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Evolution of fungal populations in corn silage conserved under polyethylene or biodegradable films

Davide Spadaro[a,b], Maria del Pilar Bustos-Lopez[b], Maria Lodovica Gullino[a,b], Serenella Piano[a], Ernesto Tabacco[a], Giorgio Borreani[a*]

[a]Department of Agricultural, Forest and Food Sciences (DISAFA), University of Torino, Largo Braccini 2, 10095 Grugliasco, Italy

[b]Centre of Competence for Innovation in the Agro-environmental Sector (AGROINNOVA), University of Torino, Largo Braccini 2, 10095 Grugliasco, Italy

[*]Corresponding Author

E-mail: giorgio.borreani@unito.it

Address: Department of Agricultural, Forest and Food Sciences (DISAFA), University of Torino Largo Braccini 2, 10095 Grugliasco, Italy

Telephone: +39-0116708783

Fax: +39-0116708798

Running Head: Dominant fungi in corn silage

Abstract

Aims: identify cultivable filamentous fungi before ensiling, after silage conservation, in farm-silos covered with two different plastic films (polyethylene vs. biodegradable), as well as after aerobic exposure of whole-crop corn silage.

Methods and Results: Molecular techniques coupled with traditional microbial counting were utilized to identify the predominant fungal species. The cultivable fungal population changed remarkably from harvesting to silo opening. Anaerobiosis and low pH reduced mold count and the presence of *Fusarium* species both under polyethylene and biodegradable film. However, in the peripheral areas of the silo, where air penetration could not be completely prevented, the fungal population did not decrease. The predominant fungal species after aerobic exposure of silage was *Aspergillus fumigatus*, without differences between the two plastic films.

Conclusions: Maintenance of anaerobiosis and a low pH also in the upper layer of the silo reduce the risk of mold growth during corn silage feed-out.

Significance and Impact of the Study: Even if the new biodegradable plastic film did not completely maintain the anaerobiosis in the upper layer of silage, the overall silage quality was not compromised and was similar to that

observed under polyethylene, indicating that the development and use of biodegradable film to cover silage is promising, but needs some improvement.

Key words: *Aspergillus fumigatus*; cultivable fungi; silage fermentation quality; forage; *Fusarium* spp.; mycotoxins.

Introduction

Food and feed losses due to mold growth and mycotoxin contamination greatly affect animal and human health, and the food safety. Whole corn silage is used throughout North America and Europe as a feed source for cattle, and it is stored in horizontal silos for long periods (Wilkinson and Toivonen 2003). Corn silage is particularly susceptible to aerobic deterioration, initiated by yeasts and molds, when exposed to oxygen or in the feed bunk (Ashbell and Weinberg 1992; Borreani and Tabacco 2010). The preservative effect against the growth of detrimental microorganisms, including fungi, is a result of anaerobiosis and acidification of ensiled forages by lactic acid-producing bacteria (Pahlow et al. 2003). Therefore, if good quality silage is prepared under anaerobic conditions and oxygen is successfully excluded, further mold growth is unlikely to occur (Scudamore and Livesey 1998). Poor storage conditions during ensiling or feed-out could lead to the entry of oxygen into the silage, with undesirable dry matter (DM) and nutritional losses, and could increase the risk of the growth of toxigenic fungi (Nout et al. 1993; Cavallarin et al. 2011; Cheli et al. 2013). If airtight sealing of the silo is not obtained, air penetrates the silage, and yeasts and molds multiply, resulting in aerobic deterioration; the DM losses in the top 0.5 m can occasionally exceed 35% (Borreani et al. 2007). Moreover, the microbiological quality of the whole silage mass could be negatively affected (Borreani and Tabacco 2014). Filamentous fungi often occur in silage as ball-sized lumps or as layers at 200 to 500 mm from the surface (Nout et al. 1993; Storm et al. 2010). Several efforts have been made to prevent air penetration during silage conservation, and plastic films utilized to seal the silo have shown to play a key role (Borreani and Tabacco 2014). In recent years, the standard polyethylene film has sometimes been replaced by oxygen barrier films, and these have been shown to prevent the growth of pathogenic and mycotoxigenic molds during conservation and to delay their growth after silage exposure to air (Dolci et al. 2011; Cavallarin et al. 2011). On the other hand, a great deal of attention has been paid to the search for cost-effective alternatives to replace petroleum-based commodity plastic films to cover silages, such as biodegradable materials with competitive mechanical properties (Borreani and Tabacco 2015). Biodegradable plastic films have proved to work well for conservation periods longer than 5 months in indoor conditions (Borreani and Tabacco 2015), but they still need to be tested in farm conditions to evaluate their ability to protect silage from the detrimental effects of molds during conservation.

The growth of filamentous fungi in silages is often associated with the production of mycotoxins (Auerbach et al. 1998; Storm et al. 2008), harmful to cattle, farm workers and dairy-product consumers (Fink-Gremmels 2008). Furthermore, some fungal species present in silage have been linked to mycotic infection in cattle, in particular *Aspergillus fumigatus* (Jensen et al. 1992; Mansfield and Kuldau 2007), which is an opportunistic human pathogen, and an agent of aspergillosis in immune-compromised individuals (Dagenais and Keller 2009).

Knowledge about microbial successions during and after the fermentation process of silage has been restricted by the lack of suitable methods that enable differentiation among individual microbial species (Stevenson et al. 2006). To the best of the authors' knowledge, no experiment that couples the traditional microbial counting with molecular identification has been performed on corn silage conserved under different plastic covers, with the aim of analyzing fungal dynamics from the pre-harvesting field phase to silage opening and the feed-out phase, in both the less anaerobic peripheral areas of the silo and in the deeper parts.

Hence, the aim of the study was to assess the effect of biodegradable plastic films, compared to a standard polyethylene film, on the fermentation quality, and the yeast and mold counts, as well as to identify the predominant mold population at opening in more and less anaerobic zones (core and outer layer) of the silo, and after 7 and 14 days of air exposure of whole-crop corn ensiled in farm-scale silos.

Material and Methods

Crop and Ensiling

Two trials were carried out at the experimental farm of the University of Turin in Carmagnola (TO), Piedmont, northern Italy (44°53'N, 7°41'E, altitude 232 m a.s.l.). Two corn hybrid cultivars (PR36B08, for Trial 1, and Eleonora, for Trial 2, Pioneer Hi-Bred Italia Srl, Gadesco Pieve Delmona, Cremona, Italy) were sown on 14 May 2010 at an intended planting density of 75,000 seeds ha^{-1}. Whole corn crops were harvested at around 3/5 and 5/6 milk-line stages for PR36B08 and Eleonora, respectively. 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 cut length of 12 mm and ensiled within 2 hours in drive-over piles (1.2 m height, 4 m width, and 6 m length), with three replications per film treatment (6 piles for each cultivar). The effects of two types of plastic sheet used to seal the silos (standard polyethylene vs. a new biodegradable film) were studied. The two sealing materials were: a) a single 200 μm thick (6 m width) black-on-white polyethylene, UV protected film (**PE**); b) a single 120 μm thick (4 m width) light green Mater-Bi® (Novamont SpA, Novara, Italy) biodegradable plastic film (**MB**). The oxygen permeability of the plastic films, determined on the basis of the American Society for Testing and Materials (ASTM) D 3985-81 standard method (ASTM, 1981), was 1,196 cm^3 m^{-2} per 24 h at 100 kPa at 23°C, 90% relative humidity (RH) for PE, and 500 cm^3 m^{-2} per 24 h for MB. The water vapor transmission rate (WVTR) was determined on the basis of the ASTM F1249-06 standard method (ATSM, 2011) and was 1.05 g m^{-2} (for 24 h at 38°C and 90% RH) for PE and 17.4 g m^{-2} for MB. Furthermore, the effect on the silage quality of the distance from the plastic film in the upper part of the silos was assessed in two zones: close to the

film, from 0 to 300 mm (**CF**), and at a depth from 600 to 900 mm (**FF**). This resulted in four treatments: silage close to the biodegradable film (MB-CF), silage far from the biodegradable film (MB-FF), silage close to the polyethylene film (PE-CF), and silage far from the polyethylene film (PE-FF). Each pile was alternatively covered with PE (three piles) or MB film (three piles), in each trial. The plastic sheets were held in place by putting gravel bags around the silage. During silo pile filling, plastic net bags (6 for each treatment) with well mixed fresh material (around 7 kg per bag) were sub-sampled (around 400 g) for pre-ensiling analyses and then the bags were closed, weighed and buried in the pile. Four bags were placed at the two depths (two for CF and two for FF) in each pile. The silos were uncovered and the bags dug from the piles after 146 d of conservation.

After sampling, the silages were subjected to an aerobic stability test, which involved by monitoring the temperature increases due to the microbial activity of the samples exposed to air. About three kilograms of each bag were allowed to aerobically deteriorate at room temperature (22 ± 1.6°C) in 17 L polystyrene boxes (290 mm diameter and 260 mm height) for 14 days. A single layer of aluminum foil was placed over each box to prevent drying and dust contamination, but also to allow air penetration. The room and silage temperatures were measured hourly by 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. The maximum temperature rise (°C) was also reported as another index of aerobic stability. The silage was sampled after 7 d and 14 d aerobic exposure in order to quantify the microbial changes in the silage during exposure to air.

Sample Preparation and Analyses

Both the pre-ensiled material and the silage were split into 4 subsamples. The first sub-sample was immediately analyzed for DM content by oven drying at 80°C for 48 h. The second sub-sample was dried for qualitative analyses in a forced-draft oven to a constant weight at 65°C, air equilibrated, weighed, and ground in a Cyclotec mill (Tecator, Herndon, VA, USA) to pass a 1 mm screen. This sample was analyzed for crude protein (CP, total nitrogen × 6.25) by combustion (Nitrogen analyzer; Primacs SN, Skalar, Breda, The Netherlands), for ash by ignition at 550°C for 3 h, for acid and neutral detergent fiber (ADF and NDF) as described by Robertson and Van Soest (1981), for ether extract (EE) by ether extraction (method number 920.39; AOAC 2005), and for starch concentration (method number 996.11; AOAC 2005) according to the AOAC International methods.

The third subsample (about 300 g) was extracted as a wet sample, using a Stomacher blender (Seward Ltd., Worthing, UK) for 4 min in distilled water at a water-to-sample material (fresh weight) ratio of 9:1 or in 0.05 M H_2SO_4 at an acid-to-sample material (fresh weight) ratio of 5:1. The water extract was immediately analysed for the nitrate

(NO3) content, through semi-quantitative analysis, using Merckoquant test strips (detection limit 100 mg NO3/kg of fresh matter), pH and ammonia nitrogen (NH_3-N) contents, determined using a specific electrode. The water activity (aw) was measured at 25°C on a fresh sample using an AquaLab Series 3TE (Decagon Devices Inc., Pullman, WA), which adopted the chilled-mirror dew point technique. An aliquot of 40 ml of silage acid extract was filtered with a 0.20-µm syringe filter and used for quantification of lactic and monocarboxylic acids (acetic, propionic, and butyric), ethanol and 1,2-propanediol by HPLC (Agilent Technologies, Santa Clara, CA) coupled to a refractive index detector, on an Aminex HPX-87H column (Bio-Rad Laboratories, Richmond, CA) (Canale et al. 1984). The analyses were performed isocratically under the following conditions: mobile phase 0.0025M H_2SO_4, flow rate 0.5 ml min^{-1}, column temperature 37°C, injection volume 100 µl. Duplicate analyses were performed for all the determined parameters. The duplicates were averaged and the six means (replicated silage bags) were considered as six observations in the statistical analysis.

Fungal isolation and morphological identification

The fourth subsample was used for microbiological analyses. A 30-g sample was transferred into sterile homogenization bags, suspended in 270 g peptone salt solution (1 g bacteriological peptone and 9 g NaCl per liter), and homogenized in a laboratory Stomacher blender for 4 min for the microbial counts. The colony-forming units (CFU) of yeasts and molds were counted using the serial plate dilution technique with 40.0 g l^{-1} of Yeast Extract Glucose Chloramphenicol Agar (YGC agar, DIFCO, West Molesey, UK) after incubation at 25°C for 3 d for the yeasts and for 5, 7 to 14 d for the molds. Mold and yeast CFUs were counted separately. During incubation, the plates were examined daily and fungal colonies were transferred on first observation on YGC agar. The direct plating method was used to isolate molds on the YGC agar. Colonies of filamentous fungi were collected from dilutions on Petri dishes with 1 to 50 CFU. Isolates growing on YGC agar were examined visually and microscopically for each silage sample.

Representative colonies of each distinct morphotype were chosen for molecular identification. Five to ten colonies per sample were streaked onto Petri dishes with potato dextrose agar (PDA, Merck, 39 g l^{-1}), medium amended with streptomycin (25 µg l^{-1}) and aerobically incubated for 5 to 7 days at 25°C, before DNA extraction, PCR amplification and sequencing.

Molecular identification

Fungal mycelium grown on PDA was scratched with a sterile blade and used for extraction. The DNA from all the isolates used in this study was extracted using the NucleoMag 96 Plant Kit (Macherey Nagel, Oensingen, Switzerland) and the Kingfisher magnetic particle processor (Thermo Labsystems, Basingstoke, United Kingdom) according to the manufacturers' instructions. About 100 mg of mycelium was ground in liquid nitrogen using mortar and pestle. The fine powder was used for DNA extraction.

Genomic DNA was amplified using universal primers ITS1 and ITS4 (White et al. 1990). PCR reactions were performed using a TGradient thermal cycler (Biometra, Göttingen, Germany). Each 20 µl PCR reaction contained 1 µl of DNA template (50 ng), 200 mM of deoxynucleotide triphosphate, 2 µl of 10X buffer (Taq DNA Polymerase, Qiagen, Chatsworth, CA, USA), 0.7 mM of primer, and 1.0 U Taq DNA Polymerase (Qiagen). PCR program followed: 95°C, 3 min; 34 cycles: 94°C, 15 s; 55°C, 45 s; 72°C, 55 s; 72°C, and a final extension at 72 °C for 7 min. A 10 µl aliquot of PCR products from each reaction was electrophoresed in 2.0% agarose gel and then stained with SYBR SAFE (Invitrogen, Eugene, OR, USA). Gel images were acquired using a Gel Doc 1000 System (Bio-Rad Laboratories, Hercules, CA, USA). PCR amplification products were cloned into the PCR4 TOPO vector (Invitrogen) using the TOPO TA cloning kit following the manufacturers' protocol and sequenced by Genome Express (Padova, Italy) using an ABI PRISM 3730XL DNA Sequencer. For some isolates belonging to the *Gibberella fujikuroi* species complex, the DNA was also amplified by using specific primers and sequenced in the portion of the coding region and introns of the translation elongation factor 1-alpha (TEF) gene (Geiser et al. 2004, Amatulli et al. 2012), to confirm the result of the ITS sequence.

Sequence analysis

ITS and EF-1α amplicon sequences were deposited in GenBank and comparison with available sequences was made using the BLAST program (www.ncbi.nlm.nih.gov). Multiple sequence alignment of nucleotide (nt) and amino acid (aa) sequences and identification of open reading frames were done by using the program AlignX (Vector NTI Suite V 5.5, InforMax, North Bethesda, Maryland, USA) with the Clustal W algorithm (Thompson et al. 1994).

Statistical analysis

Yeast and mold counts were $\log_{10}$ transformed and were presented on a fresh weight basis. The chemical compositional data and microbial counts were analyzed for their statistical significance via analysis of variance, with their significance reported at a 0.05 probability level using the general linear model of the Statistical Package for Social Science (v 17.0,

SPSS Inc., Chicago, Illinois, USA). The fermentation and microbiological characteristics of the silage at the top of the silo were analyzed separately for each trial, by ANOVA utilizing the type of plastic film (F), and the distance from the plastic film (Z) as the fixed factors.

Results

Herbage at ensiling

Table 1 shows the main characteristics of the forage prior to ensiling in the two trials. The DM content, CP, starch, and NDF contents in Trial 1 herbage were typical of whole corn forage harvested at around half of the milk line, whereas the DM content and starch were lower and the NDF was greater in Trial 2 than in Trial 1, indicating an earlier stage of maturity at harvesting. Microbial counts of the yeasts and molds were higher than 6 $\log_{10}$ cfu g^{-1} in both trials.

Silage and aerobic deterioration

The fermentative and microbiological characteristics of the silages stored under two plastic films in two silo layers (close to the film and far from the film) after 146 days of conservation in Trial 1 are shown in Table 2. The use of the MB film and the distance from the film affected most of the parameters, with the exception of acetic acid, nitrate, ammonia and butyric acid content, which was always below the detection limit (0.01 g kg^{-1} DM). The DM content was affected by the film type and sampling zone. The pH was altered in the MB-CF silage and this led to lower lactic and acetic acid contents, higher pH and higher yeast and mold counts. On the other hand, in the inner layer MB-FF, where anaerobiosis was maintained, the pH was below 4 and the lactic and acetic acid contents were higher than in the peripheral areas, with similar values to those observed below the PE film. The yeast count in the MB-FF zone was around 6 $\log_{10}$ cfu g^{-1} and the mold count was around 3 $\log_{10}$ cfu g^{-1}, indicating the beginning of aerobic microbial activity, whereas both the yeast and mold counts in the PE-FF silage were around 2 $\log_{10}$ cfu g^{-1}. The PE silages showed similar value of pH, lactic and acetic acid for the PE-CF and PE-FF zones, whereas the yeast count was higher in the PE-CF layer. Ethanol content also was influenced by the film and the sampling zone. Aerobic stability was higher in the PE silages than in MB, and higher values were observed in the FF zones.

Similar results were observed in Trial 2 (Table 3), except for a poorer microbial quality in the PE-CF silage, which showed a higher yeast count than the PE-FF zone. The mold count was not affected by zone in PE silages, as observed

for MB silages. The aerobic stability was similar in the FF zone for both films, whereas it was lower in the MB-CF than in the PE-CF silage.

The pH and yeast and mold counts of the silages after 7 and 14 days air exposure during the aerobic test in Trials 1 and 2 are reported in Tables 4 and 5. Similarly, in both trials the silage in the areas close to the film was deeply spoiled after 7 d aerobic exposure, with a pH higher than 5.5, as well as yeast and mold counts higher than 5.0 and 5.5 $\log_{10}$ cfu g^{-1}, respectively. After 14 days aerobic exposure, the mold count exceeded 8.0 $\log_{10}$ cfu g^{-1}, except for the MB-CF in Trial 2 (7.74 $\log_{10}$ cfu g^{-1} of molds).

Identification of filamentous fungi

The prevalent filamentous fungal species found in the forage at harvesting, in the silage after 146 d conservation and in the silage after 7 d air exposure are reported in Table 6. ITS and TEF sequences of representative fungal species were deposited in GenBank (Supplementary Table S1). Eleven fungal species, including several corn pathogens, were found at harvesting. After ensiling, eight species were found mainly belonging to *Galactomyces*, *Aspergillus* and *Penicillium* genera. Aerobic exposure of the silage after the conservation phase further reduced the dominant species to four, with a great prevalence of *Aspergillus fumigatus*, which became the prevalent species after 7 days of air exposure (Figures 2 and 3) and 100% after 14 d of air exposure.

The prevalent species of filamentous fungi found in the two corn forages before ensiling are reported in Figure 1. The fungal populations in the two trials were similar both in number and share of identified species. The main species were *Fusarium verticillioides* and *F proliferatum*, which accounted for 62% and 78% in Trials 1 and 2, respectively. After ensiling, both the sampling zone and the film type significantly modified the prevalent fungal population in Trials 1 and 2 (Figure 2 and 3). The silage far from the films showed the presence of *Penicillium* spp., *A. fumigatus* and *Galactomyces geotrichum*, which were always present in PE-FF and MB-FF in both trials. The zones close to the films, which are more prone to air penetration due to the permeability of the films, were characterized by the dominance of *G. geotrichum*, which ranged from 33% in the PE-CF of Trial 2 to 99% both in the PE-CF and MB-CF of Trial 1. The MB-FF silage showed a 66% and 75% presence of *G. geotrichum* out of the isolated colonies in Trials 1 and 2, respectively. In Trial 2, the two films showed different dominant fungal population, with the MB film showing a dominance of *G. geotrichum* both in the CF and FF zones; while the PE film showed a more diversified population both in the CF and FF zones.

Discussion

252

253 A large number of microbial species are living as saprophytes on the plant surface (phylloplane) already at the pre-
254 harvest stage including many bacteria and yeasts, but also filamentous fungi, some of which may be pathogenic during
255 plant development. The present study revealed that the dominant mold species (10^6 cfu g^{-1} corn) before ensiling are
256 potential plant pathogenic species with *Fusarium* species representing over 60% of the detected fungi. The dominating
257 fungal species were *F. proliferatum* and *F. verticillioides*, able to cause pink rot of corn and to produce fumonisins in
258 the field and at harvesting (Matic et al. 2013). Both species belong to the *Gibberella fujikuroi* species complex and can
259 be identified by sequencing the translation elongation factor (Amatulli et al. 2010). Other *Fusarium* species have been
260 reported at harvesting, such as *F. graminearum* or *F. culmorum*, which can cause red rot and produce trichotecenes and
261 zearalenones. Their presence requires cool and wet climatic conditions before harvesting (Lepom et al. 1998; Richard et
262 al. 2009). In our study, *Acremonium* spp. ranged from 20 to 30% of the fungal population isolated at harvesting. High
263 levels of contamination by *Acremonium zeae* and *F. verticillioides*, alone or in a combination, have frequently been
264 observed in symptomless corn kernels (Munkvold et al. 1997). Wicklow et al. (2005) have shown that *Acremonium*
265 *zeae* could sometimes act as an antagonist towards the pathogenic and mycotoxigenic fungi *Aspergillus flavus* and *F.*
266 *verticillioides*, interfering with *A. flavus* infection and aflatoxin contamination of pre-harvest corn kernels.
267 In the present experiments, the mold count after ensiling was reduced to below 3 $\log_{10}$ cfu g^{-1} in central areas of the
268 silos, where the anaerobic environment was maintained during conservation, and the dominant molds were mainly
269 *Aspergillus* and *Penicillium* species. The ensiling process, which generates a low pH and oxygen content and high
270 carbon dioxide levels, inhibits the growth of or even eliminates most fungi present at harvest (Shimshoni et al. 2013).
271 *Fusarium* spp. generally do not like low oxygen levels and acidic conditions, and their growth in silage is inhibited.
272 They may survive in silage as macroconidia, microconidia and chlamydospores, or through recolonisation after silage
273 opening, thus explaining the recovery of *Fusarium* species in silage (Driehuis and Oude Elferink 2000; Teller et al.
274 2012). The optimal pH for the growth of *F. proliferatum* and *F. verticillioides*, are 5.5 and 7.0, respectively (Marin et al.
275 1995), while the average pH of the silage samples in the present study was in most cases 3.8 to 4.0. The most common
276 fungi growing in silage which are able to produce mycotoxins are *Penicillium roqueforti*, *P. expansum* and *P. paneum*
277 (Auerbach et al. 1998). During conservation, even a small amount of oxygen can enable various facultative aerobic
278 spoilage microorganisms and microaerophilic fungi to survive in the anaerobic phase of ensiling and multiply during
279 the feed-out phase, thus causing aerobic deterioration (Woolford 1990). Borreani and Tabacco (2010), in a farm survey
280 on corn silages in Italy, found a mean mold count of 1.76 $\log_{10}$ cfu g^{-1} in well conserved central areas of the silos, 3.71
281 $\log_{10}$ cfu g^{-1} in visually unmolded peripheral areas and 8.00 $\log_{10}$ cfu g^{-1} in molded spots. Similar results were observed
282 in a farm survey in Germany: Auerbach et al. (1998) reported mold counts higher than 4 $\log_{10}$ cfu g^{-1} in more than half

of the commercial corn silages surveyed. Furthermore, they found that *Penicillium roqueforti* was the dominant mold, with a greater incidence than 60% in all the silages analyzed, while the other species identified belonged to the *Aspergillus*, *Mucor*, *Monascus*, and *Galactomyces* genera.

P. expansum, *P. roqueforti* and other *Penicillium* spp. are considered acidic fungi, that are able to tolerate low oxygen concentrations, and they are often observed in silages at a depth of 200–800 mm below the surface, where the oxygen concentration is too low for most spoilage organisms. In the present experiments, a prevalence of *Penicillium* spp. was observed at silage opening in the inner layers of the silage. *Penicillium* spp. were outcompeted by yeasts or yeast-like fungi, particularly by *G. geotrichum*, in the outer layers characterized by a microaerophilic environment.

The effect of storage conditions on mold growth and population evolution was observed in the present experiment in peripheral areas of the silo (CF zones), commonly associated with lower levels of anaerobiosis. The yeast counts were higher in the CF areas than in the deeper zones (FF zones) in both trials and under both films. Poor storage conditions, mainly due to air penetration in the silage, particularly in peripheral areas, or to an incorrect fermentation are the main factors that could lead to poor silage quality, with the possible undesirable growth of aerobic and microaerobic acid-tolerant fungi and yeasts (Garon et al. 2006; Borreani and Tabacco 2010). The oxidation of lactic acid, acetic acid and residual soluble carbohydrates, which is mainly operated by yeasts, results in the production of carbon dioxide and water, with an increase of silage temperature and pH (Pahlow et al. 2003). In the present experiment, the yeast count in the CF zones was higher than 4 $\log_{10}$ cfu g^{-1} in both trials and under both films. The highest yeast count values were found under MB film in both trials, and this was probably due to the beginning of film degradation. The formation of micro-holes in the film allowed oxygen access to the silage and alteration of the silage quality, with increased yeast and mold counts in the whole silage mass in Trial 1. Yeast and mold count increases reduced the aerobic stability of the silages (Borreani and Tabacco, 2015). As a consequence, the CF zones showed a dominance of *Galactomyces geotrichum*, a filamentous yeast-like fungus typical of microaerophilic environments (De Hoog and Smith 2004). Middlehoven and van Baalen (1998) reported that *G. geotrichum* and other yeasts are able to degrade lactic and acetic acids in the silage. Furthermore, *G. geotrichum* grows at temperatures ranging from 10 to 30°C, and is very resistant to reduced oxygen and elevated carbon dioxide levels (Marcellino et al. 2001). These characteristics could explain its development in a microaerophilic environment, which is typical of the outer layer of the silage, where slight damage to the plastic cover can occur. *G. geotrichum* growth can reduce the amount of lactic acid, increase the pH and precondition the silage, thus inducing a faster colonization by other fungi, such as *A. fumigatus*, when oxygen gains access (dos Santos et al. 2002).

During air exposure of the silage, temperature increased considerably after 7 days and *G. geotrichum* and *Penicillium* spp. were gradually substituted by *A. fumigatus* which became around 100% of the fungal population after 14 d. *A.*

fumigatus has frequently been isolated from silages throughout the world, both in temperate and warm climates (Cole et al. 1977; Pereyra et al. 2008; Dolci et al. 2011), particularly in silages where the microbial heat generated by organic matter degradation selected heat-tolerant species. Dolci et al. (2011) observed mold counts of 1.74 and 1.41 $\log_{10}$ cfu g^{-1} silage after 110 d of anaerobic conservation below PE and oxygen barrier films, and found a dominance of *A. fumigatus* after 14 days air exposure, with a mold count greater than 8 $\log_{10}$ cfu g^{-1} silage. In the present experiment, after 7 d air exposure, the temperatures ranged from 36°C to 53°C and decreased from 24°C to 41°C after 14 d. *A. fumigatus* is a saprophytic, thermophilic, microaerophilic, low pH growing fungus (Mansfield and Kuldau 2007), capable of growing between 8°C and 55°C, with an optimum at 37°C to 43°C, which is typically found on decaying organic matter and also on aerobic deteriorating silage (Gisi 2014). *A. fumigatus* resulted to be detectable after silage opening, indicating that the anaerobic and acidic environment did not affected its presence in silage. Probably it was present since the harvesting phase, but at a very low count that was masked by other potential plant pathogenic fungi present at higher counts. This is in agreement with Dolci et al. (2011) which, with culture-independent technique, found similar results with *A. fumigatus* appearing only after ensiling, when a great reduction of the mold counts was observed compared to the pre-ensiling phase. The presence of the thermophilic species *A. fumigatus* is one of the main problems in silage, as many strains can produce several mycotoxins, such as gliotoxin, fumagillin, helvolic acid, verruculogen, sphingofungin and kojic acid (Kosalec and Pepeljnjak 2005; Pereyra et al. 2008) that can affect the health of dairy cattle. In addition, the fungus is a well-known human and animal pathogen that causes aspergillosis in immuno-compromised humans (dos Santos et al. 2002; Dagenais and Keller 2009).

Culture-independent techniques, to identify microbial community via PCR-denaturing gradient gel electrophoresis (DGGE) and reverse transcription (RT)-PCR DGGE, have been used previously used by Dolci et al. (2011) on corn silage. The application of these techniques has revealed that only the dominant species of yeasts and filamentous fungi can be identified when conventional microbial enumeration shows values greater than 6 $\log_{10}$ cfu g^{-1}.

The present work highlighted the need of coupling molecular methods to traditional plate count to identify, enumerate and monitor the evolution of the fungal population when the mold count is lower than 5 $\log_{10}$ cfu g^{-1} (i.e. at forage harvesting, at silo opening, and in the first phases of aerobic deterioration). Future studies will involve also a microbiome analysis approach, to include also the unculturable component of the fungal population.

The dominant fungal population of silage dramatically changes from harvesting to silo opening. Anaerobiosis, together with acidity, can strongly reduce the mold count and the field species of *Fusarium*. Furthermore, in the peripheral areas of the silo, where the penetration of air cannot be prevented completely, the fungal population did not decrease. When silages were exposed to oxygen, the mold increased greatly over 7 days and *A. fumigatus* became the dominant species.

Several mycotoxigenic fungal genera, including *Alternaria*, *Aspergillus*, *Fusarium* and *Penicillium*, have been reported in our study. The results of the present study showed that the key factor in corn silage is the creation and maintenance of an anaerobic environment in the upper layer of the silo, in order to reduce the risk of mold growth. Therefore, air permeability and the stability of new biodegradable films should be improved to obtain silages with longer shelf life after air gains access to the silo during consumption, by delaying the growth of molds and by reducing their detrimental effect on safety and quality of silage.

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469

Figure legends

Figure 1. Filamentous fungi isolated from corn cv PR36B08 (Trial 1) and cv Eleonora (Trial 2) at harvest.  *Acremonium* sp.,  *Cladosporium* sp.,  *Fusarium proliferatum*,  *Fusarium verticillioides*, Other species (*Alternaria* sp., *Epicoccum* sp., *Penicillium* sp., *Ramularia* sp.).

Figure 2. Filamentous fungi isolated at silo opening from silage produced from corn cv PR36B08 (Trial 1) stored for 146 d under two plastic films (PE: polyethylene; MB: biodegradable) in two silo layers (CF: 0 to 300 mm from the film; FF: 600 to 900 mm from the film) and after 7 d of air exposure.  *Aspergillus fumigatus*, *Galactomyces geotricum*,  *Mucor cyrcinelloides*,  *Penicillium expansum*, *Penicillium funiculosum*, *Penicillium resedanum*,  *Penicillium roqueforti*.

Figure 3. Filamentous fungi isolated at silo opening from silage produced from corn cv Eleonora (Trial 2) stored for 146 d under two plastic films (PE: polyethylene; MB: biodegradable) in two silo layers (CF: 0 to 300 mm from the film; FF: 600 to 900 mm from the film) and after 7 d of air exposure.  *Aspergillus fumigatus*, *Galactomyces geotricum*, *Mucor cyrcinelloides*, *Penicillium expansum*, *Penicillium funiculosum*, *Penicillium resedanum*,  *Penicillium roqueforti*.

Tables

Table 1. Chemical and microbiological characteristics of the herbage prior to ensiling.

Parameters*	Trial 1 cv PR36B08	Trial 2 cv Eleonora
DM, g kg^{-1}	319 ± 5.61	271 ± 5.89
pH	5.46 ± 0.034	5.66 ± 0.093
Water activity, a_w	0.991 ± 0.001	0.992 ± 0.002
Nitrate, mg kg^{-1}	721 ± 193	1884 ± 194
NDF, g kg^{-1} DM	399 ± 7.81	431 ± 9.21
ADF, g kg^{-1} DM	194 ± 5.41	223 ± 8.00
CP, g kg^{-1} DM	68.3 ± 2.96	69.6 ± 1.71
Ash, g kg^{-1} DM	39.4 ± 2.99	41.8 ± 1.64
Starch, g kg^{-1} DM	285 ± 7.89	249 ± 8.85
Yeasts, log_{10} cfu g^{-1}	6.46 ± 0.115	6.50 ± 0.079
Molds, log_{10} cfu g^{-1}	6.37 ± 0.095	6.27 ± 0.049

* Values represent the average of three replications and the standard deviation. ADF = acid detergent fiber; CP = crude protein; DM = dry matter; NDF = neutral detergent fiber; NH_3-N = ammonia nitrogen.

Table 2. Fermentation and microbiological characteristics of the silages produced from corn cv PR36B08 (Trial 1) stored under two plastic films in two silo layers (CF: 0 to 300 mm from the film; FF: 600 to 900 mm from the film) after 146 days of conservation.

Parameters*	PE		MB					
	PE-CF	PE-FF	MB-CF	MB-FF	SEM	F	Z	FxZ
DM, g kg^{-1}	304	320	257	306	7.33	0.005	0.003	0.082
pH	3.91	3.83	5.97	3.91	0.255	0.001	0.001	0.001
a_w	0.988	0.989	0.991	0.988	0.0004	0.031	0.035	<0.001
Lactic acid, g kg^{-1} DM	50.3	59.2	6.6	45.6	5.58	<0.001	<0.001	0.006
Acetic acid, g kg^{-1} DM	15.2	21.2	10.7	29.4	2.07	0.419	<0.001	0.014
Butyric acid, g kg^{-1} DM	< 0.01	< 0.01	< 0.01	< 0.01	-	-	-	-
Propionic acid, g kg^{-1} DM	3.01	< 0.01	1.01	2.60	-	-	-	-
1,2-Propanediol, g kg^{-1} DM	0.62	1.33	< 0.01	0.33	0.137	<0.001	0.001	0.118
Ethanol, g kg^{-1} DM	16.6	21.5	0.0	7.2	2.39	<0.001	0.026	0.627
Lactic-to-acetic acid ratio	0.61	3.31	1.55	2.79	0.299	<0.001	0.240	0.009
Nitrate, mg kg^{-1} silage	0	86	165	85	21.7	0.036	0.940	0.035
NH_3-N, g kg^{-1} DM	6.59	6.57	7.04	7.32	0.47	0.571	0.901	0.887
Yeast, log_{10} cfu g^{-1} silage	4.48	2.74	7.55	5.99	0.479	<0.001	<0.001	0.664
Mold, log_{10} cfu g^{-1} silage	2.08	2.01	7.15	3.03	0.551	<0.001	<0.001	<0.001
Aerobic stability (h)	61	137	3	33	12.9	<0.001	<0.001	0.076

* a_w = water activity; LAB = lactic acid bacteria; NH_3-N = ammonia nitrogen; PE = commercial plastic film with polyethylene; F = film type; Z = sampling zone; FxZ= interaction between film and zone effects; MB-CF = zone close to biodegradable film; film; MB-FF = zone far from biodegradable film; PE-CF = zone close to polyethylene film; PE-FF = zone far from polyethylene film.

506 **Table 3**. Fermentation and microbiological characteristics of the silages produced from corn cv Eleonora (Trial 2)
507 stored under two plastic films in two silo layers (CF: 0 to 300 mm from the film; FF: 600 to 900 mm from the film)
508 after 146 days of conservation.
509

Parameters*	PE		MB					
	PE-CF	PE-FF	MB-CF	MB-FF	SEM	F	Z	FxZ
DM, g kg^{-1} DM	246	266	226	258	4.90	0.068	0.003	0.407
pH	4.00	3.83	5.88	3.88	0.313	0.075	0.050	0.091
a_w	0.991	0.989	0.994	0.990	0.0005	<0.001	<0.001	0.002
Lactic acid, g kg^{-1} DM	48.1	69.3	20.4	61.4	5.46	0.010	<0.001	0.117
Acetic acid, g kg^{-1} DM	16.8	24.9	8.9	29.8	2.30	0.524	<0.001	0.018
Butyric acid, g kg^{-1} DM	< 0.01	< 0.01	< 0.01	< 0.01	-	-	-	-
Propionic acid, g kg^{-1} DM	3.13	< 0.01	2.55	0.75	0.390	0.854	< 0.001	0.178
1,2-Propanediol, g kg^{-1} DM	0.40	1.77	< 0.01	0.46	0.176	<0.001	<0.001	<0.001
Ethanol, g kg^{-1} DM	4.79	25.68	3.79	14.30	2.63	0.055	<0.001	0.100
Lactic-to-acetic acid ratio	2.86	2.78	2.29	2.06	0.267	0.046	0.942	0.463
Nitrate, mg kg^{-1} silage	979	1414	1219	1815	160	0.324	0.124	0.801
NH_3-N, g kg^{-1} DM	8.00	7.43	5.65	7.15	0.44	0.148	0.596	0.247
Yeast, log_{10} cfu g^{-1} silage	6.58	5.11	7.61	5.03	0.351	0.343	0.001	0.266
Mold, log_{10} cfu g^{-1} silage	1.45	1.35	5.73	1.73	0.511	<0.001	<0.001	0.001
Aerobic stability (h)	24	31	6	33	3.60	0.150	0.007	0.079

510

511 * a_w = water activity; LAB = lactic acid bacteria; NH_3-N = ammonia nitrogen; PE = commercial plastic film with
512 polyethylene; F = film type; FxZ= interaction between film and zone effects; MB-CF = zone close to biodegradable
513 film; film; MB-FF = zone far from biodegradable film; PE-CF = zone close to polyethylene film; PE-FF = zone far
514 from polyethylene film; Z = zone of sampling.
515

Table 4. pH, temperature, yeast and mold count of silages produced from corn cv PR36B08 after 7 and 14 days of aerobic exposure (Trial 1).

Parameters*	PE		MB		SEM	F	Z	FxZ
	PE-CF	PE-FF	MB-CF	MB-FF				
After 7 d								
pH	5.79	4.38	7.89	7.37	0.381	<0.001	0.008	0.168
Yeast (log_{10} cfu g^{-1} silage)	8.02	7.25	6.80	5.53	0.254	<0.001	0.001	0.302
Mold (log_{10} cfu g^{-1} silage)	5.70	4.21	6.62	7.36	0.530	0.057	0.707	0.272
Temperature (°C)	43	37	28	53	3.07	0.868	0.041	0.003
After 14 d								
pH	7.37	6.27	8.07	7.48	0.180	<0.001	<0.001	0.099
Yeast (log_{10} cfu g^{-1} silage)	7.81	6.69	6.94	6.99	0.158	0.290	0.057	0.040
Mold (log_{10} cfu g^{-1} silage)	9.32	8.99	8.03	9.14	0.142	0.001	0.012	<0.001
Temperature (°C)	29	41	24	27	1.77	<0.001	<0.001	0.004

* F = film type; FxZ= interaction between film and zone effects; MB-CF = zone close to biodegradable film; film; MB-FF = zone far from biodegradable film; PE-CF = zone close to polyethylene film; PE-FF = zone far from polyethylene film; Z = zone of sampling.

Table 5. pH, yeast and mold count temperature of silages produced from corn cv Eleonora after 7 and 14 days of aerobic exposure (Trial 2).

Parameters*	PE		MB		SEM	F	Z	FxZ
	PE-CF	PE-FF	MB-CF	MB-FF				
After 7 d								
pH	7.46	6.52	7.95	7.44	0.162	0.005	0.004	0.315
Yeast (log_{10} cfu g^{-1} silage)	7.70	6.10	5.47	6.75	0.253	0.024	0.611	<0.001
Mold (log_{10} cfu g^{-1} silage)	8.09	6.73	6.88	6.94	0.149	0.001	<0.001	<0.001
Temperature (°C)	52	53	36	42	2.37	0.002	0.316	0.451
After 14 d								
pH	7.59	8.15	8.25	7.61	0.081	0.200	0.427	<0.001
Yeast (log_{10} cfu g^{-1} silage)	7.19	7.00	7.08	6.54	0.204	0.525	0.413	0.687
Mold (log_{10} cfu g^{-1} silage)	8.80	8.77	7.74	9.63	0.195	0.617	0.001	<0.001
Temperature (°C)	26	26	26	28	2.12	0.354	0.449	0.303

* F = film type; FxZ= interaction between film and zone effects; MB-CF = zone close to biodegradable film; film; MB-FF = zone far from biodegradable film; PE-CF = zone close to polyethylene film; PE-FF = zone far from polyethylene film; Z = zone of sampling.

532 **Table 6.** Predominant filamentous fungi isolated from forage at harvesting, in silage at opening and in silage after 7 d of
533 aerobic exposure.
534

Fungal species	Harvest	Ensiled	After aerobic exposure
Acremonium fusidioides	X		
Acremonium implicatum	X		
Acremonium strictum	X		
Acremonium zeae	X		
Alternaria alternata	X		
Aspergillus fumigatus		X	X
Cladosporium spp.	X		
Dipodascus australiensis		X	X
Epicoccum nigrum	X		
Fusarium proliferatum	X		
Fusarium verticillioides	X		
Galactomyces geotrichum		X	X
Mucor cyrcinelloides		X	X
Penicillium brevicompactum	X		
Penicillium roqueforti		X	
Penicillium expansum		X	
Penicillium funiculosum		X	
Penicillium resedanum		X	
Ramularia coccinea	X		
Total fungal species	11	8	4

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536

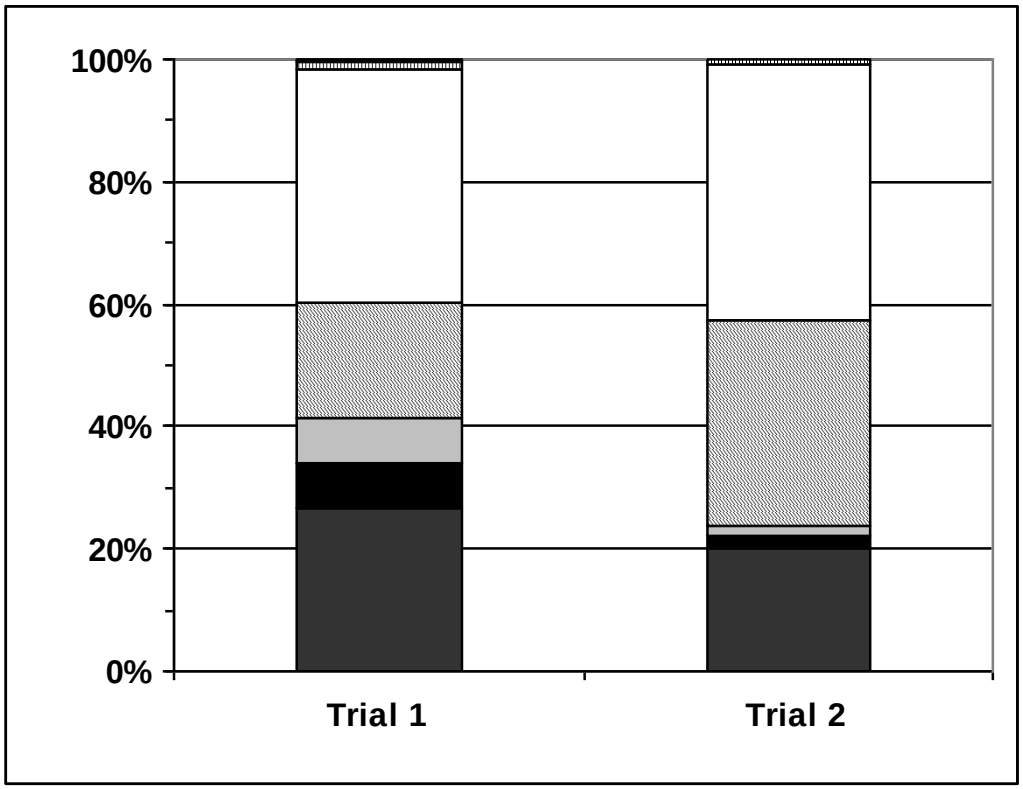
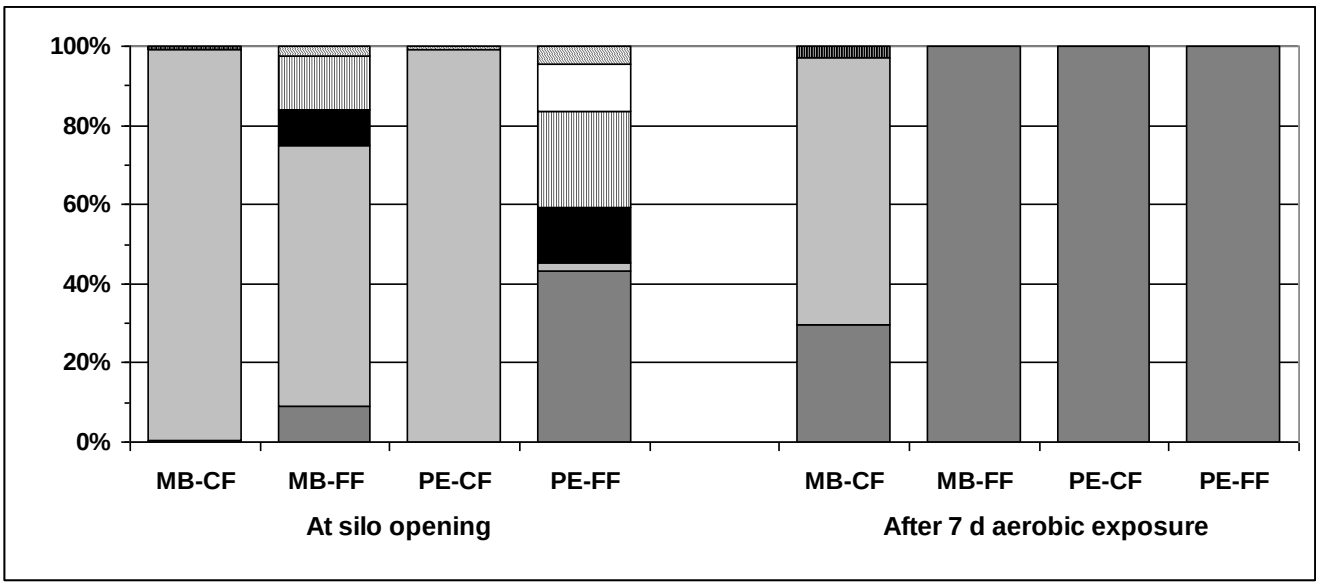


Figure 1.



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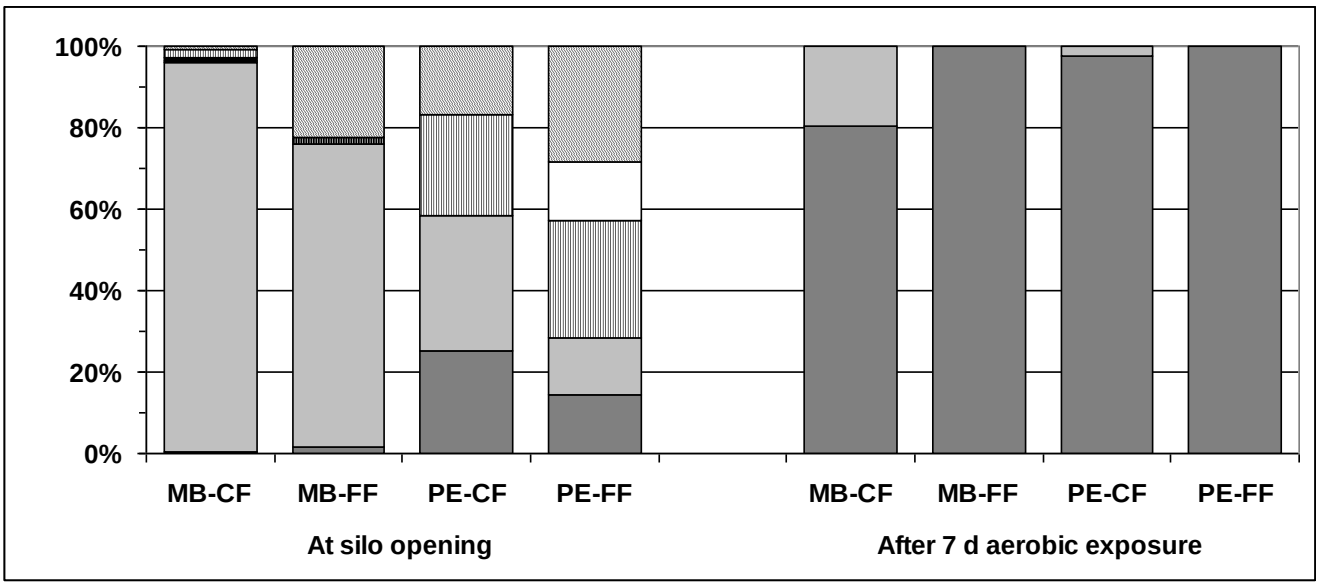
544 **Figure 2.**

545



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549 **Figure 3.**

550

Supplementary material

Supplementary Table S1. Accession numbers of the ITS (and TEF) sequences of representative fungal isolates, with indications of isolation source and time.

Species	ITS (and TEF) sequence*	Corn hybrid	Film type	Sampling time	Sampling zone
Acremonium fusidioides	KJ608089	Eleonora	-	harvest	-
Acremonium implicatum	KJ608090	Eleonora	-	harvest	-
Acremonium strictum	KJ608088	Eleonora	-	harvest	-
Acremonium strictum	KJ608083	Eleonora	-	harvest	-
Acremonium strictum	KJ608084	Eleonora	-	harvest	-
Acremonium strictum	KJ608085	PR36B08	-	harvest	-
Acremonium zeae	KJ608086	Eleonora	-	harvest	-
Acremonium zeae	KJ608087	PR36B08	-	harvest	-
Alternaria alternata	KJ608104	PR36B08	-	harvest	-
Cladosporium sp.	KJ608091	Eleonora	-	harvest	-
Cladosporium sp.	KJ608093	Eleonora	-	harvest	-
Cladosporium sp.	KJ608092	Eleonora	-	harvest	-
Epicoccum nigrum	KJ608105	Eleonora	-	harvest	-
Fusarium proliferatum	KJ608094	Eleonora	-	harvest	-
Fusarium proliferatum	KJ608095 (KM583804)	PR36B08	-	harvest	-
Fusarium proliferatum	KJ608096 (KM583805)	PR36B08	-	harvest	-
Fusarium proliferatum	KJ608097 (KM583806)	PR36B08	-	harvest	-
Fusarium proliferatum	KJ608098	PR36B08	-	harvest	-
Fusarium verticillioides	KJ608099 (KM583799)	PR36B08	-	harvest	-
Fusarium verticillioides	KJ608100 (KM583800)	PR36B08	-	harvest	-
Fusarium verticillioides	KJ608101 (KM583801)	Eleonora	-	harvest	-
Fusarium verticillioides	KJ608102 (KM583803)	PR36B08	-	harvest	-
Fusarium verticillioides	KJ608103 (KM583802)	PR36B08	-	harvest	-
Ramularia coccinea	KJ608106	PR36B08	-	harvest	-
Galactomyces geotrichum	KJ608125	PR36B08	MB	ensiled	inner

Galactomyces geotrichum	KJ608128	PR36B08	PE	ensiled	outer
Galactomyces geotrichum	KJ608127	PR36B08	PE	ensiled	outer
Galactomyces geotrichum	KJ608124	Eleonora	MB	ensiled	outer
Galactomyces geotrichum	KJ608126	Eleonora	MB	ensiled	outer
Penicillium funiculosum	KJ608114	PR36B08	MB	ensiled	inner
Penicillium funiculosum	KJ608115	Eleonora	PE	ensiled	outer
Aspergillus fumigatus	KJ608111	Eleonora	MB	ensiled	outer
Penicillium roqueforti	KJ608118	PR36B08	PE	ensiled	outer
Penicillium roqueforti	KJ608119	Eleonora	PE	ensiled	outer
Penicillium roqueforti	KJ608117	Eleonora	MB	ensiled	outer
Penicillium roqueforti	KJ608120	PR36B08	MB	ensiled	inner
Penicillium expansum	KJ608113	PR36B08	MB	ensiled	inner
Penicillium resedanum	KJ608116	Eleonora	PE	ensiled	inner
Aspergillus fumigatus	KJ608108	PR36B08	MB	ensiled	inner
Aspergillus fumigatus	KJ608107	Eleonora	PE	ensiled	inner
Mucor circinielliοides	KJ608112	PR36B08	MB	ensiled	outer
Aspergillus fumigatus	KJ608110	PR36B08	MB	aerobic exposure	inner
Diplodascus australiensis	KJ608121	PR36B08	MB	aerobic exposure	inner
Galactomyces geotrichum	KJ608122	PR36B08	MB	aerobic exposure	inner
Galactomyces geotrichum	KJ608123	Eleonora	MB	aerobic exposure	inner
Aspergillus fumigatus	KJ608109	Eleonora	MB	aerobic exposure	outer

556