the
603 present experiment we also observed, as previously reported by Cavallarin et al. (2011) that, when

604 deterioration have took place, the total aflatoxin concentration in *L. buchneri* treated silages
605 increased more than in the control or in *L. plantarum* inoculated silage.

606 The environment resulting from aerobic deterioration allowed *A. flavus* to grow and AFB_{1},
607 which was *ex-novo* produced during ensiling and air exposure, to increase. In order to verify the
608 aflatoxigenic potential of the selected colonies of *A. flavus,* four genes involved in the aflatoxin
609 biosynthesis pathway were analyzed through a quadrupex PCR, and an *in vitro* assay was
610 performed. Nine out of 14 strains of *A. flavus* showed the presence of the complete gene pattern
611 and, of these strains, 6 were able to produce aflatoxins. According to Criseo et al. (2001), some
612 atoxigenic strains could have a quadruplet pattern, and other markers are necessary to discriminate
613 aflatoxigenic strains from atoxigenic ones. Furthermore, the occurrence of S sclerotia was positively
614 related to the production of AF *in vitro*, as previously reported by Cotty (1989). On the other hand,
615 some *A. flavus* strains isolated from the corn silage of this study did not reveal the presence of the
616 complete AF gene pattern and were unable to produce AF *in vitro*. This observation could be a
617 starting point for future investigations on the selection of atoxigenic *A. flavus* to occupy the same
618 niche as the naturally occurring epiphytic toxigenic *A. flavus*, as recently proposed as a strategy to
619 reduce AF contamination in the field (Mauro et al., 2018).

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

625

626 **CONCLUSIONS**

627 Ensiling corn that was naturally contaminated by aflatoxin did not change its aflatoxin
628 concentration at the end of a long conservation period. *A. flavus* showed to be able to survive in the
629 anaerobic silage and revive when the inhibiting conditions in terms of low pH and temperature were

630 lost. Both the control and inoculated silages deteriorated during aerobic exposure and showed an
631 increase in the *A. flavus* count and AFB_1 concentration. Inoculation with LB and LB+LH increased
632 the aerobic stability of the silages and delayed the onset of aerobic microbial degradation, and this
633 in turn could indirectly reduce the risk of *A. flavus* outgrowth and AFB_1 production after silage
634 opening.

635

636 **ACKNOWLEDGMENTS**

637

638 Mention of trade names or commercial products in this article is solely for the purpose of
639 providing specific information, and does not imply either recommendation or endorsement by the
640 University of Turin, Italy.

641 This work was supported by Fondazione Cassa di Risparmio di Cuneo (CRC), Project
642 "SOST-MILK– Plant health emergencies of corn and sustainability of the Piedmontese milk chain"
643 Bando Ricerca Scientifica 2015 and by a grant (Agreement 004036) from Danstar Ferment AG
644 (Zug, Switzerland). The authors would also like to thank Mauro and Luciano Comino (Lembo Farm
645 S.S., Rocca de'Baldi, CN, Italy) for providing the corn silages used in this study; and Ilenia
646 Siciliano (Agroinnova, University of Turin, Italy) for the helping in strains characterization of *A.*
647 *flavus*.

648

649

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837 **Figure Captions**

838 **Figure 1.** Scatter plot of fermentative products in relation to hourly accumulated temperature rise
839 (°C·h) above the ambient temperature over air exposure, (A); of pH in relation to the hourly
840 accumulated temperature rise (°C·h) above the ambient temperature over air exposure, (B); dry
841 matter losses corrected starch in relation to the hourly accumulated temperature rise (°C·h) above
842 the ambient temperature over air exposure, (C); and silage temperature in relation to the hourly
843 accumulated temperature rise (°C·h) above the ambient temperature over air exposure, (D).

844
845 **Figure 2.** Scatter plot between the total mold and *Aspergillus flavus* counts and the hourly
846 accumulated temperature rise (°C·h) above the ambient temperature over air exposure.

847
848 **Figure 3.** Concentration of AFB_1, AFB_2, AFG_1, and AFG_2 from ensiling to end of conservation and
849 after 7 and 14 d of air exposure;~~, corrected for the DM losses due to fermentation and aerobic~~
850 ~~deterioration of the~~ data from different treatments are pooled together. In grey the potential amount
851 of aflatoxins derived from the field corrected for DM losses hypothesizing no new biosynthesis or
852 degradation; in black the actual measured content. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS = not
853 significant.

854
855 **Figure 4.** Agarose gel electrophoresis of quadruplex PCR products for strains used in this study.
856 Lane M: molecular marker 1kb Plus Ladder (Quiagen); Lane 1: *A. flavus* 8959; Lane 2: *A. oryzae*
857 var. *effusus* 8976; Lane 3: *A. oryzae* var. *effusus* 8931/1; Lane 4: *A. flavus* 8931/2; Lane 5: *A. flavus*
858 8931/3; Lane 6: *A. oryzae* var. *effusus* 8931/4; Lane 7: *A. oryzae* var. *effusus* 8931/5; Lane 8: *A.*
859 *oryzae* var. *effusus* 8931/6; Lane 9: *A. flavus* 8931/7; Lane 10: *A. flavus* 9002; Lane 11: *A. oryzae*
860 var. *effusus* 9004; Lane 12: *A. flavus* 9005; Lane 13: *A. flavus* 9006; Lane 14: *A. flavus* 9010; Lane
861 15: *A. flavus* 9011; Lane 16: *A. flavus* 9012; Lane 17: *A. flavus* 9015/1; Lane 18: *A. flavus* 9015/2;
862 Lane 19: *A. flavus* 9015/3; Lane 20: *A. flavus* 9016; Lane 21: Negative control; Lane 22: Positive
863 control.

864 **Tables**

865 **Table 1.** Sampling time, accession numbers of the calmodulin sequences, growth on YES, CYA and MEA media, sclerotia production and AF

866 genes pattern 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]
								aflR	*omtA*	*ver-1*	*nor-1*	µg/kg DM
8931/2	KY886401	*A. flavus*	Silo opening	4.17	3.57	3.27	S and L	+	+	+	+	++
8931/3	KY886402	*A. flavus*	Silo opening	3.90	3.80	3.38	-	+	+	+	+	-
8931/7	KY886381	*A. flavus*	Silo opening	4.13	3.97	3.53	L	+	+	+	+	-
8959	KY886377	*A. flavus*	7 d air exposure	4.20	3.77	3.60	-	-	-	-	-	-
9002	KY886382	*A. flavus*	14 d air exposure	4.13	3.53	3.63	S	+	+	+	+	+
9005	KY886383	*A. flavus*	14 d air exposure	4.03	3.77	3.33	S	+	+	+	+	++
9006	KY886384	*A. flavus*	14 d air exposure	4.10	3.60	3.44	S	+	+	+	+	++
9010	KY886385	*A. flavus*	14 d air exposure	4.33	3.70	3.61	S and L	+	+	+	+	-
9011	KY886386	*A. flavus*	14 d air exposure	4.17	3.87	3.52	-	+	+	+	+	wk
9012	KY886387	*A. flavus*	14 d air exposure	4.03	3.83	3.56	-	+	-	+	+	-
9015/1	KY886378	*A. flavus*	14 d air exposure	4.00	3.93	3.54	L	-	-	-	-	-
9015/2	KY886379	*A. flavus*	14 d air exposure	3.67	3.87	3.56	L	-	-	-	-	-
9015/3	KY886380	*A. flavus*	14 d air exposure	3.93	3.73	3.60	-	-	-	-	-	-
9016	KY886388	*A. flavus*	14 d air exposure	4.17	3.70	3.41	S and L	+	+	+	+	+

8931/1	KY886389	*A. oryzae* var. *effusus*	Silo opening	4.20	3.77	3.30	-	-	-	+	+	-
8931/4	KY886390	*A. oryzae* var. *effusus*	Silo opening	3.93	3.83	3.60	-	-	-	+	+	-
8931/5	KY886391	*A. oryzae* var. *effusus*	Silo opening	4.13	3.83	3.67	-	+	-	+	+	-
8931/6	KY886392	*A. oryzae* var. *effusus*	Silo opening	4.13	3.67	3.47	-	+	-	+	+	-
8976	KY886393	*A. oryzae* var. *effusus*	7 d air exposure	4.13	3.63	3.62	-	-	-	+	+	-
9004	KY886394	*A. oryzae* var. *effusus*	14 d air exposure	4.00	3.77	3.47	-	+	+	+	+	-

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

[2] Sclerotia production on CYA after 14 days 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.

[4] WK < 100 µg/kg DM; + = 101-1000 µg/kg DM; ++ > 1001 µg/kg DM.

873 **Table 2.** Chemical and microbiological characteristics of the herbage prior to ensiling

Parameters[1]	Value		SD
DM, %	34.0	±	1.94
pH	5.80	±	0.32
Buffering capacity, mEq kg/DM	56	±	9.31
Water activity (a_w)	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
A. flavus, $\log_{10}$ cfu/g	<1.00		-
Aflatoxin B_1, µg/kg DM	0.055	±	0.077
Aflatoxin B_2, µg/kg DM	1.050	±	1.498
Aflatoxin G_1, µg/kg DM	0.095	±	0.147
Aflatoxin G_2, µg/kg DM	0.044	±	0.059

874 [1] Values represent the average of 16 replications and the standard deviation. ADF = acid detergent
875 fiber; CP = crude protein; DM = dry matter; NDF = neutral detergent fiber; NH_3-N = ammonia
876 nitrogen; 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.

		DM[1] (%)	DM corrected (%)	pH	NH_3-N (g/kg DM)	Lactic acid (g/kg DM)	Acetic acid (g/kg DM)	Lactic-to-acetic ratio	Propionic acid (g/kg DM)	1,2-Propanediol (g/kg DM)	Ethanol (g/kg 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
LB[2]		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	-
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	-	-	-	-
LB		NS	0.17	NS	-	-	*	-	-	-	-
LH		*	0.02	NS	-	-	NS	-	-	-	-
*LB*LH*		*	0.02	**	-	-	NS	-	-	-	-

[1] C = control; DM = dry matter; LB = *L. buchneri*; LH = *L. hilgardii*; NH_3-N = ammonia nitrogen; SEM = standard error of the mean.

[2] LB = effect of LB; LH = effect of LH; LB+LH = interaction of LB and LH. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; NS = not significant.

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

		Aerobic stability 2°C (h)	Peak temperature[2] (°C)	Interval to peak temperature[2] (h)	Interval to 35°C[2] (h)	Interval with temp. >35°C [2] (h)
Opening						
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
LB[3]		*	*	***	***	***
LH		*	*	***	***	*
*LB*LH*		NS	NS	*	**	*

881 [1] C = control; LB = *L. buchneri*; LH = *L. hilgardii*; SEM = standard error of the mean.

882 [2] as observed from silo opening to 14 d of air exposure.

883 [3] LB = effect of LB; LH = effect of LH; LB+LH = interaction of LB and LH. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS = not significant.

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885

886 **Table 5.** Microbial count, DM losses, *Aspergillus flavus* count and aflatoxin B_1, B_2, G1, and G_2 of corn silage at opening (after 250 d) and after 7

887 and 14 d of air exposure.

		LAB[1]	Yeast	Mold	DM losses	*Aspergillus flavus*	Samples with *A. flavus*	Aflatoxin B_1	Aflatoxin B_2	Aflatoxin G_1	Aflatoxin G_2
		$\log_{10}$ cfu/g	$\log_{10}$ cfu/g	$\log_{10}$ cfu/g	% of DM	$\log_{10}$ cfu/g		µg/kg DM	µg/kg DM	µg/kg DM	µg/kg 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
LB[2]		***	***	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
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
LB		-	**	*	**	NS	-	NS	NS	-	NS
LH		-	NS	*	*	NS	-	NS	NS	-	NS
*LB*LH*		-	NS	NS	NS	NS	-	NS	NS	-	NS

888 [1] C = control; DM = dry matter; LAB = lactic acid bacteria; LB = *L. buchneri*; LH = *L. hilgardii*; SEM = standard error of the mean.

889 [2] LB = effect of LB; LH = effect of LH; LB+LH = interaction of LB and LH. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS = not significant.

890

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

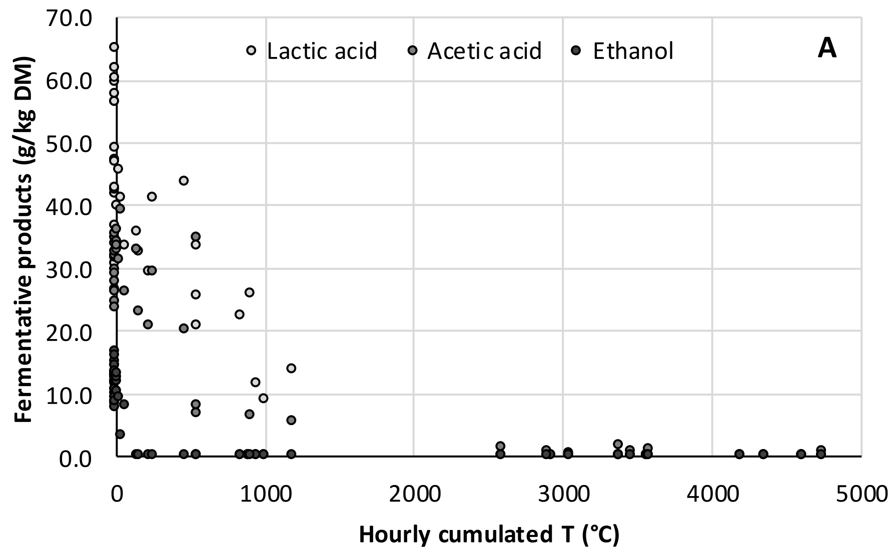
		Starch	NDF[1]	ADF	Hemicelluloses	CP	Ash
		% of DM	% of DM	% of DM	% of DM	% of DM	% of DM
Opening							
C	(LB- LH-)	34.6	37.0	19.4	17.6	8.5	4.66
LB	(LB+ LH-)	33.3	39.4	21.1	18.3	8.5	5.16
LH	(LB- LH+)	33.1	39.2	20.9	18.3	8.8	5.14
LB+LH	(LB+ LH+)	35.0	38.7	20.5	18.2	8.5	5.02
SEM		0.94	1.275	0.81	0.19	0.495	0.158
LB[2]		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
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
LB		*	*	**	***	NS	NS
LH		*	**	**	*	NS	NS
*LB*LH*		NS	NS	NS	NS	NS	NS

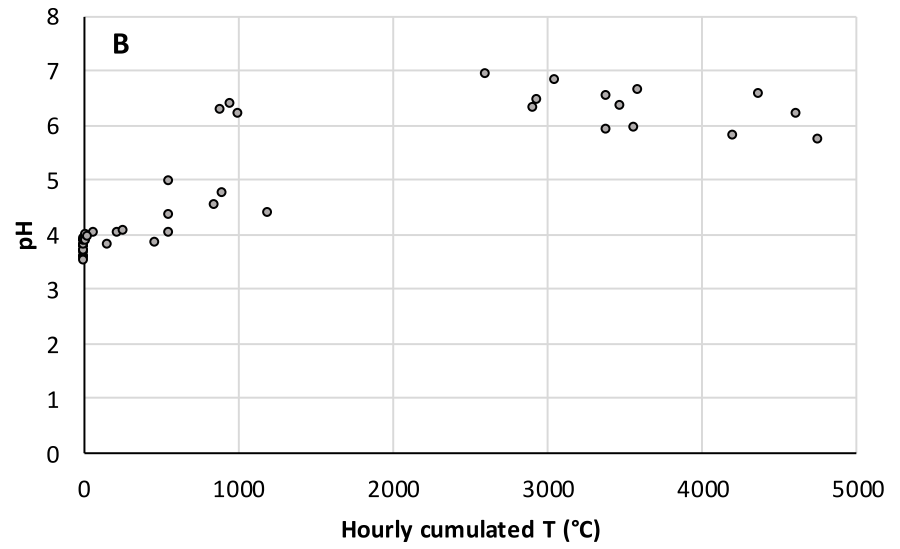
892 [1] ADF = acid detergent fiber; C = control; CP = crude protein; DM = dry matter; LB = *L. buchneri*; LH = *L. hilgardii*; NDF = neutral detergent

893 fiber; SEM = standard error of the mean.

894 [2] LB = effect of LB; LH = effect of LH; LB+LH = interaction of LB and LH. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS = not significant.

895





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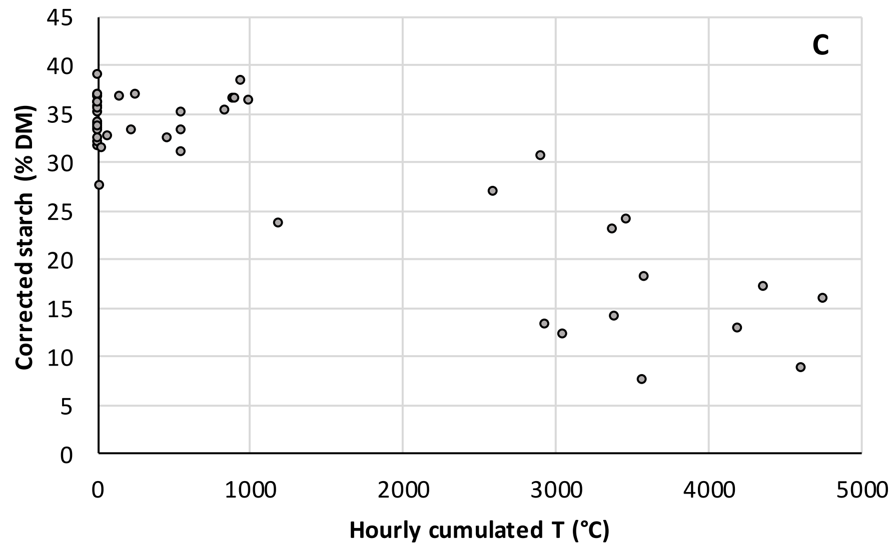

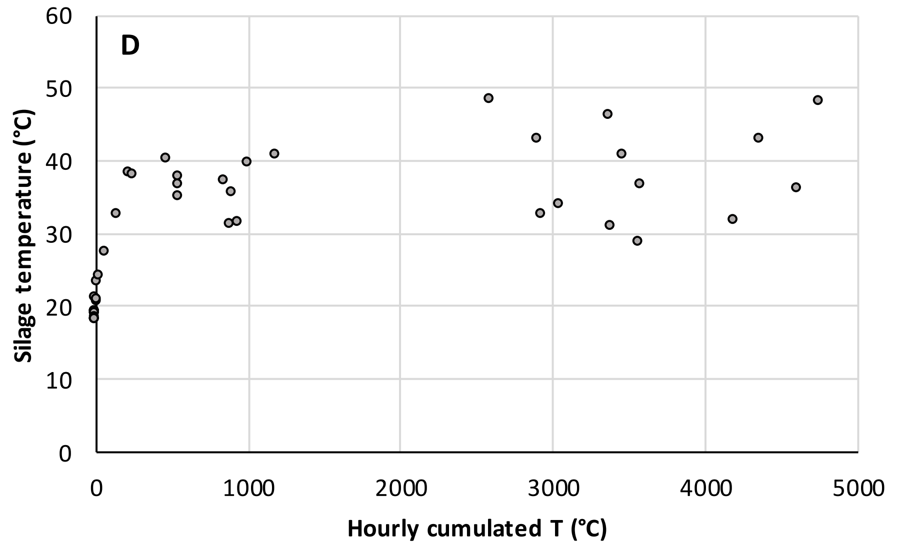

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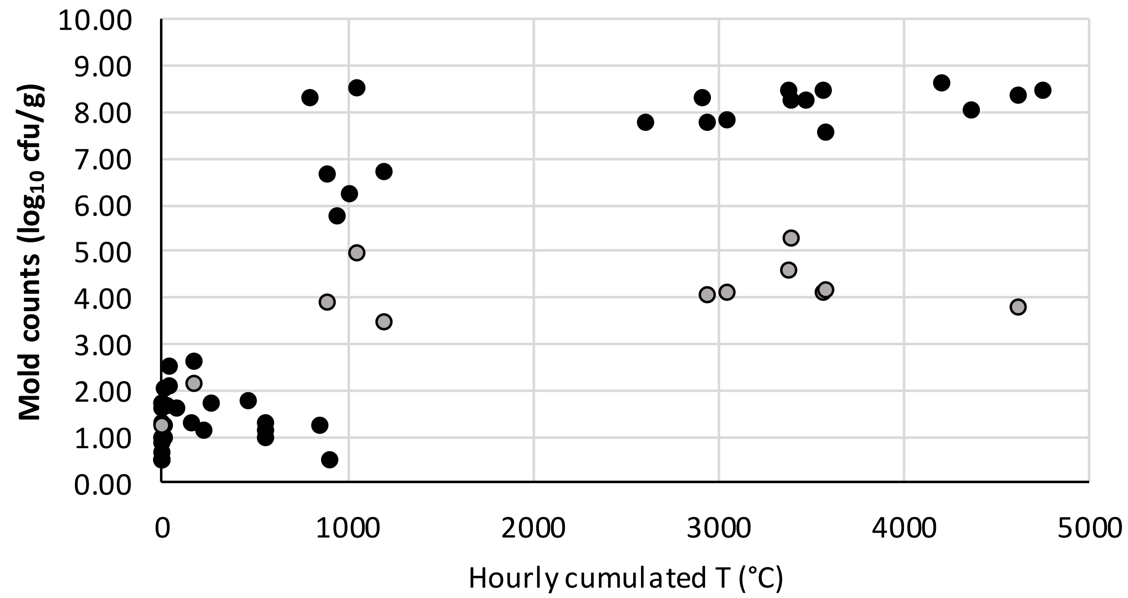
898 **Ferrero – Figure 1**



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901 **Ferrero – Figure 2**

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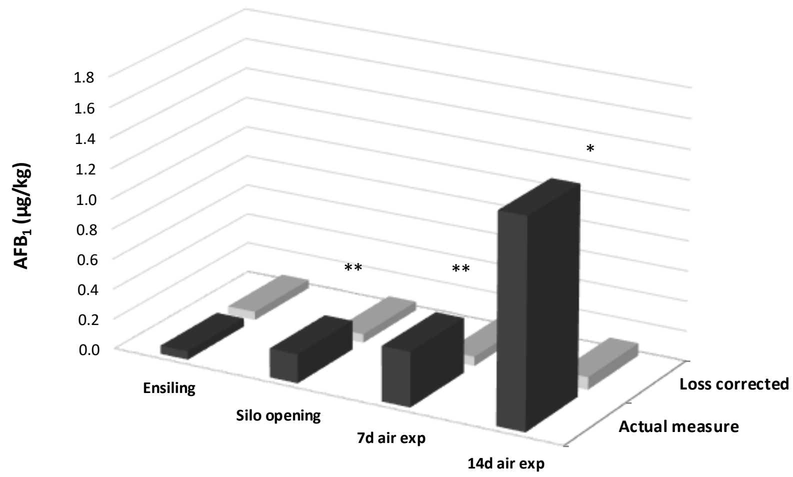

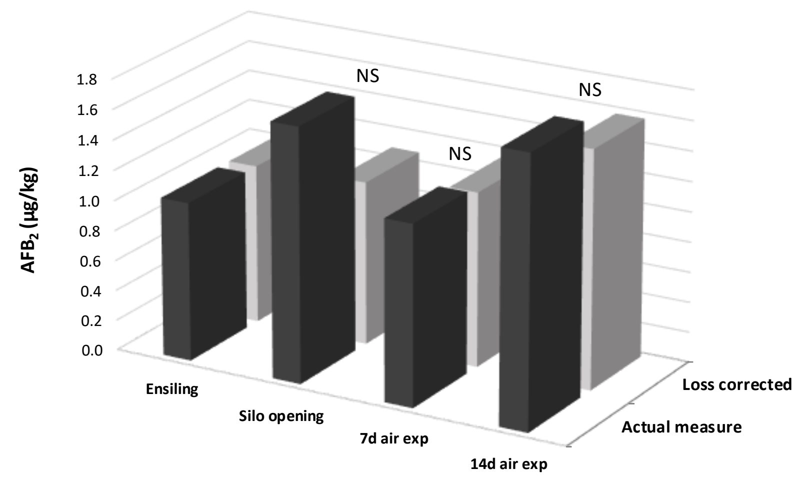

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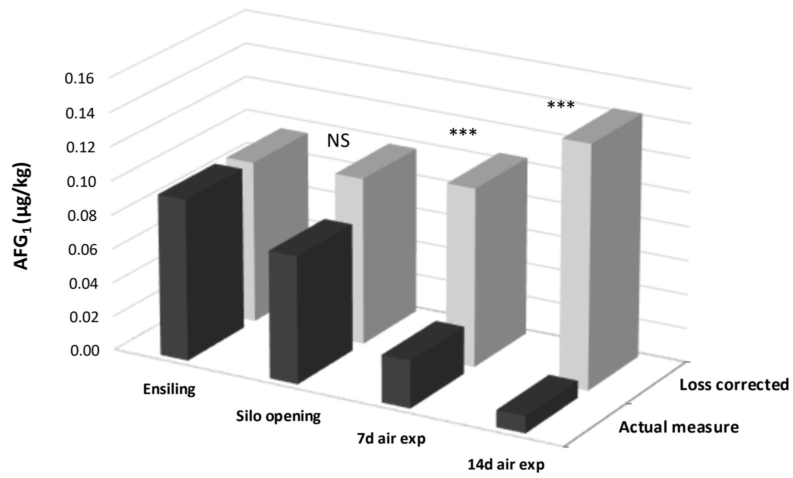

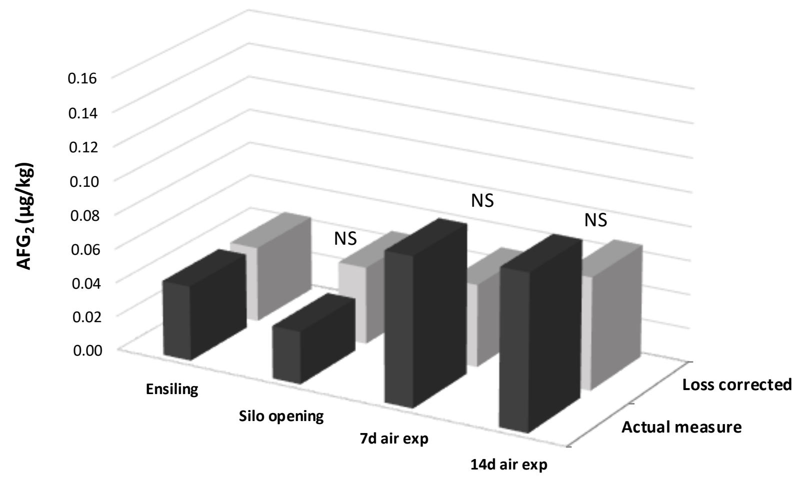

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905 **Ferrero - Figure 3**

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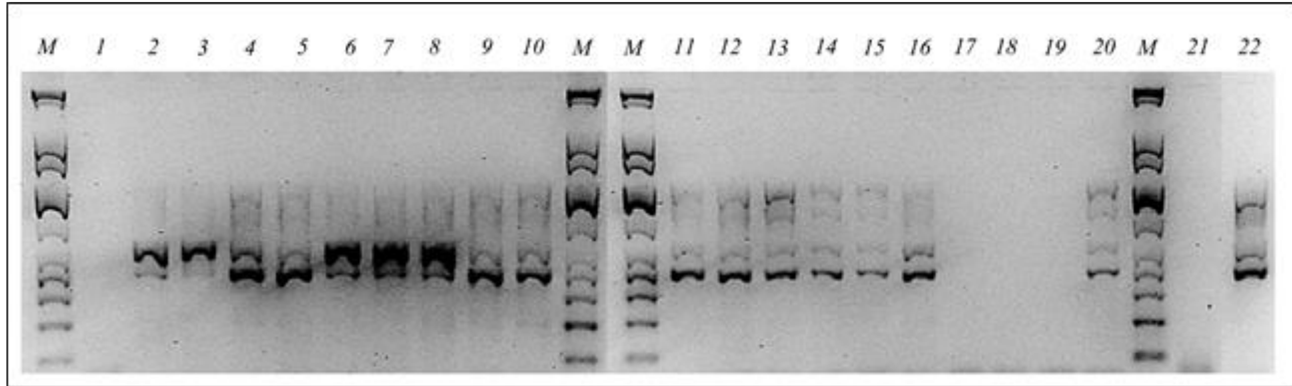

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909 **Ferrero – Figure 4**

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911 **APPENDIX**

912

913 **Supplementary Table S1.** List of primers used for quadruplex PCR amplification.

Primer name	Target gene	Sequences (5'-3')	Amplicon size (bp)
aflR-1	*aflR*	TATCTCCCCCCGGGCATCTCCCGG	1032
aflR-2		CCGTCAGACAGCCACTGGACACGG	
nor-1	*nor-1*	ACCGCTACGCCGGCACTCTCGGCAC	400
nor-2		GTTGGCCGCCAGCTTCGACACTCCG	
omt-1	*omt-A*	GTGGACGGACCTAGTCCGACATCAC	797
omt-2		GTCGGCGCCACGCACTGGGTTGGGG	
ver-1	*ver-1*	GCCGCAGGCCGCGGAGAAAGTGGT	537
ver-2		GGGGATATACTCCCGCGACACAGCC	

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